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Molecular bases of the modulation of coagulation factor levels: in vitro and in vivo study

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Chapter 1

Introduction

1.1 Hemostasis

The term *hemostasis* defines a dynamics process aimed at maintaining the blood fluid under physiological conditions, and at limiting the blood loss in case of vascular lesion. This homeostatic process involves the vascular endothelium, platelets and a number of circulating and transmembrane proteins.

In the resting state endothelial cells provide a *non-thrombogenic* surface that inhibits the platelets adhesion and blood coagulation. Moreover, the synthesis of prostacyclin and heparin-like substances, and the presence of protein complexes (thrombin-thrombomodulin), leading to generation of anticoagulant proteins (activated protein C), prevents clot formation in normal blood vessels [1].

In reaction to the vessel wall injury, damaged endothelial cells expose negatively charged phospholipids and release procoagulant proteins, platelets adhere to macromolecules in subendothelial layer and aggregate to form *the primary hemostatic plug*, necessary to temporary blocks the blood loss.
The interaction between platelets and the damaged endothelium is allowed by a large multimeric plasma protein, named von Willebrand Factor (vWF), that acts as a bridge between the sub-endothelium collagen and specific receptor on platelet surface [1].
The platelet activation that occurs in this early phase induces specific morphologic and biochemical alterations on their membrane surface and the release of molecular components necessary for the initiation and propagation of blood coagulation.
Later, as wound healing occurs, fibrin clot is broken down and removed [2].

1.2 Blood Coagulation

Blood coagulation involves a stepwise participation of a large number of plasma proteins, that progressively amplify the triggering signal in order to massively generate thrombin. Newly formed thrombin is necessary to stabilize the primary hemostatic plug.

The enzymatic proteins involved in coagulation (factor VII [FVII], factor IX [FIX],

factor X [FX], factor XI [FXI], factor XII [FXII] and prothrombin [PT]) are all vitamin K-dependent serine proteases. They circulate in plasma as inactive precursors (zymogens), and they need to be activated in order to participate in the coagulation reactions. To increase the catalytic efficiency of the serine proteases, allowing a rapid response to injury, the coagulation system also provides three cofactors: tissue factor (TF), factor V (FV) and factor VIII (FVIII).

For a long period, the blood coagulation has been described as a process where each clotting factor as proenzyme could be converted to an active enzyme, participating then to the activation of another one (*waterfall model*). In particular, this classical view divided the coagulation process into two pathways: an *intrinsic pathway*, so named because all the components are present in blood and, an *extrinsic pathway*, in which the subendothelial cell membrane protein TF is required in addition to circulating components. The initiation of both pathways finally resulted in the activation of FX and the generation of thrombin (also called FIIa), Fig. 1.1.

This classic concept is now integrated into a more comprehensive view of blood coagulation, where the cellular components acquire more importance, Fig1.2 [3].

The process of blood coagulation starts by the exposures of TF-expressing cells to the blood flow.

TF is an integral membrane glycoprotein that it is constitutively expressed on cells such as fibroblasts, smooth muscle cells, activated monocytes, activated platelets, and even cellular-microparticles but not on resting endothelium [4]. When the vessel integrity is interrupted, it becomes exposed to the blood flow, where it interacts with the circulating FVII. TF-bound FVII becomes susceptible to activation by trace amounts of proteases present in blood, among which FVIIa itself. The new formed TF-FVIIa complex activates small amounts of FX and FIX.

FXa associates with its cofactor, activated Factor V, and forms the so called *prothrombinase-complex* on the surface of the TF-bearing cells, leading the activation of small quantity of circulating PT into Thrombin.

During the *initiation phase*, the adhesion process partially activates the platelets and pro-

motes the secretion of partially activated FV from their α-granules. Moreover the small amount of newly generated Thrombin contributes to the activation of the cofactors FV, FVIII and, it is also sufficient to activates FXI, which in turn activates FIX.

The next phase, the so called *amplification of clot formation*, occurs on the surface of activated platelets. In this phase the FXa generation is supported by the prothrombinase complex on the surface of activated platelets and by FIXa-FVIIIa, the so called *tenase complex*. Additional FIXa can be supplied by platelet-bound activated-FactorXI.
More than 95% of the total amount of thrombin production takes place in the amplification phase, after initial clot formation [5]. This excess of thrombin has been proposed to play an important role in clot stabilization by activating Factor XIII [FXIII], (the fibrin stabilizing factor [6]), cleaving the platelet protease-activated receptor-4 [PAR-4] (contributes to the full activation of platelets [7]), and, activating thrombin activable fibrinolysis inhibitor [TAFI] [8]. TAFI is a carboxypeptidase that removes terminal lysine residues from fibrin, thereby removing potential binding sites for fibrinolytic enzymes and enhancing clot resistance to fibrinolysis [9].

1.2.1 Macromolecular complexes in blood coagulation

All the enzymatic proteins involved in coagulation are vitamin K-dependent serine proteases. They show an highly homologous catalytic domain and share a number of conserved structural motifs (Gla domains, EGF-like domains, kringle domains), that mediate interactions with other proteins and membranes.
Their activation, required to participate in the coagulation process, is done by limited proteolysis of determined peptide bounds. In contrast to other serine proteases, the blood coagulation proteases have evolved versatile and sensitive regulatory mechanisms for controlling the specificity of substrate recognition, mediated by specific surfaces of the enzyme far away from the catalytic site (exosites) [10].

Activation of clotting factors does not occur in the solution phase, but within membrane-bound macromolecular complexes, each comprising a vitamin K-dependent serine-protease,

a non-enzymatic protein cofactor, a zymogen substrate, and calcium ions. The protein-phospholipids and protein-protein interactions within the macromolecular complexes enhance reaction rates by several orders of magnitude. Moreover, the localization of different enzyme complexes on the same membrane surface allows product channelling between successive reaction centers, a circumstance that protects activated factors from inactivation by circulating inhibitors [11].

Finally, macromolecular complexes offer several opportunities for the control of coagulation reactions. In fact, complex assembly requires a number of simultaneous events: the conversion of a zymogen to the active serine protease, the activation of a procofactor to the active cofactor and the availability of negatively charged phospholipid membranes. This latter condition guarantees the confinement of the coagulation process to the sites of injury.

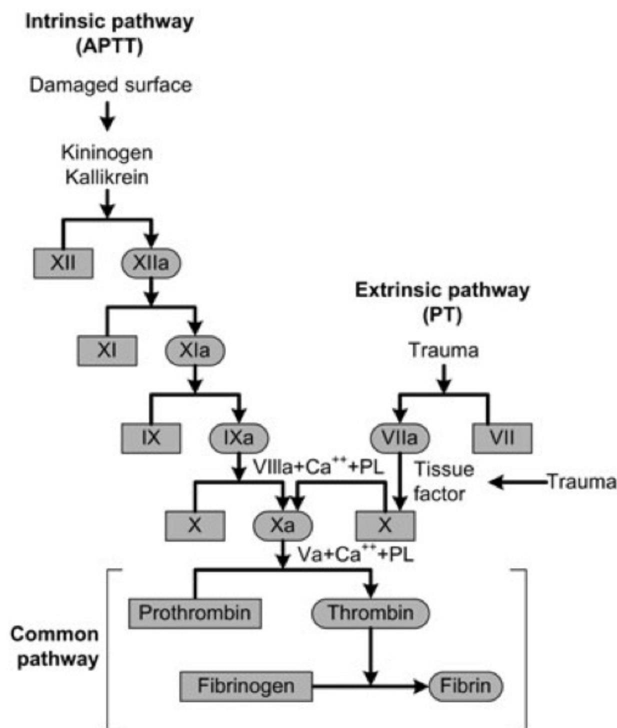

Figure 1.1: The waterfall model of blood coagulation. The point of integration between the intrinsic and extrinsic pathways in this model occurs with factor IX activation.

1.2.2 Regulation

Mechanisms of coagulation are conveniently regulated to limit the extent of activation once it has been triggered, Fig. 1.2.

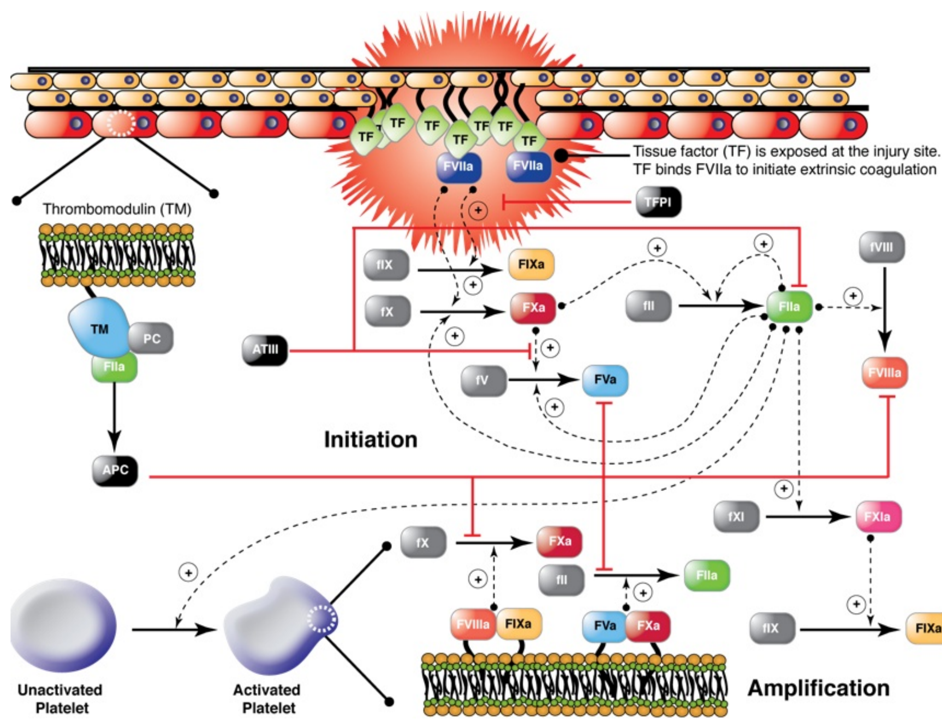

Figure 1.2: Blood coagulation *cells based model*. Upstream coagulation factors are activated by TF exposed following vessel injury. FXa and FIXa amplify the triggering signal together in order to massively generate thrombin. Thrombin (FIIa) also activates upstream coagulation factors, forming a strong positive feedback ensuring rapid activation. Thrombin itself plays a role in its own inhibition by binding the surface protein thrombomodulin (TM), catalyzing the conversion of protein C (PC) to activated protein C (APC). Other relevant inhibitory complexes are represented by the Tissue Factor Pathway Inhibitor (TFPI) and th Antithrombin (AT).

Since the active cofactors of the extrinsic pathway (TF, FVa and FVIIIa) promote thrombin production by massively enhancing the catalytic efficiency of their respective enzyme, these cofactors play important role in both the positive and negative regulation of thrombin generation.

Tissue Factor Inhibitor Pathway

The tissue-factor pathway inhibitor (TFPI) regulates the initiation phase of blood coagulation [12]. TFPI has a modest inhibitory capacity towards FXa, but it becomes a potent inhibitor of the TF-FVIIa complex.

Recently, it has been shown that Protein S lower the k_i of FXa/TFPI complex formation by ∼10 fold, thereby significantly accelerating FXa inhibition [13]. The stimulation produced by protein S explaines how full-lenght TFPI can effectively inhibits the extrinsic FXa generation at its low physiological plasma concentration.
As the TF-FVIIa complex is turned off by the TFPI-FXa complex, further generation of FXa is strictly dependent on the intrinsic tenase complex (FIXa-FVIIIa). The importance of this mechanism is underscored by the lethal phenotype of TFPI knock-out mice[14].

Protein C anticoagulant system

The activated protein C (APC) system inhibits the propagation phase of the coagulation by down-regulation the activity of the prothrombinase and intrinsic tenase complexes [15]. The severe thrombotic diseases affecting patients with homozygous protein C (PC) deficiency, show the physiological importance of the PC system.
Similar to the procoagulant serine proteases, the PC circulates in plasma as a zymogen and needs to be activate to gain its activity. Its activation is given by thrombin in complex with thrombomodulin (TM), expressed on the membrane of intact endothelial cells. The endothelial PC receptor (EPCR) is also necessary in the thrombin-TM dependent PC

activation Fig. 1.2 [15].
APC down regulates the blood coagulation by limited proteolytic cleavage of FVIIIa and FVa on the membrane surface, in the presence of cofactors. To fully inactivate FVa, APC requires the presence of the protein S. Otherwise the inhibition of FVIIIa requires the synergistic contribution of both PS and FV (see chapter 4) [16]. The inactivated forms of FVa and FVIIIa have no cofactor activity and are subsequently rapidly removed from circulation.

Serine proteases inhibitors

Blood contains a number of circulating serine proteases inhibitors, known as *Serpins* [17]. They are substrate analogues, able to form equimolar irreversible complexes with the target protease. The binding with the serpin in fact, induces a distorsion of the enzyme structure and the loss of its function. This inactive complex is then removed from circulation.
Thrombin is inhibited by the serpin Antithrombin (AT), heparin cofactor II (HCII), Protein C inhibitor (PCI), whereas FXa can be inhibited by AT and PCI only. The activated protein C (APC) is mainly inhibited by PCI and α_1-antitrypsin.

1.3 Modulation of Coagulation Factor Levels

Although the hemostatic system contains numerous controls to ensure function within the normal range, hemostasis is nevertheless sensitive to perturbation.
In the majority of the healthy population the concentrations of pro- and anticoagulant proteins vary significantly. Generally clinical coagulation laboratory values for the reference of the healthy population range from 50% to 150%.

Genetic and environmental factors contribute to this heterogeneity, influencing the biosynthesis, the utilization and the clearance of all hemostatic proteins Fig.1.3 [18].

The coagulation molecules are in fact extremely complicated proteins that required a variety of processing steps, including post-translational modifications and proteolytic cleavages. In addition, extracellular modifications by both biologically directed processes and environmental accidents may further alter the circulating product. As a consequence of these modifications, the number of variable forms of clotting proteins increases , partially explaining the variability of the so called *hemostatic proteome*.

Even among the well defined, single gene disorders of hemostasis and thrombosis, considerable variability remains to be explained. For many genetic diseases, not everyone inheriting the disease-causing mutation manifests clinical symptoms, a phenomenon referred to by geneticists as incomplete penetrance. For example, in families with typical autosomal dominant type 1 von Willebrand disease (VWD), penetrance is generally incomplete (50% or lower) [19]. In addition, among those individuals in the VWD family who do express bleeding symptoms, the severity of this bleeding can also be highly variable, a phenomenon referred to as variable expressivity.This suggests that multiple genetic, acquired and environmental factors influence the possibility of an individual to undergo a clinically significant hemorrhagic or thrombotic event [20]. Moreover the concomitant presence of genetic defects in an individual, the so called *gene-gene interaction*, also influence the normal or predicted levels of clotting factors. The best defined examples of a human modifier gene is the ABO blood group, that concerning the hemostasis is known to be a major genetic modifier of plasma VWF level, accounting for approximately 30% of its genetic variation [21].

Disease states, drug consumption, and behavior (smoking, diet, sedentariness,...), can influence the levels clotting factors, thus conditioning the hemostatic balance.

Taken together these considerations indicate that only few informations, regarding the heterogeneity of the plasma vitamin K-dependent proteins and the influence on their biological performance, are available.

1.4 Present Investigation

During the last decades there has been an increasing attention to the genetic or environmental factors that can modulate the plasma levels of blood coagulation factors.

The general objective of my work has been to shed a light on the relationships between determined acquired or genetic components and the blood coagulation phenotype. This could be instrumental to discover new parameters in the evaluation of risk for thrombotic or hemorrhagic events.

The first part of my doctoral work has been spent in evaluating the variation of several blood coagulation parameters by well defined diet or a controlled physical training, in populations with moderate to severe cardiovascular risk. The results of this research are reported on chapter 2 and 3, respectively.

In the last part, I was involved in a methodologically different project, aimed at investigating the impact of naturally occurring mutation (R1698W) of FV in its structure and function.

The FV R1698W mutation results in FV deficiency, due to a severe reduction in FV circulating levels. The clinical symptoms of FV deficiency may vary from severe bleeding

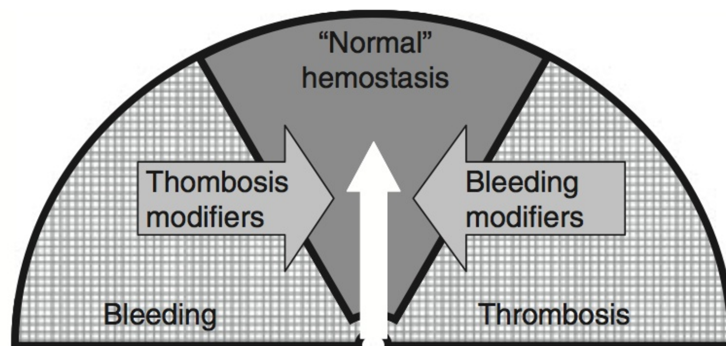

Figure 1.3: The hemostatic balance, figure from [22]. With minor fluctuation, the system is designed to operate in a wide range (grey). Genetic and environmental modifiers can cause systemic malfunction, inducing an elevated susceptibility to either bledding or thrombosis.

symptoms, to no symptoms at all.

Identifying the molecular basis underlying this disease will help explaining this variable clinical phenotype. The results of this research as well as future investigations are reported on chapter 5. Moreover a brief introduction regarding the biology and the role of FV is provided in chapter 4.

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Chapter 2

The effects on a healthy diet in the coagulation factors levels and thrombin generation in middle-aged women with moderate CVD risk.

Based on:

Reduced FVII and FVIII levels and shortened thrombin-generation times during a healthy diet in middle-aged women with mild to moderate CVD risk.

Passaro A*, Calzavarini S*, Volpato S, Caruso P, Poli A, Fellin R, Bernardi F.

Journal of Thrombosis & Haemostasis. 2008; 6(12): 2088-94.

* the authors equally contributed to the article

2.1 Introduction

Diet affects the hemostatic system [1] [2], a suitable target for interventions aimed at reducing the risk for cardiovascular disease (CVD) [3] [4].

Increased levels of hemostatic factors, including fibrinogen, factor VIII (FVIII), factor VII (FVII), tissue factor (TF) and von Willebrand factor (vWF) have been related to increased CVD risk [5]-[9], in line with the key role of coagulation factors in thrombus formation. Furthermore, the causal contribution of coagulation factor levels to CVD is supported, although indirectly, by the clinical benefit of anticoagulant therapy in patients with acute coronary syndrome [10] [11].

During the past decades the overall CVD mortality has declined but only a modest reduction has been observed among women [12], which might suggest that primary prevention programs are less effective in women than in men. Although changes of life-style in pre-menopausal women might represent an effective prevention strategy aimed at enduring risk factor modifications, little is known about haemostatic variations induced by diet in this population.

The aim of this work was to provide a detailed evaluation of the impact of a well defined intervention strategy on the hemostatic system in pre-menopausal middle-aged women with mild to moderate risk for CVD. In particular, we investigated the effects of an integrated healthy diet on a wide panel of hemostatic variables and on an overall coagulation functional test. Noticeably, the rapid turnover of most coagulation factors would enable to efficiently monitor the response to the intervention. Coagulation variations were also investigated in relation to specific metabolic and inflammatory parameters previously associated with thrombotic cardiovascular events [12] [13].

2.2 Material and Methods

2.2.1 Study Population

A group of middle-aged overweight (BMI $\geq$ 25 kg/m2) women was invited to participate in this study, Fig. 2.1. Forty-nine volunteers were screened for eligibility in accordance with the following inclusion criteria: C-reactive protein (CRP) $\geq$1 mg/l in two different assessments; and one or more of the following CVD risk factors (waist circumference $\geq$ 88 cm; triglycerides$\geq$150 mg/dL; HDL-cholesterol $<$50 mg/dL; arterial blood pressure $\geq$ 130/85 mmHg; plasma glucose $\geq$ 110 mg/dL). Exclusion criteria were: average daily ethanol intake $>$ 30 g over the last 5 years; clinical diagnosis of diabetes mellitus according to the American Diabetes Association Criteria [14]; diagnosis of menopause according to the American Association of Clinical Endocrinologists [15]; history of cardiovascular disease; current therapy with hormonal drugs, anti-thrombotic and/or anticoagulant, ACE-inhibitors, Angiotensin II Receptor Blockers, and vitamin supplementation. Fifteen women had CRP levels $<$1 mg/l; 8 were using ACE inhibitors or hormonal replacement therapy, one was on menopause state, two had diabetes, one did not have the above stated CVD risk factors, and six declined to participate. Sixteen women agreed to participate and completed an independently validated dietary questionnaire on alcoholic intake and eating habits.

2.2.2 Intervention

The protocol of this open-label, non-randomized intervention includes four phases (84 days) and are summarized in Fig. 2.2. We integrated the National Cholesterol Education Program-Adult Treatment Panel-III (NCEP-ATPIII) dietary recommendations with the guidelines of the scientific advisory committee of the American Heart Association, based on the effects induced by a Mediterranean-style diet on the risk of cardiovascular disease [16]. During the first 21 days (T0-T21) all subjects received an isocaloric diet (carbohydrates 57%, proteins 13% and lipids 30%) according to Therapeutic Lifestyle Changes

ATPIII [4]. The energy intake was calculated for every subject based on basal metabolic rate and physical activity levels. Participants were not allowed to consume any alcoholic beverage, extra virgin olive oil, fish and green and black tea, rich in omega-3, polyphenols and antioxidants. 180 mL/day of monovarietal dry white wine (11% alcoholic strength) was added at T21. This alcoholic beverage provided 20 g of alcohol/day intake. Based on the postulated anti-inflammatory effects the diet was integrated during the third phase (T42 -T63) with extra virgin olive oil, low index carbohydrates, 2 portions of salmon and 4 portions per week of nuts. In the last phase (T63-T84) participants were treated as in T0-T21.

A daily food intake diary monitored the compliance with the protocol during the study, and after each phase a clinician assessed any adverse effects. The Human Research Ethics Committee of the Azienda Ospedaliera-Universitaria of Ferrara approved the study protocol.

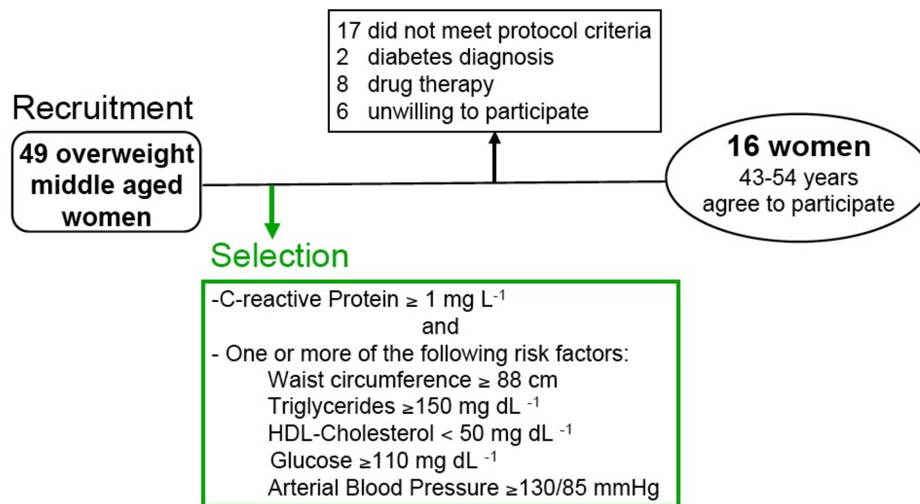

Figure 2.1: Recruitment and Screening of the subjects involved in the study.

2.2.3 Anthropometric Parameters

Anthropometric measurements were taken according to standardized procedure. Fat mass was also determined by tetrapolar bioelectric impedance analysis (Dietosystem Ltd, Milano).

2.2.4 Lipids and Inflammation Markers

Blood samples were collected at the beginning of each study phase, after overnight fasting. Serum and plasma samples aliquots were stored at 80C. Serum total cholesterol and triglycerides levels were determined using standard enzymatic techniques (Roche Diagnostics, GmbH, Basel, Switzerland). To measure HDL-Cholesterol, apo B-containing lipoproteins were precipitated from plasma by use of phosphotungstic acid and Mg2+. LDL cholesterol was calculated according to Friedewald's formula. High sensitivity CRP (hsCRP) levels were measured (inter-assay coefficient of variation 5%) by particle-enhanced immunonephelometry (Roche Diagnostics, GmbH, Basel, Switzerland). Serum Interleukin-6 (IL-6) and and Tumor Necrosis Factor-alpha (TNF-α) were quantified using commercial ELISA (BioSource International Inc., California, USA). Intra- and inter-assay variation coefficients for, IL-6 and TNF-α ranged from $1.8 to 5.4$% and from 0.9 to 9.9%, respectively.

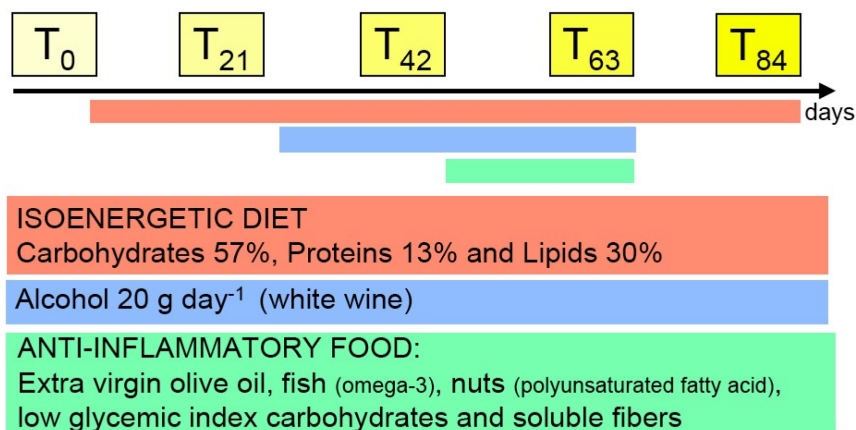

Figure 2.2: Flow chart of the different phases of the study (*courtesy of C. Casari*).

2.2.5 Plasma Coagulation Factors

Antigen levels of TF, FVII and FVIII were determined by commercial ELISA (TF American Diagnostica, Greenwich, CT; FVII and FVIII Affinity Biologicals Inc., Ancaster, Canada). The intra-assay coefficients of variation for TF, FVII and FVIII assays were 12.8%, 7% and 8.7%, respectively, while the inter assay coefficient were 14.7%, 7.3% and $7,4\%$ respectively. To evaluate the vWF antigen levels we used the ELISA protocol previously described [17]. The intra-assay and inter-assay coefficients of variation for vWF ELISA assay were 6.5% and 15%, respectively. Antigen levels of FVII, FVIII and vWF were expressed as percentage of healthy plasma in-house pool. Fibrinogen levels were measured by a prothrombin time derived method with a turbidimeter kit (Diagen, Thame, UK).

2.2.6 Thrombin Generation Measurements

Calibrated automated thrombin activity measurement was performed as described elsewhere [18]. Platelet poor plasma (PPP) samples were centrifuged at 23000 g at 4C for 1 hour before testing. Coagulation was triggered in recalcified PPP by addition of 1 pM recombinant human TF and 4 μM phospholipids (PC:PS 80:20) at 37C. All experiments were carried out in duplicate. The assay variability was lower than 3%. The slope of thrombin generation curves was calculated using the following formula: [Peak (nM)/(ttpeak (min)-lag time (min))].

2.2.7 DNA Isolation and Analysis of Genetic Polymorphisms

DNA was extracted from peripheral blood leukocytes by salting-out method. The ABO blood group of patients was determined by genotyping as previously described [19]. FVII gene polymorphisms were analyzed as described [20].

2.2.8 Statistical Analysis

Data are presented as mean standard deviation for parameters with normal distribution and as median with interquartile range for parameters with skewed distribution (TG, hsCRP, IL-6, TNF-α). In order to approximate a normal distribution, these variables were analyzed after log-transformation. Pairwise correlations were estimated using Pearson correlation coefficient. Differences in mean values across study phases were analyzed using analysis of variance (ANOVA) for repeated measures and analysis of covariance (ANCOVA). After multiple comparisons, p values were adjusted with Bonferronis method. Statistical and graphical data analyses were performed using Stata 9 (StataCorp. 2005. Stata Statistical Software: Release 9. College Station, TX: StataCorp LP) and R language (R Foundation, version 2.6.1).

2.3 Results

2.3.1 Variation in Body Composition, Lipid and Inflammatory Parameters

Variation of anthropometric, lipid, and inflammatory parameters of the 16 women who completed the study protocol are summarized in table 1. The subjects showed a clear and progressive reduction in BMI (P=0.001) throughout the study period. The BMI decline was paralleled by reduction of waist circumference (P=0.005) and, although as trend, of fat mass (P=0.096). Total and LDL cholesterol levels were significantly reduced over time with the most important changes observed after the first study phase (T21 vs T0; $P<0.05$). The degree of change of anthropometric and lipid parameters was in good agreement with previous reports [21]. HDL-cholesterol and triglyceride levels did not show appreciable variation over time, even after moderate alcohol intake, reported to affect their levels.

At baseline median CRP was 2.2, nine women had values higher than 2 mg/L and five higher than 3 mg/L. The inflammatory profile did not significantly change throughout the

Parameters	T_0	T_{21}	T_{42}	T_{63}	T_{84}	P
BMI (kg/m^2)	29.2 ± 2.7	28.4 ± 2.7	28.0 ± 2.4	27.8 ± 2.4	27.4 ± 2.6	0.001
WC (cm)	86.7 ± 6.3	85.6 ± 6.4	83.9 ± 6.4	83.1 ± 5.2	83.9 ± 5.6	0.005
TC (mg/dL)	238 ± 40	213 ± 35	209 ± 38	215 ± 45	222 ± 43	0.011
HDL-C (mg/dL)	58 ± 14	55 ± 15	53 ± 12	57 ± 13	57 ± 10	0.375
TG (mg/dL)	98 (71-118)	77 (52-112)	96 (77-106)	90 (68-114)	90 (73-109)	0.127*
hsCRP (mg/L)	2.2 (1.4-3.5)	1.9 (1.3-3.2)	1.9 (1.1-3.6)	1.8 (1.2-3.8)	1.5 (1.3-3.3)	0.517*
IL-6 (pg/mL)	0.82 (0.6-1.2)	0.67 (0.6-0.8)	0.78 (0.6-1.1)	0.68 (0.4-0.9)	0.62 (0.5-0.8)	0.121*
TNF-α (pg/mL)	4.6 (2.6-6.1)	4.3 (3.6-6.3)	4.1 (2.9-9.3)	4.6 (2.9-5.6)	4.1 (3.0-5.2)	0.626*

Table 2.1: Data expressed as mean±SD. P values refer to analyses of variance for repeated measures. Triglycerides, hsCRP, IL-6 and TNF-α are expressed as median and interquartile range (25th and 75th quartile). The asterisk on P values refers to analyses of variance for repeated measures after log-transformation of the dependent variable

study although all inflammatory markers, and particularly IL-6, tended to decrease over time.

2.3.2 Variation in Coagulation Factors Levels

In order to evaluate the effects on hemostatic components, we determined the antigen levels of clotting factors mainly synthesized in the liver (FVII, FVIII and fibrinogen) or in other tissues (vWF and TF) 2.3. FVII levels showed an appreciable variation (P=0.003) and the lowest value was observed at T63 (15.2% lower than T0; P<0.05), in the presence of both alcohol and anti-inflammatory components. However, the decrease was already present at T42 after alcohol introduction (9.5% lower comparing T42 vs T0; P<0.01). The variation pattern did not differ upon exclusion of subjects heterozygous for the -402 A and -323 10 bp insertion FVII alleles, known to be associated with increased and decreased FVII levels, respectively [20] [22] .

A positive correlation (r=0.32, P=0.004) was observed between FVII and triglyceride val-

ues, a well-known determinant of circulating FVII levels [23]. However, the FVII level variation was influenced neither by parallel variations in plasma triglyceride nor by fat mass modification, as indicated by multiple linear regression analysis (ANCOVA adjusted for triglycerides and fat mass).

FVIII antigen levels significantly decreased during dietary treatment (P=0.005). The reduction was borderline significant at T21, when all subjects had the same isoenergetic non anti-inflammatory diet, was maximal at T63 (15.1% compared to T0, P<0.05), and persisted even in the last phase when alcohol and anti-inflammatory food intake was interrupted.

We found that overall FVIII values were positively correlated with CRP (r=0.24, P=0.034), an important cytokine of the acute phase response, and TNF-α (r=0.24, P=0.043), a pro-inflammatory cytokine associated to obesity [24]. The positive relation between FVIII and TNF-α levels was also detected before the intervention (r=0.55, P=0.033). FVIII level variation was not significant after adjustment for TNF-α values (ANCOVA). Differently adjustment for other inflammation markers, CRP and IL-6, did not produce a similar effect on FVIII pattern. The FVIII variations over time were not influenced by body fat modifications. Adjustment for blood groups [25] did not affect FVIII level variation.

Fibrinogen levels, an independent cardiovascular risk factor [26], did not show appreciable variations over the study phases and were similar to those measured in Italian healthy women [27]. Among inflammatory markers, only CRP showed a strong positive relation (r=0.54, P<0.001) with fibrinogen levels at each diet phase.

Although the statistical analysis of TF levels in plasma is complicated by the pronounced inter-individual and intra-individual changes, TF levels appeared to decrease in a stepwise manner during the alcohol and anti-inflammatory food intake. White blood cells count and pro-inflammatory protein (IL-6, CRP, TNF-α) distributions during treatment did not explain the TF level variations.

vWF antigen, a potential marker of endothelial dysfunction or activation, did not show significant variations over time. A positive relation between FVIII and vWF levels was clearly detectable, with the highest values at the beginning of the study (r=0.58, P=0.018)

and in the presence of both alcohol and anti-inflammatory components (r=0.68, P=0.004). The variation of the ratio between FVIII and vWF levels showed a pattern similar to that of FVIII levels (data not shown).

2.3.3 Functional Variations of the Hemostatic System

We measured thrombin generation in plasma samples using low concentration of TF supplied with a non-limiting concentration of phospholipids, as a sensitive way to monitor the interplay of procoagulant and anticoagulant components.

The lag time and time to peak (ttPeak) were significantly modified during diet treatment 2.4. We observed markedly prolonged times, and particularly the median lag time increased from 6.3 (T0) to 8.5 minutes (T42). Adding of anti-inflammatory components (T63) did not further prolong these times, which overlapped at the beginning and end of the study. The endogenous thrombin potential (ETP) and peak parameters did not show appreciable variation during the study and were clearly correlated with FVIII antigen levels (ETP vs FVIII r=0.48, $P<0.001$; Peak vs FVIII r=0.49, $P<0.001$), as previously observed [28]. The reference plasma showed ETP and peak values (1285.2±197.4 nmol/L and 159.1±42.4 nmol respectively) similar to those observed in the study population. The modification of body composition did not influence any thrombin generation parameters considered.

In order to further evaluate the relationship between the thrombin generation rate and the dietary treatments, the slope of thrombin generation curves was calculated. As indicated by multivariate regression analysis, this parameter was determined by FVII (β=0.39, P=0.01) and FVIII (β= 0.35, $P<0.01$) levels but did not show appreciable variation over time (data not shown).

2.4 Discussion

Although several studies have been conducted on the effects of whole-diet therapies on cardiovascular risk factors [29] [30], none experimental study investigated the effect on a wide

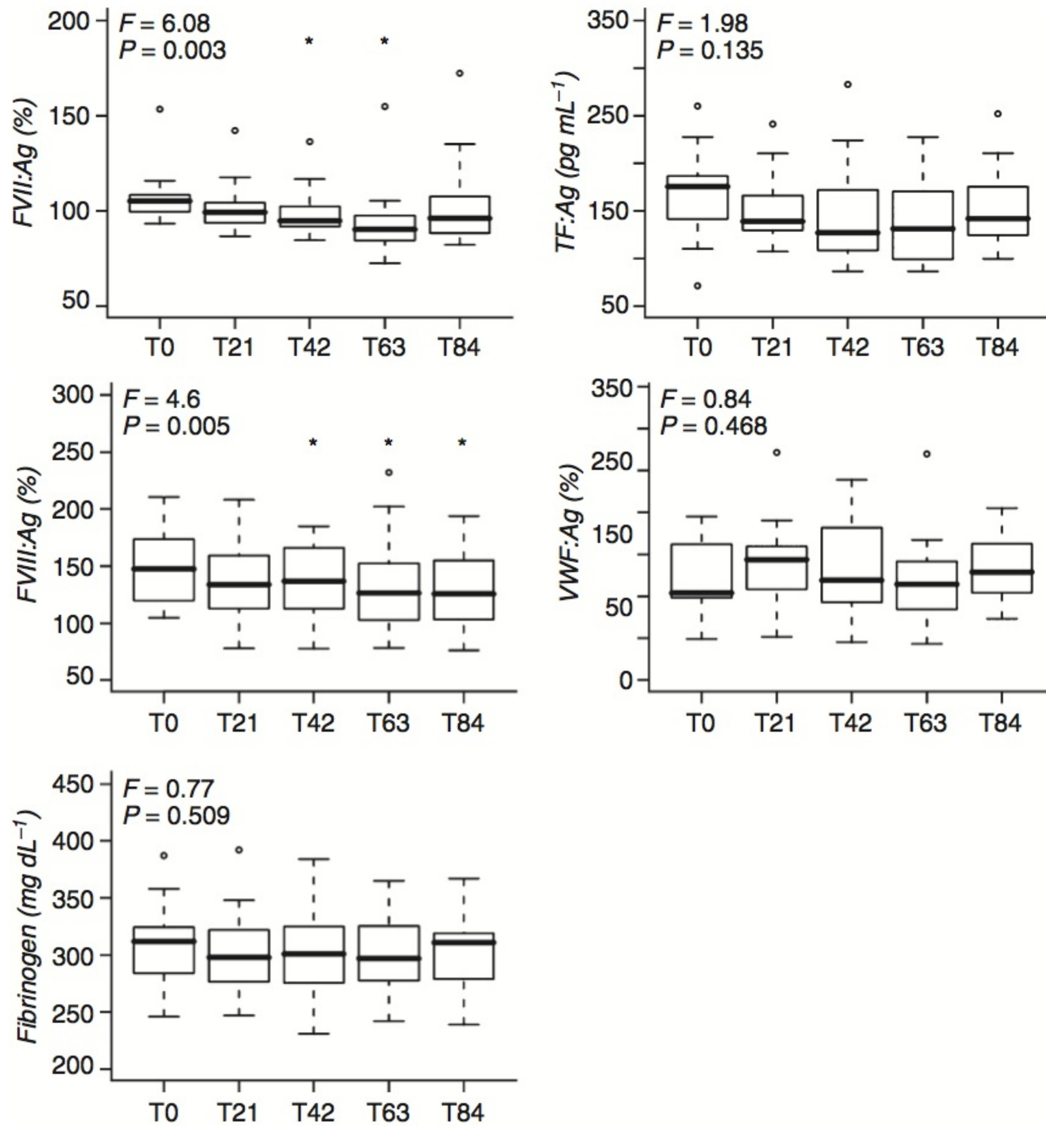

Figure 2.3: Over study variation of hemostatic factors levels. The black line inside the boxes represents the median value and distribution outliers are indicated with blank circles (o). The asterisks indicate significant variation (P<0.01) respect to T0 after Bonferronis adjustment for multiple comparisons

panel of haemostatic parameters, and their relationship with metabolic and inflammatory markers. The present study reports original observations about the beneficial effects of an integrated healthy diet in a selected middle-aged women population, characterized by high CRP levels and at least one additional risk factor for cardiovascular disease.

Biologically and clinically relevant modifications of body composition, lipids, coagulation factor levels, and thrombin generation parameters were observed. The reduction in anthropometric and lipid parameters, and particularly BMI, waist circumference, total and

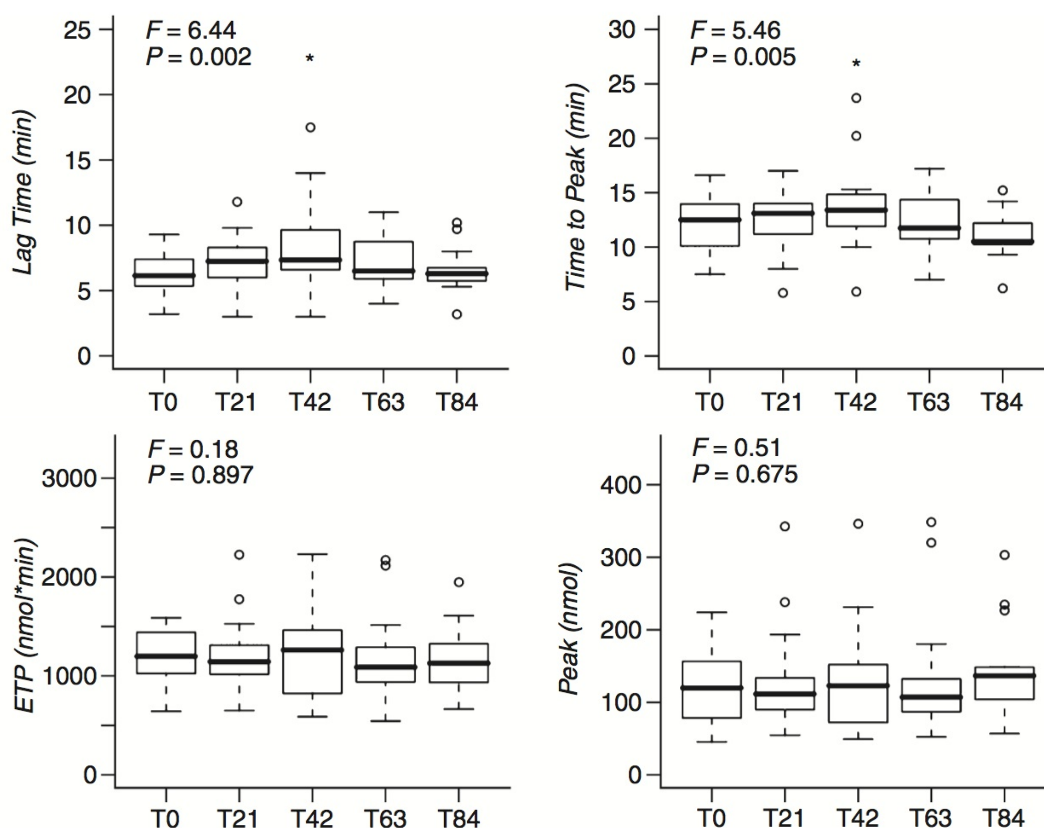

Figure 2.4: Over study variation of thrombin generation parameters. The black line inside the boxes represents the median value and distribution outliers are indicated with blank circles (o). The asterisks indicate significant variation (P<0.01) respect to T0 after Bonferronis adjustment for multiple comparisons.

LDL cholesterol levels, would translate into a remarkable decrease in risk for CVD.

The antigenic determination of the hemostatic factors, increased levels of which are recognized or candidate risk factors for CVD, favoured the quantitative evaluation of diet-induced biosynthetic changes, and avoided potential drawbacks of clotting assays.

FVII and FVIII levels were remarkably reduced, whereas fibrinogen concentration did not show appreciable changes throughout the study. These observations indicate differential effects of diet on blood coagulation factors mainly produced by the liver, a key organ involved in the metabolic changes observed. Further differences were observed between the stepwise decrease of FVII and FVIII levels, which for FVIII was not counteracted by removing alcohol and anti-inflammatory components, and persisted at the end of the study. vWF levels were strongly correlated with those of FVIII, but poorly modified by dietary treatments. This observation, and the similar pattern observed for FVIII levels and FVIII/vWF ratio, further support a role of diet on modification of FVIII expression.

Diet-responsive anthropometric and metabolic parameters were found associated with coagulation factor levels but did not appreciably influence any hemostatic factor variations, pointing toward still undiscovered regulation of coagulation factor expression by intervention.

We observed a clear association between coagulation factor levels and the inflammatory profile. Levels of TNF-α, a cytokine over-produced by adipocytes and macrophages of adipose tissue in the obese state [31], were correlated with those of FVIII and affected their variation over time, which points toward a previously unknown relationship. As comparison CRP, produced by hepatocytes, was strongly related both to fibrinogen [32] and FVIII but did not affect the FVIII variation over time. These observations suggest the presence of inflammation-driven crosstalk between hepatic and extra-hepatic sites for specific regulation of coagulation factor genes. The functional effects of the dietary treatments were clearly demonstrated by a comprehensive functional evaluation of the coagulation pathway in plasma, integrating changes in hemostatic components and potentially of clinical value to monitor the degree of blood hypercoagulability. We observed significantly prolonged initiation and propagation phases of thrombin generation, which exploit the enzymatic

and cofactor activity of FVII and FVIII respectively. In spite of the reduction of FVII and FVIII levels over time and of the estimated influence of these factors on thrombin generation rate, we did not observe significant relations between the variations thrombin generation times and of FVII and FVIII levels. The exceeding numbers of components, potentially modified by the intervention and integrated in the thrombin generation assay, could mask these relations. Some limitations have to be taken into account when interpreting our findings. The sample size, limited by restrictive inclusion criteria and particularly by alcohol assumption and the need of persistently increased CRP levels, precludes finding of small effects of the intervention (i.e IL-6 and TF levels). On the other hand, the changes observed even in a small sample are likely to be biologically and clinically significant. Finally, the study design, without a formal control group, might be prone to outcomes modification due to secular changes even in the absence of a true intervention effect. However, the finding of specific and biologically plausible relationships does not suggest the presence of this type of bias.

In conclusion, the integrated healthy diet produced a differential effect on coagulation factors, and composite effect on metabolic and coagulation parameters. The observed variations suggest novel relationships between coagulation and inflammatory components. The intervention, a feasible approach, sensibly reduced risk factors for cardiovascular disease in middle-aged pre menopausal women, an ideal target for non-pharmacologic primary prevention.

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Chapter 3

The effects of physical training on the coagulation and inflammatory factors levels in uremic patients: a pilot study

3.1 Introduction

End-stage renal disease (ESRD) is a term that broadly defines an irreversible decline in kidney function resulting in fatal outcome in the absence of hemodialysis (HD) or transplantation. The deterioration in renal function develops in parallel with a clinical syndrome (uremia) characterized by fluid electrolyte and hormonal imbalances as well as metabolic abnormalities [1].

Endothelial dysfunction and hemostatic alterations have been described in ESRD patients on maintenance HD. In fact morbidity and mortality from atherosclerotic cardiovascular disease (CVD) is greatly increased in patients with ESRD undergoing continuous HD treatment [4]. Major causes for this excess risk include higher prevalence of several traditional and uremia-related risk factors for atherogenesis such as hypertension, hyperlipidaemia, diabetes mellitus, anaemia, increased oxidative stress and thrombotic complications [10]. The annual mortality rate due to CVD in these patients is about 9%, thus 10 to 20 times higher than that of the general population [5].

Physical function is often seriously impaired in patients with ESRD and particularly in those on HD maintenance in which exercise capacity has been reported to average only about 50% compared to normal [2].

Increased physical activity and fitness are associated with a reductions in the risk of CVD in different populations.Despite a significant progress in technological aspects of renal replacement therapy and medical advances, ESRD patients remain physically limited, with a negative impact on overall health and quality of life [3] . Life expectancy in hemodialysis patients is reduced fourfold on average versus healthy age-matched individuals [8].

Albeit different studies have shown the positive effects of exercise on different morbid conditions [8], few dialysis clinics or nephrologists provide encouragement or programs as a part of their routine care of patients.

Exercise training in HD patients have been shown to improve cardiovascular risk profile as reflected by an improvement in endothelial function [9]. Besides a reduction in the atheroprotective effect of the endothelium in ESRD patients that accounts for an increased

mortality and morbidity from CVD, it is conceivable that enhanced thrombogenesis alike plays a pivotal role in such complications.
We wanted to study whether potential relationship exists between coagulation markers and physical training.

3.2 Material and Methods

3.2.1 Patients

Thirty one ESRD patients who have undergone HD three times a week for at least one year were recruited. All enrolled patients had been treated with erythropoietin and statins for at least one year. Active smokers and patients with acute illnesses or infections, recent surgery or vascular intervention, recent myocardial infarction or unstable angina were excluded from study. Informed consent was obtained from each subject prior to screening.

3.2.2 Study protocol

This non randomized intervention took in consideration 6 month of dialysis, whose phases are briefly summarized in Fig. 3.1. After reading the study protocol 17 patients gave consent to participate in the rehabilitation program study (exercise [E] group). The remaining patients (n=14) declined but agreed to undergo the clinical investigations at the baseline and at 6 months (control [C] group). Patients in group E performed a treadmill exercise based on level walking at 1.5 km/h and with speed increments (0.1 km/h every 10 m) up to the maximum treadmill speed sustainable (i.e. within the limits imposed by claudication or fatigue). Two daily indoor/outdoor walking sessions (10 min each, at 50% of the patients maximum treadmill speed) were prescribed and performed at home. A metronome was used to convert walking speed into cadence (steps/min). Patients were asked to keep a daily record of exercise and symptoms for the duration of the study. Medical controls were performed monthly for the E group, in that occasion the maximum treadmill speed

was evaluated from the patient-supplied records. The intensity of the exercise program was updated at each visit according to the changes in exercise performance so to maintain training intensity corresponding to 50% of the maximum treadmill speed. The duration of each session remained constant throughout the study period.

No exercise was prescribed for group C.

Laboratory and functional parameters were reassessed after 6 months. Members of each working group of investigators were blinded to the results of the other investigators. The Human Research Ethics Committee of the Azienda Ospedaliero-Universitaria of Ferrara approved the study protocol.

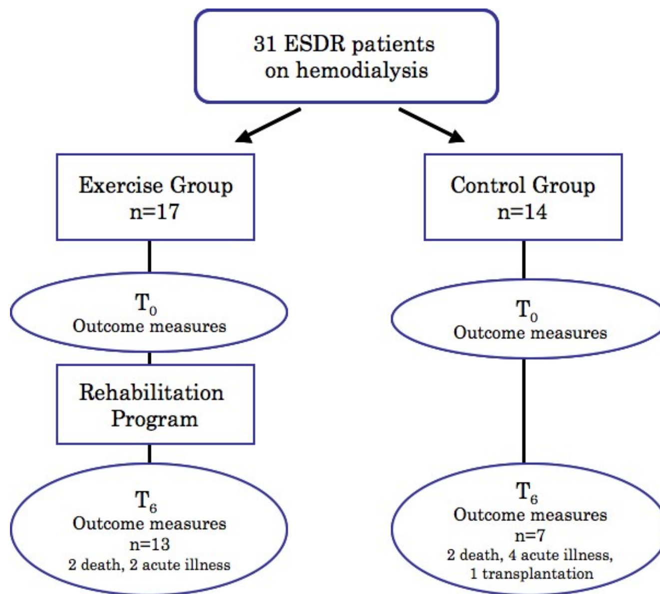

Figure 3.1: Flow chart of the study.

3.2.3 Assessment of physical capacity

Two tests validated for patients with functional limitations and a questionnaire for quality-of-life assessment were used to measure patients performance. Testing was performed in

the morning on a non-dialysis day in a temperature-controlled environment. One hour of rest between tests was fixed.

6-minute walking test

Patients were instructed to walk back and forth along a 22 m corridor alone at their own pace, without encouragement, with the aim of covering as much ground as possible in six minutes. Patients were allowed to rest in case of fatigue or pain and to continue when possible. At the conclusion the 6 minute walking distance (6 MWD) was determined.

Incremental treadmill test

The test, based on level walking, was preceded by a one-minute warm-up at a speed of 1.5 km/h. The test began at treadmill speed 1.5 km/h and it was progressively increased by 0.1 km/h every 10 meters. The test ended when the patient was unable to maintain the speed imposed for any reason (fatigue, dyspnea, claudication) thus reaching the maximal treadmill speed.

3.2.4 Laboratory examinations

Blood samples were collected pre-dialysis after overnight fasting. Plasma samples were obtained by centrifugation and stored at −80°C until tested.
Blood chemistry panel included hemoglobin concentration, high-sensitivity C-reactive protein (hsCRP), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG) levels.

3.2.5 Plasma coagulation factors

Antigen levels of TF, FVII and FVIII were determined by commercial ELISA (TF American Diagnostica, Greenwich, CT; FVII and FVIII Affinity Biologicals Inc., Ancaster, CA).

The intra-assay coefficients of variation for TF, FVII and FVIII assays were 12.8%, 7% and 8.7% respectively, while the inter-assay coefficient were 14.7%, 7.3% and 7.4% respectively. In order to evaluate vWF antigen levels we used the protocol previously described [7]. The intra-assay and inter-assay coefficients of variation for vWF ELISA assay were 6.5% and 15% respectively. Antigen levels of FVII, FVIII and vWF were expressed as percentage of healthy plasma pool.
Fibrinogen levels were measured by a prothrombin time derived method with a turbidimeter kit (Diagen, Thame, UK).

3.2.6 Thrombin generation measurement

Platelet poor plasma (PPP) samples were centrifuged at 23000 g at $4°C$ for 1 hour before testing. Coagulation was triggered in calcified PPP by addition of 1 pM recombinant human TF and 4 μM phospholipids (PC:PS 80:20) at 37C. All experiments were carried out in duplicate. Assay variability was lower than 3%.

3.3 Results

Twenty patients (13 from E group, 7 from C group) completed the study Fig. 3.1. Their baseline clinical characteristics are presented in Table 3.1.
The values of serum urea and serum creatinine are comparable to those of ESRD patients on HD. No significant differences were observed in blood values at the beginning of the study (Tab.3.1), and during the 6 months of investigation (data not shown).
The two groups did not differ significantly for the hemoglobin content and for dialysis duration. A slight but not statistical difference in age in the control group was observed.
The characteristic of patients before and after the intervention, are summarized in Tab. 3.2 and Tab. 3.3. No change in BMI neither in lipid profile were observed in both groups during the training period. The small increase in HDL-C levels in the control group was not considered clinically relevant since all patients were under statin treatment.

3.3.1 Modification of physical performance during the intervention

For an objective evaluation of functional exercise capacity we chose the *6 minute walking distance* (6MWD) test [12]. This test measures the distance that a patient can quickly walk on a flat hard surface in 6 minutes. It evaluates the global and integrated responses of all systems involved in exercise particularly the pulmonary and cardiovascular system and muscle metabolism.

At the beginning of the exercise program, subjects walked an average of 297 ± 102.4 meters, which increased significantly ($p < 0.0001$) to 334.8 ± 107.9 meters at the end of the 6 months periods. This translated into an approximate 14% individual improvement in functional performance at the end of the study, corresponding to a variation from 56% to 64% of the predicted 6MWD for healthy adults [6]. Moreover, the exercise group reported the execution of a different training duration (59±37 hours) and volume (251.136±167403 total steps) at the end of the study.

No difference in 6MWD test was observed in overall control group.

3.3.2 Modification of the coagulation proteome

In the exercise group we observed a significant decrease in FVIII antigen levels ($p< 0.05$) that is neither correlated to any performance parameters considered nor to inflammatory and anthropometric parameters. No variations were observed for vWF, a marker of endothelial disfunction, although the reported levels were higher compared to those of a healthy population used as control (data not shown). As an indication of the specificity of the observed variations in FVIII levels, a highly significant correlation between FVIII and vWF levels before ($r=0.707$, $p<0.01$) and after ($r=0.718$, $p<0.009$) the treatment was observed.

Although as a trend, FVII and FX antigen levels decreased during time too. Variations in different coagulation factor levels were also observed in the control group during the period of the study. In particular, we observed a significant decrease in FVIII levels ($p< 0.007$), parallel to FVII ($p<0.012$) and Tissue Factor ($p<0.024$). By contrast, the higher fibrinogen levels remains unchanged throughout the study in both groups.

In order to monitor the overall functional of the coagulation system, a recently developed assay that measures the thrombin generation (THG) potential in plasma samples was chosen [11]. Since many factors can perturb the hemostatic balance in ESRD, the potential of an individuals plasma to generate thrombin might represents an informative tool in highlighting the effects of exercise.

A typical THG curve is characterized by a lag phase which correlates with the plasma clotting time, a thrombin peak which reflects the propagation and subsequent termination of the coagulation cascade, and the endogenous thrombin potential (ETP), a measure of the total enzymatic work performed by thrombin during its generation.

We observed at the beginning of the study a complete different thrombin generation potential between the two groups. In particular we observed very low levels of ETP and Peak in the exercise group and higher lag time and time to peak. After the exercise period a significant increase in ETP and Peak levels and a decrease in times as a trend are observed. An increase in THG times parameters were also observed in the control group. The thrombin potential between the two groups became comparable after 6 months.

Since data about plasma coagulation inhibitors are not available the interpretation of the variation in thrombin generation parameters observed (i.e. lag time and ETP) it is even more difficult.

3.4 Study limitations and discussion

The purpose of this study was to evaluate in a pilot study the influence of a controlled physical training on a wide range of blood coagulation parameters in a population high risk of CVD. Although only twenty patients had completed the study, the sample size is

Parameter	Exercise	Control
Subjects	12	7
Age (years)	59.8±11.2	69.9±8.2
Dialysis duration (month)	69.5(47-104)	96(25.5-143)
Creatinine (mg/dL)	9.1±2.6	10.3±1.2
Urea (mg/dL)	143.6±36.8	145.4±39.6
Hb (g/dL)	12.5±1.1	12.0±1.3

Table 3.1: Clinical and serum laboratory characteristics of the exercise and the control group studied at the beginning of the study. Data are all expressed as mean±SD with the exception of the dialysis duration express as median and interquartile range.

comparable to the majority of clinical studies that have evaluated the impact of exercise on HD patients. We are aware that ESRD patients are affected by different comorbidity that can act as confounding factors in the evaluation of the above mentioned parameters, which deal together with the small sample size of the study, represents a limitation of this work.

The results suggest the presence of some common factors independent from physical exercise in the two groups that modulate the coagulation phenotype. The lack of association between physical exercise and coagulation phenotype observed in our study, cannot exclude that exercise is not able to modulate blood coagulation in the ESRD population *per se*.

Albeit independent from physical exercise, the extremely significant variations in FVIII ($p=0.002$) and to a lesser extent of FVII ($p=0.006$) and FX ($p=0.036$) levels in the whole ESRD population, prompted us to reconsider the study results by collapsing the two groups (group E and C) together Fig. 3.2. In order to have reference values for these variations, we investigated these antigen levels in pre-uremic patients (i.e. patients with uremic symptoms but not on HD treatment; group P; n=22). It is worth noticing that FVIII antigen levels in group E+C were significantly higher compared to group P at the beginning of the study ($p<0.006$) and reached levels comparable to those of group P after 6 months.

Parameter	T_0	T_6	P
BMI (kg/m^2)	25.7±4.0	25.5±3.6	–
hsCRP (mg/dL)	1.2 (0.4 - 1.4)	1.1 (0.4 - 1.18)	–
TC (mg/dL)	168.9±56.1	168.9±51.9	–
TG (mg/dL)	175 (122 - 189)	175 (117 - 244)	–
HDL-C (mg/dL)	46.8±14.9	42.8±15.5	–
Fibrinogen (mg/dL)	504.5±94.2	502.3±92.8	–
FVIII (%)	244.9±85.2	206.2±46.4	0.05
vWF (%)	229±65.1	232.7±60.8	–
Tissue Factor (pM)	5.7±2.1	5.6±1.5	–
FVII (%)	129.8±36.4	118.7±28.5	–
FX (nM)	8.6±2.4	7.8±2.0	–
Lag Time (min)	12.1±6	10.1±2.2	–
ttPeak (min)	16.5±7	13.8±2.2	–
ETP (nmol*min)	766.5±284.2	1135.6±317.9	0.006
Peak (nmol)	101.2±62	171.0±66.2	0.013
6MWD (m)	297±102.4	334.8±107.9	0.001
MT speed (km/h)	–	66.9±7.7	–
Training duration (h)	–	59.0±37	–
Training volume (steps)	–	251136±167403	–

Table 3.2: Antropometric, inflammatory, hemostatic and training parameters measure in the *exercise group* during the study. Data expressed as mean±SD or as median and interquartile range (25th and 75th quartile). P values refer to paired T test.

Parameter	T_0	T_6	P
BMI (kg/m^2)	26.9±4.0	27.1±8.1	–
hsCRP (mg/dL)	0.5 (0.4 - 1.0)	0.9 (0.5 - 1.2)	–
TC (mg/dL)	189.6±51.1	202.1±45.2	–
TG (mg/dL)	283 (155 - 337)	206(149 - 295)	–
HDL-C (mg/dL)	45.4±13.5	50.6±12.6	0.01
Fibrinogen (mg/dL)	504.5±94.2	502.3±92.8	–
FVIII (%)	209.2±63.2	117.3±38.6	0.07
vWF (%)	257.9±76.2	221.0±49.6	–
Tissue Factor (pM)	7.4±2.8	6.0±2.4	0.024
FVII (%)	129.8±36.4	118.7±28.5	0.012
FX (nM)	10±3.3	7.4±1.6	–
Lag Time (min)	6.5±1.9	10.1±1.6	0.002
ttPeak (min)	9.8±2.7	13.7±1.9	0.002
ETP (nmol*min)	1423.9±393.3	1137.6±372.3	–
Peak (nmol)	239.4±88.2	173.7±73	–
6MWD (m)	274.7±69.5	271.3±75.8	–
Training duration (h)	–	–	–
Training volume (steps)	–	–	–

Table 3.3: Antropometric, inflammatory, hemostatic and training parameters measure in the *control group* during the study. Data expressed as mean±SD or as median and interquartile range (25th and 75th quartile). P values refer to paired T test.

Increased levels of FVIII, FVII and FX have been associated with a hyper coagulability, and have been correlated to an enhanced risk for thrombotic events. These observations underlying the potential importance of FVIII, FVII and FX r reduction particularly in the CVD-prone ESRD population.

Even though the study does not provide us with elements useful for interpretation of data, and particularly the highly significant FVIII decrease, the following experimental considerations support that our observation is not spurious:

i) Antigen levels indicate differences in protein biosynthesis/half life

ii) All the tests are done at least in duplicate using plasma sample never thaw before

iii) Fibrinogen and vWF antigen values, that are known to be affected by pre-analytical variables remain constant overtime

Within all the possible explanations for the observed reductions, we do think that changes in the dialytic procedure are the most likely. Although we did not observe any difference in serum urea and creatinine, crude markers of renal function, we cannot exclude that other factors can act most specifically on clearance of coagulation factors without any difference in these parameters. An extensive evaluation of the dialysis parameters as changes in the dialytic membrane or the associated pharmacological therapies, in team with nephrologist specialists help are still necessary in order to interpret the observed results.

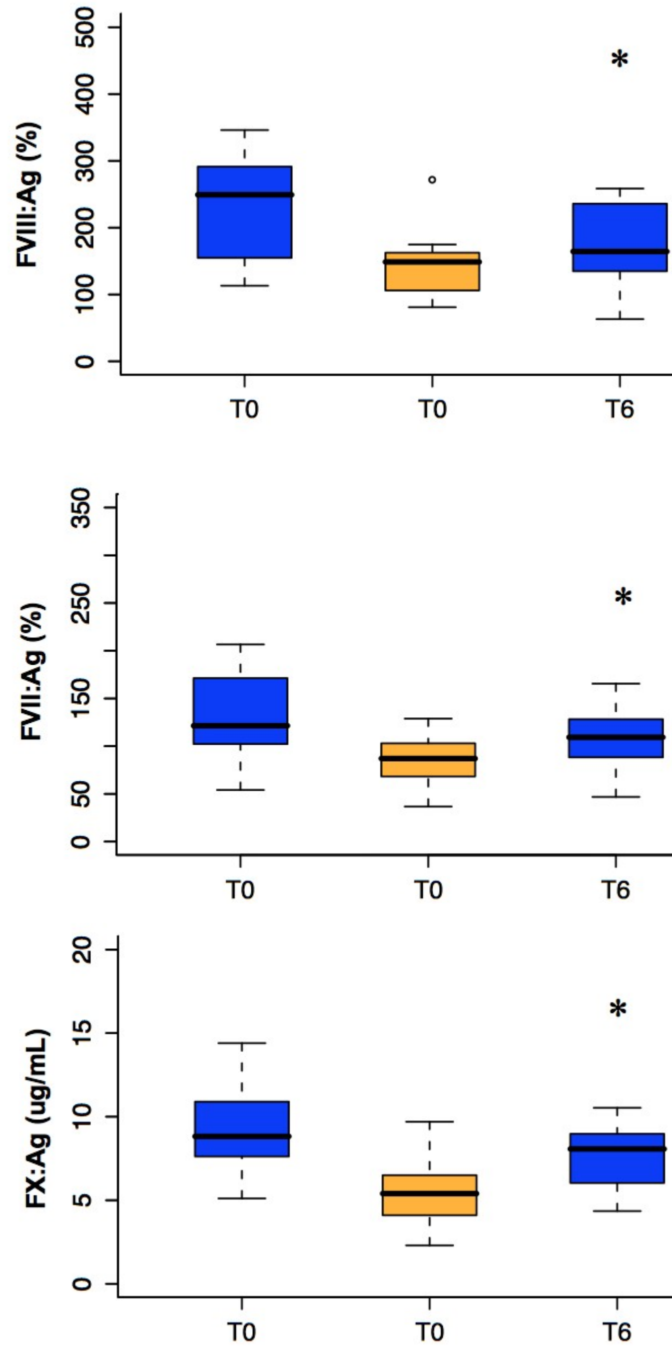

Figure 3.2: Coagulation factors levels variations overtime in a uremic (blue) and pre-uremic (orange) populations. The black line inside the boxes represents the median value and distribution outliers are indicated with blank circles (o). The asterisk * indicates a p<0.05 (paired t-test) between T0 and T6 in uremic population.

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Chapter 4

The Blood Coagulation Factor V

The coagulation Factor V (FV) was discovered by Paul Owren, the first in describing a case of FV deficency in 1947 [1].

At that time the dominant theory of blood coagulation reaction involved only four factors: fibrinogen, prothrombin (PT), thromboplastin (TF and phospholipids) and calcium. Using a relatively primitive technology, Owren deduced that the bleeding disorder affecting his patient was not explainable by deficiency of any of the four classical factors. Therefore, he postulated the existence of a new factor, called Proacellerin (Factor V), in order to explain the cause of the deficiency. In the following years lots of efforts were put on understanding the role of FV. However, due to the extreme sensitivity of FV to proteolytic enzymes, all the attempts to purify the protein kept failing. In addition, the cloning of the human FV cDNA was reported only in 1987. Therefore, the purification and characterization of both bovine and human FV are recent accomplishments.

4.1 Gene and Biosynthesis

FV is synthesized by hepatocytes, the main responsible cell type for protein secretion in plasma [2].

The coagulation FV is encoded by a gene, approximately 80 kb in size, composed by 25 exons and 24 introns and mapped on the long arm of chromosome 1 (1q23) [3].

After transcription a mature mRNA (6.8 kb), is encoded in a protein of 2224 amino acids (aa), called pre-procofactor. The pre-procofactor includes a 28 aa signal peptide that is cleaved off after translocation to the endoplasmic reticulum (ER) where it undergoes multiple post-translational modifications, Figure 4.1. The bovine, murine FV cDNA and amino acid sequences were found to have similar characteristics [4] [5].
The FV molecule has 37 potential sites for N-linked glycosilation, known to influence FV cofactor function. Different glycosilation of the C2 domain results in the formation of two functionally different forms of FV, called FV1 and FV2 [6]. Both these iso-forms circulates in plasma and in platelets at a 33 : 67 molar ratio [7].
Like different secreted proteins FV undergoes tyrosine sulphation, a post-translational modification shown to influence FV activation by thrombin. Its inhibition results in a FV molecule with one fifth of the activity compare to the native molecule [8].
Correctly folded FV leave the ER for the ER Golgi intermediate compartment (ERGIC). Trafficking of FV (and FVIII) from ER to ERGIC is dependent on a heterodimeric protein consisting of *mannose binding lectin 1* (LMAN1) and *multiple coagulation factor deficiency 2* (MCFD2) [9]. The heterodimer LMAN1-MCFD2 interacting with B domain, specifically captures correctly folded FV in the ER lumen. This cargo-containing complex is then packaged into COPII-coated vesicles for budding. Deficiency of either LMAN1 or MCFD2 causes combined FV and FVIII deficiency.
In the Golgi apparatus FV is additionally modified by the formation of disulfide bridges. FV has 19 cysteine residues. Five of them present as free SH and the remaining 14 involved in disulfide bridges by the formation of several loops. These seven disulfide bridges are conserved in human CP and FVIII as well as in bovine and murine FV. The C1085 has been shown to be involved in the interaction with Multimerin-1, in the platelet α-granules. Once FV leave the Golgi, it is secreted by the cell.
Following secretion, plasma FV is then phosphorylated. In particular, FV is phosphorylated at the S692 residue by a membrane-associated platelet casein kinase II (CKII) enzyme. This covalent modification increases the rate of inactivation of the FV by activated protein C (APC) [10]. FV is also phosphorylated by an intra-platelet protein kinase

C (PKC) and does not appear to affect either the FVa activity in the prothrombinase complex or its sensitivity to APC [11] .

FV circulates in plasma as a free inactive procofactor at a concentration of $20-25\ nmol/L$. In *whole blood* FV is distributed between two pools; approximately 80% is found in plasma, while the remaining 20% is found within the α-granules of platelets.

Platelet FV originates from the plasma pool, and particularly from a specific internalization process by megakaryocytes. After endocytosis, FV gains several unique modifications that make platelet FV structurally and functionally different from plasma FV [12].

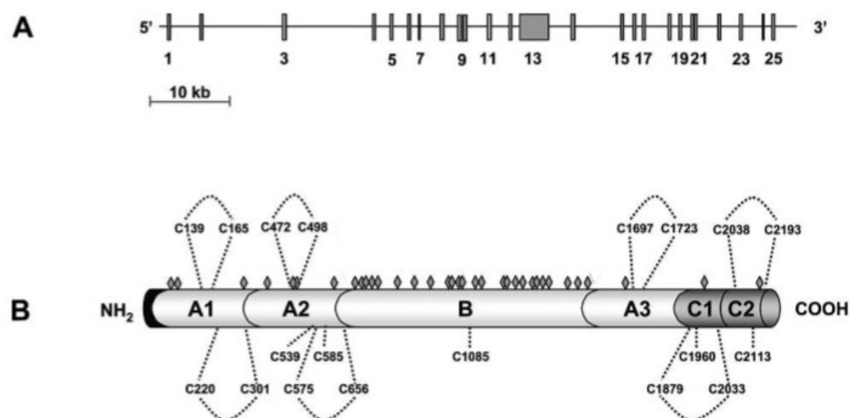

Figure 4.1: FV gene and post translational modifications occuring inside the cell, figure from [7].

A. Schematic representation of the exons (grey boxes) introns (lines) structure of the FV gene. B. Organization of FV protein with indicated post translational modification sites: N-glycosilation sites (grey rhombs) and disulfide bridges (dotted loops).

4.2 Structure

The coagulation FV consists of a single chain glycoprotein (molecular weight 330 kDa), $13-25\%$ of which is accounted for by the carbohydrate moiety.

FV has a mosaic-like structure, composed by three homologous A domains, two smaller C domains and a large B domain, Figure 4.2.

The same domains organization (A1-A2-B-A3-C1-C2) is well conserved in the coagulation factor VIII (FVIII) and is similar to that of ceruloplasmin (CP), a copper binding plasma protein, Figure 4.2 [13] [14]. Moreover, the primary structure of FV and FVIII A domains

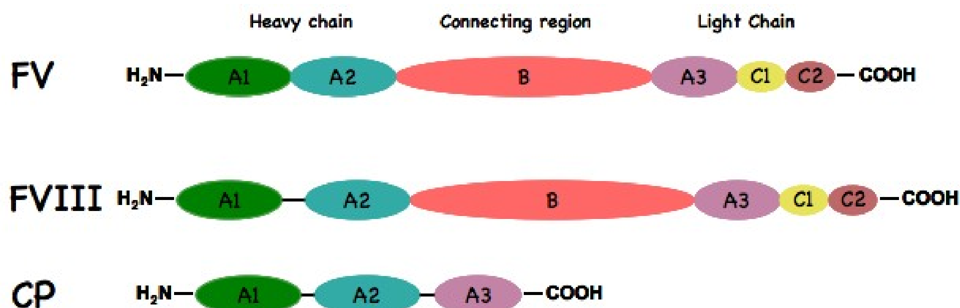

Figure 4.2: Diagram of domains structure of human FV, FVIII and CP

are highly homologous and share about 40% sequence identity with CP; while the primary structure of FV C domains share 46% sequence identity with their FVIII counterparts and belong to the discoidin family of phospholipids binding lectins [14].

The B domain is poorly conserved among the various species in which FV has been studied, and share low sequence identity (15%) with the FVIII B domain, while it is missing in CP [3].

The high homology and the well conserved domains organization indicate that these proteins evolved from the same common ancestral protein [15] .

Although the three-dimensional structure of the FV A domains has not been solved yet, a model has been developed using the X-ray structure of human CP as template [16]. The A domains (~350 aa) of FV are arranged in a triangular fashion and consists of two linked cupredoxin-like folds (a typical copper-binding protein motif also present in CP), which are essentially β-barrels structures. Like CP, FV is able to bind with high affinity, a single copper ion per molecule although an effect of Cu^{2+} binding on function has not been reported yet.

The two C domains (∼150 aa) of FV consists of eight anti-parallel strands arranged in two β-sheets of five and three strands which together form a β-barrel structure [17]. The upper surface of the barrel is composed by several salt bridges, whereas the lower part is rich in basic residues and characterized by three adjacent loop-structures consisting of a β-hairping structures with hydrophobic aa residues (W2063, W2064, L2116) located at their tips.

The isolated FV C2 domain is known to crystallize in two distinct conformations [18]. In the open conformation, the hydrophobic residues are solvents exposed, whereas in the closed conformation they are tilted inwards to form a hydrophobic ridge, in order to minimize solvent-exposure of apolar side-chains. It has been suggested that the membrane bound, C2 domain, acquires the open conformation, while the closed conformation corresponds to the C2 domain of free circulating FV.

In this way the A domains are present at a height above the phospholipid membrane whereas the two C domains, arranged "side-by-side", provide the interaction platform with a phospholipid membrane, that is essential in the formation of the macromolecular complexes of the blood coagulation, Figure 4.3.

When the coagulation cascade is activated, the circulating FV is specifically cleaved by FXa or Thrombin. This cleavage removes the B domain and releases the activated form of FV (FVa), which consist of a heavy chain (A1-A2, 105 kDa) and a light chain (A3-C1-C2, 71-74 kDa) non covalently associated in the presence of Ca^{2+} ions, Figure 4.3.

4.3 Activation

FV circulates in plasma as inactive procofactor, expressing less than 1% of its maximal procoagulant activity. The activation of the intact FV procofactor is an essential step for the expression of its full activity in the prothrombinase complex (see next section) [19].

Thrombin and FXa are considered to be the most potent physiological activators of FV, although several other proteolytic enzymes can be used to activate FV in vitro. These include α-chymotrypsin, plasmin, cathepsin, elastase, the TF:FVIIa complex, different snake

venoms, and several proteases derived from platelets, endothelial cells and neutrophils [].
The activation involves the elimination of the B domain and the Ca2+ dependent association of the resulting amino-terminal and carboxyl-terminal peptides in a heterodimer, Figure 4.3.
The two principal FV activators differ for various functional features (activation conditions, mechanism of action and rate of catalysis). Nonetheless they give rise to molecular species with the same biological activity [12].

4.3.1 FV activation by Thrombin

The principal and most potent activator of circulating plasma FV is thrombin. Thrombin-mediated activation of human FV does not require phospholipids. It takes place via three

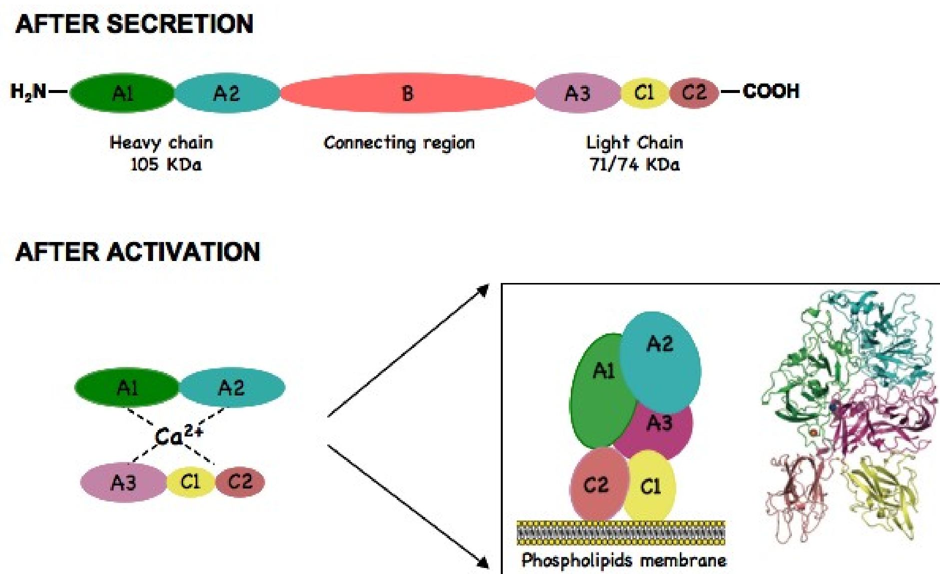

Figure 4.3: Diagram of FV domain organization after secretion from the cell (single-chain 330 kDa), and after activation (heterodimer composed of a heavy and a light chain held together by a single calcium ion). Three-dimensional model of membrane-bound FVa, obtained by molecular dynamics simulations [55]. The domain color code is the same: A1, green; A2, cyan; A3, magenta; C1 yellow; C2, pink.

consecutive proteolytic cleavages (R709, R1018 and R1545) and the formation of two intermediates, Figure 4.4.

The first cleavage cuts the peptide bond between R709 and S710 and separates a 105 kDa amino-terminal peptide, from a 280 kDa intermediate. The latter is further cleaved between R1018 and T1019, giving rise to a 71 kDa activation peptide and a second 220 kDa intermediate.

The single cleavage at R709 or R1018 bring about very small increases in the FV procoagulant activity, whereas the combination results in partial FV activation (30%). Cleavage at these sites appears to be required for rapid thrombin mediated cleavage at R1545, which correlates with the expression of full FVa activity.

The proteolysis between R1545 and S1546 produces 71/74 kDa carboxyl-terminal portion of the protein from a heavily glycosilated 150 kDa activation peptide.

Thrombin-catalyzed activation of FV obeys Michaelis-Menten kinetics according to the parameters shown in Table 4.1.

Inhibition of thrombin-catalyzed FV activation by bothrojaracin and hirugen (both bind specifically thrombin-exosite I), but not by heparin (that binds specifically thrombin exosite II), indicate that FV binds directly to thrombin exosite I. This interaction, which is mediated by the FV heavy chain and involves the Tyr-O-sulfate-containing region, upstream the FV activating cleavage sites, contributes to specific substrate recognition by

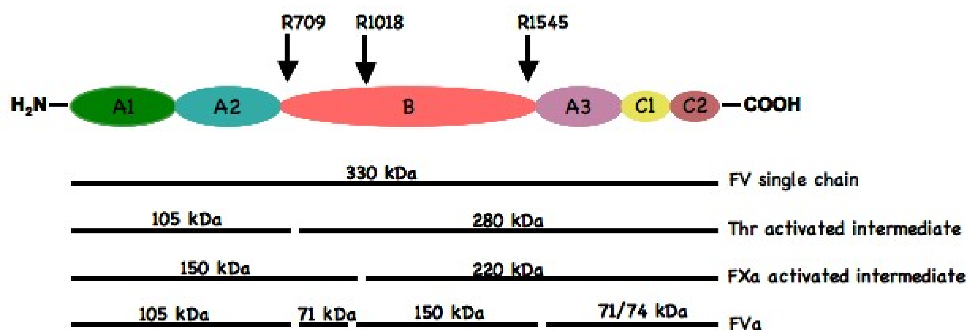

Figure 4.4: Schematic representation of FV activation intermediates produced by cleavage at one or more of the thrombin/FXa cleavage sites.

thrombin and accelerates cleavage at R709 and at R1545.

4.3.2 FV activation by FXa

Differently from thrombin, FXa has the ability to activate FV only in the presence of Ca2+ ions and negatively charged phospholipids.

The activation process proceeds via initial cleavage of R1018, producing a 150 kDa intermediate and a 220 kDa peptide. It is followed by cleavage at R709 that liberates the 105 kDa heavy chain, as well as a 71 kDa activation peptide from the 150 kDa fragment. Differently from α- thrombin, the cleavage at R1545 appear to be absent or very slow. FXa-mediated activation of human FV occurs at a 5-fold lower rate than the thrombin-catalyzed reaction, Tab. 4.1.

Activator	Km (nM)	kcat (min^{-1})	kcat/Km ($M^{-1}s^{-1}$)
α-Thrombin	71.1	14.0	$3.26x10^6$
FXa	10.4	2.6	$4.14x10^6$

Table 4.1: Kinetic parameters of FV activation by Thrombin and FXa. The kcat of FV is five-fold higher for thrombin than for FXa, whose affinity for the substrate, however is significant higher: thus the catalytic efficiency of the two enzymes turn out to be similar.

4.3.3 FVa interaction with metal ions

The resulting FVa is a non-covalent heterodimer, composed by an HC and LC, held together in the presence of divalent cations (Ca^{2+} and Cu^{2+}). Bovine FV and FVa have a single high affinity site for Ca^{2+} [21], which must be occupied for the FVa subunit association and consequent activity [22]. Ca^{2+} does not interact with either FVaH or FVaL alone [23], suggesting that the Ca^{2+}-binding pocket is formed by subunit association. FV also binds with high affinity to one Cu^{2+} atom per mole of protein [24], although an effect of Cu^{2+} binding on function has not yet been reported.

Although the FVa HC and LC interaction is required for prothrombinase function, little is known about how the divalent cations precisely play their biochemical role.
Based on several theoretical models for intact human FVa, there is consensus that an acidic A1 domain loop is involved in Ca^{2+}-binding. The loop encompasses E96, D102, E108, D111 and D112, and is predicted to be located at the A1-A3 domain interface, Fig.4.5. Most of the structural modeling studies also suggest that the bound Ca^{2+} and Cu^{2+} are located close to each other.
More recently it has been shown that a single point mutation that substitute D111 into an alanine, affects both Ca^{2+} and Cu^{2+} binding. This consequently weaken the inter subunit stability (fast heterodimer dissociation, $2.1 \pm 0.05\ min^{-1}$) and the FVa activity [25].

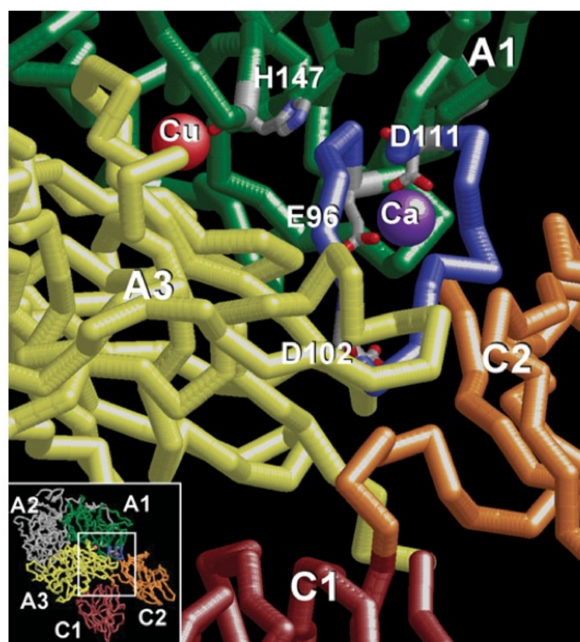

Figure 4.5: Structural model of FVa metal binding, figure from [25].

4.4 Procoagulant FV activity

The activated FV contributes to the blood clotting reaction by binding with FXa on a membrane surface to form the prothrombinase complex, the essential activator of prothrombin.

FVa is considered the essential FXa cofactor, inasmuch as its presence in the prothrombinase complex enhances the catalytic efficiency by several order of magnitude, Table 4.2.

According to a kinetic model, FXa and FVa would bind independently to negatively charged phospholipid membranes. These associations represent the rate-limiting step of prothrombinase assembly, however the FVa-FXa protein-protein interaction is relatively weak in the absence of a membrane surface (Kd 0.8 $\mu mol/L$) and probably not relevant at biological concentrations [26].

Both FV and FVa bind with high affinity (Kd 10^{-9}, 10^{-10} mmol/L, respectively) to negatively charged phospholipid membranes in a calcium-independent way [27] [28].

Specific phospholipid-binding site are found in the C1 domain (Y1956-L1957), as well as in the C2 domain (W2063-W2064). Site-specific mutagenesis on C2 domain binding sites has been reported to result in impaired ability to interact with phospholipids and promote the assembly of a functionally competent prothrombinase complex. However, when phospholipids binding residues in both C domains are mutated FVa activity is abolished.

Both anionic (PS) and neutral (PC) phospholipids must be present in the membrane for optimal binding, phospholipids layer penetrance of FVa and correspondingly for an optimal activity of prothombinase complex. Impairment of PS exposure on the outer surface of activated platelets, known as Scott syndrome, is associated with inefficient prothrombin activation and bleeding.

After the interaction with the membrane, FVa and FXa associate to form the active prothrombinase complex, which consists of FVa and FXa in a 1 : 1 molar ratio.

A fundamental contribution of FVa to prothrombinase function is the retention of FXa on the membrane surface. Both FVa and FXa interact with PC / PS vesicles at diffusional controlled rates ($10^7 - 10^8$ $M^{-1}sec^{-1}$). However FXa has an affinity (Kd) for membranes of

approximately 0.1 $\mu mol/L$, corresponding to a dissociation rate constant of approximately 3.3 sec^{-1}. As a consequence, the duration of retention of FXa alone on a membrane is short lived even though the membrane association process is rapid. Moreover the interaction of FVa with the membrane surface and FXa effectively increases the affinity of the enzyme for the phospholipid bilayer.

The interaction of FXa and FVa involves two regions in the FVa A2 domain. One FXa-binding site maps to the FVa surface between the APC-cleavage sites at R306 and R506, whereas the other is located proximal to R506, but on the opposite side of A2 domain.

During the assembly of the prothrombinase complex there is no direct interaction between FXa and PT, and substrate recognition is mediated by FVa heavy chain (residues 683-709). After inactivation of FVa by activated protein C (APC), the A2 domain dissociates and the PT binding is lost.

Prothrombin promotes the stability of the prothrombinase complex only if it is assembled on a phospholipid surface containing a low mole fraction of PS. On optimal phospholipid membrane, prothrombin affects neither the assembly, nor the stability of the complex.

Ca^{2+} ions play a key role in the organization of the prothrombinase complex. In fact, Ca2+ is required for both FXa and PT binding to phospholipids and ensures the association of the FVa heavy and light chains, which is essential for the interaction with FXa. The optimal Ca2+ concentration for the assembly and activity of the prothrombinase complex is 2-3 mmol/L.

In summary, the mechanism by which FVa executes its procoagulant role is by:

1. increasing the binding of FXa to the membrane surface,
2. increasing the catalytic efficiency of FXa,
3. enhancing the binding of Prothrombin to the complex.

Activating component	k_m (μM)	V_{max} ($mol^{-1}s^{-1}$)	k_{cat}/k_m (M^{-1} s^{-1})
Xa	84	0.011	131
Xa, PL	0.06	0.038	6.3x10^5
Xa, PL, FVa	0.21	32	1.5x10^8

Table 4.2: The effects of different components of prothrombinase complex on the kinetic of prothrombin activation [56], [57]. PL, phospholipid membrane.

4.4.1 FVa inactivation

Accelerating the PT conversion of several orders of magnitude, the activity of FVa needs to be tightly regulated. This is the task of the system of activated protein C (APC) [29]. Interacting with the light chain of FV (aa residues 1865-1874) [30], APC inactivates it *via* limited proteolysis of the heavy chain (R306, R506 and R679), Figure 4.8 [31].

The R506 cleavage is kinetically favored, but only partially inactivates FVa by reducing the binding affinity of FXa, around 40 fold. The procoagulant activity of this form is dependent on the amount of FXa present and is around 40% at high concentration of FXa (5 nM) [31]. APC cleavage at R506, has been postulated to facilitate the cleavage at R306. This causes a ~7 fold reduction in FXa binding affinity and no changes in the catalytic efficiency in prothrombin activation. However, when both cleavage have taken place, the FXa affinity is decreased by about 100 fold and the k_{cat} by about 10 fold. The biological importance of the third cleavage at Arg679 is not fully understood, but appears to be of less importance for the physiological inactivation of FVa. The reduction in FXa affinity and in the catalytic efficiency of the prothormbinase complex explains the inhibitory effect of APC on FVa activity. The dissociation of A2 domain following R506 and R306 cleavage, represents only a modest contribution to this inactivating mechanism. However, in case of R506Q mutation (see next subsection), the A2 dissociation becomes more important.

APC catalyzed FVa inactivation is a calcium-dependent reaction which can be modulated by several other molecular components, such as phospholipids, FXa, PT, heparin, platelets

and the FV isoforms (FV1/FV2).

The presence of PS containing phospholipid vesicles increases the rate of cleavage at R306 ∼1000 fold and the rate of R506 ∼100 fold. Moreover the incorporation of phosphatidylethanolamine (PE) into vesicles does also enhance the inactivation of FVa.

The rate of APC-catalysed FVa inactivation is increased ∼ 200 fold in the presence of phospholipids and is also modulated by the composition of phospholipids [32] [33]. Another important modulator of APC function is the protein S, that as nonenzymatic cofactor of APC [34]. The protein S effect is highly phospholipid-dependent and is mediated by a selective 20-fold stimulation of APC-catalysed cleavage at R306, and only a weak stimulation (∼ 2 fold) at R506 . Therefore, the role of protein S become relevant in the inactivation of the natural variant R506Q.

The selective potent stimulation of the R306 cleavage by protein S has been suggested to be due to a protein S-mediated relocation of the active site of APC closer to the membrane Figure 4.7. According to this hypothesis, the R306 is located at a shorter distance to the membrane surface than the R506 site, a distance that is favorable for cleavage by the APC-protein S complex. The efficient kinetics of the R506 cleavage and its independence of protein S have been proposed to be explained by a positive cluster of lysines in the 37- and 60- loops of the serine protease domain of APC interacting with an undefined negatively charged area in FVa close to R506.

Incorporation of FVa in the prothrombinase complex partially protects FVa from APC-catalyzed inactivation.

In particular when FVa is incorporated in the prothrombinase complex, it is protected from APC-mediated inactivation by interactions of FXa [34] [35], which specifically protects the R506, and prothrombin[36], which inhibits cleavage at both R306 and R506. However these protective effects are abrogated by protein S that influences the cleavage at R506.

Alternative mechanisms of FVa inactivation are also induced by thrombin or by the fibrinolytic protease plasmin. In particular, it has been recently described that the cleavage at Arg643 by thrombin, in the presence of endothelial cells, results in reduced affinity between the heavy and the light chains [37]. Plasmin inactivation of FVa was analyzed only

in the bovine FVa, demonstrating the critical role of the amino acid region 307348 (bovine numbering) [38].

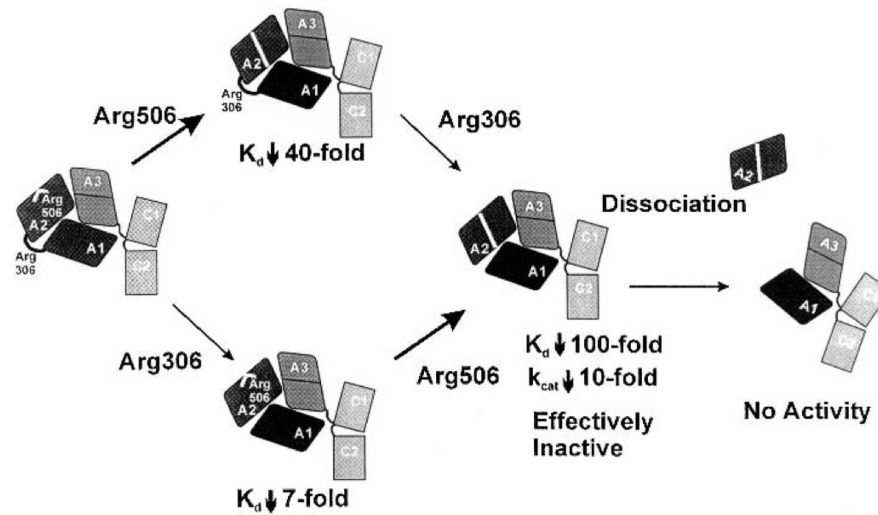

Figure 4.6: Model of the mechanism of inactivation of FVa by APC, figure from [58].

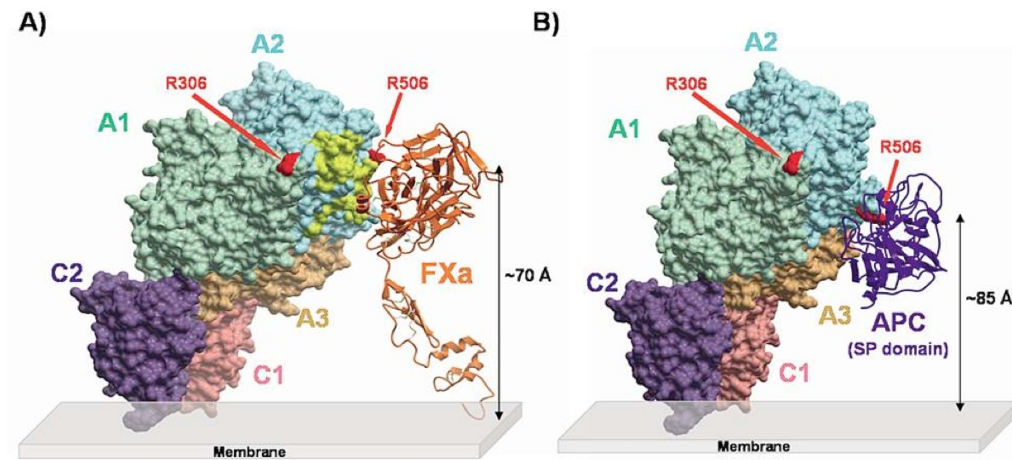

Figure 4.7: Three-dimensional model of the FVa-FXa (A) and FVa-APC (B) complexes, figure from [7].

4.4.2 APC resistance

Resistance to activated protein C is defined as a poor anticoagulant response of plasma to exogenously added APC and is inherited in an autosomal dominant mode. The prevalence of of this condition in the european population is from 5% to 10% while among patients with venous thrombosis is 20% to 60%. For this reason APC resistance is considered the most common risk factor for venous thrombosis [39].

Hereditary APC resistance is most often associated with carriership of the FV R506Q (FV Leiden) mutation, however there are several other conditions (both genetic [40] [41] or acquired [42] [43]) that can interfere with the expression of APC activity, causing APC resistance.

The molecular mechanisms underlying FV Leiden-associated APC resistance have been well characterized. The FV Leiden mutation ,due to the substitution of adenine to guanine at nucleotide 1691, abolishes the predominant APC-cleavage site (R506) in FVa [44]. Experiments in model systems have indicated that FVa Leiden can be fully inactivated by APC via slow cleavage at R306. Although the inactivation rate is slower compare to the normal FVa, this difference is largely eliminated in the presence of protein S. On the other hand, the absence of the R506 cleavage site prevents the conversion of single-chain FV Leiden into a functional APC-cofactor, resulting in an inefficient inactivation of FVIIIa (see next section).

Therefore, the FV Leiden mutation interferes with both FVa and FVIIIa inactivation.

Impaired APC-cofactor activity of FV Leiden also importantly contributes to the thrombosis risk associated with FV Leiden carriership in vivo, as suggested by the rare condition known as *pseudo-homozygous* APC resistance [45].

FV Leiden pseudo-homozygotes are heterozygotes whose non-FV Leiden allele is not expressed due to a null mutation, resulting in the presence of 50% FV Leiden (and no normal FV) in plasma. Although FV Leiden pseudo-homozygotes have as much FV Leiden in their plasma as heterozygotes, their thrombosis risk resembles that of FV Leiden homozygotes, suggesting that thrombosis risk is determined not so much by the absolute amount

of FV Leiden, but rather by the presence or absence of normal FV capable of expressing APC-cofactor activity.

4.5 Anticoagulant FV activity

In addition to its role as a precursor of procoagulant FVa, circulating FV has an anticoagulant role, Figure 4.8. This new function was discovered observing that the addition of FV to APC resistant plasma, dose dependently restore the sensitivity to APC [46].
FV acts together with protein S, as enzymatic cofactor of APC in the inactivation of FVIIIa. 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.
The anticoagulant APC cofactor function of FV is lost after FV activation by FXa or thrombin. In particular, the dissociation of the B domain resulting from the cleavage at R1545, is responsible for the complete loss of anticoagulant activity, whereas the other two cleavages (R709 and R1018) do not affect this activity. Using recombinant FV variants, the highly acidic C-terminal part of the B domain (residues 1477 to 1545) was found to be essential for the anticoagulant APC-cofactor activity of FV. Structural data suggest that this region forms a solvent-exposed loop that may interact with APC and that would be disrupted following thrombin-mediated FV cleavage at R1545 [47].
While APC-catalyzed cleavage of FV at R306 and R679 has no significant effect on the anticoagulant FV role, cleavage at R506 increase the APC-cofactor activity of FV of 10 fold. This indicates that APC cleavage at R506 is essential for the expression of the APC cofactor activity of FV and explains why FV Leiden is a poor APC cofactor in FVIIIa inactivation.
In summary, FV is either activated to procoagulant FVa by thrombin or FXa or render anticoagulant APC cofactor by APC catalyzed cleavage at R506 (FV_{ac}).
In this pathway, the local concentrations and availability of procoagulant and anticoagu-

lant enzymes, such as thrombin, FXa and APC, determine the fate of each FV molecule, Figure 4.8 [48].

Thus, FV acts as a local sensor of procoagulant 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 procoagulant or an anticoagulant cofactor.

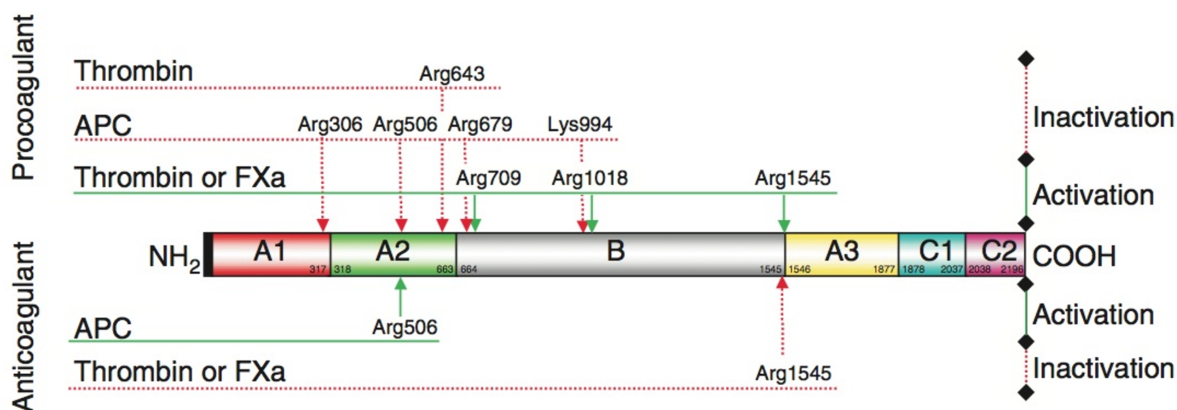

Figure 4.8: Schematic representation of all the proteolytic cleavages of FV responsible for its activation and inactivation, figure from [50].

4.6 FV deficiency

FV deficiency, or para-hemophilia, is an autosomal recessive bleeding disorder, caused by loss-of-function mutations in the FV gene [49].The prevalence is likely $1 : 10^6$ individuals in the general population, however it seems to be underestimated because mild cases often go undetected [50]. The majority of FV decient individuals shown a parallel reduction of FV antigen and activity levels (type I deficiency). The only example of a qualitative (type II deficiency) FV deficiency known to date is FV New Brunswick [51], which is characterized by decreased stability of FVa [52]. Moreover the congenital FV deficiency can be classified 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.

At the present more than 100 mutations were found to be associated with reduced FV levels (online F5 mutation database, January 2009 release).

These causative mutations include missense, nonsense and splicing mutations as well as small in frame insertions/deletions covering the whole F5 gene. While nonsense and frame-shift mutations are uniformly distributed throughout the gene, missense mutations, which usually impair folding and/or secretion, tend to cluster in the A and C domains and are characteristically absent from the B domain.

The phenotypic expression of FV deficiency is variable; heterozygotes are usually asymt-pomatic, whereas homozygous patients show mild, moderate or severe bleeding symptoms. The most common symptoms associated with FV deficiency are bleeding from mucous membranes (e.g. epistaxis, menorrhagia) and after surgery or delivery, which occur in approximately half of all FV decient individuals. Severe bleeding manifestations (e.g. intracranial or gastro-intestinal haemorrhages) are rare and conned to patients with undetectable FV levels.

Premature stop codon mutations, account for as much as two thirds of all FV mutations and are signicantly over-represented in the FV gene as compared to other genes [50].

Traditionally these mutations were considered null mutations, as their mRNAs are normally degraded by nonsense-mediated decay. However these defects may be actually compatible with the expression of traces of FV sufcient for minimal haemostasis [53].

Considering the pivotal role of FV in prothrombin activation, large F5 deletions or chromosomal rearrangements involving the FV gene,inducing a real FV defect are expected to be incompatible with life. Accordingly, it is currently accepted that all FV decient patients actually have some residual FV [54]. Still, it remains puzzling that many patients with undetectable FV experience only mild-to-moderate bleeding and even have a more favuorable prognosis than severe haemophiliacs.

The variable phenotype associated with low or undetectable FV levels strongly suggests the existence of a compensatory mechanisms present in human blood, which either bypass or reduce the need for FV in generating levels of thrombin required for survival.

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Chapter 5

Modulation of Factor V levels by intragenic components

5.1 Introduction

Several gene mutations have been described to affect the levels of the corresponding coagulation protein. These variations can lately result in a tendency towards a hemorrhagic or thrombotic state [1]. However, it is evident from both the literature and clinical practice that even within the same monogenic hemostatic disorder considerable phenotypic variability exists [1].

In this regard, inherited factor V (FV) deficiency presents the most variable phenotype among the different coagulation factors [3].

FV plays a pivotal role at the crossroads between the procoagulant and the anticoagulant pathway, acting as a cofactor both in prothrombin activation (procoagulant pathway) and in FVIIIa inactivation (anticoagulant pathway) [4]. This dual nature contributes to the variability in FV-associated disorders and makes the modulation of its levels in plasma of importance to maintain the hemostatic balance.

FV deficiency is typically classified as an anticoagulant defect; nevertheless, its combination with FV mutations associated with APC resistance has been associated with a more enhanced APC resistance. This rare condition is known as *pseudo-homozygous* state of APC resistance (APCR) and represents another mechanism by which the combination of different genetic defects in the same gene can contribute to the modulation of FV phenotype [5].

At present, many more deficiency-causing mutations in the FV gene have been described, both in patients with a hemorrhagic or thrombotic symptoms (*Online FV Mutation Database*). The possibility to express recombinant FV mutants in eukaryotic cells is increasingly widening our knowledge on the physiological and pathological properties of the molecule however, most of them are not characterized at the molecular level. Moreover, the FV deficiency classification in use (Type I, quantitative and Type II qualitative deficiency) is rather simplified and thus unable to include the gradient of quantitative and functional effects produced by the wide spectrum of molecular mechanisms altering FV expression. It is predicted that the gradient of effects, and their combination in the doubly heterozygous

condition, is of relevance for the clinical phenotypes associated with mutations, both for the pro-thrombotic condition and for the pro-hemorhagic defects.

Here we report the molecular characterization of a new natural variant of FV (R1698W) found *in-trans* with FV APC resistance mutations (R506Q and H1299R), in a small FV deficiency cohort. The higher FV:Ag/FV:C ratio found for the recombinant R1698W variant, suggested the presence of a dysfunctional FV *cross-reacting material negative* (CRM-). The *in vitro* characterization of this variant shows the pleiotropic effect of R1698W missense mutation, that act not only affecting FV secretion and function, but also influencing the stability of the activated FV.

5.2 Material and Methods

5.2.1 Patients and identification of new mutation on FV gene

A small group of unrelated patients affected by FV deficiency was recruited. The patients were referred for genetic analysis because of either bleeding or asymptomatic deficiency of FV:C detected during routine coagulation testing.

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 [8]. Primers located in intron 14 FIVS14 (5'- TAACCAGCCATTTTGACTTA -3') and 15 RIVS15 (5'-GAAATAACCCCGACTCTTC -3') respectively, were used to amplify a 411 bp DNA fragment spanning the whole exon 15.

A restriction protocol for the detection of the 5266 C/T mutation was obtained by means of the 15F primer (5'-AATTTAGCATCCAGACCGTATT-3') with a mutagenized primer R15TaqI (5'-AGTAGTAGGCCCAAGCTC-3'), introducing a TaqI restriction site in the normal allele (192 + 19 bp). Genomic DNA (100 ng) was amplified with Taq polymerase (0.25U) in 30 cycles in the following conditions: 30 s denaturation at 95C; 30 s annealing at 54°C; 20 s extension at 72°C. PCR products (211-bp) were digested with TaqI and separated on a 3% agarose gel by electrophoresis.

The FV R506Q and FV H1299R polymorphisms were detected as reported (Bertina 94; Lunghi 96).

Factor V antigen (FV:Ag) in plasma was measured by Laurell's technique (specific polyclonal antiserum, Istituto Behring, Scoppito, Italy). FV activity (FV:C) was measured by a one-stage clotting method (Thromborel S and FV-depleted plasma, Dade Behring, Marburg, Germany). Normal ranges for FV:Ag and FV:C levels were $70 - 130\%$. APCR was measured according to de Ronde and Bertina, [9]. Normalized APC sensitivity ratio (nAPC-sr) values >0.81 were considered as normal, while values <0.57 are found for homozygous R506Q FV condition.

5.2.2 Site-directed Mutagenesis

Mutations were introduced into the expression vector pMT2 containing the full-length cDNA of human FV and a variant lacking of part of B domain (deletion of 2441-4546 bp of FV cDNA that correlate to the in frame deletion of 756 through 1458 aa (PMT2-702)) using the QuikChange site-directed mutagenesis kit (Stratagene). For each mutant, two complementary oligonucleotide, containing the mutation, were properly design.The mutated fragments were then isolated by restriction enzymes and used to replace corresponding fragments in the template.

The sequences of the fragments were confirmed by DNA sequencing. A summary of all the FV variants, primers and the restriction system used, is shown in Tab.5.1 and Tab.5.2.

5.2.3 Expression and Quantification of rFV

Expression plasmids containing the various FV cDNA constructs were transfected into COS-1 cells using the diethylaminoethyl (DEAE)-dextran method.

After 72 h, the proteins were harvested in serum-free medium (Optimem Glutamax) and concentrated in Amicon-Ultra with a molecular weight cutoff of 100,000 (Millipore, Cork, Ireland). Aliquots were frozen at $80°C$. The concentrations of recombinant proteins were determined by ELISA.

Mutant Name	Template	Primer (5'- -3')
1698W	PMT2	TCCTGGCTCTGCCTGTTGGGCTTGGGCCTA
1698W-702	PMT2-702	TCCTGGCTCTGCCTGTTGGGCTTGGGCCTA
1698A	PMT2	TCCTGGCTCTGCCTGTGCGGCTTGGGCCTA
1698A-702	PMT2-702	TCCTGGCTCTGCCTGTGCGGCTTGGGCCTA
1698Q-702	PMT2-702	TCCTGGCTCTGCCTGTCAAGCTTGGGCCTA
1698E-702	PMT2-702	TCCTGGCTCTGCCTGTGAAGCTTGGGCCTA
1691K 1698E-702	PMT2 1698E-702	TGAGCGATCAGGGCCAAAGAGTCCTGGCTCTGC
611/610K 1698E-702	PMT2 1698E-702	CTATGGAAAGAGGCATAAGAAGACCTTGACCC

Table 5.1: The recombinant FV variants produced by site direct mutagenesis using the reported forward primers.

Mutant Name	Restriction Enzymes	Mutated Insert (bp)
1698W	SnaBI/NaeI	3000
1698W-702	SnaBI/NaeI	3000
1698A	SnaBI/NaeI	3000
1698A-702	SnaBI/NaeI	3000
1698Q-702	SnaBI/NaeI	3000
1698E-702	SnaBI/NaeI	3000
1691K 1698E-702	SnaBI/NaeI	3000
611/610K 1698E-702	SnaBI/Bsu36I	1700

Table 5.2: The recombinant FV variants and restriction enzymes used for isolating the mutated fragments. The mutated inserts were then ligate on the WT PMT2 vector, lacking the same fragment. To check the correct ligation process and to verify the presence of the wanted mutations, the vectors were then sequenced.

A polyclonal antibody (Ab8806) was used to coat microtiter plates, after a overnight incubation the plates were washed and then quenched. Samples were properly diluted and incubated at $4°C$ overnight. Standard curves were created using recombinant FV wt, using the concentration evaluated by PTH-ase assay. Biotinylated monoclonal MK30 (against the B domain, for full length FV variants) or HV-1 (against light chain, for FV B domain lacking variants) were used as secondary antibody. After streptavidin-peroxidase was added, then the plates were developed adding OPD substrate, and the absorbance was measured at 490 nm.

5.2.4 Western Blot Analysis of Recombinant Protein

Based on the ELISA evaluation, the same amount of the recombinant FV variants in HN buffer ($25mM$ Hepes, $150mM$ NaCl, pH 7.5) with 5 mM $CaCl_2$ before and after thrombin activation were subjected to SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes. Two different antibodies were used to detect the proteins, a polyclonal (Ab299, DAKO) and a monoclonal antibody against the heavy chain (AHV5146, Hematologic Tech.).
To develop the Western blots, Supersignal West Dura Extended Substrate (Pierce) was used for enhancement according to the manufacturer's instructions. The membrane developed with the chemiluminescent technique was exposed and visualized using a LAS 3000 CCD camera (Fuji Film, Tokyo, Japan).

5.2.5 Phospholipids vesicles preparation

Phospholipids dissolved in chloroform/methanol ($9 : 1, v/v$) were dried in a glass tube under a mild flow of nitrogen. The phospholipids were suspended in HN Buffer, vigorously vortexed for 2 min, and then sonicated for 10 min at room temperature at amplitude 3, using an XL 2020 sonicator (Misonix).

5.2.6 Thrombin activation of recombinant FV

Recombinant FV variants (final concentration, 1.25 nM) were incubated with increasing amounts of thrombin (0-20 U/mL) at 37C for 5-10 minutes, and the FVa-activities were measured in the PTase assay. In this case, Pefabloc (1 μM), an inhibitor of thrombin, was included to avoid the activation of FV during the assay. Part of the activated samples was taken and loaded on western blot to further evaluate the FV activation.

5.2.7 Prothrombinase-based FVa assay

FVa cofactor activity was measured by determining the rate of FXa-catalyzed thrombin generation. FVa, FXa ($5nM$), and phospholipid vesicles ($50\mu M$, PS/PC $10:90, mol/mol$) were preincubated at $37°C$, and the thrombin generation was started by addition of preheated prothrombin ($0.5\mu M$). The reactions were stopped after 1 minute by dilution in ice-cold EDTA buffer. The generated thrombin was quantified using the chromogenic substrate S-2238.

5.2.8 Determination of Apparent k_d of FXa for FVa using the Prothrombinase Assay

The formation of membrane-bound FXa FVa complexes was measured by determining the rates of prothrombin activation, at increasing concentrations of FXa and a fixed concentration of FVa.

FVa ($50pM$) was preincubated for 4 min with FXa (0.5 - $20000pM$) and phospholipid vesicles ($50\mu M$ PS/PC 10 : 90, mol/mol) at $37°C$. The thrombin generation was started by addition of $0.5\mu M$ preheated prothrombin and allowed to continue for 1 min before being stopped using ice-cold EDTA buffer. The generated thrombin was quantified using chromogenic substrate S-2238.

The apparent Kd for the binding of FXa to FVa was obtained from plots of the rate of thrombin generation as a function of the FXa concentration. The k_d was obtained by fit-

ting the data to the following equation for a single site binding isotherm using non-linear least squares regression analysis [6].

$$V = \frac{V_{max} - \frac{1}{2}([FVa] + k_d - [FXa]) + \sqrt{\frac{1}{4}([FVa] + k_d - [FXa])^2 + [FXa]k_d}}{k_d - \frac{1}{2}([FVa] + k_d - [FXa] + \sqrt{\frac{1}{4}([FVa] + k_d - [FXa])^2 + [FXa]k_d}} \tag{5.1}$$

The maximal values of thrombin generation obtained by curve fitting, were used to evaluate the catalytic activity of the prothrombinase complex, using the following formula:

$$k_{cat} = \frac{V_{max}}{[FVa]} \tag{5.2}$$

5.2.9 Determination of k_m for Prothrombin activation by Prothrombinase Assay

The K_m for prothrombin activation by the prothrombinase complexes containing the different FVa variants was determined by varying the prothrombin concentrations. FVa ($50pM$) was preincubated for 4 min with increasing concentrations of prothrombin (0.5-5000nM) and $25\mu M$ phospholipid vesicles (PC/PS 95 : 5, mol/mol). Thrombin generation was started by the addition of preheated FXa ($5nM$) and allowed to continue for 1 min before being stopped by dilution with ice-cold EDTA buffer. Thrombin was quantified using chromogenic substrate S-2238. The k_m was obtained by fitting the data to the following equation using non-linear least squares regression analysis [6].

$$V = \frac{V_{max}[PT]}{([PT] + k_m)} \tag{5.3}$$

5.2.10 FV/FVa stability

The recombinant FV variants (150 pM) were incubated at $37°C$ both before and after thrombin activation (0.5 U/mL). After 10 minute at intervals, aliquots were drawn and inactivated using a 2 μM thrombin inhibitor Pefablock TH. The remaining FVa activity

were then determined in the prothrombinase assay, as described before. The FV aliquots that were drawn from the nonactivated incubation mixture were activated with thrombin (0.5 U/mL, for 10 minutes at $37°C$) prior to the determination of residual FVa activity.

5.3 Results

5.3.1 Mutation Screening

We reported the identification in a FV Leiden carrier (patient A) of a novel FV gene mutation, a C/T transition at nucleotide 5266 in the exon 15.
This mutation, located at the A3 domain, results in a substitution of an arginine at the aminoacidic (aa) residue 1698 into a tryptophan.
A restriction system was designed in order to have a rapid test to screen a group of mild to severe FV deficient subjects (n=38 , FV:c range $< 0.4 - 58\%$) for the R1698W mutation. In this small population another subject (patient B) was found heterozygous for the R1698W FV mutation, Tab.5.3. Moreover patient B was doubly heterozygous for the H1299R mutation (HR2 haplotype). A summary of the available clinical features of these patients is provided in Tab. 5.3.
To verify the presence of this mutation in the normal population, a group of 38 subjects (FV:C range $64 - 104\%$) were screened, but no carrier of the R1698W change was identified.
A coagulation screening on FV, showed antigen and activity levels reduced to a half. The lower FV activity in patient B is probably due to the presence of the H1299R mutation, that is associated to slightly lower FV levels and could thus explain the FV levels well below the 50%. The APCR data (0.65 ± 0.02) in patient A showed a APC-resistance phenotype that is slightly above the range of FV R506Q homozygotes (<0.57), despite heterozygosity at the genetic test.
These data suggest that 1698W mutation is responsible for a quantitative FV deficiency

(CRM-). Moreover, the data collected for patient A could suggest a condition of pseudo-homozygosity for APC resistance. Routine coagulation tests including PT, aPTT, antithrombin III, protein C (PC), protein S (PS), factor VIII:C and fibrinogen levels excluded any other defect in these patients (data not shown).

	Patient A	Patient B	Normal Range
FV:Ag (%)	46%	na	$70 - 130$ %
FV:C (%)	41%	29%	$70 - 130$ %
APCR	0.65 ± 0.02	na	> 0.81
Other FV mutations	R506Q	H1299R	–
Clinical features	piastrinopenia	epistaxis	–

Table 5.3: Laboratory and genetic characteristics of the patients analyzed in this study.

5.3.2 Expression and Characterization of the recombinant FV variants

To study the functional property of the R1698W mutation, this mutant FV variant was created by site-direct mutagenesis of the wt FV cDNA and transiently expressed in COS-1 cells. The FV variants were quantified by ELISA and analyzed by western blotting before and after thrombin activation, Fig 5.1.

The activation fragments of FVwt and the mutant variants have identical molecular mass thus suggesting that the aa substitution does not introduce new thrombin cleavage sites. Before activation, two bands of approximately 250 and 150 kDa and corresponding to the intracellularly cleaved forms of FV are visible [12]. These forms are present in different quantities independently of the FV variant and transfection. Both these fragments are recognized and cleaved by thrombin as the full length FV, but it is not clear if they have cofactor activity in the prothrombinase complex.

The concentration of these fragments varies in the different samples. Moreover, since the

different FV forms affect the evaluation of concentration by ELISA testing, we decided to produce all FV variants lacking a part of the B domain. These new variants are not recognized and cleaved by the intracellular proteases, and hence prompted us to have a more accurate evaluation different FV variants concentrations. In addition to 1698W FV, we created another FV variant that present at the same aa position an alanine residue (1698A), Tab.5.1.

Antigen levels in conditioned media were determined by ELISA, and are presented in Tab.5.4. Although a parameter quantifying the efficiency of transfection is missing, the expression level of both the mutant variants is relevantly reduced compared to the FV wt even if 1698A mutant is slightly higher compare to the tryptophan variant, Fig. 5.2.

The same expression pattern is also observed using PMT2 FV-702, suggesting that the substitution of an positive charged aa (Arginine) with an hydrophobic one (Alanine or Tryptophan), impairs the intracellular processing and secretion of the mutant FV. The secretion improvement observed for the 1698A is likely due to the higher steric hindrance of tryptophan compared to alanine. Nevertheless additional experiments are required to investigate the differences in the secretion pattern of all FV variants.

FVa activity was determined after activation by thrombin, through the evaluation of FVa cofactor effect on the rate of prothrombin activation. Relative specific activity (activity/antigen ratio) based on this assay and ELISA, was calculated for each FVa, Tab.5.4. In contrast to the FVa wt, FV1698W had lower specific activity that is more relevant for FV1698A, raging from 0.2 to 0.09 respectively, Tab.5.4.

To further characterize the recombinant variants, they were analyzed by western blotting using a monoclonal antibody against the heavy chain, Fig.5.3.

Before thrombin activation, all the FV variants contained a 330 or 250 kDa band, corresponding to FV full length or FV -702, respectively. After thrombin activation a band of approximately 105 kDa, corresponding to the heavy chain of FVa, appeared.

To ensure that activation was not affected by the introduced mutations, FV variants were incubated with increasing amount of thrombin and tested via an activity assay and western blotting: all the variants appear completely activated (data not shown).

5.3.3 Stability of the recombinant FV variants

The R1698W mutation is located at the interface between the A2 and A3 domains. The replacement of a polar, positively charged aa (arginine) with a non-polar, bulky aa (tryp-

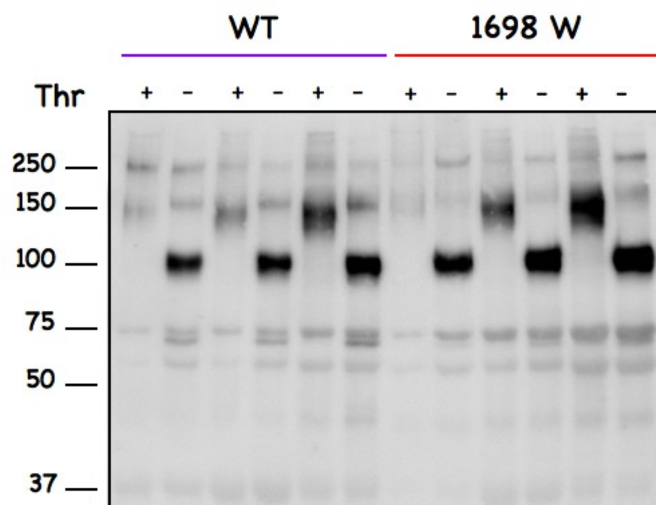

Figure 5.1: Analysis of FV wt and FV 1698W by western blotting, before and after thrombin activation. For each FV variants, three different transfections were loaded on the gel, and detected on the membrane using a polyclonal antibody against FV (A299).

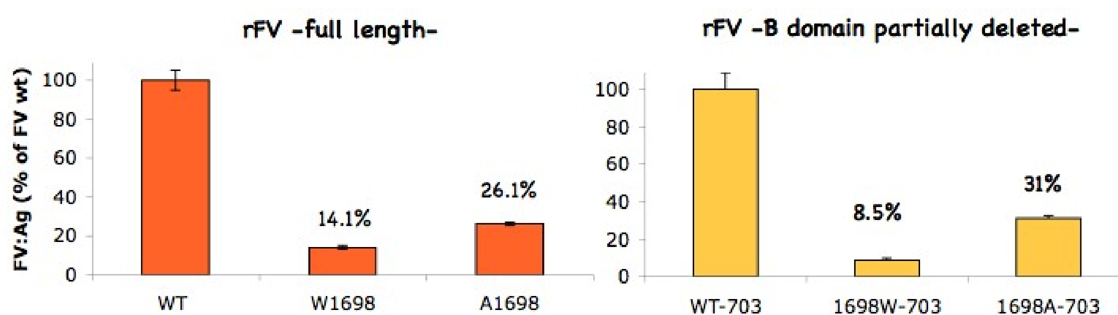

Figure 5.2: Differences in the secretion patterns of FV variants, expressing both the full length or partially deleted B domain. The percentage of expression compared to the wt are indicated as mean.

FV variant	FV:Ag (nM)	FV:C (nM)	Specific Activity (FV:C/FV:Ag)
WT	99.0±5.6	nd	/
W1698	13.9±0.9	nd	/
A1698	25.9±0.8	nd	/
WT-702	51.0±1.6	40.9±3.6	0.8±0.1
W1698-702	3.5±0.6	0.7±0.03	0.2±0.04
A1698 -702	12.7±0.5	1.09±0.24	0.09±0.02

Table 5.4: Expression and activity levels of FV variants. Data are average ± the standard deviation of three independent experiments.

tophan) could affect the interaction with the surrounding residues at the interface and the local intramolecular structural integrity. A decreased stability could hence provide an explanation for the reduced amount of 1698W FV in the plasma of the subjects carrying this mutation.

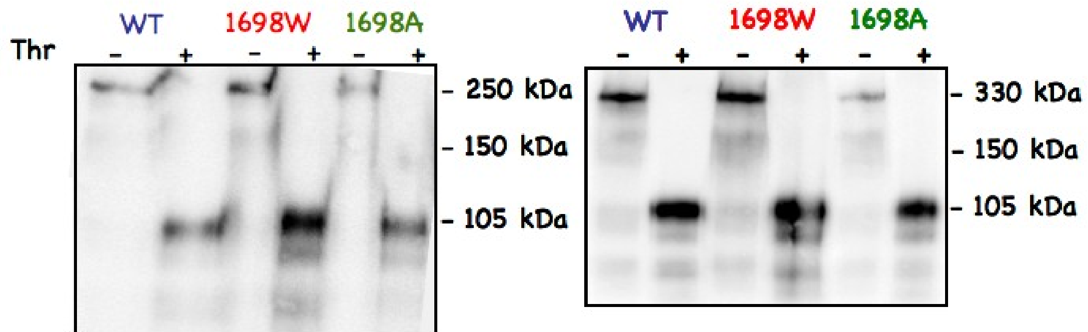

Figure 5.3: Characterization of the rFV variants by western blotting before and after thrombin activation. $rFV-702$ (left panel) and rFV full length (right panel)were detected by using a monoclonal antibody against the heavy chain (AHV5146).

These observations prompted us to investigate the FV stability overtime by evaluating its residual activity at different time points (at 37°C, before and after thrombin activation). We have started by using the full length FV variants, Fig.5.4.
Although a slight decrease was observed for FV 1698W, both unactivated forms were quite stable at 37°C in the time range. However, after thrombin activation FVa1698W activity rapidly decreased overtime, suggesting that 1698W mutant is unstable only after becoming activated.
To analyze more in detail the instability of the activated form of FV1698W, we monitored the FV activation, using a low and high concentration of thrombin (molar ratio FV:Thr 1 : 3 and 1 : 30, respectively). At different times, the FVa activity of both variants were tested in a prothrombinase assay.
Unlike FVa wt, that reaches a maximal and stable activity after 5-10 minute, FVa 1698W shows an increase in its activity during the first 5 minutes with a subsequent rapid decline characterized by a $\sim$ 50% loss in activity already after 20 minutes, Fig.5.5.
The stability of the activated recombinant variants partially lacking the B domain (FV-702) was also tested. The decay pattern of FVa 1698W was conserved for the variant FVa1698W-702, Fig.5.6. The presence of an alanine instead of a tryptophan at the 1698 seems to affect FVa stability even more. After full activation ($\sim$ 2.5 min), the activity of 1698A rapidly decays reaching levels comparable to the mock control after 25 min.

The same samples were also loaded on western blot to follow FV activation, Fig. 5.7. This experiment was performed to exclude that the decay in FV activity was due to a different sensitivity of FV mutants to thrombin compare to the wt. The 250kDa single chain band disappeared quite rapidly after 2.5 minute in all samples indicating a full activation of FV variants. However, a residual band at 250 kDa for FV 1698W-702 persisted overtime suggesting the presence of a thrombin-resistant conformation is some molecules. The amount of this conformation, evaluated by densitometry, is not relevant to affect the result of the prothrombinase assay.

5.3.4 Functional properties of the recombinant FV variants

In order to better understand the reasons for the reduced activity of FV1698W and FV1698A, their abilities to support prothrombin activation were tested at increasing FXa concentrations, Fig.5.8. The dose response-curves obtained were used to calculate the apparent k_d of FXa binding to the different FVa variants ,Tab.5.5.

The FVa wt had an apparent k_d of $\sim 85pM$ while both mutants had a ~ 3 fold higher k_d compared to wt. We also observed a reduction of k_{cat} for prothrombin activation using FVaW1698 and FVa1698A of ~ 1.5 and 5.7 fold respectively, compared to FVa wt.

These data suggest that the aa change at the A3-A2 interface has a deep impact on FV cofactor activity both by lowering FXa affinity and its catalytic function in the prothrombinase complex.

The affinity of FVa variants to prothrombin was also investigated, Fig.5.9. Suboptimal phospholipid composition (PC:PS 95 : 5, mol/mol) and concentration ($25\mu M$) were used in order to assure that the k_m would not reflect only the binding of prothrombin to the phospholipid membrane. A negative control lacking FVa was tested as well.

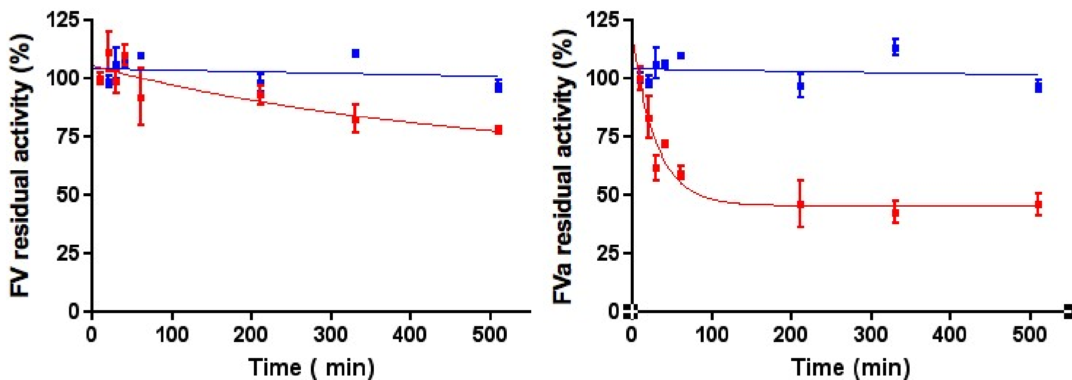

Figure 5.4: Stability overtime at $37°C$ of the rFV wt (blu) and rFV1698W (red) before (letf panel) and after (right panel) thrombin activation. The value are referred as % of activity compared to that obtained after 10 min of activation. The data presented are expressed as mean±SD of a duplicate.

Prothrombinase complexes generated using the different FVa variants yielded k_m values for prothrombin similar to that calculated for the FVawt. This observation suggests that the 1698 residue and the conformational modifications induced by tryptophan or alanine replacement at 1698 are not involved in prothrombin interaction, Tab.5.5.

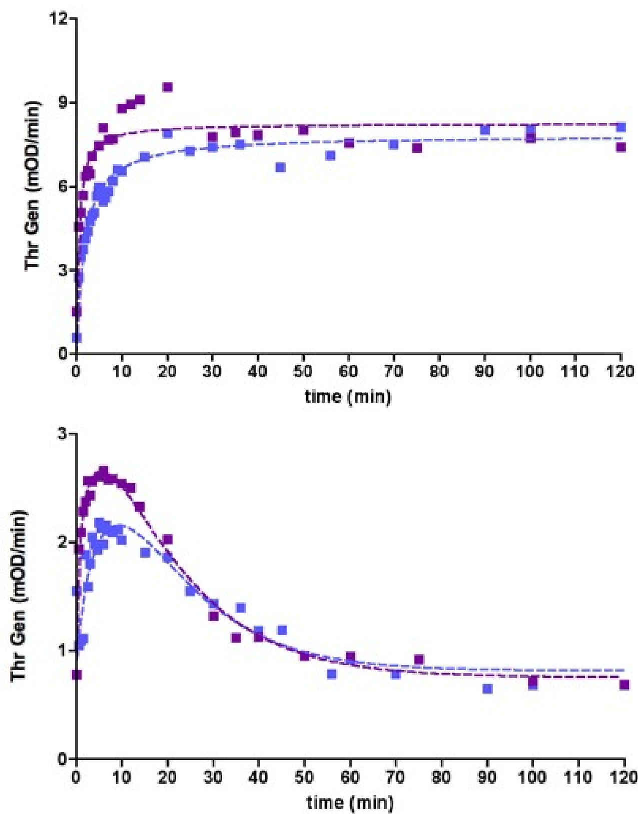

Figure 5.5: Time course of thrombin catalyzed rFV activation. rFV wt (on the left panel) and rFV1698W (on the right panel) were activated using 0.5 U/mL (purple) and 0.05 U/mL (blue) thrombin. The FV activity was tested at different time points using the prothrombinase assay.The plots are representative of two independent experiments.

5.4 Discussion

Among the several acquired and genetic factors which could influence the APC resistance in FV Leiden carriers and thereby modulating the thrombotic risk [7], we decided to investi-

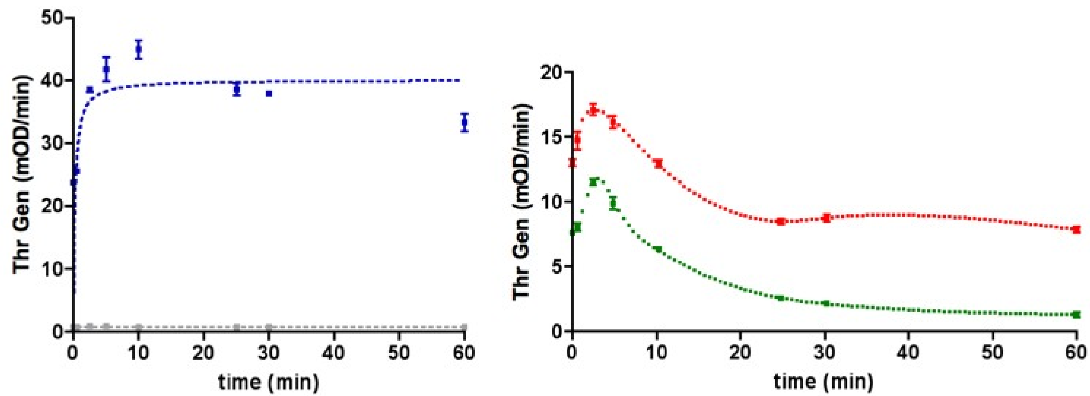

Figure 5.6: Time course of thrombin catalyzed rFV activation. rFV wt -702 (blue) and mock (grey), on the left panel, while rFV1698W -702 and (red) and rFV1698A-702 (green), on the right panel. All samples were activated using 0.5 U/mL thrombin, and then tested at different time points in the prothrombinase assay. The data used for these plots are representative of two independent assay.

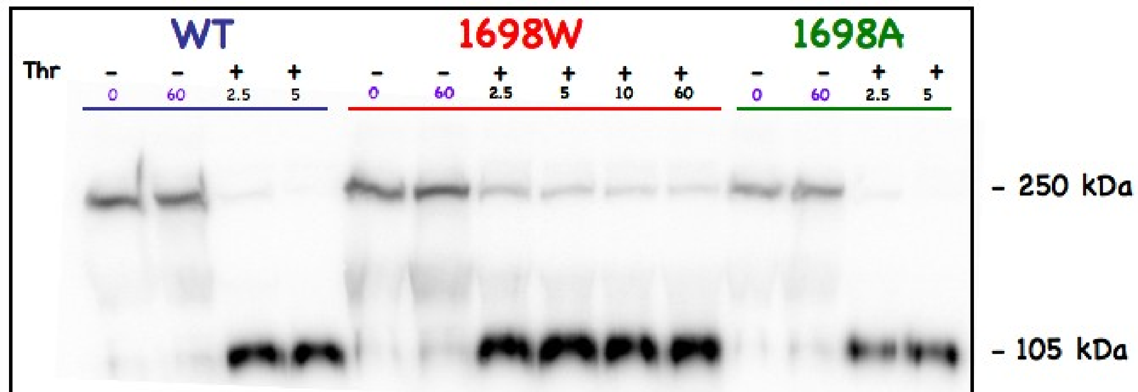

Figure 5.7: Time course of thrombin catalyzed rFV activation. FV variants are detected using a monoclonal antibody (5146) against the heavy chain of FV.

FVa variant	$k_d(pM)$	$k_{cat}(sec^{-1})$	$K_m(nM)$
FV $wt - 702$	84.1 ± 7.8	58.7 ± 0.7	285.3 ± 44.5
FV1698$W - 702$	210.9 ± 7.8	37.8 ± 0.3	274.7 ± 40.2
FV1698$A - 702$	229.8 ± 41.6	10.3 ± 0.4	267.3 ± 33.6

Table 5.5: Binding constant of rFVa variants in the prothrombinase assay. The apparent k_d and k_m values were calculated by fitting the curves shown in Fig.5.9 and Fig.5.8.

gate the intragenic determinants of FV levels. In particular we checked for polymorphisms and mutations in the FV counterpart gene in a selection of FV Leiden heterozygotes with low FV levels.
In two unrelated italian subjects we found a new missense mutation, resulting in a arginine-to-tryptophan substitution at the 1698 of A3 domain of FV (R1698W). More recently the same mutation was reported in a cohort of FV deficient German patients [10].

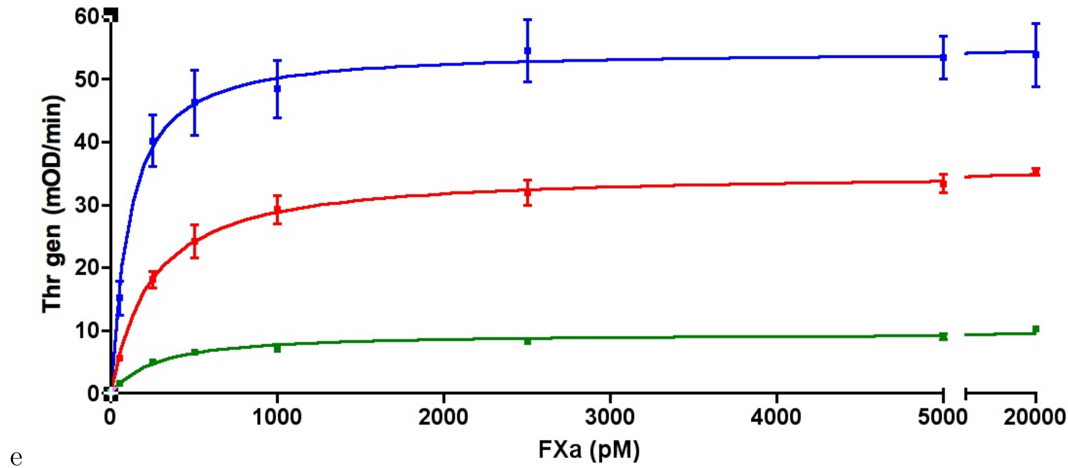

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Figure 5.8: FXa titration in a prothrombinase assay using activated forms of FV wt - 702 (blue), FV1698W -702 (red), rFV1698A-702 (green) and mock (grey).Each data point represents the mean of three independent experiments performed in duplicate, error bars represent the standard deviation.

The analysis of FV levels in these patients suggests a causative role of the R1698W mutation in determining an severe reduction on FV secretion and function FV causing a FV deficient phenotype (CRM-). Although data available on the APC resistance sensitivity phenotype in patients plasma are limited, the compound heterozygote for R1698W and FV Leiden seems to confer a phenotype similar to the pseudo-homozygous APC resistance.
Here we report the molecular characterization of the recombinant FV 1698W in order to understand which molecular mechanisms are at the basis of the FV deficiency.
In vitro expression of recombinant FV 1698W reveals a remarkably reduction in FV antigen levels (∼10% vs FV wt) and its specific activity (0.20±0.04 vs FV wt 0.80±0.1). The reduced amount of the altered FV in media has been also observed using the B-domain less variants. Moreover, the expression of A1698 is markedly reduced (∼30 % vs FV wt) confirming the essential role of the arginine at 1698 for the biosynthesis of normal amounts of FV molecules. Although the reduced ability in supporting prothrombin activation can

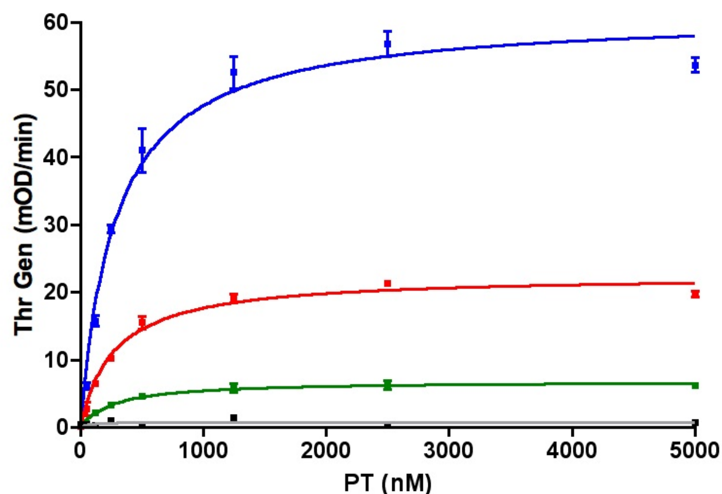

Figure 5.9: PT titration in a prothrombinase assay using activated forms of FV wt -702 (blue), FV1698W -702 (red), rFV1698A-702 (green) and mock (grey). Each data point represents the mean of three independent experiments performed in duplicate, error bars represent the standard deviation.

be partially due to a decreased FXa affinity and catalytic efficiency of the resulting prothrombinase complex, these results cannot completely explain the FV 1698W phenotype. These results support both the CRM red FV deficiency and the pseudo homozygous APC resistance condition described for patient A, highlighting an additional mechanism characterized by a specific combination of mutations.

Since another possible mechanism contributing to FV deficiency can be an impaired molecular stability, we checked FV stability before and after thrombin activation at physiological temperature. Although the use B domain less FV variant in studying the effects of FV deficiency mutations can induces difference in protein processing and secretion, thus representing a study limitation, here we show similar results independently from the presence of B domain full length or partially deleted which further support our findings.

FV1698W showed a reduced stability after thrombin activation only, with a complete loss of activity after 20 minutes compared to the wt. FVa instability was even more relevant in FV1698A. This suggests that the cause of the instability is not related to the bulky nature of the tryptophan, but probably to a loss of an inter-domains interaction at this residue. However, because of the fast decay of those variants, further investigations of the molecular bases of the mutant FVa instability represent a difficult task.

Additional considerations can be drawn from A3 domain protein sequences alignment [11]. The residue 1698 is maintained both in the human and bovine FV, moreover its surroundings are highly conserved in the A3 domains of different species, Fig.5.10. In FVIII the 1833 residue, aligned to 1698 on FV, is conserved only as a charge (K) and a homologous missense mutation resulting in a lysine-to-glutamic acid substitution at this site (K1833E), has been associated to a severe FVIII deficiency (FVIII:C <1%) [Bogdanova N. et al. unpublished data].

Taken together, these data suggest that the 1698 residue plays an important role in FVa A3 and A2 domains interaction and that the tight packing in this region is important for the stabilization of the active hetorodimer. In particular, the replacement of an arginine with a tryptophan or an alanine, affects the local intramolecular structural integrity of the FVa creating clashes or destabilizing cavities at A3-A2 interface. This structural variation

deeply affects the FXa binding affinity and facilitates the A2 dissociation from the FV light chain. In this respect, the role of B domain is not known but we could speculate that its presence stabilizes FV inter-domain interfaces, more in particularly at the A3-A2 interface. Moreover, delayed stabilization of interaction between A domains could have implication in the folding process and thus in quality control, which could ultimately contribute to decrease biosynthesis of the altered FV molecules. It is worth noting that even wild type FV biosynthesis requires a number of dedicated chaperons.

Preliminary data on APC-dependent FVa inactivation show an increase in APC sensitivity of both 1698 mutants compared to the wt, Fig. 5.11. This observation indicates that the APC resistance state found in patient A is only due to the combination *in trans* of FV Leiden and FV deficient associated mutation. However, the contribution of APC and of the intrinsic instability of the mutants on the inhibition FVa activity still remains to be defined.

Multiple approaches have been used in this study to evaluate the pleiotropic effect of the R1698W missense mutation, enabling us to dissect the molecular mechanisms at the basis of the FV deficiency. In particular, we have found several mechanisms by which R1698W affects FV levels:

- reduction in the amount of secreted FV by cells,
- impaired FXa binding affinity,
- reduced catalytic efficiency of the prothrombinase complex,
- reduced FV half-life after activation.

Taken together these information would indicate that A domain interactions stabilization is crucial for the biosynthetic process, FV function and FVa stability and clearance. Although the influence in vivo of the altered biosynthetic process seems to be prevalent and significantly contributes to FV deficiency, the other mechanisms have very likely an essential role to produce moderate to severe FV deficiency. On the other hand the reduced but not absent biosynthesis and function indicate that this mutation produces a deficiency clearly distinguishable from a null conditions.

5.4.1 Future investigations

Other FV mutants have been created and are being subjected to molecular characterization Tab.5.1. These new variants were created using two different models of FV A domains [13] [14].

Based on the FV model reported by Orban et al., the arginine at 1698 is directly involved in salt bridge, or important hydrogen bonds, with aspartic and glutamic acids at 611 and 610, located in A2 domain. However, in the FV model proposed by Villoutreix et al., the arginine at 1698 is involved in a network of interactions at A3-A2 interface, and particularly in the interaction with the glutamic acid at 1691.

The next step of our research will be to evaluate the role of the 1698 residue in the inter-domain interaction by replacing arginine with an uncharged aa (glutamine). This will likely prevent the formation of potential salt bridge or hydrogen bonds at the A3-A2 interface without clashes creation. Moreover, we are willing to investigate more in details the interactions of R1698 by reversing the nature of the aa residues involved. For this task we will take advantage of the two above mentioned structural models of FV. In order to

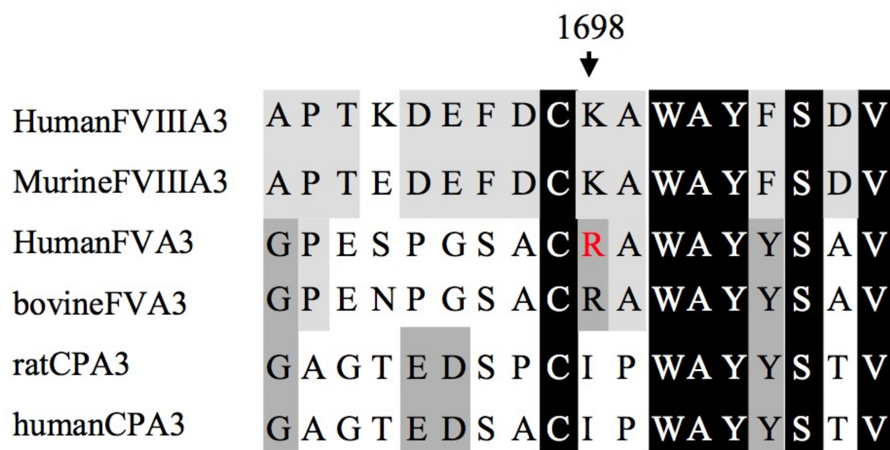

Figure 5.10: Aminoacids sequence alignement of A3 domain from different proteins modified from [11]

recover the full FV activity and stability, we have already created two recombinant FV variants: *FV R1698E-D611K-E610K* and *FV R1698E-E1691K* according to the models of Orban model and Villoutreix, respectively.

These new FV variants will likely help to shed a light on the potentially unique role of 1698R in the stabilization of the FVa heterodimer at A3-A2 interface. Although a direct structural information for the A2 domain of the precursor protein is not available, these experiments could also provide valuable information for validation of the FV model in this particular region.

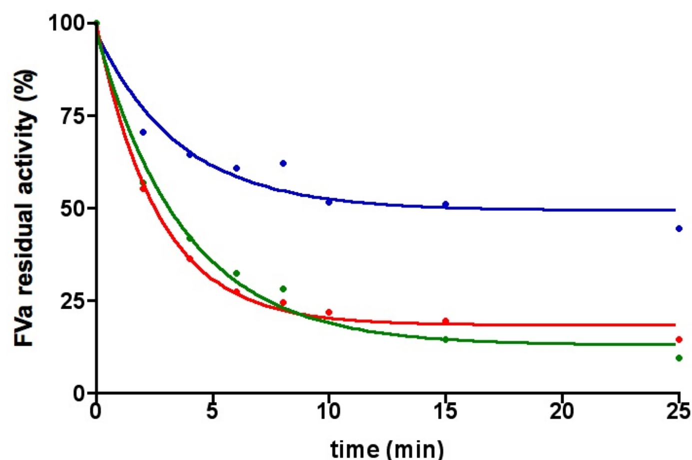

Figure 5.11: APC-mediated FVa inactivation. FV-702 wt (blue), FV-702 1698W (red) and FV-702 1698A (green) were incubated with thrombin (molar ratio 1:5) for 3 minutes at 37°C. After activation, FVa variants (0.8 nM) were added to a solution constituted by 0.05 nM APC, 100 nM protein S and 25μM phospholipids vesicles(PS:PE:PC10:20:70). The FVa degradation was followed for 25 minutes. The subsamples were withdrawn at different time points and stopped in ice-cold HN buffer. The residual activity was immediately determined by prothrombinase assay using saturated FXa concentrations (5 nM). The residual activity is expressed as percentage of the activity generated by each FVa variant in the absence of APC.

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Chapter 6

Conclusion

Hemostasis is a finely tuned mechanism that balance two opposing functions that point towards a hemorrhagic or thrombotic condition. Several genetic and environmental factors affect this balance through different molecular mechanisms that can finally result in the modulation of the levels of coagulation proteins [1].
The general purpose of this PhD project was to shed a light on complex regulative network providing three different examples of induced clotting factors modulation. The study of coagulation factor levels variations, due to acquired or inherited components, could establish novel determinants or new relationships for a better understanding of the pathophysiology of the hemostatic disorders.

Acquired Components

It is well-established that acquired components, also related to human behavior, have a strong influence on the expression of several coagulation factors and might represent independent risk factor for various disease. In particular, intensive lifestyle changes have been shown to improve cardiovascular risk profiles. Several studies in fact have reported strong evidences for benefits of life style intervention in populations with an elevated risk of cardiovascular disease (CVD) [2] [3].

In this regard, we report two studies aimed at evaluating the hemostatic variations induced by two life-style (e.g. diet and exercise) interventions, in two population characterized by moderate to high CVD risk.
In the first study (chapter 2), we evaluated the effects of a well defined whole-diet therapy on a wide panel of hemostatic and inflammatory parameters. We chose an overweighted premenopausal women population with high levels of C-Reactive Protein and at least another CVD risk factor. The overall CVD mortality in the last decades have shown only a modest reduction among women, indicating that primary prevention programs are less effective in women than in men. Our aim was to verify if determined changes in dietary habits could induce positive hemostatic variations in a moderate CVD risk population.
After progressive introduction of proved anti-inflammatory dietary elements (such as wine

and fish) all inflammatory markers, in particularly IL-6, showed the decrease over time, albeit not significantly. Moreover, we observed remarkably reduced of FVII and FVIII levels during the course of the study.
A new association between coagulation factor levels and the inflammatory profile was found too. Levels of Tumor Necrosis Factor-α, a cytokine over-produced by adipocytes and macrophages of adipose tissue in the obese state, were correlated with those of FVIII and affected their variation over time.
Also temporal parameters of thrombin generation, lag time and time to peak were significantly prolonged, perhaps reflecting changes related with initial stages of coagulation cascade.

In summary, the changes observed in coagulation initiation and amplification phases together with those of body composition and lipid profile could translate into a remarkable decrease in the risk for CVD, hence representing an effective prevention strategy in this population.
In the second study (chapter 3), we evaluated the effect of exercise training on end-stage renal disease (ESRD) patients on hemodialysis (HD) maintenance. In particular we wanted to study whether a potential relationship exists between coagulation markers and physical training in a population characterized by high CVD risk and a seriously impaired physical function [4].
The results that we have obtained suggest the presence of factors common to the two groups and independent from physical exercise capable of modulating the coagulation phenotype. The lack of an association between the physical exercise and the coagulation phenotype, cannot exclude that exercise alone is not able to modulate blood coagulation in the ESRD population. Albeit independent from physical exercise, the extremely significant reduction in FVIII (p=0.002) and to a lesser extent of FVII (p=0.006) and FX (p=0.036) levels found, are clinically worth of note in a high CVD risk population. More efforts in collaboration with specialist nephrologists are required to interpret these variations.

Intragenic Components

The presence of several missense variants of FV suggest a pivotal role of this protein in orchestrating the hemostatic balance [5]. In this respect the research on the genetic components involved in the modulation of FV levels acquires a huge importance.

In the chapter 5, we show that the extensive sequencing of FV gene in a small cohort of FV deficient subjects revealed a new missense mutation (R1698W) on FV gene. Multiple approaches have been used to evaluate the molecular mechanisms by which R1698W modulates FV levels *in vitro* . In particular, R1698W has a pleiotropic effect on FV by reducing its cellular secretion, impairing its binding affinity to FXa, reducing the prothrombinase catalytic efficiency and deeply increasing its instability after activation. These data provide an interpretation of the *CRM-* phenotype shown in R1698W carriers.

In previous works, the stability of the FVa heterodimer has been associated to the presence of divalent cations binding (calcium and copper ions) at the A1-A3 domains interface [6][7][8] [9]. No natural FV variants affecting the calcium binding sites have been described to date. Until now, only one FV mutation (A221V) located at A1-A2 interface, has been demonstrated to be able to modify FVa stability [11]. The molecular characterization of FV New Brunswich, the only variant associated to a qualitative FV deficiency, has shown that the conformational changes induced by the A211V mutation affect the strength of the interaction between the heavy and light chain, resulting in an increased of the dissociation of FV heterodimer. Molecular dynamics simulations suggested that A221V could induce structural changes at relatively large distance, toward the A3-A2 domains interface [11].

In our study, we characterized for the first time a natural mutation (R1698W) located at the A3-A2 domains interface. Our results suggest that the tight packing at A3-A2 interface is also required for the stabilization of the active heterodimer. The replacement of the arginine with a relatively large and hydrophobic residue (tryptophan or alanine) at this site is not tolerated since it probably abolishes an important inter-domains interaction at the 1698 residue. A direct structural information for the A2 domain of the FV is lacking. Moreover, since we have two different models of FV A domains presenting several

differences particularly at this region, the next step of our research will be focused at understanding the potentially unique role of 1698R in FVa stability by the creation of new FV mutants.

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Chapter 7

Summary

Hemostasis is a finely tuned status of equilibrium between procoagulant and anticoagulant activities. Despite the presence of several regulatory factors, hemostatic balance is nevertheless sensitive to perturbation.
In this thesis, we have analyzed the effects of some acquired and inherited components that could modulate specific coagulation factors. The study of coagulation factor level variations could be instrumental to discover new parameters for the evaluation of the risk of thrombotic and hemorrhagic events.

In the first study we evaluated the effects of a whole-diet therapy on a wide panel of hemostatic and inflammatory parameters in a population of overweighted premenopausal women with moderate CVD risk. We observed specific changes on proteins important in coagulation initiation (factor VII, [FVII]) and amplification phases (factor VIII, [FVIII]). Levels of Tumor Necrosis Factor-α, a cytokine over-produced by adipocytes and macrophages of adipose tissue in the obese state, were correlated with those of FVIII, thus suggesting new relations between coagulation and cellular components of inflammation. Also temporal parameters of thrombin generation (lag time and time to peak) were significantly prolonged, perhaps reflecting changes related with initial stages of coagulation cascade.

In the second study, we evaluated the effect of exercise training on end-stage renal disease (ESRD) patients on hemodialysis (HD), a population characterized by high CVD risk and a seriously impaired physical function. The results we have obtained suggest the presence of factors capable of modulating the coagulation phenotype in both the two groups of patients, albeit independently of physical activity.
The lack of association between physical exercise and the coagulation phenotype, cannot exclude that exercise alone is not able to modulate blood coagulation in the ESRD population. We have found an extremely significant reduction in the levels of FVIII, FVII and FX levels, that are of potential clinical importance, even though they proved to be independent from lifestyle change. For a better understanding of these results, we will need the expertise of clinicians, and particularly of nephrologists.

Finally, we have investigated the molecular mechanisms induced by a new mutation (R1698W) of coagulation factor V (FV), in the modulation of its protein levels. Our results indicate that the R1698W mutation has a pleiotropic effect on FV by reducing its cellular secretion, impairing its binding affinity to FXa, reducing the prothrombinase catalytic efficiency and deeply increasing its instability after activation. These data also provide an interpretation of the *cross-reacting material negative* (CRM -) phenotype shown in R1698W carriers. This study gives new structural information at the A3-A2 domains interface, a region presenting several differences between the two available FV models. The replacement of the arginine with a relatively large and hydrophobic residue (tryptophan or alanine) at 1698 is not tolerated since it probably abolishes an important inter-domains interaction. The next step of our research will be focused at understanding the potentially unique role of 1698R in FVa stability by the creation of new FV mutants.

Abstract in Italian

L'emostasi é un processo finemente regolato in uno stato di equilibrio tra attività procoagulanti e anticoagulanti. Nonostante la presenza di numerosi meccanismi di regolazione,

risulta essere piuttosto sensibile alla perturbazione. In questa tesi, abbiamo analizzato gli effetti di alcune componenti genetiche e ambientali che potrebbero modulare specifici fattori della coagulazione. Lo studio delle variazioni dei livelli di questi fattori potrebbe essere utile nella scoperta di nuovi parametri per la valutazione del rischio trombotico ed emorragico.

Nel primo studio abbiamo analizzato gli effetti di una dieta completa su diversi parametri emostatici e infiammatori, in una popolazione di donne in pre-menopausa con un modesto rischio cardiovascolare. Specifiche variazioni sono state osservate nei livelli delle proteine coinvolte nella fase di inizio (fattore VII, [FVII]) e in quella di amplificazione (fattore VIII, [FVIII]) della coagulazione. Inoltre i livelli di TNF-α, una citochina sovra-espressa da adipociti e macrofagi del tessuto adiposo in soggetti obesi, presentano una significativa correlazione con i livelli di FVIII durante lo studio. Questa nuova correlazione suggerisce nuove relazioni tra coagulazione e componenti cellulari dell'infiammazione. I parametri che definiscono i tempi di generazione di trombina (lag time e time to peak) risultano anch'essi significativamente aumentati. Ciò riflette le variazioni antigeniche osservate nelle fasi di inizio della coagulazione.

Nel secondo studio abbiamo valutato l'effetto dell'esercizio in pazienti affetti da end-stage renal disease (ESRD) in emodialisi, una popolazione caratterizzata da un elevato rischio cardiovascolare e una ridotta capacita' fisica. I risultati ottenuti suggeriscono la presenza in entrambi i gruppi, di fattori capaci di modulare il fenotipo coagulativo, indipendentemente dall'attività fisica sostenuta. La mancanza di una associazione tra l'esercizio fisico e i parametri coagulativi considerati, non può escludere un ruolo dell'esercizio nella modulazione del fenotipo coagulativo nella popolazione ESRD. Le riduzioni osservate nei livelli di FVIII, FVII e FX sono considerevoli e di interesse clinico anche se indipendenti dall'esercizio fisico sostenuto. Tuttavia l'esperienza clinica di specialisti nefrologi e' necessaria per una interpretazione dei risultati descritti.

Infine, sono stati studiati i meccanismi molecolari di modulazione dei livelli del fattore V (FV), indotti da una nuova mutazione (R1698W) presente nel suo gene. I risultati ottenuti indicano un effetto pleiotropico della mutazione R1698W sui livelli di FV. Abbiamo osservato infatti, che la riduzione dei livelli di proteina é ottenuta attraverso molteplici meccanismi: riduzione della secrezione cellulare della proteina matura, compromissione della sua affinitá di legame con il FXa, riduzione dell'efficienza catalitica del complesso protrombinasico e un'aumentata instabilitá dopo la sua attivazione. Questi dati costituiscono un'interpretazione del fenotipo *cross-reacting material negative* (CRM -) osservato nei portatori della mutazione R1698W. Questo studio fornisce inoltre nuove informazioni strutturali all'interfaccia tra i domini A3-A2, una regione che presenta diverse differenze nei due modelli strutturali di FV disponibili. In particolare noi sosteniamo che la sostituzione dell'arginina in 1698 con un residuo idrofobico relativamente grande (triptofano o alanina) non sia tollerata poiché impedisce la realizzazione di una importante interazione inter-domini. Le prossime ricerche saranno rivolte alla valutazione del ruolo del residuo 1698 nella stabilitá del FVa attraverso la creazione di nuovi mutanti.

Curriculum vitae et studiorum

The author of this thesis was born in Rovigo (Italy) on August 20, 1981 and accomplished the secondary education at the Scientific High School *L. Ariosto* in Ferrara. From 2000 to 2003 she studied Biological Science at University of Ferrara. After she started her master degree in Biomolecular and Cellular Science and graduated *cum laude* in 2006 under the supervision of Prof. Francesco Bernardi on the coagulation protein Tissue Factor. In 2007 she entered a Ph.D. program in Biochemistry, Molecular Biology and Biotechnology in the same lab, working on some projects presented in this thesis. At the end of her second year of PhD course, she started a new project on coagulation factor V at the Department of Clinical Chemistry of Lund University (Sweden), in the frame of a collaboration with Prof. Dr. Bjorn Dalhback.

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