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DOTTORATO DI RICERCA IN
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# Analysis of the **c**o-inheritance between FV Leiden and/or FV**H**is1299**A**rg and/or other **m**utations as a **p**ossible **i**ndividual risk factor of thr**o**mboembolic ve**n**ous disease (CHAMPION study).

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# - 1- INTRODUCTION

## 1.1 THROMBOSIS: A MULTIFACTORIAL DISEASE

The clinical definition of thrombosis is a pathological presence of a clot (thrombus) in a blood vessel or at the level of the heart, which cause the obstruction of flow through the circulatory system. The formation of a clot obstructing veins and arteries is the end product of an imbalance between pro-coagulating, anticoagulant and fibrinolytic factors.

Based on the location in which the thrombus formed (i.e. in the venous or arterial circulatory system), the thrombotic disease can be classified as venous thrombosis or arterial thrombosis. Both types of thrombosis are seen as distinct diseases, characterized by different pathogenic mechanisms and risk factors[1-4].

We distinguish various clinical forms of arterial thrombosis depending on the involved districts, leading to different pathologies: myocardial infarction (coronary district), ischemic stroke (brain districts), intermittent claudication (obstructive arterial disease of the lower limbs). The arterial thrombi tend to be “white thrombi” with their content largely contributed by aggregated platelets with relatively little fibrin or red cells. Arterial thrombi usually develop in areas of high shear stress, which are more prone to endothelial damage, such as stenotic arteries[1-3].

The venous thromboembolism also recognizes different clinical forms depending on the involved districts: deep vein thrombosis of the lower limbs (i.e. iliac-femoral-popliteal-twin), deep vein thrombosis of the upper limbs (i.e. subclavian-axillo-humeral), deep vein thrombosis of other organs (i.e. splanchnic) and pulmonary thromboembolism (identified as primary and secondary). It is widely accepted that venous thrombi are “red thrombi” being predominantly formed from red blood cells and fibrin.

“Red thrombi” usually develop in areas with slow blood flow, such as veins or left atrial appendage of patients with atrial fibrillation[1-3].

Thrombosis is a multifactorial disease that may appear as arterial thrombosis and/or venous thrombosis. Several inherited and acquired risk factors are involved in the pathogenesis of a thrombotic event. Venous thrombosis may be triggered by inherited or acquired thrombotic risk factors. Acquired thrombotic risk factors for venous thrombosis are represented by recent surgery, prolonged bed rest, cancer and its therapies, oral contraceptives use, pregnancy/puerperium and aging. However, also for venous thrombosis the presence of familial history of venous thrombosis is a well recognised thrombotic risk factor.

### 1.1.1 Venous Thromboembolism

Haemostasis is a normal physiological process following damage of the vascular system. In various diseases, however, the haemostatic mechanisms are inappropriately activated with pathological consequences known as thrombosis. Venous thromboembolism (VTE) including deep vein thrombosis (DVT), pulmonary embolism (PE) or both, represents one of the most common health problems. More than 2 million Americans suffer from acute VTE each year. In the European Union (EU), incidence rates of DVT and PE are assumed to be slightly higher, but are generally consistent with those in the United States (US)[5,6]. Deep vein thrombosis and PE are a burden for healthcare systems as they are associated with high mortality and considerable morbidity in terms of recurrent events, the post-thrombotic syndrome, and chronic thromboembolic pulmonary hypertension[7-9]. Treatment of DVT and PE aims at prevention of worsening of the existing thrombus, as well as prevention of recurrent VTE[10].

VTE is the third most common vascular disease after myocardial infarction and ischemic stroke. The annual incidence of symptomatic and objectively confirmed VTE, is 2 to 3 per thousand inhabitants. The incidence varies strongly with age from 0.1 in adolescence to 8 per 1000 in those above the age of 80 years[11].

## 1.2 PATHOPHYSIOLOGY OF VENOUS THROMBOSIS

The mechanistic framework that helps to understand and group the causes of VTE is an extension of the Virchow's triad (Fig. 1.1), which postulates that thrombosis is caused by changes in (1) blood flow, (2) the state of the vessel wall, and/or (3) the composition of blood[12].

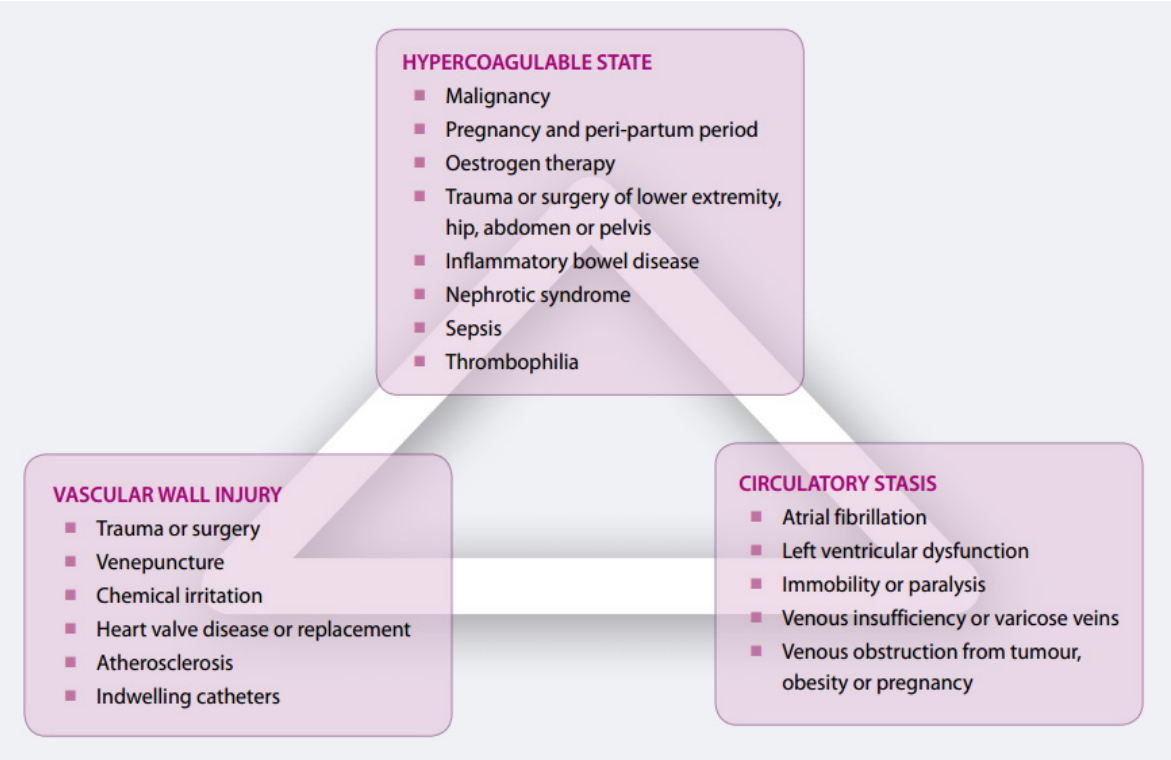



*Figure 1.1 - Virchow's triad*

In a more current reductionist's view, stasis and low oxygen tension, activation of the endothelium, activation of innate (involving monocytes and granulocytes) and acquired immunity, activation of blood platelets, the concentration and nature of microparticles (MPs), and the individual concentrations of pro- and anticoagulant proteins all claim a role (Fig. 1.2). In addition, red blood cells are present in venous clots[13].

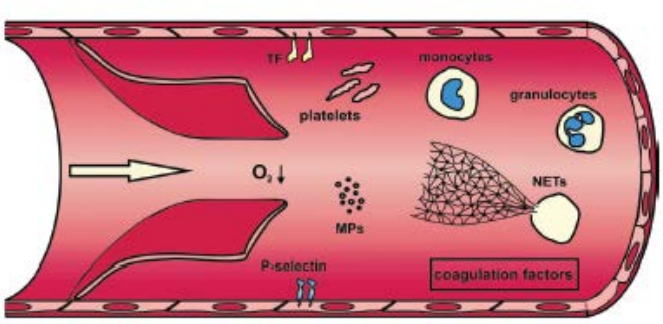



*Figure 1.2 – A venous segment near a valve. Oxygen tension may become particularly low in the pocket of the valve when blood flow is impaired. This will result in pro-coagulant and pro-inflammatory conditions involving tissue factor (TF), P-selectin, platelets, microparticles (MPs) monocytes, and granulocytes. Damaged granulocytes may release neutrophil extracellular traps (NETs). The strength of the pro-coagulant response is strongly dependent on the concentration of coagulation factors in the blood.*

Haemostasis is the process that maintains the integrity of a closed, high-pressure circulatory system after vascular damage. Vessel-wall injury and the extravasation of blood from the circulation rapidly initiate events in the vessel wall and in blood that seal the breach. Circulating platelets are recruited to the site of injury, where they become a major component of the developing thrombus; blood coagulation, initiated by tissue factor, culminates in the generation of thrombin and fibrin. These events occur concomitantly, and under normal conditions, regulatory mechanisms contain thrombus formation temporally and spatially. When pathologic processes overwhelm the regulatory mechanisms of haemostasis, excessive quantities of thrombin form, initiating thrombosis[14].

Although Virchow's triad of stasis, vein injury and hypercoagulability has framed the factors that predispose to DVT formation since the mid-19$^{th}$ century, current thinking suggests that these events occur at the level of the vein wall, including the inflammatory response to, and often the cause of, thrombogenesis. The inflammatory response determines both the ultimate resolution of the thrombus and the fibrosis of the vein wall and vein valves leading to post thrombotic syndrome (PTS)[15].

### 1.2.1 Blood Flow, Oxygen Tension and Endothelial Activation

Blood is, for the purpose of flow analysis, essentially a fluid (plasma) with particles in suspension (mostly red blood cells). In addition, local flow conditions are made up of the viscosity of the blood and the resistance offered by the vessel, as well as a turbulence at vessel bifurcations and stenotic regions[16].

Abnormal blood flow, applies to patients who undergo prolonged immobilization, or those who have varicose veins or atrial fibrillation. In these conditions, blood is allowed to pool or stagnate, increasing the chance that platelets and coagulation factors will meet up and start doing their thing.

Prolonged stasis in a vein, in particular in deepest parts of the pocket of a venous valve, causes lowered oxygen tension[17]. This oxidative stress will lead to the up-regulation of multiple stress-response genes including hypoxia inducible factor 1-alpha, P-selectin (CD62), and other adhesion receptors[18]. The resulting pro-inflammatory state of the endothelium supports the local recruitment of monocytes, granulocytes, platelets, and MPs. The recruitment of these actors and their activation may lead to the local exposure of tissue factor (TF)[19], thus initiating the extrinsic pathway of coagulation. When damaged granulocytes start releasing neutrophil extracellular traps, DNA, and RNA, factor XII may become activated thus triggering the intrinsic pathway of coagulation and further facilitating the formation of a thrombus[20,21]. The intrinsic pathway can also be triggered when activated platelets release inorganic polyphosphates[22].

Low blood flow in veins occurs in conjunction with several classical risk factors for VTE, including bed rest and plaster cast, and in some forms of orthopaedic surgery in which the blood flow is temporarily interrupted in order to facilitate the procedure[23-25].

Travel-related VTE is to some extent due to impaired blood flow in the limbs[13], and the thrombotic risk that is associated with obesity may in part be due to the fact that individuals with a high body mass index have a chronically raised intra-abdominal pressure and decreased blood velocity in the femoral vein, and are more likely to have a sedentary lifestyle than non-obese individuals[26].

In pregnant women, venous stasis already begins in the first trimester and is assumed to be caused by progesterone-induced venodilation, whereas compression of the pelvic veins by the gravid uterus becomes more important in the second and third trimester of pregnancy[27]. Smoking is a risk factor for VTE, although the effect is much less than for arterial thrombosis

and the evidence less consistent[28]. There are no mechanistic studies on the relationship between VTE and smoking, but it may be safe to postulate an inflammatory state of the endothelium, also involving components of innate immunity. Blood coagulation reactions and platelet activation are in part dependent on blood flow. How and to what extent this dependence influences thrombotic risk when flow is impaired is unknown[13].

### 1.2.2 Vessel Wall and Endothelial Damage

Endothelial damage, increases the risk of thrombus formation through many mechanisms. One thing that happens when endothelium gets ripped up is that tissue factor is exposed (which initiates the coagulation cascade). Conditions falling under this category of risk include atherosclerosis and bacterial sepsis[14].

The vessel wall, with its inner lining of endothelium, is crucial to the maintenance of a patent vasculature. The endothelium contains three thromboregulators — nitric oxide, prostacyclin, and the ectonucleotidase CD39 — which together provide a defense against thrombus formation. Collagen in the sub-endothelial matrix and tissue factor facilitate the maintenance of a closed circulatory system. When the vessel wall is breached or the endothelium is disrupted, collagen and tissue factor become exposed to the flowing blood, thereby initiating formation of a thrombus (Fig. 1.3). Exposed collagen triggers the accumulation and activation of platelets, whereas exposed tissue factor initiates the generation of thrombin, which not only converts fibrinogen to fibrin but also activates platelets[14].

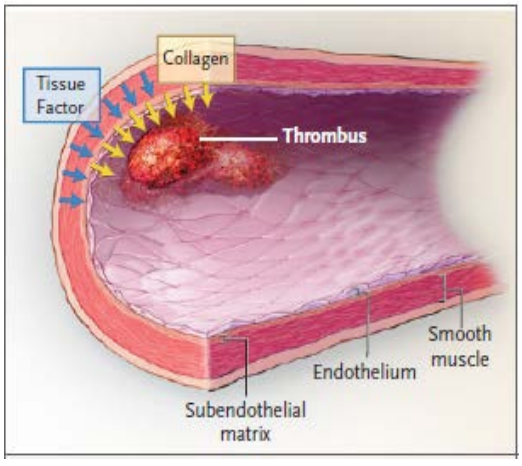



*Figure 1.3 – Response to Vascular Injury*

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

***Platelets***

Platelets are anucleate cells that circulate in blood in a resting form. Upon stimulation at the site of tissue injury, platelets adhere to the injured surface. This interaction requires von Willebrand factor (vWF), a large multimeric plasma protein that binds to a specific receptor (glicoprotein Ib) on the platelet membrane, and acts as a bridge between sub-endothelial collagen and specific receptors (GPIb) on the platelet membrane[29]. The phospholipid composition of the platelet membrane changes, resulting in the exposure of negatively charged phopshatidylserine on the outer leaflet of platelet membrane[30-31]. The activation of platelets by thrombin, ADP, thromboxane $A_2$ or epinephrine triggers characteristic morphological and biochemical alterations in the platelet. Activated platelets secrete α-granules, containing fibrinogen, Factor V (FV), Factor VIII (FVIII), vWF and other proteins involved in hemostasis, and δ-granules, containing calcium ions and ADP, and aggregate at the site of injury, forming a sort of plug that provisionally blocks blood loss. The expression on the platelet surface of a receptor (glycoprotein IIb-IIIa) for plasma proteins (fibrinogen) mediates platelet aggregation[29].

Venous blood clots are structures consisting of successive layers of fibrin, platelets, red bloods cells, and leukocytes[17]. Compared to arterial clots, the number of platelets is relatively low. Older work also suggests that primary venous thrombi are platelet free, confirming the notion that platelets are not involved in the formation of the original nidus[13]. Based on these structural characteristics, platelets have historically been ignored in studies of VTE. Several lines of research indicate though that platelets play a determining role. First, the older work shows that as the formation of the thrombus propagates, the successive layers do contain platelets[13]. Second, (activated) platelets are important catalysts of both intrinsic and extrinsic thrombin generation and thus fibrin production[13]. Third, the platelet collagen receptor glycoprotein 6 was recently identified in a genome-wide association study that searched for novel risk factors for VTE[32]. Fourth, the use of aspirin may decrease the risk of first and recurrent VTE[33].

Platelets thus may play a mechanistic role in supporting the formation of a venous clot, and some of the common risk factors for VTE can be ascribed to alterations in platelet number or function. The predisposition of obese individuals to VTE is in part determined by increased ADP-induced platelet aggregation, which in turn may be determined by the increased leptin levels that are often found in obese individuals[13]. Also, platelet activation may also play a role in antiphospholipid syndrome. Myeloproliferative diseases such as polycythemia vera and essential thrombocytosis lead to increases in platelet number and function and VTE occurs in

a large proportion of patients[13]. It is likely that this risk increase is a direct consequence of the change in platelet number rather than the presence of the JAK2V617F mutation that is present in virtually all patients with polycythemia and in about half of those with essential thrombocytosis[34].

***Red Blood Cells***

Primary erythrocytosis is related to venous thrombosis, but the mechanisms are complex and debated[13]. With respect to the association of haematocrit with venous thrombosis, the data are scarce and conflicting[35,36]. The most recent study, a large prospective study in the general population, shows a hazard ratio of 1.25 per % rise in hematocrit[37]. Whether the relation is causal or not remains unanswered[38].

***Microparticles***

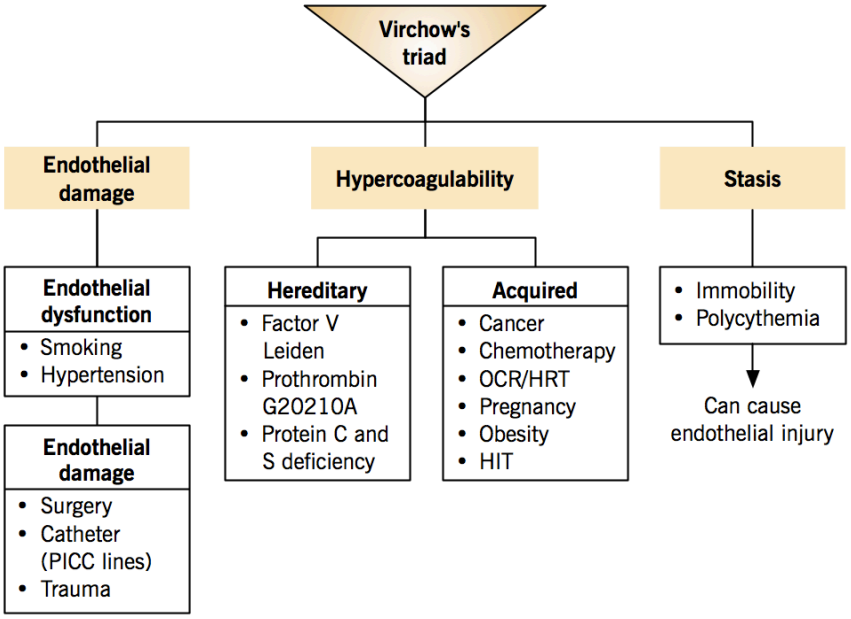
Over the past 10 years, MPs have emerged as a potential key player in thromboembolic events[39]. MPs are submicron vesicles (sizes ranging from 50–1000 nmol/L) that are shed from the surface of intravascular cells, among which platelets, endothelial cells, and leukocytes. The repertoire of MPexposed membrane proteins reflects that of the cell of which they were shed and one of these proteins is TF. TF is the primary initiator of the coagulation cascade, especially the association between TF-positive MPs (TF-MPs), and VTE has been studied. Although TF is normally not expressed by intravascular cells, certain pathological states such as sepsis upregulate TF expression on these cells and consequently on MPs[40]. MPs generated from platelets form the majority (approximately 80%) of all MPs found in plasma[41], but pro-coagulant activity of these MPs is limited. Both platelets and platelet MPs were recently suggested to express TF[42-44], but this remains controversial. More likely, platelet MPs acquire TF through fusion with TF-positive MPs from other cellular sources, such as monocytes. Indeed, monocyte- and endothelium- derived MPs expose TF in vitro and show significant clotting activity[45,46]. Several, but not all studies, found that either plasma TF-MP concentration or TF-MP pro-coagulant activity positively correlates with the risk of VTE [44,46-49]. An explanation for these inconsistent findings may be that detection of MPs is often inaccurate because of their small size. With regard to the recurrence of VTE, a clearer picture emerges. TF-MP activity and concentrations in plasma unambiguously correlate with the risk of recurrent VTE[44,50]. Patients suffering from malignancy have a clearly elevated risk of VTE. Several studies indicate that cancer patients with VTE have significantly higher TF-MP activity and levels than cancer patients without VTE[51-54], and such an association between TF-

MP and VTE is confirmed by mechanistic studies[55]. Chemotherapy further increases the risk of VTE in cancer patients and a correlation with TF-MP activity has been suspected. Nevertheless, TF-MP activity in these patients does not appear to correlate with VTE[51].
Surgery, trauma and delivery are risk factors for VTE, but any link between TF-MPs and surgery/trauma/delivery remains largely unexplored. However, blood from patients undergoing cardiopulmonary bypass surgery contains substantial amounts of MP-TF activity[56]. As detection methods for MPs in plasma continue to improve, it may be expected that the near future will see an accumulation of literature on TF-MPs in VTE.

### 1.2.3 Coagulation Factor Concentrations and Hypercoagulability

Thrombophilia is a hereditary or acquired condition (Fig. 1.4), characterized by clinical tendency to thrombosis or molecular abnormalities of haemostasis, that predisposes to thromboembolic disease. It is now accepted that the combination of stasis and hypercoagulability, much more than endothelial damage, is crucial for the occurrence of VTE[57].
The best-studied determinants of venous thrombotic risk are concentrations of individual coagulation factors. These concentrations are in part, up to 50%, determined by genetic factors, but also lifestyle and other environmental factors play an important role[13].

*Figure 1.4 – The Virchow's triad and the Hypercoagulability state*



**Acquired Factors**

This group includes a lot of different disorders, which act by perturbing the haemostatic balance in pro-thrombotic way. Some of them may be persistent over time, as antiphospholipid antibodies and chronic diseases as infections, inflammations, cancer and dysmetabolic disease; other are transitional, such as trauma, surgery, pregnancy and puerperium, oral contraceptive use, obesity and smoking.

- ***Antiphospholipid Antibody Syndrome***

The antiphospholipid antibody syndrome is one of the most important acquired risk factors for thrombosis. Characterised by the presence of circulating antiphospholipid antibodies in plasma, it is associated with arterial or venous thrombosis and/or pregnancy complications, including foetal loss. The clinically relevant antiphospolipid antibodies include lupus anticoagulant, anticardiolipin and anti-β2-glycoprotein I antibodies[57].

The term "antiphospolipid antibodies" is widely used even if it is not correct, because antibodies are not directed against phospholipids per se, but against a wide variety of protein co-factors acting on phospholipid membrane surfaces (β2-glycoprotein I, prothrombin, protein C, protein S, annexin V, coagulation factor XII and others). The resulting complexes interact with several cell types, including endothelial cells, monocytes and platelets, all of which play important roles in haemostasis and thrombogenesis[57]. The indirect activation of these cells results in the release of prothrombotic and pro-inflammatory mediators (e.g. TF-bearing microparticles, interleukin-6, proteins of the complement system), leading to the activation of platelet and coagulation pathways[58]. Recent observations show that antiphospholipid antibodies interact directly with vessel wall and cause alterations of plasma lipoprotein [i.e. high density lipoprotein (HDL)] function leading to increased atherothrombotic risk[59].

- ***Cancer***

Cancer is one of the most important acquired risk factors for VTE[60]. Some authors stimate an annual incidence of VTE of 1 in 200 patients with cancer[61], and 20% of VTE cases occur in patients with cancer[62]. Conversely, of all patients with cancer, 15% will develop symptomatic VTE[62], 50% asymptomatic VTE[63], and 50% will have VTE diagnosed at autopsy[60]. The risk of VTE is higher at diagnosis (OR 53.5; 95% CI 8.6-334.3) and in patients with distant metastases (OR 19.8; 95% CI 2.6-149.1)[64]. If a patient with cancer survives an initial VTE event, he or she has an increased risk of recurrence (OR 1.72; 95% CI 1.31-2.25) compared

with that in a patient without cancer. The cancer patient with VTE also has a significantly increased risk of death (OR 8.1; 95% CI 3.6-18.1), which persists for as long as the malignancy persists[65]. In addition, VTE is the second leading cause of death in hospitalised patients with cancer, after infections[66].

Chemotherapy increases the risk of thrombosis 6.5-fold[67]. The proposed mechanisms for chemotherapy-related risk of VTE probably include both direct drug-induced damage of the endothelium and an increased expression of TF procoagulant activity by macrophages and monocytes, thus inducing a procoagulant response by host cells[68]. Another prothrombotic mechanism of antitumour therapy is likely related to the direct hepatotoxicity of radio- and chemo-therapy, which can cause a reduction in the plasma levels of natural anticoagulant proteins (antithrombin, protein C and protein S)[69].

- ***Trauma, Surgery and Immobilisation***

These transient conditions are associated with an increased risk of venous thrombosis. The incidence of DVT associated with major trauma is up to 58%, PE occurs in 2% of these individuals and is the third cause of death among patients who survive the first 24 hours after trauma[70,71]. Considering minor trauma, the incidence of DVT is 28% when lower limbs are involved and the risk is higher in proximal than in distal fractures[72]. In a large, population-based, case-control study the relative risk of VTE associated with previous minor injury was 3.1 (95% CI 2.5-3.8)[73]. Minor injuries in a leg were strongly associated with VTE (OR 5.1; 95% CI 3.9-6.7), whereas minor injuries in other parts of the body were not[73].

Major orthopaedic surgery involving the lower extremity is a major risk factor for VTE. Rates of DVT without prophylaxis range from 40% to 60% in the 2 weeks after major orthopaedic surgery[74].

The mechanism by which these conditions lead to VTE is a combination of stasis and local accumulation of TF (i.e., hypercoagulability). Stasis locally concentrates haemostasis activation factors (cytokines and other mediators of inflammation), favours cellular margination and interaction of circulating blood cells with endothelium, and is responsible for local hypoxia which is one of the principal mechanisms of endothelial activation[75]. TF is expressed by cells in the subendothelial compartment. Thus, physical disruption of the endothelium, as occurs in trauma or surgery, may lead to exposure of blood to extravascular TF. However, the vast majority of venous thrombi occur in the context of an intact endothelium. In these cases, TF may be expressed on the surface of activated endothelial cells

and/or mononuclear cells which have been stimulated by any number of inflammatory mediators[57,75].

- ***Pregnancy/Puerperium***

VTE remains the major cause of maternal mortality world-wide (the rate of maternal deaths from VTE is 0.12 per 10,000 live births and stillbirths)[76]. Results from studies in which either all or most pregnant women underwent accurate diagnostic testing for VTE report an incidence of VTE ranging from 0.6 to 1.3 events per 1,000 deliveries, confirming a 5- to 10-fold increased risk in pregnant women compared to that in non-pregnant women of comparable age[77].

The MEGA study showed that the risk of VTE is nearly 5-fold increased during pregnancy and up to 60-fold during the first 3 months after delivery[78]. A 14-fold increased risk of DVT of the legs and a 6-fold increased risk of PE were reported, with this risk being higher in the third trimester (OR 3.3; 95% CI 2.2-5.0) and during puerperium (OR 11.0; 95% CI 8.1-15.1), and highest in the 2 days before and the day after delivery (OR 77.6; 95% CI 52.4-114.8)[79].

The hormonal changes in pregnancy lead to a hypercoagulable state caused by decreased anticoagulant activity, increased procoagulant activity, and decreased fibrinolysis[13].

These changes help to maintain placental function during pregnancy and minimise blood loss at delivery. However, they may also predispose to maternal thrombosis and placental vascular complications[57].

The decrease in anticoagulant activity is mainly due to a decrease in protein S concentration and an increase in activated protein C resistance. The increase in procoagulant activity is a result of an increase in plasma levels of factors V, VII, VIII, IX, and X and fibrinogen. Finally, increases in levels of plasminogen activator inhibitor-1 and -2 activity and a decrease in tissue plasminogen activator lead to a hypofibrinolytic state[13].

- ***Oral Contraceptives and Hormone Replacement Therapy***

Combined oral contraceptives contain both an estrogen and a progestagen. Hormonal contraceptive use is associated with changes in the coagulation system at different levels. Crossover studies have demonstrated an increase in coagulation factors II, VII, VIII, and X in women using oral contraceptives[80], a decrease of the levels of the natural anticoagulant protein S[81], and a decrease of fibrinolytic activity, mainly through an increase of thrombin-activatable fibrinolysis inhibitor[82]. The use of oral contraceptive leads to increased resistance to the natural anticoagulant activity of activated protein C, which is partly explained by

decreases of free protein S and free TF pathway inhibitor[83]. The prothrombotic state as measured by coagulation and fibrinolysis assays directly translates into observed epidemiological risks associated with different components of oral contraceptives. For example, combined oral contraceptives that contain desogestrel, gestodene, drosperidone, or cyproterone as a progestagen induce a more pronounced activated protein C resistance than those containing levonorgestrel[84], and there is convincing evidence that users of such oral contraceptives have an increased risk of VTE as compared to users of contraceptives with levonorgestrel[85].

*Table I - Haemostatic changes during oral contraceptive (OC) use and pregnancy[57].*

| | Change during OC use | Change during pregnancy |
|---|---|---|
| **Procoagulant factors** | | |
| fibrinogen, V, VII, VIII, IX, X, XII | ↑ | ↑ |
| XI | = or ↑ | ↓ |
| von Willebrand factor | = | ↑ |
| **Anticoagulant proteins** | | |
| antithrombin | ↓ | = |
| protein C | = or ↑ | = or ↑ |
| protein S | ↓ | ↓ |
| resistance to activated protein C (ratio) | ↓ | ↓ |
| **Markers of thrombin formation** | | |
| F1+2, TAT complexes, fibrinopeptide A | ↑ | ↑ |
| D-dimer | ↑ | ↑ |
| **Fibrinolytic factors** | | |
| TAFI, PAI 1 and 2 | ↑ | ↑ |
| t-PA | ↓ | ↓ |

↑ increase, ↓ decrease, = no change, compared to non-use of oral contraceptives and to the non-pregnant state.

Oral hormonal replacement therapy has very similar effects on coagulation and fibrinolysis variables as use of oral contraceptives, all pointing toward a prothrombotic effect. In particular oral estrogen-containing hormone replacement therapy decreases the levels of the natural coagulation inhibitors antithrombin, protein C, and protein S and increases activated protein C resistance[13]. However, a systematic review of trials comparing the effects of

transdermal hormone replacement therapy with oral hormone replacement therapy on markers concluded that these effects are absent or at least lower with transdermal hormone replacement therapy use[13]. The effects of tibolone on markers of VTE risk are also less than in oral hormone replacement therapy or absent[13]. These findings are in line with the observed VTE risk[86].

- ***Infiammatory Disease, Obesity, Smoking, Dyslipidaemia***

Chronic inflammatory diseases like rheumatoid arthritis, inflammatory bowel disease, and psoriasis, but also smoking, increase the risk of VTE, and again the involvement of activated leukocytes, although not proven, is a likely mechanism[87].

Obesity is also associated with a chronic inflammatory state, probably involving the production of adipokines, and it is conceivable that this will increase thrombotic risk[13]. The relationship between obesity and VTE has been established in several epidemiological studies[26]. Obese subjects have increased plasma levels of procoagulant factors VII, VIII, and XII and fibrinogen, whereas fibrinolysis is decreased as reflected by increased levels of plasminogen activator inhibitor-1[88]. On the other hand, levels of the anticoagulant factors protein C and protein S are higher, and tissue plasminogen activator levels are lower under obese conditions, which might be considered to be a compensatory response to the hypercoagulable state[89]. It is noteworthy that studies evaluating the effect of weight loss on haemostatic parameters showed that levels of TF, factor VII, plasminogen activator inhibitor-1, and tissue plasminogen activator decreased on weight loss, resulting in a decrease in thrombin generation[90].

Finally, also dyslipidaemia may exert a mild influence on the risk of VTE[91], as determined by a recent meta-analysis in which patients with VTE had high triglyceride and low HDL cholesterol levels, while no effect of total cholesterolaemia on VTE was seen[92]. Moreover, preliminary evidence shows that statins may be protective against VTE[93], supporting the hypothesis of dyslipidaemia influencing the risk of VTE.

- ***Hyperhomocysteinaemia***

Hyperhomocysteinaemia is a mild risk factor for thrombosis due to an impairment of the metabolic pathway that transforms the amino acid methionine into cysteine, leading to an abnormal elevation of plasma concentrations of homocysteine, an intermediate product of this pathway. Genetic factors (e.g., gene mutations in methylenetetrahydrofolate reductase and cystathionine β-synthase) and acquired factors (e.g., deficiencies of folate, vitamin B12 or

vitamin B6, advanced age, chronic renal failure, and the use of anti-folic drugs) interact to determine plasma homocysteine concentrations, so that hyperhomocysteinaemia is a "mixed" (i.e., genetic and/or acquired) risk factor for both arterial and venous thrombosis[57]. The possible mechanisms by which hyperhomocysteinaemia contributes to thrombosis are multiple and still under study; they include a toxic effect on endothelial cells, smoothmuscle-cell proliferation and intimal thickening, impaired generation of nitric oxide and prostacyclin, increased platelet adhesion, activation of factor V, interference with protein C activation and thrombomodulin expression, induction of tissue factor activity and inhibition of tissue plasminogen activator (t-PA)[94].

**Genetic or Inherited Factors**

We know 6 (moderately) strong genetic risk factors for VTE[13].

- ***Antithrombin, Protein C and Protein S***

The first 3 are heterozygous deficiencies of the natural anticoagulants antithrombin, protein C, and protein S. The prevalence of these deficiency states is low in the general population (in all races), and their genetic architecture is complex with several hundred documented mutations in the human gene mutation database (http://www.hgmd.org/)[95]. In some populations particular mutations occur often because of founder effects[96]. Venous thrombotic risk may be increased up to 10-fold in these deficiency states[95].

Antithrombin directly inhibits several activated coagulation factors, particularly thrombin and activated factor X, and the inhibitory effect is amplified by its binding to glycosaminoglycans of the endothelial surface which carry heparin-like activity. Antithrombin deficiency results in significantly reduced inhibition of thrombin and activated factor X and an increased tendency to clot formation, particularly in the venous system where the coagulation pathway (as distinct from platelets) plays a major role in thrombus formation[97]. The protein C anticoagulant pathway, localised on the surface of the endothelium, is essential in the down-regulation of thrombin generation. Thrombin activates protein C; the presence of thrombomodulin, together with endothelial protein C receptor (EPCR), accelerates the catalytic efficiency of this activation. Activated protein C proteolytically inactivates factor Va and factor VIIIa, the two most important activated co-factors of the coagulation cascade, dramatically slowing the rate of thrombin and fibrin formation. The inhibitory effect of activated protein C is accelerated by its main cofactor, protein S[98]. The inherited deficiency of one of these inhibitors leads to a critical reduction of the natural anticoagulant system and enhances thrombin generation,

increasing susceptibility to VTE[97]. There is no consistent evidence that deficiencies of other natural anticoagulants – such as TF pathway inhibitor, thrombomodulin, endothelial protein C receptor, and heparin cofactor II – are also strong risk factors for VTE. Partial deficiency of these natural anticoagulants are associated with other episodic or chronic diseases, like atypical hemolytic uremic syndrome in the case of thrombomodulin[99].

- ***Prothrombin G20201A, Blood group non-0 and Factor V Leiden***

The other 3 moderately strong genetic factors are associated with an increase, directly or indirectly, of the pro-coagulant potential of the blood: factor V Leiden, prothrombin G20201A, and blood group non-O[95]. The genetic architecture of these risk factors is simple.

The prevalence in Caucasians varies from 3% to 15% for factor V Leiden and prothrombin G20210A, depending on the geographical location; in other races these 2 risk factors are extremely rare. The increase in thrombotic risk is about 2- to 3-fold for prothrombin G20210 and 3- to 5-fold for factor V Leiden. Blood group non-O is the most common pro-thrombotic genetic risk factor and approximately doubles the risk of VTE and does so in all races[100].

The G20210A mutation in the prothrombin gene is a G to A transition at nucleotide position 20210 in the 3'-untranslated region of the coagulation factor II (prothrombin) gene, which increases plasma prothrombin levels[101]. The factor V Leiden gain-of-function mutation consists of the substitution of an arginine by glutamine at position 506 of coagulation factor V (R506Q), which is the cleavage site for activated protein C in the factor V molecule[102]. Mutant factor V is partially resistant to inactivation by activated protein C, leading to a hypercoagulable state. Factor V Leiden explains more than 90% of cases of activated protein C resistance[103]. These two mutations also increase the risk of atherothrombosis, but to a lesser extent[104]. The prevalence of inherited thrombophilia in the general population and in patients with VTE are shown in Table II[57].

*Table II - Prevalence (%) of inherited risk factors for VTE in the general population and in patients*[57]

| Abnormality | General population | Patients with VTE | Patients with recurrent VTE or age < 45 years |
|---|---|---|---|
| Antithrombin deficiency | 0.02 - 0.17 | 1.1 | 0.5 - 4.9 |
| Protein C deficiency | 0.14 - 0.5 | 3.2 | 1.4 - 8.6 |
| Protein S deficiency | ? | 2.2 | 1.4 - 7.5 |
| Heterozygous factor V Leiden | 3.6 - 6.0 | 21.0 | 10 - 64 |
| Heterozygous prothrombin G20210A | 1.7 - 3.0 | 6.2 | 18 |

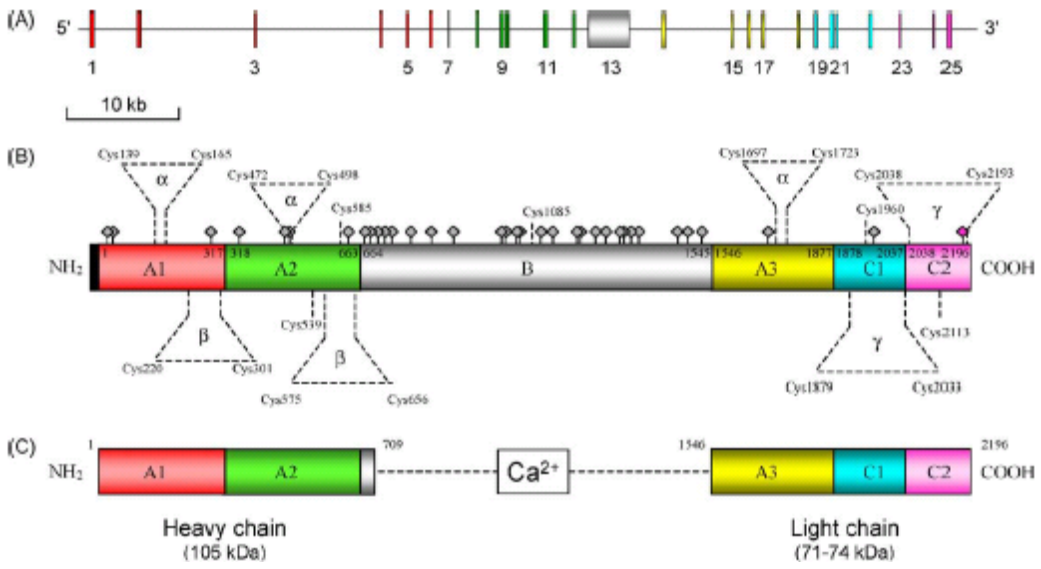
## 1.3 COAGULATION FACTOR V

Coagulation FV, also known as proaccelerin or labile factor, is an enzyme cofactor performing central and pivotal functions in maintaining a normal haemostatic balance. FV was discovered by Paul Owren in 1947 when, using relatively primitive technology, he was able to deduce the existence of a fifth component required for fibrin formation, that he named "factor V", thus beginning the era of Roman numerology for coagulation factors[105].

### 1.3.1 Structure

FV is a large single-chain glycoprotein with a molecular weight 330 kDa, 13%-25% of which is accounted for by the carbohydrate moiety. Cloning of the human 6,9 kb FV cDNA was reported in 1987, revealing a 6672 bp coding region. The corresponding FV gene, isolated in 1992, maps on chromosome 1q23 and is composed by 25 exons, spanning a chromosomal region of about 80 kb (Fig. 1.5A). The encoded 2224 amino acids pre-procofactor includes a 28- residue signal peptide[106-107]. This leader peptide is cleaved off after translocation to the endoplasmic reticulum, leaving a mature protein of 2196 amino acids (Fig. 1.5B).
The proteolytic removal of the B domain generates activated FV (FVa), which is composed of two non-covalently associated chains stabilized by a Ca2+ ion[108-109] (Fig.1.5C).

*Figure 1.5 – Structure of human FV gene and protein*[110]

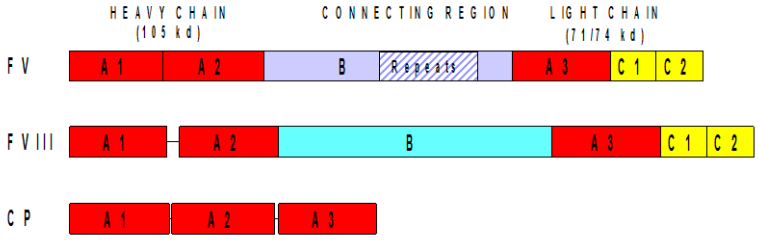


*(A) Schematic representation of the exon–intron structure of FV gene. Exons: represented by boxes, are colored according to the encoded domain. Introns are depicted as lines. (B) Diagram of the domain organization of the single-chain 330 kDa FV. The black box at the N-terminus represents the 28-residue signal peptide. Numbers within boxes denote N- and C-terminal residues of each domain. The position of N-glycosilation sites are indicated by grey rhombs, except for Asn2181 depicted in violet. All cysteines, either involved in disulphide bridges or present as free residues, are also indicated. (C) Domain structure of FVa. Following activation by thrombin or FXa, the active FV is a heterodimer composed of a heavy (A1 and A2 domains) and a light chain (A3, C1, and C2 domains) held together by a single calcium ion.*

### *Domain Structure*

FV has a mosaic-like structure, with a domain organization (A1-A2-B-A3-C1-C2), that is similar to that of FVIII[110] (Fig. 1.5). The A domains of FV and FVIII, together with those of ceruloplasmin (CP)[111], a copper-binding plasma protein, have evolved from a common ancestral protein (Fig. 1.6). Overall, the two coagulation cofactors FV and FVIII share ~40% sequence identity in their A and C domains[112].

*Figure 1.6 - Domain structure of human FV, FVIII and CP[113].*




*Coagulation FV and FVIII show identical domain organizations, encompassing three homologous A domains, one poorly conserved B domain and two homologous C domains. The copper-binding protein CP consists only of three A domains, which are ~30% homologous to those of FV and FVIII..*

The three A domains (~350 amino acids each) of FV are highly conserved between species (>80%) and ~40% homologous to the corresponding A domains of FVIII and CP[106-107].

The two C domains (~150 amino acids each) of FV show 35-50% homology with each other and with the C domains of FVIII[111], and are 20% homologous to discoidin I of the slime mold *Dictyostelium discoideum*. The C2 domains of FV and FVIII have been shown to be involved in the interaction of the respective cofactors with negatively charged phospholipid membranes (Fig. 1.7).

The sequence of the B domain of human FV does not show any appreciable homology to its FVIII counterpart (only 14%, restricted to the amino- and carboxyl-terminal regions), and is

poorly conserved even between different mammalian species (only 35% overall amino acid identity among the human, bovine and murine sequences[114]), suggesting rapid divergence during evolution.

Functional characterization of FV mutants lacking the whole B domain or parts of it has provided evidence for an important role of the B domain in the regulation of FV activation, and more recent experiments support the involvement of the FV B domain in the APC anticoagulant pathway.

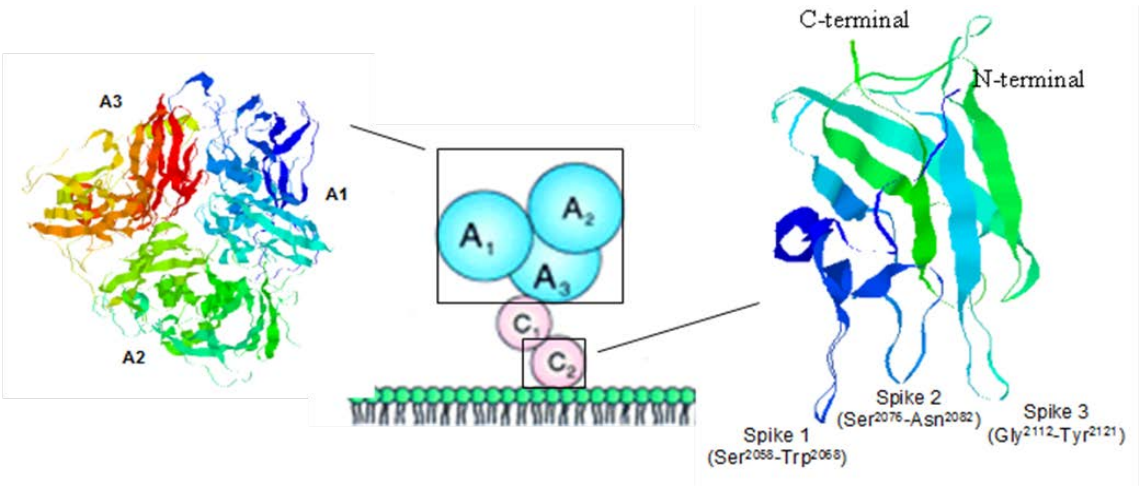



*Figure 1.7 - Three-dimensional structure: human ceruloplasmin and C2 domain of human FV*
*On the left the structure of the three FV A domains has been developed using the X-ray structure of human CP[115] as a template. In the middle schematic representation of the human FVa. On the right three-dimensional structure of the C2 domain of human FV in the "open" (i.e. membrane-bound) conformation. The typical β-barrel structure is apparent. The three loops (spikes 1-3) that extend from the lower part of the domain constitute the primary membrane-binding site of the C2 domain.*

### 1.3.2 Biosynthesis

Approximately 80% of blood FV circulates in plasma as a free protein at the concentration of 7 µg/ml (~21 nmol/L)[116]. The remaining 20% is stored in the α-granules of platelets[116-117]. Plasma-derived FV is synthesized by hepatocytes[118-119], and is constituted by a single-chain procofactor of 330 kDa; the platelet fraction of FV, which is partially proteolyzed and stored in association with the binding protein multimerin, is partly synthesized in the megakaryocyte and partly adsorbed from plasma via endocytosis[120]. Release of platelet FV upon platelet activation is responsible for an increase in the local concentration of the cofactor at injury

sites. FV synthesis has also been reported to occur in other cellular types, such as endothelial cells[121], vascular smooth muscle cells[122] and T-lymphocytes[123].

FV stability in plasma was studied in a primate model by infusing iodinated FV or FVa in baboons[49]. Single-chain FV showed a half-life of ~13 hours and was cleared from the circulation without being degraded. After activation by thrombin, the FV heavy and light chains were removed with $t_{½}$ ≤20 min, whereas the B domain-derived activation peptides remained detectable in plasma for several hours[124].

FV biosynthesis is rather complex and similar to that of FVIII. Nascent FV is co-translationally translocated to the lumen of the endoplasmic reticulum and directed to the secretory pathway. Immediately after translocation, the 28-amino acid leader peptide is cleaved off by an intra-lumenal signal peptidase. The FV molecule undergoes multiple posttranslational alterations on its way through the endoplasmic reticulum and Golgi apparatus, including sulfation, phosphorylation and glycosylation.

Phosphorylation of plasma FV occurs after secretion and targets both the heavy and the light chain. The heavy chain of FV is phosphorylated at a single residue ($Ser^{692}$ in human FV) by a membrane-associated platelet casein kinase II (CKII) enzyme; this covalent modification increases the rate of inactivation of the FV by APC[125]. Differently, phosphorylation of the FV light chain is catalyzed by an intra-platelet protein kinase C (PKC) and appeared to affect neither FVa activity in the prothrombinase complex nor its sensitiviy to APC[126].

### 1.3.3 FV Isoforms

FV in human plasma and platelets is present in tow isoforms, $FV_1$ and $FV_2$, having slightly different molecular masses and charges[127]. The $FV_1$ isoform is slightly bigger than $FV_2$, and the $FV_1/FV_2$ ratio in blood is approximately 30/70. The heterogeneity has been found to reside in the light chain, which on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) migrates as a closely spaced 71 kDa and 74 kDa doublet[128]. The two FVa forms $FVa_1$ (74 kDa) and $FVa_2$ (71 kDa) can be separated by cationic exchange chromatography, suggesting that the molecular mass difference between the two isoforms is associated with a surface charge difference.

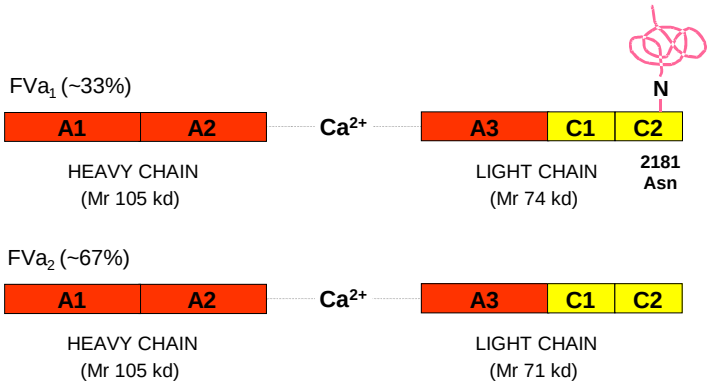
The two FV isoforms are functionally distinct, which is related to different phospholipids binding properties[127,129]. $FVa_1$ binds negatively charged phospholipid membranes 45-fold less efficiently ($K_d$ 24 nM) than $FVa_2$ ($K_d$ 0.53 nM)[127]. Since membrane-binding capacity affects

both FVa activity in the prothrombinase complex and FVa susceptibility to APC-mediated inactivation, at limiting concentrations of phosphatidylserine the two FV isoforms will show different functional properties[129]. The lower procoagulant potency of the $FVa_1$ is particularly pronounced at low phospholipid concentrations or a low percentage of phosphatidylserine in the membrane, whereas at high phospholipid levels and/or with a high phosphatidylserine content, little difference in phospholipid binding and procoagulant activity is observed[129].

Since an optimal APC-mediated FVa inactivation requires more phosphatidylserine than prothrombinase activity, in the presence of vesicles with the same phospholipid composition as activated platelets (10% phosphatidylserine) the two FVa isoforms express the same procoagulant activity, but $FVa_1$ is inactivated at a considerably slower rate than $FVa_2$. This circumstance makes $FVa_1$ 7-fold more thrombogenic than $FVa_2$ in physiological conditions[129].

It has been suggested[127] that the molecular weight difference between the two FV isoforms may be due to differential glycosylation of the C2 domain, which contains a single N-linked glycosylation site at $Asn^{2181}$ (Fig. 1.8). Formal evidence came from site-directed mutagenesis experiments[130] that demonstrated that replacement of $Asn^{2181}$ by a Gln yields a single FV species, which is structurally and functionally identical to $FV_2$ (71-kd light chain). The crystal structure of the FV C2 domain[131] indicates that $Asn^{2181}$ is located in the vicinity of a phospholipid-binding loop (spike 1 in Fig.1.7) and that its glycosylation in $FV(a)_1$ may indeed interfere with membrane-binding[130].

*Figure 1.8 – FV isoforms[113]*

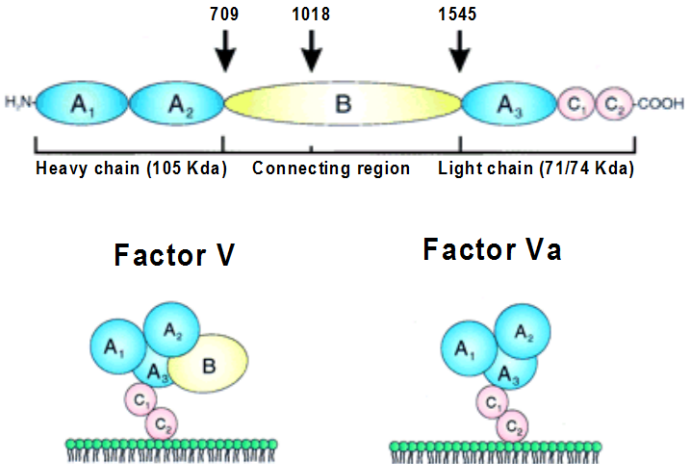



*The two FV isoforms differ for the presence ($FV_1$) or absence ($FV_2$) of an N-linked carbohydrate moiety on $Asn^{2181}$ in the C2 domain. As a result of glycosylation at $Asn^{2181}$, $FVa_1$ has a heavier light chain (74 kDa) than $FVa_2$ (71 kDa). Normal plasma contains approximately 33% $FV_1$ and 67% $FV_2$.*

### 1.3.4 FV activation

Circulating single-chain FV is an inactive procofactor, expressing <1% of the procoagulant enzyme-activated FXa-cofactor activity that it can maximally obtain[132]. An increase in FXa-cofactor activity is associated with limited proteolysis of several peptide bonds in FV mediated by procoagulant enzymes such as thrombin and FXa[133]. As a result, the large connecting B domain (Fig. 1.9) dissociates from FVa, which is formed by non-covalently associated heavy (A1-A2) and light (A3-C1-C2) chains.

*Figure 1.9 – FV activation sites and FVa structure[108]*




*Schematic models of FV illustrating the mosaic-like structure of the molecule and the spatial arrangement of the FV domains. On activation of FV, 3 peptide bonds (upper black arrows) at positions Arg709, Arg1018 and Arg1545 are cleaved, thereby releasing the B domain.*

The prothrombinase complex comprises FXa and FVa, which in the presence of calcium ions assemble on negatively charged phospholipid membranes. FVa is considered an essential FXa cofactor, since its presence in the prothrombinase complex enhances the rate of prothrombin activation by several orders of magnitude[132].

The principal and most potent physiological activator of FV is represented by thrombin. Thrombin-mediated activation of human FV does not require phospholipids and takes place *via* three consecutive proteolytic cleavages and the formation of two intermediates[128]. Thrombin cleaves sequentially at Arg709, Arg1018 and Arg1545 to generate the non-covalently associated light and heavy chains. The first cleavage cuts the peptide bond between Arg709 and Ser710 and separates a 105 kDa amino-terminal peptide (heavy chain of the activated cofactor), from a 280 kDa intermediate. The latter is further cleaved between

Arg1018 and Thr1019, giving rise to a 71 kDa activation peptide and a second 220 kDa intermediate. Although FV activity is already detectable at this stage, thrombin proteolyzes a third peptide bond between Arg1545 and Ser1546, thus detaching the carboxyl-terminal portion of the protein (the 71/74 kDa light chain) from a heavily glycosylated 150 kDa activation peptide. Functionally competent FVa results from the non-covalent association of the heavy and the light chains *via* a $Ca^{2+}$ ion and hydrophobic interactions.

Single-chain FV does not bind FXa, thus the activation of FV to FVa is essential to its biologic function. To ensure FVa activation at the onset of coagulation, before the first traces of thrombin become available, an alternative mechanism for FV activation is necessary. This function is fulfilled by FXa, which has the ability to cleave membrane-bound FV (no appreciable activation is observed in the absence of phospholipid vesicles) in the presence of $Ca^{2+}$ ions[134]. FXa-mediated activation of human FV occurs at a 5-fold lower rate than the thrombin-catalyzed reaction, but the end-product expresses the same cofactor activity in PT activation[134-135]. Human FV is initially cleaved at Arg1018, producing a 150 kDa intermediate and a 220 kDa peptide. A second cleavage, taking place at Arg709, liberates the 105 kDa heavy chain, as well as a 71 kDa activation peptide, from the 150 kDa fragment. Increasing activity was reported to correlate with accumulation of the 220 kDa fragment, and the active species was shown to comprise the 105 kDa and 220 kDa peptides.

### 1.3.5 FVa inactivation

APC down-regulates thrombin generation, and as consequence the coagulation process, by proteolytically inactivating FVa and FVIIIa, in the presence of negatively-charged phospholipids and $Ca^{2+}$-ions[136]. The proteolytic activity of APC is highly specific for membrane-bound FV/FVa and FVIIIa. APC binds to the FVa light chain in a competitive manner with FXa[137]. Thus APC provides inhibition of coagulation by noncovalent competition with FXa and by cleavage and inactivation of FVa[137]. FVa has been shown to contact APC through the light chains[136]; in particular, the APC-interaction site has been mapped to amino acids 1865-1874[138] (Fig. 1.10).

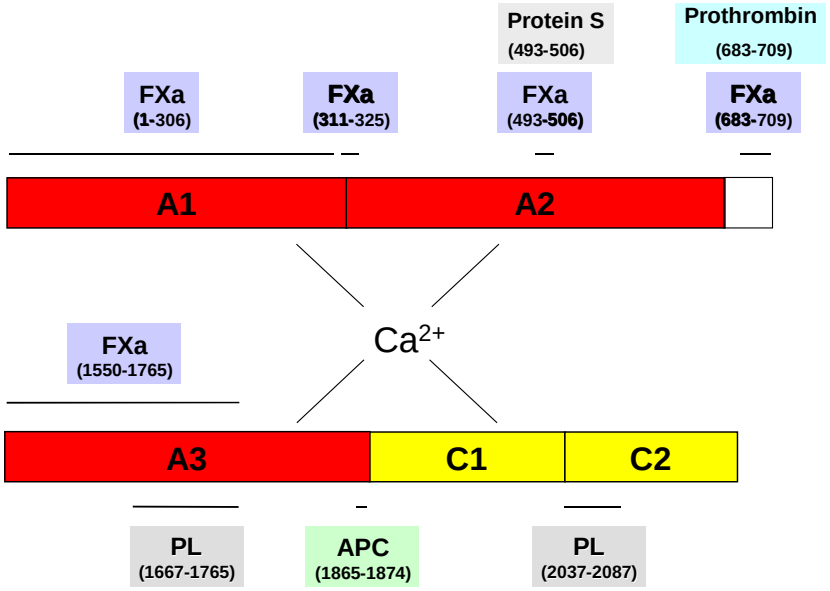
Inactivation of FVa by APC is a $Ca^{2+}$-dependent reaction which is enhanced 230-fold by negatively charged phospholipids[139]. APC cleaves human FVa heavy chain at Arg506, Arg306 and Arg679[140], while the light chain anchors the cofactor to the surface of phospholipid membranes. Cleavages at these positions are under strict kinetic control;

cleavage at Arg506 is kinetically favoured over that at Arg306, less dependent on phospholipid composition and on presence of protein S; however it yields only partial inactivation of FVa, and cleavage at Arg306 is necessary for the complete inactivation of FVa activity[141]. The third cleavage at Arg679 is likely of lesser importance to FVa inactivation.

FVa inactivation is greatly enhanced by protein S. For a long time protein S was thought to increase the rate of Arg306 cleavage about 20-fold, while no effect on the cleavage at Arg506 was observed[142]. Recently it has been shown that protein S stimulates both cleavages at Arg506 and Arg306 in a phospholipid-dependent manner of 5-fold and 27-fold respectively[143]. No effect of PS is observed in the absence of phospholipid vesicles[139].

The human procofactor FV is also inactivated by APC, but only when bound to a membrane surface. FV is inactivated by cleavages at Arg306, Arg506, Arg679 and Lys994. In this instance, cleavage at Arg306 appears to be the sole requirement for inactivation[140].

*Figure 1.10 – FV interactions*[113]




*Map of putative protein-protein and protein-phospholipid interaction sites on human FVa. PL, phospholipids.*

### 1.3.6 Anticoagulant FV

In addition to its role as a precursor of pro-coagulant FVa, circulating FV has an anticoagulant role, functioning as a synergistic APC cofactor to protein S in the inactivation of FVIIIa[108].

The molecular mechanism by which FV can exert its APC-cofactor activity in down-regulation of FVIIIa is largely unknown, however it has been demonstrated that FV can accelerate the rate of FVIIIa inactivation by APC, only when protein S is present[144].

The anticoagulant APC cofactor function of FV is lost on the pro-coagulant activation of FV, which is the result of FXa or thrombin-mediated cleavages at Arg709, Arg1018 and Arg1545[145]. Cleavage at Arg1545, resulting in dissociation of the B domain from the light chain, is responsible for the complete loss of anticoagulant activity, whereas the other two cleavages do not affect this activity. These results suggest the involvement of the B domain in this function. Moreover, studies using recombinant FV variants have shown the C-terminal of the B domain (last 70 amino acids) to be essential for the anticoagulant APC-cofactor activity of FV[145]. It has been proposed that FV has to be cleaved by APC at Arg506 to express its anticoagulant function[144]. Thorelli and coworkers concluded that cleavage at Arg506 by APC is essential for expression of full anticoagulant properties of FV, whereas Arg to Gln substitution at the other APC cleavage sites did not affect APC-cofactor function[146]. The APC-mediated cleavage of intact FV at Arg506 indicates a delicate balance between the pro-coagulant and the anticoagulant properties of FV. In fact, FV activation leads to a pro-coagulant protein that is devoid of anticoagulant properties, whereas APC-catalyzed proteolysis of intact FV generates an anticoagulant protein, that has virtually no pro-coagulant properties. In this pathway, the local concentrations and availability of pro-coagulant and anticoagulant enzymes, such as thrombin, FXa and APC, determine the fate of each FV molecule. Thus, FV acts as a local sensor of pro-coagulant and anticoagulant forces, inasmuch as it is able to sustain ongoing reactions through its susceptibility to limited proteolysis and its ability to function as either a pro-coagulant or an anticoagulant cofactor.

### 1.3.7 The FV gene

The FV gene has been mapped to chromosome 1q23[147], and spans more than 80 kb. It consists of 25 exons and 24 introns. Its organization is almost identical to that of the FVIII gene, with most of the exon-intron boundaries occurring at the same locations in the two genes. Intron length ranges between 0.4 and ~11 kb. Splicing junctions are characterized by the usual GT-AG donor/acceptor sequences (apart from intron 6, GC-AT)[148]. FV gene exons range in size from 72 to 2820 bp.

The messenger RNA (mRNA) encodes a leader peptide of 28 amino acids and a mature protein of 2196 amino acids, composed by three A domains, a connecting B domain and two C domains, highly homologous to their FVIII counterparts (Fig. 1.6). Roughly the heavy chain is encoded by exons 1 to 12, and the light chain by exons 14 to 25. The entire B domain

is encoded by exon 13, which contains 2 tandem repeats of 17 amino acids and 31 tandem repeat of 9 amino acids, that are absent in the B domain of FVIII[107,148]. The whole gene has been sequenced and the sequence is available from GenBank under the accession number Z99572.

## 1.4 FACTOR V DEFICIENCY

FV deficiency, or parahemophilia (Mendelian Inheritance in Man, OMIM *227400)[149], was first described in 1947 by Owren[105] and causes a hemorrhagic phenotype. It is inherited as an autosomal recessive trait with an incidence of about 1 in 1 million. Due to the low prevalence of FV defects, affected individuals are often the offspring of consanguineous marriages.

The phenotypic expression of FV deficiency is variable; heterozygotes are usually asymtpomatic, whereas homozygous patients show mild, moderate or severe bleeding symptoms. The most common symptoms[150] include easy bruising, epistaxis, spontaneous bleeding from the oral cavity, and menorrhagia in females. Haemarthroses and haematomas are also fairly common, whereas gastrointestinal and urinary tract haemorrhages are rare. Intracranial haemorrhages, which tend to occur at birth or in the neonatal period[151], appear to be confined to patients with undetectable FV levels (<1%)[150]. However, humans with FV deficiency are alive, whereas in mice the total lack of FV is not consistent with survival[152]. In empirical models conducted with purified reaction components and computer models of the blood coagulation system, thrombin cannot be generated in the absence of FV when all inhibitors are present in the reaction system[153]. Conversely, humans with severe truncations in the FV molecule, that do not allow any synthesis of FV by known conventional mechanisms, have been observed to have either manageable or no pathology associated with bleeding. These observations suggest that there may be compensatory mechanisms present in human blood, which either bypass or reduce the need for FV in generating levels of thrombin required for survival.

FV deficiency results in the simultaneous prolongation of the prothrombin time (PT) and the activated partial thromboplastin time (APTT), however specific diagnosis of FV deficiency relies on the measurement of FV activity (by a one-stage functional assay using FV-deficient plasma) and antigen (by ELISA or radioimmunoassay). Congenital FV deficiency can be classified[154] as either CRM- (cross reacting material negative) (type I deficiency), with low or

unmeasurable antigen levels, or CRM+ (type II deficiency), showing normal or mildly reduced antigen levels associated with reduced coagulant activity[154].

The management of patients with severe FV deficiency is complicated by the unavailability of FV concentrates, which makes the daily administration of fresh or fresh-frozen plasma the treatment of choice. Further difficulties are related to the short half-life of FV in plasma (about 20 hours) and to the risk of volume overload in case of prolonged treatment[154].

The molecular bases of FV deficiency are still largely unexplored. In contrast to FVIII deficiency, for which more than 300 causative mutations have been described (cf. Haemophilia A database), only few intragenic mutations impairing FV gene expression exist on record[155-157], mostly private mutations. In addition, a frequent FV gene polymorphism predicting slightly reduced FV levels has been described[158].

## 1.5 APC RESISTANCE

Resistance to activated protein C (APC-resistance)[159] is defined as a poor anticoagulant response of plasma to exogenously added APC. It was firstly described in 1993, in one patient with venous thrombosis[103]. This procoagulant condition turned out to be rather frequent in the general population (~5%)[160-161] and to be inherited within families according to an autosomal dominant pattern[160,162].

APC resistance has been recognized as the most important cause of venous thrombosis, which is present in 20% to 60% of patients with venous thromboembolism. The relative thrombotic risk conferred by APC-resistance was estimated to be 6.6[160].

### 1.5.1 The FV Leiden mutation

**Pathogenic mechanisms and molecular basis**

The genetic background for the APC resistance phenotype was demonstrated in 1994, when a single nuclear polymorphism in the FV gene was found to be associated with APC resistance[102]. The term “Factor V Leiden” refers to the specific guanine to adenine substitution (a missense mutation) at nucleotide 1691 in exon 10 of FV gene, which predicts the substitution of glutamine for arginine at the Arg 506 APC cleavage site. Because of this single amino acid substitution, Factor Va is resistant to APC and is inactivated at a 10-fold slower rate than normal, resulting in increased thrombin generation[163].

Over 90% of cases of APC-resistance in the Caucasian population are attributable to the FV R506Q mutation[102]. Factor V Leiden is the most common genetic risk factor for VTE, found in 20–25% of patients with VTE and 50% of patients with familial thrombophilia[164-165]. Carriership of the FV R506Q mutation confers a life-long risk of venous thrombosis. It has been estimated that heterozygous and homozygous carriers of the mutation experience 7-fold and 80-fold increased risk, respectively, as compared to individuals with normal genotype[164-165].

The Arg506Gln substitution results in the loss of one of the important cleavage sites for APC in FVa. FV Leiden is thus inactivated by APC after cleavages at Arg306 and Arg679, leading to FVa fragments of 60 and 54 kDa (Fig. 1.11).

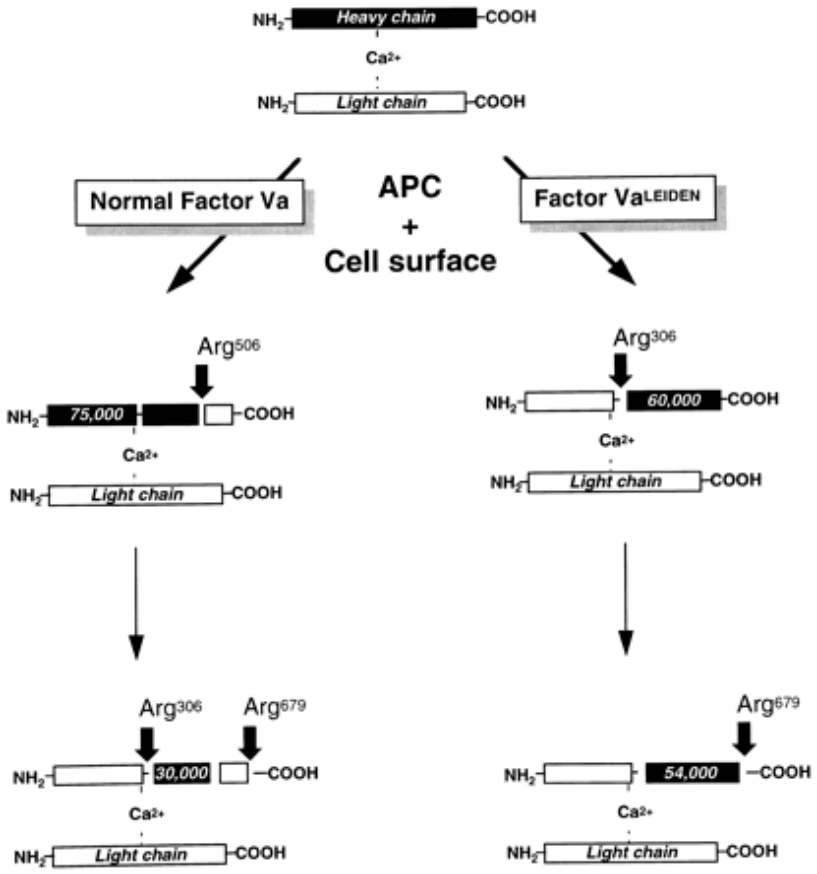



*Figure 1.11 – FVa inactivation by APC*

*Cleavage and inactivation of normal FVa (left) and FVa Leiden (right) by APC. FVa is inactivated by APC after cleavages at Arg506, Arg306 and Arg679. FVa Leiden is inactivated by APC after cleavages at Arg306 and Arg679. Fragments of 75000 and 30000 Da identify normal FV, whereas fragments of 60000 and 54000 Da identify the FV Leiden molecule[167].*

The impaired down-regulation of FXa cofactor activity of FVa contributes to the increased risk of thrombosis. The abolition of the APC-cleavage site at Arg506 appeared a satisfactory explanation for the APC resistance associated with the mutation. Only when it was reported that FV Leiden is a poor cofactor in APC-catalysed FVIIIa inactivation[145], it was realized that reduced APC-cofactor activity may also contribute to FV Leiden-associated APC resistance. Recently, it has been unequivocally demonstrated that FV Leiden exhibited no cofactor activity in FVIIIa inactivation[166]. Poor susceptibility to APC and impaired APC-cofactor activity equally contributed to FV Leiden associated APC resistance[166]. These results show that the anticoagulant activity of FV plays a pivotal role in the regulation of thrombin formation.

**Prevalence**

Factor V Leiden is the most common inherited form of inherited thrombophilia, accounting for 40–50% of cases. The prevalence varies by population (Table III)[163]. Heterozygosity for Factor V Leiden occurs in 3–8% of the general US and European populations. The highest eterozygosity rate is found in Europe; the mutation is extremely rare in Asian, African, and indigenous Australian populations. Within Europe, the prevalence varies from 10 to 15% in southern Sweden and Greece to 2–3% in Italy and Spain.[10] The mutation is found in 3.8% of individuals in France, but the frequency ranges from 1.3% in south-western regions to 7.1% in north-eastern France[168]. In the United States, the prevalence reflects the world distribution of the mutation[169]. It is present in: 5.2% of white Americans; 2.2% of Hispanic Americans; 1.2% of African Americans; 0.45% of Asian Americans; 1.25% of native Americans.

The frequency of homozygosity for Factor V Leiden in white populations is approximately 1 in 5000. Haplotype analysis of the Factor V gene strongly suggests that the mutation was a single event that occurred 20,000–30,000 years ago, after the evolutionary separation of whites from Asians and Africans[170]. The high prevalence of Factor V Leiden among whites suggests a balanced polymorphism with some type of survival advantage associated with the heterozygous state. Some investigators speculate that the mild prothrombotic state conferred by the mutation could have reduced mortality from bleeding associated with childbirth or trauma in premodern times[170]. In several studies, Factor V Leiden heterozygotes had significantly less blood loss during menses, childbirth, and cardiac surgery[163]. Some evidence suggests that severe hemophiliacs who are heterozygous for Factor V Leiden have less severe bleeding and reduced clotting factor concentrate utilization, also consistent with this

hypothesis[171]. However, an evolutionary survival benefit of Factor V Leiden has not been confirmed.

*Table III - Worldwide prevalence of Factor V Leiden (a,b)[163]*

| Population | Prevalence (%)[9–11,12–17] |
|---|---|
| European whites | 3–15 |
| Spain | 3.3 |
| France | 3.8 |
| Germany | 4 |
| Iceland | 5.2 |
| United Kingdom | 8.8 |
| Greece | 15 |
| Sweden | 11 |
| Africa | Absent |
| Southeast Asia | Absent |
| Asia minor | 1.2 |
| Australia (indigenous) | Absent |
| Japan | Absent |
| Jordanian Arabs | 12.2 |
| Lebanon | 14 |
| Western Iran | 2.97 |
| Canada | 5.3 |
| United States | |
| Whites | 5.2 |
| Hispanic Americans | 2.2 |
| African Americans | 1.2 |
| Asian Americans | 0.45 |
| Native Americans | 1.25 |

[a]*Healthy individuals with no history of venous thromboembolism.*
[b]*Includes heterozygous and homozygous individuals.*

**Natural History and Clinical manifestations**

The clinical expression of Factor V Leiden thrombophilia is variable. Many individuals with a Factor V Leiden allele never develop thrombosis. Although most affected individuals do not experience their first thrombotic event until adulthood, some have recurrent VTE before the age of 30 years. Factor V Leiden homozygotes are more likely to develop their first VTE at a younger age[163].

- ***Venous thromboembolism***

The primary clinical manifestation of Factor V Leiden is VTE. The mutation is found in 25% of patients with a first idiopathic VTE and up to 40–50% of those with recurrent VTE or an estrogen-related thrombosis[172,173] (Table IV)[163]. DVT most commonly develops in the legs, but upper extremity thrombosis also occurs. Some evidence suggests that pulmonary embolism is less common than DVT in individuals with a Factor V Leiden allele[73,174]. The explanation for a differential effect of Factor V Leiden on the risk for DVT and pulmonary embolism is unclear.

*Table IV - Estimated prevalence of Factor V Leiden among patients with thrombotic complications[163]*

| Thrombotic complication | Prevalence (%) |
|---|---|
| First idiopathic VTE[3] | 25 |
| Recurrent VTE[40,42] | 30–50 |
| Upper extremity thrombosis[43–45] | 9–20 |
| Cerebral vein thrombosis[46,47] | 10–20 |
| Pregnancy-associated VTE[48–51] | 20–46 |
| Oral contraceptive-associated VTE[49,52] | 20–60 |
| Pregnancy loss[11,53–55] | 8–30 |

VTE, venous thromboembolism.

- ***Risk for a first VTE***

The relative risk for VTE is increased 3- to 8-fold in Factor V Leiden heterozygotes (Table V)[163]. Lower relative risks are reported in heterozygotes identified from general population screening[175]. The thrombotic risk is increased 10- to 80-fold in homozygotes[95] (Table VI)[163].

- ***Recurrent VTE***

Approximately 30% of individuals with an incident VTE develop recurrent thrombosis within the subsequent 8 years[65]. There are conflicting data on the risk of recurrent VTE associated with Factor V Leiden heterozygosity. The risk for recurrent VTE in Factor V Leiden homozygotes is not well defined but presumed to be higher than in heterozygotes. A systematic review found a 2- to 3-fold increased risk for recurrent VTE in Factor V Leiden homozygotes[176]. However, not all studies found a high risk for recurrence in homozygotes and double heterozygotes. Risk estimates were limited by the small number of homozygotes and double heterozygotes included in these studies[163].

*Table V - Estimated risk of thrombotic complications: Factor V Leiden heterozygotes*[163]

| Thrombotic complication | Estimated risk (odds ratio)[a] |
|---|---|
| First VTE[61–64] | 3–8 |
| Cerebral vein thrombosis[46,59] | 3–5 |
| Primary upper extremity thrombosis[b][43,45] | 3–6 |
| CVC-associated thrombosis[65] | 2–3 |
| Superficial vein thrombosis[60] | 6 |
| Pregnancy-associated VTE[66,67] | 8–52 |
| Recurrent VTE[42,68] | 1.4–1.6 |
| Pregnancy loss[66,69–71] | 2–4 |

[a]Risk relative to individuals without Factor V Leiden.
[b]Not related to malignancy or a central venous catheter.
VTE, venous thromboembolism; CVC, central venous catheter.

*Table VI - Estimated risk of thrombotic complications: Factor V Leiden homozygotes*[163]

| Thrombotic complication | Risk (odds ratio)[a] |
|---|---|
| First VTE[4,33,62] | 10–80 |
| Pregnancy-associated VTE[66,73,74] | 20–40 |
| Oral contraceptive-associated VTE[75] | 100 |
| Recurrent VTE[33] | 2–3 |
| Surgery-associated VTE[76] | 20 |
| Early fetal loss[66] | 3 |
| Late fetal loss[b][77] | 11 |

[a]Risk relative to individuals without a Factor V Leiden allele.
[b]Occurring after 12 weeks gestation.

- ***Pregnancy complications***

The available data indicate that Factor V Leiden is associated with a 2- to 3-fold increased relative risk for pregnancy loss and possibly other complications such as preeclampsia, intrauterine growth restriction and placental abruption. However, a Factor V Leiden mutation is at most one of multiple predisposing factors contributing to these adverse outcomes. Other genetic and environmental triggers in addition to Factor V Leiden are likely necessary for the development of pregnancy complications. Overall, the probability of a successful pregnancy outcome is high, even in homozygous women[177].

**Clinical Expression of FV Leiden**

The clinical expression of Factor V Leiden is influenced by coexisting genetic and acquired thrombophilic disorders and circumstantial risk factors (Table VII)[163]. The presence of a Factor V Leiden allele increases the risk associated with other inherited and acquired thrombophilic disorders. The combination of Factor V Leiden heterozygosity and most thrombophilic disorders has a supra-additive effect on overall thrombotic risk[163].

*Table VII - Estimated risk of venous thromboembolism in Factor V Leiden heterozygotes with coexisting risk factors[163]*

| Coexisting risk factor | Risk of VTE (odds ratio)[a] |
|---|---|
| PG20210G>A double heterozygote[48,108] | 20<br>100 (pregnancy-associated VTE) |
| Hyperhomocysteinemia[109] | 22 |
| Obesity[110] | 8 |
| Oral contraceptives[46,111–114] | 11–41<br>30 (cerebral vein thrombosis) |
| Third generation oral contraceptives[b]114 | 50 |
| HRT[115–118] | 7–16 |
| Air travel[112,119] | 14–16 |
| Minor injury[57] | 50 |
| Malignancy[45,120] | 12<br>20 (upper extremity thrombosis) |

[a]Risk relative to risk of individuals without either risk factor.
[b]Oral contraceptives containing the third-generation progestagen desogestrel.
VTE, venous thromboembolism; PG 20210G>A, prothrombin 20210 G>A mutation; HRT, hormone replacement therapy.

- ***Coexisting thrombophilic disorders***

There is an increased prevalence of Factor V Leiden among symptomatic patients with deficiencies of Protein C, Protein S, and antithrombin and the prothrombin 202010G_A mutation[163]. Individuals with two defects have a higher risk for thrombosis than family members with a single defect. In a pooled analysis of eight case-control studies, individuals with a single Factor V Leiden or prothrombin 20210G_A allele had a 5-fold and 4-fold increase in thrombotic risk, respectively. In contrast, individuals doubly heterozygous for both mutations had a 20-fold increase in relative risk, illustrating the multiplicative effect of these two mutations on overall thrombotic risk[178]. A recent study found that individuals with a Factor V Leiden mutation who had a first-degree relative with a history of thrombosis had a

3-fold higher risk for VTE than Factor V Leiden carriers with a negative family history. The risk was increased 18-fold in those with two or more symptomatic relatives.
The family history had additional value in predicting risk regardless of Factor V genotype, suggesting the presence of unknown genetic risk factors[32]. The risk of thrombosis is also increased in patients with Factor V Leiden and hyperhomocysteinemia. In the Physicians' Health Study, individuals with either a Factor V Leiden allele or hyperhomocysteinemia had a 3- to 4-fold increased risk for idiopathic VTE compared with those with neither abnormality. The relative thrombotic risk was increased 22-fold in individuals with both abnormalities[169].
It is still unclear to what extent Factor V Leiden increases the thrombotic risk associated with malignancy. A large population based case-control study found that Factor V Leiden heterozygotes with malignancy had a 2-fold higher risk for VTE than patients with cancer without the mutation and a 12-fold higher risk than those with neither risk factor[179].

- ***Circumstantial risk factors***

Other predisposing factors for VTE include central venous catheter use, pregnancy, estrogen contraception, HRT, SERMs, travel, injury, age, obesity, and surgery. Factor V Leiden interacts with these environmental risk factors to increase the risk of VTE. At least 50% of thrombotic episodes in individuals with Factor V Leiden are provoked by additional predisposing factors[180]. In a large cohort of symptomatic Factor V Leiden homozygotes, the initial VTE was associated with circumstantial risk factors in 81% of women and 29% of men. Oral contraceptives and pregnancy were the most common predisposing factors in symptomatic women. Thirteen percent of major surgeries were complicated by VTE, suggesting an approximately 20-fold increase in risk[181].

### 1.5.2 Other FV mutations causing APC resistance

Two different mutations at the Arg306 APC cleavage site in Factor V have been reported, only one of which is associated with APC resistance (Table VIII)[163]. A G-to-C point mutation, which predicts the replacement of Arg with Thr at position 306 (Factor V Cambridge), was identified in a British individual with a history of thrombosis and APC resistance in the absence of a Factor V Leiden mutation[182]. However, the mutation was not found in several series of individuals with VTE or healthy controls, suggesting it is a very rare Factor V variant[182]. A different mutation in the same codon predicting an Arg to-Gly substitution was identified in two Chinese individuals with a history of thrombosis. The Arg306Gly mutation

(Factor V Hong Kong) was not associated with APC resistance in the single individual tested with a coagulation screening test[183]. The clinical significance of the Arg306Gly mutation is unclear because it was found in a similar percentage of healthy Hong Kong Chinese blood donors (4.5%) and individuals with a history of thrombosis (4.7%)[183]. A missense mutation in Factor V, Ile359Thr (Factor V Liverpool) was identified in several family members with recurrent thrombosis[184]. Factor V Liverpool showed resistance to APC and impaired cofactor activity for APC similar to that of Factor V Leiden[163]. Although the available evidence suggests that the Arg306Thr, Arg306Gly, and Ile359Thr mutations are not major risk factors for thrombosis, they may contribute when combined with other genetic or acquired risk factors. There are anecdotal reports of double heterozygosity for Factor V Leiden and Factor V Cambridge[185].

*Table VIII - Selected Factor V allelic variants[163]*

| Allelic variant | DNA nucleotide change | Protein amino acid change | Clinical significance |
|---|---|---|---|
| R2 polymorphism[171,172] | 4070 A>G | His1299Arg | Increases APC resistance and VTE risk associated with Factor V Leiden |
| Factor V Cambridge[6,173] | 1091 G>C | Arg306Thr | Uncertain; rare variant; confers APC resistance |
| Factor V Hong Kong[6,174,175] | 1090 A>G | Arg306Gly | Uncertain, found in 4.7% Hong Kong Chinese with and 4.5% without VTE |
| Factor V Liverpool[6,176] | 1250 T>C | Ile359Thr | Uncertain; confers APC resistance |
| Factor V Leiden[61,62] | 1691 G>A | Arg506Gln | APC resistance and 3- to 8-fold increased risk of VTE |

### 1.5.3 HR2 Haplotype

A common FV allele, marked by the 4070 A/G transition (FV H1299R) and by a unique FV gene haplotype, has been reported to contribute to APC-resistance and thrombotic risk both *per se* and in combination with the FV R506Q mutation.

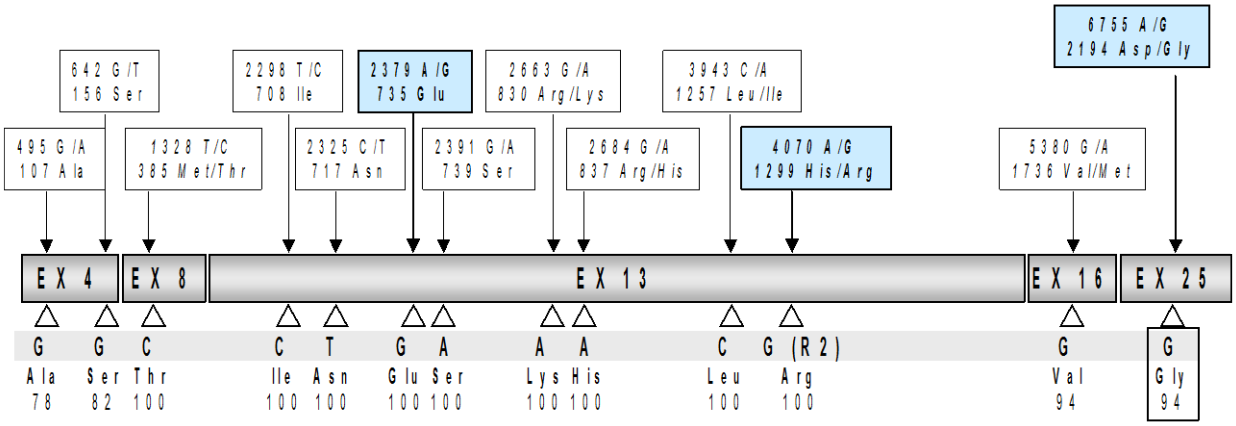
The 4070 A to G transition in FV exon 13, causing a His to Arg substitution at position 1299 of the B domain, has been identified in 1996 in our laboratory[158]. Further analysis revealed that this substitution is tightly linked to a number of 13 different polymorphisms in the FV gene[156,186], which has been called “R2 haplotype” (FVHR2). Seven of the 13 base change s predict an amino acid change in FV, and lead to functional modifications of the protein[187].

The HR2 haplotype is rather frequent, with an allele frequency throughout Asia, Europe and native African populations ranging from 5 to 17%[156,186]. The very high prevalence (up to 50%) in Indian tribes from Costa Rica is notable[187]. The fact that the R2 allele is well represented in an African population (Somali) hints at a very ancient origin, pre-dating the

out-of-Africa radiation of human populations that took place about 100000 years ago[188]. The overall prevalence of the HR2 is estimated to be approximately 10% in the European population, but can differ greatly between populations[186,189].

Among all gene variations included in the FV HR2 haplotype, only three (2379G, 4070G and 6755G) are hallmarks of this haplotype, whereas all others can also be encountered on wild-type allele.

*Figure 1.12 – The FVHR2[113]*




*The 4070G allele in exon 13 is genetically linked to other gene variations in the FV gene, which define the FV HR2 haplotype. The three transitions boxed in light blue distinguish the R2 allele from all other FV alleles.*

The FVHR2 has been associated with slightly reduced FV levels in plasma, both at the coagulant activity[158] and antigen levels[190].

The FVHR2 has decreased co-factor activity for APC-mediated degradation of FVIIa, and an increased ratio of $FV_1$ to $FV_2$, the former being the more pro-coagulant isoform[156,187].

Increased resistance to APC and reduced FV antigen and/or coagulant activity have been associated with FVHR2, though not consistently[186-187].

The thrombotic risk associated with the FVHR2 is still matter of debate[187]. Data from a meta-analysis suggest that FVHR2 could be a very mild pro-thrombotic factor[187]. However, it is generally accepted that FVHR2 enhances the risk of venous thrombosis conferred by FV Leiden mutation[190-192], and compound heterozygous carriership of both defects is indeed associated with a higher risk for venous thrombosis than heterozygous FV Leiden alone.

### 1.5.4 Glu1608Lys, Tyr1702Cys and Arg2080Cys

On 2004 was described a novel FV gene mutation (c. 4996G>A), predicting the Glu1608Lys substitution in the A3 domain and responsible for FV deficiency, which was identified in thrombophilic patients. The heterozygous mutation was detected in three unrelated patients, two carriers of the FV Leiden mutation, and one of theFVHR2 haplotype. The same mutation was also found in two unrelated subjects with partial FV deficiency, one of whom was also a carrier of FVHR2. Overall the FVGlu1608Lys mutation was identified in five unrelated subjects, all coming from the Po Valley (northern Italy) (Table VIV).

The FV1608Lys carriers showed reduced mean FV activity (42% ± 12%) and antigen (53% ± 18%) levels and, in Western blot analysis, reduced amounts of intact platelet FV. The restriction fragment length polymorphism (RFLP) study identified two haplotypes underlying the mutation, which suggests that it is recurrent. Molecular genetics data indicate that the FVGlu1608Lys mutation is associated with two different FV gene haplotypes, which, as inferred from known haplotypes of FV Leiden or FVHR2, differ in one subject for the intron 9 single nucleotide polymorphism (SNP) and intron 11 microsatellite alleles (Table VIV)[193].

*Table VIV - Clinical features, FV genotype and phenotype in carriers of the FVGlu1608Lys mutation*

| Patient | Pedigree | Gender | Clinical features | 1608 (Glu/Lys) | 506 (Arg/Gln) | HR2 | 2120 (Met/Thr) | C > T (intron 9) | GT repeat (intron 11) | FV:C | FV:Ag | nAPC-sr |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | 1 (proband) | M | DVT | Glu/Lys | Arg/Gln | R1R1 | Met/Met | CC | 13/14 | 55 | 54 | 0.37 |
| B1 | 1 (sister) | F | Recurrent phlebitis | Glu/Lys | Arg/Gln | R1R1 | Met/Met | CC | 13/14 | 57 | NA | 0.37 |
| C2 | 2 (proband) | M | Recurrent DVT | Glu/Lys | Arg/Gln | R1R1 | Met/Met | CC | 13/14 | 20 | NA | 0.52 |
| D3 | 3 (proband) | F | Recurrent DVT | Glu/Lys | Arg/Arg | R1R2 | Met/Met | CC | 13/12 | – | – | – |
| E3 | 3 (sister) | F | Asymptomatic | Glu/Lys | Arg/Arg | R1R2 | Met/Met | CC | 13/12 | 40 | 36 | 0.73 |
| F4 | 4 (proband) | F | Metrorrhagia | Glu/Lys | Arg/Arg | R1R1 | Met/Thr | CT | 13/14 | 38 | 50 | 0.80 |
| G4 | 4 (daughter) | F | Epistaxis | Glu/Lys | Arg/Arg | R1R1 | Met/Met | CT | NA | 39 | 44 | 0.81 |
| H5 | 5 | M | Asymptomatic | Glu/Lys | Arg/Arg | R1R2 | Met/Met | CT | 12/14 | 44 | 83 | NA |

*DVT, deep venous thrombosis; FV:C, factor V activity; FV:Ag, factor V antigen (both expressed as percentage of PNP); nAPC-sr, normalized APC sensitivity ratio; NA, not available; PNP, pooled normal plasma; APC, activated protein C. The intron 9 polymorphism is located 164 bp 5¢ to exon 10. The GT repeat starts 234 bp 3¢ to exon 11.*

However, it could not detected the FVGlu1608Lys mutation in 200 healthy individuals, which precludes an estimate of its frequency in the general population. This mutation could contribute to thrombophilia in carriers of FV Leiden or FVHR2 via a pseudo-homozygosity mechanism that enhances the prothrombotic features of these molecules[193].

On 2000, while studying the molecular bases of FV deficiency in a thrombophilic family, a novel A/G transition, which predicting a remarkable amino acid substitution of Y1702 by a

cysteine in the A3 domain of FV, was identified at nucleotide 5279 in exon 15 in the heterozygous state[157]. This missense mutation is a common cause of FV deficiency in the Italian population[194].

Alignment of the FV A domains with the homologous counterparts[107,148] of factor VIII (FVIII) and ceruloplasmin (CP) showed the absolute conservation of the tyrosine residue affected by the mutation and a high degree of homology among the surrounding amino acid sequences (Figure 1.13)[157]. The FV Y1702C substitution causes protein instability by disrupting the A3 domain scaffold. In addition, the exposure of a novel Cys residue at position 1702 may interfere with correct disulfide bridge formation between nearby Cys residues (Cys1697-Cys1723)[194].

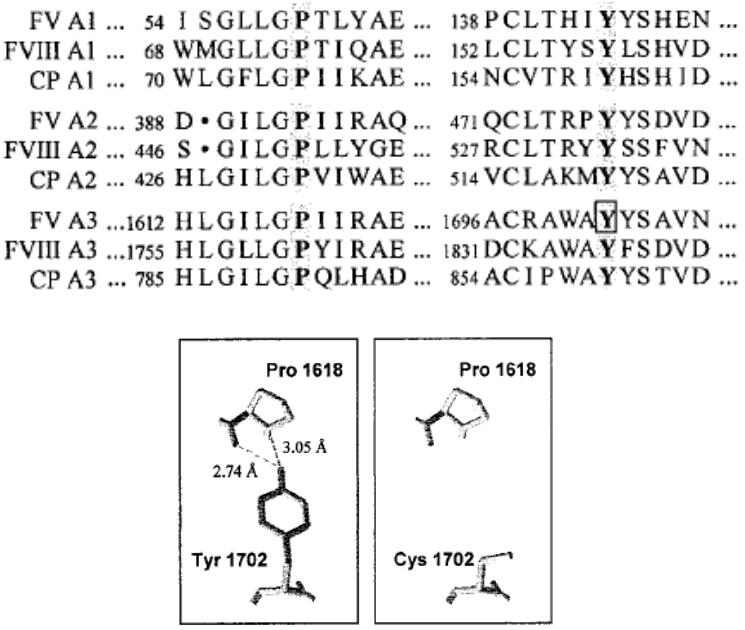



*Figure 1.13 - Conservation and structural role of the mutated tyrosine (FV Y1702) in the A domains. (Top) Alignment of a portion of the A domains of human FV, FVIII, and CP. The conserved tyrosine and proline residues are highlighted. The tyrosine residue affected by the FV 5279A/G mutation is boxed. Three-dimensional model of a portion of the A3 domain of FV based on the coordinates of CP. The highly conserved Y1702 residue forms 2 hydrogen bonds with Pro1618. The substitution of the tyrosine residue by a cysteine, predicted by the FV 5279A/G mutation, causes the loss of these interactions.*

Although a disulfide bridge formed between this cysteine and one of the other free cysteines in FV would stabilize the abnormal structure, the FV antigen levels and Western blot analyses indicate that the product of the FV Y1702C allele is not detectable in plasma, although its expression at the mRNA level is normal[157].

CRM- FV deficiency caused by the Y1702C mutation turned out to contribute to shape the thrombotic risk profile in the family. In carriers of the FV R506Q mutation and of the FV H1299R mutation, quantitative FV deficiency due to the FV Y1702C mutation, far from protecting from thrombosis, proved to increase APC resistance and thus thrombotic risk via a pseudo-homozygous APC resistance mechanism[167]. As suggested by the Western blot, increased APC resistance is attributable to the exclusive presence of R506Q or H1299R FV molecules in plasma[157].

The Arg2080Cys mutation was found in combination with the FV Glu1608Lys mutation, which it has to be responsible for quantitative FV deficiency. The FVArg2080Cys mutation, associated with FV deficiency and thrombophilia, in the absence of known thrombophilic mutations, represents the first FV natural variation affecting phospholipid binding.

The physiological role of phospholipid membranes in blood clotting has been extensively investigated[123]. The protein-phospholipids interactions within the macromolecular complexes enhance reaction rates of several orders of magnitude, and allow the localization of different enzymes and cofactors on the same surface. FVa cofactor activity in the prothrombinase complex[124] as well as FVa inactivation by APC[109] require interactions with negatively charged phospholipids. The molecular mechanism involved in binding of FVa to negatively charged phospholipid surface is poorly understood, although several FV structural and functional studies[124] have elucidated the crucial role of the C2 domain in the phospholipid membrane binding and cofactor activity. Very few natural FV mutation were identified in the C2 domain, and most of them resulted in quantitative FV deficiency[125]. By investigating thrombotic patients, the Arg2080Cys mutation was identified in the FV C2 domain, which is responsible for a CRM+ FV deficiency. Alignment of FV and FVIII sequences in mammalian species showed a complete conservation of the Arg2080 residue (FVIII, Arg2215), belonging to the motif “NAQG<u>R</u>VNAW” (Fig. 1.14), suggesting a functional role of this residue in the FV C2 domain.

Functional analysis of this alteration in the C2 domain showed that it affects APC-mediated inactivation to a higher extent than procoagulant cofactor activity, thus increasing survival of FVa molecules in plasma. This mutation could enhance the thrombotic risk, particularly in the doubly heterozygous condition with the Glu1608Lys.

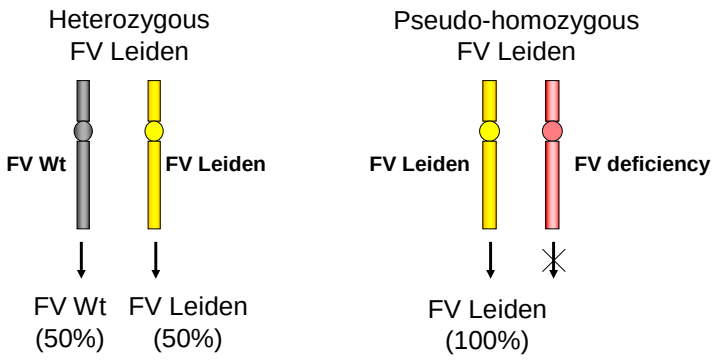
*Figure 1.14 - Alignment of FV and FVIII amino acid sequences surrounding the FVArg2080 residue*

| Species | Aminoacid sequence | GenBank accession number |
|---|---|---|
| FV C2 *(Homo sapiens)* | 2075 L N A Q G **R** V N A W 2084 | Z99572 |
| FV C2 *(Sus scrofa)* | 2075 L N A Q G R V N A W 2084 | AF191308 |
| FV C2 *(Bos taurus)* | 2075 L N A Q G R V N A W 2084 | M81440 |
| FV C2 *(Mus musculus)* | 2075 L N A Q G R V N A W 2084 | U52925 |
| FVIII C2 *(Homo sapiens)* | 2075 L H L Q G R S N A W 2084 | M88648 |
| FVIII C2 *(Canis familiaris)* | 2075 L H L Q G R T N A W 2084 | AF049489 |

### 1.5.5 Pseudo-homozygous APC-resistance

"Pseudohomozygous" Factor V Leiden occurs in individuals who are doubly heterozygous for Factor V Leiden and a Factor V null mutation on different alleles. Coinheritance of a Factor V null allele occurs in approximately 1:1000 individuals heterozygous for Factor V Leiden.
Rather than attenuating the effect of a Factor V Leiden allele, a coexisting Factor V deficiency enhances it, producing a more severe APC-resistant phenotype, reflected by an extremely low APC resistance ratio, indistinguishable from that of a homozygous mutation[187].
The diagnosis of pseudohomozygous Factor V Leiden is based on the combination of a heterozygous Factor V Leiden mutation, low-plasma Factor V activity level (approximately 50% of normal), and a low APC resistance ratio in the range typical for a homozygous mutation.

*Figure 1.18 – Pseudo-homozygous APC resistance[67]*



*Comparison between simple heterozygosity (left) and pseudo-homozygosity (right) for the FV R506Q mutation. In both cases, FV R506Q is in the heterozygous state. In patients with pseudo-homozygous APC-resistance, however, lack of expression of the counterpart allele leads to the presence of only FV Leiden molecules in plasma.*

Because pseudo-homozygous APC resistance is rare, it will be difficult to find enough individuals to achieve statistical power in studies of thrombosis risk in the affected subjects. However, almost all cases of pseudo-homozygous APC resistance reported to date have clinical manifestations[155] as severe as those of homozygous individuals.

Factor V Leiden pseudohomozygotes seem to have an increased thrombotic risk and clinical phenotype similar to that of Factor V Leiden homozygotes[191]. Pseudohomozygous APC resistance has also been reported in individuals doubly heterozygous for Factor V Leiden and Factor V Cambridge[192].

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# - 2- MATERIALS AND METHODS

## 2.1 COAGULATION LABORATORY ASSAYS

Venous blood was drawn in 9 mL BD Vacutainer® sodium citrate 3,2% and immediately centrifuged at 4000rpm for 20 minutes at 4°C. Plasma was separated, snap-frozen, and stored in aliquots at –20°C. The formed elements ("buffy coat" - white blood cells and platelets - and red blood cells) were frozen at -20 ° C for the following search of the FV Leiden mutation in patient with positive APCR.

### 2.1.1 Measurement of FV activity

FV coagulant activity (FV:C) was measured by a one-stage clotting method, based on the ability of the test plasma to normalize the prothrombin time (PT) of a FV-deficient plasma sample.
One volume of test plasma, diluted 1:20 in imidazole buffer (Factor Diluent), was mixed with one volume of undiluted normal plasma immunologically depleted of FV (FV:Ag <1%). As a consequence of pre-dilution, all coagulation factors needed for plasma clotting during the test are mainly provided by the FV-deficient plasma, except for FV itself, which is contributed by the test plasma. This ensures reproducibility of the test conditions for different plasma samples and eliminates the effects of other coagulation alterations possibly present in the test plasma. After 1 min incubation at 37 °C, the extrinsic pathway of coagulation was activated by adding two volumes of a calcium-containing thromboplastin solution (RecombiPlasTin) and the time necessary for clot formation was recorded. This time, which reflects FV activity in the test plasma, was reported on a calibration curve obtained by testing progressive dilutions of pooled normal plasma, in order to identify the dilution of pooled normal plasma having the same FV activity as the test plasma. Dilution was then converted into percentage FV activity assuming the clotting time of undiluted pooled normal plasma as a reference (100% FV activity).

Factor Diluent (IL Coagulation Systems), FV deficient plasma, RecombiPlasTin and HemosIL Calibration Plasma were purchased from Instrumentation Laboratory S.p.A, Milano (Italy). Coagulation analyzer used HemosIL$^{TM}$ ACL TOP 500).

### 2.1.2 Measurement of APC resistance

APCR was measured according to de Ronde and Bertina[1]. The APC-resistance test was originally introduced by Dahlbäck[2] and requires the measurement of the activated partial thromboplastin time (APTT) in the presence and in the absence of APC. It is based on the principle that the presence of APC will considerably prolong the clotting time in normal plasma, whereas it will only mildly affect the APTT in APC-resistant plasma.
The selectivity for the factor V:$Q^{506}$ or other mutations in the Factor V gene rendering the protein resistant to inactivation by APC is increased by normalizing the concentrations of other plasma proteins involved in formation and regulation of thrombin. By performing the APTT-based APC resistance assay in the presence of an excess of Factor V Reagent Plasma, the sensitivity and specificity for the Factor V:$Q^{506}$ mutation is significantly increased. Further, this modification allows for the analysis of plasma from patients who are on Oral Anticoagulant Therapy.[3-8]
The result of the test is expressed as a ratio (APC-sensitivity ratio, APC-sr) between the APTT measured in the presence of a carefully standardized amount of APC and that measured in its absence[9]. To make it possible to compare results between different laboratories, the APC-sr is usually normalized to that of a pool of normal plasmas[1] (APTT measured by addition of APC in the plasma pool/APTT measured in its absence in the plasma pool), yielding the so called normalized APC-sensitivity ratio (nAPC-sr). APC-sr's were normalized against the APC-sr of a pool of 60 plasmas from healthy individuals (30 males and 30 females) not carrying FV R506Q. An algorithm calculates the geometric mean of the last 20 samples of pool. Normalized APC-sr values greater than 0.84 were considered as normal.

Sample plasma is pre-diluted with Factor V Reagent Plasma and incubated with the APTT reagent for a standard period of time (30'). Coagulation is triggered by the addition of $CaCl_2$ in the absence and presence of APC and the time of clot formation is recorded. Clotting times were determined on an coagulation analyzer (HemosIL™ ACL TOP 500).

## 2.2 DNA ANALYSIS

Venous blood was drawn in 3 mL BD Vacutainer® K3 EDTA 5,4mg and stored at -20°C.

### 2.2.1 Extraction of genomic DNA from whole blood

Genomic DNA was extracted from whole blood samples using a kit QIAamp DNA blood mini kit ® - QIAGEN. This procedure comprises 4 step:

- cells lysis with QIAGEN Protease or proteinase K (its activity is 600 mAU/ml solution or 40 mAU/mg protein);
- binding of genomic DNA present in the cell lysate to the membrane of a QIAamp Mini spin column (DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane);
- washing of QIAamp membrane to remove residual contaminants DNA bound to the membrane (2 centrifugation and the use of 2 different wash buffers, Buffer AW1 and Buffer AW2, significantly improves the purity of the eluted DNA);
- elution of pure nucleic acids using Buffer AE or water.

In order to obtain genomic DNA from blood pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube, add 200 µl of whole blood and 200 µl of Buffer AL to the sample. Mix by pulse-vortexing for 15 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. All samples were incubated at 56°C for 10 min. DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA. After a briefly centrifuge (to remove drops from the inside of the lid), 200 µl ethanol (96–100%) were added to the sample, that was mixed again by pulse-vortexing for 15 s. The 1.5 ml microcentrifuge tubes were still briefly centrifuged. Carefully the mixture was applied in a 2 ml collection tube without wetting the rim, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded. Each spin column was closed to avoid aerosol formation during centrifugation. Centrifugation is performed at 6000 x g (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation,

centrifuge again at higher speed until the QIAamp Mini spin column is empty. 500 µl Buffer AW1 were added to the QIAamp Mini spin column, without wetting the rim and sample was centrifuged at 6000 x g (8000 rpm) for 1 min. One more time, the QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the collection tube containing the filtrate was discarded. Carefully, 500 µl Buffer AW2 were added the QIAamp Mini spin column, without wetting the rim, and sample was centrifuged at full speed (20,000 x g; 14,000 rpm) for 4 min. This step helps to eliminate the chance of possible Buffer AW2 carryover. For the last time, the QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube (not provided), and the collection tube containing the filtrate was discarded. 200 µl Buffer AE or distilled water were added to the QIAamp Mini spin column, that was incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min. DNA samples were stored at –20°C.

A 200 µl sample of whole human blood (approximately 5 x 106 leukocytes/ml) typically yields 6 µg of DNA in 200 µl water (30 ng/µl) with an A260/A280 ratio of 1.7–1.9.

### 2.2.2 Polymerase chain reaction (PCR) for the detection of FV Leiden mutation

DUPLICα$^{\text{Real Time}}$ FACTOR V Genotyping Kit is an in vitro nucleic acid amplification test for the detection of the nucleotide substitution G1691A of the blood coagulation Factor V gene in human genomic DNA extracted from peripheral whole blood samples collected in EDTA. Two reaction mixes are provided for the amplification:

- AMPLIFICATION MIX: with Hot Start Taq DNA polymerase, nucleotides, $MgCl_2$ and buffer;
- OLIGO MIX: with primers and fluorogenic probes.

The polymerase chain reaction (PCR)[10] is a reactions that allows to amplify a target sequence of DNA creating several copies of a template. The reaction is generally composed of three phases: DNA denaturation (separation of the two strands of template DNA), annealing (primers hybridization to their complementary sequences on the template DNA) and extension (elongation of the primers by DNA-polymerase activity). These phases, which are realized through automated heating and cooling steps (denaturation: 95 °C; annealing: 45-60 °C, according to the characteristics of the primers; extension: 70 °C) with a Thermal Cycler, represent an amplification cycle; the repetition in continuum of these cycles brings to the production of billions of fragments of the DNA in study. After the denaturation of the DNA, that occurs at high temperature, follows the hybridization of primers specific to the target

DNA: the Taq polymerase enzyme (the thermostable DNA-polymerase I of the thermophilic bacterium *Thermus aquaticus*) recognize the 3' end of those primers and using triphosphate nucleotides (dNTPs) polymerize the DNA, so creating several copies of the template.
The DUPLICα$^{\text{Real Time}}$ FACTOR V Genotyping Kit allows to detect the genomic DNA through sequence-specific fluorogenic probes, that are composed by a oligonucleotidic sequence presenting at the 5' end a fluorescent marker called "reporter", and at the 3' end a second marker called "quencher". The assay is able to detect the specific product of the amplification, monitoring the increase in fluorescent signal that is directly proportional to the quantity of amplification product; the high specificity of the system allows to the probe to discriminate between fragments that differ by only one nucleotide. In particular, the probe designed to detect the Wild Type allele carries the fluorophore FAM (6-carboxyfluorescein) at the 5' end, while the probe detecting the Mutated allele, is labelled with the fluorophore HEX (hexachloro-fluorescein). Both the probes have a non fluorescent black quencher at the 3' end. If excited, the whole probe doesn't emit fluorescence, since the proximity of the quencher to the reporter prevents the emission of the fluorescence from the reporter (quenching effect).

The total reaction volume was 25 µl. For each experiment was prepared a PCR mix for the 2 controls C1 (wild type control) and C2 (mutated control), 1 Reaction Blank (BM) and n+1 samples.
The reagents of the PCR mix have to be mixed as indicated in the table below. The PCR mix has to be freshly prepared every time. After its preparation, aliquot 20 µl of Master Mix in the tubes for PCR then add in each tube 5 µl (100-250 ng/reaction) from the extracted DNA.

| **Reagents** | **Volume (µl)** |
|---|---|
| Amplification mix | 10 µl |
| Oligo Mix | 10 µl |
| Extracted DNA | 5 µl |
| Control 1 WT | Control 1 WT 5 µl |
| Control 2 MUT | Control 2 MUT 5 µl |
| Reaction blank (B) | Reaction blank (B) 5 µl |

A negative control (*i.e.* a reaction carried out in the absence of template DNA) was always included to check for reagent contamination with template DNA. The Thermal Cycler used is Software Rotor-Gene Q version 1.7.94.

**Results interpretation**

The fluorescence signal detected by the instrumentation identifies the presence of DNA, in particular the fluorescence detected in the GREEN CHANNEL (FAM) identifies the Wild Type genotype, while the fluorescence detected in the YELLOW CHANNEL (HEX) identifies the Mutated genotype. The results are interpreted through the analysis of the Threshold Cycle (Ct) for each sample, using the "allelic discrimination" function.

The Threshold Cycle is defined as the cycle at the level of which the amplification curve intersects the threshold (threshold line). The threshold line is calculated from the mean of fluorescence of the background for three times the standard deviation; during the initial cycles of amplification prior to a significant accumulation of target DNA. During the first few cycles of PCR amplification, the fluorescence of the background is calculated subsequently, it will be used for the calculation of the fluorescence baseline.

For each sample, we will have the fluorescence data combined: the continue line represents the fluorescence signal in Green (channel 1 - wild type) and the line with the circles the fluorescence signal in Yellow (channel 2 - mutated). If a sample shows a fluorescence in FAM, the sample has Wild Type allele. If a sample shows a fluorescence in HEX, the sample has Mutated allele. Therefore, if only a FAM signal is detected the sample is Homozygous Wild Type, whereas if only a HEX signal is detected the sample is Homozygous Mutated. Finally, if both FAM and HEX are detected the sample is Heterozygous.

Control 1 WT, Control 2 MUT and reaction blank (B) are provided in order to properly set the threshold line before samples analysis. After the run, the threshold line has to be set so that: Control 1 is detected in FAM and not detected in HEX, whereas Control 2 is detected in HEX and not detected in FAM. The Reaction Blank must not be detected in any channel.

### 2.2.3 Polymerase chain reaction (PCR) for the detection of H1299R, E1608K, Y1702C, R2080C

The DNA polymerization reaction follows the same fundamentals as described in the previous paragraph.

Primers for the PCR-amplification of all fragments mutations of the FV gene (Table 2.1) were designed on the basis of the published FV gene sequence (GenBank accession number Z99572) using the computer programme OLIGO 4.1 Primer Analysis Software. Amplification reactions (25 µl total volume) were carried out using 1 unit of *Taq* polymerase (BioTherm GenCraft, Germany) in the buffer provided by the supplier (10 mmol/L K-phosphate buffer pH 7.0, 100

mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L DTT, 0.01% Tween 20, 50% glycerol v/v). Reaction conditions were as follows: 0.1 ng genomic DNA (template), 7 pmol each primer, 200 µmol/L each nucleotide precursor and 2.0 mmol/L $MgCl_2$. A negative control (*i.e.* a reaction carried out in the absence of template DNA) was always included to check for reagent contamination with template DNA. Thermal cycles comprised 5 min initial denaturation at 95 °C, 10 min hot start at 65 °C (during which the enzyme was added to the reaction mixtures), 30 cycles of denaturation, annealing, extension as described above, and 10 min final extension at 70 °C.

The qualitative and quantitative outcome of the amplification reaction was checked by running 3 µl PCR product on a 2% agarose gel in parallel to an appropriate molecular weight marker.

*Table 2.1 - Primers for PCR amplification of the FV gene mutations were designed on the basis of the FV gene sequence, GenBank accession number Z99572. For each primer pair is indicated the temperature of annealing. The restriction site is artificially created by means of a mutagenic primer (in bold)*

| *Fragment* | *Primer* | *Sequence (5'-3')* | *Amplicon lenght (bp)* | *Annealing (°C)* |
|---|---|---|---|---|
| His1299Arg | F<br>R | CAAGTCCTTCCCCACAGATATA<br>AGATCTGCAAAGAGGGGCAT | 703 | 60 |
| Glu1608Lys | F<br>R | CAAACGTGATCCTCGAG**A**G<br>TCCTGGAAAACAAACAAATC | 196 | 54 |
| Tyr1702Cys | F<br>R | CTGTCGGGCTTGGG**T**CT<br>GAAATAACCCCGACTCTTC | 184 | 60 |
| Arg2080Cys | F<br>R | CCCGTCTGAATGCCCAGG**T**A<br>GGACTCTTGGACCTAGGATTT | 120 | 54 |

### 2.2.4 Restriction analysis

Restriction enzymes are endonucleases of bacterial origin able to recognize and reproducibly cut specific sequences of DNA (recognition sites). Single-nucleotide polymorphisms can create/disrupt a restriction endonuclease recognition site, making it possible to distinguish the two alleles by restriction analysis of PCR products spanning the polymorphism (Table 2.2). When this is not the case, a mutagenic primer (*i.e.* a primer containing a mismatch with

respect to the template) can be used to artificially introduce a restriction site in one of the two alleles during the amplification reaction (cf. polymorphisms 1608, 1702 and 2080, Table 2.2). Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, MA, USA. 100 ng amplified DNA were incubated with 3-4 units restriction enzyme in the appropriate buffer for 2 hour at the recommended temperature. The products of DNA digestion were then analyzed by agarose gel electrophoresis. All the digestion product (30 µl) were loaded on the gel (Polymorphisms *E1608K, Y1702C* and *R2080C* are separated on 3% Agarose gel; polymorphism *H1299R* on 2% Agarose gel).

*Table 2.2 – Experimental protocols for the detection of FV gene polymorphism by restriction analysis*

| *Polymorphism #* | *Nucleotide change* | *Location* | *Recognition Site* | *Restriction Enzyme* | *Digestion conditions* |
|---|---|---|---|---|---|
| 4070A/G (H1299R) | 4070A > G | Exon 13 | GT↓AC | RsaI | 37°C 2 hour |
| 4996G/A (E1608K) | 4996G > A | Exon 14 | GAGGAG (10/8) | BseRI* | 37°C 2 hours |
| 5279A/G (Y1702C) | 5279A > G | Exon 15 | GT↓MKAC | AccI* | 37° C 2 hours |
| 6412C/T (R2080C) | 6412C >T | Exon 23 | GT↓AC | RsaI* | 37°C 2 hours |

*# numbering according with Jenny et al (Jenny)*

** the restriction site is artificially created by means of a mutagenic primer (in bold)*

### 2.2.5 Gel electrophoresis

Due to their numerous phosphate groups, nucleic acids are negatively charged at neutral pH and tend to migrate towards the anode if subjected to an electric field. Their migration rate is inversely proportional to the logarithm of their length in bp. These properties make it possible to separate DNA fragments according to their size. At the end of the electrophoretic run, the positions of the DNA fragments in the gel are visualized by ethidium bromide, an intercalating dye that fluoresces when bound to DNA.

Agarose gels (able to separate fragments ranging from 200 bp to 50 kb) were prepared by dissolving the desired amount of agarose in 1×TAE buffer (40 mmol/LTris-acetate, 1 mmol/L EDTA) and heating this mixture in a microwave oven till complete clarification. A 1% agarose gel contains 1 g agarose in 100 ml buffer. Ethidium bromide was added directly to the melted gel before casting, in the proportion of 5 µl of a 10 mg/ml stock to 100 ml gel. Agarose gels were run horizontally in 1× TAE buffer, by applying a voltage of 5 V/cm.

Stained gels were viewed under UV transillumination at 254 nm, the picture was imported with a Gel Doc 1000 UV-gel camera (Bio-Rad Laboratories, Hercules, CA, USA) and stored on a computer as an image file. Gel images were manipulated with the software Molecular Analyst (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.2.6 Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) and Gel Electrophoresis

PCR-restriction fragment length polymorphism (RFLP)-based analysis, also known as cleaved amplified polymorphic sequence (CAPS), is a popular technique for genetic analysis. It has been applied for the detection of intraspecies as well as interspecies variation. There exist several techniques that are related with PCR-RFLP and also involve gel electrophoresis including techniques for DNA fingerprinting and expression profiling.

There are different types of genetic variations. The so-called small-scale genetic variation includes single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs) and microindels. MNPs are multiple, consecutive nucleotide variations of a single common length such as double nucleotide polymorphisms (DNPs) and triple nucleotide polymorphisms (TNPs) with two and three variable nucleotides, respectively. Microindels are deletions, duplications and combinations thereof involving the gain or loss of 1 to 50 nucleotides[11].

The human genome contains more than 3 million SNPs located with an average distance of approximately 1000 bp[12,13]. The frequency of DNPs and TNPs, the most common forms of MNPs, amounts to ~1% of the total number of SNPs[14]. Most likely, the genome-wide occurrence of small insertions and deletions has been underestimated, probably reflecting an inaccuracy of the current sequencing techniques[15].

PCR-restriction fragment length polymorphism (RFLP)-based analysis is a popular technique for genotyping. The technique exploits that SNPs, MNPs and microindels often are associated with the creation or abolishment of a restriction enzyme recognition site[16]. The first step in a

PCR-RFLP analysis is amplification of a fragment containing the variation. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolvement of the fragments. Important advantages of the PCR-RFLP technique include inexpensiveness and lack of requirement for advanced instruments. In addition, the design of PCR-RFLP analyses generally is easy and can be accomplished using public available programs. Disadvantages include the requirement for specific endonucleases and difficulties in identifying the exact variation in the event that several SNPs affect the same restriction enzyme recognition site. Moreover, since PCR-RFLP consists of several steps including an electrophoretic separation step, it is relatively time-consuming. Finally, the technique is not suitable for the simultaneous analysis of a large number of different SNPs due to the requirement for a specific primer pair and restriction enzyme for each SNP. This limits its usability for high throughput analysis.

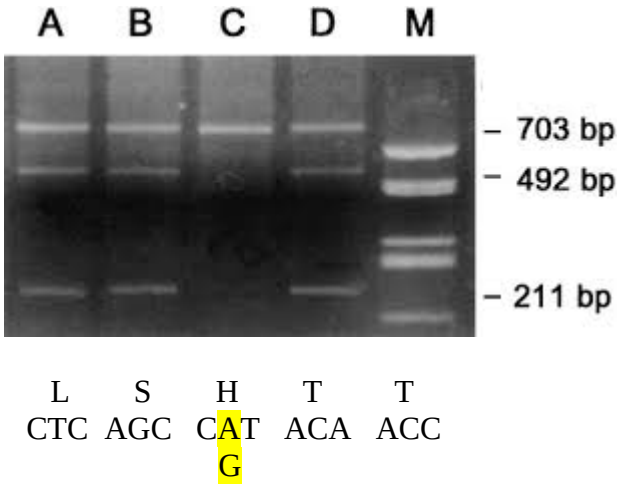
***Detection of the FVHis1299Arg mutation and DNA study***

This polymorphism in the exon 13 of FV gene (A4070G transition, referred to as R2 allele) predicting the substitution of Histidine by Arginine at aminoacid 1299 of mature FV[26]. Primers for exon 13 of FV gene have been previously described (F primer 5'-CAAGTCCTTCCCCACAGATATA-3'; R primer 5'-AGATCTGCAAAGAGGGGCAT-3')[27]. The amplification involved 30 cycles of 93°C (20s), 57°C (25s) and 70°C (1 min). Digestion of PCR products was carried out by incubating at 37°C with RsaI (Promega, Madison, USA)[26].

*Tab. 2.6 - Pattern of RsaI-restriction analysis and relative genotype*

| | ***4070 A>G (allele G is cut)*** |
|---|---|
| **Genotype** | **Restriction Fragment obtained** |
| FV Wt | R1 (A) undigested (703bp) |
| FV mutated | R2 (G) 493bp and 210bp |

*Fig. 5 – Detection of the polymorphism R2. The undigested fragment 1 identifies the R1 allele (703 bp), and fragments 2 (492 bp) and 3 (211 bp) the R2 allele.*




***Detection of the FVGlu1608Lys mutation and DNA study***

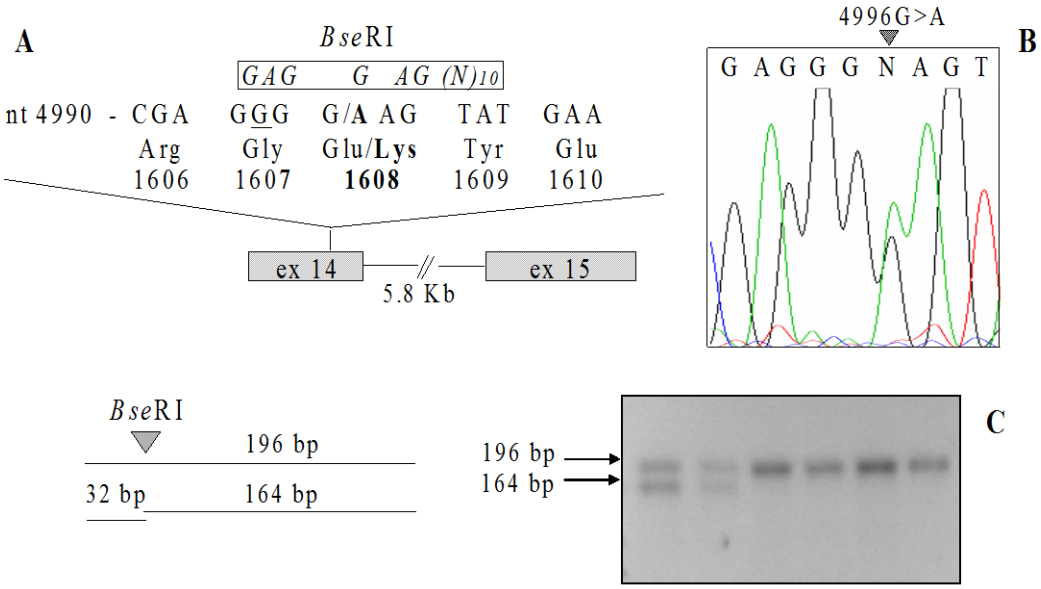
As described in previous literature, in order to identify the mutation responsible for FV deficiency, coding regions and splicing junctions of the FV gene were sequenced in the probands[17]. A heterozygous G to A transition at nucleotide 4996 in exon 14 has been detected, predicting the Glu1608Lys amino acid substitution in the A3 domain of FV[17] (Table 2.3).

Exon scanning of the FV gene was performed by automated sequencing with the ABI-Prism 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA), as previously described[18]. Nucleotide numbering was in accordance with Jenny et al. (accession number M16967)[19]. The FVGlu1608Lys (4996G>A) mutation was detected by PCR-mediated amplification of FV exon 14 followed by BseRI restriction analysis. The mutagenized primer 3 (5'-CAAACGTGATCCTCGAGAG-3', nucleotides 4977–4995) was used to introduce a BseRI restriction site in the normal allele[17;20-22] (Figure 1).

*Tab. 2.3 - Pattern of BseRI-restriction analysis and relative genotype*

| | ***4996 G>A (allele G is cut)*** |
|---|---|
| **Genotype** | **Restriction Fragment obtained** |
| Homozygous Wt - GG | 164 + 32 bp |
| Homozygous mutated - AA | 196 bp |
| Heterozygous mutated - GA | 196 + 164 + 32 bp |

*Fig.1 - (A) Nucleotide and amino acid sequence in exon 14 and schematic diagram of the genomic region. The mutation and the BseRI restriction site obtained by mutagenesis (G to A) are indicated. (B) Nucleotide sequencing of exon 14 in a patient. The heterozygous pattern is indicated by N. (C) Schematic diagram of the amplified DNA and electrophoretic pattern of BseRI-restriction*

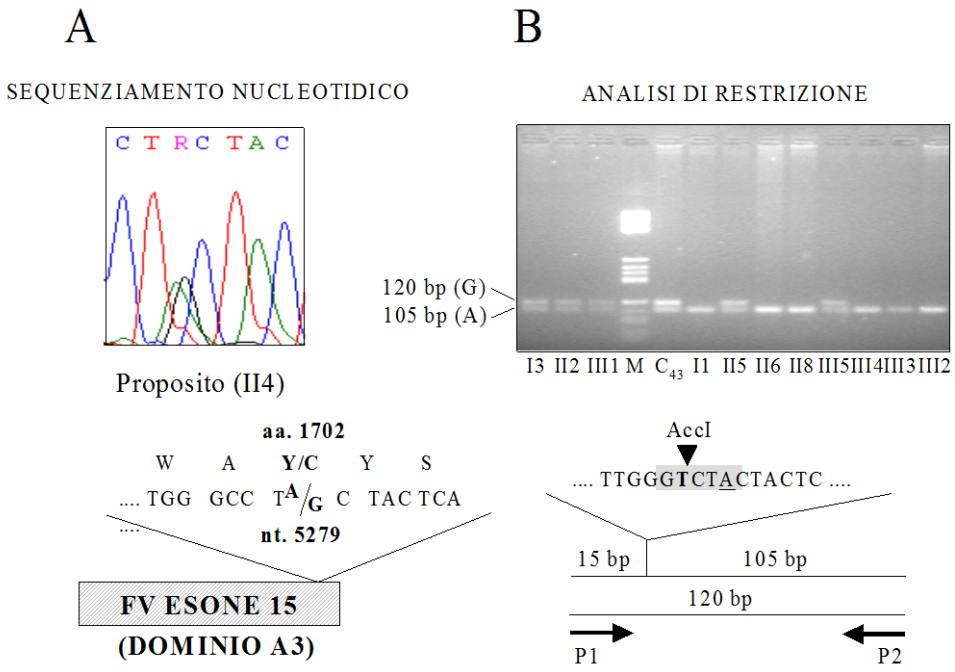



***Detection of the FVTyr1702Cys mutation and DNA study***

Exon scanning of the FV gene was performed by direct sequencing as described[23].

Primers located in introns 14 (5'-AACCAGCCATTTTGACTTA-3') and 15 (5'-GAAATAACCCCGACTCTTC-3'), respectively, were used to amplify and sequence (Figure 2A) a 410–base pair (bp) DNA fragment spanning the whole exon 15. A restriction protocol for the detection of the 5279A/G mutation (Figure 2B) was obtained by means of a mutagenized primer (5'-CTGTCGGGCTTGGGTCT-3', P1, nucleotides [nt.] 5262-5278) introducing an *Acc*I restriction site in the normal allele[24].

*Fig. 2 - Detection of the 5279A/G mutation (FV Y1702C). (A) Detection by direct sequencing: The heterozygous sequencing pattern of the propositus is shown. The "R" in the nucleotide sequence indicates that either an A or a G may be present at this position. (B) Detection by restriction analysis: Mutagenized (bold) primer P1, which creates an AccI restriction site (highlighted in gray) in the normal (A, underlined) allele, is used in combination with reverse primer P2 to amplify a 120-bp DNA fragment. The fragments obtained by AccI digestion of the polymerase chain reaction products are visualized by agarose gel electrophoresis. C43, individual from the general population; M, molecular weight marker.*




Forward primer P1 was used in combination with reverse primer P2 (5'-GAAATAACCCCGACTCTTC-3', intron 15) to amplify a 120-bp DNA fragment, which was subsequently digested with *Acc*I (New England Biolabs, Beverly, MA) under the conditions recommended by the manufacturer[24].

The mutated allele gives rise to a 120-bp undigested band, whereas the wild-type allele gives rise to a 105-bp band[25] (Table 2.4).

*Tab. 2.4 - Pattern of AccI-restriction analysis and relative genotype*

| | ***5279 A>G (allele A is cut)*** |
|---|---|
| **Genotype** | **Restriction Fragment obtained** |
| Homozygous mutated - GG | 120 bp |
| Homozygous Wt - AA | 105 + 15 bp |
| Heterozygous mutated - AG | 120 + 105 + 15 bp |

***Detection of the FVArg2080Cys mutation and DNA study***

As described in previous literature[28], FV sequencing of the coding region and the exon-intron boundaries of FV gene (primers listed in Table 2.1) detected the novel 6412C>T transition in exon 23, resulting in the Arg2080Cys substitution in the C2 domain (Figure 3).

*Fig. 3 - (A) Nucleotide sequencing of exon 23 in patient A. (B) Nucleotide and amino acid sequence. The mutation is located in exon 23 and causes the substitution of the Arg2080 with a Cys in the C2 domain.*

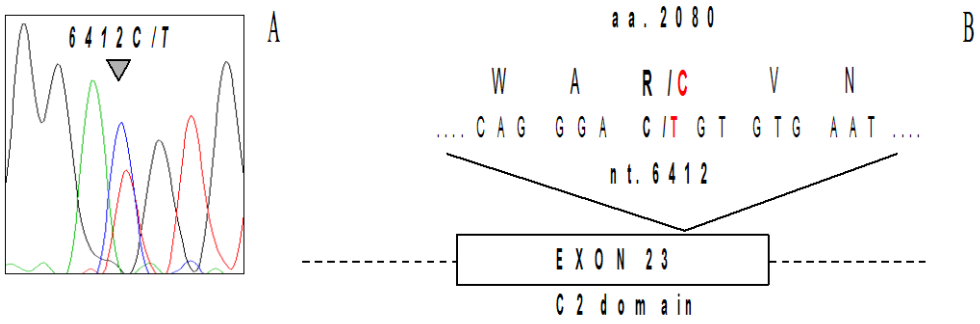
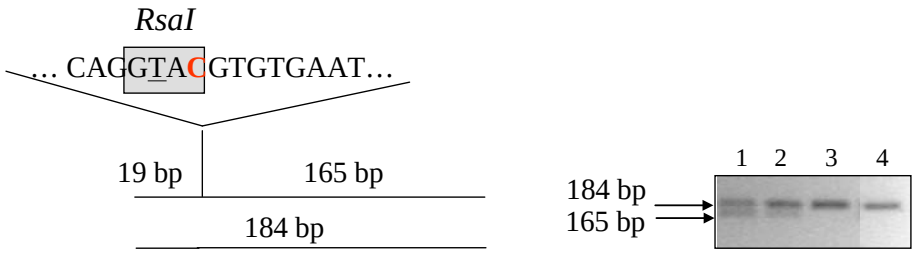



*Fig. 4 - DNA study of the Arg2080Cys mutation. Detection of the Arg2080Cys mutation by RsaI-restriction analysis. The FIVS2A primer is used in combination with the mutagenic R3MboI reverse primer, which creates a RsaI-restriction site in the normal (C, in red) allele, to amplify a 184 bp DNA fragment. The fragments obtained by RsaI-digestion of the PCR products are visualized by 3% agarose gel electrophoresis. Lane 1, patient A; lane 2, patient B; lanes 3-4, normal controls.*




*Tab. 2.5 - Pattern of RsaI-restriction analysis and relative genotype*

| | *6412 C>T (allele C is cut)* |
|---|---|
| **Genotype** | **Restriction Fragment obtained** |
| Homozygous mutated - TT | 184 bp |
| Homozygous Wt - CC | 165 + 19 bp |
| Heterozygous mutated - CT | 184 + 165 + 19 bp |

## 2.3 STATISTICAL ANALYSIS

Statistical analysis were conducted by the Unit of Clinical Trials and Statistics Department "Infrastructure Research and Statistics" IRCCS "Arcispedale Santa Maria Nuova" in Reggio Emilia, ensuring all privacy criteria, in accordance with the Italian laws (Guarantor authorization, n.72 of March 26, 2012).

Statistical analyses were performed by the IBM software SPSS 20.0. Qualitative variables (i.e. sex, blood type, thromboembolism, FVL, HR2 haplotype, polymorphisms E1608K, Y1702C and R2080C), and quantitative variables (i.e. age, APCRnr and FVc) were collected to demonstrate the objectives of the study.

Due the exploratory nature of the study, statistical analysis will be limited to a description of the variables collected through the usual descriptive statistics tools in accordance with the variables collected. The quantitative variables were represented by mean, median, standard deviation, minimum, maximum; while qualitative variables were described using absolute and relative frequencies and supported by graphical representations.

Differences between FV levels were assessed by the Student's t-test.

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# - 3 – RESULTS AND DISCUSSION

Since FV plays a pivotal role at the crossroads of procoagulant and anticoagulant pathways[1], FV levels are thought to be crucial for the maintenance of the haemostatic balance.

Thrombin-activated FV is a procoagulant molecule that greatly enhances thrombin formation by the prothrombinase complex and it is subsequently inactivated by activated protein C (APC). Differently, APC-cleaved FV is an anticoagulant molecule that stimulates the APC-mediated inactivation of coagulation FVIIIa.

Frequent mutations in the FV gene have been found to play an important role as genetic risk factors[2] for venous thromboembolism. The FVArg506Gln (FV Leiden) mutation has been found to cause activated protein C resistance (APCR), a recognized risk factor for venous thrombosis[3].

Although it is well known that intragenic and extragenic mutations, and functional polymorphisms, influence plasmatic FV levels. When these studies were initiated, the FVHR2 haplotype[4] was the only gene variation associated with modulation of FV activity[4] and antigen[5] levels. The molecular bases of FV deficiency are still largely unexplored. To date, 153 identified cases of mutations in the FV gene were described in FV-deficient patients in the homozygous or compound heterozygous state[6]. In addition, two common FV gene polymorphisms predict a slight reduction (25%) in FV levels: FVAsp2194Gly, which is part of the FVHR2 haplotype[7], and FVMet2120Thr[8]. Co-inheritance of heterozygous FV deficiency with FV Leiden enhances the APCR associated with this mutation, resulting in pseudo-homozygous APCR[9]. The role of FV deficiency in modulating thrombotic risk in this rare condition is poorly understood. Whether co-inheritance of FV Leiden mutation, or of FVHis1299Arg, with FV deficiency also increases the thrombotic risk is a matter of debate.

The aim of our study was to observe the behavior of the plasma FV levels and the APC resistance in a small cohort of subjects, most of them suffering from venous thromboembolism (VTE), and to analyze the possible role of FV mutations co-inheritance as thrombotic risk factors.

We investigate the role of FV deficiency in venous thrombosis by screening Italian patients from Reggio Emilia area (in northern Italy), carrying frequent thrombophilic mutations.

Preliminary data show that in Reggio Emilia heterozygosis for FV R506Q is most likely associated with venous thrombotic events with a particular tendency to recurrence in case of discontinuation of anticoagulant therapy. These preliminary observations may be suggestive of co-inheritance of FV Leiden, or FVHis1299Arg, or other mutations (i.e. Glu1608Lys, Arg2080Cys, Tyr1702Cys).

## 3.1. STUDY POPULATION

103 subjects (55 males and 48 females, mean age 60.3 ± 15.8 years - Figure 3.1) living in Northern Italy (Reggio Emilia and neighboring districts) were enrolled by the Department of Medicina II – Angiologia of the Arcispedale Santa Maria Nuova – IRCCS of Reggio Emilia in the frame of a regional thromboembolic disease survey.

We collected, for each patient, personal and family medical history, including thrombophilic test results (anamnestic data). All subjects were characterized for FV activity (FV:C) and APCR ratio (nAPC-sr). Ranges from 70% to 130% for FV:C were considered as normal. Range from 0,8 to 1,2 for nAPCR-sr are usually considered as normal. However, for the purpose of the present thesis, the ratio range from 0,8 to 0,85 was also investigated. FV coagulant activity was measured by a one-stage clotting method, based on the ability of the test plasma to normalize the prothrombin time (PT) of a FV-deficient plasma sample. The APC-resistance test requires the measurement of the activated partial thromboplastin time (APTT) in the presence and in the absence of APC. It is based on the principle that the presence of APC will considerably prolong the clotting time in normal plasma, whereas it will only mildly affect the APTT in APC-resistant plasma (refer to section number 2 -"*Materials and Methods"*).

Finally we performed genomic DNA extraction from whole blood samples - using a kit QIAamp DNA blood mini kit ® - QIAGEN - , and factor V mutations analysis, by PCR-amplification followed by restriction analysis on gel electrophoresis, to identify these polymorphisms: H1299R, E1608K, Y1702C, R2080C.

We have selected 3 groups of subjects with the following characteristics:

i) patients heterozygotes for FV Leiden mutation suffered from VTE (FV R506Q + VTE),

ii) subjects carriers of FV R506Q but asymptomatic, otherwise who have never had VTE events (FV R506Q – VTE),

iii) patients FV wild-type (FVWt, absence of the FV Leiden mutation), otherwise without any inherited hypercoagulability state, but suffered from VTE (FV R506R + VTE).

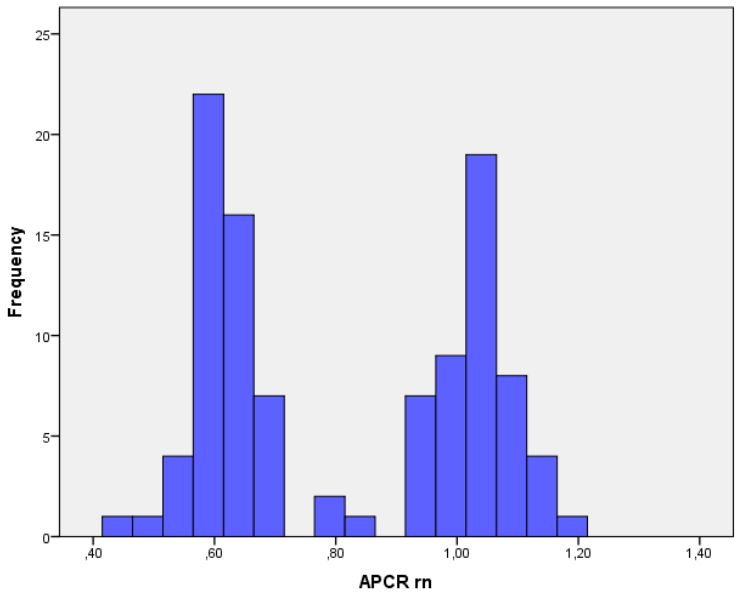
From anamnestic data we knew that none of patients enrolled had other known thrombophilic conditions (such as antithrombin, LAC positivity, anti β-2 glycoprotein 1 antibodies, anti-cardiolipin antibodies (ACA), protein C and protein S deficiency, or G20210A mutation of the prothrombin gene).

Within the population study we enrolled 43 patients (41,7%) in the group FV R506Q + VTE, 10 patients in the group FV R506Q – VTE (9,7%) and 50 patients (48%) in the group FV R506R + VTE. Unfortunately the groups were not homogeneous, especially with regard to the second group (FV R506Q – VTE): in this case, being patients who don't attended our department, the privacy issues have made the enrollment very difficult.

## 3.2. APC RESISTANCE AND PLASMA FV LEVELS IN THE ENROLLED CASES

In the total population we found average values of APCR ratio and FV plasma levels amounting to 0,82 *ratio* ± 0,22 (Figure 3.1) and 99,28% ± 15,87, respectively (Figure 3.2).

*Fig. 3.1 – nAPCR-sr distribution in the enrolled cases*




Referring to each group, we found average values of APCR ratio amounting to 0,62 ± 0,07, 0,62 ± 0,03 and 1,03 ± 0,08, respectively (Table 3.1), in agreement with the presence or the absence of FV R506Q mutation.

*Fig. 3.2 – FV:C distribution in the enrolled cases*

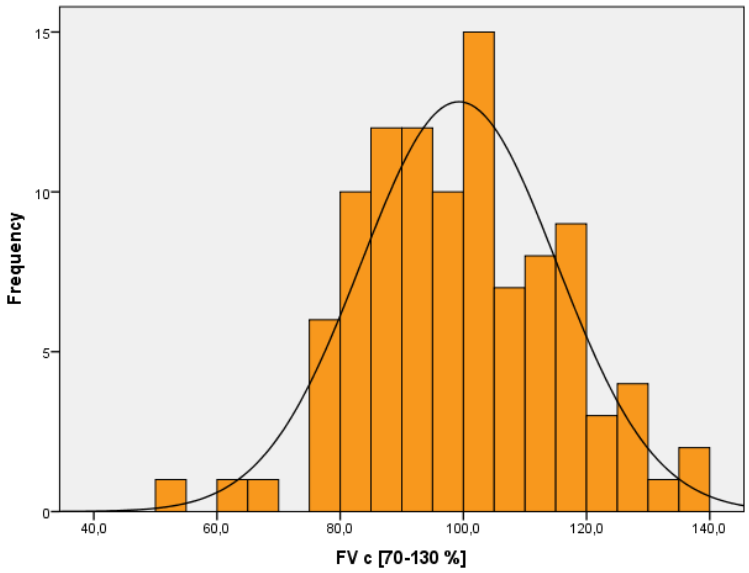



*Table 3.1 – nAPCR-sr and FV:C average distribution in the three groups*

| GROUP | | | nAPCR-sr | FV:C |
|---|---|---|---|---|
| **FV R506Q + VTE** | N | Valid | 43 | 43 |
| | | Missing | 0 | 0 |
| | Average | | ,6195 | 101,040 |
| | Median | | ,6100 | 98,400 |
| | Standard Deviation | | ,06969 | 16,7795 |
| | Minimum | | ,44 | 50,3 |
| | Max | | ,83 | 136,2 |
| **FV R506Q – VTE** | N | Valid | 10 | 10 |
| | | Missing | 0 | 0 |
| | Average | | ,6240 | 97,350 |
| | Median | | ,6150 | 98,900 |
| | Standard Deviation | | ,03273 | 6,9822 |
| | Minimum | | ,58 | 84,2 |
| | Max | | ,67 | 109,0 |
| **FV R506R + VTE** | N | Valid | 49 | 49 |
| | | Missing | 1 | 1 |
| | Average | | 1,0308 | 98,120 |
| | Median | | 1,0500 | 95,700 |
| | Standard Deviation | | ,07874 | 16,4168 |
| | Minimum | | ,67 | 62,5 |
| | Max | | 1,19 | 136,4 |

*FV activity was measured as % of PNP (= pooled normal plasma).*

Also, the average values of FV:C were 101,04% ± 16,78, 97,35% ± 6,98 and 98,12% ± 16,42 respectively (Table 3.1).

Observing the individual frequencies of each patient within each group, interesting aspects arise (Table 3.2 and 3.3). In the first group (FV R506Q + VTE), patient n°61, carrier of FV Leiden mutation and suffering from idiopathic pulmonary embolism (PE) combined with deep vein thrombosis (DVT), has a normal/uncertain APCR ratio (nAPCR-sr = 0,83) and normal FV plasma levels (FV:C=92,8%).

Patients n°93 and 106, both carries of FV Leiden affected by secondary PE and idiopathic DVT respectively, have high FV level, that exceed the upper limit normal (FV:C=136,2% and 133,5%, ULN values) .

Finally, always in the same group, patient n°33 affected by recurrent superficial vein thrombosis, shows very low FV activity (FV:C=50,3%; LLN value) and an APCR ratio in accordance with FV Leiden heterozygosis (nAPCR-sr = 0,70). This may suggest the co-inheritance with a FV polymorphism which might be responsible for FV reduced coagulant activity, as for example one of the 4 FV mutations that we investigated in our population (i.e. His1299Arg, or Glu1608Lys, or Tyr1702Cys, or Arg2080Cys, found to be associated with reduced FV activity).

In the third group (FV R506R + VTE), patient n°10 developed idiopathic DVT at the age of 20. She has a lower APCR ratio, under the normal limit (nAPCR-sr = 0,67) compatible with a FV R506Q heterozygosis but which is not carrier. We known that co-inheritance of heterozygous FV deficiency with FV R506Q enhances the APCR associated with this mutation, resulting in pseudo-homozygous APCR, nevertheless this is not the case. This may suggest that maybe there is another FV mutation causing APC resistance, or a FV deficiency which is responsible alone to enhance the APCR. The patient indeed also shows FV plasma levels under the normal limit (FV:C=62,5%).

Two patients (n°20 and 25), both FV506R suffering from idiopathic DVT and recurrent superficial vein thrombosis respectively, show low FV activity (FV:C=66% and 62,5%, LLN values) and normal APCR ratio. In these cases it is reasonable to assume the inheritance of a FV polymorphism, which might be responsible for FV reduced coagulant activity.

*Table 3.2 –FV R506Q + VTE Group: one patient carrier of FV R506Q mutation and affected by VTE with an high APCR ratio (normal/uncertain value). FV R506R + VTE Group: one patient FVR506 with very low APCR ratio, and affected by VTE.*

| GROUP | Patient n° | nAPCR-sr | Frequency | % | Valid % |
|---|---|---|---|---|---|
| **FV R506Q + VTE** | 61 | 0,83 | 1 | 2,3 | 2,3 |
| | 33 | 0,70 | 1 | 2,3 | 2,3 |
| | 106 | 0,64 | 6 | 14,0 | 14,0 |
| | 93 | 0,60 | 3 | 7,0 | 7,0 |
| **FV R506R + VTE** | 10 | 0,67 | 1 | 2,0 | 2,0 |
| | 20 | 1,03 | 3 | 6,0 | 6,1 |
| | 25 | 1,05 | 5 | 10,0 | 10,2 |

*Table 3.3 – FV R506Q + VTE Group: two patients carriers of FV R506Q mutation and affected by VTE with a FV:C ULN value; one patient with FV:C LLN value. FV R506R + VTE Group: two patients FVWt suffering from VTE with FV:C LLN values.*

| GROUP | Patient n° | FV:C | Frequency | % | Valid % |
|---|---|---|---|---|---|
| **FV R506Q + VTE** | 93 | 136,2 | 1 | 2,3 | 2,3 |
| | 106 | 133,5 | 1 | 2,3 | 2,3 |
| | 61 | 92,6 | 1 | 2,3 | 2,3 |
| | 33 | 50,3 | 1 | 2,3 | 2,3 |
| **FV R506R + VTE** | 25 | 62,5 | 1 | 2,0 | 2,0 |
| | 20 | 66,0 | 1 | 2,0 | 2,0 |
| | 10 | 107,3 | 2 | 4,0 | 4,1 |

*ULN = upper limit normal; LLN = lower limit normal*

## 3.3. DISTRIBUTION OF FVHR2 IN THE ENROLLED CASES

It was identified a high percentage (86.4%, 89/103 of enrolled cases) of subjects FVHR2 wild-type (R1R1) homozygotes, .

13 patients, which represent the 12,6% of cases, were also found to be carriers of FVHR2 (R1R2). Finally, only one patient is homozygous for the R2-haplotype (R2R2) (Table 3.4).

*Table 3.4 – FVHR2 genotype distribution in the population enrolled*

| | FVHR2 genotype | Frequency | Percent (%) |
|---|---|---|---|
| Valid | **R1/R1** | 89 | 86,4 |
| | **R1/R2** | 13 | 12,6 |
| | **R2/R2** | 1 | 1,0 |
| | Total | 103 | 100,0 |

Differently from the FV 506Q gene, which is confined to Caucasoid populations[10], the invariant HR2 haplotype was found with similar frequencies in the Italian population and in subjects of Somali and Indian origin, suggesting a worldwide distribution. Because modern Somali are representative of the original population that spawned the migration from Africa more than 100,000 years ago[11], our results indicate ancestral mutational events as the origin of the HR2 haplotype, dating it further back than the R506Q mutation.

The HR2 haplotype is very ancient and rather frequent, its prevalence throughout Asia, Europe and in native African populations ranging from 8 to 12%[5,12-16]. This is also reflected in our data. The very high prevalence (up to 50%) in Indian tribes of Costa Rica is notable[17].

The homozygous condition for the R2 allele is rare (2 to 5/1000)[16]; however, in our population we found one patient carrier of R2R2 genotype.

The 93% (n=40) of patients carrier of FV Leiden mutation (FV Leiden heterozygotes) and affected by at least one venous thromboembolic event (VTE), are FVHR2 wild-type (R1R1), while 7% (n=3) are doubly heterozygous for FV Leiden and FVHR2 (Table 3.5).

Because HR2 resides on the allele that does not carry the R506Q mutation, patients inherit each factor V gene defect independently[16]. The 100% (n=10) of subjects belonging to the second group (FV R506Q – VTE) are carrier of only the Leiden mutation; however it is not a very reliable result, due the small sample size. In contrast, the 78% (n=39) of patients affected by VTE with negative thrombophilia examination, are FVHR2 wild-type, but the 20% (n=10) are carrier of the heterozygous genotype (R1R2) and 1% is homozygous (R2R2) for the R2-haplotype.

*Tab. 3.5 - FVHR2 genotype in the three groups (FV R506Q + VTE, FV R506Q – VTE and FV R506R + VTE)*

| GROUP | | FVHR2 genotype | | | |
|---|---|---|---|---|---|
| | | R1/R1 | R1/R2 | R2/R2 | Total |
| FV R506Q + VTE* | Counting | 40 | 3 | 0 | 43 |
| | % in GROUP | 93,0% | 7,0% | 0,0% | 100,0% |
| | % in HR2 | 44,9% | 23,1% | 0,0% | 41,7% |
| FV R506Q – VTE | Counting | 10 | 0 | 0 | 10 |
| | % in GROUP | 100,0% | 0,0% | 0,0% | 100,0% |
| | % in HR2 | 11,2% | 0,0% | 0,0% | 9,7% |
| FV R506R + VTE | Counting | 39 | 10 | 1 | 50 |
| | % in GROUP | 78,0% | 20,0% | 2,0% | 100,0% |
| | % in HR2 | 43,8% | 76,9% | 100,0% | 48,5% |
| Total | Counting | 89 | 13 | 1 | 103 |
| | % in GROUP | 86,4% | 12,6% | 1,0% | 100,0% |
| | % in HR2 | 100,0% | 100,0% | 100,0% | 100,0% |

**The apparently reduced percentage of HR2 is explained by the 100% FV Leiden carriership*

Has previously been reported that the HR2 haplotype in the FV gene, which includes 13 different polymorphisms throughout the gene (seven of these base changes predict an amino acid change in FV and lead to functional modifications of the protein), is associated with increased APC resistance both in normal subjects and in thrombophilic patients, independently of carriership of FV R506Q[16]. FVHR2 has also increased ratio of factor V1 to factor V2, the former being the more procoagulant isoform[18]. Based on these results, it is reasonable to expect that carriership of FVHR2 would be associated with an increased risk of VTE. The R1R2 and R2R2 genotypes are a rather high percentage within their group, but they can't be considered a representative sample, due to the low number of patients and particularly of controls enrolled.

Till now, the published data say that the association of FV Leiden and HR2 is however rare, being expected in approximately 3 in 1000 individuals in the general population and 3 in 100 unselected patients with thrombosis[16]. Moreover, HR2 by itself is not associated with VT, because a similar prevalence was found in normal individuals and in consecutive patients referred for a venous thromboembolic event[16]. The conclusions relating to the effect of the coinheritance of HR2 on the relative risk of venous thromboembolism are uncertain. However, it is of interest that one patient out of 50 is homozygous for the HR2 haplotype, a rare condition.

## 3.4. DESCRIPTIVE STATISTICAL ANALYSIS OF nAPCR-sr AND FV:C IN THE FVHR2 GENOTYPE

APC resistance is known as the most common inherited risk factor for venous thrombosis. Frequent mutations in the FV gene have been found to play an important role as genetic risk factors[2;15;19-20] for venous thromboembolism.

FV Leiden mutation is the most common cause of APC resistance in the Caucasian population, accounting for up to 95% of cases[21]. The high frequency of the FV Leiden mutation in the general population makes it possible to observe the combination with other genetic defects affecting coagulation system. The doubly heterozygous condition for FV Leiden and quantitative FV deficiency, known as "pseudo-homozygous condition"[22], enhances APC resistance and thus the thrombotic risk[23]. In the doubly heterozygous condition with FV Leiden, the FVHR2 could produce, through a partial pseudo-homozygous mechanism, a relative increase in plasma levels of APC resistant molecules.

*Tab. 3.6 – FV:C in the FVHR2 genotype*

| FVHR2 genotype | | | FV:C |
|---|---|---|---|
| **R1R1** | N | Valid | 88 |
| | | Missing | 1 |
| | Average | | 101,263 |
| | Median | | 101,300 |
| | Standard Deviation | | 15,6641 |
| | Minimum | | 50,3 |
| | Max | | 136,4 |
| **R1R2** | N | Valid | 13 |
| | | Missing | 0 |
| | Average | | *88,654 |
| | Median | | 88,800 |
| | Standard Deviation | | 8,7726 |
| | Minimum | | 76,5 |
| | Max | | 102,4 |
| **R2R2** | N | Valid | 1 |
| | | Missing | 0 |
| | Average | | *62,500 |
| | Median | | 62,500 |
| | Standard Deviation | | 62,5 |
| | Minimum | | 62,5 |

**significantly different from the "Wt" group R1R1 (Student's t-test, P<0.05).*

*FV activity was measured as % of PNP (= pooled normal plasma).*

The HR2 variant genotypes offered the opportunity to test the contribution of the His1299Arg change to the plasma FV phenotype. Differences between FV levels were assessed by the Student's t-test. FV activity was significantly (t-test, $P<0.05$) lower in subjects heterozygous for the HR2 allele (R1R2) than in homozygous for the wild-type R1R1 allele. These results confirm what has previously been reported in literature by Lunghi and co-workers: Hisl299Arg variant, linked with a number of potentially functional polymorphisms, is responsible for the reduction in FV levels[4-5]. Otherwise the FVHR2 has been associated with slightly reduced FV levels in plasma, both at the coagulant activity[4] and antigen levels[4-5].

*Tab. 3.7 – nAPCR-sr and FV:C in the FV genotype: co-inheritance FVR506Q + R2 allele*

| FV genotype | | nAPCR-sr | FV:C |
|---|---|---|---|
| **FV R506Q + R1R1** | N | 50 | 50 |
| | Average | *0,6224 | *100,878 |
| | Median | 0,615 | 100,8 |
| | Standard Deviation | 0,0654205 | 15,5858 |
| | Minimum | 0,44 | 50,3 |
| | Max | 0,83 | 136,2 |
| **FV R506R + R1R1** | N | 38 | 38 |
| | Average | *1,031842 | *101,7684 |
| | Median | 1,05 | 102 |
| | Standard Deviation | 0,084848 | 15,96166 |
| | Minimum | 0,67 | 66 |
| | Max | 1,19 | 136,4 |
| **FV R506Q + R1R2** | N | 3 | 3 |
| | Average | *0,586667 | *91,43333 |
| | Median | 0,59 | 96,5 |
| | Standard Deviation | 0,015275 | 10,46438 |
| | Minimum | 0,57 | 79,4 |
| | Max | 0,6 | 98,4 |
| **FV R506R + R1R2** | N | 10 | 10 |
| | Average | *1,025 | *87,82 |
| | Median | 1,02 | 86,65 |
| | Standard Deviation | 0,058166 | 8,656122 |
| | Minimum | 0,94 | 76,5 |
| | Max | 1,12 | 102,4 |

*FV activity was measured as % of PNP (= pooled normal plasma).*

** Student's t-test applied, but the number of patients is not sufficient to reach a significant difference.*

### 3.4.1. nAPCR-sr and FV:C distribution in the FVHR2 genotype – First group (FV R506Q + VTE)

Patients (n=40) heterozygous for the FV Leiden show average values of nAPCR-sr and FV:C in the amount of 0,62 ± 0,72 and 101,76% ± 17,03; nevertheless, patients (n=3) found to be doubly heterozygous for the FV Leiden and the His1299Arg polymorphism show lower average values of both nAPCR-sr and FV:C: 0,59 ± 0,02 and 91,43% ± 10,46 respectively (Table 3.8).

Carriership of the HR2 haplotype has been reported to enhance APC resistance *per se*[16,18,21,24] and in association with FV Leiden mutation[25,26]. However, probably due to the reduced number of subjects in our population (R1R1=40 pt; R1R2=3 pt), the APC ratios in the 3 doubly heterozygous for FVL and R1R2 genotype did not significantly differ from that of the simple FVHR2 wild-type, in spite of a slightly decreased nAPC-sr. Moreover, what we know from the literature is that the product of the FVHR2 gene has been characterized extensively: it possess decreased co-factor activity for APC in the degradation of FVIIIa, and increased ratio of FV1 to FV2, the former being the more procoagulant isoform[18,27-28].

The three patients with compound heterozygosity for FVL and for R2 allele, also show reduced coagulant activity, as proven by lower average values of FV:C 91,43% ± 10,46.

Furthermore, has previously been reported in literature that Hisl299Arg variant linked with a number of potentially functional polymorphisms, is responsible for the reduction in FV levels[4-5]. Otherwise the FVHR2 has been associated with slightly reduced FV levels in plasma, both at the coagulant activity[4] and antigen levels[4-5].

*Tab. 3.8 – nAPCR-sr and FV:C distribution in FVHR2 genotype. First group: FVR506Q + VTE ((heterozygotes for FV Leiden mutation affected by VTE)*

| FVHR2 genotype | | | nAPCR-sr | FV:C |
|---|---|---|---|---|
| **R1/R1** | N | Valid | 40 | 40 |
| | | Missing | 0 | 0 |
| | Average | | ,6220 | 101,760 |
| | Median | | ,6150 | 102,400 |
| | Standard Deviation | | ,07162 | 17,0283 |
| | Minimum | | ,44 | 50,3 |
| | Max | | ,83 | 136,2 |
| **R1/R2** | N | Valid | 3 | 3 |
| | | Missing | 0 | 0 |
| | Average | | ,5867 | 91,433 |
| | Median | | ,5900 | 96,500 |
| | Standard Deviation | | ,01528 | 10,4644 |
| | Minimum | | ,57 | 79,4 |
| | Max | | ,60 | 98,4 |

*FV activity was measured as % of PNP (= pooled normal plasma).*

### 3.4.2. nAPCR-sr and FV:C distribution in the FVHR2 genotype – Second group (FV R506Q – VTE)

The enrollment of patients in this group has been very difficult. Unfortunately, this happened because these subjects, being asymptomatic for venous thromboembolic events, didn't approach into our department, and due to issues concerning the Privacy laws, it wasn't possible to recruit patients from other departments. The few patients enrolled (n = 10) are actually first degree relatives of subjects belonging to group FV R506Q + VTE or to group FV R506R + VTE.

On these premises, we found that all patients (n=10) asymptomatic for VTE and carrier of FV Leiden mutation are HR2 wild-type and show average values of nAPCR-sr and FV:C in the amount of 0,62 ± 0,03 and 97,35% ± 6,98 respectively (Table 3.9).

*Tab. 3.9 – nAPCR-sr and FV:C distribution in FVHR2 genotype. Second group: FVR506Q – VTE (heterozygotes for FV Leiden mutation, asymptomatic)*

| FVHR2 genotype | | | nAPCR-sr | FV:C |
|---|---|---|---|---|
| **R1/R1** | N | Valid | 10 | 10 |
| | | Missing | 0 | 0 |
| | Average | | ,6240 | 97,350 |
| | Median | | ,6150 | 98,900 |
| | Standard Deviation | | ,03273 | 6,9822 |
| | Minimum | | ,58 | 84,2 |
| | Max | | ,67 | 109,0 |

*FV activity was measured as % of PNP (= pooled normal plasma).*

### 3.4.3. APCR-sr and FV:C distribution in the FVHR2 genotype – Third group (FV R506R + VTE)

Patients wild type for FV gene (FVWt) and affected by almost one venous thromboembolic event have been enrolled in this group.

Most of them (n=38) have a wt genotype for the R2-haplotype (R1R1) and show average values of nAPCR-sr and FV:C of 1,03 ± 0,08 and 101,77% ± 15,96 respectively. However, a good 20% (n=10) found to be doubly heterozygous for the FV Leiden and the FVHR2 show average values of 1,03 *ratio* ± 0,06 and 87,82% ± 8,66, in relation to APCR and FV:C respectively. The only one patient carrier of homozygous genotype (R2R2) for the R2-haplotype has 1,05 nAPCR-sr and 62,5% FV:C (Table 3.10).

In this groupthe His1299Arg polymorphism shows to significantly predict the plasma FV phenotype. Differences between FV levels were assessed by the Student's t-test. FV activity was significantly (t-test, $P<0.05$) lower in the group of subjects heterozygous for the HR2 allele (R1R2) and homozygous for the same mutation, than in homozygous for the wild-type R1R1 allele. It is to remember that these patients aren't FV Leiden carriers (we can see normal values for nAPCR-sr as FV Wt) but they are affected by TEV.

*Tab. 3.10 – nAPCR-sr and FV:C distribution in FVHR2 genotype. First group: FVR506R + VTE (FVWt affected by VTE)*

| FVHR2 genotype | | | nAPCR-sr | FV:C |
|---|---|---|---|---|
| **R1/R1** | N | Valid | 38 | 38 |
| | | Missing | 1 | 1 |
| | Average | | 1,0318 | 101,768 |
| | Median | | 1,0500 | 102,000 |
| | Standard Deviation | | ,08485 | 15,9617 |
| | Minimum | | ,67 | 66,0 |
| | Max | | 1,19 | 136,4 |
| **R1/R2** | N | Valid | 10 | 10 |
| | | Missing | 0 | 0 |
| | Average | | 1,0250 | *87,820 |
| | Median | | 1,0200 | 86,650 |
| | Standard Deviation | | ,05817 | 8,6561 |
| | Minimum | | ,94 | 76,5 |
| | Max | | 1,12 | 102,4 |
| **R2/R2** | N | Valid | 1 | 1 |
| | | Missing | 0 | 0 |
| | Average | | 1,0500 | *62,500 |
| | Median | | 1,0500 | 62,500 |
| | Standard Deviation | | 1,05 | 62,5 |
| | Minimum | | 1,05 | 62,5 |

**significantly different from the "Wt" group R1R1 (Student's t-test, P<0.05)*

*FV activity was measured as % of PNP (= pooled normal plasma).*

## 3.5. DETECTION OF Glu1608Lys, Tyr1702Cys AND Arg2080Cys MUTATIONS

Using PCR-restriction fragment length polymorphism (RFLP)-based analysis, also known as cleaved amplified polymorphic sequence (CAPS), a popular technique for genetic analysis, wegenotyped for candidate mutations predicting alteration of the FV gene expression., Specifically, we were interested in detecting Glu1608Lys, Tyr1702Cys and Arg2080Cys mutations. The Glu1608Lys mutation was found for the first time in 2004 and was detected in 5 unrelated patients (two carriers of the FV Leiden mutation, one carrier of the FVHR2 haplotype, two with partial FV deficiency - one of whom was also a carrier of FVHR2), all

coming from the Po Valley (northern Italy). The FV1608Lys carriers showed reduced mean FV activity (42% ± 12%) and antigen (53% ± 18%) levels and, in Western blot analysis, reduced amounts of intact platelet FV[9].

The Tyr1702Cys mutation was found on 2000, while studying the molecular bases of FV deficiency in a thrombophilic family. In these years it was established that this missense mutation is a common cause of FV deficiency in the Italian population[29].

Finally, the Arg2080Cys mutation was found in combination with the FV Glu1608Lys mutation, which it has to be responsible for quantitative FV deficiency. The FVArg2080Cys mutation, associated with FV deficiency and thrombophilia, in the absence of known thrombophilic mutations, represents the first FV natural variation affecting phospholipid binding.

On the basis of this background, and particularly on geographic distribution of these mutations, we were interested in understanding how theywere distributed in our population, in order to analyze the possible role of FV mutations co-inheritance as thrombotic risk factors. However, none of them have been found in enrolled patients.

It is however of interest that a few patients (n° 10, 20, 25, 33, 61, 93 and 106) are characterized by low FV levels and borderline APCr values, potentially produced by additional mutations in the FV gene.

## 3.6. BLOOD GROUP ANALYSIS

It is now known that ABO blood group and high von Willebrand factor (VWF) and factor (F)VIII levels have been associated with thrombotic disease. An excess of non-O blood group has long been recognized in patients with ischemic heart disease[30] and venous thrombosis[31].

In 1995, Koster and co-workers demonstrated that non-O blood group, high VWF levels and high FVIII levels all increased the risk of deep vein thrombosis[32-33]. Usually blood group phenotypes are used to study the association between blood group and venous thrombosis. Blood group genotypes may be more informative since genotypes can distinguish between heterozygous and homozygous carriers of A, B and O alleles and between A1 and A2 alleles.

Therefore Koster and co-workers studied the effect of ABO genotype on thrombosis risk in a large population-based case–control study of venous thrombosis (Leiden Thrombophilia Study, LETS), which included 474 patients and 474 control subjects[32-33].

*Table 3.11 - Thrombosis risk for ABO blood group genotype*[33]

| Genotypes | N. of patients N = 471 | (%) | N. of controls N = 471 | (%) | OR | 95% CI |
|---|---|---|---|---|---|---|
| **00 and FVL (-)** | 113 | 24 | 193 | 41 | 1* | |
| **00 and FVL (+)** | 24 | 5,1 | 9 | 1,9 | 4,6 | 2,0 - 10,1 |
| Non 00 and **FVL (-)** | 266 | 56,5 | 264 | 56 | 1,7 | 1,3 - 2,3 |
| Non 00 and **FVL (+)** | 68 | 14,4 | 5 | 1,1 | 23,2 | 9,1 - 59.3 |

*FVL, Heterozygous (1691AG) and homozygous (1691AA). *Reference category.*

They found that the risk of the combination of non-OO blood group genotypes and FV Leiden, compared with subjects with OO genotypes and without FV Leiden was 23-fold increased (Table 3.11). This is higher than expected on the basis of the effects of non-OO genotype (OR 1.7) and FV Leiden (OR 4.6) separately[32-33].

On the basis of this background, we investigated the distribution of the ABO blood groups genotype in the population enrolled. We could ascertain that our data agreed upon those reported previously in the literature. The 70% (n=30) of patients (Table 3.12) heterozygous for FV R506Q and suffering from VTE have been found to have non-0 blood group genotype (43%A, 37%AB, 25%B; Table 3.12), compared with the 30% (n=13) who have 0 blood group. Interestingly 87% (n=40) of subjects FVWt but suffering from almost one thromboembolic event, have been found to have non-0 genotype.

*Table 3.12 - 0 and non-0 blood group in the enrolled population*

| | Blood Group | | | |
|---|---|---|---|---|
| | 0 | | ***non* 0** | |
| **GROUP** | **N** | **(%)** | **N** | **(%)** |
| **FV R506Q + VTE** | 13 | 30% | 30 | 70% |
| **FV R506Q - VTE** | 3 | 33% | 6 | 67% |
| **FV R506R + VTE** | 6 | 13% | 40 | 87% |
| **CTR*** | 30 | 33% | 62 | 67% |

*CTR = control group (males from Ferrara)*

*Table 3.13 - ABO blood group genotypes in the enrolled population*

| | Blood Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | A | | AB | | B | |
| **GROUP** | **N** | **(%)** | **N** | **(%)** | **N** | **(%)** | **N** | **(%)** |
| FV R506Q + VTE | 13 | 59% | 24 | 43% | 3 | 37% | 3 | 25% |
| FV R506Q - VTE | 3 | 14% | 4 | 7% | 0 | | 2 | 17% |
| FV R506R + VTE | 6 | 27% | 28 | 50% | 5 | 63% | 7 | 58% |

It is worth noting that in a control group of male subjects from Ferrara, the 67% have non-0 blood group genotype (Table 3.12), a value overlapping to that observed in our small group of asymptomatic subjects and lower than that observed in our FV Leiden carriers and particularly in patients without the FV Leiden mutation (87%)

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# - 4 - CONCLUSIONS

The aim of our study was to observe the relation between the plasma FV levels and the APC resistance in a small cohort of subjects, most of them suffering from venous thromboembolism (VTE), and to analyze the possible role of FV mutations co-inheritance as thrombotic risk factors. We investigated the role of FV deficiency in venous thrombosis by screening Italian patients from Reggio Emilia area (in Northern Italy) for frequent thrombophilic mutations.

First of all, it was enrolled a small cohort of subjects (n=103), distributed into three subgroups which, unfortunately, were not homogeneous, especially with regard to the second group (FV R506Q – VTE): in this case, being patients who don't attended our department, the privacy issues have made the enrollment very difficult. This represents a limitation of our study.

Preliminary data show that in Reggio Emilia heterozygosis for FV R506Q is most likely associated with venous thrombotic events with a particular tendency to recurrence in case of discontinuation of anticoagulant therapy. These preliminary observations may be suggestive of co-inheritance of FV Leiden, or FVHis1299Arg, or other mutations such as Glu1608Lys, Arg2080Cys and Tyr1702Cys. The interest for the last three FV mutations was due in particular to their geographic distribution, pointing out to their onset in the Po Valley or Northern Appennini. However, none of them have been found in enrolled patients.

This can be explained in two ways: the reduced number of patients enrolled in the study and the immigration from central and southern Italy into the past and the ethnic variation of the Emilian population, which have added further difficulties for the selection of native people.

Despite this, for some patients, interesting aspects arose:

one patient (n°61) FV 506Q suffering from VTE showed a normal/uncertain APCR ratio (nAPCR-sr = 0,83), pointing toward an abnormal expression of the FV Leiden gene;

one patient (n°33), FV 506Q affected by recurrent superficial vein thrombosis, showed very low FV activity (FV:C=50,3%; LLN value), which could indicate a pseudo-homozygous condition. His APCR ratio is in accordance with FV Leiden heterozygosis (nAPCR-sr = 0,70), this may suggest a co-inheritance of FV null mutation (FV deficiency) or FV gene polymorphisms, predicting a slight reduction (about 25%) in FV levels.

one patient (n°10), FV 506R suffering from idiopathic DVT has a lower APCR ratio and FV plasma levels, both under the normal limit (nAPCR-sr = 0,67; FV:C=62,5%), again suggesting a partial pseudo-homozygous condition;

and one patient (n°20) FV 506R affected by idiopathic DVT showed FV:C=66%, LLN value, which further suggest involvement of FV alterations in this small sample.

These aspects deserve to be explored by further studies, both in the coagulation and genetics laboratories.

The APC resistant phenotype, resulting from FV Leiden mutation, is fully reflected in our data.

The first two groups (FV R506Q + VTE and FV R506Q – VTE) show average values of nAPCR-sr 0,62 ± 0,07 and 0,62 ± 0,03 respectively.

In the doubly heterozygous condition with FV Leiden, the FVHR2 could produce, through a partial pseudo-homozygous mechanism, a relative increase in plasma levels of APC resistant molecules. Nevertheless, the number of patients was not sufficient to reach a significant difference.

Indeed, testing the contribution of the His1299Arg change to the plasma FV phenotype we found that FV activity was significantly (t-test, $P<0.05$) lower in subjects heterozygous for the HR2 allele (R1R2) than in homozygous for the wild-type R1R1 allele. These results confirm what has previously been reported for the first time, in 1996 by Lunghi and co-workers: Hisl299Arg variant, linked with a number of potentially functional polymorphisms is responsible for the reduction in FV levels. Otherwise the FVHR2 has been associated with slightly reduced FV levels in plasma, both at the coagulant activity and antigen levels. The mechanism which underlies the reduction of FV levels has been investigated and Scanavini et al have indicated that the aminoacid change at 2194 could be the causative change

Based on our results, we observed that the R2 allele, and particulary the heterozygous form, appeared with a frequency (R1R2=12,6%) similar to that expected, indeed its prevalence throughout Asia, Europe and in native African populations ranging from 8 to 12. Of note is the presence of the homozygous form, especially considering the small sample size; the homozygous condition for the R2 allele is rare (2 to 5/1000); however, in our population we found one patient (n°25) homozygous R2R2 genotype. This patient, FV 506R suffering form recurrent superficial vein thrombosis, shows FV:C=62,5%, LLN values.

Overall, the R2 allele was found only in subjects affected by VTE, both carriers or not of Leiden mutation. However, we must consider the limitation caused by the non homogeneity of three groups. A similar prevalence was apparently found in normal individuals and in consecutive patients referred for a venous thromboembolic event. However, the evaluation of the frequency requires to consider that in FV Leiden carriers one FV gene is shared between all patients. As a consequence the R2 allele is more frequent than expected in the population under study.

Although we cannot affirm that the R2 allele is alone a risk factor for VTE, its co-inheritance with the FV Leiden - because HR2 resides on the allele that does not carry the R506Q mutation, could determine an increased degree of APC resistance, even we did not observe a statistically significant difference regarding the nAPCR-sr value, evident only in carriers of FV R506Q. The APCr test at present used in the coagulation laboratory may not favour the contribution of the R2 allele.

Finally, investigating the distribution of the ABO blood groups genotype in the population enrolled we found that the 70% (n=30) of patients FV R506Q carriers and suffering from VTE has non-0 blood group genotype, in agreement with previous literature. Interestingly, in a control group the 67% have non-0 blood group genotype, a value overlapping to that observed in our small group of asymptomatic subjects but lower than that observed in our FV Leiden carriers (70%) and particularly in patients without the FV Leiden mutation (87%).

Overall our observations further suggest that rare and frequent genetic conditions, both FV gene-related or involved in other biological pathways (i.e. blood group) could contribute to modulate the risk of venous thrombosis in FV Leiden carriers and in patients without major thrombophilic mutations.