UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Course in Experimental Medicine

Department of Medical Biotechnologies and Translational Medicine (BioMeTra)

XXXV CYCLE

PhD Thesis

"Role of endothelial dysfunction in the pathogenesis of unprovoked venous thromboembolism"

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Academic Year 2021-2022

Abstract

Background and Aim

Venous thromboembolism (VTE) is a multifactorial disease that includes deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE patients can be classified on the basis of the risk factor(s) that trigger the thromboembolic event. Indeed, up to 50% of VTE events occur in absence of predisposing conditions and are therefore classified as unprovoked VTE (uVTE). Being uVTE pathogenesis still unknown, uVTE patients are characterised by a high risk of recurrence, and the therapeutic management of these patients to prevent VTE recurrence is still controversial. A better comprehension of uVTE pathogenesis and the identification of the molecular mechanisms underlying VTE triggering in these patients are needed to guide patient treatment through the definition of new clinicopathological entities and the definition of molecular targets for the development of novel therapeutic strategies. Endothelial dysfunction (ED) plays a crucial role in promoting thrombus formation, yet its presence in uVTE patients has been poorly investigated, so far. Therefore, in this study we investigated ED in uVTE, using patient-derived endothelial colony-forming cells (ECFCs) that represent an optimal non-invasive model to assess the functionality of the endothelial compartment. In particular, in this project we aimed to:

- set-up and optimize ECFC-based functional assays aimed at assessing thrombogenic endothelial properties;
- investigate the presence of constitutive ED in uVTE patients and the involved underlying molecular mechanisms;
- investigate whether ED in uVTE patients is further promoted by pro-inflammatory stimuli;
- evaluate whether ED contribute to VTE recurrence risk in uVTE patients;
- assess whether ECFC features of ED are restricted to uVTE or shared with secondary VTE.

Methods

Optimization of functional assays. ECFC-based TGA and thrombogenesis assay were preliminarily set up on commercially-available human umbilical venous endothelial cells (HUVECs), then tested on ECFCs isolated and expanded from 8 healthy donors (HDs). ECFCs were used at passages 4-6, in basal conditions or after TNFα-stimulated activation. TNFα–induced EC activation was assessed as upregulation of the adhesion molecule VCAM-1 and the pro-coagulant molecule Tissue Factor (TF) by flow cytometry. EC function was assessed by analyzing: a) thrombin generation assessed by TGA on ECs - a modification of Hemker's method; b) platelet deposition and fibrin formation under flow conditions, assessed in thrombogenesis assay.

Assessment of ED role in uVTE pathogenesis. 78 VTE patients and 27 HDs were enrolled in the study. VTE patients were classified as: i) provoked VTE (pVTE) (n=28) when the VTE event was associated with major risk factors, ii) weakly provoked VTE (wpVTE) (n=28) when the VTE event was associated with minor risk factors; iii) unprovoked VTE (uVTE) (n=22) when the VTE event occurred in the absence of any provoking risk factors. A peripheral blood sample was collected at a single timepoint from all the enrolled subjects for ECFC isolation and plasma collection. In VTE patients, blood withdrawal was executed 3 months after the index VTE event, along with simultaneous assessment of validated predictors of VTE recurrence (namely, post thrombotic syndrome, D-dimer levels, and presence of residual venous obstruction). To investigate the presence of constitutive ED, ECFCs obtained from the different subgroups of VTE patients and HDs were characterized by assessing: i) their immunophenotype; ii) their ability to promote thrombosis in ECFC-based TGA and thrombogenesis assay; iii) their secretion of soluble ED markers (soluble adhesion molecules including the soluble forms of ICAM-1, VCAM-1, PECAM-1 and E-Selectin) in culture supernatants; to assess the *in vivo* relevance of these markers, they were also measured in the plasma of the same patients; iv) their transcriptomic profile assessed by RNA sequencing to investigate possible molecular

pathways underlying ED in uVTE. To investigate whether ED in uVTE patients is further promoted by pro-inflammatory stimuli, the phenotype and functionality of TNFα-treated ECFCs were assessed by using the same functional assays used to study constitutive ED. To investigate the role of ED in VTE recurrence, ECFC features of ED were analyzed in patients stratified according to their recurrence risk.

Results

Optimization of functional assays. TGA and the thrombogenesis assay were optimized to the use of ECs as a substrate. Both assays demonstrated the ability to discriminate between unstimulated and activated ECs, either HUVECs or ECFCs, whose activation was confirmed by the upregulation of VCAM-1 and TF expression. In particular, TNFα-activated HUVECs and ECFCs were similarly characterized by significantly higher thrombin generation, platelet deposition and fibrin formation compared with their unstimulated counterparts. Based on these results, both ECFC-based functional assays were used for subsequent experiments addressing ED in uVTE, using patient-specific ECFCs.

Assessment of ED role in uVTE pathogenesis. ECFCs were isolated from the three subgroups of VTE patients with a similar efficiency as HDs, but they were characterized by increased early senescence. The presence of constitutive ED was observed only in uVTE patients, as assessed as: a) a faster and higher peak of thrombin generation observed in TGA; b) increased platelet deposition observed in the thrombogenesis assay, in uVTE patients compared with HDs. Relevant to the increased platelet deposition, higher levels of soluble ICAM-1 and PECAM-1 were detected in the culture supernatants of ECFCs obtained from uVTE patients, the *in vivo* relevance of this finding being suggested by the observation of increased levels of soluble ICAM-1 also in the plasma of the same patients. Based on the preliminary analysis of the transcriptomic ECFC profiling, the upregulation of these adhesion molecules in uVTE patients may be supported by a downregulated gene expression of GSTM1 and PTGIR that were downregulated in uVTE ECFCs compared with ECFCs obtained from both HDs and patients with secondary VTE.

A higher propensity of ECFCs obtained from uVTE patients to promote thrombus formation was also confirmed in inflammatory conditions, as only TNFα-stimulated ECFCs from these patients showed a shorter lag time in TGA and higher platelet deposition in the thrombogenesis assay, compared with HDs. Notably, ECFC treatment with TNFα increased the amount of thrombin generated in TGA only in HDs but not in uVTE, wpVTE or pVTE patients, likely related to direct oral anticoagulants (DOACs) taken by the vast majority of VTE patients at the time of blood collection.

Finally, by stratifying patients according to their risk to develop VTE recurrences based on the presence of residual venous obstruction, we showed that several ECFC features of ED were broadly higher in uVTE and wpVTE patients with a high risk of recurrence compared with low-risk patients, thus suggesting that ED may also play a role in VTE recurrence in these patients.

Conclusions

In this study, we validated the use of ECFCs for assessing endothelial functions relevant to the study of human thromboembolic diseases. By applying properly optimized assays, we performed a functional characterization of patient-derived ECFCs, demonstrating that uVTE patients have endothelial prothrombotic alterations that may play a relevant role in the pathogenesis of the disease and in the risk of VTE recurrence. By extending the ECFC characterization on a larger cohort of patients, deepening the molecular characterization of ED, and by addressing the impact of anticoagulation on ED and VTE recurrence, this experimental approach has the potential to further dissect the molecular pathways underlying ED in uVTE patients, with the final aim to identify molecular targets for the development of novel therapeutic strategies aimed at preventing VTE recurrences in these patients.

Disclosure for research integrty

The research proposed in this manuscript was conducted following the European Code of Conduct for Research Integrity, which includes the values of reliability, rigor, honesty, respect and transparency.

Table of contents:

1. INTRODUCTION

1.1 Haemostasis: steps and process

The term haemostasis derives from the combination of the ancient Greek words *"haeme"* and *"stasis"* and indicates the stopping of bleeding (Thornton P. et al., 2010). In particular, the physiological balance aimed to prevent and stop bleeding and haemorrhagic events maintaining normal blood flow elsewhere in the circulation is provided by the complex interactions between the blood coagulation process and the fibrinolytic process along with platelets and the vessel wall. In addition, inhibition of the coagulation system is mediated by numerous factors that prevent clot formation and subsequently thrombus promotion. Table 1.1 summarizes some of the main thrombogenic and antithrombogenic elements.

Site	**Thrombogenic elements**	**Antithrombogenic elements**
Vessel wall	Exposed endothelium Tissue Factor (TF) Collagen	Heparin Thrombomodulin Tissue plasminogen activator
Circulating elements	Platelets Platelet activating factor Clotting factor Prothrombin Fibrinogen von Willebrand factor (vWF)	Antithrombin Protein C and S Plasminogen

Table1.1 | Thrombotic and anti-thrombotic elements *(Adapted from Palta S. et al., 2014).*

Wherever coagulation factors characterized by a pro-coagulant effect are increased or the function of inhibitors is disrupted, this subtle balance is broken thus leading to thrombosis (Previtali E. et al., 2011). On the contrary, opposite alterations are on the basis of pathological conditions characterized by excessive bleeding (Furie B. & Furie BC., 2008).

In this section, to better understand the entire coagulation system, primary haemostasis, coagulation pathways involved in secondary haemostasis, and the fibrinolytic system will be described.

1.1.1 Primary Haemostasis

Platelets are cell fragments originating from megakaryocytes in the bone marrow, characterized in their resting state by a discoidal structure with a diameter of 2-3 μm. Platelets - whose count in the blood range from 150 to 400x 10^9/L in adults - play a crucial role in clot formation to stop bleeding. In fact, they participate in the early stages of haemostasis, known as primary haemostasis, which consist in the interaction between platelets and the vessel wall resulting in the development of an early "platelet plug" (Andrews RK. et al., 2004).

In physiological conditions, endothelial cells (ECs) lining the vascular wall have antithrombotic properties due to the expression of various components, such as negatively charged heparin-like glycosaminoglycans, neutral phospholipids, and the synthesis and secretion of platelet inhibitors, coagulation inhibitors, and fibrinolysis activators. Components that support platelet adhesion, such as collagen, von Willebrand factor (vWF), and other proteins such as laminin, are localized in the subendothelial layer that is therefore endowed with thrombogenic properties (Bernardo A. et al., 2004).

In case of vascular wall damage that cause the subendothelial matrix exposure or in the presence of endothelial alterations that impaired the antithrombotic properties of ECs, the platelet adhesion to the vessel wall occurs thus leading to the formation of a platelet plug. The generation of the platelet plug involves several steps (Figure 1.1):

1. Platelet adhesion. Following vascular injury, platelets adhere to exposed collagen and vWF in the subendothelial layer and undergo morphological changes, presenting an irregular surface with pseudopodia that increase the surface area (Andrews RK. et al., 2004; Heemskerk JW. et al., 2002). In case of slow blood flow, platelets binding to collagen is mediated by the platelet glycoprotein Ia (GpIa) complex; when the blood flow is faster, vWF fosters platelet adhesion by acting as a bridge between endothelial collagen and platelet by interacting with surface receptors GpIb expressed by platelet (Andrews RK. et al., 2004; Heemskerk JW. et al., 2002; Lasne D. et al., 2006; Triplett DA. et al., 2000). In the case of platelet adhesion to

ECs, this interaction is mediated by several molecules expressed by activated endothelium (i.e. during inflammatory processes) such as by by intercellular adhesion molecule 1 (ICAM-1), P-selectin (CD62P), and P-selectin glycoprotein ligand-1 (PSGL-1), Platelet endothelial cell adhesion molecule (PECAM-1), integrins (e.g. αvβ3 integrin) and ADAM-15, a transmembrane cell-surface protein member of the ADAM (a disintegrin and metalloproteinase) family that binds to platelets through the GIIb/IIIa receptor (Hamilos M. et al., 2018).

2. Platelet secretion. Platelets contain two types of granules:
    - α-granules that contain P-selectin, fibrinogen, fibronectin, factor V, factor VIII, platelet-derived factor IV, platelet-derived growth factor and tumor growth factor-α (TGF-α) (Heemskerk JW. et al., 2002);
    - δ-granules or dense granules, that carry adenosine triphosphate (ATP), adenosine diphosphate (ADP), calcium (Ca), serotonin, histamine, and hepinephrine (Heemskerk JW. et al., 2002).

    The release of these different factors from both types of granules contained in platelets follows the adhesion phase. In addition, calcium is released and interacts with phospholipids, which are exposed after platelet activation, forming a surface that enables the assembly of clotting factors.
3. Platelet aggregation. Any vascular alteration triggers activation of platelets that release a local mediator, thromboxane A2 (TxA2) (Heemskerk JW. et al., 2002; Lasne D. et al., 2006) that further support platelet aggregation. The platelet plug arises from the action between TxA2 and ADP, increasing platelet aggregation, and temporarily folds vascular damage. On the other hand, the inhibition of platelet aggregation is provided by prostacyclin, and a proper balance between prostacyclin and TxA2 leads to local platelet aggregation, preventing clot expansion and vessel lumen permeability (Heemskerk JW. et al., 2002). The association between TxA2 and ADP causes also a conformational change in GpIIb/IIIa receptors on the surface of platelets, resulting in fibrinogen deposition. Its conversion to fibrin is

catalyzed by thrombin, providing more stabilization of the platelet plug and thus initiating secondary hemostasis (Heemskerk JW. et al., 2002).

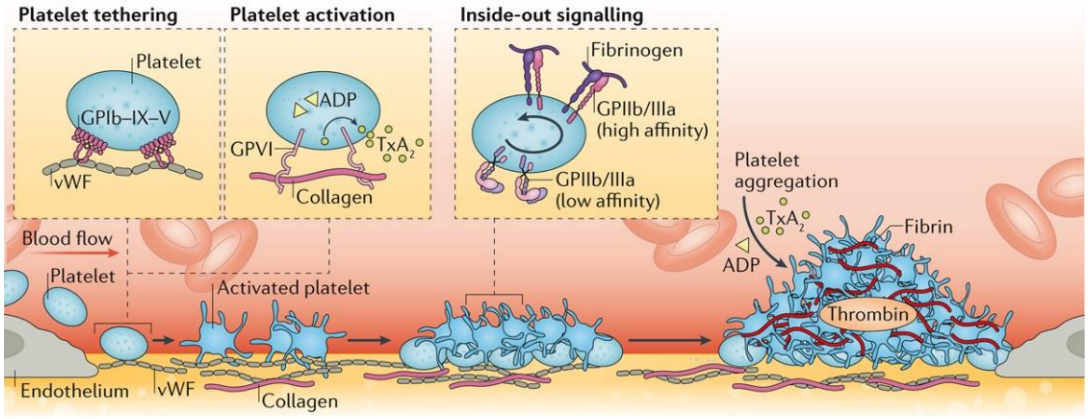

Figure 1.1 | Platelet adhesion and aggregation steps. After vascular injury, the thrombogenic subendothelial matrix proteins, von Willebrand factor (vWF) and collagen are exposed. vWF binds collagen and uncoils, providing numerous binding sites for platelet glycoprotein (GP)Ib–IX–V. Interaction between collagen and GPVI promote platelet activation and the release of the soluble agonists ADP and thromboxane A_2 (TxA_2), inducing GPIIb/IIIa activation through inside-out signaling. In the high affinity state, GPIIb/IIIa bind to its major ligand, fibrinogen, forming a bridge for platelets to aggregate. Platelet thrombus is intensified by the release of soluble agonists, such as ADP and TxA_2 *(Adapted from: McFadyen JD. et al., 2018).*

1.1.2 Secondary haemostasis

Blood coagulation was defined in the 1960s by Davie, Ratnoff and Macfarlane who described the "waterfall" or "cascade" theories, introducing the essential concept of several cascading proenzymes that orchestrate the activation of downstream enzymes (Achneck HE. et al., 2010) with the final aim to convert soluble fibrinogen into its insoluble form represented by fibrin. In particular, coagulation factors are indicated by Roman numbers and a main group is present in circulation as zymogens which are the inactive precursors of the proteolytic enzymes. The activation of the coagulation factors allows their participation in the coagulation cascade and occurs through an initial post-translational modification, a specific vitamin K-dependent Ɣ carboxylation of the glutamic acid residues, that is required for the binding of calcium and other essential bivalent cations (Monroe DM. et al., 2010). The suffix "a" indicate the active form of the coagulation factors. The different nomenclatures and functions of coagulation factors are shown in Table 1.2.

Coagulation factors

Number	*Name*	*Function*
I	Fibrinogen	Clot formation
II	Prothrombin	Activation of I, V, VII, VIII, XI, XIII, protein C, platelets
III	TF	Co factor of VIIa
IV	Calcium ion	Facilitates coagulation factor binding to phospholipids
V	Proacclerin, labile factor	Co-factor of X-prothrombinase complex
VI	Unassigned	
VII	Stable factor, proconvertin	Activates factors IX, X
VIII	Antihaemophilic factor A	Co-factor of IX-tenase complex
IX	Antihaemophilic factor B or Christmas factor	Activates X: Forms tenase complex with factor VIII
X	Stuart-Prower factor	Prothrombinase complex with factor V: Activates factor I
XI	Plasma thromboplastin antecedent	Activates factor IX
XII	Hageman factor	Activates factor XI, VII and prekallikrein
XIII	Fibrin-stabilising factor	Crosslinks fibrin
XIV	Prekallikerin (F Fletcher)	Serine protease zymogen
XV	HMWK- (F Fitzgerald)	Co factor
XVI	vWF	Binds to VIII, mediates platelet adhesion
XVII	Antithrombin III	Inhibits IIa, Xa, and other proteases
XVIII	Heparin cofactor II	Inhibits IIa
XIX	Protein C	Inactivates Va and VIIIa
XX	Protein S	Cofactor for activated protein C

Table 1.2 | Nomenclature of the coagulation factors. *HMWK – High molecular weight kininogen; vWF – Von Willebrand factor; TF – Tissue factor (Adapted from Palta S. et al., 2014).*

Traditionally, coagulation cascade was subdivided into intrinsic and extrinsic pathways, both converging in factor X activation.

Intrinsic pathway is the longer pathway of secondary haemostasis and is activated by the exposed endothelial collagen. Intrinsic pathway involved plasmatic mediators, such as factor XII, XI and IX as shown in Figure 1.2. In addition, prekallikrein and factor XII reciprocally activate each other. Through a cascade mechanism, each factor activates the downstream factor and in particular the resulting factor XIIa activates FXI to FXIa which in turn acts as a

catalyst to activate factor IXł to factor IXa. Factor IXa, together with its cofactor (factor VIIIa) composed the intrinsic tenase complex that is assembled on a negatively charged phospholipid surface and in which factor X in converted in factor Xa (Hall JE., 2010; Kumar V. et al., 2010).

The extrinsic pathway is a rapid process activated by the Tissue Factor (TF, also known as factor III or CD142) released by ECs in case of vascular damage (Lasne D. et al., 2006; Monroe DM. et al., 2010). TF expression depends not only on mechanical injury but also on various physical and biochemical conditions such as hypoxia, sepsis, neoplasia, and inflammation (Mackman N. et al., 2007; Manly DA. et al., 2011). Under physiological conditions, ECs express no or minimal amounts of TF whereas TF expression is significantly enhanced in presence of EC activation or dysfunction (Yang Y. & Loscalzo J., 2000). TF activates factor VII to factor VIIa. The extrinsic tenase complex thus formed also includes Ca2+ as an activating ion and promotes factor X activation in factor Xa (Owens AP. & Mackman N., 2010).

The two pathways converge in the common pathway in which the prothrombinase complex - that arises from the combination of factor Xa with its cofactor (factor V), tissue phospholipids, platelet phospholipids, and calcium - converts prothrombin to thrombin. The resulting thrombin further cleaves the free form of fibrinogen into insoluble fibrin and stimulates activation of factor XIII to factor XIIIa, which binds fibrin polymers located in the platelet plug (Palta S. et al., 2014; Kamath S. et al. 2003). The cross-linked fibrin forms a network that stabilizes the clot and transforms it into a secondary hemostatic plug (Hall JE., 2010; Kumar V. et al., 2010).

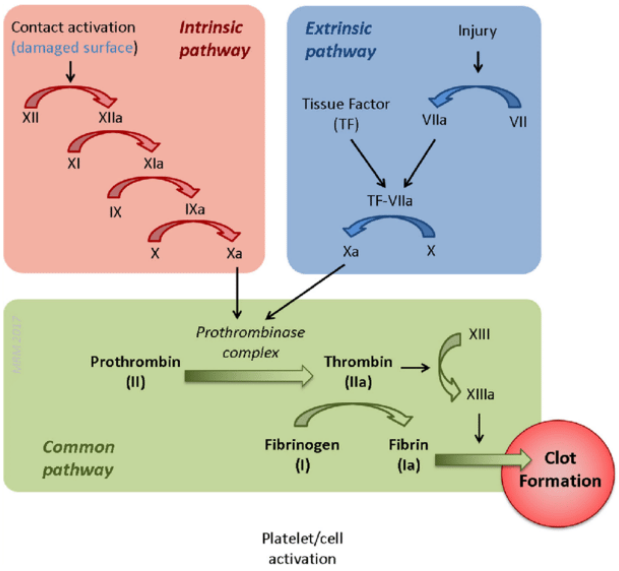

Figure 1.2 | The intrinsic, extrinsic and common pathways of the coagulation (clotting) cascade converging in factor X activation *(Adapted from Robertson S. & Miller M., 2018).*

Recent studies demonstrated that these two pathways are not parallel but they are closely interconnected. In particular, it has been shown that factors and complexes of the intrinsic pathway can be activated by extrinsic pathway factors (Triplett DA. et al., 2000; Lasne D. et al., 2006; Owens AP. & Mackman N., 2010; Kumar V. et al., 2010; Hall EJ. & Hall EM., 2020). Accordingly, the current definition of coagulation involves four stages: initiation, amplification, propagation, and stabilization (Figure 1.3).

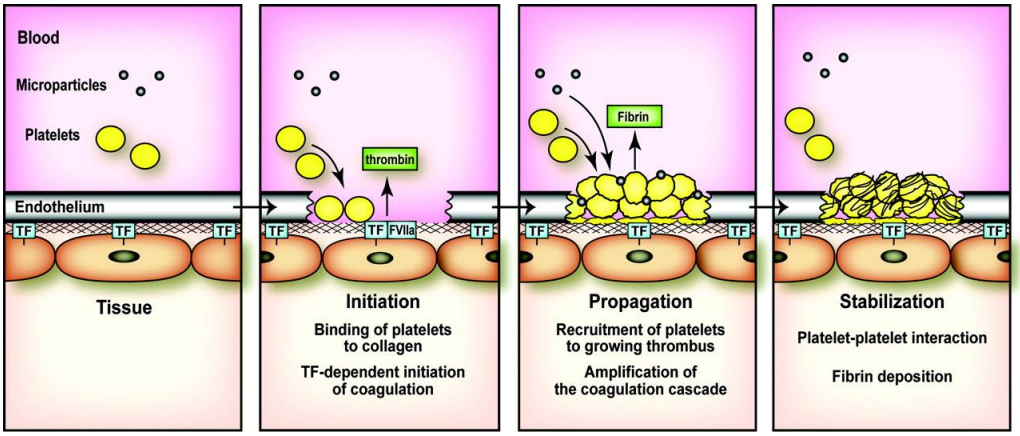

Figure 1.3 | Formation of a platelet clot at the site of blood vessel damage. In a physiological condition, TF expression is physically separated from its ligand FVII/FVIIa by the endothelium. Vessel damage leads to the rapid binding of platelets to the subendothelium and activation of the coagulation cascade by TF. Propagation of the thrombus involves recruitment of additional platelets and amplification of the coagulation cascade by the intrinsic pathway. Finally, fibrin deposition stabilizes the clot. *De novo* synthesis of TF by platelets may also play a role in stabilization of the clot *(Adapted from Mackman N. et al., 2007).*

Initiation. Initiation is mediated by TF expressed by ECs and/or exposed on the subendothelial structures. TF, by interacting with factor VIIa, mediated the activation of factor IX and factor X. Factor IXa binds to phospholipid-rich structures of the vascular wall and, together with factor Va, forms the first activation core known as the prothrombin complex. Factor IXa thus represents a bridge between the classical extrinsic and intrinsic pathways. Factor Xa interacts with prothrombin (factor II), promoting the generation of thrombin (factor IIa). This process does not robustly support thrombin production, and inhibitors of the TF pathway can easily inhibit it (Mackman N. et al., 2007).

Amplification. The thrombin produced in the initiation phase activate multiple positive feedback loops that foster the haemostatic process. In particular, it induces the activation of factor V and factor VIII, which are used as cofactors in the prothrombinase complex, and promotes the thrombin generation by FXa and the activation of FXa by FIXa, respectively. In addition, thrombin produced in the initiation phase stimulate the activation of the platelets themselves, inducing both phenomena of aggregation and the transformation of the platelets into a catalytic substrate at the level of which all subsequent coagulation phenomena occur.

Propagation. Prothrombin complexes located on platelet surface, in the presence of factor VIIIa (a cofactor in the formation of the complex itself), determine the generation of a high quantity of thrombin and platelet activation. Thrombin, in turn, converts the highly soluble fibrinogen in fibrin thus supporting the formation of the clot scaffold.

Stabilization. Factor XIII after being activated by the generated thrombin, covalently binds fibrin polymers to consolidate fibrin meshwork in the platelet plug. In addition, thrombin activates thrombin-activated fibrinolysis inhibitor (TAFI), protecting the clot from fibrinolysis (Bombeli T. & Spahn DR., 2004; Lasne D. et al., 2006). All these processes required both the presence of phospholipid surfaces and the presence of calcium ions which are also necessary for platelet activation (Bombeli T. & Spahn DR., 2004; Lasne D. et al., 2006).

To avoid excessive intravascular thrombus propagation and limit clotting to the location of the injury, three key anti-coagulant processes counteract the mechanisms described above (Colvin BT., 2004):

- Antithrombin. Antithrombin plays a key role in thrombin inhibition. This serine protease interacts with thrombin, factor IXa, Xa, XIa, and XIIa and inhibits them. The enzymatic activity of antithrombin is potentiated by heparin. Specifically, heparin sulfate on the surface of ECs binds and activates antithrombin. In turn, antithrombin interacts with coagulation factors in a 1:1 ratio, and the formed complexes are then removed by the reticuloendothelial system. Besides antithrombin, Heparin II cofactor, α2 macroglobulin, and α1-antitrypsin are indicated as additional thrombin inhibitors (Opal SM. et al., 2002; Ejiofor JA., 2013).
- Tissue factor plasminogen inhibitor (TFPI). TFPI is a polypeptide originated from ECs that is able to inhibit the initiation of coagulation through direct binding and inhibition of factor Xa (FXa). In the presence of calcium and phospholipids, the binding and the subsequent inhibition of factor Xa is intensified by Protein S that act as cofactor of TFPI (Dahm AE. et al., 2008). Moreover, TFPI-FXa complexes also inhibit the TF/factor VIIa (FVIIa) complex by forming a quarternary TF/FVIIa/FXa/TFPI complex (Ejiofor JA., 2013; Price GC. et al., 2004).
- Activated Protein C pathway. Protein C is a serine protease that exhibits anticoagulant, profibrinolytic, and anti-inflammatory characteristics. Protein C activation is enhanced by its interaction with the Endothelial protein C receptor (EPCR) that binds protein C on the endothelial surface and presents it to the thrombin:thrombomodulin complex for its activation (Thiyagarajan M. et al., 2007). In its activated form (activated protein C APC) that is induced by thrombin, protein C inactivate factors Va and VIIIa to down-regulate thrombin generation. In this pathway, Protein S, a vitamin K-dependent glycoprotein, acts as APC cofactor by fostering the inactivation of factor Va and VIIIa (Dahlbäck B. & Villoutreix BO., 2005). Protein S is syntesised by ECs and hepatocytes and also causes direct and reversible inactivation of the prothrombinase complex (FVa-FXa). It is

present in plasma both in a free form (40%) and in a bound form (60%), interacting with the complement protein C4b. While the former form exhibits an anticoagulant capacity, the bound form – which is typical of inflammatory states - possesses inhibitory activity against the complement system and support a procoagulant condition by reducing the levels of free protein S (Rigby AC. & Grant MA., 2004).

1.1.3 Fibrinolytic system

To circumscribe the size of the clot, the fibrinolytic system is activated. During fibrinolysis, the fibrin clot is dissolved into fibrin degradation products (FDP) by the enzymatic action of plasmin. Tissue occlusion, thrombin, epinephrine, vasopressin, and intense exercise promote fibrinolysis since they stimulate the production of tissue plasminogen activator (t-PA), which together with the plasminogen activator urokinase (u-PA) generate plasmin from the zymogen plasminogen. Generated plasmin in turn catalysed the degradation of fibrin into FDP (Figure 1.4). For the diagnosis of pulmonary embolism, disseminated intravascular coagulation and deep vein thrombosis, the activity of the fibrinolytic system is assessed by measuring FDPs and evaluating the plasmatic concentration of the D-dimer derived from the digestion of cross-linked fibrin (Colvin BT., 2004).

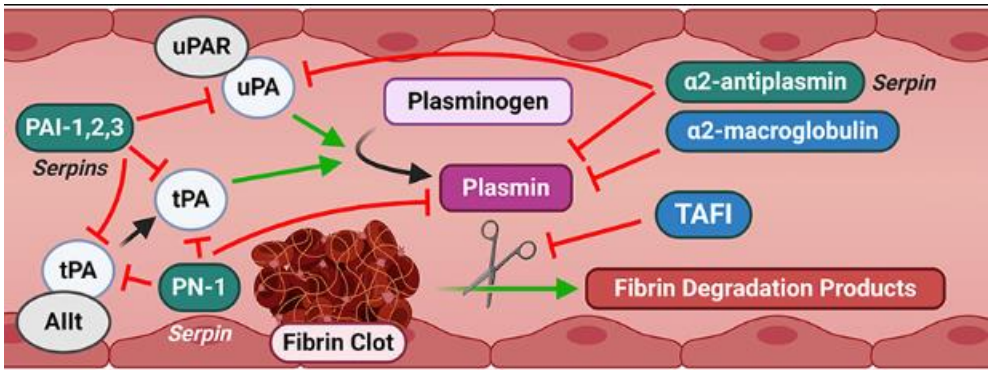

Figure 1.4 | Signaling of the fibrinolysis pathway. Fibrinolysis is characterized by the degradation of a fibrin clot into degradation products by plasmin. Plasmin derived from plasminogen by uPA and tPA activity. Several serpins and other inhibitors provide a tight regulation of this cascade. A promiscuity remains across multiple elements of the pathway, with redundant controls against inappropriate activation. AIIt and uPAR are representative canonical fibrinolytic receptors for brevity. AIIt, Annexin II tetramer; PAI-1,2,3, Plasminogen Activator Inhibitor-1, 2, 3; PN-1, Protease Nexin-1; TAFI, Thrombin activatable fibrinolysis inhibitor; tPA, Tissue-type plasminogen activator; uPA, Urokinase-type plasminogen activator; uPAR, Urokinase-type plasminogen activator receptor *(Adapterd from Yaron JR.et al., 2021).*

As shown in Figure 1.4, the regulation of plasmin activity is mediated by its inhibitors, α-2 antiplasmin and macroglobulin α2, that prevent excessive fibrinolysis (Cesarman-Maus G. & Hajjar KA., 2005). In addition, fibrinolytic process is also limited by Plasminogen activator inhibitor (PAI), which physiologically inhibits t-PA and u-PA irreversibly and by TAFI a plasma proenzyme activated by thrombin that blocks plasmin activity (Yaron JR. et al., 2021).

1.2 The endothelium and its progenitors

To deeply study the alteration of the endothelial compartment, a general understanding of the morphology and function of ECs is necessary, taking also into consideration how different stimuli can affect endothelial function (Krüger-Genge A. et al., 2019).

1.2.1 Endothelial compartment: structure and functions

The vascular endothelium consists of a monolayer of ECs anchored to the basal lamina lining the innermost part of arteries, capillaries and veins. ECs together with the basal lamina form the vascular intima, a haemocompatible surface which cover an area of about 3000-6000 m^2 in human body (Krüger-Genge A. et al., 2019). ECs have a general size of about 30-50 μm long, 10-30 μm wide, and a thickness of 0.1-10 μm. *In vitro* ECs are endowed with a typical cobblestone-like morphology while *in vivo* are usually thin and slightly elongated and their orientation in the vessel wall follows the axis of the vessels due to the shear stress exerted by the blood flow (Krüger-Genge A. et al., 2019). Whereas until the early 1970s, this single-cell layer of ECs was considered a simple inert wall dividing blood cells from adjacent tissue, now the endothelium is defined as a dynamic organ that controls the environment and responds to external stimuli playing an active role in regulating several functions such as vascular tone, coagulation, the exchange of fluids and solutes, inflammation, and angiogenesis, among others. In particular, healthy ECs regulate antioxidants, have anti-inflammatory and anticoagulant action, and, thus, control vascular relaxation and contraction, thrombogenesis, fibrinolysis, and platelet activation and inhibition. Therefore, ECs emerge to be crucially involved in cardiovascular physiology and pathophysiology (Medina-Leyte DJ. et al., 2021) and the maintaining of the functional integrity of this complex organ is critical for preserving blood flow and preventing thrombosis (Stanek A. et al., 2018).

Since their first description, ECs have been considered polarized cells. In particular, at the level of their basolateral surface ECs secrete the glycoproteins that composed the basal membrane on which ECs are anchored and that separate the EC basal surface from the surrounding tissues; on the other hand, the apical surface of ECs faces the blood space and is therefore exposed to blood components and circulating cells. The apical surface of the endothelium is constituted by a mosaic of glycoproteins and proteoglycans which form the so-called endothelial glycocalyx (GCX) (Li Z. et al., 2023). The synthesis of the glycocalyx is a complex process involving multiple enzymatic pathways (Krüger-Genge A. et al., 2019; Reitsma S. et al., 2007) and glycocalyx composition and dimension are regulated by local pH and mechanical stimuli deriving from the shear forces due to turbulent blood flow (Krüger-Genge A. et al., 2019; Reitsma S. et al., 2007). Plasma proteins and soluble glycosaminoglycans (GAGs) also bind to the GCX that therefore become physiologically active. Among the GAGs, heparan sulfates – which are found in syndecans, perlecans and glypicans – are the most frequent ones but also hyaluronic acid and chondroitin-, dermatan-, and keratan-sulfates are GAGs that interact with the GCX (Krüger-Genge A. et al., 2019; Reitsma S. et al., 2007). Therefore, the complex structure that composed the surface layer of the endothelium in physiological conditions minimize the EC contact with blood components, contributes to vascular permeability regulation and forms a barrier against pathogens. In fact, glycoproteins, proteoglycans, and GAGs in the GCX are negatively charged and therefore are able to repel erythrocytes, leukocytes and platelets (Medina-Leyte DJ. et al., 2021; Krüger-Genge A. et al., 2019). Moreover, in addition to its structure, GCX possesses several vascular functions, creating a complex barrier (Curry FE. & Adamson RH., 2012) that acts as a macromolecular filter (van den Berg BM. et al., 2006). The barrier function played by ECs is granted by the endothelial continuity that in turn is maintained by the presence of protein binding complexes (tight junctions, adherens junctions and gap junctions) (Medina-Leyte DJ. et al., 2021). Considering the heterogeneity of the vascular system, under physiological condition the vessel permeability differs among the different type

of vessels. Permeability is low in vessels with big diameter since their main function is to conduct the blood from the heart to organs and tissues whereas permeability is high at the levels of capillaries where the exchange of fluids and solutes mainly occurred (Krüger-Genge A. et al., 2019).

Another function regulated by ECs is represented by the vascular tone since ECs produce and release various factors with relaxing and contractile potential in order to regulate blood flow rate thus regulating also the oxygen supply to surrounding tissues. The factors involved are subdivided in the following groups: i) endothelium-derived hyperpolarizing factors, such as nitric oxide (NO) and endothelium-dependent hyperpolarizing factor (EDHF); ii) arachidonic acid-derived metabolites involved in cyclooxygenases, lipoxygenases, and cytochrome P450 pathways; iii) various peptides such as endothelin, urotensin, C-type natriuretic peptide (CNP), adrenomedullin, adenosine, purines, reactive oxygen species and others. The balance among these factors is maintained under normal conditions, whereby vascular homeostasis promotes mild vasodilation. In larger arteries, the major vasodilator in the vascular wall is NO while in smaller vessels, the situation is more heterogeneous and involves also EDHF or prostacyclin (PGI2) (Sena CM. et al., 2022). Under physiological conditions, NO is atheroprotective, a potent vasodilator, inhibits proliferation of vascular smooth muscle cells (VSMCs), platelet aggregation and reduces inflammation. In the vascular wall NO production in ECs, platelets, VSMCs, and activated macrophages is dependent on the activities of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) (Sena CM. et al., 2022). In particular, increased shear stress affects EC gene expression that results in eNOS expression and rapid activation and in the consequent NO release (Zhou J., 2014). The microparticles shedded of from the glycocalys are also involved in the modulation of vasomotor tone through the release of nitric oxide (NO) (Lipowsky HH. & Lescanic A., 2017).

NO and PGI2 are also the major antiplatelet agents that in physiological conditions allow ECs to regulate platelet functions and blood coagulation. In particular, they increase the platelet cAMP contents thus preventing

inadequate platelet adhesion and aggregation. In physiological conditions, platelet aggregation is also decreased by the presence on the EC luminal surface of enzymes such as the ecto-nucleotidases that hydrolyze into AMP the potent platelet aggregating agents ATP and ADP (Krüger-Genge A. et al., 2019).

In addition, ECs regulate the blood fluidity through a proper balance between pro- and anti-coagulant factors. In particular, an intact and healthy endothelium expresses various anticoagulants, such as the previous described TFPI, thrombomodulin, EPCR, and heparin-like proteoglycans (Yau JW. et al., 2015).

1.2.2 Endothelial colony forming cells (ECFCs)

In 1971, the presence of postnatal circulating endothelial progenitor cells (EPCs) was firstly hypotesized by Kennedy and Weissman who showed in allograft patients that part of the ECs lining the coronary arteries belonged to the recipient and not the donor thus suggesting the presence of circulating endothelial precursors (Kennedy LJ. & Weissman I., 1971).

Nevertheless, the existence of postnatal EPCs was not taken into account in the following years since vasculogenesis - defined as the formation of primitive vascular structures starting from the differentiation of endothelial precursors - was believed to occur only in embryogenesis from angioblasts or mesodermal progenitors whereas tissue growth and wound healing in adulthood was believed to be sustained only by the formation of new blood vessels from existing one through a mechanism called angiogenesis (Barrasa-Ramos S. et al., 2022).

Only in 1997, Asahara and colleagues described the isolation of putative EPCs from peripheral blood demonstrating that a subset of circulating CD34+ VEGFR2+ progenitors were endowed with the ability to differentiate *ex vivo* into cells that had an endothelial phenotype. In particular, they expressed PECAM-1 and the Tie-2 receptor, incorporated acetylated LDL, and produced NO upon VEGF stimulation (Asahara T. et al., 1997).

The implications deriving from the existence of EPCs in adulthood were huge, since opened to the existence of postnatal vasculogenesis and to the possibility of using these cells for therapeutical purposes (Rafii S. & Lyden D., 2003).

Starting from this seminal work, several approaches have been used to study and characterize EPCs. In particular, the approaches reported in literature can be divided in two main strategies: i) quantification of circulating EPCs by flow cytometry in which EPCs are identified on the basis of cell surface markers expression; ii) *in vitro* culture of EPCs isolated from peripheral blood (Hirschi KK. et al., 2008; Fadini GP. et al, 2012; Medina RJ. et al., 2017). In particular, in the present work we focused our attention to the second strategy to study and characterize EPCs *in vitro*.

Concerning the *in vitro* culture of EPCs, after Asahara publication, for years there was no precise and common definition of EPCs. The confusion in the scientific community was alimented by the fact that different studies were published in which the generic term EPCs was used to define cell populations that were identified with different approaches and that were endowed with different characteristics (Medina RJ. et al., 2017). Only recently a consensus has been achieved and now endothelial colony-forming cells (ECFCs; also called as Late-EPCs or blood endothelial outgrowth cells, BOECs) are considered the true endothelial progenitors (Medina RJ. et al., 2017) whereas other cell types are now defined as myeloid angiogenic cells or MACs (Vaughan EE. & O'Brien T., 2012; Medina RJ. et al., 2017) and are no longer considered EPCs since despite expressing endothelial markers and supporting neovascularisation processes through their paracrine activity, they expressed hematopoietic markers (i.e. CD45 and CD14) and are not directly involved in the formation of a functional endothelium (Rehman J. et al., 2003; Yoon CH. et al., 2005; Yoder MC. et al., 2007).

1.2.2.1 Characteristics and phenotype of ECFCs

ECFCs are a population of progenitor cells that circulate in the peripheral blood and, when cultured, give rise to colonies. As described in the previous paragraph, after a decades-long debate, a consensus has been reached in the scientific community and ECFCs are considered to be the true EPCs as such they fulfil three specific characteristics: i) endowed with endothelial phenotype, not expressing hematopoietic markers; ii) robust clonal proliferative potential; iii) ability to directly incorporate at the sites of vascular damage and formation of blood vessels *in vivo* (Ingram DA. et al., 2004; Tasev D. et al., 2016; Medina RJ. et al., 2017) (Figure 1.5). In the absence of any of these three criteria the isolated cells cannot be considered ECFCs.

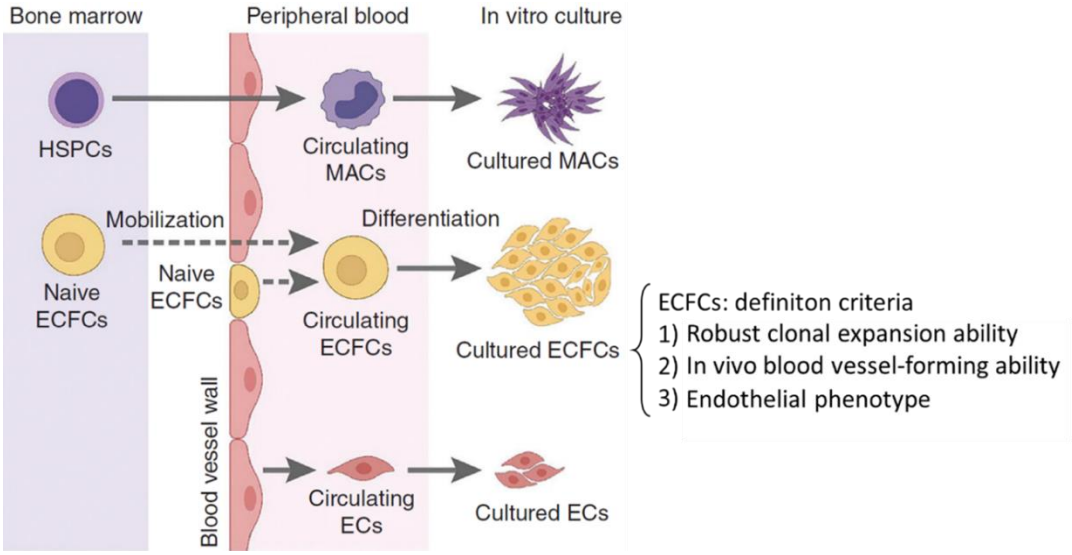

Figure 1.5 | Properties of human ECFCs *(Adapted from: Melero-Martin JM., 2022).*

Concerning the first criterion, ECFCs are characterized by endothelial phenotype (Yoder MC. et al., 2007; Melero-Martin JM., 2022) expressing typical endothelial markers, such as CD31 (or PECAM1-1), VE-cadherin, vWF, CD146, and the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) that is the main VEGF receptor expressed on vascular endothelial cells. Notably, such endothelial markers are expressed by ECFCs both at the protein and transcript level (Ingram DA. et al., 2004; Lin Y. et al., 2000; Melero-Martin JM. et al., 2007). The fact that the endothelial lineage of cultured ECFCs was demonstrated also by genome-wide transcriptional profiling and

ultrastructural analysis confirmed the differences between ECFCs and MACs (Medina RJ. et al., 2010; Wang K. et al., 2020; Rehman J. et al., 2003). In this context, Prokopi and colleages showed that MACs expressed endothelial antigens on the cell surface but they did not express mRNA encoding for the same surface proteins thus demonstrating that MACs are monocytes that in culture acquire an endothelial-like phenotype after the uptake of platelets and platelet-derived microparticles and the consequent transfer of platelet proteins - such as PECAM-1, integrins and Ulex Europaeus lectin I (UEA-I) – that are known to be expressed by both platelets and ECs (Prokopi M. et al., 2009).

In addition, ECFCs lack the expression of hematopoietic markers such as the pan-leukocyte marker CD45, and the monocyte/macrophage markers CD14 and CD115. This feature is also critical to distinguish ECFCs from MACs, which are cells of hematopoietic origin that sustain angiogenesis only through paracrine mechanisms (Medina RJ. et al., 2017; Melero-Martin JM. et al., 2007; Mund JA. et al., 2012; Timmermans F. et al., 2007; Case J. et al., 2007). In particular, Gulati and colleagues demonstrated that ECFCs developed only from the CD14- fraction of peripheral blood mononuclear cells (Gulati R. et al., 2003).

From a functional point of view, ECFCs also differ from MACs, as they show functions typical of competent vascular ECs. In fact, they are endowed with high affinity for AcLDL and agglutinin I (Ingram DA. et al., 2004; Moreno-Luna R. et al., 2014). Moreover, after exposure to pro-inflammatory cytokines (such as TNFα, IL-1), ECFCs upregulate the expression of E-selectin, VCAM-1, ICAM-1, and promote leukocyte adhesion (Melero-Martin JM. et al., 2007; Wang K. et al., 2020). On the contrary, MACs showed some features typical of monocytes/macrophage as they are able to phagocyte bacteria and possess a nonspecific esterase activity (Yoder MC. et al., 2007).

Since ECFCs and mature ECs show many phenotypic and functional similarities, the identification of a unique marker that allows to identify ECFCs is still matter of investigation. In fact, ECFCs are not specifically identified by a set of unique cell surface markers (Medina RJ. et al., 2017), and a unique cell surface marker useful for ECFC isolation and enrichment is not yet available.

In this context, one promising marker is represented by neuregulin-1 (NRG-1) since recent studies showed that is highly expressed in ECFCs but is absent in mature ECs (Hong X. et al., 2021). Further studies are required to confirm its potential role as specific ECFC marker.

Concerning the second criterion, ECFCs exhibit a high clonal expansion potential (Yoder MC., 2012), with a single cell derived from adult peripheral blood being able to form colonies of > 2000 cells within 10 days of culture (Lin Y. et al., 2000; Melero-Martin JM. et al., 2007). This characteristic is even more marked in ECFCs derived from umbilical cord blood (Ingram DA. et al., 2008; Melero-Martin JM. et al., 2007; Smadja DM. et al., 2019). This clonal capacity differentiates ECFCs from both MACs and mature ECs since MACs do not give rise to ECs progeny per se whereas mature ECs are characterized by a limited proliferative capacity whereas (Medina RJ. et al., 2017; Melero-Martin JM. et al., 2007; Mund JA. et al., 2012; Timmermans F. et al., 2007; Case J. et al., 2007). In fact, despite mature ECs are able to develop small colonies, ECFCs show a high proliferative potential and form more than 100 population doublings. Moreover, ECFCs can also regenerate into secondary and tertiary colonies and mainly maintain high levels of telomerase activity, resulting in prolonged lifespan *in vitro* (Ingram DA. et al., 2004; Toupance S. et al., 2021).

The last criterion consists in to form a functional vascular network *in vivo* (Melero-Martin JM. et al., 2007). This characteristic allows to discriminate true ECFCs from the other cells type that despite being endowed with endothelial phenotype are not able to support vasculogenesis (Yoder MC., 2012). In accordance with this ability, ECFCs form capillary-like structures when seeded on Matrigel and give rise to vascular networks when seeded in three-dimensional scaffolds (Melero-Martin J M. et al., 2007; Wang K. et al., 2020; Wu X. et al., 2004).

1.2.2.2 Strategies to isolate ECFCs

In 2004, Ingram and colleagues described for the first time a culture method that allowed to obtain ECFCs thus opening to their use in studies of translational and experimental medicine (Ingram DA. et al., 2004). In particular, Ingram and his co-workers proposed a protocol for isolating ECFCs starting from peripheral blood-derived mononuclear cells (PBMCs) seeded in plates coated with collagen – an extracellular matrix component that facilitate EC adhesion and culture – and fed with a cell culture medium consisting of several growth-promoting factors and foetal bovine serum (FBS) (Yoon CH. et al., 2005; Ingram DA. et al., 2004; Melero-Martin JM. et al., 2007; Timmermans F. et al., 2007). In the days after PBMC seeding, non-adherent cells were removed and then plates with adherent cells underwent microscope inspection in order to identify ECFC colonies appearing starting from the second week after PBMC seeding. ECFC colonies were identified as well-defined colonies composed of at least 50 adherent cells characterized by cobblestone-like morphology and high proliferation ability.

After this publication, other studies on ECFCs were published by different groups despite the absence of clear consensus on the protocols that should be used for ECFC isolation and cultured limited a more in-depth study of these cells. In this regard, several protocols related to the method of isolation and culture of ECFCs have been reported all mainly differing from each other for cell seeding density, culture medium formulation and the type of substrate used as coating (Mauge L. et al., 2014; Smadja DM. et al., 2019; Melero-Martin JM. & Bischoff J., 2008; Colombo E. et al., 2013; Kim HD. et al., 2021). Regarding the last point, a previous study developed in our laboratory demonstrated that fibronectin supports ECFC colony isolation more efficiently than collagen whereas collagen supports ECFC colony expansion more efficiently than fibronectin (Colombo E. et al., 2013).

In recent years, efforts have been made to standardise the method of isolation and culture of ECFCs thus facilitating their widespread use and overcoming the generation of conflicting results deriving from the comparison of studies

performed using different procedures for ECFC isolation and culture (Medina RJ. et al., 2017; Smadja DM. et al., 2019).

Depending on the source of the blood used as starting sample for ECFC isolation, the number of colonies and the time of colony appearance is highly variable. In particular, a higher number of ECFC colonies can be obtained from cord blood compared to adult blood (Gulati R. et al., 2003; Yoder MC. et al., 2007; Ingram DA. et al., 2004). In addition, ECFCs obtained from cord blood also appear faster and are characterized by an increased proliferative potential compared with ECFCs isolated from adult blood (Ingram DA. et al., 2004; Melero-Martin JM. et al., 2007; Smadja DM. et al., 2019).

In adult population, circulating ECFCs are considered a rare population (< 0.2 cells/mL) and no significant differences in ECFC isolation efficiency emerged among subjects aged 20 to 73 years. Even today, the variation of ECFC levels from young age to adulthood remains undefined (Ingram DA. et al., 2004; Melero-Martin JM. et al., 2007; Smadja DM. et al., 2019). Since ECFCs are considered a rare population the occurrence of unsuccessful isolation ("zero colonies") is expected. In particular, the expected success rate in isolating ECFCs from peripheral blood is 70% (Smadja DM. et al., 2019).

The methods that allow the isolation of ECFCs have some intrinsic complexities, due mainly to the fact that the isolation of specific colonies starts from a highly heterogeneous population such as PBMCs. Nevertheless, the use of a culture medium that foster endothelial cell differentiation and growth favours the isolation of ECFC that thanks to their high proliferative potential easily exceed any other haematopoietic colony in culture (Melero-Martin JM. & Bischoff J., 2008; Colombo E. et al., 2013). In particular, it was shown that ECFC colonies have a homogeneous phenotype and by the second culture passage the colonies are entirely composed of ECFCs and any other cell lines are lost (Colombo E. et al., 2013).

Alternative approaches aimed to identify and isolate ECFCs based on cell-sorting and/or cell enrichment are under investigation. However, sorting strategies has proven difficult to pursue due to the rarity of ECFCs in adult peripheral blood that makes it difficult to select them directly from blood without

an enrichment strategy. Another difficulty is related to the fact that a specific marker (or combination of markers) for ECFCs has not yet been identified since the use of above mentioned NRG-1 for the direct isolation of ECFCs from peripheral blood still require further studies to confirm its potential role (Hong X. et al., 2021). In addition, another element that should be taken into account is represented by the fact that the expression of certain molecules in cultured ECFCs does not guarantee that these markers are also expressed by ECFCs *in vivo* – either by naive ECFCs that are located in their original niche within the bone marrow and vasculogenic sites in the vessel wall (Ingram DA. et al., 2005; Patel J. et al., 2013; Goligorsky MS. et al., 2013), or by circulating ECFCs that are present in the peripheral blood and whose true phenotype is still matter of debate (Hebbel RP., 2017; Melero-Martin JM., 2022). Initially, early studies suggested that ECFCs can be enriched on the basis of CD34, CD133, and VEGFR2 expression since they have been proposed as markers for the identification of circulating ECFCs (Gehling UM. et al. 2000; Peichev M. et al. 2000). Concerning VEGFR2 and CD34, this hypothesis was supported by the fact that isolated ECFCs express both VEGFR2 and CD34, with the level of the latter marker that changes over time, with high expression levels characterizing early passage ECFCs and lower expression levels of CD34 observed in ECFCs at later culture passages (Lin et al. 2000; Ingram et al. 2004; Melero-Martin et al. 2007; Medina et al. 2010; Wang et al. 2020). In contrast, there is no convincing evidence for significant expression of CD133 on cultured ECFCs (Timmermans F. et al. 2007). In addition, further studies demonstrated that this antigen combination was not suitable for ECFC isolation and/or enrichment (as review in Yoder MC., 2012). In this regard, Case and collegues showed that the selection on the basis of CD34, CD133 and VEGFR2 expression allowed the enrichment of cells with hematopoietic progenitor activity but did not allowed the isolation of endothelial colonies (Case J. et al., 2007). In conclusion, only the expression of CD34 has been consistently reported to enable enrichment of circulating ECFCs within mononuclear cells (Timmermans F. et al. 2007; Lin RZ. et al., 2015). In particular, Timmermans et al. demonstrated that endothelial cell colonies with

high proliferative potential originated only from a population of CD34+CD45- cells from umbilical cord blood or human bone marrow and not from CD34+CD45+ cells that were enriched in hematopoietic colony-forming cells (Timmermans F. et al. 2007).

1.2.2.3 ECFC applications

Starting from their identification, increasing interest for ECFCs raised in the scientific community for their possible applications. On the one hand, ECFC therapeutic potential is under investigation especially in the context of regenerative medicine, tissue bioengineering and gene therapy (Paschalaki KE. & Randi AM., 2018; Melero-Martin JM., 2022). On the other hand, blood-derived ECFCs has become a valuable research tool to study endothelial compartment since they provide easy access to patient-specific ECs (Paschalaki KE. & Randi AM., 2018; Melero-Martin JM., 2022). This last aspect will be described more in details in the section dedicated to endothelial dysfuncion.

1.2.2.3.1 Therapeutic potential of ECFCs

Considering the high proliferative potential of ECFCs, their possible use for therapeutic application is under investigation. In fact, the possibility to obtain high amounts of highly vasculogenic autologous ECs from the *ex vivo* expansion of patient-specific ECFCs is extremely appealing in this context. The therapeutic potential of ECFCs have been shown in several diseases in preclinical studies and in animal models (O'Neill CL. et al., 2018; Paschalaki KE. & Randi AM., 2018; Melero-Martin JM., 2022).

The majority of the studies investigated the use of ECFCs for the revascularization of ischemic tissues. Although the mechanisms underlying ECFC effects are not fully understood, ECFC ability in promoting revascularization appeared to be sustained either by directly integrating in new vessel walls, or by paracrine mechanisms through the secretion of pro-angiogenic growth factors.

In particular, studies have been performed in mouse models of ischemic retinopathies (Medina RJ. et al., 2010; Reid E. et al., 2018) demonstrating that

the intravital injection of ECFCs promoted vascular regeneration by integrating within intraretinal microvasculature and engrafting within neovessels emerging within the ischemic areas (Medina RJ. et al., 2010). In addition, retinal regeneration was promoted by ECFCs by supporting the vascular development sustained by bone marrow-derived CD34 hematopoietic stem cells, as recently demonstrated in a mouse model of retinopathy, named oxygen-induced retinopathy (OIR) (Li Calzi S. et al., 2019). By using the same murine model of retinopathy, a follow-up study also revealed that the injection of ECFC-derived extracellular vesicles restored the vascular network by stimulating local angiogenesis through the delivery of a combination of multiple microRNAs (miRNAs) (Dellett M. et al., 2017).

Moreover, both mouse and rat models were used to demonstrate the potential therapeutic effect of ECFCs in vascular repair after cerebrovascular accidents (i.e. ischemic stroke, focal cerebral ischemia and middle cerebral artery occlusion, and cerebral aneurysm) (Ding J. et al., 2016; Moubarik C. et al., 2011; Huang XT. et al., 2013; Li S. et al., 2014). In this regard, Ding and colleagues used bioluminescence imaging to investigate the reparative effect of ECFCs in a mouse model of ischemic stroke (Ding J. et al., 2016). It was observed that ECFCs settled and remained in the brain on the infarct border for up to one week, with evidence that ECFCs successfully settled in the ischemic brain, enhancing angiogenesis, decreasing neuronal apoptosis, and increasing neurogenesis (Ding J. et al., 2016). In addition, ECFCs have been shown to be a suitable cellular substrate to promote vascular repair in a rodent model of traumatic brain injury (TBI). After intravenous infusion, ECFCs were detected in the inner cell lining of microvessels crossing the injured area at 24 hours and further increased at 72 hours after TBI. This process resulted in increased capillary density and paracrine release of pro-angiogenic factors, such as VEGF and stromal-derived factor-1α (SDF-1α) (Zhang Y. et al., 2013). The potential therapeutic effect of ECFCs was further supported by the demonstration that ECFC infusion in a rat model of cerebral aneurysm (CA) attenuated vascular remodeling and inflammation. In this context, ECFCs were able to protect against aneurysmal wall degeneration by preventing matrix metalloproteinase (MMP) activation, attenuating inflammatory signaling (e.g.,

nuclear factor κB (NF-κB), and expression of VCAM-1, inducible NO synthase (iNOS)), and protecting vascular smooth muscle cells (VSMCs) from apoptosis (Lin RZ. et al., 2014). Taken together, this evidence supports the use of ECFCs as a promising cellular source to promote vascular repair in the brain.

In addition, having the highest clonogenic potential among CD34+ cells, ECFCs represent the most promising therapeutic tool for restoring the cardiac vasculature in ischemic diseases (Schwarz TM. et al., 2012). In this context, ECFC ability in supporting revascularization has been shown also in models of ischaemic heart disease (Deutsch MA. et al., 2020), myocardial infarction (MI) (Hong X. et al., 2021; Dubois C. et al., 2010; Brunt KR. et al., 2012) and hind limb ischaemia (Schwarz TM. et al., 2012; Lin RZ. et al., 2014; Prasain N. et al., 2014). In these studies, ECFCs mainly acted by directly incorporating into the newly formed vessels thus improving the blood flow and therefore promoting the functionality of affected tissues. In particular, Dubois and colleagues showed that infusion of autologous ECFC-like cells attenuated myocardial remodeling in a porcine model of acute MI (AMI), reducing the infarct size and promoting microvessel density. Interestingly, beta-galactosidase-labeled ECFCs engrafted within the neovessels that appeared in the border zone of the infarcted area and did not induce cardiomyogenesis, but enhanced cardiac neovascularization by releasing pro-angiogenic mediators, such as placental growth factor (PlGF) (Dubois C. et al., 2010). A recent study showed that the injection of ECFCs into the peri-infarct region of an AMI model in mice with severe combined immunodeficiency attenuated adverse post-AMI remodeling, possibly due to paracrine effects (Deutsch MA. et al., 2020).

Moreover, Prasain and colleagues also showed that iPSC-derived ECFCs supported a higher improvement of blood perfusion and limb recovery compared to cord blood-derived ECFCs (Prasain N. et al., 2014).

In the context of tissue engineering, ECFCs are under investigation to assess their ability to integrate into functional vascular networks within the bioengineered tissue constructs, thus fostering nutrient and oxygen supply, and proper elimination of waste products (Wang K. et al., 2019). The ECFC ability to incorporate into 3D scaffolds and generate functional vessels has

been described for complex tissues such as myocardium (Hong X. et al., 2021; Riemenschneider SB. et al., 2016) and skin (Athanassopoulos A. et al., 2012; Traktuev DO. et al., 2009; Kim KL. et al., 2009). The use of ECFCs in engineered human skin substitutes induced the formation of a vascular network that promotes the survival of bioengineered human tissues (Kung EF. et al., 2008). In addition, it has been described that wound contraction was prevented by ECFC integration into dermal fibroblast sheets related to the ability of ECFCs to induce robust vasculogenesis (Hendrickx B. et al., 2010).

Moreover, Critser et al. demonstrated that the physical properties of collagen matrices influence ECFC-dependent vasculogenesis *in vivo* (Critser PJ. et al., 2010). In particular, by modifying the biochemical and physical properties of the scaffold or by adding growth factors such as VEGF, angiopoietin-1 or BMP-2 it has been observed that it is possible to further foster the pro-angiogenic capabilities of cells (Tasev D. et al., 2016).

Finally, the use of ECFCs as a source of autologous cells for gene therapy is also under investigation, with ECFCs mainly used in gain-of function studies. In particular, preclinical studies addressed ECFC overexpression of factor VIII as possible treatment for haemophilia A (Lin Y. et al., 2002; Matsui H. et al., 2007; Olgasi C. et al., 2021), ECFC overexpression of erythropoietin in the context of anaemia (Lin RZ. et al., 2011), and ECFC overexpression of tumor growth inhibitors in cancer (Dudek AZ. et al., 2007; Bodempudi V. et al., 2010). Studies based on loss-of-function modifications are fewer. Among them, the possibility to block ECFC expression of genes encoding for molecules that sustain the activation of T-cells (Abrahimi P. et al., 2016) was investigated as a possible strategy to be used in the context of allogeneic transplantation to prevent allograft rejection.

1.3 Endothelial Dysfunction

Functional alteration of the endothelial compartment is deeply implicated in the pathogenesis of cardiovascular disease (Boulanger CM., 2016). Recently, robust study has shown that endothelial dysfunction (ED) is triggered by the loss of vascular tone and redox balance, and the establishment of a pro-inflammatory environment in the blood vessel wall (Ooi BK. et al., 2018). In this scenario, endothelial cell activation also plays a notable role in ED, involving the upregulation of chemokines, adhesion molecules, and other proteins that relate to the cell-cell connection (Weber C. & Noels H., 2011) thus culminating in a pro-thrombotic and pro-inflammatory condition of blood vessels.

1.3.1 Role of oxidative stress in Endothelial Dysfunction

NO and prostacyclin are vasodilating agents that maintain the endothelium in a quiescent condition. In the presence of cardiovascular risk factors or in the context of host defence, a switch from NO-mediated signaling to redox signaling occurs activating ECs and promoting vasoconstriction through the release of endothelin-1, angiotensin II, thrombin and other molecules (Poredos P., 2002). In this context, ED – resulting not only in the loss of vasodilation but also in remodeling, platelet aggregation, inflammation and smooth muscle cell growth – relies on the altered balance between NO and reactive oxygen species (ROS) that can be due to a reduced NO availability and to an increased in ROS supported by their increased production/decreased degradation.

The reduced biological availability of NO can be due to the reduction in NO production caused by a decreased expression of endothelial cell NO synthase (eNOS) (Wilcox JN. et al., 1997), In particular, ROS are able to trigger the PI3K/ras/Akt/MAPK pathway which through redox transcription factors promote the expression of redox genes thus resulting in the inhibition of eNOS mRNA expression and eNOS activity (Cai H. & Harrison DG., 2000; Münzel T. et al., 2010). A reduction in NO production is also sustained by the absence of eNOS substrate or cofactors and by changes in cell signaling (Münzel T. et al.,

2010). Tetrahydrobiopterin (BH4) is an essential cofactor of eNOS required for the coupling of the oxidase and reductase domains. BH4 absence leads to eNOS uncoupling in which eNOS reduces oxygen instead of L-arginine, thus producing superoxide rather than NO (Figure 1.6) (Münzel T. et al., 2008). Numerous hypotheses attempt to explain the absence of BH4. These include activation of the vascular superoxide complex, which can induce the formation of superoxide and/or ONOO-, which in turn can oxidize BH4 to BH3 radical and release additional ROS and formation of reactive nitrogen species ("kindling radicals") with positive feedback (Kuzkaya N. et al., 2003). The importance of BH4 for the proper eNOS function is supported by the fact that BH4 supplementation improved endothelial function in smokers, patients with diabetes, hypertension, hypercholesterolaemia and chronic heart failure, and elderly subjects (Kuzkaya N. et al., 2003; Stroes E. et al., 1997; Setoguchi S. et al., 2002; Moat SJ. et al., 2005; Higashi Y. et al., 2006; Hayden MR. et al., 2003).

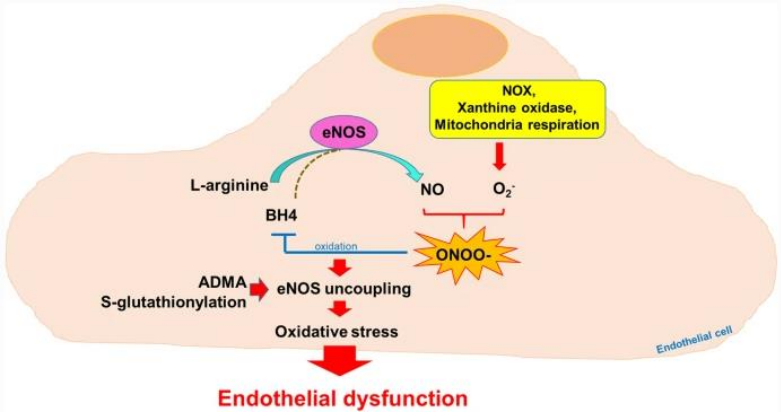

Figure 1.6 | "eNOS uncoupling" in endothelial dysfunction. NO is generated from L-arginine with the presence of cofactor tetrahydrobiopterin (BH4) via eNOS catalysis. Superoxide anion (O−2) is generated in endothelium from various sources such as NOX activity, xanthine oxidase and mitochondria respiratory chain. Reaction between NO and O−2 leads to ONOO− formation, causing inhibition of BH4 oxidation. The reduction of BH4, the presence of asymmetrical dimethylarginine (ADMA) and eNOS S-glutathionylation are factors attributed to eNOS uncoupling. Uncoupling eNOS reduces not only NO generation, but also produce oxidative stress, which takes part into ED *(Adapted from: Huynh DTN. & Heo KS., 2019).*

In addition, the reduction of NO availability can be caused by ROS that are able to directly inhibit NO activity (Ogita H. & Liao J., 2004). Recent direct (i.e., elevated levels of ROS and/or evidence of ROS-derived damage) and indirect evidences (i.e., improvements in endothelial vasomotor responses following acute intravascular administration of high-dose vitamin C) support the ROS-driven NO degradation as central mechanism (Münzel T. et al., 2008; Amponsah-Offeh M. et al., 2023). The interaction between superoxide (O2–) and NO reduces NO availability and forms the superoxide anion peroxynitrite (ONOO–). ONOO- has been linked to ED in several cardiovascular diseases (i.e. diabetes, hypertension, heart failure and atherosclerosis) and exerts its cytotoxic function by inducing DNA damage through oxidative modification of the sugar-phosphate backbone and nucleobases (Heo KS. et al., 2011; Neves KB. et al., 2018). However, superoxide do not represent the unique radical which binds NO. Indeed, lipid radicals (LO· and LOO·) which interact with NO produced LONO and LOON (Rubbo H. et al., 2000) and the oxidized LDL interacting with isolated vessels blocks endothelium-dependent vascular relaxation (Liu C. et al., 2021).

As previously mentioned, increased levels of ROS are sustained either by an increased ROS production and a decreased ROS degradation. Such mechanisms are due to various pathophysiological processes. The first one, consists in the above mentioned eNOS uncoupling due to BH4 oxidation that leads to superoxide production. The second one, consists in the ROS production by the activation of enzymes such as nicotinamide- adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase (XO), and cyclooxygenase. Among these ROS-producing enzymes, NOX is the most important source of vascular ROS. NOX is a superoxide-producing enzyme first described in neutrophils but also observed in endothelial cells, smooth muscle cells and adventitia. Four isoforms of the NOX family, including Nox1, Nox2, Nox4 and Nox5, have been found to be widely expressed in vessels. In an atherosclerotic state, ED associated with the activation of the renin-angiotensin system is supported by the Angiotensin II induced-activation of NOX (de Queiroz TM., et al., 2013). NOX- derived ROS stimulate transcription

factors that modulate the expression of inflammatory cytokines and chemokines, causing the recruitment of inflammatory cells (De Silva TM. et al., 2018; Libby P. et al., 2015). For these reasons, selective NOX inhibitors to suppress ROS overproduction in pathological conditions are under investigation (Vermot A. et al., 2021).

The third mechanism responsible for the increased levels of ROS that lead to ED is represented by the inactivation of antioxidant systems, including superoxide dismutase (SOD), glutathione peroxidase and catalase (Wang Y. et al., 2018). SOD isoforms (including SOD1, SOD2 and SOD3) mediate the conversion of O2– into H2O2, while catalase is responsible for the elimination of H2O2 by decomposing it into oxygen and water (Chen Q. et al., 2018). Antioxidant enzymes are therefore considered possible targets for the regulation of vascular ROS.

Finally, the increased ROS production occurring in ED also supports an increase production of asymmetric dimethyl arginine (ADMA) (Sydow K. & Münzel T., 2003). In fact, ADMA synthesis and its hydrolysis are mediated by enzymes characterized by redox susceptibility. Therefore, oxidative stress in the vascular system may lead to ADMA generation of and/or inhibition of ADMA degradation. High concentrations of ADMA in turn inhibit NOS activity or uncouple the enzyme further promoting ROS production by activating a mechanism with a positive feedback (Sydow K. & Münzel T., 2003).

Overall, these results support the correlation between vascular ROS generation and atherosclerosis, suggesting that minimization of oxidative stress positively affects not only vascular function but also the prognosis of a cardiovascular disease event.

1.3.2 Role of inflammation in Endothelial Dysfunction

Following infection or vascular injury, functional and structural changes occur in ECs, resulting in endothelial activation. This condition is caused by various stimuli, such as bacterial-derived endotoxins, inflammatory cytokines, or activation of pattern recognition receptors (PRRs) when they recognize

pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Gong T. et al., 2020). This context stimulates increased expression of inflammatory cytokines, chemokines, growth factors through the activation of NF-κB signaling that contributes to the production of inflammatory mediators that modulate smooth muscle cell activation, inflammation, and the development of atherosclerosis (Mehrhof FB. et al., 2005). Notably, a chronic low-grade inflammation is also linked to oxidative stress and to increased ROS production. In fact, ROS are able to activate NF-κB pathway that in turn sustain the expression of a number of pro-inflammatory cytokines, such as TNFα and interleukin-1 (IL-1) (Naik E. & Dixit VM., 2011).

In the presence of a stressful microenvironment, the inflammatory and pro-coagulative process of ECs is mediated by activation of the NF-Kb pathway (Rahman A. & Fazal F., 2011) through increased downstream expression of genes encoding for adhesion molecules, particularly vascular cell adhesion molecule 1 (VCAM-1), intercellular Adhesion Molecule 1 (ICAM-1), endothelial (E)-selectin, platelet (P)-selectin, and chemokines (Turner MD. et al., 2014; Sprague AH. & Khalil RA., 2009; Xue J. et al., 2009). This upregulation induces platelet adhesion and mediate firm adhesion and/or transendothelial migration of leukocytes to inflamed tissues.

Regarding adhesion molecules, the major player in the endothelial activation cascade is VCAM-1 (or CD106) which is involved in the firm adhesion and transmigration of leukocytes expressing the α4-integrin ligand (very late antigen-4, VLA-4) (Chakraborty S. et al., 2015; Imai Y. et al., 2010). In addition, ICAM-1 on ECs by interacting with its ligands LFA-1 (lymphocyte function-associated antigen-1) and MAC-1 (macrophage antigen-1) facilitates leukocyte adhesion and migration (Frank PG. & Lisanti MP., 2008). ICAM-1 is also involved in platelet adhesion by interacting with platelet αIIbβ3 integrin in a fibrinogen-dependent bridging mechanism (Etulain J. & Schattner M., 2014).

Another I-type lectin expressed on the endothelium, platelets and leukocytes is represented by Platelet– endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31. PECAM-1 forms both homophilic interactions with PECAM-1 and heterophilic interaction $\alpha_V\beta_3$ integrin expressed on ECs and

platelets. The functional relevance of PECAM-1 on platelet–endothelium interactions is still object of study but studies performed in mouse models showed PECAM-1 involvement in platelet adhesion–aggregation at sites of minor endothelial damage (intact monolayer) in brain arterioles (Etulain J. & Schattner M., 2014).

Selectins are cell surface lectins that mediate the adhesion of leukocytes to ECs and platelets under flow. Within this family, three selectins have been identified: platelet (P)-selectin, endothealial (E)-selectin and leukocyte (L)-selectin. Among the selectins, E-Selectin expressed by ECs is able to slow the rolling of leukocytes to a halt and is therefore central to inflammatory responses (Silva M. et al., 2018). P-selectin is stored in the alpha granules of platelets and in the Weibel-Palade bodies of ECs. Exposure to an activating stimulus leads to EC activation and to rapid translocation of P-selectin to the cell surface. Once expressed on EC surface, P-selectin mediate the initial adhesion and rolling of platelets and leukocytes that express the P-selectin receptor, P-selectin glycoprotein ligand-1 (PSGL-1). Davies et al. demonstrated the role in atherosclerosis of both E- and P-selectins describing the presence of selectins on the ECs that form atherosclerotic plaques (Davies PMJ. et al., 1993; Theofilis P. et al., 2021). In addition, knockout mice for P- and E-selectins had attenuation of the atherosclerotic disease (Collins RG. et al., 2000; Tvaroška I. et al., 2020).

Also, chemokines play a key role in the inflammatory response. In particular, monocyte chemoattractant protein (MCP-1/CCL2) is produced by a variety of cell types – including ECs and monocytes/macrophages - either constitutively or after induction by oxidative stress, cytokines, or growth factors. CCL2 regulates the migration and infiltration of monocytes, memory T lymphocytes, and natural killer (NK) cells expressing the CCR2 receptor. CCL2-CCR2 axis is associated with the advancement of several disorders such as atherosclerosis (Niu J. & Kolattukudy PE., 2009) as demonstrated in mouse models in which the absence of MCP-1 or CCR2 induces a reduction in lipid deposition and slows down the atherosclerotic process (Gu L. et al., 1998; Boring L. et al., 1998; Schober A. et al., 2004) whereas in contrast, MCP-1

overexpression promotes more macrophages migration and rapid development of atherosclerosis (Namiki M. et al., 2002) and supported by the elevated circulating levels of MCP-1 detected in patients who experienced restenosis after coronary angioplasty (Cipollone F. et al., 2001).

Inflammatory stimuli as cytokines and superoxide anions have also been correlated with pro-thrombotic effects such as the downregulation of thrombomodulin (Theofilis P. et al., 2021) and the increase TF expression on the cell surface thereby initiating the extrinsic coagulation pathway (Borissoff JI. et al., 2011). The role of EC activation in promoting the intrinsic coagulation pathway is not clearly defined yet. Nevertheless, Cohen and colleagues demonstrated by treating HUVECs with TNFα, the potential effect of inflammatory stimuli in inducing factor IX production (Cohen CT. et al., 2020).

Activated ECs are also charactized by increased expression of vWF (Vischer UM. et al., 2000; Bernardo A. et al., 2004). vWF shows a dual function since on one hand contributes to thrombotic formation through the promotion of platelet adhesion, aggregation and prevention of proteolytic degradation of factor VIII, and on the other hand contributes to inflammation through fostering leukocyte mobilization, complement activation and NETosis (Gragnano F. et al., 2017). In this context, the role played by neutrophil extracellular traps (NETs) on endothelial function is under investigation since NETs - which include nuclear chromatin, histones, and proteins - have been described to be associated with the inflammatory milieu that characterizes cardiovascular disease (Döring Y. et al., 2020). In particular, it has been reported that overproduction of NETs can cause vascular changes and endothelial-mesenchymal transition (EndoMT) through VE-cadherin degradation (Pieterse E. et al., 2017) and complement activation thus leading to endothelial injury (Wang H. et al., 2015).

Finally, thrombosis resulting from ED is also common in severe bacterial and viral infections. Immunothrombosis, which is characterized by increased levels of inflammatory cytokines, ROS, and NETs, resulting in vascular leakage (Delabranche X. et al., 2017), has been shown to be central to the coronavirus disease pandemic started in 2020, where endotheliitis associated with severe

acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been correlated with increased risk of thrombosis (Bonaventura A. et al., 2021).

1.3.3 Role of shear stress in Endothelial Dysfunction

Throughout blood flow, the endothelium is subjected to mechanical and hemodynamic forces, such as: i) radial forces due to intravascular network pressure; ii) tangential forces generated by the balance of cell-cell interactions and vasomotion of the vessel, and iii) axial shear forces, due to the friction of blood flow on the vessel wall. ECs are able to identify and respond to blood shear stress forces (SS) through cell-cell junctions (Tzima E. et al., 2005), heterotrimeric G proteins (Gudi S. et al. 2003), primary cilia (Hierck BP. et al., 2008), caveolae (Yu J. et al., 2006), integrins (Jalali S. et al., 2001) and GCX (Pahakis MY. et al., 2007). In recent research, a mechano-sensory complex composed by PECAM-1, VE-cadherin and VEGFR-2 was observed in cell-cell junctions, which is useful for triggering SS-dependent signaling pathways, aligning cells under flow and stimulating atherosclerosis (Tzima E. et al., 2005). Among the major components, PECAM-1 is activated by external forces to initiate signaling. VEGFR-2 is shifted to PECAM-1 through the activity of VE-cadherin as an adaptor, so as to promote VEGFR-2 transactivation through a tyrosine kinase pathway. PI3-kinase is then involved by VEGFR-2 and regulates the activation of a protein kinase B (Akt) and eNOS (Tzima E. et al., 2005; Jin ZG. et al., 2003).

Typically, ECs are exposed to two different types of flow, unidirectional/lamellar shear stress (USS) and oscillatory shear stress (OSS) or disturbed flow (Chiu JJ. et al., 2011).

USS promotes EC survival and enhances vasodilation and anticoagulation by exerting an anti-inflammatory function as, through KLF2 (SenBanerjee S. et al., 2004), it induced the upregulation and the activation of Transcription factor EB (TFEB). TFEB has been shown to inhibit the formation of atherosclerotic plaques by inhibiting endothelial inflammation (Lu H. et al., 2017). In this context, it has been shown that TFEB overexpression can alleviate diabetic

ED by restoring autophagy flux and eNOS dimerization (Zhao L. et al., 2022). Besides activating TFEB, KLF2 is also a negative regulator of the NF-κB signalling pathway, further supporting the suppressive effect of USS on NF-κB activation at the endothelial level.

In vitro, USS exerted on ECs results in increased NO generation due to eNOS overexpression that is mediated by Akt phosphorylation and the subsequent phosphorylation of eNOS (Boo YC. et al., 2006; Dimmeler S. et al., 1999) (Figure 1.7).

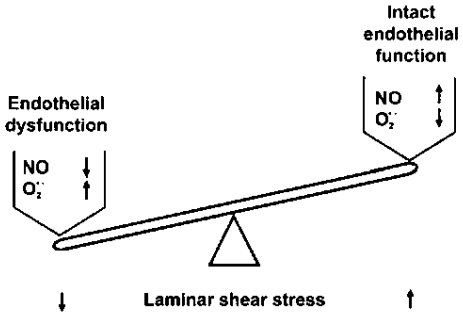

Figure 1.7 | Regulation of laminar blood flow of the endothelial NO/·O2 – balance and endothelial function. Activation of ·O2- formation by shear stress, followed by a downregulation of endothelial NAD(P)H oxidase in response to laminar shear stress. NO-mediated downregulation by shear stress preferentially affects the NAD(P)H oxidase complex, involved in the shear stress-dependent regulation of the endothelial NO/·O2- balance *(Adapted from Morawietz H., 2007).*

On the contrary, OSS is considered a harmful mechanism that damages the endothelium and the cardiovascular system since is one of the main local mediators of inflammation in the vascular endothelium. In areas characterized by bifurcation, blood differentiates its flow toward turbulence, described with an altered phenotype that induces increased monocyte adhesion, proliferation, and apoptosis. In fact, the exposure to OSS activates the NF-κB or MAPK pathways thus inducing the expression of genes encoding for TNFα, IL-1, MCP-1, P-selectin, ICAM-1, VCAM-1 and E-selectin (Chiu JJ. et al., 2011). These adhesion molecules and chemokines trigger atherogenesis by promoting immune cells attachment and transendothelial migration. Moreover, OSS also supports EndoMT, which further exacerbates ED and atherogenesis (Cheng CK. et al., 2023). Furthermore, it has recently been shown that SS can modulate angiopoietin (Ang)/ Tie signalling pathway. In fact, in a mouse model

has been described that Ang2 expression was increased by atherogenic low laminar flow whereas it was suppressed in the presence of protective high SS (Hayashi SI. et al., 2020). Considered the Ang2 ability to antagonize the Ang1/TIE2 pathway, blood flow alterations-related inflammation and ED may be mediated by the Ang2 upregulation (Hayashi SI. et al., 2020). For these reasons, OSS, combined with local hypoxia and other as yet unknown causes, is often the origin of venous thrombus formation.

1.4 Evaluation of Endothelial Dysfunction

Despite the current knowledge about cardiovascular risk factors, the cardiovascular mortality rate is still high today, and the evaluation of ED is considered an optimal prognostic tool (Green DJ. et al., 2011; Matsuzawa Y. et al., 2015). In the present section, strategies for ED evaluation will be described.

1.4.1 Flow mediated dilation

Noninvasive assessment of endothelial function can be performed with flow mediated dilatation (FMD) of the brachial artery. This technique involves occluding the brachial artery by inflating a blood pressure cuff to about 50 mmHg above the patient's systolic blood pressure for 5 minutes. The induced hyperemia ensures an increase in endothelial shear stress that stimulates NO generation (Thijssen DHJ., 2019). In a patient with vascular dysfunction – such as patients with coronary artery disease (CAD) - dilation is reduced or absent. Assessment of FMD can therefore provide prognostic data for cardiovascular risk prevention (Takishima I. et al., 2012; Kitta Y. et al., 2009) and also provide mechanistic insight into the role of ED in several pathological conditions (Weissgerber TL., 2014).
The information about endothelial function deriving from FMD assessment in several studies on atherosclerosis, heart failure and deep vein thrombosis have been integrated with the information deriving by the assessment of biomarkers known to be associated with the studied disease and with ED in general (Dimitropoulos S. et al., 2020; Mourouzis K. et al., 2021).

1.4.2 Soluble biomarkers of endothelial damage

The measurement of blood biomarkers is a simple and non-invasive method for assessing the integrity of endothelial function. Conventional markers of ED are the plasmatic levels of pro-inflammatory molecules, and the soluble form of adhesion molecules (i.e. E-selectin, ICAM-1, VCAM-1) (Raffray L. et al.,

2017). Molecules that participate in the coagulation pathway, such as vWF and soluble thrombomodulin, are also evaluated (Constans J. & Conri C., 2006). Among them, vWF seems to be the most consistent predictor of cardio- and cerebrovascular conditions, used in hypertensive and hyperlipidemic patients and in patients with previous chronic cardiac status (Constans J. & Conri C., 2006).

Given the complexity of endothelial function, measurement of these biomarkers alone cannot provide sufficient information about the endothelium since the majority of them are not generated only by ECs (Goncharov NV. et al., 2017). For these reasons, the quantification of conventional biomarkers alone to assess the risk of developing CVD is still questionable (Nozaki T. et al., 2009) and other biomarkers are needed to be used alone or in combination with conventional biomarker of ED to assess more specifically endothelial injury/ED (Blann AD., 2015; Nozaki T. et al., 2009).

To improve patient stratification and patient management, new biomarkers of ED – such as Endocan, and Endoglin - are emerging (Leite AR. et al., 2020). Endoglin, also known as CD105, is predominantly expressed by proliferating ECs (Santibañez JF. et al., 2011; Zhu W. et al., 2017). It is a homodimeric transmembrane accessory receptor for transforming growth factors β-1 (TGF-β1) and β-3 (TGF-β3) thus modulating TGF-β signaling that is known to play a crucial role during angiogenesis, cardiac and vascular development, and in maintaining vascular homeostasis (Santibañez JF. et al., 2011; Zhu W. et al., 2017). Two endoglin isoforms - long-form (L-Endoglin) and short-form (S-Endoglin) – can be distinguish on the basis of cytoplasmic tail length and phosphorylation capacity (López-Novoa JM. & Bernabeu C., 2010). High levels of S-Endoglin were described in hypercholesterolemia, hypertension, diabetes mellitus, and inflammatory states (Blann AD. et al., 2015). In addition, since high variability in S-Endoglin were described across the studies, it has been hypothesized that S-Endoglin is an early indicator of vascular alterations, which decreases as the disease develops (Li CG. et al., 2000). For these reasons, further studies are needed to clarify the role of S-Endoglin in CVD and to assess its predictive value as marker of damage, inflammatory status,

activation, and endothelial senescence in different diseases (Li CG. et al., 2000).

Finally, Endocan also referred to as EC-specific molecule-1 (ESM-1) is a proteoglycan secreted by vascular ECs and other cell types (Zhang SM. et al., 2012) that is emerging as marker of ED. Its expression on activated ECs is fostered by inflammation triggers such as lipopolysaccharide and pro-inflammatory cytokines such as TNFα and IL-1β (Sarrazin S. et al., 2006). Once expressed, Endocan promotes leukocyte transendothelial migration (Canpolat U. et al. 2020; Rocha SF. et al., 2014). Endocan expression also supports in ECs the production of pro-inflammatory cytokines and the expression of adhesion molecules (i.e. ICAM-1 and VCAM-1) as well as the adhesion between monocytes and ECs (Lee W. et al., 2014; Sun H. et al., 2019). Moreover, Endocan promotes both the expression of VEGF-A and its binding to its receptor VEGFR-2 thus increasing vascular permeability (Lee W. et al., 2014). The interaction between Endocan and VEGF signaling is bidirectional, - in fact also VEGF signaling directly induces Endocan expression (Sun H. et al., 2019) – and is crucial in angiogenesis, inflammation, and permeability either in physiological and pathological conditions. For these reasons, the interaction between Endocan and VEGF signaling represents a valuable therapeutic target (Sarrazin S. et al., 2006; Rocha SF. et al., 2014).
Because of its immunomodulatory role in EC activation and dysfunction, increased serum levels of Endocan have been observed in autoimmune and systemic inflammatory diseases (Balta I. et al, 2013; Icli A. et al., 2016; Balta S. et al., 2015). In addition, high levels of endocan have been described in atherosclerosis (Balta I. et al., 2013; Icli A. et al., 2016) and in CV conditions, such as hypertension, coronary heart disease, and coronary flow restriction (Zhao T. et al., 2018) thus supporting its use as marker od ED in the clinical setting (Balta S. et al., 2015; Walczak M. et al., 2015).

1.4.3 Cellular Biomarkers of endothelial dysfunction

In addition to soluble biomarkers, another emerging stategy to evaluate ED is represented by the assessment of microparticles (MPs). MPs deriving from the plasma membrane of leucocytes, platelets, and ECs, have been extensively studied as biomarkers of ED and predictors of CV diseases (Leite AR. et al., 2020). MPs allow the communication among the cell of origin and the other cells thus regulating cellular events. In fact, the biological contents of the MPs that include not only components of the cell origin plasma membrane (i.e adhesion molecules and pro- and anti-coagulant molecules) but also enzymes and microRNAs are able to regulate the gene expression in target cells (Alexandru N. et al., 2016; Santilli F. et al., 2016). Exosomes represent another group of vesicles, smaller than MPs, generated from the fusion of intracellular microvesicular bodies with the plasma membrane and involved in inter-cellular communication (Dignat-George F. & Boulanger CM., 2011; Théry C. et al., 2009). Notably, MPs content is influenced either by the type and by the activation state of the origin cells. MPs derived from activated ECs may regulate monocyte/macrophage function stimulating the production of pro-inflammatory cytokines (Sena CM. et al., 2022).

Endothelial-derived MPs released by activated and damaged endothelium can be quantified in plasma by cytofluorimetric analysis (Lacroix R. et al., 2010), atomic force microscope (AFM) visualization, nanoparticle tracking analysis (van der Pol E. et al., 2010), and electron microscope observations (Favretto G. et al., 2019). In presence of ED, the levels of EC- and platelet- derived MPs increased. In particular, it has been showed that TNFα, thrombin, uremic toxins, plasminogen activator inhibitor-1, and ROS stimulate the release of MPs and exosomes by ECs (Lekakis J. et al., 2011). In accordance, elevated level of MPs and/or exosomes has been found in association with CV risk factors such as smoking (Mobarrez F. et al., 2014), obesity (Esposito K. et al., 2006) and in several disorders, such as stroke, hypertension, pre-eclampsis, and renal and heart failure (Yong PJ. et al., 2013; Preston RA. et al., 2003; Marques FK. et al., 2013). High levels of MPs have also been directly related to thrombogenesis (Sena CM. et al., 2022). It has been therefore proposed

that MP evaluation can be used to stratify patients for ED and also monitor ED in patients with CVD over time this evaluating therapy effectiveness (Alexandru N. et al., 2016; Jung KH. et al., 2011).

In addition to MPs, other strategies that allow the evaluation of the balance between vascular endothelial damage and repair consist in the quantification of circulating endothelial cells (CECs) and in the quantification of circulating EPCs by flow cytometry (Sena CM. et al., 2022). CECs derived from the detachment of mature ECs and their amount in circulation is increased in the case of EC activation or injury as in patients with vascular inflammation, atherosclerosis, and COVID-19 (Guervilly C. et al., 2020; Szmitko PE. et al., 2003). Concerning EPCs they are recruited from the bone marrow in response to stimuli such as ischaemia including myocardial infarction, ischaemic stroke, vascular trauma, sickle cell anaemia, vasculitis and pulmonary hypertension. EPCs are typically identified on the basis of absent/weak CD45 expression (unlike haematopoetic cells) combined with the expression of immaturity (CD34) and endothelial markers (CD146, VEGFR-2/KDR) (Bradbury C. et al., 2019). In several studies, it has been shown that EPC levels seem to predict the occurrence of cardiovascular events and death from cardiovascular causes and may help to identify patients at increased cardiovascular risk (Lee PS. et al., 2014).

1.4.4 ECFCs as a tool to assess endothelial dysfunction

All the biomarkers here described provide information about the presence of ED by providing an indirect indication of endothelial function. In order to study more in deepth the mechanism(s) underlying ED, there is an increasing interest of the scientific community in the use of patient-derived ECFCs as non-invasive approach to study alteration of the endothelial compartment.

The use of ECFCs as liquid-biopsy of the endothelium is supported by the fact that: i) ECFCs can be easily isolated from blood, a biological specimen of easy access; ii) ECFCs are characterized by a higher proliferative potential than mature ECs thus allowing their use to perform extensive *in vitro* characterization of patient-specific cells.

ECFC numeric and functional alterations have been described in pregnancy-related diseases and associated comorbidities such as pre-eclampsia (Muñoz-Hernandez R. et al., 2014; Baker CD. et al., 2009), infants with intrauterine growth restriction (Sipos PI. et al., 2013) and diabetes (Moreno-Luna R. et al., 2014; Javed MJ. et al., 2008; Blue EK. et al., 2015; Ingram DA. et al., 2008). Considering that many CV diseases originate during development, an aspect that deserve to be further investigated is whether the ECFC alterations observed during pregnancy can foster in offspring the development of CDV in the long term (Adam K., 2017).

The ECFC characterization was also used to study the role of ED in the pathology of pulmonary arterial hypertension (PAH), highlighting the role of type I interferon and the role of tumour translational control protein (TCTP) in PAH (George PM. et al., 2014; Lavoie JR. et al., 2014). In addition, in PAH patient ECFCs it has been described the presence of metabolic abnormalities, and aberrant expression of the chloride intracellular channel 4 (CLIC4) (Caruso P. et al., 2017; Wojciak-Stothard B. et al., 2014). Finally, ECFCs were also used to study the possible use of BMP9 signaling as therapeutic target in PAH (Dunmore BJ. et al., 2013).

In addition, an impairment in the anti-coagulant function of ECFCs in patients with Idiopathic Pulmonary Fibrosis (IPF) that is mediated by a reduction in the expression of the anti-coagulant proteins thrombomodulin and EPCR was described by Billoir and colleagues (Billoir P. et al., 2021).

In the context of CVD, increased levels of ECFCs were observed in patients with myocardial infarction (MI) although the mechanisms regulating this mobilisation remain unknown (Massa M. et al., 2009; Huang L. et al., 2007; Meneveau N. et al., 2011).

Altogether, the studies described in literature support the use of ECFC characterization as a strategy that allow not only the detection of ED but also allow to investigate in depth the mechanisms underlying ED.

1.5 Venous Thromboembolism (VTE)

Venous thromboembolism (VTE), stroke and ischemic heart disease are the three most common cardiovascular disorders, all three having thrombosis as common element. The relevance of these pathological conditions is highlighted by the fact that in the 20th century, cardiovascular diseases are one of the major cause of mortality and disability worldwide (Raskob GE. et al., 2014).

1.5.1 Epidemiology

VTE includes both deep vein thrombosis (DVT) and pulmonary embolism (PE) with PE being the complication endowed with the highest mortality regardless the primary site of the thrombus (Kim HY. et al., 2021). VTE commonly occurs in legs; nevertheless, VTE events can affect any venous circulation, occurring also in the arms or in the veins of central nervous system as well as in abdominal veins (i.e. portal and renal veins) (Cushman M. et al., 2004; Oger E., 2000; Rocha AT. et al., 2007). Especially in western regions of the world, VTE is the major health problem having an annual incidence of 1-2 subjects per 1000 person-years in the general population (Tagalakis V. et al., 2013; Rothwell PM. et al., 2004; Cushman M. et al., 2004; Oger E., 2000; Naess IA. et al., 2007; Palareti G. et al., 2019) that is similar to the incidence described for stroke (Heit JA., 2005b). In addition, considering that asymptomatic VTE can occur, it is highly probable that the VTE incidence is underestimated.

Studies performed in United States showed that the incidence of VTE varies among the different ethnic groups, with reduced rates in Asians, Pacific Islanders, and Hispanics compared with the Caucasian population, whereas African-Americans have a higher VTE incidence compared with the Caucasian (+30%) (Tsai AW. et al., 2002; White R. et al, 1998; Stein PD. et al, 2004). Information on thrombosis in Africa is limited while more informations are available for Asian states. In particular, two studies showed that the rates of in-hospital or postoperative VTE in Asian states are similar those observed in

Western states (Piovella F. et al., 2005; Leung V. et al., 2006) and Cheuk and colleagues estimated at 0.17 per 1000 the annual incidence of DVT in the Chinese population thus confirming the lower incidence in Asians compared with Caucasian population (Cheuk BL. et al., 2004). A different genetic background could explain the different VTE risk observed among the different ethnic groups since high levels of FVIII observed in African Americans could explain their higher VTE incidence (Zakai NA. et al., 2014; Patel RK.et al., 2003), and some forms of inherited thrombophilia (i.e. those related to prothrombin G20210A and factor V Leiden gene mutation) that are frequent in Caucasian are hardly detectable in Asians (Saeed A. et al., 2015).

Notably, in addition to ethnic differences, VTE incidence is also affected by age and sex. As shown in Figure 1.8, VTE is a disease characterized by an almost exponential age-dependent increase both in men and women. In fact, VTE is rare among children whereas its annual incidence in people older than 80 years attests to 4.5-6 cases per 1000 individuals (Rocha AT. et al., 2007; Oger E., 2000). The increased VTE risk associated with the increasing age can be appreciated also when DVT and PE are separately evaluated (Heit JA., 2015).

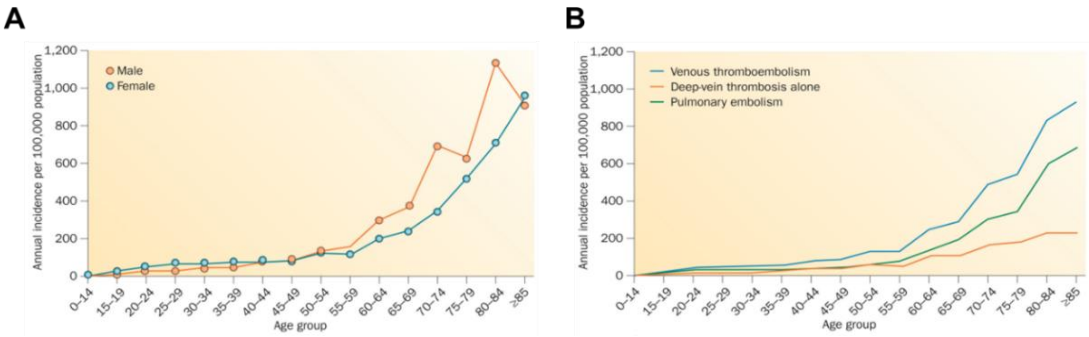

Figure 1.8 | VTE annual incidence. A) VTE annual incidence per 100,000 population in age group of males and females; B) VTE annual incidence per 100,000 population in age group, along with the incidence of deep-vein thrombosis alone, and pulmonary embolism (with or without deep-vein thrombosis) *(Adapted from: Heit JA., 2015).*

Concerning sex, the age-adjusted annual incidence rate described for men is higher compared with the one observed in women (1.30 vs 1.1 cases per 1000 individuals). Nevertheless, some studies described a higher incidence in women when subjects between the ages of 16 to 44 years were taken into account (Oger E., 2000; Heit JA. et al., 2005a; Heit JA., 2015). These results

could be due to the fact that women in childbearing age are exposed to VTE risk factors such as pregnancy and treatment with oral contraceptive (Oger E., 2000; Heit JA., 2015; Bell EJ. et al., 2016). Those VTE risk factors may explain the rapid increase in cumulative incidence in women around age 20, with almost half of all VTEs occurring before age 50 (Bell EJ. et al., 2016). These observations are further supported by the fact that the VTE risk of was observed to be twice as high in men as in women without reproductive risk factors (Arnesen CAL. et al., 2022). Recently, based on the MEGA, Hokusai-VTE, and RIETE studies, Scheres et al. documented in the context of VTE differences between male and female in the anatomical location of the first VTE showing that a greater proportion of women than men have a PE event (Scheres LJJ. et al., 2017).

1.5.2 VTE classification

Considering the importance of VTE patient classification to provide a guide for patient treatment and prognosis evaluation, several efforts have been made in order to define a consensus on the framework that should be used to classify VTE patients (Konstantinides SV. et al., 2020; Kearon C. et al., 2016a). As mentioned above, VTE can be triggered by the presence of specific conditions that are therefore considered risk factors for VTE. Such factors reflect those pathological conditions that are summarized in the Virchow's triad and known to underlie VTE pathogenesis. Such VTE events are therefore classified as “provoked” (pVTE). Factors known to concur to VTE can be classified as hereditary or as acquired factors, and will be described more in detail in the dedicated subsection. Acquired VTE risk factors can also be subgrouped on the basis of their being transient or permanent conditions (Palareti G., 2012; Weitz JI. Et al., 2020). In patients who experience a provoked VTE event, the recurrence risk after the end of anticoagulant treatment is low when the trigger factor is transient whereas it is high when the risk factor consists in a permanent and/or progressive condition (Prandoni P. et al., 2002; Heit JA. 2000). Moreover, different risk factors are also characterized by having a different “weight” in favouring VTE occurrence. This aspect is described by the

odd ratio that is a statistical measure of the relationship between the exposure to a risk factor and the development of VTE (Table 1.3) (Wang H. et al., 2021; Pastori D. et al., 2023). Compelling evidence confirms that the risk of thrombosis increases proportionally with the number of predisposing factors that affect the subject. Nevertheless, it is still unclear how these factors combine thus generating the VTE risk in a patient-specific way (Wang KL. et al., 2016).

The identification of conditions that are risk factors for VTE and their proper assessment are crucial elements for foster VTE prevention by guiding the use of prophylaxis strategies aimed to prevent VTE in those subjects that are classified at high risk (Henke PK. & Pannucci CJ., 2010). In this context, not only major risk factors deserved to be considered since antithrombotic prophylaxis may also be taken into consideration when 2 or more moderate and/or weak factors are present in combination (Geerts WH. et al., 2004).

Strong risk factors (odds ratio > 9)	Moderate risk factors (odds ratio 2-9)	Weak risk factors (odds ratio < 2)
• Fracture (hip or leg) • Hip or knee replacement • Major general surgery • Major trauma • Spinal cord injury	• Arthroscopic knee surgery • Central venous lines • Chemotherapy • Congestive heart or respiratory failure • Hormone replacement therapy • Malignancy • Oral contraceptive therapy • Paralytic stroke • Pregnancy/postpartum • Previous venous thromboembolism • Thrombophilia • Mutation of factor V Leiden • Mutation of G20210-A • Antithrombin/protein C/protein S deficiency	• Bed rest > 3 days • Immobility due to sitting (e.g. prolonged car or air travel) • Increasing age • Laparoscopic surgery (e.g. cholecystectomy) • Obesity • Pregnancy/puerperium • Varicose veins • Arterial hypertension • Dyslipidemia

Table 1.3 | VTE risk factors. VTE risk factors classidied on the basis of the odd ratio *(Adapted from: Anderson FA. Jr* & Spencer FA*., 2003)*.

Notably, in up to 50% of the cases, VTE event occurs in absence of known risk factors, such events are therefore classified as unprovoked VTE (uVTE). In uVTE patients the pathogenic mechanism(s) underlying the VTE event are unknown and therefore the recurrency risk is high in this class of patients (Kearon C. et al, 2012; Iorio A. et al., 2010). The identification of triggers involved in uVTE events represents an unmet clinical need that deserves to be investigated in depth to better stratify patients, identify possible therapeutic strategies and provide indications for type and duration of the anticoagulant treatments that in uVTE patients are still controversial (Eichinger S. et al., 2010; Anderson FA. Jr & Spencer FA., 2003).

1.5.2.1 Inherited VTE Risk Factors

Concerning the inherited VTE Risk Factors, the most common ones are:

Antithrombin deficiency. The hereditary defect in the antithrombin activity in association with thrombotic disease was initially described by Egeberg in 1965 (Stevens SM. et al., 2016). In the coagulation cascade, antithrombin inhibits

thrombin (factor IIa), factor Xa, and other serine proteases. Deficiency of this natural anticoagulant determines a highly thrombogenic condition, associated with an odds ratio of VTE of 16.3 compared to a non-thrombophilic state (Di Minno P. et al., 2015). In absence of other acquired risk factors, it has been shown that a percentage ranging from 50% to 90% of the individuals affected by hereditary AT deficiency will develop thrombosis in their life (Vossen CY. et al., 2005; Haemostasis and Thrombosis Task Force, 2001). In particular, up to 50% of the subject characterized by this condition experienced a VTE event by the age of 50 (Patnaik MM. & Moll S., 2008).

Factor II (Prothrombin) G20210A genetic variant. Subjects having prothrombin genetic variant known as G20210A are characterized by increased prothrombin production that in turn foster blood clotting (Jadaon MM., 2011).

APC resistance. During last years, anticoagulant activated protein C (APC) resistance has been considered as one of the major risk factors that foster VTE being present in 5% of US white population (Folsom AR. et al., 2002). APC resistance is mainly associated with a mutation of factor V gene, Arg506Gln, also called factor V Leiden (FVL). This mutation alters one of the APC cleavage sites in factor V thus decreasing the efficiency of APC-mediated FVa inactivation (Castoldi E. & Rosing J., 2010). FVL was observed to induces more DVT than PE, (Bounameaux H., 2000; Brink A. et al., 2023; Corral J. et al., 2010; Ordóñez AJ. et al., 2000). A new variant of factor V gene haplotype – defined HR2 and consisting consists of an 4070A>G polymorphism in exon 13 – was lately identified that can contribute to APC resistance (Mingozzi F. et al., 2003). Nevertheless, additional studies are required to deeply understand the correlation with HR2 haplotype and VTE risk (Alhenc-Gelas M. et al., 1999; Oztürk A. et al., 2013).

Protein C and protein S deficiencies. Due to the role exerted by protein C and protein S in regulating coagulation, Protein C and protein S deficiencies alter the proper balance between procoagulant and anticoagulant proteins thus inducing a prothrombotic state that results in excessive formation of blood clots (thrombophilia) and VTE (Aydin S. et al., 2021). The role of Protein C and

protein S deficiencies in fostering thrombosis is corroborated by the fact that up to 50% of the subjects affected by these deficiencies in heterozygosis experience a VTE event within the age of 50 years (Tang L. et al., 2012).

Coagulation Factors. Increased incidence of VTE has been also associated with alterations in the levels of coagulation factors, such as factors VIII, IX, and XI despite molecular basis underlying the high levels of these factors are still unknown and deserves to be investigated more in depth (Wang H., 2021). By comparing patients who experienced a first VTE event with healthy controls, increased plasmatic levels of Factor VIII were associated with increased thrombotic risk (Basaric D. et al., 2023). Similarly, elevated plasmatic levels of factors IX and XI seem to increase thrombotic risk (van Hylckama Vlieg A. et al., 2000).

1.5.2.2 Acquired VTE risk factors

The most common known acquired risk factors for VTE are:

Immobilization. The increased risk of VTE associated to immobilization is mainly mediated by stasis. As general rule, longer is the immobilization higher is the VTE risk. The VTE risk further increases when immobility occurs in combination with other risk factors. This occurs in case of hemiplegia (i.e. in the case of spinal cord injuries) (Geerts WH et al., 2001; Kamphuisen PW. et al., 2005), prolonged hospitalization/immobilization (i.e. in case of Fracture of the Pelvis, Hip, or Long Bones) (Beam DM. et al., 2009; Flanders SA. et al., 2014) and long-distance travel (Lapostolle F. et al., 2001). This last case also defined as “the economy class syndrome”, is a rare event that mainly occurred in subjects that already experienced a VTE event previously or that also have other predisposing factors (Scurr JH. et al., 2001).

Surgery and Trauma. Major general surgery and orthopedic surgery increase the risk of VTE (Shin WC. et al., 2016; Kearon C. et al., 2012). The term "major general surgery" refers to patients undergoing thoracic or abdominal surgery with a total anesthesia of at least 30 minutes (Roderick P. et al., 2005). In this context, high risk of developing VTE has been associated with coronary artery bypass (Ho KM. et al.,2015; Ayatollahzade-Isfahani F. et al., 2013), surgery

for gynecologic malignancies (Yuk JS. et al., 2021) and major urologic surgery (Dyer J. et al., 2013). Also in the case of minimally invasive (laparoscopic) procedures a coagulation activation was described (Lindberg F. et al., 2000). In case of orthopedic surgery, surgeries of the lower extremities (i.e. total hip or knee replacement) are characterized by a high risk of VTE and the majority of thrombotic events occur on the operated extremity (Shin WC. et al., 2016). The increased VTE risk is mediated by immobilization during and after surgery, as well as by direct venous damage and inflammation during surgery (Kearon C. et al., 2012).

Malignancy. The VTE risk is sevenfold higher in subjects with active malignancies due to the release of pro-coagulants factors (Blom JW. et al., 2005) while VTE risk decreases when cancer is in remission (Chew HK. Et al., 2006). Moreover, it has been shown a higher VTE risk in patients affected with high-grade tumours compared with patients with low-grade tumours (Ahlbrecht J. et al., 2012). In addition, chemotherapy administration has been associated with increased risk of thrombosis in patients with multiple myeloma and in patients with breast cancer (Zangari M. et al., 2001; Bowcock SJ. et al., 2002; Castaldi M. et al., 2021).

Obesity. A linear relationship has been described between body mass index (BMI) and VTE risk. The association between overweight and VTE is weak whereas in subjects with severe obesity (BMI >34) the VTE risk is six times higher than in normal weight subjects (Heit JA., 2000; Stein PD. et al., 2011).

Age. As described in the previous paragraph, the association between older age and higher incidence of VTE events has been demonstrated in several studies (Rocha AT. et al., 2007). The increased VTE occurrence observed in elderly is due to either the alterations in pro- and anti-coagulant balance occurring during aging (Lowe GDO. et al., 2022) and the increased presence of lower mobility, malignancies and obesity (Silverstein RL. et al., 2007).

Pregnancy and the puerperium. The increased VTE risk associated with pregnancy and puerperium is linked to the state of natural hypercoagulation - characterized by increased levels of factors VII, VIII, X, vWF and fibrinogen,

and decreased levels of protein S with an acquired APC resistance - that aims to prevent the risk of haemorrhage during the delivery (Heit JA. et al., 2005b; Hellgren M., 2003). Additional factors, such as smoking, prior VTE and inherited thrombophilia, can further increased the VTE risk in pregnant women (Gerhardt A. et al., 2016).

Combined oral contraceptives (COCs). Estrogen-based therapies are associated with increased VTE risk. Since the high dosage of estrogen has been identified as the main contributor to this risk, their dosage was progressively reduced in new drugs (Morimont L. et al., 2021). In particular, low- dose second- generation oral contraceptives are associated with a lower VTE risk compared with drugs of the first generation (Lidegaard Ø et al., 2002). VTE risk associated with oral contraceptives is higher in the first 3 months of therapy and ceases with the end of the therapy (Lidegaard Ø. et al., 2009).

Varicose veins. Varicose veins are considered a weak risk for development of VTE. The role of varicose veins as independent risk factors is controversial, due to paucity of studies and their subjective severity assessment. In addition, Heit and colleagues demonstrated that VTE risk associated with varicose veins decrease with aging (Heit JA., 2000).

Arterial Hypertension and Dyslipidemia. Those conditions are considered a weak risk factors for VTE since they are among the common cardiovascular risk factors that have been associated with VTE in meta-analyses despite these associations are heterogeneous among the considered studies (Ageno W. et al., 2008; MacDonald CJ. et al., 2021). In a recent work, MacDonald and colleagues also suggest that Hypertension and dyslipidaemia may not be risk-factors for VTE since in their study performed on both provoked and uVTE patients no association was observed between these traditional cardiovascular risk factors and the VTE risk after adjustment for BMI (MacDonald CJ. et al., 2021). Nevertheless, since a high incidence of atherosclerotic lesions and cardiovascular events has been reported in uVTE patients, these factors can be more associated with uVTE events (Migliacci R. et al., 2007).

Antiphospholipid antibody syndrome (APS). This autoimmune disease is characterized by the presence of antiphospholipid antibodies (aPLs) and by the occurrence of VTE and pregnancy morbidity (Ruiz-Irastorza G. et al., 2010; Levine JS. et al., 2002). This disease is characterized by recurrent venous or arterial thrombosis occurring in one third of patients (Cervera R. et al., 2002). Among APS patients, thrombotic risk is higher in triple positive patients and in patients that are positive for lupus anticoagulant (LAC) (Chayoua W. et al., 2018).

Prior VTE event. The VTE risk is increased in patients who showed a previous VTE event. This is true especially when the trigger(s) of the first VTE events cannot be removed (Ozen G. et al., 2021).

1.5.3 VTE pathogenesis

Starting from mid-twentieth, the so-called Virchow's Triad has formed the basis for understanding the pathogenesis of VTE and is still widely used to assess VTE risk (Figure 1.9) (Kumar DR. et al., 2010; Haley MP., 2017). Virchow's Triad was first described in 1856 and is composed by stasis, hypercoagulability and alteration/damage of the endothelium (Kumar DR. et al., 2010). Those three elements all contribute to alter the physiological vascular balance aimed to prevent excessive blood clotting thus concontributing to thrombus formation (Kumar DR. et al., 2010; Haley MP., 2017). In addition, these elements can also trigger VTE by acting alone or in combination as described in the "multiple hit hypothesis" (Bertina RM. 2003).

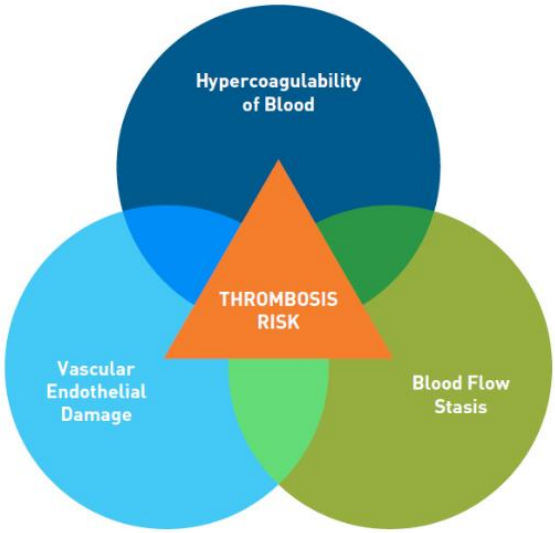

Figure 1.9| Virchow's triad *(Adapted from: Haley MP., 2017)*

Considering the first two elements of the Triad – namely the blood flow stasis and the presence of a state of hypercoagulability - they represent the mechanisms underlying the majority of VTE risk factors that are responsible for provoked VTE.

Stasis is involved in thrombosis triggered by immobilisation and those conditions that generate alterations in the blood flow (Bahloul M.et al., 2020). In particular, Stasis and the deriving hypoxia can induce endothelial activation and therefore ED that in turn promotes thrombosis (Akrivou D. et al., 2022; Bahloul M.et al., 2020; Borissoff JI., et al., 2011; van Hinsbergh VW., 2012).

Hypercoagulability is a state due to abnormalities in platelets as well as in the coagulation and fibrinolytic pathways (Chung I. & Lip GY., 2003) is characteristic of patients that carry genetic variance associated with thrombophilia (Di Minno P. et al., 2015).

Concerning the third element of the Virchow's Triad, which consists in the alteration of the endothelium, it was initially associated more to arterial thrombosis rather that to venous one especially when the term endothelial alteration is referred to vessel wall injury. In fact, Sevitt and colleagues showed in an autopsy study that the injury of the vessel wall was not a necessary prerequisite for venous thrombosis (Sevitt S., 1974) wheras its involvement in arterial thrombus formation has been observed (Chen J. & López JA., 2005) being mediated by the exposure of vWF and TF which in turn initiate the

coagulation processes (Chen J. & López JA., 2005). In accordance, arterial thrombi differ from venous ones. The first are characterized by a nucleus constituted by platelets (Chen J. & López JA., 2005) whereas venous thrombi are mainly characterized by fibrin with platelets being involved in the later stages of thrombi formation through their interaction with fibrin (Stone J. et al., 2017). Nevertheless, in the last years, the idea that arterial and venous thrombosis are two different disease entities has been challenged by emerging clinical evidence showing that VTE and arterial cardiovascular events are often associated and that these two conditions share common risk factors (Gresele P. et al., 2010; Riva N. et al., 2015). Moreover, emerging evidence indicates that alteration of the endothelial compartement consisting in the presence of ED are involved also in VTE pathogenesis. In particular, it was described that VTE patients have reduced flow mediated vasodilation and increased plasmatic levels of the endothelial damage-associated molecules P-selectin and vWF (Wolberg AS. et al., 2015; Migliacci R. et al., 2007; Jezovnik MK. et al., 2017). In addition, inflammation, ED, and thrombosis are strictly linked elements with inflammation that can be either the consequence or the cause of the VTE event (Mandel J. et al, 2022; Pendu R. et al., 2006).

Therefore, the presence of constitutive ED and its possible role in promoting thrombosis is an aspect that deserve to be investigated more in depth since it has been poorly characterized, so far. This aspect is particularly relevant especially in patients who experienced an uVTE event in whom the involvement in VTE pathogenesis of the other two elements of the Virchow's Triad (stasis and hypercoagulability) is excluded by the absence of kwnon VTE risk factors.

Studies demonstrated impaired endothelial function in patients characterized by uVTE events, compared with healthy donors (Migliacci R. et al., 2007) by reporting increased plasma levels of markers of endothelial activation/ endothelial damage markers, such as vWF and soluble P-selectin in uVTE patients (Migliacci R. et al., 2007; Jezovnik MK. et al., 2017). In a recent study, Bradbury and colleagues showed that EPC levels were lower in uVTE patients who later developed VTE recurrence (Bradbury C. et al., 2019).

In addition, by characterizing ECFCs obtained from uVTE patients it was also observed a pro-inflammatory profile and the presence of mitochondrial dysfunction (Alvarado-Moreno JA. et al., 2016). In further studies, ECFCs derived from such patients also showed reduced cell proliferation, increased senescence, increased ROS levels, and altered gene expression related to venous and arterial endothelial markers, particularly ephrin-B2/Eph-4, which promote vascular regeneration (Hernandez-Lopez R. et al., 2017). Finally, in a previous study we also confirmed the presence of increased early senescence in ECFCs obtained from uVTE patients and we identified the pathological upregulation of the TNFSF15-TNFRSF25 axis as possible mechanism involved in the observed ECFC alterations (Della Bella S. et al., 2020). Further studies based on the use of ECFC characterization as a tool to investigate ED in a patient-specific setting are needed to better understand the role of ED in the pathogenesis of uVTE, and to investigate whether ED is specifically involved in uVTE only, or it is also involved in the pathogenesis of provoked VTE.

1.5.4 VTE diagnosis

In order to make a diagnosis of VTE, it should be taken into account that both DVT and PE cannot be diagnosed only on the basis of signs and symptoms because the clinical presentation of VTE is usually nonspecific (Kearon C., 2016). In fact, the majority of VTE cases are often characterized only by pain and in case of PE the list of possible symptoms includes dyspnea, chest pain, presyncope or syncope, or hemoptysis (Michota F., 2005).

To diagnose VTE overcoming this criticism, clinicians therefore combine several elements.

Firstly, clinical anamnesis is taken into account to identify the possible presence of factor(s) known to trigger VTE (see in the dedicated section) and pre-test probability scoring systems based on a number of clinical features (i.s. Wells Pretest Probability Score) are applied to stratify patients on the basis of having either a PE or DVT (Cennamo E. et al., 2023; Bates SM. et al., 2012).

Then blood tests are performed to investigate the presence of genetic variants known to be associated with thrombophilia, such as FVL and PT variants (Di Nisio M. et al., 2007; EGAPP Working Group, 2011) and to measure the circulating levels of molecules known to be altered in case of thrombosis, such as D-dimer (Di Nisio M. et al., 2007). D-dimer - which is the product of cross-linked fibrin degradation - is a sensitive marker of thrombosis whose increased levels correlate with the presence of VTE (Di Nisio M. et al., 2007). Since it has been observed that D-dimer levels are increased in elderly, the use of test that apply an age-adjusted D-dimer threshold has been proposed to further increase the specificity of D-dimer level evaluation and its validation is still ongoing (Van Es N. et al., 2016). Finally, to confirm VTE diagnosis, imaging examination is also performed. In these regards, compressive ultrasonography represents the imaging test mostly used for the diagnosis of DVT because of its accuracy, simplicity, reproducibility and relatively low costs (Dronkers CE. et al., 2016). An alternative imaging technique for DVT assessment is represented by magnetic resonance that in a meta-analysis has been reported having for DVT diagnosis an estimated sensitivity of 93% and specificity of 96%, respectively (Abdalla G. et al., 2015). However, this diagnostic technique is not routinely used today.

Concerning PE, computed tomography pulmonary angiography (CTPA) represents a main test to diagnose PE and it allows to verify the presence of blood clots in the lungs (Cronin P. et al., 2008). Alternative imaging techniques for PE diagnosis also include magnetic resonance imaging (Konstantinides SV. et al., 2014) and V˙/Q˙ Scintigraphy and V˙/Q˙ Single-Photon Emission Computed Tomography (Bajc M. et al., 2019).

1.5.5 VTE Treatment

Regardless patient classification, VTE treatment can be subdivided in three main phases: the initial management in the days after VTE diagnosis, the primary treatment that can last up to 3 or 6 months and the secondary prevention that applies to those patients that required an anticoagulant treatment of indefinite duration (Beckman MG. et al., 2010; Palareti G et al., 2005; Kearon C., 2012) (Figure 1.10).

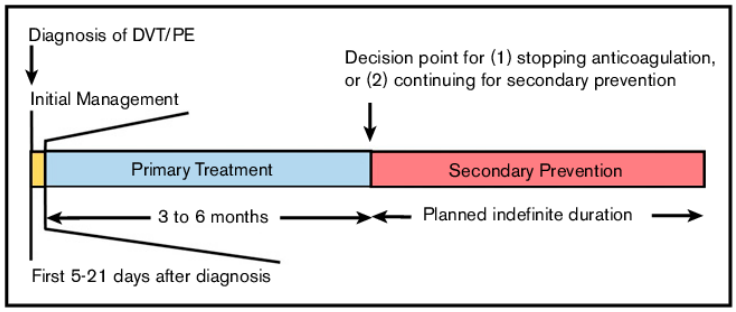

Figure 1.10 | Time frame of VTE management *(Adapted from: Ortel TL. et al., 2020).*

The initial management of VTE aims to either reduce the risk of recurrence and the risk of embolization (Roy PM. et al., 2023; Kearon C. et al., 2000). Besides anticoagulant treatments – that will be described later more in details - other therapies that are used for VTE patient treatment include thrombolytic drugs that are mainly indicated in case of large blood clots that can cause severe symptoms and complicantions such as in high risk PE patients with haemodynamic instability (Konstantinides SV. et al., 2020). Only in rare cases, Catheter-directed thrombolytic therapy is performed to remouved the blood clot (Imai K. et al., 2006).

Primary treatment and secondary prevention are aimed to further reduce the risk of VTE recurrence and this aspect is particularly relevant in uVTE patients and in patients whose VTE event has been provoked by a permanent risk factor (Beckman MG et al., 2010; Kearon C et al., 2012). In this context, anticoagulant therapies are the main treatment used and several class of drugs are available (Figure 1.11) (Ageno W. et al., 2012).

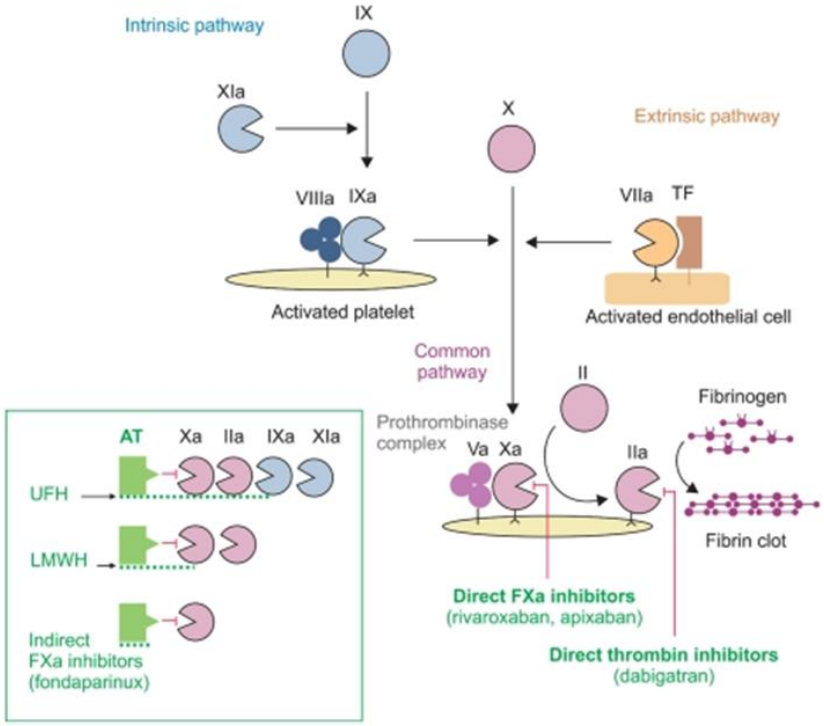

Figure 1.11 | Mechanisms of action of antithrombotic agents in the coagulation pathways *(Adapted from: Ageno W., 2010)*.

Heparin. Heparin exerts its anticoagulant activity by creating a complex with antithrombin 3 thus inactivating clotting factors. In its Unfractionated form (Unfractionated heparin - UFH) discovered more than a century ago, it is still considered the primary choice drug to prevent and to treat acute VTE (Bates SM, 2005; Bartholomew JR., 2017). It is considered the most used parenteral anticoagulant for ease of administration, rapid onset, short duration, and immediate reversal by protamine. Moreover, its action can be monitored with activated partial thromboplastin (aPTT), activated clotting time and factor Xa activity (Hirsh J. et al., 2001). Compared with UFH, Low-molecular-weight heparins (LMWH) are characterize by showing a higher ratio of anti–factor Xa to anti–factor IIa activity, greater bioavailability, a longer half-life, and a longer predictable anticoagulant response (Newall F. et al., 2013; Akhtar F. et al., 2018).

Due to its rapidity of action, heparin is often used in the phase of initial management alone or in combination of other anticoagulant drugs that are characterized by a slower activity during the transition to other therapies (i.e.

Vitamin K antagonists) (Kearon C. et al., 2012; Torbicki A. et al., 2008). In addition, guidelines for VTE treatment recommend the use of LMWH in patients with cancer and in pregnancy (Kearon C. et al., 2016b).

Vitamin K antagonists (VKAs). Since the active form of vitamin K is required for the synthesis of several clotting factors - such as II, VII, IX and X – VKAs (i.e. warfarin or dabigatran) are able to act as anticoagulants by exerting their activity 72-96 hours after administration (Bristol-Myers Squibb, 2014; Schein JR., et al., 2016). In particular, VKAs inhibit the recycling of inactive vitamin K epoxide back to its active reduced form through the inhibition of the enzyme vitamin K epoxide reductase (Bristol-Myers Squibb, 2014). Limiting factors for VKA treatment are represented by its narrow therapeutic window and by the fact that several factors including diet, drugs and genetic mutations can altered the VKA effect (Heestermans M. et al., 2022). For these reasons, VKAs and in particular Warfarin that in the past were mainly used for the long-term secondary prevention in VTE patients have been progressively replaced by direct oral anticoagulants (DOACs) (Heestermans M. et al., 2022; Kearon C. et al., 2012). Nevertheless, Warfarin remains the first choice for patients with renal disease, liver dysfunction, or those that are unable to undergo anticoagulat therapy with DOACs (Heestermans M. et al., 2022).

Fondaparinux. Fondaparinux is a synthetic penthasaccaride which binds antithrombin with high affinity and thus indirectly inhibits factor Xa (Ageno W., 2010). On the basis of MATISSE Study results, its use in combination with VKAs is approved for acute VTE treatment (van Doormaal FF. et al., 2009). Buller and colleagues showed its efficacy, safety and practicity and also demonstrated that the rates of VTE recurrence and major bleeding observed in patients treated with Fondaparinux were similar to thosed observed in patients treated with UFH (Büller HR. et al., 2004; Büller HR. et al., 2003). Fondaparinux has also been approved for VTE prophylaxis in case of undergoing hip fracture, hip or knee replacement, and abdominal surgery, but it is contraindicated in severe renal impairment and bacterial endocarditis (Bergqvist D., 2006).

Direct oral anticoagulants (DOACs). DOACs are anticoagulant drugs that specifically inhibit thrombin (i.e. Dabigatran) (Muñoz-Corcuera M. et al., 2016; Schulman S. et al., 2009; Schulman S. et al., 2014) or factor Xa (i.e. Rivaroxaban, Apixaban, Edoxaban) (Kubitza D. et al., 2005; Prins MH. et al., 2013; EINSTEIN Investigators, 2010; EINSTEIN–PE Investigators, 2012; Turpie AG., 2007; Hokusai-VTE Investigators, 2013; Agnelli G. et al., 2013; Ogata K. et al., 2010). Starting from their introduction, there are progressively replacing traditional anticoagulants especially for long-term therapies (Prandoni P. et al., 2002). In fact, DOAC dosing is easier since they are characterized by a lower susceptibility to drugs and food interactions compared with VKAs (Burnett AE. et al., 2016). This allows for DOACs a fixed-dose administration, avoiding continuous coagulation monitoring and subsequent dose adjustment, with predictable pharmacokinetics and pharmacodynamics (Burnett AE. et al., 2016). In addition, DOACs are characterized by a lower risk of bleeding than VKAs (Prandoni P. et al., 2002; Schulman S. et al., 2009; Schulman S. et al., 2014) and it has been recently shown that DOACs represent an alternative to LMWH for the treatment of cancer-associated VTE since the two treatments resulted in similar efficacy and safety (Agnelli G. et al., 2020; McBane RD. et al., 2020). Finally, the good pharmacodynamic profile and favourable response curve also make DOACs an excellent drug for the acute treatment of VTE (Connors JM., 2018).

Finally, other anticoagulant drugs targeting coagulation factors IX, XI and XII are also currently under development in order to obtain antithrombotic drugs characterized by a further reduction in bleeding risk (DeLoughery EP. et al., 2019; Rai V. et al., 2019; Büller HR. et al., 2015). In this context, Verhamme and collegues showed that Abelacimab by inhibiting factor XI successfully prevented VTE in subjects who underwent total knee arthroplasty and observed that the treatment was associated with a low risk of bleeding (Verhamme P. et al., 2021).

1.5.5.1 Duration of anticoagulant treatment and risk of VTE recurrence

The anticoagulant therapy after an acute episode of VTE last at least three months since it has been observed that if the treatment is discontinued before the completion of the active phase the risk of VTE recurrence doubled (Kearon C. 2012; Boutitie F. et al., 2011). After the initial acute treatment, clinicians have to decide whether to continue anticoagulation treatment to prevente VTE recurrence since it has been shown that anticoagulants effectively reduce the risk of recurrent VTE by 80% to 90% (Castellucci LA. et al., 2013).

In this scenario, the definition of anticoagulant therapy duration for secondary prevention of VTE represents one of the most important therapeutic challenges in VTE patient managment. In particular, it depends on two factors: the patient individual risk of thrombosis recurrence after anticoagulant discontinuation and the risk of major bleeding related to anticoagulant treatment (Rodger MA. & Le Gal G., 2018). In addition, in their decision, clinicians take also into account the type of index event, and the association between anticoagulation burden and quality of life.

Despite discrepancies in risk classification and guideline recommendations exist, current guidelines prescribe different duration of anticoagulation therapy based on each patient's risk of recurrence (Konstantinides SV. et al., 2020). In addition to the assessment of VTE risk factor presence and belonging category, other factors considered indicators of VTE recurrence (i.e. presence of Post-thrombotic syndrome, D-dimer levels and presence of residual obstruction) can be taken into account to define the therapy duration (Carrier M. et al., 2011; Notten P. et al., 2020; Kearon C. et al., 2016 a). In this context, scoring systems - such as the MenContinue and HERSOO2 scores, the Vienna risk model and the DASHP prediction score – have been proposed to assess the VTE recurrence risk on the basis of several patient-specific factors. Neverthelss, these scores do not appear to be used in clinical daily practice (Rodger MA. et al., 2008; Eichinger S. et al., 2010; Tosetto A. et al., 2012).

Considering the risk of recurrence, according to the guidelines proposed by the International Society on Thrombosis and Haemostasis (ISTH) the

anticoagulant treatment can be discontinued when the VTE recurrence risk is lower than 5% (Ebraheem M. et al., 2020). Accordingly, a long-term anticoagulation is recommended for patients with VTE episodes sustained by persistent risk factors that cannot be removed, as these patients have a high risk of VTE recurrence. Anticoagulant therapy can be discontinued after three months treatment in VTE patients with episodes caused by transient risk factors that can be removed, as these patients are characterized by a lower risk of VTE recurrence (Baglin T. et al., 2004). Notably, patients with uVTE, in which possible risk factors have not yet been identified and therefore cannot be removed, are characterized by a high risk of recurrence once anticoagulant therapy is discontinued (Khan F. et al., 2019). In particular, after the first episode of VTE the risk of recurrence attests to ~10% in the first year, ~25% in the first 5 years, and ~36% in the first 10 years (Agnelli G. et al., 2001; Rodger MA. et al., 2016). Therefore, long-term anticoagulant therapy should be carefully evaluated in these patients (Agnelli G. et al., 2001; Rodger MA. et al., 2016).

Indeed, recommendations on the duration of anticoagulant treatment for uVTE patients are still debated and controversial because in the evaluation of the risk/benefit ratio of a prolonged anticoagulant treatment the bleeding risk deriving from the therapy has to be taken into account. In these regards, in accordance with the 2016 American College of Chest Physicians (ACCP) guidelines, anticoagulation treatment should be prolonged in uVTE patients with a low-to-moderate bleeding risk, whereas prolonged anticoagulation should be avoided in patients with an annual risk of major bleeding higher than 6% (Kearon C. et al., 2012).

Continued anticoagulation carries the potential harm of major haemorrhage, which is fatal in about 11% of cases (Carrier M. et al., 2010). In recent years, the low bleeding risk that characterizes DOACs compared with VKAs has led to consider prolonged rather than limited duration anticoagulation therapies for uVTE (Chai-Adisaksopha C. et al., 2015).

In this scenario, the identification of pathogenic mechanisms involved in uVTE events is a key point that deserves to be deeply investigated, as the deriving information will be useful for patient stratification and will improve patient management by providing indications for the optimal type and duration of the therapy thus reducing the risk of recurrence without exposing patients to excessive risk of major bleeding derived by the prolonged anticoagulant treatments.

2. AIM OF THE STUDY

Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common and potentially fatal condition (Carrier M. et al., 2011). Due to VTE complexity as a multifactorial disease (Heit JA., 2015), VTE patients can be classified on the basis of the risk factor(s) that trigger the thromboembolic event. Indeed, up to 50% of VTE events occur in the absence of predisposing conditions and are therefore classified as unprovoked VTE (uVTE). Being uVTE pathogenesis still unknown, uVTE patients are characterized by a high risk of recurrence and their therapy management in terms of the optimal treatment duration is still controversial. In fact, on one hand the benefits deriving from anticoagulant treatment fall in uVTE patients upon anticoagulation discontinuation (Couturaud F. et al., 2015), on the other hand a prolonged treatment with anticoagulants carries a non-negligible risk of major bleeding of about 1-3%/year (Kearon C. et al., 2016b) with a significant associated morbidity and mortality (Linkins LA. et al., 2003).

Considering the central role of endothelial dysfunction (ED) in promoting thrombus formation, investigating possible alterations of the endothelial compartment in uVTE patients is crucial to investigate the role of ED in uVTE pathogenesis. The identification of endothelial compartment alterations underlying the pathogenesis of uVTE events may thus possibly provide indications useful to guide patient treatment through the definition of new clinicopathological entities and the definition of molecular targets for the development of novel therapeutic strategies.

Endothelial colony-forming cells (ECFCs) represent an optimal model for the non-invasive functional characterization of the endothelial compartment. Therefore, in this study we *in vitro* characterized patient-derived ECFCs to investigate ED in uVTE patients. In particular, we aimed to:

- Set up and optimize ECFC-based functional assays aimed at assessing thrombogenic endothelial properties:

In particular, we modified the commonly used thrombin generation assay (TGA) and thrombogenesis assay, by using ECFCs as a substrate.

- Investigate the presence of constitutive ED in uVTE patients

  To this aim, ECFCs obtained from uVTE patients and healthy donors (HDs) were characterized by assessing: i) their immunophenotype; ii) their ability to promote thrombosis in ECFC-based—TGA and thrombogenesis assay; iii) their secretion of soluble ED markers (soluble adhesion molecules including ICAM-1, VCAM-1 and PECAM-1) in culture supernatants; to assess the *in vivo* relevance of these markers, they were also measured in the plasma of the same patients; iv) their transcriptomic profile assessed by RNA sequencing to investigate possible molecular pathways underlying ED in uVTE.

- Investigate whether ED in uVTE patients is further promoted by pro-inflammatory stimulation

  To this aim, the phenotype and the ability of the ECFCs obtained from uVTE patients and HDs to promote thrombosis were assessed after stimulation with TNFα by using the same functional assays used to study constitutive ED.

- Investigate ED contribution to the VTE recurrence risk in uVTE patients

  To this aim, possible correlations between EFCF features of ED and validated clinical predictors of VTE recurrence were investigated.

- Investigate whether EFCF features of ED are restricted to uVTE or shared with secondary VTE

  To investigate this issue, patients with secondary VTE – classified as weakly provoked (wp) and provoked (p) VTE patients – were also enrolled in the study, and ECFCs obtained from these groups of patients were characterized by using the same strategy applied to uVTE ECFCs.

3. MATERIAL AND METHODS

3.1 Subjects

3.1.1 Subjects enrolled to set-up ECFC-based thrombin generation assay (TGA) and thrombogenesis assay

In collaboration with Thrombosis and Haemostasis Center at Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi (Varese) and with the Thrombosis and Haemorragic Diseases Center at Humanitas Research Hospital (Rozzano, Milan), 8 healthy donors were enrolled in the study and underwent a peripheral blood draw that was used to isolate ECFCs and to perform *in vitro* functional assays. Subjects with no history of thrombosis or haemorrhage and without having taken anti-inflammatory drugs for at least 10 days were included in the study.

The study protocol was approved by the institutional review boards (IRB) of Humanitas Research Hospital (ICH 2014/1297) and informed consents were provided to all participants before inclusion in the study in compliance with the Declaration of Helsinki.

3.1.2 Subjects enrolled to investigate ED in uVTE pathogenesis

In collaboration with Thrombosis and Haemostasis Center at Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi (Varese) and with the Thrombosis and Haemorragic Diseases Center at Humanitas Research Hospital (Rozzano, Milan), a total of 78 VTE patients were recruited in this study and were classified as:

- pVTE when the VTE event was associated with the following risk factors occurring during 3 months before VTE: major surgery, admission to hospital with an acute illness, estrogen therapy, confinement to bed/ reduced mobility for at least 3 days for an acute illness, major trauma/leg injury (Kearon C. et al., 2016a);

- wpVTE when the VTE event was associated with minor surgery; recent long-haul flight/road travel or reduced mobility not qualifying for major risk, minor injuries (all during 1 month before VTE); arterial hypertension, dyslipidemia, varicose veins/venous insufficiency, chronic renal impairment (Heit JA. et al., 2002);
- uVTE when the VTE event was associated with no predisposing risk factors described above.

The following exclusion criteria were considered:

- factors interfering on endothelial function: age > 65 years; active cancer; chronic inflammatory diseases; autoimmune disorders; diabetes mellitus; drugs known to influence ECFC number and function (i.e. statins, angiotensin-converting enzyme inhibitors, calcium antagonists and metformin);
- previous VTE event(s) with a different pathogenesis than the index event;
- known major thrombophilia;
- pregnancy and puerperium.

VTE patients' characteristics are summarised in Table 3.1.

Characteristics		uVTE	wpVTE	pVTE
Number n		22	28	28
Sex Male/Female		18/4	14/14	15/13
Age Years; Mean (Range)		48.5 (21-59)	45.9 (22-60)	45.0 (19-62)
Type of Thrombotic Event	DVT	14	17	14
	PE	5	6	7
	DVT+ PE	3	5	7
Therapy at the moment of ECFC assessment	AVK	0	0	2
	DOAC	19	28	19
	LMWH	1	0	1
	No therapy	2	0	6

Table 3.1 | VTE patients' characteristics and therapy at the moment of ECFC assessment. *DVT, deep vein thrombosis; PE, pulmonary embolism; AVK, anti-vitamin K; DOAC, Direct oral anticoagulant; LMWH, Low-molecular-weight heparin.*

Three months after the index VTE event, all recruited VTE patients underwent blood sampling for ECFC isolation and plasma collection and assessment of the following validated predictors of VTE recurrency:

- Post Thrombotic Syndrome (PTS). PTS was assessed by Villalta scale (Kahn SR. et al., 2009). PTS is diagnosed on the basis of the presence of clinical signs and typical symptoms that can differ in character and severity among patients (Kahn SR., 2006). In this context, the Villalta scale, evaluated 6–36 months after venography-confirmed DVT (Villalta S. et al., 1994), consists of 5 patient-rated venous symptoms (pain, cramps, heaviness, paresthesia, pruritus) and 6 clinician-rated physical signs (pretibial edema, skin induration, hyperpigmentation, pain during calf compression, venous ectasia, redness). They are each assessed on a 4-point scale (0 = none, 1 = mild, 2 = moderate, 3 = severe). The amount of different points led to a final score, with a range equal to 0-33. Patients were associated to PTS if the final score was ≥ 5, or in presence of a venous ulcer in a leg with previous DVT. The

severity of PTS was classified as “mild/moderate” in case of the score was 5–14, and as “severe” in case of the score was ≥ 15 or in presence of a venous ulcer, despite the total score. In recent scientific works, the “mild/moderate” class has been subdivided as “mild” if the score is 5–9 and “moderate” if the score is 10–14 (Kahn SR. et al., 2008; Caprini JA et al., 2003).

- D-dimer levels. Plasmatic concentration of D-dimer was assessed and D-dimer levels were considered altered when higher than 250 µg/L (Rodger MA et al., 2017).
- Residual obstruction. The presence of a residual obstruction was assessed with different techniques according to the district in which the index event occurred. In particular:
    - in DVT patients, Residual Venous Obstruction (RVO) was assessed by ultrasound examination. According to the method of Prandoni (Donadini MP. et al., 2014, Prandoni P. et al., 2002), RVO was evaluated by compression ultrasonography (CUS) of the common femoral and popliteal veins in DVT patients to measure the diameter of any residual thrombus in the common femoral, superficial femoral and popliteal veins. Veins were defined recanalized if they were 2.0 mm or less in diameter on a single test or 3.0 mm or less in diameter on two consecutive tests.
    - in PE patients Residual Pulmonary Vascular Obstruction (RPVO) was assessed by perfusion lung scan (Le Pennec R. et al., 2022). RPVO is based on planar scintigraphy of lung ventilation/perfusion V/Q through the Meyer score, validated using pulmonary angiography as a reference standard (Le Pennec R. et al., 2022). Each lung lobe is assigned a weight depending on the local distribution of pulmonary blood flow. Perfusion within each lobe is assessed with a semi-quantitative perfusion score based on the extent and severity of perfusion impairment. The perfusion score of each lobe is then calculated by multiplying the lobe weight by the perfusion score and the overall perfusion score is evaluated.

Considering the same exclusion criteria, 27 sex- and age- matched healthy donors (HDs) were enrolled and subjected to a blood draw dedicated to ECFC isolation and plasma collection.

The study protocol was approved by the institutional review boards (IRB) of Humanitas Research Hospital (595/21) and Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi (75/19) and informed consents were provided to all participants before inclusion in the study in compliance with the Declaration of Helsinki.

3.2 Endothelial cells

3.2.1 Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs are mature ECs obtained from Human Umbilical Veins and are considered one of the most commonly used model in cardiovascular research. Commercially-available HUVECs (Merck Millipore, Billerica, MA) grown in adhesion in culture plates (Sigma Aldrich, Missouri, USA) and flasks (Sigma Aldrich, Missouri, USA) precoated with type-I collagen (Corning, USA), and were cultured in Endothelial growth Medium-2 (EGM2, Lonza, Basel, Switzerland) supplemented with 5% foetal bovine serum (FBS, Avantor, VWR International; Radnor, USA).

Specifically, collagen coating was performed using collagen diluted in 0.02 N acetic acid at a final concentration of 50 µg/mL. Diluted collagen was incubated for 90 minutes at RT for its polymerisation. Just before seeding the cells, the excess liquid was removed from the surface and the culture plates were washed twice with PBS without Ca2+ and Mg2+.

Concerning the culture medium, EGM-2 was composed by Endothelial Cell Basal Medium- (EBM^{TM}-2) and EGM^{TM}-2 SingleQuots^{TM} supplements - hydrocortisone, hFGF, VEGF, R3-IGF-1, hEGF, heparin and ascorbic acid - (Lonza, Basel, Switzerland) and is a culture.

3.2.2 Endothelial Colony Forming Cells (ECFCs)

The isolation and expansion of ECFCs were performed according to a protocol previously optimized in our laboratory (Colombo E. et al., 2013) and already used to study ECFCs under different physiological and pathological conditions (Della Bella S. et al., 2020; Abdel Hadi L. et al., 2018; Calcaterra F. et al., 2017). In particular, ECFCs were isolated and expanded starting from peripheral blood mononuclear cells (PBMCs) derived from fresh heparinized blood samples of both HDs and VTE patients (Figure 3.1).

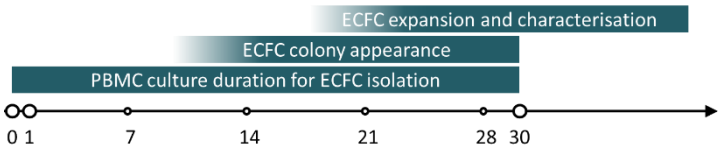

Figure 3.1 | ECFC culture timeline. PBMCs isolated by density-gradient centrifugation were seeded in fibronectin-coated mw24 culture plates in EGM2-5% FBS. The first day after seeding, debris and non-adherent cells were removed. For 30 days, plates were daily visually inspected for identifying ECFC colonies that usually appears starting from day 10 of PBMC culture. Once identified, ECFC colonies can be detached and then expanded over several passages culturing them in EGM-2+5% FBS on collagen-coated culture plates/flasks. Obtained ECFCs can be characterized and used in *in vitro* assays.

3.2.2.1 Mononuclear cell isolation from peripheral blood

In this project, about 50 mL of peripheral blood were drawn from either HDs and VTE patients. PBMCs were isolated by density-gradient centrifugation using Lympholyte®-H Cell Separation Media (Cedarlane, Burlington, North Carolina, USA) according to manufacturer's instructions. For this purpose, the following protocol was used:

1. Heparinized blood samples were centrifuged (1400 rpm for 10 minutes) to separate blood cellular components from plasma.
2. After plasma removal, blood was diluted with Hanks' Balanced Salt solution (HBSS) -/- 1x (Lonza, Basel, Switzerland) in 50mL Falcon tubes (BD, New Jersey, USA) at a volume ratio 1:2.
3. *Lympholyte*®-H Cell Separation density gradient solution (Cederlane Laboratories, Burlington, Canada) was then stratified below the

blood/HBSS solution obtaining two separated phases (Volume ratio between Lympholyte and blood/HBSS solution: 1:3).

4. Samples were then centrifuged for 30 minutes at 400 rcf at 20°C without neither acceleration nor brake.
5. After centrifugation, PBMCs that stratified forming an interphase ring at the interface between *Lympholyte*®-H and HBSS -/- 1x, were collected in a new 50mL Falcon (Figure 3.2).
6. Collected PBMCs were washed twice with HBSS-/-.
7. After centrifugation, the supernatant was removed and the PBMCs were resuspended in EGM-2 + 5% FBS and counted in a Burker counting chamber. Cell viability was tested with trypan blue (Sigma-Aldrich, Missouri, USA).

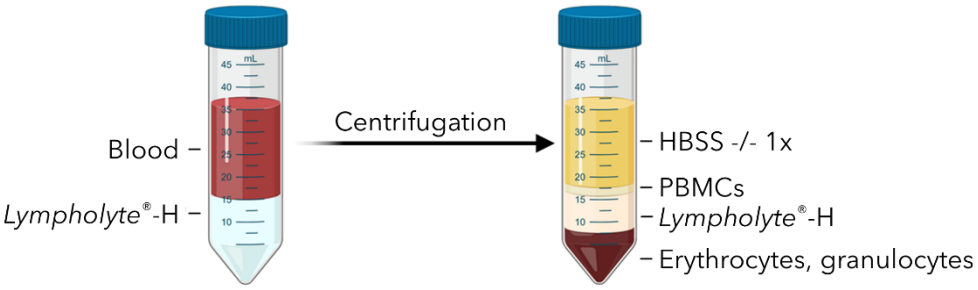

Figure 3.2 | Illustration of the density gradient procedure used to isolate PBMCs. On the left, the stratification of the diluted blood on top of the Lympholyte®-H; on the right, the separate layers observed after centrifugation.

3.2.2.2 ECFC isolation

Once isolated, PBMCs derived from fresh peripheral blood samples were resuspended in EGM2 + 5% FBS and seeded at a density of 2.5 x 10^6 cells/cm^2 in mw24 culture plates pre-coated with fibronectin (Sigma Aldrich, Missouri, USA). Fibronectin was first diluted in phosphate-buffered saline, pH 7.4 without Ca2+ and Mg2+ (PBS, Euroclone, Milan, Italy) to obtain a final concentration of 1 µg/cm^2. The fibronectin coating was incubated for 45 minutes at room temperature (RT) to allow its polymerisation. Before seeding the cells, excess liquid was removed from the surface.

Seeded PBMCs were maintained in culture at 37°C with 5% CO_2. One day after the start of PBMC culture, non-adherent cells and debris were removed,

while cells adhering to the substrate were washed with EGM-2 + 5% FBS medium and fresh medium was added to each well. For the following 30 days, the culture medium was changed every two days and the appearance of ECFC colonies was monitored daily with an inverted microscope. ECFCs colonies were identified as well-circumscribed monolayers of adherent cells endowed with *cobblestone-like morphology*.

In order to determine the efficiency of ECFC isolation, the number of PBMCs seeded, the number of colonies isolated and the time of colony appearance - estimated as the number of days from the day of PBMC seeding - were reported for both HDs and VTE patients.

3.2.2.3 ECFC expansion

After identification, ECFC colonies were detached using Trypsin-EDTA 1x w/o Ca_{2+} and Mg_{2+}, w/o Phenol Red (Euroclone, Milan, Italy) and transferred in EGM-2 + 5% FBS into 24-well tissue culture plates (Corning, USA), previously coated with type-I collagen (Corning, USA). Collagen coating was prepared as previously described in paragraph 3.2.1.

The sub-confluent cells were further expanded in collagen-coated culture plates and/or flasks in EGM-2 + 5% FBS and seeded at a density of 5000 cells/cm^2 until the cells became senescent.

3.3 EC characterization

To characterize ECFCs, the frequency of early sencescent colonies was assessed. Moreover, the activation status of ECs – both HUVECs and ECFCs - was assessed by evaluating the expression of the adhesion molecule VCAM-1 and the pro-coagulant factor TF, and by performing functional assays aimed to evaluate thrombin generation in TGA on ECs and to assess fibrin formation and platelet deposition on EC layers (thrombogenesis assay). To assess the response to pro-inflammatory stimulation, ECs - both HUVECs and ECFCs - were incubated with TNFα (20 ng/mL, PeproTech, Massachusetts, USA) for 4 hours.

3.3.1 Early senescence evaluation

ECFC senescence was assessed by using the same criteria that we already used in a previous work (Della Bella S. et al., 2020). In particular, ECFC senescence was characterized by growth cell arrest and a change in morphology - assessed by visual inspection with optical microscope (Florido R. et al., 2011). Senescence was also confirmed by the positivity for senescence-associated β-galactosidase (SA- β -Gal) activity, one of the most widely used markers of senescence. In particular, a commercial kit (Cellular Senescence Assay Kits, Merck Millipore) was used to perform the senescence-associated β-galactosidase assay, in line with the manufacturer's instructions. In the Cellular Senescence Assay, the substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) is cleaved by beta-galactosidase in senescent cells generating 5-bromo-4-chloro-3-hydroxy-1H-indole, which immediately dimerizes to give an intensely blue product. The blue precipitate thus allowing the identification of senescent cells by using an inverted microscope (Figure 3.3).

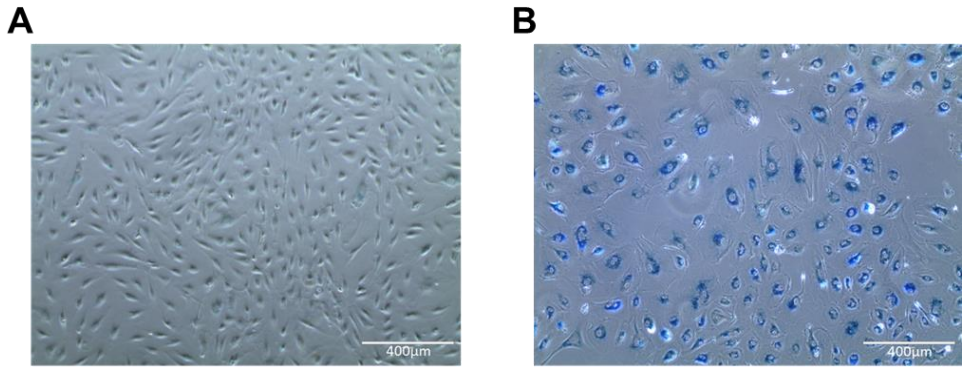

Figure 3.3 | Senescence-associated β-galactosidase activity. A) Representative image of viable cells where no blue precipitate was formed when senescence-associated β-galactosidase activity was tested. B) Representative image of senescent cells characterized by altered morphology and expression of high levels of senescence-associated β-galactosidase activity, identified by the blue/green precipitate. Images were obtained with Olympus IX51 inverted microscope at 10x magnification.

3.3.2 EC phenotypic characterization by flow cytometry

In order to investigate the activation state of ECs, the expression levels of VCAM-1 and TF were assessed by flow cytometry. Experiments were performed on both HUVECs and ECFCs cultured either in basal condition or after TNFα stimulation (Peprotech, 20 ng/ml, 4h).

In order to proceed with the cell staining, ECs were first collected by detaching them from the culture plate using Accutase Cell Detachment Solution (Merck-Millipore, Billerica, MA). The harvested cells were stained with the viability marker Zombie Aqua dye (BioLegend, San Diego, CA) for 15 min at RT. ECs were then washed with MACS buffer (HBSS 1x -/-, 2% FBS, 2mM EDTA) and stained with FITC-conjugated anti-human VCAM-1 monoclonal antibody (AbD Serotec, UK) and PE-conjugated anti-human TF monoclonal antibody (BD PharMingen, San Diego, CA) for 20 minutes at RT. After incubation, cells were washed using MACS buffer and then fixed with 1% paraformaldehyde (PFA, Sigma-Aldrich, Missouri, USA) for 20' at RT. The fixed cells were acquired with the FACSCanto II flow cytometer (Becton-Dickinson) within 24 hours.

Standard Flow Cytometry (.fcs) files were analysed with FlowJo software (v 10.8.1, TreeStar Inc, Ashland, Oregon, USA) and compensated using single-staining antibody capture beads (CompBeads, BD Biosciences). Cells were

electronically gated based on light scattering properties to ignore cell debris. FACS data were evaluated as frequency of positive cells gated on viable ECs or as Mean Fluorescent Intensity (MFI) (Figure 3.4). Fluorescence Minus One (FMO) controls were used as negative controls.

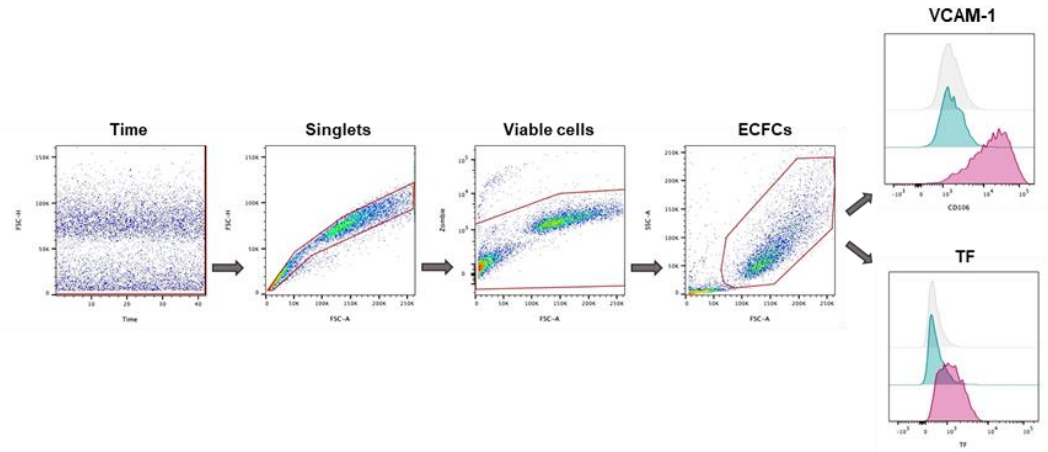

Figure 3.4 | Gating strategy. Gating strategy applied to detect VCAM-1+ and TF+ ECFCs by dot plot and representative flow cytometry histograms. In histograms: in gray FMO, in green Stained cells cultured in basal condition, in purple Stained cells stimulated with TNFα.

3.3.3 EC functional characterization

To better understand ED involvement in thrombus formation, a modified thrombin generation assay (TGA) and a thrombogenesis assay were performed on ECs. In particular, both assays were first tested on HUVECs and ECFCs isolated from HDs cultured either in basal condition and after TNFα stimulation to set up the assays and evaluate whether ECFCs can be used as substrate. Then ECFCs isolated from HDs and from the different subgroups of VTE patients – cultured either in basal condition and after TNFα stimulation – were used as substrate in the assay to investigate ED in uVTE patients.

3.3.3.1 Thrombin generation assay (TGA) on ECs

Thrombin generation assay (TGA) represents a test used to evaluate coagulation and thrombotic risk. It allows to evaluate the potential ability of plasma to produce thrombin over time once coagulation has been activated by the addition of TF (Hemker HC. et al., 2006). In order to study ED, this standard

method reported by Hemker and co-workers, was modified using ECs as endogenous substrate and source of TF in association with Platelet rich plasma (PRP) derived from blood samples collected from HDs. In particular, to study ED in the context of uVTE, ECFCs isolated from HDs and from VTE patients were used as substrate in the assay.

Platelet rich plasma (PRP) preparation. Venous whole blood samples obtained from HDs were collected in tubes containing sodium citrate (blood:sodium citrate ratio 9:1) and platelet's count was estimated with hematology analyzer DXH 800 (Beckman Coulter, Germany). In order to obtain PRP, whole blood was centrifuge (150g for 10 minutes). Platelet's count in PRP was also estimated with hematology analyzer DXH 800 (Beckman Coulter, Germany). An aliquot of PRP was further centrifuge (2500g for 10 minutes) in order to obtain Platelet poor plasma (PPP) that was used to adjust platelet count in PRP at the original platelet concentration before using PRP samples in TGA on ECs. PRP samples were analyzed within 3 hours after collection.

TGA on ECs. ECs were detached with Accutase Cell Detachment Solution (Merck-Millipore) and, once counted, cell concentration was adjusted with DPBS (Sigma-Aldrich) in order to place 20.000 ECs/well in a 96-well-plate by adding 20 µl of cell suspension/well. 80 µl of PRP were then added in each well of the TGA plate. Calibrated Automated Thrombography and Fluoroskan Ascent Fluorometer (Thermoscientific, Finland) measured thrombin generation, after the addition of a mixture of $CaCl_2$ and fluorogenic substrate (Fluo kit, Diagnostica Stagò, France). Thrombin calibrator (Thrombin Calibrator, Diagnostica Stagò, France), in which the concentration of thrombin was set, defined each condition. Thrombin generation (TG) or "*thrombogram*" curve was defined by the following three parameters (Figure 3.5):

- Lag time, corresponding to clotting time and defined as the time required to initiate thrombin generation;
- thrombin Peak, the maximum thrombin concentration and thus associated with hyper- or hypo-coagulability;

- endogenous thrombin potential (ETP), which represents the area under the curve and thus the net amount of thrombin that can be generated under the experimental conditions.

Those parameters were calculated by Thrombinoscope software (version 5.0, Thrombinoscope BV, The Netherlands).

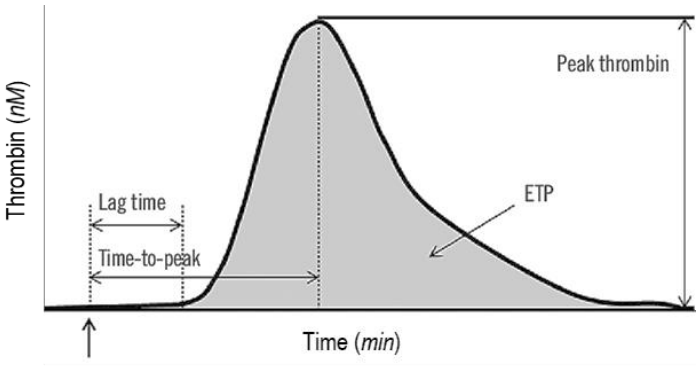

Figure 3.5 | Thrombin generation curve with related parameters. Tissue factor generates thrombin along the reaction time, resulting in a thrombin generation curve characterized by: Lag Time (minute, min); thrombin Peak (nM); ETP (nM•min) *(Adapted from: Kim JA. et al., 2015).*

3.3.3.2 Thrombogenesis assay

Endothelial function was assessed by means of a modified thrombogenesis assay in *ex vivo* perfusion experiments. In this context, platelet deposition and fibrin formation occurring on EC monolayers during thrombus formation under blood flow conditions were specifically evaluated both in ECs cultured under basal condition and after TNFα incubation.

EC preparation and chamber characteristics. A glass- coverslip (24 X 50 mm, Deckglaser Fisher scientific, Germany) was coated with type I collagen and used as a substrate to seed the ECs. Once confluent, the seeded cells were stained with AF647-conjugated anti-human CD31 monoclonal antibody (BD PharMingen, San Diego, CA) or with BV421-conjugated monoclonal anti-human CD31 antibody (BioLegend, San Diego, CA) diluted 1:100 in HEPES buffer (20mM Hepes, 135m NaCl, pH 7.4) additioned with 5% FBS. ECs were contained in a flow chamber (Wang YX. et al., 2016), with a flow channel defined by a silicon layer of 3 cm length, 0.3 cm width and 0.0125 cm height,

in which whole blood perfused the cells through an inlet and an outlet (Figure 3.6).

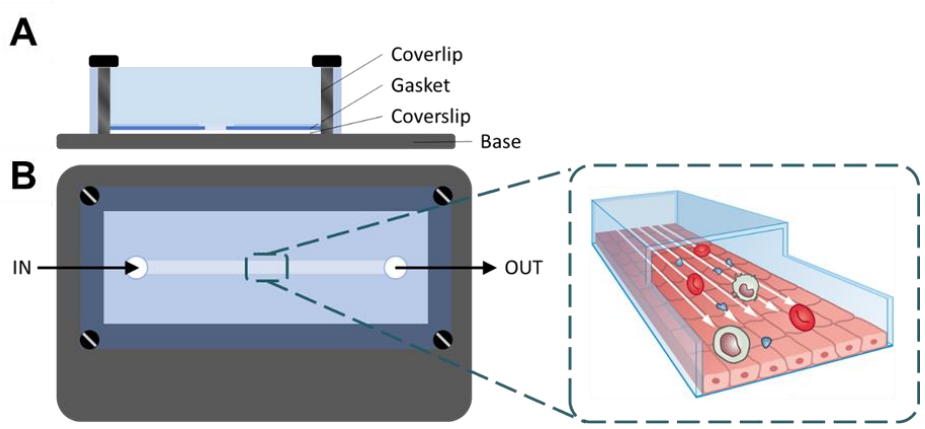

Figure 3.6 | Perfusion chamber. A) Side view of the perfusion chamber. The chamber comprises a metal base in which it is installed the coverslip on which the ECFCs were previously grown. The central channel is defined by a silicone gasket placed over the coverslip. Finally, the entire chamber is protected by a transparent plastic coverslip. B) Top view of the perfusion chamber, with illustration of the channel details (*Adapted from:* Cell in focus, ibidi).

Preparation of whole blood samples for perfusion. Blood samples that were perfused in thrombogenesis assays were drawn from healthy donors and collected in sodium citrate that prevents the clotting of the blood by binding calcium. Before perfusion, 5 µl of FITC conjugated mouse anti-human GpIβ (CD42b, Beckman Coulter, Germany), and 1 µg/ml of R-PE or APC conjugated mouse anti-human fibrin (MERU VasImmune, USA) monoclonal antibodies were used to stain 500 µl of whole blood, for 10 minutes. The anti-GpIβ antibody specifically bind GpIb on platelets thus allowing the detection of platelet aggregates. The anti-fibrin antibody used targets a human fibrin neo-epitope that generates only at the moment of fibrinogen cleavage by thrombin; this reaction thus allows a precise and specific measurement of fibrin and the real-time monitoring of its production. Stained whole blood was then reconstituted with 10 mM of $CaCl_2$ immediately before perfusion.

Thrombogenesis assay. To detect platelet deposition and fibrin formation, the previous described chamber was installed on an inverted confocal microscope (TCS Sp5, Leica Microsystems, Germany). To mimic the physiological shear rate, the ECs were perfused with stained whole blood in the flow chamber for

5 minutes at a regular rate of 300^{-1}sec at 37°C by using a syringe pump (New Era Pump System Inc., USA). Immediately after perfusion of the cells, platelet and fibrin deposition, derived from endothelial activation and TF exposure, were assessed by Z-stacks acquisition. Laser intensity, voltage, magnification and pinhole aperture, which constitute the microscope setup, were kept constant to improve the matching of the different experimental sessions (Guglielmini G. et al., 2016). The assessment of adhesion and confluence of stained ECs was performed before and after perfusion and the corresponding images were acquired using the same position for z-stack acquisitions. Several z-stacks were acquired along the channel to average the measured values at 3 locations.

Image analysis. Platelet deposition and fibrin formation were examined using ImageJ software (NIH, USA). The images, 512 X 512 pixels, were acquired at 40X magnification in LIF (.lif) format and then converted to 8-bit greyscale binary images. Z-stacks were acquired with a size of 0.97 µm. To analyse the obtained planes along the frame, thresholds were set for GpIβ and Fibrin signals in order to exclude the background (Machin M. et al, 2006). Images collected from the same samples before perfusion were used as negative controls. The analysis was based on the area occupied in each plane by the platelet deposits and fibrin, to assess the percentage of covered area; moreover, through the sum of the areas multiplied by the z-stack pitch (0.97 µm), the volume (μm^3) was calculated.

3.4 Evaluation of soluble markers of ED

In order to further investigate ED in uVTE, a bead-based immunofluorescent assay (Human Adhesion Molecule- Mix and Match Subpanel; LEGENDplex™, Biolegend) was used according to manufacturer instructions to evaluate the concentration of the soluble forms of ICAM-1, VCAM-1, E-selectin, and PECAM-1 (CD31) either in the ECFC culture supernatant and in the plasma samples of VTE patients and HDs. These markers were selected since the soluble form of the adhesion molecules are considered soluble markers of ED (Kjaergaard AG. et al., 2013).

LEGENDplex™ exploits the same principles as sandwich immunoassays but allows the simultaneous analysis of multiple analytes by flow cytometry since the different analytes are bound by different set of beads that can be discriminated on the basis of their size and APC fluorescence levels. Each detection antibody binds to its specific analyte bound on the capture beads. Streptavidin-phycoerythrin (SA-PE), binding to the biotinylated detection antibodies, was then added and incubated thus allowing the production of fluorescent signal with intensities which is proportional to the amount of bound analyte. For each bead population, the PE signal fluorescence intensity was measured using LSRFortessa™ (BD). The concentration of every single soluble molecules was defined based on a known standard curve using the LEGENDplex™ data analysis software. Concentration data in supernatant were normalized on basis of cell number.

3.5 Transcriptome profiling of ECFCs

In order to investigate the mechanisms underlying ED in uVTE patients, ECFC transcriptome profile was evaluated by performing a RNA sequencing analysis on a total of 15 samples (3 uVTE, 4 wpVTE, 4 pVTE, and 4 HDs).

RNA sequencing (RNA-seq) represents an application of Next Generation Sequencing (NGS) that allows a deep investigation of gene expression. Therefore, RNA-seq technology enables the quantification of the abundance level or relative changes of each transcript under specific condition (Ritchie ME. et al., 2015), thus providing the comparative analysis of the global expression level in different conditions, as in this project.

RNA-seq workflow included substantially three sections: Experimental Biology, Computational Biology, and Systems Biology (Subramanian A. et al., 2005) (Figure 3.7).

Experimental Biology includes RNA extraction and reverse transcription followed by library construction. Libraries were then sequenced producing millions of short reads per sample. Computational Biology analyzes the raw reads generated by experimental procedures which are the starting point for further bioinformatics analysis. It includes the pre-processing and quality check of the reads, followed by the alignment to a reference genome and the normalization of the data. Finally, the systems biology approach analyses both variability within experiments and differential gene expression. It implements pathway-level analyses to obtain a biological understanding of RNA-seq data. In this regard, several computational programs in constant development were required due to the complexity of databases generated by RNA-seq technology. In the following section, these steps will be described in detail.

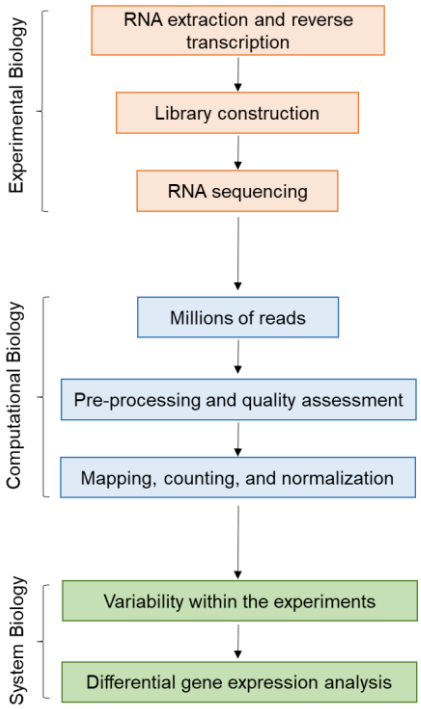

Figure 3.7 | RNA-sequencing (RNA- seq) workflow. RNA-seq workflow can be divided in three sections: Experimental Biology, Computational Biology, and Systems Biology *(Adapted from Han Y et al., 2015).*

3.5.1 RNA isolation

25000 cells per sample were used to prepare cell lysates. ECFCs were first lysed and then homogenized by using a denaturing buffer, which immediately inactivates RNAses to allow the isolation of whole RNA. In order to extract RNA, the RNeasy® Plus Micro Kit™ (Qiagen®) was used according to the manufacturer's instructions. Briefly, since the purified RNA is RNase-rich, β-Mercaptoethanol (β-ME) (Sigma- Aldrich, USA) was added to the RLT buffer before use. Then a solution composed by RLT lysis buffer + β-ME additioned with Recombinant RNAsin Ribonuclease Inhibitor (Promega, USA) was added to samples to lysate cells. The sample lysates were then homogenised by vortexing for 1 minute.

The homogenized lysates obtained were transferred to a gDNA Eliminator spin column. Indeed, each column was centrifuge and then discarded, saving the flow-through thus removing genomic DNA from samples. Since purification of total RNA containing small RNAs from cells was performed, ethanol 100% was

added to the flow-through deriving from the gDNA Eliminator spin column. After mixing, samples were transferred to an RNeasy MinElute spin column and RPE buffer was added; finally, the samples were centrifuged. In these steps, the liquid collected in the tube below the column is discarded. During centrifugation, it is essential to dry the spin column membrane, as ethanol interference can interfere with downstream reactions.

Finally, the tube below the column was changed to a new 1.5 mL RNAse-free tube (Bisosphere®) and 14 µL of RNAse-free H_2O were added to the column. After 1 minute, columns were then centrifuged to elute the RNA. The extracted RNA was collected in the tube below and the column was discarded.

3.5.2 RNA sequencing

3.5.2.1 Pre-processing and quality assessment

Sample quality assessment is the first step to allow sequencing analysis. RNA quality control was performed with the Agilent 4200 Tape Station system by High Sensitivity RNA screen tape and only RNAs having a RIN >6 were used for library preparation using SMART-Seq Stranded kit (Takara). All samples were sequenced on an NextSeq 2000 Illumina Platform generating at least 40 million 75bp-Paired End reads per sample (i.e. 80 million reads per sample). Illumina sequencing instruments generated per-cycle BCL basecall files as primary sequencing output, which required conversion to FASTQ format through the BCL Convert (Illumina, Cambridge, UK). Then, the quality control check was performed on fastq files for each sample by using FastQC tool. Indeed, FastQC tool assess several informations, such as reads' length and quality score across all bases.

3.5.2.2 Reads mapping and counting, and data normalization

Once high-quality data were generated from pre-processing, the short reads were mapped to the reference genome GRCh38.104 (Homo sapiens) using the STAR aligner, by setting --runMODE alignReads, with default parameters (version 2.7.9). Once the reads are mapped to the reference genome, they are

counted by using STAR aligner with the option --quantMode GeneCounts (Liao Y., et al. 2014) and Homo_sapiens.GRCh38.104.gtf annotation.

The read counts were imported into R statistical software (version 4.1.2), and differential gene expression analysis was performed using the DESeq2 package (version 1.38.3) (Anders S. & Huber W., 2010).

Therefore, for pair-wise comparisons, raw read counts were normalized to minimize the differences between samples and library size using the Relative Log Expression method (RLE). On the other hand, within-sample variability was assessed using principal component analysis (PCA). From the PCA graph, we observed a separation of our samples on the first principal component that captured sex-related variables. Therefore, we decided to adjust this variable in order to better compare the subgroups. The PCA plot was generated using the plotPCA function of the DESeq2 package. P-values were adjusted using the Benjamini-Hochberg method.

3.5.2.3 Differential gene expression analysis

Differential expression analysis between condition was performed by using DESeq function (version 1.38.3) (Anders S. & Huber W., 2010). A negative binomial generalized linear model was used to fit each gene and pairwise comparisons were performed using a Wald test. The outlier genes were detecting using Cooks's distance and removed from the analysis. Moreover, genes whose mean of normalized counts were below a threshold determined by an optimization procedure were removed. Finally, to control for false positives due to multiple comparisons in the genome-wide differential expression analysis, the false discovery rate (FDR) was computed using the Benjamini-Hochberg procedure. Only genes with FDR-adjusted *p*-value < 0.05 were considered significant.

3.5.2.4 Pathway analysis

Pathway enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA) tools (GSEA, version 4.3.2). For each comparison, the list of expressed genes and their log_2FC values were used to identify significantly

enriched Gene sets. The gene signature was retrieved from GO, in particular from GO Biological Process and Molecular Function. GSEA was performed in pre-ranked mode with “weighted” scoring scheme and 1,000 permutations. The maximum Gene set size was set at 500 genes, and the minimum size was set at 5 genes. Only pathways with a nominal p-value lower than 0.01 were selected as enriched

3.6 Statistical analysis

Data were shown as mean±SEM or as frequency. The χ^2 test, Uncorrected Dunn's test, Wilcoxon matched-pairs signed rank test were used for comparisons between samples, as indicated. All statistical analyses assumed a two-sided significance level of 0.05. Data analyses were performed with Openstat software (version 11.9.08; Softonic International, Barcelona, Spain) and GraphPad PRISM (version 9.0, La Jolla California, USA).

4. RESULTS

Due to their high proliferative potential and their unique ability to generate new vessels *in vivo* and *in vitro*, ECFCs are now considered as the true endothelial progenitors (Yoder MC., 2012). Being involved in maintaining endothelial homeostasis, ECFCs were studied in several physiological and pathological settings (Poredos P., 2002; Yoder MC., 2012; Melero-Martin JM., 2022; Colombo E. et al., 2013; Della Bella S. et al., 2020).

In particular, they are now considered an optimal tool to fully understand the alterations affecting the endothelial compartment in patient-specific settings. For these reasons, in the present study we took advantage of *in vitro* ECFC characterization to investigate the role of ED in the pathogenesis of uVTE.

To do that we first set up the TGA on ECFCs and the thrombogenesis assay, functional assays dedicated to investigate the ECFC activation state and in particular their ability to promote thrombosis. Then, we isolated ECFCs from patients with VTE (sub-grouped in uVTE, wpVTE and pVTE patients) and from HDs and then the expanded ECFCs were characterized for the parameters described in the following chapters.

4.1 Evaluation of validated predictors of VTE recurrence in the study cohort

In collaboration with our clinical collaborators, 3 months after the index VTE event, VTE patients were re-evaluated by assessing the following validated predictors of VTE recurrency: presence of Post Thrombotic Syndrome (PTS), altered plasmatic levels of D-dimer and presence of residual obstruction (either RVO and/or RPVO).

In particular, by assessing the possible presence of PTS with Villalta score, we observed that no significant differences emerged among the different subgroups of VTE patients as the vast majority of the patients enrolled in the Study did not have PTS (Figure 4.1 A-B). Only 3 VTE patients (2 wpVTE and 1 pVTE) presented a mild PTS among all the VTE patients enrolled in the study. The low frequencies of patients with PTS observed in our cohort was probably due to the fact that the VTE patients enrolled in the study were mainly under treatment with DOAC. In fact, studies have shown that the use of DOACs for the initial and long-term treatment of DVT in place of VKAs reduces the risk of PTS by approximately 50% (Prandoni P., 2022).

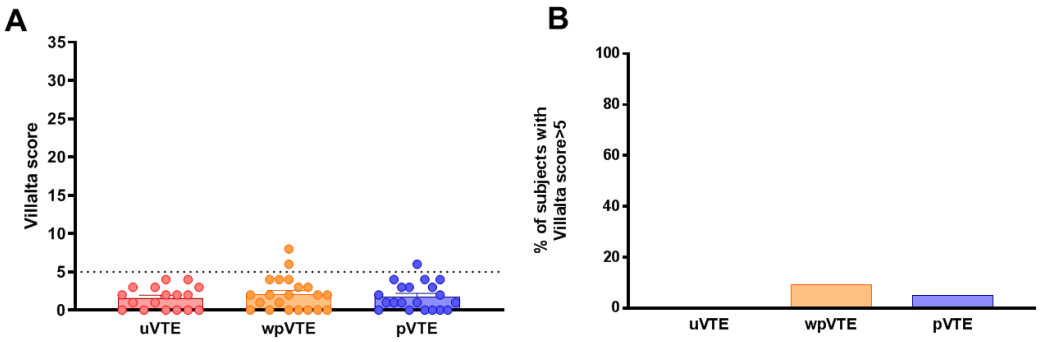

Figure 4.1 | PTS assessment in each subgroup of VTE patients. The presence of PTS in VTE patients who experienced a DVT (uVTE=17; wpvte=20; pVTE=20) was assessed by using Villalta scale. A) The presence of PTS was confirmed when Villalta score was ≥5; B) percentage of subjects with Villalta score>5%, data shown as frequency. *Data are presented as mean ± SEM. Statistical significance was calculated using A) the Uncorrected Dunn's test and B) χ^2 test.*

Concerning D-dimer levels, no significant differences in the average D-dimer levels were observed among the different subgroups of VTE patients (Figure

4.2 A). Subdividing patients on the basis of D-dimer levels and considering patients with D-dimer levels higher that 250 µg/L being the ones with higher recurrence risk, we observed that uVTE and pVTE groups had similar frequencies of patients with D-dimer >250 µg/L (45% and 44% respectively) whereas the frequency was lower in wpVTE group (32%) (Figure 4.2 B) Also for this parameter, the similar frequency of patients characterized by altered D-dimer levels observed in the three subgroups of VTE patients could be due to the fact that the vast majority of VTE patients enrolled in the cohort were under anticoagulant treatment at the moment of follow-up evaluation.

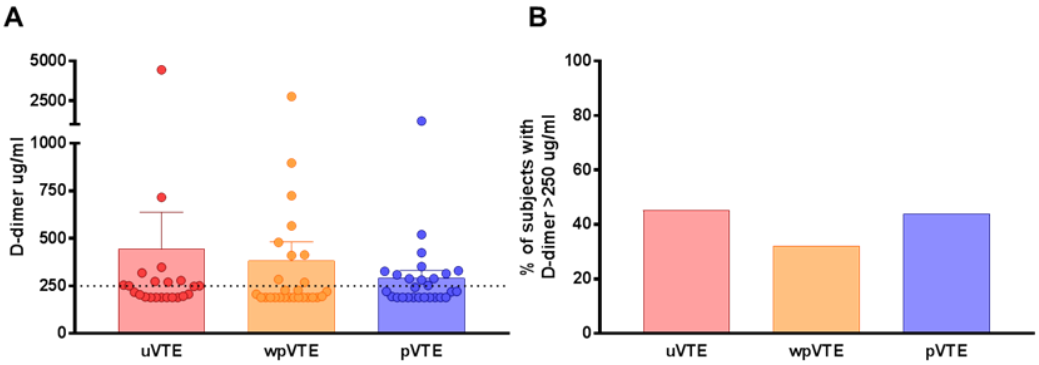

Figure 4.2 | Evaluation of plasmatic D-dimer levels in each subgroup of VTE patients. Plasmatic D-dimer levels were measured in VTE patients (uVTE n=22, wpVTE n=28, pVTE n=27). A) D-dimer levels, data shown as mean ± SEM; B) percentage of subjects with D-dimer levels higher than 250 µg/L, data shown as frequency. *Statistical significance was calculated using A) Uncorrected Dunn's test and B) χ^2 test.*

Finally, we took into consideration as last validated predictor of recurrence the presence of residual obstruction. The frequency of subjects who had a residual obstruction – evaluated as RVO and/or RPVO - was assessed in each subgroup of VTE patients considered in this study. As shown in Figure 4.3, the frequency of subjects who still had a RVO and/or RPVO 3 months after the index event is significantly higher in uVTE group than patients with wpVTE and pVTE groups.

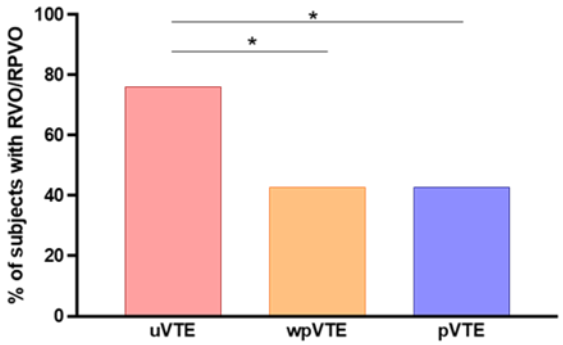

Figure 4.3 | Presence of residual obstruction assessed in each subgroup of VTE patients. The presence of residual obstruction 3 months after the index VTE event in VTE patients (uVTE=21; wpvte=28; pVTE=28) was assessed as the presence of RVO and/or RPVO. *Data are presented as frequency. *p<0.05. Statistical significance was calculated using χ^2 test.*

Considering the results obtained by evaluating these predictors of VTE recurrence on the present cohort, in the following paragraphs the three subgroups of VTE patients were divided in high and low risk patients for VTE recurrence on the basis of the presence/absence of the residual obstruction at the follow-up evaluation.

4.2 Set up of ECFC-based assays to investigate ED

To shed light on the role of ED in the pathogenesis of thrombus formation in a patient-specific *in vitro* setting, a thrombin generation assay (TGA) on ECFCs and a thrombogenesis assay that allowed the evaluation of platelet deposition and fibrin formation on ECFC layers under flow conditions were setup. Both assays were first optimised on human umbilical vein ECs (HUVECs) - the "gold standard" in vascular research - and then tested on ECFCs isolated from HDs. To assess whether the assays allowed a proper assessment of ED, both HUVECs and ECFCs were used as substrates in the assays both under basal conditions and after *in vitro* activation with TNFα. Activation of ECs was first assessed by evaluating the expression of the adhesion molecule VCAM-1 and the pro-coagulant molecule TF by flow cytometry.

4.2.1 EC activation upon inflammatory stimulation

In the set of experiments dedicated to set up the protocols of the functional assays, activated ECs were obtained incubating cells with TNFα, a pro-inflammatory cytokine known to promote ED. In order to confirm the ability of TNFα treatment in inducing endothelial activation, the expression levels of VCAM-1 and TF were assessed by flow cytometry on both TNFα -treated HUVECs and ECFCs and their untreated counterparts.

As expected, in basal conditions VCAM-1 and TF were not expressed by HUVECs whereas TNFα treatment significantly increased the expression of both VCAM-1 (Figure 4.4 A) and TF (Figure 4.4 B). Similar results were observed when the expression of VCAM-1 and TF were assessed on ECFCs. In fact, VCAM-1 and TF were barely absent on untreated ECFCs and their expression levels were significantly increased in TNFα-treated ECFCs compared to untreated ones (Figure 4.4). These results were confirmed both in terms of frequency of positive cells and MFI.

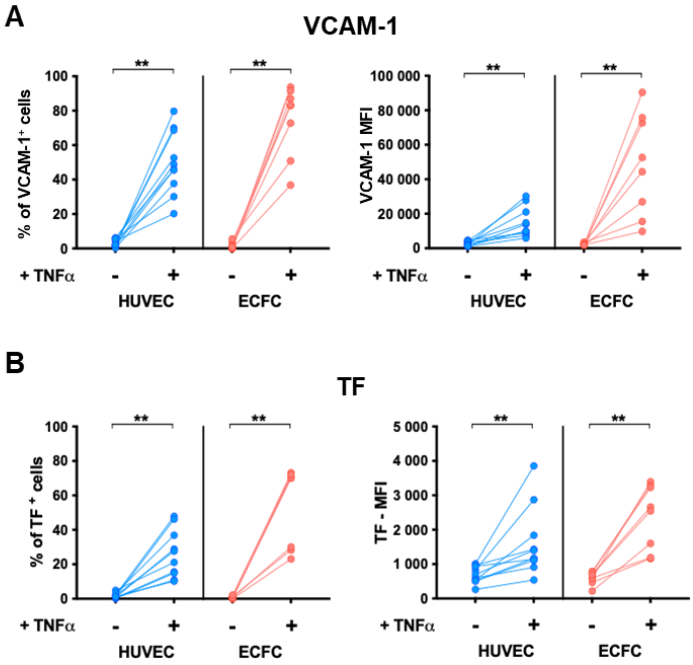

Figure 4.4 | Evaluation of EC activation in response to TNFα treatment. The ability of TNFα stimulation in activating HUVECs and ECFCs was confirmed by flow cytometry evaluating the expression of A) the adhesion molecule VCAM-1 and B) the pro-coagulant molecule Tissue Factor (TF). Data were expressed as frequency of positive cells (left) and as Mean fluorescence intensity (MFI) (right). **p < 0.01 treated ECs vs untreated ECs. *Statistical significance was calculated using Wilcoxon matched-pairs signed rank test.*

The results obtained confirmed that TNFα can be used as stimuli to activate both HUVECs and ECFCs and allowed us to use TNFα-treated HUVECs and ECFCs to verity that the functional assays were able to discriminate between activated ECs from resting ones both HUVECs and ECFCs.

4.2.2 Setting ECFCs as a source of TF in the TGA assay

To assess the presence of alteration of the endothelial compartment, a modified version of the TGA was set up to test the ECFC ability to induce thrombin formation in a TGA that use ECs as source of TF.

The assay was first set up on HUVECs and then tested on ECFCs isolated and expanded from HDs. In particular, both HUVECs and ECFCs cultured under basal conditions and in the presence of TNFα were used as substrate in the assay to assess whether the assay allowed to distinguish between untreated and activated ECs. In TGA, thrombin generation was monitored over time by evaluating the lag time, the peak and the ETP measured in the thrombogram.

Thrombin generation was significantly increased when TNFα-treated HUVECs were used as a source of TF compared to HUVECs cultured in basal conditions. In particular, the lag time was significantly reduced, while thrombin production - measured either as Peak and ETP - was significantly increased in TNFα-treated HUVECs compared to HUVECs cultured in basal conditions (Figure 4.5). Similar results were obtained when ECFCs were used as substrates in the TGA. As shown in Figure 4.5, thrombin generation was significantly increased when TNFα-treated ECFCs were used as substrate in the assay compared to ECFCs cultured in basal conditions. In particular, the lag time was significantly reduced, whereas thrombin peak and ETP were significantly higher in TNFα-treated ECFCs than in ECFCs cultured under basal conditions (Figure 4.5). Altogether, our results indicate that ECFCs are a suitable substrate for the TGA on ECs and that our setting allowed us to distinguish between basal and activated cells not only on HUVECs but also when using ECFCs.

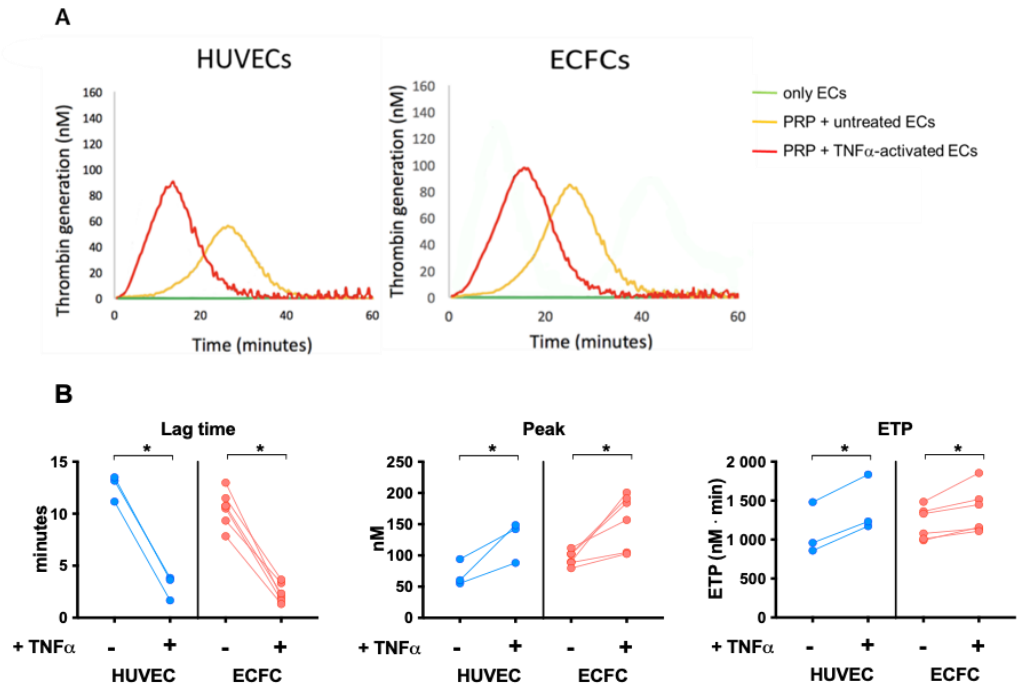

Figure 4.5 | TGA on ECs. A) Representative thrombogram of TGA performed on HUVECs and ECFCs as a source of TF. In both plots, the following conditions were shown: only ECs (green line), PRP + untreated ECs (yellow line), PRP + TNFα-activated ECs (red line). B) Lag Time, thrombin formation Peak and endogenous thrombin potential (ETP) were assessed on TGA using as TF source HUVECs and ECFCs cultured either in basal conditions and after incubation with TNFα. *p<0.05 treated ECs vs untreated ECs. *Statistical significance was calculated using Wilcoxon matched-pairs signed rank test.*

4.2.3 Setting ECFCs as a substrate for the thrombogenesis assay

To further investigate the ability of ECFCs in sustaining thrombosis, a thrombogenesis assay that allows to assess fibrin formation and platelet deposition on ECFC layers was optimized. The assay was first set up on HUVECs and then tested on ECFCs. In particular, both HUVECs and ECFCs were evaluated under basal conditions and after activation with TNFα, in order to assess whether the assay allowed to distinguish between untreated and activated ECs.

In order to assess that measured fibrin and platelet deposition occurred on ECs and not on the exposed collagen used as substrate for cell adhesion, cell confluence was confirmed prior and after blood perfusion by identifying ECs on the basis of CD31 expression. As shown in Figure 4.6 A, the layers of both HUVECs and ECFCs remained intact after blood perfusion.

After perfusion, fibrin formation and platelet deposition on ECs were assessed by analysing z-stack images acquired on confocal microscopy. As shown in Figure 4.6 B and C, fibrin formation and platelet deposition were significantly increased when TNFα-activated HUVECs were used as substrate in the assay compared to their basal counterpart. When ECFCs were used as substrate in thrombogenesis assay, similar results were obtained. In fact, both fibrin formation (Figure 4.6 B) and platelet deposition (Figure 4.6 C) were significantly increased on TNFα-treated ECFCs compared to ECFCs cultured in basal conditions. Altogether, our results indicate that ECFCs are a suitable substrate for the thrombogenesis assay and that our setting allowed us to distinguish between basal and activated cells not only on HUVECs but also when using ECFCs.

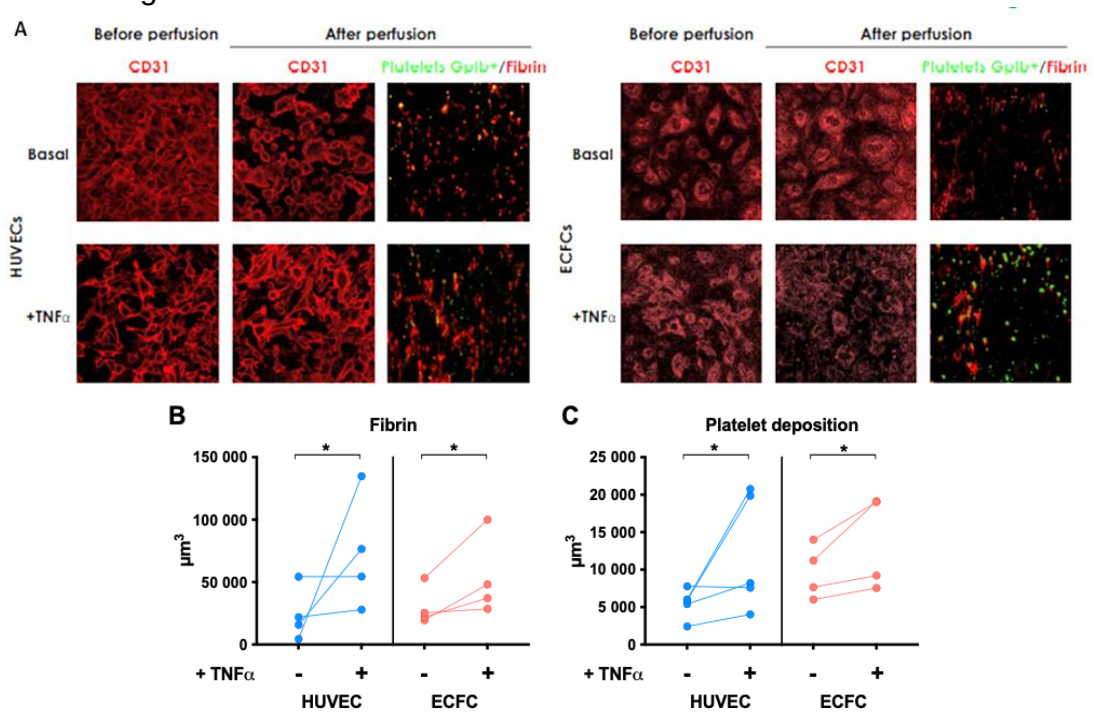

Figure 4.6 | Evaluation by confocal microscopy of fibrin formation and platelet deposition on endothelial layer under flow conditions. A) Representative photographs of HUVECs and ECFCs seeded on glass coverslips before (first column) and after perfusion (second column). Cell confluence was assessed by CD31 staining (in red). Third column: representative photographs showing fibrin deposition (assessed by fibrin staining, in red) and platelets adhesion (assessed by GpIb staining, in green) on HUVECs and ECFCs cultured in basal condition (upper line) and after incubation with TNFα (lower line). Photographs were acquired with confocal microscopy (Leica TCS SP5; magnification 60x). B) fibrin formation and C) platelet deposition were assessed on HUVECs and ECFCs cultured in basal conditions and after incubation with TNFα. Data were expressed as volume (µm3) of fibrin and platelet deposition. *p<0.05 treated ECs vs untreated ECs. *Statistical significance was calculated using Wilcoxon matched-pairs signed rank test.*

Finally, since thrombin acts as an enzyme and convert fibrinogen into fibrin, we evaluated whether there was a correlation between the thrombin generated in TGA on ECFCs and the amount of fibrin formed in ECFCs layers in thrombogenesis assay. In order to do that we took into account the data obtained by performing the two assays on 3 different ECFC colonies analysed either in basal condition or after TNFα treatment. As shown in Figure 4.7, the amount of fibrin detected in thrombogenesis assay directly correlated with the amount of thrombin generated in TGA assessed either as Peak and as ETP, thus further supporting the combined used of these assays to investigate the ability of ECFC in promote thrombus formation in patient-specific settings.

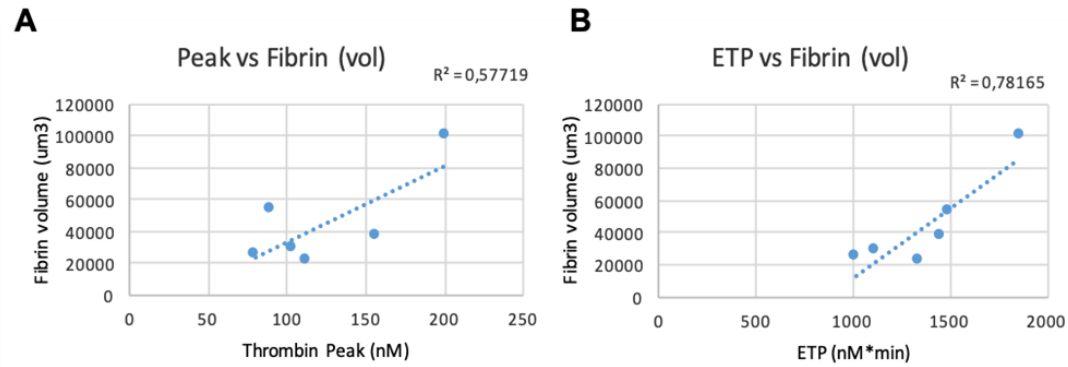

Figure 4.7 | Correlation between thrombin generation in TGA on ECFCs and fibrin formation on ECFC layers in thrombogenesis assay. A) Correlation between the volume of fibrin formed on ECFC layers in thrombogenesis assay and the amount of thrombin generated in TGA in ECFCs assessed as Peak. B) Correlation between the volume of fibrin formed on ECFC layers in thrombogenesis assay and the total amount of thrombin generated in TGA in ECFCs assessed as ETP. Data obtained from 3 ECFC colonies cultured either in basal conditions and after TNFα activation were used to assess the correlations.

4.3 Characterization of ECFCs isolated from uVTE patients compared with HDs and patients with secondary VTE

4.3.1 Assessment of the efficiency of ECFC colony isolation

In order to evaluate the efficiency of ECFC isolation, PBMCs were isolated by density gradient centrifugation from fresh peripheral blood samples collected from both HDs and VTE patients. PBMCs were then seeded on fibronectin substrate and cultured in EGM-2 + 5% FBS medium and PBMC cultures were monitored for one month by daily microscopic observation in order to ensure the detection of the ECFC colonies identified as group of adherent cells with the typical cobblestone-like morphology. The number of seeded PBMCs, the time of appearance of the ECFC colonies, and the number of ECFC colonies isolated were recorder for each individual.

By considering the rate of success in isolating ECFC colonies, we observed that the percentage of subjects who gave rise to at least one ECFC colony was similar among the three sub-groups of VTE patients and HDs (Figure 4.8 A). Furthermore, the number of isolated ECFC colonies and their time of appearance were assessed in subjects who generated at least one ECFC colony. As shown in Figure 4.8 B, no significant differences were observed when comparing the HDs and VTE subgroups with regard to the number of isolated ECFC colonies, expressed as the number of ECFC colonies per 10^8 seeded PBMCs. Concerning the time of ECFC appearance, no significant differences were observed among HDs, uVTE and pVTE patients, whereas ECFC colony appearance was significantly delayed in wpVTE patients compared to HDs and uVTE patients (Figure 4.8 C). The time of ECFC colony appearance was expressed as days occurring from PBMC seeding to ECFC colony identification.

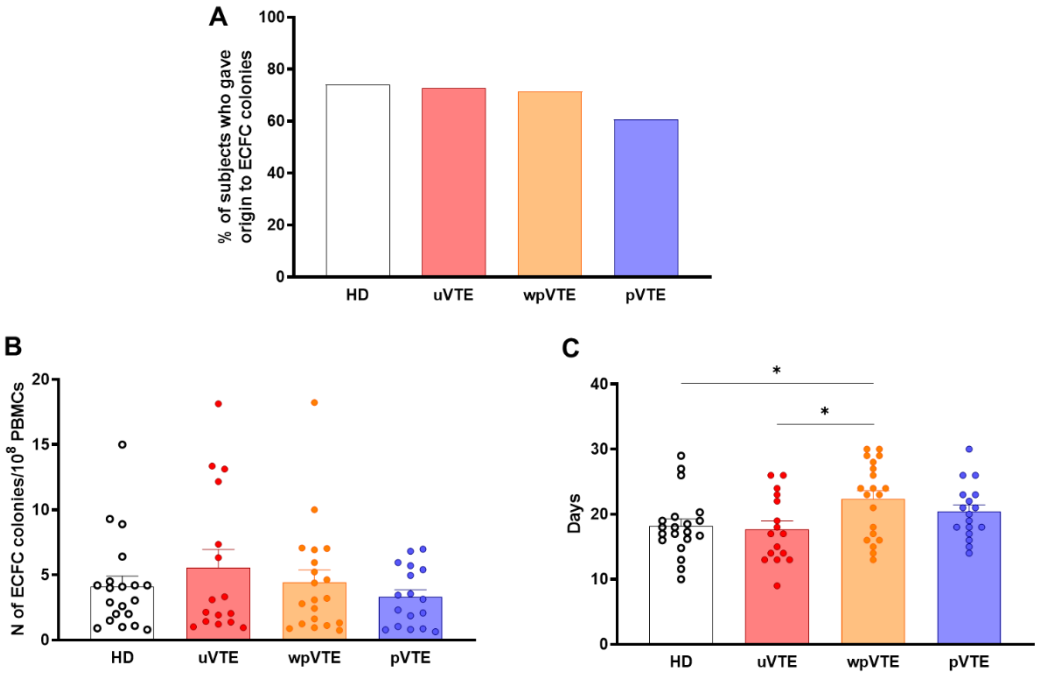

Figure 4.8 | ECFC colony isolation from HD and VTE patients. A) Frequency of subjects generating at least one ECFC colony within 30 days after PBMC seeding in healthy donors (HD n=27) and VTE patients (uVTE n=22, wpVTE n=28, pVTE n=28). B) Number of ECFC colonies isolated from healthy donors (HD n=20) and VTE patient subgroups (uVTE n=16, wpVTE n=20, pVTE n=17). The number of isolated ECFC colonies was expressed as the number of isolated ECFC colonies/10^8 PBMCs seeded. C) Time of appearance of the ECFC colonies isolated from healthy donors (HD n=20) and VTE patients (uVTE n=16, wpVTE n=20, pVTE n=17). Data are presented as days after PBMC seeding. *Data are presented as (A) frequency or (B-C) mean ± SEM. *p<0.05. Statistical significance was calculated using (A) χ^2 test and (B-C) Uncorrected Dunn's test.*

4.3.2 Assessment of cellular senescence of ECFCs

After identification of ECFC colonies, cell expansion was performed in culture plates using collagen as substrate. Considering that in our previous study an increased early senescence was observed in ECFCs derived from patients with uVTE (Della Bella, S. et al., 2020), this aspect was evaluated also in this independent cohort of VTE patients. Early senescence refers to cells that undergo senescence by the third culture passage with cells being considered senescent when underwent growth cell arrest combined with senescence-associated (SA) morphological changes. Furthermore, ECFC senescence was further confirmed the β-galactosidase assay (Della Bella S. et al., 2020). In accordance with our previous study, an increased early senescence in ECFCs

isolated from patients with uVTE was observed compared to ECFCs isolated from HDs (Figure 4.9). In addition, having enrolled in this new independent cohort also patients with wpVTE and pVTE, we were able to extend our knowledge on ECFC early senescence also in patients with secondary VTE. As shown in Figure 4.9, similarly to what observed in uVTE patients, the frequency of early senescent ECFC colonies was significantly increased also in patients with secondary VTE compared with HDs.

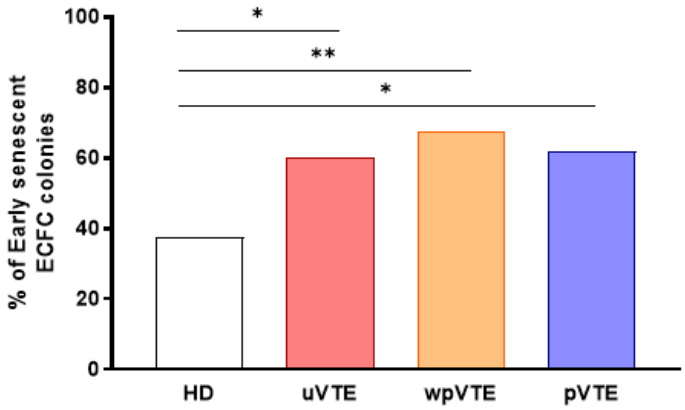

Figure 4.9 | Evaluation of early senescence in ECFC cultures. Percentage of early senescent ECFC colonies in healthy donors (HDs, n=56 ECFC colonies) and VTE patients (uVTE n=30, wpVTE n=49, pVTE n=34 ECFC colonies). Early senescence was considered as senescence occurring within the 3rd culture passage. *Data are presented as frequency. *p<0.05, **p<0.01. Statistical significance was calculated using χ^2 test.*

4.3.3 Assessment of constitutive ED in uVTE patients

In order to investigate the possible presence of constitutive ED in uVTE patients the expression levels on VCAM-1 and TF as well as the ability to promote thrombosis was investigated in ECFCs isolated from uVTE patients and HDs. The ECFC ability to promote thrombosis was investigated by performing TGA on ECFCs and thrombogenesis assay that were previously set up. ECFCs isolated from patients with pVTE and wpVTE were also considered in the analyses, to assess whether the presence of constitutive ED was typical of uVTE or shared with secondary VTE.

4.3.3.1 ECFC immunophenotype by flow cytometry

In order to evaluate ECFC expression of VCAM-1 and TF, the frequency of VCAM-1^+ and TF^+ cells was assessed on viable ECFCs by flow cytometry.

As shown in Figure 4.10 A-B, expression of VCAM-1 and TF was barely detectable on ECFCs derived from HDs, as expected. Furthermore, no significant differences in VCAM-1 expression were detected when ECFCs obtained from the three subgroups of VTE patients were compared with ECFCs isolated from HDs (Figure 4.10 A). Similar results were obtained when the frequency of ECFCs expressing TF was taken into account (Figure 4.10 B). Nevertheless, only in VTE patients some colonies were characterized by a higher expression of VCAM-1 and TF. Therefore, by setting an arbitrary threshold, for both markers we assessed the proportion of ECFC colonies in which the frequency of cells expressing the evaluated marker was higher than 5%. As shown in Figure 4.10 C, a higher frequency of ECFC colonies with a percentage of VCAM-1+ cells > 5% was observed patients with VTE compared with HDs. In addition, only among ECFC colonies obtained from VTE patients ECFC colonies containing more than 5% of TF^+ cells were identified (Figure 4.10 D).

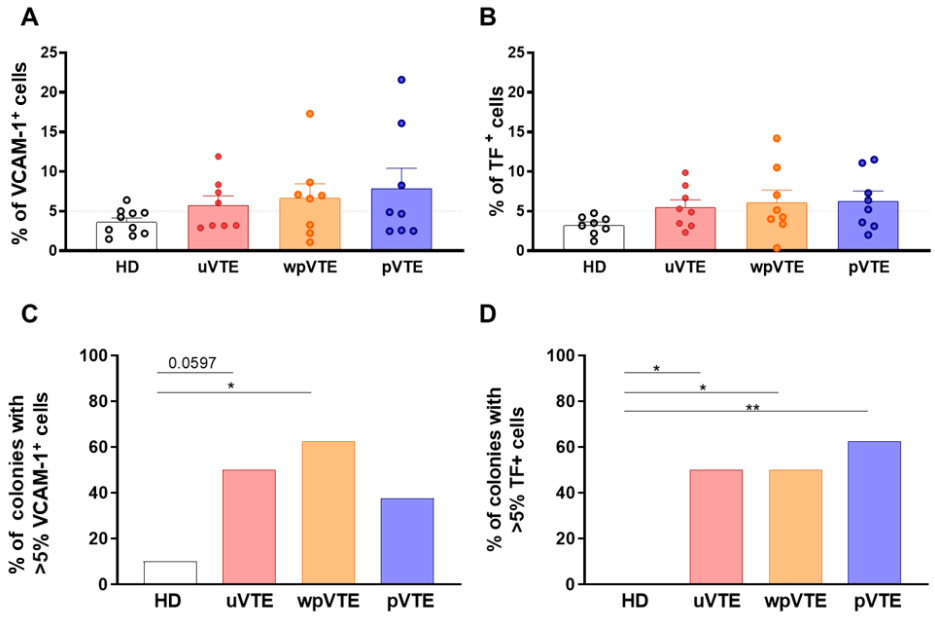

Figure 4.10 | Evaluation of the expression levels of the endothelial activation markers VCAM-1 and TF in ECFCs. A) Frequency of VCAM-1+ cells in ECFC colonies isolated from healthy donors (HD n=10) and VTE patients (uVTE n=8, wpVTE n=8, pVTE n=8). B) Frequency of TF+ cells in ECFC colonies isolated from healthy donors (HD n=8) and VTE patients (uVTE n=8, wpVTE n=8, pVTE n=8). C) Frequency of ECFC colonies with a percentage of VCAM-1+ cells >5% in healthy donors (HD n=10) and VTE patients (uVTE n=8, wpVTE n=8, pVTE n=8). D) Frequency of ECFCs colonies with a percentage of TF+ cells >5% in healthy donors (HD n=8) and VTE patients (uVTE n=8, wpVTE n=8, pVTE n=8). *Data are presented as (A-B) mean ± SEM and as (C-D) frequency. *p<0.05, **p<0.01. Statistical significance was calculated using (A-B) Uncorrected Dunn's test and (C-D) χ^2 test.*

Moreover, since in the group of uVTE patients the frequency of patients having a residual obstruction was significantly higher compared with wpVTE and pVTE patient groups, and considering that the presence of a residual obstruction is a validated predictor of VTE recurrence we evaluated the expression of the activation markers VCAM-1 and TF also in uVTE patients stratified as low- and high-risk patients on the basis of residual obstruction presence at the time of follow-up evaluation. Patients were classified at high risk of VTE recurrence when a residual obstruction was identified either as RVO and/or RPVO.

As shown in Figure 4.11, no significant differences in the expression levels of VCAM-1 and TF emerged between uVTE patients with low risk of VTE recurrence and uVTE patients with high risk of VTE recurrence. Only in wpVTE

group an increasing trend can be observe in high risk patients compared with low risk ones. In addition, in accordance with the results obtained without stratifying patients according to the presence of a RVO and/or RPVO, in high risk patients no significant differences were observed among the VTE subgroups both in the frequency of cells expressing VCAM-1 and in the frequency of cells expressing TF. Similar results were obtained when the expression levels of VCAM-1 and TF were evaluated on patients who did not have a residual obstruction (Figure 4.11).

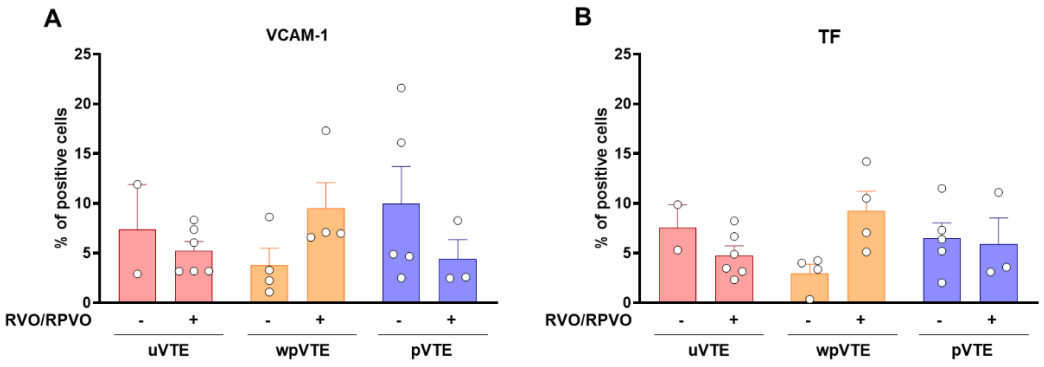

Figure 4.11 | VCAM-1 and TF expression in ECFCs of VTE patients subgrouped on the basis of Residual obstruction presence at the follow-up evaluation. A) Frequency of VCAM-1+ cells and B) Frequency of TF+ cells in ECFC colonies obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=2, wpVTE n=4, pVTE n=5) and from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=6, wpVTE n=4, pVTE n=3). Patients were considered positive for a residual obstruction when a RVO and/or a RPVO were detected at the follow-up. *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

4.3.3.2 TGA on ECFCs

To study constitutive ED in uVTE, functional assays aimed to investigate ECFC ability to promote thrombosis were performed. In particular, in order to investigate whether uVTE patients ECFCs were able to support higher thrombin formation, a TGA on ECFCs was performed by using as substrate the ECFCs obtained from HDs and from the 3 subgroups of VTE patients. Since in this experimental setting the used PRP derives from HDs and ECFCs were obtained from the groups compared in the study, the measure of the thrombin produced allowed to assess the activation status of the tested

ECFCs. In the analysis of thrombin generation or "*thrombogram*" curves the following parameters were considered: lag time, peak and ETP.

As shown in Figure 4.12 A, a faster thrombin generation occurred when uVTE patient ECFCs were used as substrate as demonstrated by the lag time that was significantly reduced in uVTE patients compared to HDs and compared to wpVTE. In addition, the highest thrombin peak was detected when ECFCs obtained from uVTE patients were used as substrate in the assay, being significantly higher compared with the peaks recorded when ECFCs isolated from HDs and pVTE patients were used as substrate in TGA (Figure 4.12 B). No significant differences were observed in the net amount of thrombin produced – evaluated as ETP - among the groups considered (Figure 4.12 C).

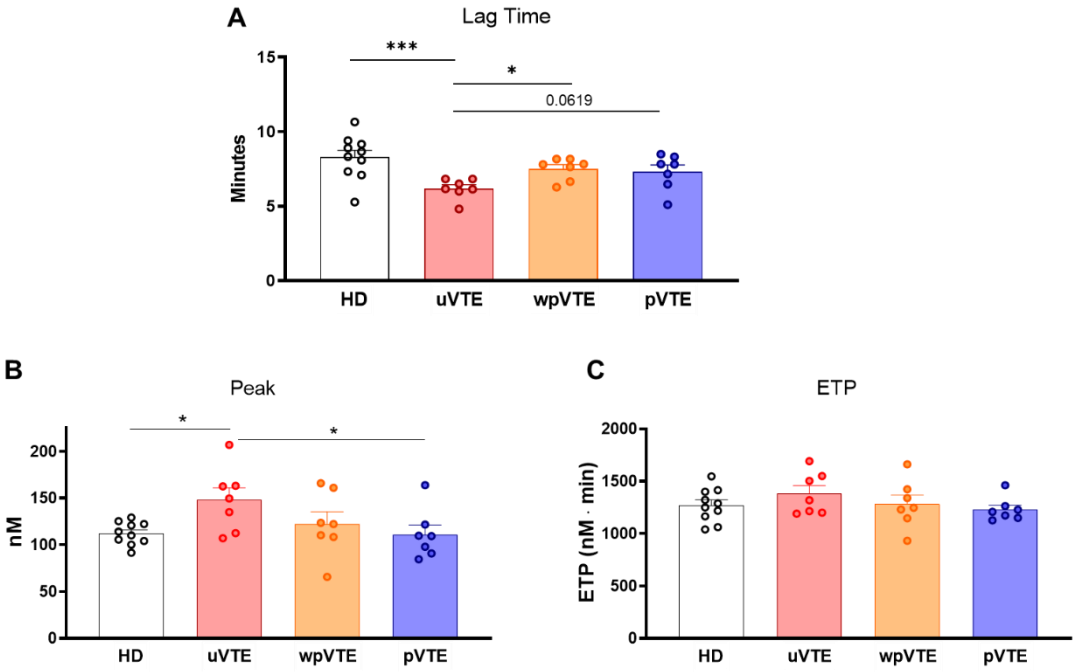

Figure 4.12 | Thrombin formation in TGA performed on ECFCs. Thrombin generation was evaluated performing TGA on ECFCs isolated from healthy donors (HDs n=10) and VTE patients (uVTE n=7, wpVTE n=7, pVTE n=7). Three parameters were assessed: A) Lag time, expressed in minutes, B) Peak thrombin, expressed in nM and C) ETP, expressed in nM of thrombin x minute. *Data are presented as mean ± SEM. *p<0.05, ***p<0.001. Statistical significance was calculated using Uncorrected Dunn's test.*

Stratifying uVTE patients as low- and high-risk patients for VTE reccurrence according to the presence of a residual obstruction, an increased thrombin

generation assessed either as Peak and as ETP was observed in the group of high-risk patients compared with low-risk ones despite the limited number of patients analyzed in the comparison (Figure 4.13). Similar results were observed for wpVTE group when patients were subdivided on the basis of residual obstruction presence. In addition, a trend in faster thrombin generation can be observed comparing the group of wpVTE patients with high risk of VTE recurrence with the group of low risk ones. Finally, in pVTE patients, similar thrombin generation occurred in low and high-risk patients.

Restricting the analysis to patients with high risk of VTE recurrence, trends similar to those observed on all patients were observed (Figure 4.13). In fact, considering high risk patients the fastest thrombin formation occurred when ECFCs obtained from uVTE patients were used as substrate in the assay. Moreover, by evaluating the amount thrombin produced – assessed either in term of Peak and ETP – the greatest thrombin formation was observed when ECFCs obtained from uVTE patients were used as substrate with pVTE patient being the one characterized by the slowest and lowest thrombin formation (Figure 4.13). In low risk patients, thrombin formation assessed in TGA on ECFCs was similar among groups (Figure 4.13).

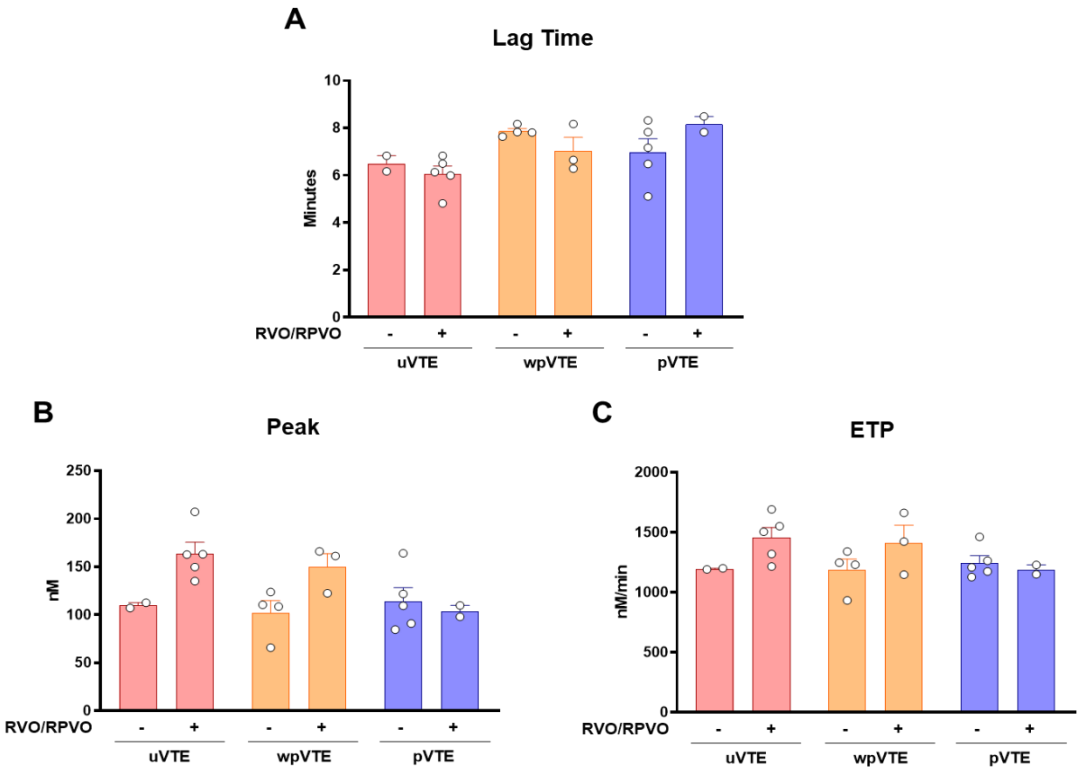

Figure 4.13 | Thrombin formation in TGA on ECFCs of VTE patients subgrouped on the basis of Residual obstruction presence at the follow-up evaluation. Thrombin generation was evaluated performing TGA on ECFCs obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=2, wpVTE n=4, pVTE n=5) and on ECFCs obtained from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=5, wpVTE n=3, pVTE n=2). Patients were considered positive for a residual obstruction when a RVO and/or a RPVO were detected at the follow-up. The following parameters were evaluated: A) Lag time, expressed in minutes B) Peak thrombin, expressed in nM and C) ETP, expressed as nM of thrombin x minute. *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

4.3.3.3 Thrombogenesis assay on ECFCs

To further investigate the potential role of ED in thrombus formation in uVTE patients, a thrombogenesis assay was performed under flow conditions to assess fibrin formation and platelet deposition on layers of ECFCs isolated from the three subgroups of VTE patients and HDs. For both fibrin formation and platelet deposition the percentage of covered area and the total volume were evaluated in Z-stacks acquired in confocal microscopy.

Concerning fibrin formation, no significant differences were detected between uVTE patient and HD groups either in terms of percentage of area covered

(Figure 4.14 A) or considering the volume of fibrin generated (Figure 4.14 B). Considering the three subgroups of VTE patients, the highest amount of fibrin was detected when ECFCs obtained from uVTE patients were used as substrate whereas the lower fibrin formation occurred on ECFCs obtained from pVTE patients, despite the differences were not statistically significant (Figure 4.14).

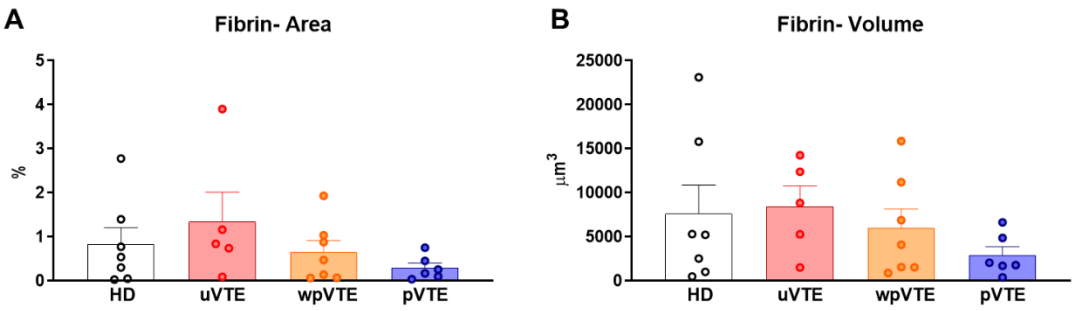

Figure 4.14 | Thrombogenesis assay on ECFCs. Fibrin formation on ECFC layers was evaluated as A) percentage of covered area and B) volume (μm^3) in thrombogenesis assay. ECFCs isolated from healthy donors (HD n=7) and VTE patients (uVTE n=5, wpVTE n=7, pVTE n=6) were used as substrate. *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

Stratifying uVTE patients as low- and high-risk patients according to the presence of a residual obstruction, an increasing trend in fibrin formation was observed in the group of high-risk patients compared with low-risk ones despite only one of the analyzed uVTE patients was classified as low-risk for VTE recurrence (Figure 4.15). Similar results were observed when fibrin formation occurring on ECFCs obtained from low and high-risk patients were compared within the group of wpVTE patients whereas no differences in fibrin formation were observed by comparing low and high-risk patients within the group of pVTE ones.

Restricting the analysis to patients with high risk of VTE trends similar to those observed on all patients were observed (Figure 4.15) with pVTE patient group being the one characterized by the lowest fibrin formation. In low risk patients, fibrin formation on ECFCs was similar among groups (Figure 4.15).

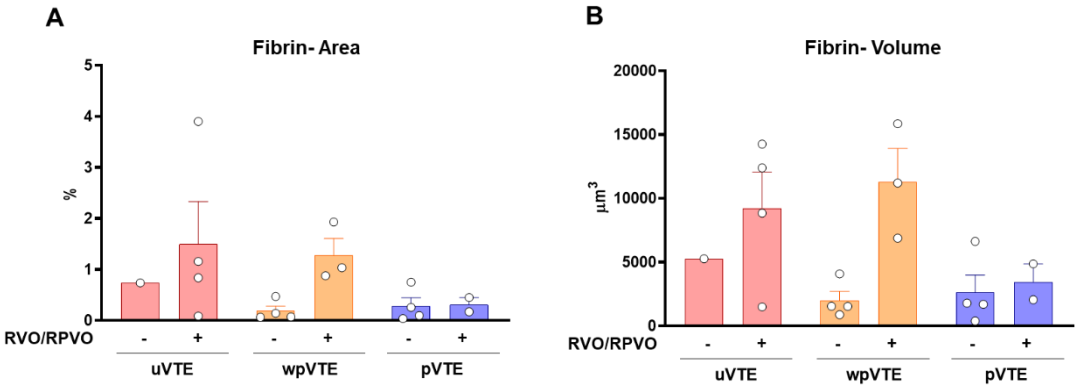

Figure 4.15 | Fibrin formation on ECFCs of VTE patients subgrouped on the basis of residual obstruction presence at the follow-up evaluation. Fibrin formation was evaluated performing Thrombogenesis assay on ECFCs obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=1, wpVTE n=4, pVTE n=4) and on ECFCs obtained from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=4, wpVTE n=3, pVTE n=2). Patients were considered positive for a residual obstruction when a RVO and or a RPVO were detected at the follow-up. Fibrin formation was assessed as A) percentage of covered area and B) volume (μm^3). *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

In thrombogenesis assay, the amount of platelet deposition occurring on ECFCs layers perfused with HD whole blood was also assessed. The highest amount of platelet deposition was detected when ECFCs obtained from uVTE patients were used as substrate in the assay (Figure 4.16). In particular, the total volume of platelet aggregates was significantly higher in uVTE patients compared with the ones formed on HD ECFCs (Figure 4.16 B).

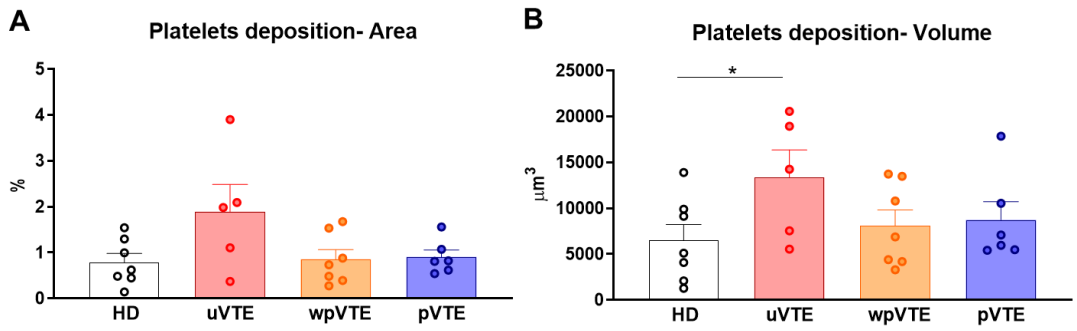

Figure 4.16 | Platelet deposition on ECFCs from VTE patients. Platelet deposition on ECFC layer under flow conditions was assessed by thrombogenesis assay performed using as substrate ECFCs isolated from healthy donors (HD n=7) and VTE patients (uVTE n=5, wpVTE n=7, pVTE n=6). Platelet deposition was evaluated as A) percentage of covered area and B) volume (μm^3). *Data are presented as mean ± SEM. *p<0.05. Statistical significance was calculated using Uncorrected Dunn's test.*

Stratifying uVTE patients as low- and high-risk patients for VTE recurrence according to the presence of a residual obstruction, an increasing trend in platelet deposition was observed in the group of high-risk patients compared with low-risk ones despite only one of the analyzed uVTE patients was classified as low-risk for VTE recurrence (Figure 4.17).

Similar a trend in increasing the platelet deposition was observed when low and high-risk patients were compared within the group of wpVTE patients whereas no differences in platelet deposition were observed by comparing low and high-risk patients within the group of pVTE ones.

Restricting the analysis to patients with high risk of VTE trends similar to those observed on all patients were observed (Figure 4.17). In fact, in high risk patients, the highest platelet deposition occurred on ECFCs isolated from uVTE patients and it progressively decrease either in term of covered area and volume in wpVTE and pVTE patient groups. In low risk patients, a similar platelet deposition on ECFCs was observed among groups (Figure 4.17).

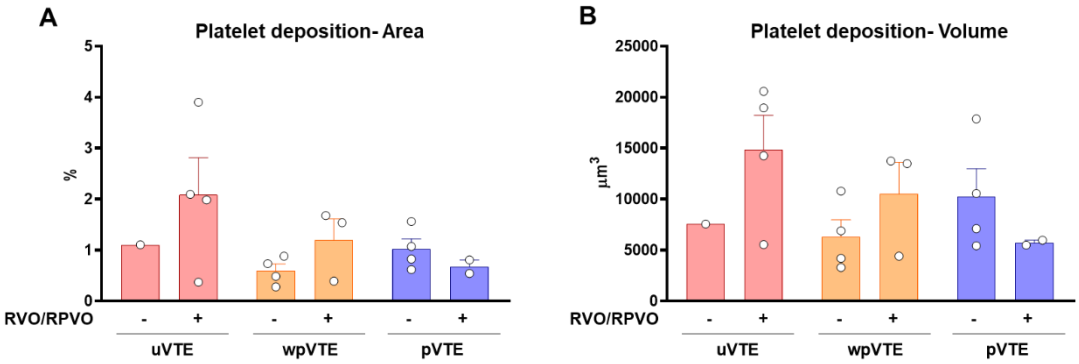

Figure 4.17 | Platelet deposition on ECFCs of VTE patients subgrouped on the basis of residual obstruction presence at the follow-up evaluation. Platelet deposition was evaluated performing the thrombogenesis assay on ECFCs obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=1, wpVTE n=4, pVTE n=4) and on ECFCs obtained from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=4, wpVTE n=3, pVTE n=2). Platelet deposition was assessed as A) percentage of covered area and B) volume (μm^3). *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

4.3.3.4 Soluble adhesion molecules as markers of ED

To further investigate the presence of ED, the concentration of soluble adhesion molecules in the supernatant of ECFC cultures was assessed. In particular, the concentration of the soluble form of ICAM-1, VCAM-1, E-selectin and PECAM-1 was measured. As shown in Figure 4.18, sICAM-1, sVCAM-1, and sPECAM-1 were detected in the culture supernatant of ECFC obtained from both HDs and VTE patients whereas sE-selectin was undetectable in ECFC culture supernatants (data not shown). In particular, the highest levels of sICAM-1 and sPECAM-1 were detected in the supernatant of ECFCs obtained from uVTE patients being statistically significant compared with the levels detected in the culture supernatant of ECFCs obtained from HDs (Figure 4.18 A-C). No significant differences were observed in the levels of sVCAM-1 among the considered groups (Figure 4.18 B).

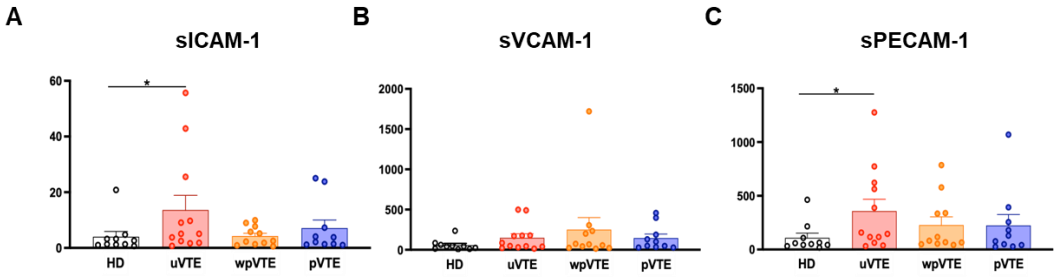

Figure 4.18 | Soluble markers of ED in ECFC culture supernatants. The levels of the soluble forms of the adhesion molecules (A) ICAM-1 (sICAM-1), (B) VCAM-1 (sVCAM-1), (C) PECAM-1 (sPECAM-1) were assessed in the culture supernatant of ECFC colonies obtained from healthy donors (HD n=11) and VTE patients (uVTE n=12, wpVTE n=11, pVTE n=10). *Data are expressed as concentration/10^5 cells. Data are presented as mean ± SEM. *p<0.05. Statistical significance was calculated using Uncorrected Dunn's test.*

Stratifying uVTE patients as low- and high-risk patients according to the presence of a residual obstruction, an increasing trend in the concentration of the soluble form of the adhesion molecules assessed was observed in the group of high-risk patients compared with low-risk ones despite only two of the analyzed uVTE patients were classified as low-risk for VTE recurrence (Figure 4.19). Similar results were observed only in pVTE group. Restricting the analysis to patients with high risk of VTE trends similar to those observed on all patients were observed (Figure 4.19). In fact, considering high risk patients,

the highest levels of sICAM-1 and sPECAM-1 were detected in culture supernatants of uVTE ECFCs while the lowest ones were observed in the culture supernatants of wpVTE ECFCs. Moreover, the levels of sVCAM-1 was similar among the VTE patient subgroups. In low risk patients, the levels of sICAM-1 and sVCAM-1 were similar among groups whereas an increasing trend in the levels of sPECAM-1 was observed in wpVTE group compared with uVTE and pVTE groups (Figure 4.19).

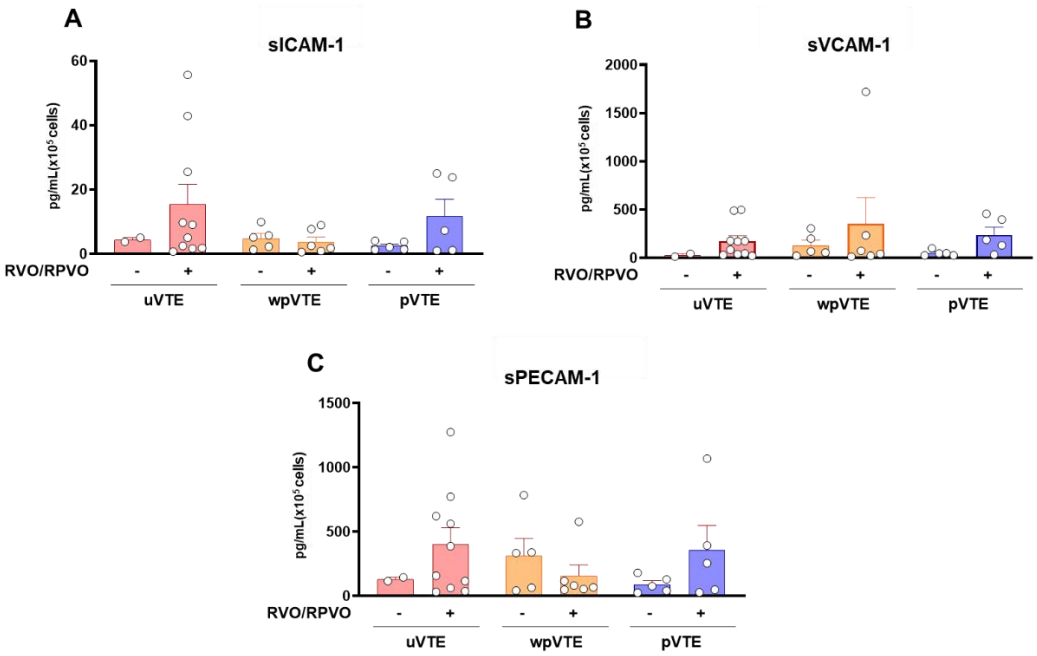

Figure 4.19 | Soluble markers of ED in culture supernatants of ECFCs of VTE patients subgrouped on the basis of residual obstruction presence at the follow-up evaluation. The levels of the soluble forms of the adhesion molecules (A) ICAM-1 (sICAM-1), (B) VCAM-1 (sVCAM-1), (C) PECAM-1 (sPECAM-1) were assessed in the culture supernatant of ECFC colonies obtained from patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=2, wpVTE n=5, pVTE n=5) and from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=10, wpVTE n=6, pVTE n=5). Data are expressed as concentration/10^5 cells. *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

Since the elevated plasmatic levels of the soluble forms of the adhesion molecules are considered markers of ED, the levels of same molecules were also assessed in the plasma of HDs and VTE patients.

As shown in Figure 4.20, all the evaluated molecules were detectable in the plasma samples of the analysed groups. In particular, the plasmatic levels of

sICAM-1 were significantly increased in uVTE patients compared with HDs. Moreover, the plasmatic levels of sICAM-1 of uVTE patients were also significantly higher compared with the levels detected in the plasma of patients with secondary VTE. No significant differences were observed among the groups for the other analysed molecules.

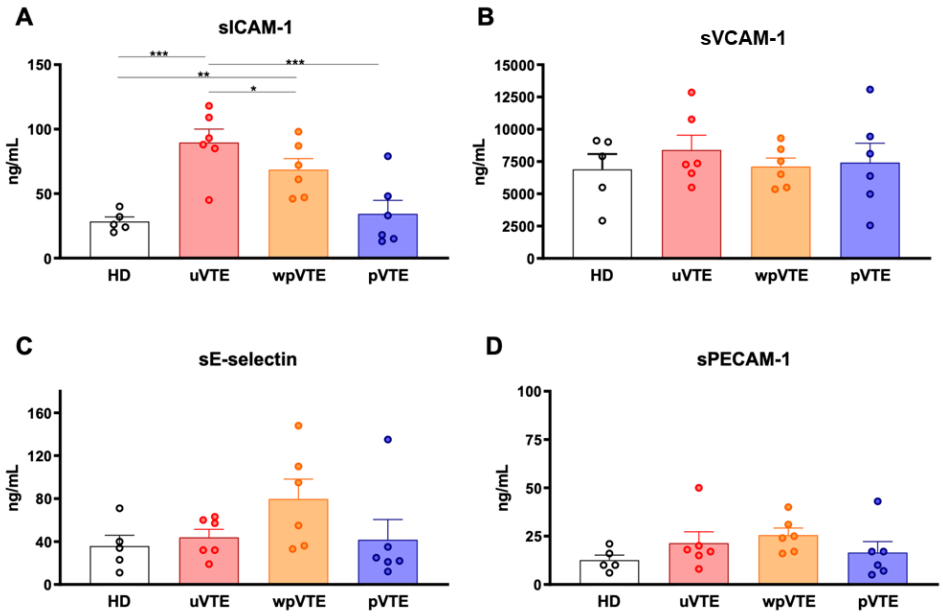

Figure 4.20 | Plasmatic levels of soluble markers of ED. The levels of the soluble forms of the adhesion molecules (A) ICAM-1 (sICAM-1), (B) VCAM-1 (sVCAM-1), (C) E-Selectin (s E-selectin), and (D) PECAM-1 (sPECAM-1) were assessed in the plasma of healthy donors (HD n=5) and VTE patients (uVTE n=6, wpVTE n=6, pVTE n=6). *Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical significance was calculated using Uncorrected Dunn's test.*

Stratifying uVTE patients as low- and high-risk patients according to the presence of a residual obstruction, only an increasing trend in the plasmatic concentration of sPECAM-1 was observed in the group of high-risk patients compared with low-risk ones despite only two of the analyzed uVTE patients were classified as low-risk for VTE recurrence (Figure 4.21). Considering wpVTE patients, increasing trend in sICAM-1 were observed in patients with high risk of VTE recurrence. Finally, similar plasmatic levels of the considered molecules were observed in low-risk and high-risk pVTE patients.

As shown in Figure 4.21, restricting the analysis to patients with high risk of VTE, for both sICAM-1 and sPECAM-1 the lowest plasmatic levels of sICAM-

1 were detected in pVTE patients while similar levels were observed in uVTE and wpVTE patients. No differences were observed in the plasmatic levels of sVCAM-1 among the groups whereas concerning sE-selectin, the highest plasmatic concentration of was detected in wpVTE patients and the lowest one was detected in pVTE patient group (Figure 4.21).

In low risk patients, the highest plasmatic levels of sICAM-1 were detected in uVTE patients (Figure 4.21), whereas the concentration of the other considered molecules was similar among the groups.

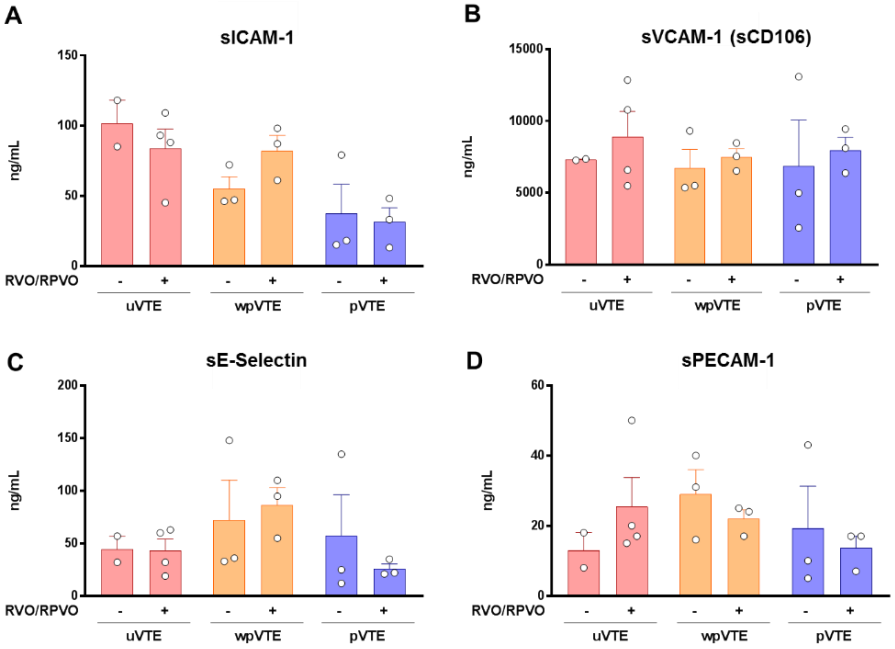

Figure 4.21 | Plasmatic levels of soluble markers of ED in VTE patients subgrouped on the basis of residual obstruction presence at the follow-up evaluation. The levels of the soluble forms of the adhesion molecules (A) ICAM-1 (sICAM-1), (B) VCAM-1 (sVCAM-1), (C) E-Selectin (s E-selectin), and (D) PECAM-1 (sPECAM-1) were assessed in the plasma of patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=2, wpVTE n=3, pVTE n=3) and from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=4, wpVTE n=3, pVTE n=3). Patients were considered positive for a residual obstruction when a RVO and or a RPVO were detected at the follow-up. *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

4.3.4 Assessment of ECFC activation after inflammatory stimulation

Considering the known role played by inflammation in promoting ED, in the present study we also assessed the ECFC ability to activate in the presence of a pro-inflammatory stimulation in order to investigate another possible mechanism underlying ED in uVTE patients. To do that, ECFCs obtained from HDs and the three subgroups of VTE patients were stimulated with TNFα and then their activation status was assessed by using the tests applied in the previous section.

4.3.4.1 Immunophenotype of TNFα-treated ECFC by flow cytometry

To define the activation state of ECFCs after TNFα treatment, the expression levels of VCAM-1 and TF were assessed by flow cytometry. As expected, TNFα treatment induced the activation of ECFCs obtained from HDs as confirmed by the significantly higher frequency of VCAM-1 and TF expressing cells detected in TNFα-treated cells compared with untreated ones (Figure 4.22 A-B). In addition, TNFα treatment induced activation also of ECFCs obtained from the three subgroups of VTE patients as confirmed by the significantly higher frequency of VCAM-1+ and TF+ cells detected in TNFα-treated cells compared with untreated ones (Figure 4.22 A-B).

In addition, after stimulation with TNFα, the frequency of VCAM-1+ cells was significantly higher in ECFCs obtained from uVTE and wpVTE patients compared with HD ECFCs (Figure 4.22 C). Similar results were observed when in TNFα-treated ECFCs was assessed the frequency of TF+ cells (Figure 4.22 D).

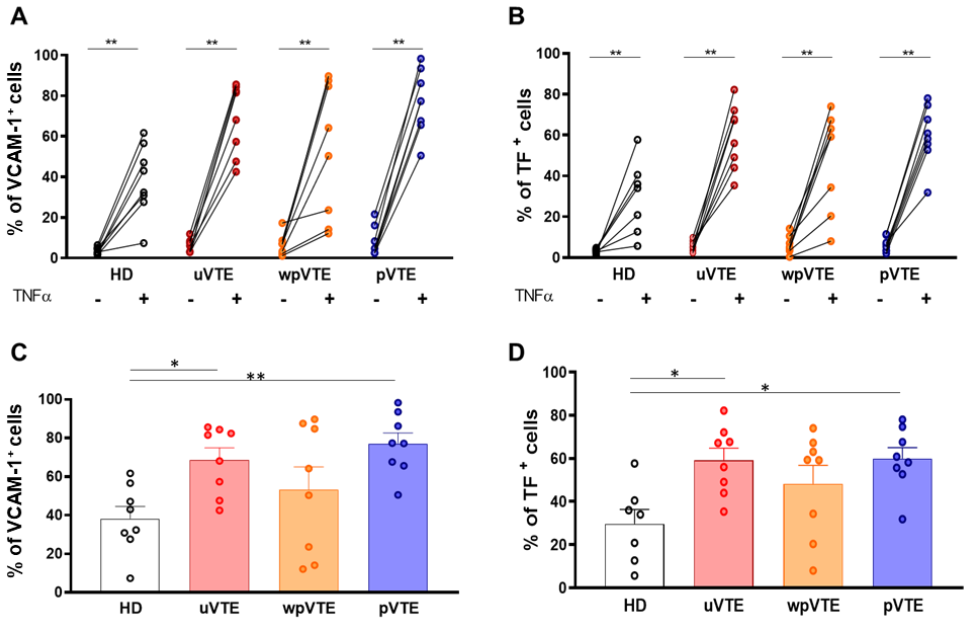

Figure 4.22 | Evaluation of the endothelial activation marker expression levels in ECFCs after pro-inflammatory stimulation. The frequencies of VCAM-1+ and TF+ cells were evaluated at baseline and upon TNFα-treatment in ECFCs isolated from healthy donors (VCAM-1: HD n=8; TF: HD n=7) and VTE patients (uVTE n=8, wpVTE n=8, pVTE n=8). A-B) *Data are presented as dot plot with paired data.* C-D) The frequencies of VCAM-1+ and TF+ cells in TNFα-treated ECFCs, data shown *as mean ± SEM.*p<0.05, **p<0.01. Statistical significance was calculated using (A-B) Wilcoxon matched-pairs signed rank test, and (C-D) Uncorrected Dunn's test.*

By stratifying patients for the VTE recurrence risk on the on the basis of RVO and/or RPVO presence at follow-up evaluation we observed that in uVTE patients the frequencies of VCAM-1+ and TF+ cells in TNFα-treated ECFCs were similar between low and high-risk patients (Figure 4.23). Similar results were obtained when wpVTE patients and pVTE patients were considered.

In addition, in high risk patients the frequency of VCAM-1+ and the frequency of TF+ cells were similar among patient groups (Figure 4.23) whereas trend more similar to those observed in unstratified patients were obtained in low risk patients with wpVTE ECFCs being the ones with the lowest percentages of VCAM-1+ and of TF+ cells after TNFα treatment among patient groups (Figure 4.23).

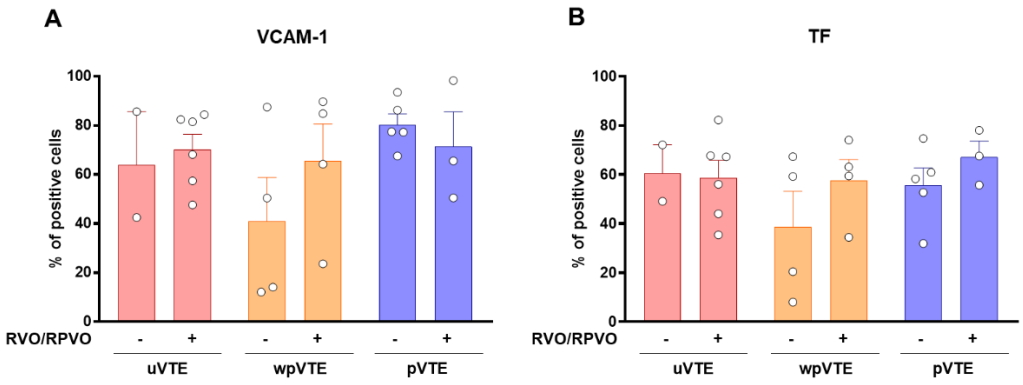

Figure 4.23 | VCAM-1 and TF expression in TNFα-treated ECFCs of VTE patients subgrouped on the basis of Residual obstruction presence at the follow-up evaluation. A) Frequency of VCAM-1+ cells and (B) frequency of TF+ cells in ECFCs obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=2, wpVTE n=4, pVTE n=5) and from VTE patients in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=6, wpVTE n=4, pVTE n=3). Patients were considered positive for a residual obstruction when a RVO and or a RPVO were detected at the follow-up. *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

4.3.4.2 TGA on TNFα-treated ECFCs

To further investigate the activation state of uVTE patient ECFCs in presence of pro-inflammatory stimulation, ECFCs ability to sustain thrombin formation was assessed performing a TGA on TNFα-treated ECFCs.

Firstly, we compared in each analysed group the thrombin formation sustained by TNFα-treated ECFCs with the results obtained by using untreated cells as substrate in the assay. As shown in Figure 4.24 A, TNFα treatment was able to induce ECFC activation in all the considered groups as demonstrated by the significantly reduced lag time recorded when in TGA TNFα-treated ECFCs were used as substrate. In addition, in HD group, a significantly higher amount of thrombin – assessed either as peak (Figure 4.24 B) and ETP (Figure 4.24 C) – was generated when TNFα-treated ECFCs were used as substrate compared with their untreated counterpart. Concerning VTE patients, in all subgroups analysed no significant differences were observed in thrombin Peak and ETP when TNFα-treated ECFCs were compared to their untreated counterpart (Figure 4.24 B-C).

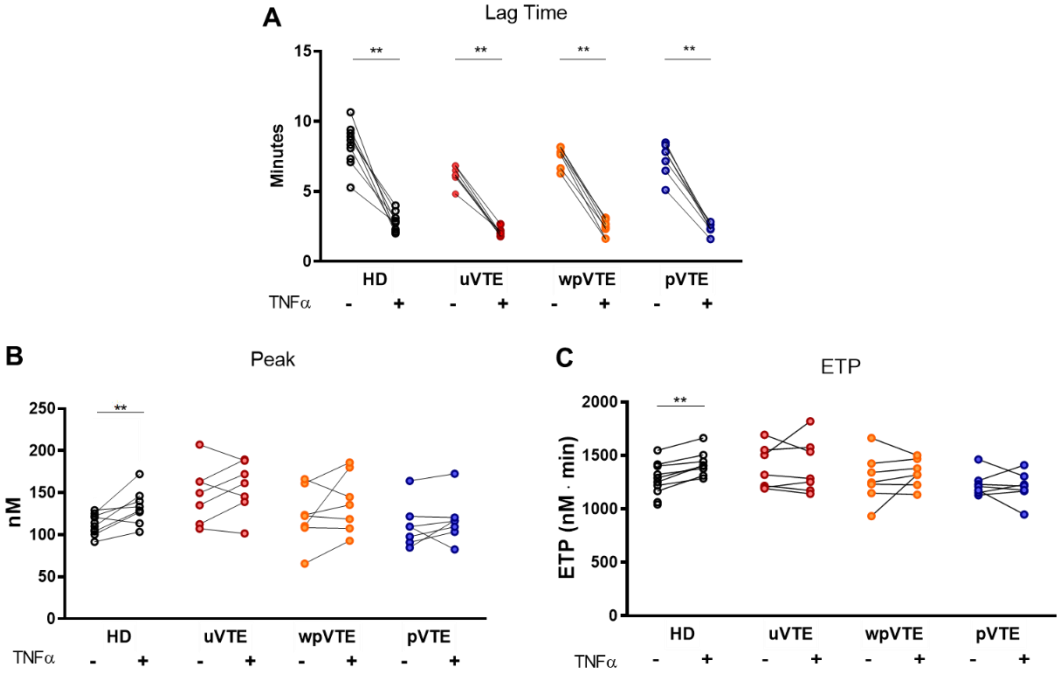

Figure 4.24 | Evaluation of Thrombin generation on ECFCs cultured in basal conditions and after TNFα treatment. Thrombin generation was evaluated by TGA on ECFCs isolated from healthy donors (HD n=8) and VTE patients (uVTE n=7, wpVTE n=7, pVTE n=7) in untreated condition and after treatment with TNFα. The following parameters were considered: A) Lag time, expressed in minutes, B) Peak thrombin, expressed in nM and C) ETP, expressed in nM of thrombin x minute. *Data are presented as dot plot with paired data. *p<0.05, **p<0.01. Statistical significance was calculated using Wilcoxon matched-pairs signed rank test.*

Considering the thrombin generation sustained by TNFα-treated ECFCs, the Lag time that was significantly lower in uVTE patients compared to HDs thus indicating that a faster thrombin generation occurred when TNFα-treated uVTE ECFCs were used as substrate in the assay whereas no significant differences were observed between the patients with secondary VTE and HDs (Figure 4.25 A). Concerning the amount of thrombin formed, no significant differences in the Peak and in the ETP were observed between TNFα-treated ECFCs obtained from uVTE patients and HDs (Figure 4.25 B-C). Comparting the three subgroups of VTE patients we observed that the highest peak of thrombin was produced when TNFα-treated uVTE ECFCs were used as substrate with the peak produced by TNFα-treated uVTE ECFCs being significantly higher compare to the peak produced by TNFα-treated ECFCs obtained from pVTE patients (Figure 4.25 B). Moreover, a significantly lower ETP was observed

when TNFα-treated ECFCs isolated from pVTE patients were used as substrate compared with TNFα-treated ECFCs isolated from HDs (Figure 4.25 C).

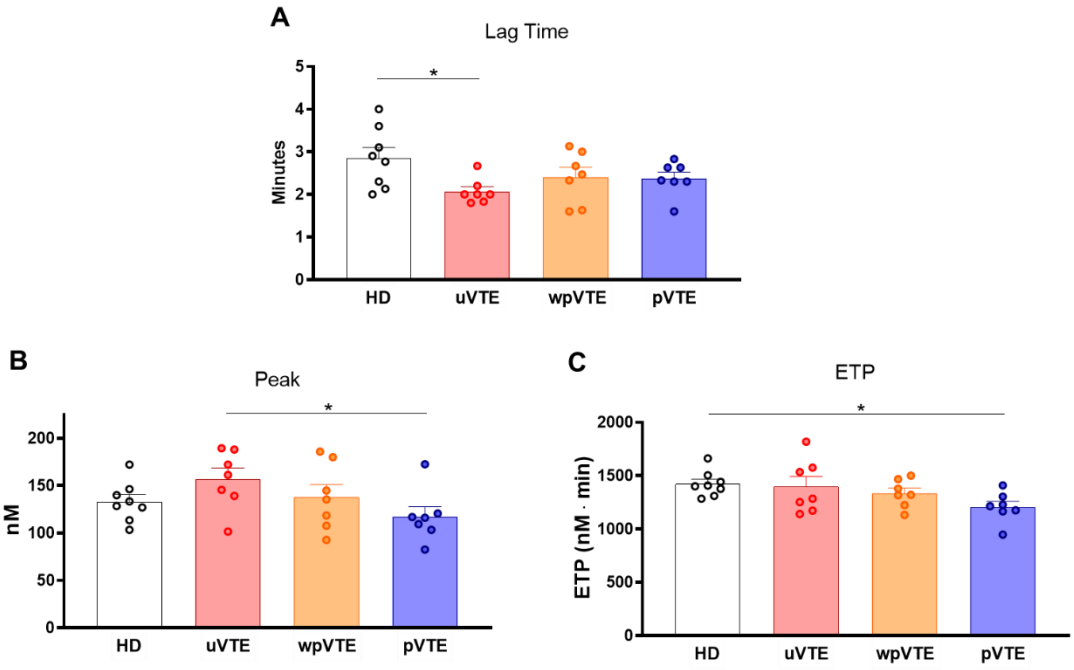

Figure 4.25 | Evaluation of thrombin generation on TNFα-treated ECFCs. Thrombin generation on TNFα-treated ECFCs obtained from healthy donors (HDs n=8) and VTE patients (uVTE n=7, wpVTE n=7, pVTE n=7) was evaluated considering the following parameters: A) Lag time, expressed in minutes, B) Peak thrombin, expressed in nM and C) ETP, expressed in nM of thrombin x minute. *Data are presented as mean ± SEM. *p<0.05. Statistical significance was calculated using Uncorrected Dunn's test.*

Stratifying uVTE patients as low- and high-risk patients for VTE reccurrence according to the presence of a residual obstruction, an increased thrombin generation assessed either as Peak and as ETP was observed in the group of high-risk patients compared with low-risk ones despite the limited number of patients analyzed in the comparison (Figure 4.26). In wpVTE and pVTE groups, similar thrombin formation was detected when TNFα-treated ECFCs obtained from low and high-risk patients were compared.

Restricting the analysis to patients with high risk of VTE trends similar to those observed on all patients were observed (Figure 4.26). In fact, in high risk patients the fastest thrombin formation occurred when ECFCs obtained from uVTE patients were used as substrate in the assay. Moreover, by evaluating

the amount thrombin produced – assessed either in term of Peak and ETP – the greatest thrombin formation was observed when ECFCs obtained from uVTE patients were used as substrate (Figure 4.26). In low risk patients, thrombin formation assessed in TGA on ECFCs was similar among groups, only a trend of faster thrombin formation was observed when uVTE ECFCs were used as substrate compared with ECFCs isolated from patients with secondary VTE (Figure 4.26).

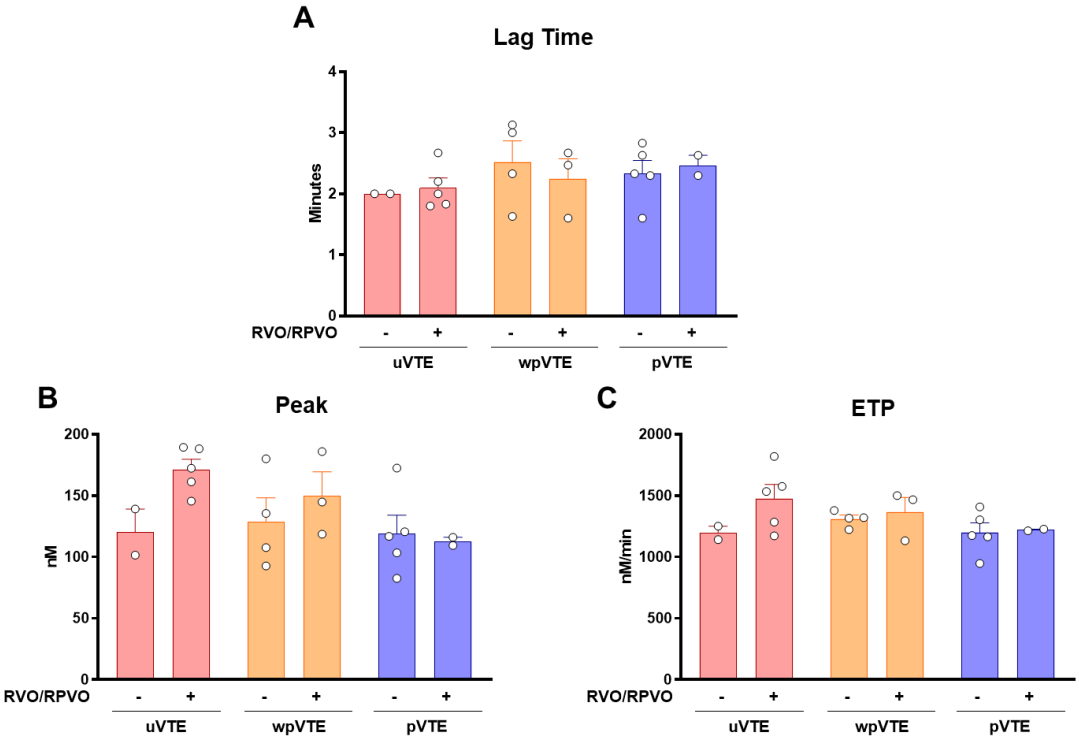

Figure 4.26 | Thrombin formation in TGA on TNFα-treated ECFCs of VTE patients subgrouped on the basis of Residual obstruction presence at the follow-up evaluation. Thrombin generation was evaluated performing TGA on ECFCs obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=2, wpVTE n=4, pVTE n=5) and on ECFCs obtained from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=5, wpVTE n=3, pVTE n=2). Patients were considered positive for a residual obstruction when a RVO and or a RPVO were detected at the follow-up. The following parameters were evaluated: A) Lag time, expressed in minutes B) Peak thrombin, expressed in nM and C) ETP, expressed in nM of thrombin x minute. *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

4.3.4.3 Thrombogenesis assay on TNFα-treated ECFCs

To conclude the evaluation of the activation state of ECFC following pro-inflammatory stimulation, the thrombogenesis assay was performed on ECFCs treated with TNFα. As previously described, the area and the volume of fibrin formation and platelet deposition occurring on layers of ECFCs obtained from the three subgroups of VTE patients and HDs were assessed.

Firstly, we assessed whether TNFα-treatment affect the ECFC ability to sustain fibrin formation and platelet deposition in thrombogenesis assay by comparing in each analysed groups the results obtained on TNFα-treated cells with the ones obtained using as substrate their untreated counterpart. As shown in Figure 4.27, both fibrin formation and platelet deposition on HD ECFCs were significantly increased in TNFα-treated cells compared with untreated ones. Similar results were observed when ECFCs isolated from the VTE patients were taken into account despite the TNFα treatment did not induce a statistically significant increase of all the assessed parameters in all the three VTE subgroups (Figure 4.27).

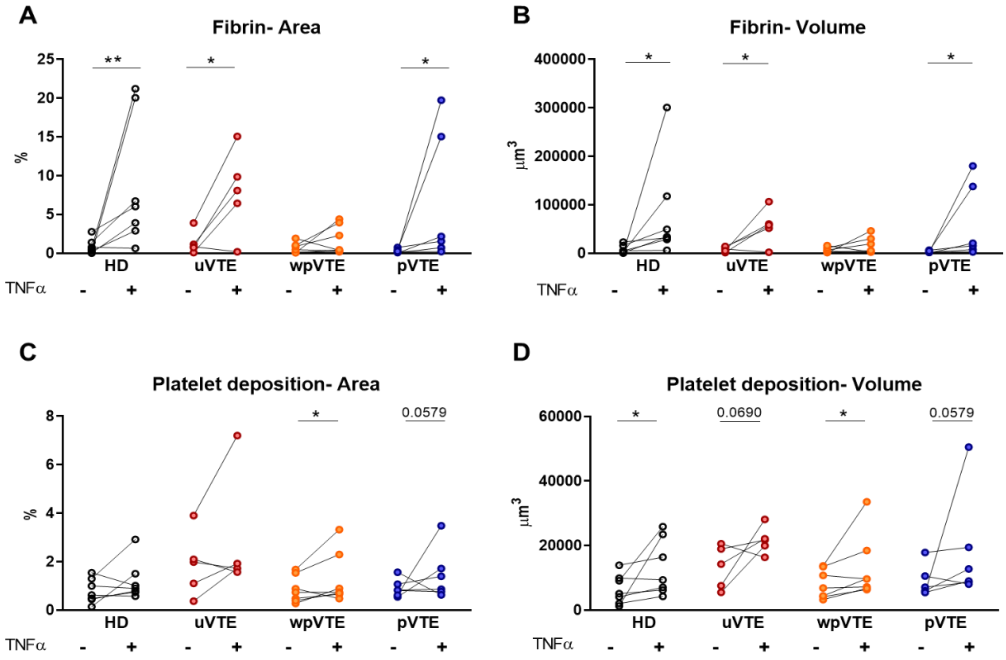

Figure 4.27 | Evaluation of fibrin formation and platelet deposition in thrombogenesis assay on ECFCs cultured in basal conditions and after TNFα treatment. (A-B) Fibrin formation and (C-D) platelet deposition on ECFC layer were assessed in thrombogenesis assay. Either untreated and TNFα-treated ECFCs obtained from HDs and the three subgroups of VTE patients were used as substrate. Data are expressed as percentage of covered area (A-C) and as volume (B-D). *Data are presented as dot plot with paired data. *p<0.05, **p<0.01. Statistical significance was calculated using Wilcoxon matched-pairs signed rank test.*

By evaluating the results obtained in thrombogenesis assay perfomed using TNFα-treated ECFCs as substrate, we observed that similar fibrin formation occurred on TNFα-treated ECFCs obtained from uVTE and HDs (Figure 4.28). No significant differences were observed among VTE patient groups despite ECFCs from wpVTE patients were characterized by the lowest fibrin formation being significantly reduced compared with the amount formed on layers of HDs ECFCs either in terms of covered area covered and in terms of volume (Figure 4.28).

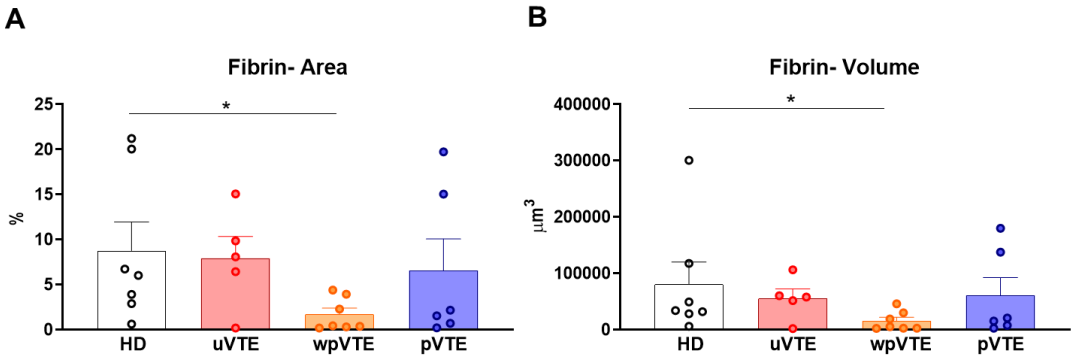

Figure 4.28 | Assessment of fibrin formation on TNFα-treated ECFCs. Fibrin formation on ECFC layers was evaluated as A) percentage of covered area and B) volume (μm^3) by thrombogenesis assay. ECFCs isolated from healthy donors (HD n=7) and VTE patients (uVTE n=5, wpVTE n=7, pVTE n=6) were used as substrate upon incubation with TNFα. *Data are presented as mean ± SEM. *p<0.05. Statistical significance was calculated using Uncorrected Dunn's test*

Stratifying uVTE patients as low- and high-risk patients for VTE recurrence according to the presence of a residual obstruction, no differences were observed in fibrin formation when the area was assessed yet only one uVTE patient was classified as low risk. (Figure 4.29). Similar fibrin formation on TNFα-treated ECFCs was observed when low- and high-risk patients were compared within wpVTE patient group and within pVTE patient group.

Restricting the analysis to patients with high risk of VTE recurrence, trends similar to those observed on all patients were observed (Figure 4.29). In fact, also in high risk patients, ECFCs from wpVTE patients were characterized by the lowest fibrin formation either in term of area and volume. A similar trend was observed in low risk patients yet only one of the analysed uVTE patients was classified as low-risk (Figure 4.29).

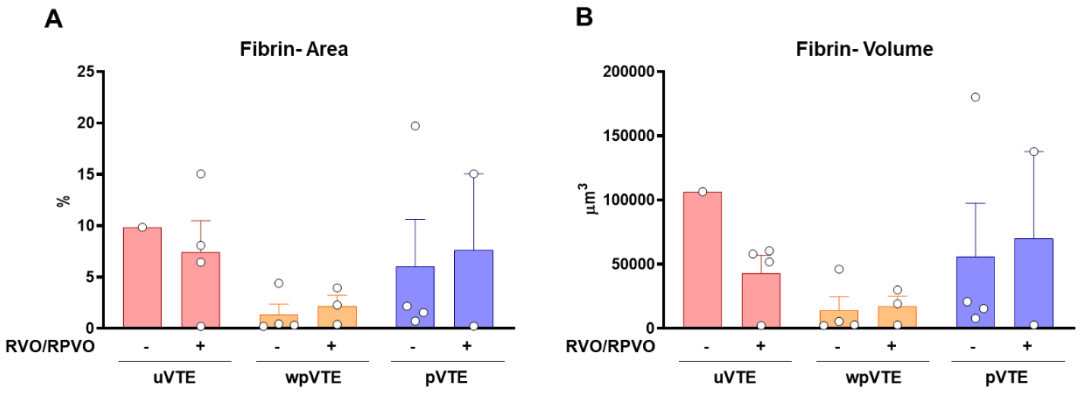

Figure 4.29 | Fibrin formation on TNFα-treated ECFCs of VTE patients subgrouped on the basis of residual obstruction presence at the follow-up evaluation. Fibrin formation was evaluated performing Thrombogenesis assay on TNFα-treated ECFCs obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=1, wpVTE n=4, pVTE n=4) and on TNFα-treated ECFCs obtained from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=4, wpVTE n=3, pVTE n=2). Patients were considered positive for a residual obstruction when a RVO and or a RPVO were detected at the follow-up. Fibrin formation was assessed as A) percentage of covered area and B) volume (µm^3). *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

Furthermore, in the thrombogenesis assay the platelet deposition occurring on layers of TNFα-treated ECFCs was also evaluated.

As shown in Figure 4.30, among the considered groups, the highest platelet deposition occurred when in the thrombogenesis assay TNFα-treated ECFCs obtained from uVTE patients were used as substrate. In particular, the area covered by platelet aggregates was significantly increased on ECFCs obtained from uVTE compared with ECFCs obtained from wpVTE (Figure 4.30 A).

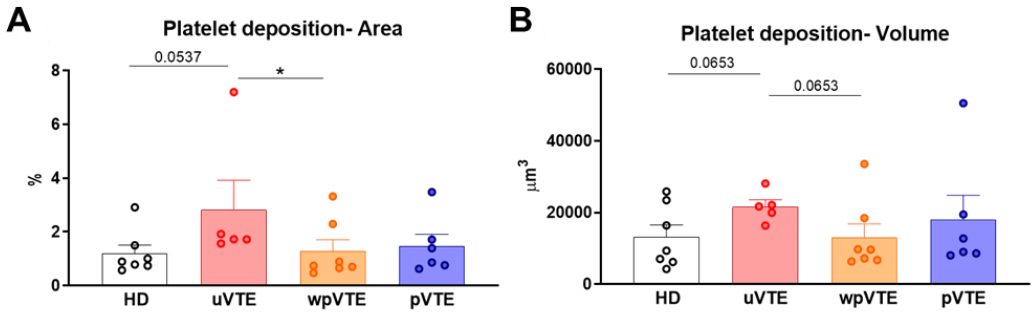

Figure 4.30 | Platelet deposition on TNFα-activated ECFCs. Platelet deposition on ECFC layer was evaluated as A) percentage of covered area and B) volume (µm^3) in thrombogenesis assay. TNFα-treated ECFCs isolated from healthy donors (HD n=7) and VTE patients (uVTE n=5, wpVTE n=7, pVTE n=6) were used as substrate in the assay. *Data are presented as mean ± SEM. *p<0.05. Statistical significance was calculated using Uncorrected Dunn's test.*

Stratifying uVTE patients as low- and high-risk patients for VTE recurrence according to the presence of a residual obstruction, no differences were observed in platelet deposition yet only one uVTE patient was classified as low risk. (Figure 4.31). Similar results were observed in pVTE patient group whereas a trend in increasing platelet deposition was observed on TNFα-treated ECFCs when high-risk patients were compared low-risk ones within wpVTE patient group. (Figure 4.31). Restricting the analysis to patients with high risk of VTE recurrence, trends similar to those observed on all patients were observed (Figure 4.31). In fact, in high-risk patients, ECFCs from uVTE patients were characterized by the highest platelet deposition compared with ECFC obtained from patients with secondary VTE, especially compared with pVTE patients (Figure 4.31). A similar trend was observed in low risk patients yet the evaluation was performed on a limited number of patients.

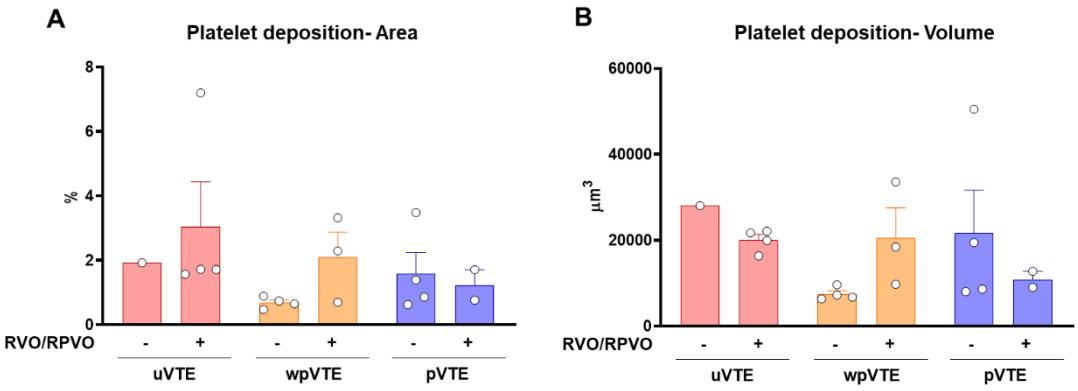

Figure 4.31 | Platelet deposition on TNFα-treated ECFCs of VTE patients subgrouped on the basis of residual obstruction presence at the follow-up evaluation. Platelet deposition was evaluated performing the thrombogenesis assay on TNFα-treated ECFCs obtained from VTE patients who did not have a residual obstruction (RVO/RPVO-) (uVTE n=1, wpVTE n=4, pVTE n=4) and on TNFα-treated ECFCs obtained from VTE patients at high risk of VTE recurrence in whom a residual obstruction was detected during the follow-up evaluation (RVO/RPVO+) (uVTE n=4, wpVTE n=3, pVTE n=2). Platelet deposition was assessed as A) percentage of covered area and B) volume (μm^3). *Data are presented as mean ± SEM. Statistical significance was calculated using Uncorrected Dunn's test.*

4.3.5 Transcriptome profiling of ECFCs

To identify the mechanisms underlying ED in uVTE patients, we performed an RNA sequencing analysis of the ECFCs obtained from HDs and the three subgroups of VTE patients in order evaluate their transcriptome profile. In this first set of analysis we sequenced ECFCs obtained from 4 HDs, 3 uVTE patients, 4 wpVTE patients, and 4 pVTE patients.

By performing a principal component analysis (PCA) we assessed whether the different subgroups clusterized separately. As shown in Figure 4.32 A, the analysed samples clusterized in two groups in the basis of subject sex. Therefore, we corrected our analysis for sex thus obtaining the clustering shown in Figure 4.32 B.

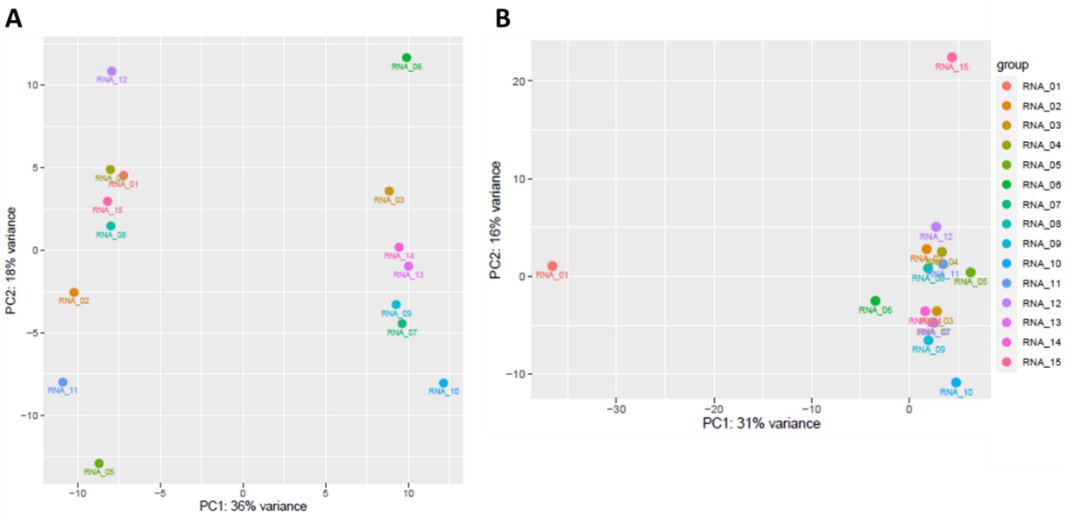

Figure 4.32 | Principal component analysis (PCA). PCA plot of the normalized expression matrix. Each point represents an individual sample. A) PCA performed on the three subgroups of VTE patients and HD subjects. B) PCA performed on the three subgroups of VTE patients and HDs corrected for sex.

In accordance with the fact that no clear clusters can be defined in PCA plot, only few differentially expressed genes (DEGs) emerged by the comparison of the analysed groups. Considering that also in functional experiments ECFCs obtained from patients with secondary VTE were similar to those obtained from HDs, few differences were expected between HDs and patients with secondary VTE.

In particular, by setting a threshold for the p-value adjusted for the FDR to 0.05, we identified 13 DEGs comparing uVTE patients with HDs, 21 DEGs comparing wpVTE patients with HDs, and only 4 DEGs comparing pVTE patients with HDs (Table 4.1).

Differentially Expressed Genes (DEGs)					
uVTE vs HDs		w pVTE vs HDs		pVTE vs HDs	
Gene name	log_2FoldChange	Gene name	log_2FoldChange	Gene name	log_2FoldChange
MIR216A	20,703	*CCL8*	25,256	*TDRD9*	22,621
TDRD9	16,123	*TDRD9*	23,271	*MYPN*	3,979
DHRS9	9,423	*MIR216A*	20,381	*FOSB*	-2,855
CNTNAP3	1,525	*LINC00615*	18,099	*ZNF736P9Y*	-22,114
HES1	-2,112	*SIX2*	17,850		
IRF6	-2,872	*SPINK2*	17,691		
FOSB	-3,006	*DGKB*	17,490		
FOS	-3,018	*PCDH10-DT*	15,277		
HEY1	-3,930	*MYPN*	3,277		
FAM86FP	-7,127	*MMP10*	2,837		
PTGIR	-19,163	*EFNA5*	2,177		
KCNB2	-20,295	*PLEC*	0,782		
GSTM1	-25,017	*COLGALT1*	0,721		
		PLPP3	-1,171		
		DUSP1	-1,558		
		HES1	-1,939		
		ARIH2OS	-2,234		
		FOS	-2,813		
		FOSB	-3,149		
		HEY1	-3,664		
		CDH4	-19,326		

Table 4.1 | List of DEGs in the uVTE, wpVTE and pVTE subgroup of patients compared to HD subjects. DEGs were identified on the basis of the *p-value adjusted for FDR with the threshold set to < 0.05.*

Since the focus of our study was to investigate the mechanism(s) underlying ED in uVTE, we compared the DEGs deriving from the comparison of the 3 subgroups of VTE patients with HDs to identify those genes that are DEGs specific for uVTE patients and not shared with the DEGs emerging comparing secondary VTE with HDs. As shown in Figure 4.33, 7 genes out of the 13 DEGs were specific for uVTE patients whereas 6 genes were differentially expressed not only by ECFCs obtained from uVTE patients but also by ECFCs obtained from wpVTE and/or pVTE patients when compared with healthy donor ECFCs.

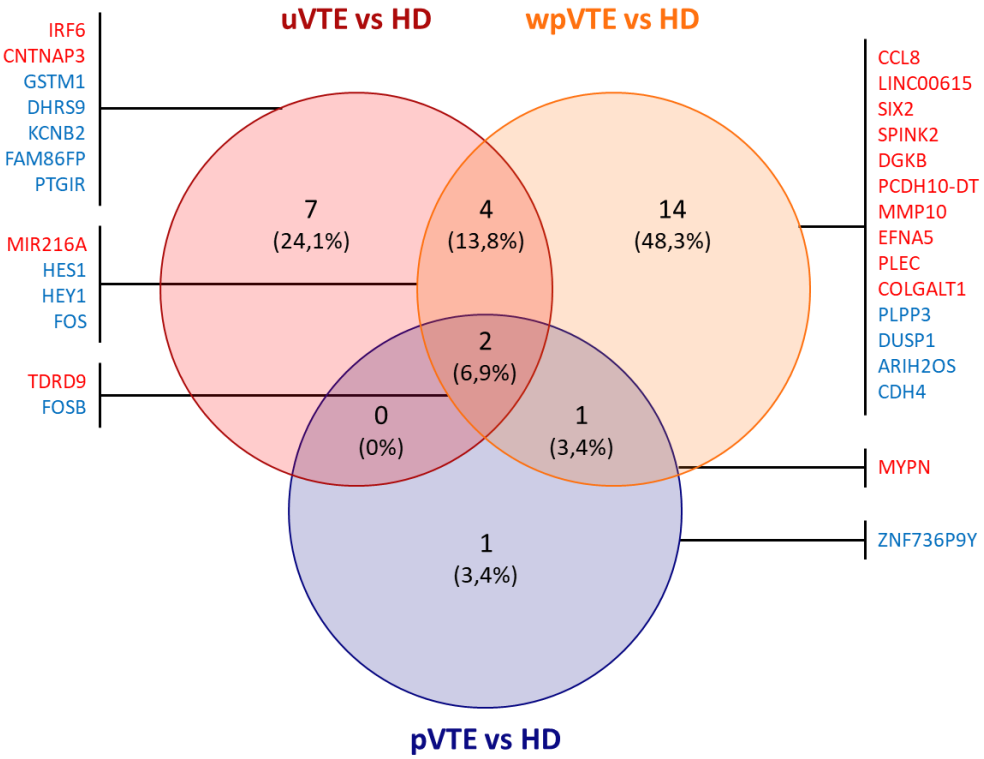

Figure 4.33 | Intersection of Differentially expressed genes (DEGs) in each subgroup of VTE patients compared to HD subjects by using the Venn diagrams. Downregulated DEGs are shown in blue, upregulated DEGs are shown in red. *Statistical significance p-value adjusted for FDR < 0.05. The diagrams were drawn using Venny v. 2.1.0.*

Considering that from the *in vitro* functional assay the presence of ED emerged as a feature of uVTE patients, in this preliminary analysis we focused on those genes that not only resulted as DEGs specific of the uVTE group when patients were compared with HDs but that also emerged as being differentially expressed among the three VTE patient subgroups. To do this, we identified also the DEGs that emerged comparing uVTE patients with wpVTE patients and by comparing uVTE patients with pVTE patients respectively (Table 4.2) and the resulting DEGs were compared with the ones that emerged by comparing uVTE patients with HDs.

Differentially Expressed Genes (DEGs)					
uVTE vs HDs		uVTE vs wpVTE		uVTE vs pVTE	
Gene name	log_2FoldChange	Gene name	log_2FoldChange	Gene name	log_2FoldChange
MIR216A	20,703	*CDH4*	22,420	*MIR216A*	32,409
TDRD9	16,123	*AQP1*	7,150	*ZNF736P9Y*	21,621
DHRS9	9,423	*ADAMTS1*	1,334	*RPS3AP3*	7,997
CNTNAP3	1,525	*DYNLT5*	-2,394	*CCDC74B*	6,547
HES1	-2,112	*MMP10*	-3,448	*DHRS9*	5,796
IRF6	-2,872	*FAM86FP*	-6,993	*INSIG1*	-1,263
FOSB	-3,006	*HOXA-AS3*	-7,106	*ANXA3*	-1,333
FOS	-3,018	*LINC01811*	-7,319	*RSPO3*	-3,927
HEY1	-3,930	*NRK*	-9,067	*HOXA-AS3*	-6,914
FAM86FP	-7,127	*KCNB2*	-19,382	*LINC01811*	-7,377
PTGIR	-19,163	*PTGIR*	-22,109	*NRK*	-8,067
KCNB2	-20,295	*GSTM1*	-23,405	*PTGIR*	-19,505
GSTM1	-25,017			*KCNB2*	-22,164
				GSTM1	-23,117

Table 4.2 | List of DEGs in the uVTE, wpVTE and pVTE subgroup of patients compared to HDs. Differentially expressed genes (DEGs) were identified on the basis of the *p-value adjusted for FDR with the threshold set to < 0.05.*

As shown in the Venn diagrams depicted in Figure 4.34, three genes (GSTM1, PTGIR, and KCNB2) were specifically deregulated in ECFCs obtained from uVTEs and in particular they were downregulated in uVTE patients in comparison not only with HDs ECFCs but also with ECFCs obtained from patients with secondary VTE.

Considering the increased adhesiveness for platelet demonstrated by uVTE ECFCs in thrombogenesis assay and the increased levels of s-ICAM-1 and sPECAM-1 detected in the culture supernatant of uVTE ECFCs, among these three genes we focused our attention of GSTM1 and PTGIR.

GSTM1 encodes for Glutathione S-Transferase Mu 1, a detoxifying and antioxidant enzyme involved in the metabolism of a wide type of xenobiotics (Lawrence SE. et al., 2002). GSTM1 downregulation has already been described in association with altered expression of adhesion molecules and is at least partly responsible for the increased susceptibility of patients with end-stage renal disease (ESRD) to develop cardiovascular disease (Jerotic D. et

al., 2021). In addition, Jerotic and colleagues showed that GSTM1 knockdown in HUVECs induced the upregulation of monocyte chemoattractant protein-1 (MCP-1), a cytokine involved in the regulation of monocyte migration and adhesion and the upregulation of ICAM-1 and VCAM-1 expression (Jerotic D. et al., 2021).

PTGIR encodes for Prostaglandin I2 Receptor or PGI2 receptor a member of the G-protein coupled receptor family 1, shown to be a receptor for prostacyclin (PGI2) (Majed BH. et al., 2012). This receptor can be localised to different cell types and has physiological effects by mediating the effects of PGI2 that has been shown to be involved in cardiovascular health, with potent vasodilator effects through smooth muscle relaxation and inhibition of platelet aggregation (Camacho M. et al., 2011; Mohite A. et al., 2012).

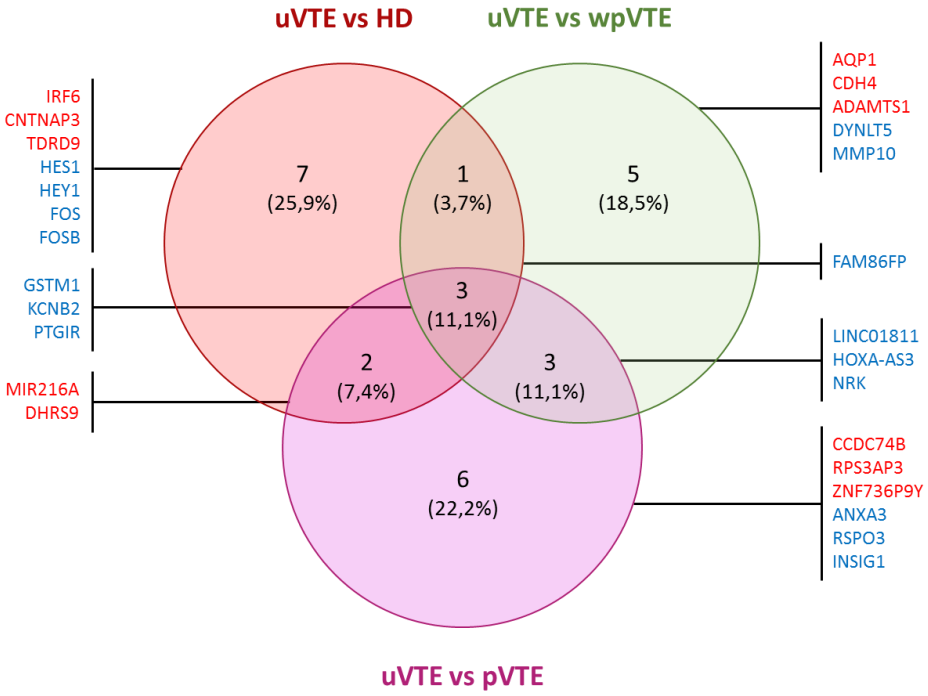

Figure 4.34 | Intersection of Differentially expressed genes (DEGs) in uVTE patients compared to wpVTE patients, pVTE patients and HD subjects by using the Venn diagram. Downregulated DEGs are shown in blue, upregulated DEGs are shown in red. *Statistical significance p-value adjusted for FDR < 0.05. The diagrams were drawn using Venny v. 2.1.0.*

A more detailed analysis of the ECFC transcriptome profile is now in progress in order to extend the analysis of the DEGs to the ones that:

1. emerged from the comparison between uVTE and HDs, but are not differentially expressed when uVTE patients are compared with wp and pVTE;
2. are shared by the different VTE groups;
3. are specific of patients with secondary VTE.

This approach will allow us to identify other mechanism(s) responsible of ED that despite not being specific for uVTE patients can contribute to endothelial alterations - such as the increased early senescence - that we observed not only in ECFCs obtained from uVTE patients but also in ECFCs isolated from patients with secondary VTE.

4.3.5.1 Pathway analysis

In order to elucidate the molecular pathway(s) underlying ED in uVTE patients and disclose whether they are specific or shared with secondary VTE, we assessed the specific enrichment in gene signatures (Gene Set) by using GSEA and comparing each subgroup of VTE patients with HDs, and also performing the comparison among the 3 subgroups of VTE patients.

In this analysis, we took into consideration those GSEA collections that encompass Gene sets containing genes annotated by the same ontology term. In particular, we used Gene sets referred to GO molecular functions (MF) and GO biological processes (BP), setting the Gene set size between 5 and 500 genes, and the threshold for the nominal p-value <0.01, to identify the enriched Gene sets.

In the analysis of MF terms, by comparing ECFCs obtained from uVTE, wpVTE or pVTE patients with ECFCs obtained from HDs we identified the Gene sets that were enriched in HD ECFCs (Reported as “DOWN” in Table 4.3) and the Gene sets that were enriched in VTE patient ECFCs (Reported as “UP” in Table 4.3).

MOLECULAR FUNCTIONS	nominal p-value < 0.01		
	TOT	UP	DOWN
uVTE vs HD	6	4	2
wpVTE vs HD	18	8	10
pVTE vs HD	13	5	8

Table 4.3 | Number of GO Molecular Function (MF) Gene sets that resulted significantly enriched in the analyzed comparisons setting the threshold for the nominal p-value < 0.01. UP: number of Gene sets enriched in ECFCs from uVTE, wpVTE and pVTE patients compared to HDs. DOWN: number of Gene sets enriched in HD ECFCs in the comparisons with the three subgroups of VTE patients.

Because the focus of our study was to investigate the mechanism(s) underlying ED in uVTE, we compared the enriched MF Gene sets deriving from the comparison of the 3 subgroups of VTE patients with HDs to identify those Gene sets that were specifically enriched in the comparison between uVTE and HD ECFCs, but not in the comparison of ECFCs obtained from secondary VTE with HDs ECFCs. As shown in Figure 4.35, five out of six Gene sets were specifically enriched in the comparison between uVTE patients and HDs, whereas only one Gene set resulted enriched in both uVTE and wpVTE ECFCs compared with HD ECFCs. Notably, four of the Gene sets enriched in uVTE ECFCs (namely: *GOMF -Oxygen Binding; GOMF – Cullin Family Protein Binding; GOMF – Steroid Dehydrogenase Activity Acting On The Ch Oh Group Of Donors Nad Or Nadp As Acceptor; GOMF –Steroid Dehydrogenase Activity;* Figure 4.35) were related to oxidative stress that is known to be associated with ED.

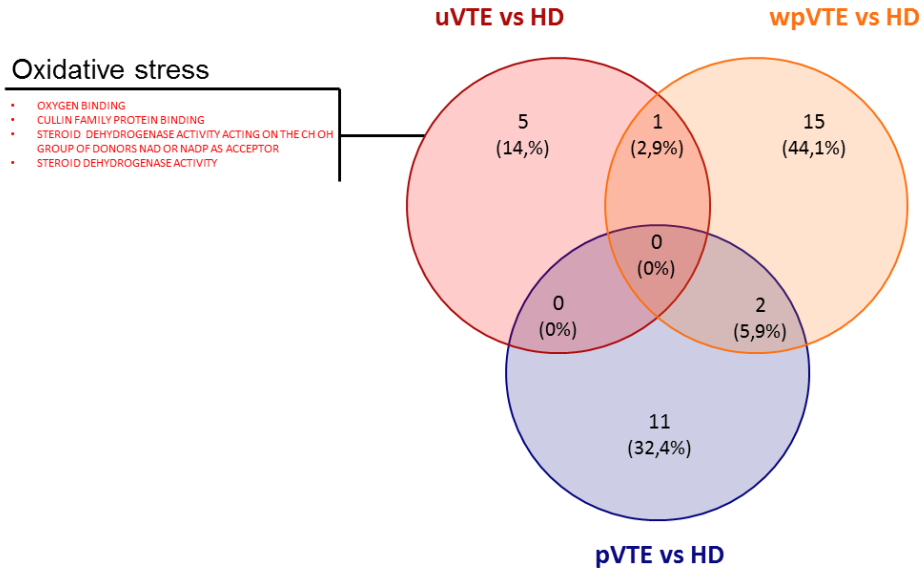

Figure 4.35 | Intersection of differentially enriched Molecular Function (MF) Gene sets that resulted in the comparisons between ECFCs from each subgroup of VTE patients and HD ECFCs. *Statistical significance was considered by setting a threshold for nominal p-value < 0.01. In red: selected Gene sets enriched in uVTE patients compared with HDs. The diagrams were drawn using Venny v. 2.1.0.*

In order to investigate Gene sets specifically enriched in ECFCs obtained from uVTE patients, GSEA analysis was also performed by comparing uVTE ECFCs with ECFCs obtained from wpVTE patients or pVTE patients. As shown in Table 4.4, we identified MF Gene sets that were enriched in uVTE patients ECFCs (Reported as "UP" in Table 4.4) and Gene sets that were enriched in ECFCs obtained from wpVTE patients or pVTE patients (Reported as "DOWN" in Table 4.4).

MOLECULAR FUNCTIONS	nominal p-value < 0.01		
	TOT	UP	DOWN
uVTE vs wpVTE	8	4	4
uVTE vs pVTE	10	1	9

Table 4.4 | Number of GO Molecular Function (MF) Gene sets that resulted as significantly enriched in the analyzed comparisons considering a nominal *p-value < 0.01.* *UP:* number of Gene sets enriched in uVTE patients when compared to wpVTE and pVTE subgroups of patients, respectively. DOWN: number of Gene sets enriched in wpVTE or pVTE patients when compared to uVTE patients.

As shown in the Venn diagrams in Figure 4.36, the MF Gene sets related to oxidative stress were not further enriched in uVTE ECFCs compared with ECFCs obtained from secondary VTE patients. However, the presence of a more pronounced oxidative stress in uVTE ECFCs was suggested by the enrichment of MF Gene sets involved in anti-oxidative pathways (namely: GOMF – Transferase activity Transferring Alkyl Or Aryl Other Than Methyl Groups; GOMF – Glutathione Transferase Activity; GOMF – Glutathione Binding) in the intersection of wpVTE and pVTE ECFCs.

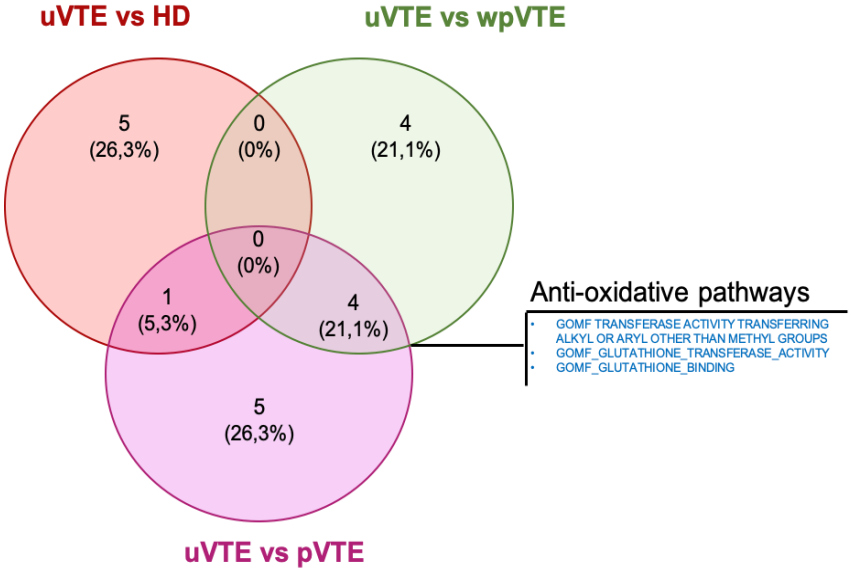

Figure 4.36 | Intersection of differentially enriched Molecular Function (MF) Gene sets that resulted in the comparisons between uVTE patients and HDs, uVTE and wpVTE patients, uVTE and pVTE patients, respectively. *Statistical significance was considered by setting a threshold for nominal p-value < 0.01. In red: selected Gene sets enriched in uVTE patients compared with HDs. In blue: selected Gene sets downregulated in uVTE patients and enriched in both wpVTE and pVTE patients. The diagrams were drawn using Venny v. 2.1.0.*

A similar analysis was then performed taking into consideration Gene sets related to GO Biological processes (BP) terms. In particular, by comparing HD ECFCs with ECFCs obtained from the three different groups of VTE patients we identified Gene sets that were enriched in HD ECFCs (Reported as "DOWN" in Table 4.5) and BP Gene sets that were enriched in ECFCs from VTE patients (Reported as "UP" in Table 4.5).

BIOLOGICAL PROCESSES	nominal p-value < 0.01		
	TOT	UP	DOWN
uVTE vs HD	49	4	45
wpVTE vs HD	165	93	72
pVTE vs HD	103	92	11

Table 4.5 | Number of GO Biological Processes (BP) Gene sets that resulted significantly enriched in the analyzed comparisons considering a nominal *p-value < 0.01*. *UP:* number of Gene sets enriched in uVTE, wpVTE and pVTE patients respectively when compared to HDs. DOWN: number of Gene sets enriched in HD ECFCs in the comparisons with the three subgroups of VTE patients.

As shown in Figure 4.37, 33 out of 49 Gene sets were specifically enriched in uVTE patients, whereas 16 Gene sets were enriched also in wpVTE and/or pVTE ECFCs compared with HD ECFCs. Among the Gene sets specifically enriched in uVTE ECFCs, we observed three Gene sets involved in vesicle formation (namely: GOBP – Organelle Membrane Fusion; GOBP –Positive Regulation Of Vesicle Fusion; GOBP – Lytic Vacuole Organization; Figure 4.37). An increased release of extracellular vesicles may be related to the increased oxidative stress observed in uVTE patient ECFCs and the consequent condition of increased ED observed in this group of VTE patients.

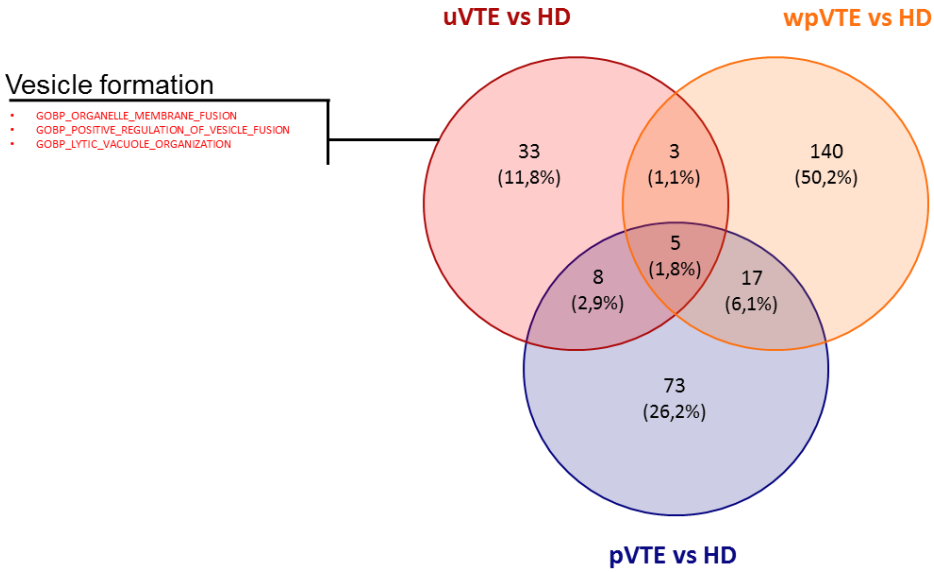

Figure 4.37 | Intersection of enriched Gene sets in Biological processes (BP) in each subgroup of VTE patients compared to HD subjects by using the Venn diagrams. *Statistical significance was considered by setting a threshold for nominal p-value < 0.01. The diagrams were drawn using Venny v. 2.1.0.*

In order to investigate whether these alterations were specifically observed in uVTE ECFCs, a GSEA analysis of the Gene sets related to BP terms was also performed by comparing uVTE ECFCs with ECFCs obtained from wpVTE patients and pVTE patients. As shown in Table 4.6, we identified in both comparisons BP Gene sets that were enriched in uVTE patients ECFCs (Reported as "UP" in Table 4.6) and Gene sets that were enriched in wpVTE and pVTE ECFCs, respectively (Reported as "DOWN" in Table 4.6).

BIOLOGICAL PROCESSES	nominal p-value < 0.01		
	TOT	UP	DOWN
uVTE vs wpVTE	73	24	49
uVTE vs pVTE	77	6	71

Table 4.6 | Number of GO Biological Processes (BP) Gene sets that resulted significantly enriched in the analyzed comparisons considering a nominal *p-value < 0.01.* *UP:* number of Gene sets enriched in uVTE patients when compared to wpVTE and pVTE subgroups of patients, respectively. DOWN: number of Gene sets enriched in wpVTE and pVTE patients respectively when compared to uVTE patients.

As shown in the Venn diagrams in Figure 4.38, BP Gene sets related to release of extracellular vesicles did not result enriched when uVTE ECFCs were compared with ECFCs obtained from secondary VTE patients.

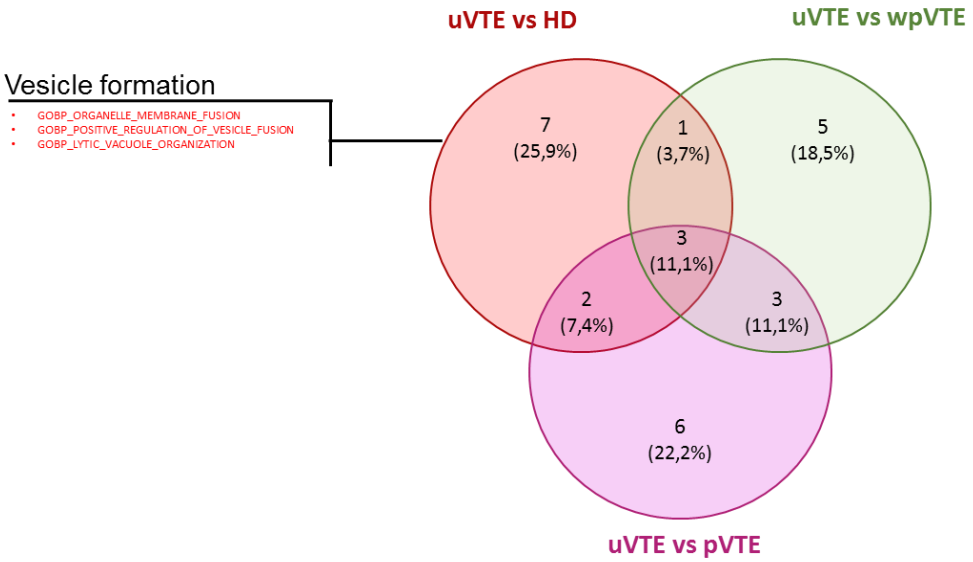

Figure 4.38 | Intersection of enriched Gene sets in Biological processes (BP) in uVTE patients compared to HDs, wpVTE patients, and pVTE patients by using the Venn diagram. *Statistical significance was considered by setting a threshold for nominal p-value < 0.01. The diagrams were drawn using Venny v. 2.1.0.*

A more detailed pathways analysis is still in progress in order to extend the analysis also to the other pathways that emerged as enriched in GSEA analysis.

5. DISCUSSION

Venous thromboembolism represents a condition in which blood clots inappropriately (Almodaimegh H. et al., 2017). Encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), VTE is considered a “silent killer” due to its high morbidity and mortality (Schulman S. et al., 2017; Beckman MG. et al., 2010). Despite several risk factors have been identified as triggers of VTE, up to 50% of VTE events occurs without an identifiable cause; these events are therefore defined unprovoked VTE (uVTE). Since the pathogenesis of uVTE events is unknown, this group of VTE patients have a higher risk of recurrence after stopping anticoagulant treatment (AT) (Áinle FN & Kevane B., 2020) and for these reasons the recommendations for their treatment are controversial, in particular concerning the duration of AT. Shedding the light on the pathogenic mechanism(s) underlying uVTE events is therefore crucial to provide indications to improve treatment strategies for uVTE patients. In this context, considering that in physiological conditions the endothelium exerts an antithrombotic and anticoagulant action (Reichenbach G. et al., 2005), and considering that several studies demonstrated that endothelial damage increases the thrombotic risk in venous compartments (Jezovnik MK. et al., 2010; Poredos P., 2002) it is conceivable that endothelial dysfunction (ED) may contribute to the pathogenesis of uVTE. For these reasons in the present study we investigated ED in uVTE patients, as this aspect has been poorly studied so far.

To study ED in uVTE patients, we characterized patient-derived endothelial colony-forming cells (ECFCs). ECFCs are endothelial progenitors that can be isolated and expanded from the peripheral blood that are endowed with typical endothelial morphology, express endothelial markers, exhibit clonal proliferative potential and directly contribute to neovascularization (Medina RJ. et al., 2017; Mund JA. et al., 2011; Moschetta M. et al., 2014; Yoder MC., 2012). Because of their properties, ECFCs are increasingly used as an optimal non-invasive model that allows to investigate the endothelial compartment in a patient-specific setting (Melero-Martin JM., 2022). Notably, current animal models recapitulating the onset of spontaneous uVTE are lacking, whereas models to study the pathogenesis of pVTE are available (Wolberg AS. et al.,

2015). Therefore, the characterization of patient-derived ECFCs is particularly useful in the context of uVTE to identify molecular pathogenic mechanisms that may be further validated in *in vivo* experiments by assessing the role of candidate pathways in promoting a pro-thrombotic condition.
In addition, to assess whether ECFC features of ED were observed only in uVTE patients or were also observed in secondary VTE, in our study we also included patients with weakly provoked VTE (wpVTE) and provoked VTE (pVTE), and characterized their ECFCs as described for uVTE patients.

In a previous study investigating ECFCs in uVTE patients, we reported that ECFC isolation efficiency in uVTE patients was similar to healthy donors, although ECFC colonies obtained from these patients were characterized by an increased proportion of ECFCs undergoing early senescence (Della Bella S. et al., 2020). A similar propensity of ECFCs obtained from uVTE patients to undergo early senescence was also described by Hernandez-Lopez and colleagues (Hernandez-Lopez R. et al., 2017). In the present study, we confirmed the same ECFC behaviour on a new independent cohort of uVTE patients, and we extended our observation to patients with secondary VTE demonstrating an increased frequency of early senescent ECFC colonies also in patients with wpVTE and pVTE. In the above-mentioned studies, early senescence in uVTE ECFCs was associated to a pathological upregulation of the TNFSF15-TNFRSF25 axis (Della Bella S. et al., 2020) and to increased oxidative stress (Alvarado-Moreno JA. et al., 2016; Hernandez-Lopez R. et al., 2017), both mechanisms likely contributing to an impairment of endothelial function that can represent a permanent risk of recurrence in uVTE patients. The mechanism(s) involved in the increased early senescence observed in patients with secondary VTE is an issue that needs to be further investigated. In this regard, concerning the pathological upregulation of TNFSF15-TNFSRSF25 axis, we previously reported that the upregulation of TNFSF15 was observed in uVTE patients but not in patients with secondary VTE, thus supporting the hypothesis that the endothelial alterations observed in patients with secondary VTE may be mediated by different mechanisms (Della Bella S. et al., 2020). Concerning the role of oxidative stress in promoting ED in VTE

patients, by assessing the ECFC transcriptome profile in this study, we further confirmed in our cohort that ECFCs obtained from uVTE patients were characterized by increased oxidative stress. Moreover, although the presence of increased oxidative stress in ECFCs from patients with secondary VTE could be likely considering that oxidative stress has been associated with known VTE risk factors (i.e. aging, venous stasis, chronic inflammation) (Wang Q. et al., 2020; Pilard M. et al., 2022) our preliminary analysis showed that Gene sets related to oxidative stress were enriched only in uVTE ECFCs, and that Gene set associated with anti-oxidative functions were specifically downregulated in uVTE ECFCs compared with both wpVTE and pVTE ECFCs. Moreover, we observed that Gene sets associated with vesicle production were also specifically enriched in uVTE ECFCs, but not in ECFCs obtained from secondary VTE. This finding may be related to the up-regulation of oxidative stress pathways observed in uVTE ECFCs, as it is well known that the release of extracellular vesicles is increased by conditions of cellular stress, injury, activation, or apoptosis (Burger D. et al., 2013; Viera AJ. et al., 2012; Badimon L.et al., 2016) in response to cell exposure to inflammatory cytoknes, infectious agents, lipoproteins, oxidative stress, coagulation mediators, hypoxia, or shear stress (Burger D. et al., 2013). Therefore, an increased release of extracellular vesicles by endothelial cells may be considered as a biomarker of ED in uVTE patients. Moreover, because of the crucial role played by extracellular vesicles in intercellular communication and their ability to affect the function of their target cells (Sanchez GB. Et al., 2021), it is likely that endothelial extracellular vesicles may heavily contribute to ED in uVTE patients. Further experiments will be needed to: i) quantify the extracellular vesicles released by ECFCs in culture supernatants to validate *in vitro* the increased vesicle production by uVTE ECFCs and their possible use as a marker of ED; ii) characterize the phenotype of ECFC-derived extracellular vesicles by assessing their protein expression; iii) investigate in functional *in vitro* assays the impact of uVTE ECFC-derived microparticles on the behaviour of control ECFCs.

In this study, we aimed to investigate ED by using two functional assays – the TGA and the thrombogenesis assay – properly modified to evaluate the ability of ECFCs to promote thrombosis by monitoring their contribution to the different phases of thrombus formation. In the thrombogenesis assay performed on ECFC layers we evaluated the platelet adhesion - the first phase of haemostasis – and the fibrin formation – the last phase of haemostasis. In the TGA on ECFCs we assessed the formation of thrombin that in turn is involved in fibrin formation. In this modified version of the TGA originally proposed by Hemker (Hemker HC. et al., 2006; Kintigh J. et al., 2017), the amount of generated thrombin is assumed to reflect the activation status of the tested ECs.

In order to adapt the TGA and the thrombogenesis assay to the assessment of ECFC function, we first set up both assays on HUVECs and then moved to ECFCs isolated from healthy donors. In agreement with previous studies in which TNFα has been successfully used as a pro-inflammatory stimulus in *in vitro* experiments on both HUVECs and ECFCs (Jain A. et al., 2016; Green LA. et al., 2016; Rossi E. et al., 2021), TNFα-treated HUVECs and ECFCs were compared with their untreated counterparts to verify that the assays were able to discriminate between activated and non-activated cells. In our experiments, the HUVEC and ECFC activation in response to TNFα treatment was confirmed by the increased expression of VCAM-1 and TF that are both molecules typically expressed by activated ECs (Liu X. et al., 2012). By comparing cells cultured in basal conditions with cells activated *in vitro* with TNFα, we observed that the set up of the assays allowed the assessment of the activation status of the cells used as a substrate. In particular, increased thrombin generation and increased platelet deposition and fibrin formation were observed when either TNFα-treated HUVECs or TNFα-ECFCs were used as a substrate in the assays compared to their untreated counterparts. Our results thus confirmed that ECFCs can be used as a substrate in TGA on ECs and in thrombogenesis assay and are in line with previous studies in which ECFCs were used as a substrate in this type of functional assays. In particular, concerning the assessment of ECFC ability to modulate thrombin

generation, Cuccini and colleagues reported that increased expression of TF in ECFCs from healthy donors induce a strong thrombin-generating capacity without affecting their non-coagulant properties (Cuccini W. et al., 2010). Moreover, in other studies the ECFCs were added in the assay in the presence of exogenous TF thus demonstrating an impairment in the anti-coagulant function of ECFCs in patient with Idiopathic Pulmonary Fibrosis (IPF) that is mediated by a reduction in the expression of the anti-coagulant proteins thrombomodulin and EPCR (Billoir P. et al, 2021; Billoir P. et al., 2018; Collier MEW et al., 2017).

Concerning the thrombogenesis assay, our results are in line with the observations of Marthur and colleagues that using a different vessel-on-chip model assessed the activation status of HUVECs and healthy donor-derived ECFCs comparing TNFα-treated with untreated cells (Mathur T. et al., 2019). Moreover, by using as a substrate ECFCs obtained from type 1 diabetic pigs the Authors confirmed the presence of vascular dysfunction and thrombogenicity in pathological ECFCs compared with control ECFCs (Mathur T. et al., 2019). In our study, by combining both ECFC-adapted assays on the same cells, we demonstrated that the amount of thrombin generated in TGA on ECFCs – assessed either in term of peak and ETP - positively correlated with the amount of fibrin detected in the thrombogenesis assay, thus further supporting the reliability of these assays in the estimation of the thrombotic risk in pathological conditions. Following the successful set up of both assays, we used the ECFC-adapted TGA and thrombogenic assay to investigate ED the three groups of VTE patients, namely uVTE, wpVTE and pVTE.

By performing ECFC *in vitro* characterization we observed the presence of constitutive ED in uVTE patients. In particular, in thrombogenesis assay the highest platelet deposition occurred when uVTE ECFCs were used as a substrate, uVTE being the only group of VTE patients characterized by a statistically significant increased platelet deposition compared with healthy donor group. Considering that in our assay we perfused peripheral blood obtained from healthy donors on a monolayer of patient-derived ECFCs, the observed alterations can be ascribed to ECFCs only. We may hypothesize that

the increased adhesiveness of uVTE ECFCs for platelets may be mediated by an increased expression of adhesion molecules such as glycosaminoglycan-binding proteins or lectins. Indeed, it is known that lectin surface expression on ECs is low or even absent in homeostatic conditions, whereas it increases on activated/dysfunctional endothelium, being fostered by oxidative stress and inflammation (Etulain J. & Schattner M., 2014). In particular, P-selectin, ICAM-1 and PECAM-1 are lectins that allow the interaction between endothelium and platelets and foster platelet degranulation (Etulain J. & Schattner M., 2014). Notably, we detected significantly higher concentrations of the soluble form of ICAM-1 and PECAM-1 in the culture supernatants of ECFCs obtained from uVTE patients compared to healthy donors. Considering that elevated levels of the soluble forms of the adhesion molecules are considered ED markers correlating with the increased surface expression of such molecules on activated endothelium, we could hypothesize that the increased platelet adhesion observed in the thrombogenesis assay when using uVTE ECFCs could be mediated by an increased expression of ICAM-1 and PECAM-1 on the cell surface. Further experiments aimed at assessing the surface expression levels of ICAM-1 and PECAM-1 on ECFCs obtained from the different groups of VTE patients will be needed to investigate this issue. Supporting the relevance of our results, other adhesion molecules not involved in platelet aggregation were not upregulated in uVTE patients. In particular, the soluble form of E-selectin was not detectable in ECFC culture supernatants, and no significant differences were observed in VCAM-1 expression among the analysed groups neither when the soluble form was measured in ECFC culture supernatants, nor when VCAM-1 surface expression was evaluated on ECFCs. This is particularly relevant, as e-Selectin and VCAM-1 are adhesion molecules expressed by activated endothelium that do not directly mediate platelet aggregation but are more involved in the control of leukocyte recruitment to the site of inflammation by slowing down leukocyte rolling and mediating leukocyte adhesion (Timmerman I. et al., 2016).

Because elevated plasmatic levels of the soluble form of the adhesion molecules are considered circulating markers of ED, we measured the plasmatic levels of the same molecules that we measured in ECFC culture supernatants, and confirmed that sICAM-1 levels in uVTE patients were significantly increased not only compared with healthy donors but also compared with the other groups of VTE patients. No significant differences were observed in the plasmatic levels of s-PECAM-1 probably because the assessment of s-PECAM-1 levels in plasma is a less specific approach than evaluating it in ECFC culture supernatant. In fact, the systemic levels of s-PECAM-1 assessed in the plasma are the result of the PECAM-1 shedding not only from ECs but also from other cell types expressing PECAM-1, such as neutrophils, monocytes, T cell subsets, and platelets (Baumann C. et al., 2004).

Notably, the upregulation of ICAM-1 and PECAM-1 in uVTE ECFCs may be sustained by a downregulation of PTGIR and GSTM1, as suggested by the results of the transcriptome profiling we performed on ECFCs. Indeed, the preliminary analysis of the results obtained by RNA sequencing indicated that few genes were differentially expressed in uVTE ECFCs compared with ECFCs obtained from the other subject groups. Among these genes, PTGIR and GSTM1 were downregulated in uVTE patients compared with healthy donors, wpVTE and pVTE patients. Notably, downregulation of these genes has been reported to be associated with increased platelet aggregation and increased expression of the adhesion molecules on ECs in different settings. In particular, PTGIR encodes for Prostaglandin I2 (PGI2) Receptor, and it has been reported to play a role in cardiovascular health, specifically by inhibiting platelet aggregation and promoting vasodilation via relaxation of smooth muscle cells (Dorris SL. et al., 2012). GSTM1 encodes for glutathione S-transferase M1, and it has been reported to modulate the activation by inhibiting the expression of several molecules including ICAM-1 (Jerotic D. et al., 2021). Altogether, these observations support the hypothesis that an increased endothelial expression of adhesion molecules such as ICAM-1 and PECAM-1, at least in part sustained by GSTM-1 and PTGIR downregulation,

may contribute to an increased ability of ECs to initiate platelet adhesion and aggregation, thus representing a pro-thrombotic mechanism possibly involved in the pathogenesis of uVTE.

By performing the TGA on ECFCs, we further confirmed the presence of constitutive ED in uVTE patients. In particular, thrombin generation occurred faster, as assessed by a lower lag time, and a higher amount of thrombin was generated, as assessed by a higher Peak, when ECFCs isolated from uVTE patients were used as a substrate in the assay. These alterations that sustain increased thrombogenicity to ECFCs were specific of uVTE patients, as they were not observed when ECFCs obtained from secondary VTE patients were challenged in the assay. The increased thrombin generation promoted by uVTE ECFCs in TGA did not rely on a higher expression of TF, the key molecule used by ECs to trigger the extrinsic pathway of the coagulation cascade upon activation. In fact, when we analyzed TF expression by ECFCs, we did not observe significant differences between uVTE patients and the other analyzed groups. As ECs can also affect the intrinsic pathway of the coagulation cascade and express various anticoagulants such as thrombomodulin, EPCR, TFPI and heparin-like proteoglycans (Yau JW. et al., 2015), further studies will be needed to investigate the molecular mechanisms underlying the increased thrombin generation sustained by uVTE ECFCs. Notably, a reduction of thrombomodulin and EPCR expression has been associated with impaired anti-coagulant function of ECFCs in a different clinical setting (Billoir P. et al., 2021).

As inflammation plays a crucial role in triggering a pro-thrombotic behavior of ECs, we further investigated the *in vitro* behavior of ECFCs upon stimulation with the prototypical pro-inflammatory cytokine TNFα. As expected, we observed that upon TNFα exposure ECFCs isolated from healthy donors and the three subgroups of VTE patients significantly up-regulated the expression of the activation markers VCAM-1 and TF. In line with this endothelial activation, when we challenged TNFα-treated ECFCs in TGA we observed a faster thrombin generation in all groups compared with their untreated counterparts. However, the amount of thrombin generated in the TGA,

assessed as peak height and ETP, was upregulated by TNFα-treatment only in the group of healthy donors, and not in uVTE, wpVTE or pVTE patients. Because the vast majority of VTE patients enrolled in this study were receiving AT at the time of peripheral blood collection for ECFC isolation, we speculate that this behaviour of patient-derived ECFCs may be influenced by the effects of anticoagulation. Due to the negligible number of patients without AT in our cohort of patients, we cannot compare treated with untreated patients in this study, and further studies will be needed to investigate whether AT may at least in part counteract endothelial thrombin generation in inflammatory conditions.

The results of the thrombogenesis assay performed using TNFα-treated ECFCs broadly indicated a general increase in fibrin formation and platelet deposition compared with untreated cells. Notably, TNFα-treated ECFCs obtained from uVTE patients sustained a higher platelet deposition than TNFα-treated ECFCs obtained from healthy donors, suggesting that uVTE endothelial cells maintain their higher thrombogenic potential during inflammation, and further confirming the presence of ED in uVTE patients.

A critical issue in the management of uVTE patients is the prevention of thrombotic recurrences (Douketis J. et al., 2011; Douketis J. et al., 2010; Stain M. et al., 2005; Áinle FN. & Kevane B., 2020). Understanding the relevance of ED in VTE recurrence may provide useful information for the comprehension of the mechanisms underlying the risk of recurrence and the development of adequate prevention strategies. Therefore, in this study we further analyzed ED features in ECFCs obtained from VTE patients stratified according to their recurrence risk. To this aim, we used three validated clinical predictors of VTE recurrence – namely the presence of the post thrombotic syndrome (PST), the presence of increased D-dimer levels, and the presence of residual obstruction. PST was assessed by using the widely used Villalta Score, and we observed that only 3 VTE patients within the entire cohort – 2 in the wpVTE group and one in the pVTE group - had mild PTS having a score higher that 5. The low frequencies of patients with PTS observed in our cohort was probably

due to the fact that the VTE patients enrolled in this study were mostly under treatment with DOACs. In fact, several studies have shown that the use of DOACs in place of VKAs for the initial and long-term treatment of DVT reduces the risk of PTS by approximately 50% (Prandoni P., 2022). The DOAC treatment still ongoing in the vast majority of VTE patients at the time of study enrolment may also explain why no significant differences were observed among the subgroups of VTE patients in the average D-dimer levels nor in the frequency of patients having altered D-dimer levels. The third validated predictor of VTE recurrence that we analyzed in our patients was the presence of a residual obstruction (RO), defined as the presence of a residual venous obstruction (RVO) and/or a residual pulmonary vascular obstruction (RPVO). In particular, we evaluated the presence of RO three months after the index event in accordance with a study performed by Donadini and colleagues, demonstrating that the strength of RVO as VTE recurrence predictor varies on the basis of the timing chosen for RVO assessment with respect to the time of DVT event (Donadini MP. et al., 2014). In particular, the Authors showed that RVO was a stronger predictor of recurrence if assessed at three months after the diagnosis of DVT, whereas it was a weaker predictor or even no longer predictive in case of longer time interval occurring between the diagnosis of DVT and RVO assessment (Donadini MP. et al., 2014). Notably, the presence of a RO not only allowed us to divide VTE patients into low and high-risk patients, but it was also the only recurrence predictor significantly increased in uVTE compared with secondary VTE patients, as expected based on the knowledge that uVTE is endowed with a higher recurrence risk. Therefore, to investigate whether ED is involved in VTE recurrence in uVTE patients, we stratified patients on the basis of RO that was apparently the only recurrence predictor unaffected by DOAC treatment.

Within the group of uVTE patients, ED appeared to be higher in patients classified at high risk compared with low risk of VTE recurrence. In fact, although the low number of patients within each subgroup did not allow to perform a statistical analysis, most ED parameters tended to be increased in ECFCs obtained from uVTE patients with high recurrence risk, namely the

amount of thrombin generated in TGA in basal conditions and upon TNFα exposure, fibrin formation and platelet deposition in thrombogenesis assay, levels of sICAM-1, sVCAM-1 and sPECAM-1 in ECFC culture supernatants, and plasmatic levels of sPECAM-1. Restricting the analysis to high-risk patients only, we confirmed that uVTE patients were still characterized by higher levels of ED compared with patients with secondary VTE, thus indicating that uVTE patients with high recurrence risk have the most marked features of ED.

Notably, also within the group of wpVTE patients, but not pVTE patients, some ECFC features of ED appeared to be higher in patients classified at high risk compared with low risk of VTE recurrence, suggesting that ED could be involved in VTE recurrence also in these patients. Further studies will be needed to elucidate the role of ED, distinct transient minor predisposing factors and their reciprocal interactions in triggering VTE events in wpVTE patients.

In conclusion, through the *in vitro* characterization of patient-specific ECFCs, in this study we demonstrated the presence of endothelial alterations in uVTE patients that support the valuable use of ECFCs as a 'liquid biopsy' to functionally characterize the endothelial compartment. In particular, our results confirmed that ECFCs undergo early senescence in VTE and demonstrated the presence of ED in uVTE patients. By combining *in vitro* functional characterization and ECFC transcriptome profiling we could identify mechanisms of ED likely contributing to uVTE pathogenesis. We further showed that ED could also play a role in sustaining VTE recurrence.

Several additional issues may deserve to be investigated in order to deepen our comprehension of uVTE pathogenesis. Indeed, further experiments are in progress to extend the ECFC characterization on a larger cohort of patients, progress in the analysis of RNAseq (still preliminary), improve the molecular characterization of ED, and address the impact of anticoagulation on ED and VTE recurrence. In addition, as future perspectives of this study, we are planning to investigate further issues:

- as mentioned above, an extensive characterization of ECFC phenotype will be performed in order to assess the surface-expression of EC activation markes (i.e. adhesion molecules as ICAM-1 and PECAM-1) and anti-coagulant molecules (i.e. TM and EPCR) on ECFCs obtained from the three groups of VTE in order to improve the knowledge on the mechanisms that are involved in platelet adhesion and thrombin generation, respectively.
- because in previous studies a reduced proliferation and a reduced ability to form capillary-like structures in Matrigel matrix were described in ECFCs isolated from uVTE patients (Della Bella S. et al., 2019; Alvarado-Moreno JA et al., 2016), the assessment of these ECFC functions will be extended on this novel cohort of patients, in order to assess whether these impaiments are specific of uVTE patients or are present also in ECFCs isolated from patients with secondary VTE. Moreover, other ECFC functions that deserve to be assessed include migratory properties and control of permeability. Indeed, on the one hand an impaired migratory capability could be suggestive for an impaired ECFC ability to properly maintain endothelial integrity; on the other hand, alterations in endothelial permeability would be indicative of ED. In fact, EC permeability represents an important factor in tissue homeostasis, with increased vascular permeability being one of the main markers of blood vessel damage that has been demonstrated in various pathological contexts, including diabetes (Rask-Madsen C. et al., 2013).
- to address the increased early senescence that we observed in VTE patient ECFCs, it is worthy to consider that Patel and colleagues showed that ECFCs expressing CD34 – a cell surface glycoprotein that enables cell-cell adhesion – were characterized by higher proliferative ability and underwent senescence later than $CD34^-$ ECFCs (Patel J. et al., 2016). Therefore, an aspect that could be further investigated in our cohort of patients is the frequency of $CD34^+$ ECFCs to evaluate whether a reduced frequency of $CD34^+$ ECFCs may contribute to the increased frequency of early senescent ECFC colonies in the different groups of VTE patients.

- considering the elevated levels of soluble adhesion molecules detected in plasma and ECFC supernatants from uVTE patients, another aspect that may deserve investigation is represented by the secretome of uVTE ECFCs. Indeed, the precise characterization of soluble molecules involved in ED in uVTE patients would be very useful to identify soluble ED biomarker(s) to be used in patient follow-up, and novel therapeutic targets to be used for uVTE treatment and prevention of VTE recurrence. In this context, previous studies reported a significant increase in the concentration of pro-coagulant, inflammatory mediators such as IL-6, TNF-α and IFN-γ in the plasma and supernatants of ECFC-ECs from VTE patients (Alvarado-Moreno JA. et al., 2016). By performing experiments where ECFCs from healthy donors will be incubated with plasma samples and ECFCs supernatants obtained from uVTE, wpVTE and pVTE patients, we plan to investigate the impact of endothelial-derived soluble molecules on ED, and assess whether the ED observed in uVTE patients is an intrinsic endothelial feature or it may be induced through autocrine and paracrine mechanism(s) or by factors not necessarily EC-derived present in the microenviroment. Multiplex assays will be used for a detailed characterization of the molecular composition of different samples.
- according to our observation that Gene sets associated with vesicle production are specifically enriched in uVTE ECFCs, we plan to similarly investigate the impact of extracellular vesicles on ED. Notably, we plan to investigate not only EC-derived extracellular vesicles, but also vesicles released by other cell types - such as platelets and monocytes – that may play a role in uVTE pathogenesis.

Altogher, the information obtained in the present study, combined with the information that will derive form the planned studies, will allow us to further dissect the molecular mechanism(s) and altered pathways underlying ED in uVTE patients, with the final aim to identify molecular targets for the development of novel therapeutic strategies aimed at preventing VTE recurrences in these patients.

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