RESEARCH ARTICLE

Quantitative measurement of APC-Resistant factor V clotting activity (FV-Leiden) and potential graduation of the associated thrombo-embolic risk

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Abstract:

Coagulation Factor V (FV) is a key factor for regulating blood coagulation cascade, and it acts at the crossroads of the intrinsic and extrinsic pathways. It shows a dual activity as the procoagulant cofactor for Factor Xa in the prothrombinase complex, but it also supports an anticoagulant activity in combination with TFPI and Protein S. Its rapid cleavage by Activated Protein C (APC) complexed with Free Protein S (FPS), in presence of phospholipids and calcium, inhibits its activity and limits the propagation of blood coagulation, keeping it to where it is beneficial. Rapid inactivation of active FV by APC-FPS is essential for preventing the risk of thrombosis development. In 1993, Dahlbäck and coworkers reported an inherited disorder characterized by activated protein C resistance (APC-R) and associated to an increased occurrence of thromboembolic events in affected families. In 1994 Bertina demonstrated that this diathesis resulted from a Factor V mutation (R506Q), rendering this factor resistant to inactivation by APC. This mutated Factor V was called Factor V Leiden (FV-L). APTT based assays and molecular biology methods for detecting the mutation were developed, but these methods are only qualitative and classify tested individuals as normals, heterozygous or homozygous for the coagulation defect. Our group developed a quantitative assay for FV-L, which is described in this report, along with its performances. This assay allows to quantitate specifically FV-L coagulant activity, and to graduate its amount in heterozygous or homozygous patients. FV-L is absent in normal individuals and present in homozygous or heterozygous patients, accounting respectively for 100 % or 50 % of blood FV. Its amount is compared with FV clotting activity or antigenic concentration. Measured FV-L activities overlap between heterozygous patients with high FV and homozygous ones with low FV levels. This assay allows to better discriminate for the FV-L associated thrombotic risk, which depends on the effective FV-L concentration rather than on patients' genetic status. This expectation is supported by literature review, which shows that FV-L concentrations correlate with presence of platelet released microparticles in patients carrying that mutation.

Key words: Factor V Leiden; Quantitative assay; Activated Protein C; APC Resistance; Thrombosis.

Introduction:

Factor V (FV) is a key cofactor, known since a long time as accelerin, and essential for the activity of the prothrombinase complex, involving activated Factor X (FXa), phospholipids and calcium, which generates thrombin from prothrombin [1]. During normal physiology, in-vivo coagulation is a site-targeted mechanism, which is initiated, amplified, and propagated where its action is beneficial, and is then regulated at this site. In pathology, coagulation can be activated in blood circulation by abnormal components induced by disease states, tissue injuries, metabolism abnormalities, inflammation, or infection [2]. When activated, coagulation process ends by inducing a clot formation, which prevents from bleeding and allows healing in presence of vascular wounds, but it can be deleterious when it is provoked by pathology. An efficient regulation process must control clot formation, to keep it at the healing sites, and to prevent propagation of coagulation pathways in blood circulation, where it could provoke undesired disseminated activation and fibrin formation, and then thrombosis can occur. Regulation of hemostasis involves intact endothelium, serine esterase inhibitors (such as Antithrombin and Heparin Cofactor II), the Protein C (PC)-Protein S (PS)-Thrombomodulin (TM) system, which regulates and inhibits activated factors V (FVa) and VIII (FVIIIa), and fibrinolysis. The complementary action of these functions is of essence for keeping the procoagulant pathways at the right sites, and for a delayed dissolution of the clot once the healing function is achieved. Abnormal concentration or activity of these anticoagulant mechanisms favors occurrence of thromboembolic diseases [3, 4]. FV plays then a key role in this process, being at the crossroads of blood coagulation pathways.

Regulation of FV/FVa clotting activity:

The PC-PS-TM system has a double action: i) it captures thrombin in micro-circulation through its binding to endothelium exposed thrombomodulin: its coagulant activity is transformed into an anticoagulant one; ii) PC is activated to APC, and in presence of Free Protein S (FPS), forms an APC-PS complex that inactivates thrombin activated Factor VIII (FVIIIa) and FV (FVa). These actions stop any blood hemostasis activation pathway, which could occur in blood circulation away from the initial activation site and they inactivate the blood coagulation cascade when the beneficial clot is formed [5, 6]. This regulatory mechanism operates similarly in presence of diffuse blood activation, triggered by developing pathologies (even occult) or by metabolic abnormalities. In this disease context, the protective intra-vascular endothelial surface and the blood content are altered. For example, cancer, traumatisms, atherosclerosis, diabetes, infections, autoimmune diseases, immun-complexes can activate hemostasis and mobilize the blood regulatory system to prevent thrombus formation. Only when these body defenses are overwhelmed because blood activation exceeds body regulation capacity, thrombo-embolic diseases can develop [2]. Efficient inactivation of FVIIIa, and FVa by APC-PS-TM system is then essential for maintaining blood fluidity, and its dysfunction induces an increased risk of thrombosis. In practice, FVa has a higher survival time in blood circulation than FVIIIa, and its prolonged survival was reported many years ago in patients with Lupus Anticoagulant (LA) [7]. If not readily controlled, FVa can disseminate activation of blood coagulation pathways away from the beneficial clotting site. FV has a complex implication in the coagulation cascade. Recent studies have shown that it has a dual role and can also be anticoagulant [8-11]. A small amount of FV is truncated (FV short) and behaves as the PS and Tissue Factor Pathway Inhibitor (TFPI) cofactor for the inhibition of FXa [8]. This additional function of FV, which is anticoagulant, will not be discussed further in that report, which focuses on the procoagulant potential of FV and its control. In contrast,

when FV carries the R506Q mutation [12], it becomes resistant to the action of APC-PS and it has then a prolonged survival following activation of blood coagulation pathways, and this increases the risk of developing thrombo-embolic diseases (TED).

APC-Resistance and Factor V-Leiden:

Many years ago, the key role of APC to inactivate FVa for preventing thrombosis was already described [5, 13]. A breakthrough occurred in 1993, when Dahlbäck and coworkers reported the elevated incidence of familial thrombosis associated with APC resistance (APC-R) in Sweden [14]. Laboratory testing for APC-R was performed by measuring the Activated Partial Thromboplastin Time (APTT) in presence or absence of APC, then calculating the clotting times (CT) ratios [14, 15]. Inherited thrombosis tendency, unprovoked, was associated with a decreased APC-R ratio [15]. One year later, Bertina's group in Leiden (NL) showed that this defect is the consequence of a FV mutation at position 506, where arginine is replaced by glutamine (noted R506Q), and the mutated factor was named Factor V Leiden (FV-L) [12]. This FV mutation is located on one of the FV cleaving sites by APC [6, 16-19]. Thrombin activated FV-L has then an increased activity survival time in plasmas from patients with this R506Q mutation, and this can propagate activation of coagulation pathways. APC resistance and FV-L were rapidly identified as the major hereditary diathesis associated with an increased thrombotic risk [15, 20-23], although for patients with the R506Q mutation odds ratios for occurrence of thrombosis are lower than those associated with AT, PC or PS deficiencies [4, 23-26]. Since these discoveries, testing for APC-R with clotting assays and for the FVa mutation with molecular biology, have become major parameters of the thrombosis check-up. Affected patients can be homozygous (100 % of FV is mutated), and are then at the highest thrombotic risk [20, 25], or heterozygous (50% of FV is mutated). Control and regulation of FVa is essential for a normal coagulation homeostasis, and the APC-PS-TM system has a major role in this process [17].

Factor V variations:

The FV concentrations present a high distribution among normal individuals and they can range from < 70% to > 150% [26, 27, 28]. In patients with FV-L mutation, the same variation range is present: therefore, homozygous FV-L patients can have FV-L concentrations from < 70% to > 150%, and heterozygous patients from < 35% to > 75% [9]. There is some overlapping of FV concentrations between heterozygous patients with high levels and homozygous patients with low levels. Thrombosis risk results from the APC-resistant FVa concentration, i.e. FV-L, and not from the patient's genetic status [26]. Conventional APC-R clotting assays, performed with or without APC, are based on clotting time ratios, and allow an approximate classification of patients in heterozygous or homozygous [14, 29]. Assay performance and specificity was improved by introducing a dilution of tested plasma in FV deficient plasma [30]. The molecular biology approach gives a similar classification, which is definite, and it is fully specific for the R506Q mutation [12, 15]. The quantitative FV-L assay is expected to allow graduating FV-L levels and analyzing their association with thrombotic diseases [26].

Measurement of FV-L and association with thrombosis:

Thrombotic diseases associated with FV-L are frequently reported as unprovoked, as they occur in the absence of any major inducing cause [21, 22, 31]. In any case, the thrombotic risk of individuals carrying the FV-L mutation remains linked to the balance between coagulation triggers and body's capacity to control and regulate hemostasis [2]. However, the presence of a low-level trigger, which has no pathogenic effect on normal individuals, can induce thrombosis in patients with decreased antithrombotic defenses, because an

anticoagulant protein is deficient or in presence of FV-L [2-4, 20, 25]. The quantitative concentration of FV-L is then a very useful information for graduating the thrombotic risk in affected patients [26]. For this objective, we developed a quantitative assay for measuring FV-L clotting activity. We report the assay principle, its validation and performances, and the FV-L concentrations in normals, heterozygous or homozygous patients. In few cases, in presence of congenital or acquired FV deficiencies the FV concentration is reduced. Then, comparison of FV-L levels with the conventional FV clotting activity or the FV antigenic content is necessary for confirming the patient status.

Development of a quantitative assay for FV-L:

Material and methods:

*Plasma samples***:** normal human citrated plasmas were obtained from healthy blood donors collected at Etablissement Français du Sang (EFS), Pontoise, France, or from Precision Biologics Inc. (PBI), Halifax, Canada; citrated plasma pouches or apheresis plasma pouches were obtained either from EFS Normandie, Rouen, France, or from Biomex, Heidelberg, Germany; citrated plasmas from patients carrying homozygous or heterozygous FV-L mutation, duly characterized with molecular biology, were kindly provided by Dr Leroy-Matheron from Henri Mondor Hospital, Créteil, France, as part of a collaborative study (some plasmas were from patients anticoagulated with Vitamin K antagonists); alternatively, they were selected from the plasma pouches supplied by EFS or Biomex, and screened for the presence of FV-L with the FV-L clotting assay.

Purified proteins and enzymes: highly purified human fibrinogen, prothrombin (FII), factor X (FX), PS, factor Xa (FXa), and APC were all from Hyphen BioMed (Neuville sur Oise, France); factor X (FX) was tested for the absence of FXa, and of trace amounts of thrombin and APC; APC was tested for the absence of trace amounts of FXa.

Factor V Deficient plasma was from Affinity Biologicals (Ancaster, Canada) and supplied frozen, in bulk: the various coagulation factors (excepted FV) are within the normal range.

Cephalin was extracted from rabbits' brain, supplied by Pelfreez (Rogers, Arkansas, USA) and qualified upon receipt. All coagulation reagents used (Owren Koller or imidazole buffer, calcium solution, FV coagulation reagent, Zymutest Factor V for FV antigen) were from HYPHEN BioMed (Neuville sur Oise, France).

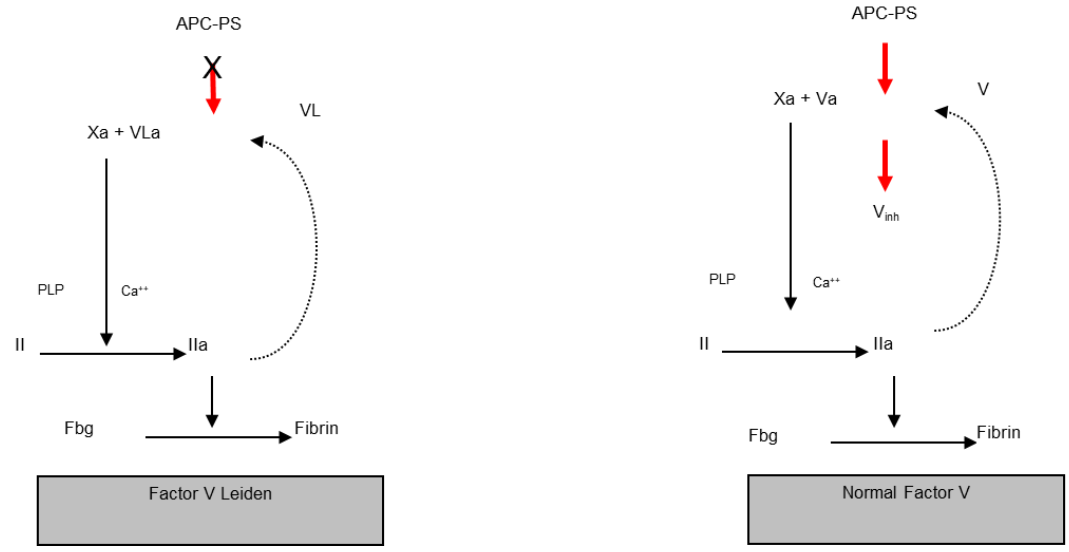
Coagulation instruments used were semi-automated (ST4 from Stago, Asnières, France; KC 10 from Amelung, Lemgo, Germany; MC10 from Merlin Medical, Lemgo, Germany), or automated (STA-R from Stago, Asnières, France; CS-2100i or CS-5100 from Sysmex, Kobe, Japan; ACL-Top from IL-Werfen, Le Pré Saint Gervais, France).

Quantitative clotting assay for FV-L: the assay principle is depicted on figure 1, and performed at 37°C. In a first step, tested plasma or calibrator (100 µL), diluted 1:20 in Owren Koller or imidazole buffer, is added to a clotting mixture (100 µL) containing human APC (about 15 µg/ml), purified Prothrombin, Fibrinogen, and purified PS, all at a constant concentration close to that in normal human plasma and in excess, and a heparin neutralizing substance (Polybren). Then, a purified mixture (50 µL) of human factor Xa and phospholipids is added, and coagulation is triggered with 100 µL of 0.025 M calcium chloride. The clotting time is recorded. A calibration curve is obtained by mixing plasmas from patients with the FV-L mutation and normal plasma, all with a known FV concentration. In heterozygous patients, 50% of total FV is FV-L, and all is FV-L (i.e. 100%) in homozygous. Measured clotting time (CT, sec) is inversely proportional to the FV-L concentration, whilst normal factor V is not measured (it is fully inactivated by APC-PS complex). This assay is available as

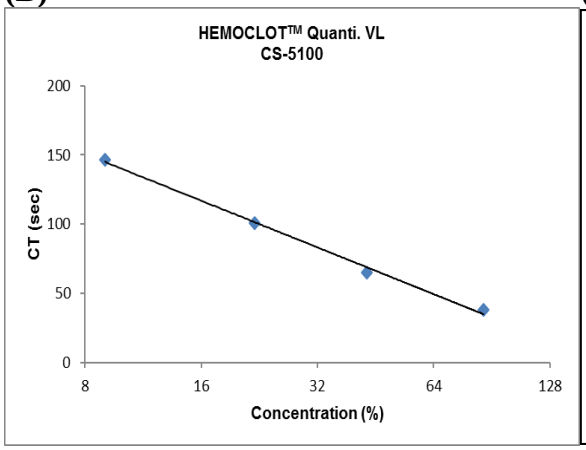
HEMOCLOT™ Quanti-VL (HYPHEN BioMed). A single clotting test is required, conversely to the APC-R method, which involves 2 clotting tests, with or without APC, for establishing the ratio. In addition, the result is quantitative, whilst it is only qualitative for APC-R assay.

Figure1: (A) Assay principle for the FV-L quantitative clotting assay, performed in presence of a constant concentration of Activated Protein C (APC). Normal FV is inactivated by APC in presence of PS, phospholipids, and Calcium, whilst FV-L is resistant to inactivation. There is an inverse relationship between the clotting time (CT) measured and the FV-L concentration. CT shortens when FV-L concentration increases. The dose-response curve obtained between CT and FV-L concentration is shown in (B). The linearity range is presented in (C).

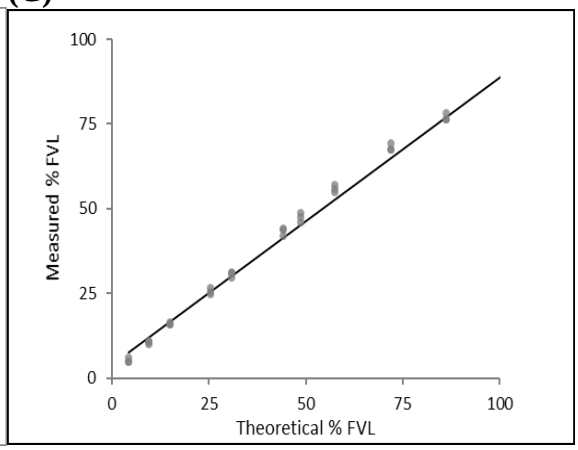
(A)



(B)



(C)



Other laboratory materials used: Plasma WHO coagulation factors' International Standard and SSC/ISTH Secondary Coagulation Standard were from NIBSC (Potters Bar, UK), COATEST™ APC™ Resistance V was used on ACL TOP® instrument (both from IL-Werfen, Le Pré Saint Gervais, France), as per manufacturer's

recommendations: for the lot and the instrument application used, the normal range cut-off value was for a ratio > 2.39.

Interferences: substances used for testing assay interferences were spiked in citrated plasmas, comparatively to a control sample added with the same volume of the corresponding dilution buffer. These products were: WHO International Standards from NIBSC (Potters Bar, UK) for Unfractionated Heparin (UFH 07/328) and Low Molecular Weight Heparin (LMWH 11/176); Rivaroxaban powder from Bayer Pharma (Germany); Apixaban powder from BMS (Princeton, NJ, USA); and Dabigatran powder (active form) from Boehringer Ingelheim (Ingelheim, Germany). Hemoglobin and bilirubin were from Sysmex (Japan), and Intralipids were from Sigma (St Louis, Mo, USA).

***Results*:**

Quantitative FV-L assay design:

The quantitative FV-L assay is performed at 37°C by incubating first 100 µL of the 1:20 diluted tested plasma (in imidazole, or Owren Koller's dilution buffer, or physiological saline) first with 100 µL of a mixture of fibrinogen, FII, PS, APC, and polybren (to neutralize heparin), for exactly 1 minute at 37°C; then 50 µL of a FXa-phospholipids solution are added, and incubated for another minute exactly at 37°C, and finally, 100 µL of 0.025 M CaCl2 are introduced and clotting time is recorded. Fibrinogen, FII, and PS are at a constant concentration and in excess, close to that present in normal human plasma, and APC is used at about 15 µg/ml. FXa is adjusted to produce a clotting time of about 30 seconds for 100% FV-L. The assay can be performed on any laboratory instrument, semi-automated or automated. We used successfully that method with KC 10, MC 10, ST4, STA-R, and CS-5100 instruments. Clotting times obtained are dependent on the clot detection mode of the instrument, optical or mechanical, and they can present some variations depending on the instrument type used. A Log-Lin linear and inverse relationship between the concentration of FV-L and the corresponding clotting time is obtained (Figure 1B).

Calibration is performed by mixing a pool of plasmas from heterozygous FV-L patients (which are available in a rather abundant way, whilst homozygous patients are rare) with a normal plasma. This pool of heterozygous FV-L patients (> 15 individual plasmas) has a FV-L concentration corresponding to 50% of the total FV clotting activity, which can be exactly measured by reference to the international WHO International Standard for plasma FV. When this pool is used diluted 1:20, its FV-L concentration is close to 50%, and it is exactly measured. The 1:10 dilution of this pool allows obtaining a concentration twofold higher, close to 100 % of FV-L, and exactly defined. The other FV-L concentrations used for calibration are obtained by diluting the FV-L plasma pool with a normal plasma, then 1:20 in the assay dilution buffer. In practice, a FV-L concentration close to 25% (2 volumes normal plasma and 2 volumes FV-L plasma pool) and another one close to 10 % (4 volumes normal plasma and 1 volume FV-L plasma pool) are prepared. The 0% FV-L concentration is obtained with a normal citrated plasma pool. The standard calibration curve generated on the CS-5100 instrument is presented on figure 1B. The clotting time is measured for the tested plasma, and the FV-L concentration is directly deduced from the calibration curve, or directly calculated by the instrument. In normals, FV-L concentration is always expected < 5% and it is frequently undetectable. The basic clotting time for normal plasmas is always higher than that corresponding to a FV-L concentration of 5% and it can be variable from plasma to plasma depending on the specimen characteristics and possible presence of activated factors. As FV-L is absent in normal plasmas clotting time can be very long and correspond to unmeasurable FV-L concentrations. Therefore, to keep a sufficient margin, a cut-off value of FV-L < 10 % in normals is defined, although in our experience

all normals have a FV-L < 5%, and assay is often unclottable. In practice, using automated instruments, the setting needs to be adjusted to 5%, as below this cut-off value it is unnecessary to record, all samples being without FV-L. With the assay conditions reported, normal FV is totally inhibited for concentrations > 200 %, and there is then no impact of samples with a high factor V concentration.

Assay interferences:

These interferences were tested by spiking concentrated solutions of UFH, LMWH, rivaroxaban, apixaban, dabigatran, bilirubin, hemoglobin, or intra-lipids in normal or FV-L patients, and by testing plasmas with LA, coagulation factor deficiencies or from vitamin K antagonist treated patients. No interferences were observed for UFH or LMWH up to 2 IU/mL in assayed samples, or for Dabigatran up to 200 ng/mL. Rivaroxaban and apixaban (but also other anti-FXa DOACs, not tested in that study) showed an interference from concentrations from 50 ng/mL and above and can generate underestimated FV-L concentrations in tested patients. This is expected as the clotting reaction is triggered by FXa in presence of phospholipids and calcium. No interference was noted when testing a few patients with Anti-Phospholipid-Antibodies (APA and LA: 5 with normal FV and 2 with FV-L mutation) or coagulation factor deficiencies, nor in VKA treated patients, without or with the FV-L mutation. No interference of hemoglobin (up to 1,000 mg/dL), bilirubin (up to 60 mg/dL) or intra-lipids (up to 1,000 mg/dL) was detected.

Limiting factor:

The assay limiting factor concerns the very rare cases of patients carrying the R506Q mutation and with an acquired or congenital FV deficiency. If FV concentration is low (≤ 10%), tested FV-L patients can erroneously be classed as normals. This pitfall can be overcome by measuring FV clotting activity and calculating the ratio between FV-L and FV: Clotting activity, or FV-L and FV: Antigen concentration. This ratio is always < 0.10 for normal individuals (and usually < 0.05), as no FV-L is present; it is of about 0.50 for heterozygous patients (50 % of FV is in the FV-L form) and of about 1.00 for homozygous patients (all FV is in the FV-L form). Comparative studies of FV-L, FV clotting activity and FV-Antigen are presented later in this article.

Assay linearity:

The assay linearity was checked by serially diluting plasmas from homozygous patients with a high FV-L concentration with a normal plasma pool, as shown on figure 1C. The measuring range obtained is then from < 5% to > 100 % FV-L. No matrix effect is noted whether the assayed plasma is diluted in assay dilution buffer or in 1:20 diluted normal plasma.

FV-L distribution:

The incidence of the R506Q mutation is highly linked to the ethnic group tested. It is mainly distributed in Caucasians with a prevalence > 12% in Sweden, in Greece, and some North-Africa or Middle East countries, but < 5% in Mediterranean European countries, and with intermediary values in other European countries, as referenced in discussion. This mutation is absent in Africans, native Americans, and Asians. In America, its distribution is closely linked to immigration history. As an example, we tested 557 individual plasma pouches from EFS Normandy (France) and found 13 donors with FV-L concentrations corresponding to a heterozygous mutation (i.e. an occurrence of 2.3 %), and 324 pouches from Biomex (Heidelberg, Germany) and found 16 donors with a FV-L concentration in line with a heterozygous mutation (i.e. an occurrence of 4.9%). This does not represent however population statistics, because carriers of the R506Q mutation are usually known and this can impact their participation to blood donation; plasma pouches from APC-R patients can also be selected by blood banks for specific uses. FV-L concentrations ranged

from 24 to 71% for EFS Normandy and from 22 to 53 % for Biomex. These concentrations are only indicative, as they were measured on apheresis plasma pouches, frozen for a variable time and thawed just before use. Plasma preparation, storage conditions and duration can impact the FV-L concentration measured, as some loss of FV activity can occur. Nevertheless, this shows that FV-L plasma distribution is wide, with some overlapping of ranges between heterozygotes with high FV/FV-L and homozygotes with low FV-L.

Precision:

Intra-assay, and inter-assays reproducibility (5-days, 2 runs per day, 3 repetitions per run) evaluated on CS-5100 yielded coefficients of variation (CV%) below 3% as reported on Table 1. The FV-L measurement with that quantitative assay is accurate and reliable.

Table 1: Intra- and inter-assay reproducibility obtained for the FV-L quantitative clotting assay on the CS-5100 automated coagulation instrument, with 2 plasmas at different FV-L concentrations.

Plasma	Intra assay			Inter assays		
	n	Mean %	CV%	n	Mean %	CV%
Level 1	40	10.8	2.9	30	11.1	2.8
Level 2	40	43.1	2.0	30	44.7	2.4

Measurements on patients with heterozygous or homozygous FV-L mutations:

Plasma samples from heterozygous patients (n=61, including 20 patients treated with VKA) had a FV-L concentration ranging from 24 to 76 % with a mean FV-L concentration of 49 % when untreated, and of 53 % when VKA treated; this concentration ranged from 73 to 118 % in FV-L homozygous patients, with a mean value of 92%. There is a clear discrimination of patients carrying the R506Q mutation from normals as presented on Figure 2. However, there is a slight overlapping of FV-L concentrations between the heterozygous group with high FV concentration and the homozygous one with low FV concentration

Comparison with the APC-R ratio and molecular biology:

Comparison of FV-L concentrations with the APC-R ratio (COATEST™ APC™ Resistance V) on 53 normal citrated plasmas (from Precision Biologics, or from EFS) and 63 plasmas from patients with APC-R, yielded a 100% agreement (Table 2). In this comparison study, the 53 normal plasmas from Precision Biologics were confirmed as normals (FV-L was <5% with HEMOCLOT™ Quanti V-L and APC-R ratio > 2.90, for a reported cut-off ratio of 2.39). The 63 APC-R plasmas were identified as positive for FV-L, and all measured with a FV-L concentration > 25%, and up to 64% with HEMOCLOT™ Quanti V-L kit, whilst all had an APC-R ratio < 2.30 with the Coatest® APC-R kit (Table 2).

Figure 2: FV-L concentrations in normals and various groups of patients (untreated or VKA treated heterozygous, or homozygous) carrying the R506Q (FV-L) mutation.

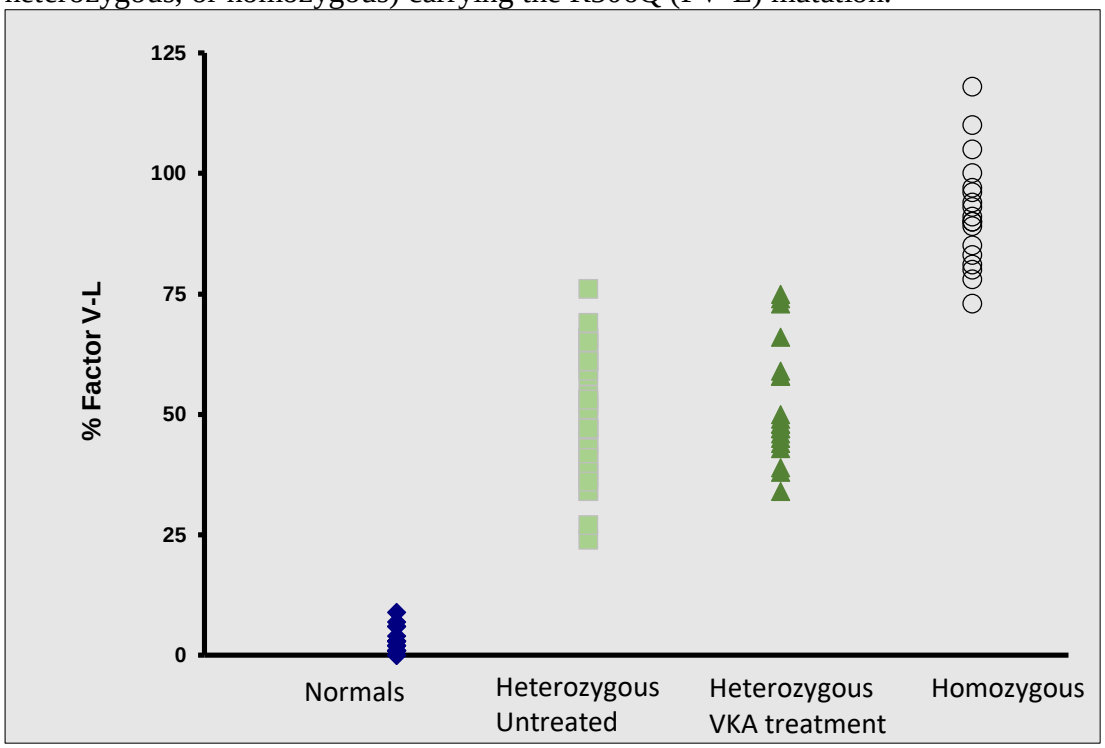


Table 2: Comparison study of HEMOCLOT™ Quanti V-L on Sysmex CS-5100 versus COATEST™ APC™ Resistance V on ACL TOP® (qualitative assay, based on the APTT assay performed with or without APC and calculating the CT ratio). Study was performed on 116 citrated plasmas tested with the COATEST™ APC™ Resistance V qualitative method (cut-off for normal range for the lot and application used: 2.39) or the quantitative Hemoclot™ Quanti VL assay. There is a full consistence between both assays.

		HEMOCLOT™ Quanti. V-L (CS-5100)		
		FV-L +	Normal	Total
COATEST™ APC™ Resistance V (ACL TOP)	FV-L +	63	0	63
	Normal	0	53	53
	Total	63	53	116

We also tested 21 citrated healthy normal plasmas (French Blood Bank, EFS), and 79 citrated plasmas from patients carrying the FV-L, R506Q mutation, from Créteil Hospital (of which 61 were heterozygous, including 20 with a current VKA therapy, and 18 homozygous). They were all duly characterized with molecular biology. FV-L was measured with HEMOCLOT™ Quanti VL, on the Stago STA-R system. Normals were all measured with a

FV-L < 5%. An excellent consistency of FV-L concentrations with molecular biology status was obtained. In patients, the FV-L concentration ranged respectively from 24 to 76% (mean value of 50.3%) for heterozygotes, and from 73 to 118% (mean value of 92.0%) for homozygotes (Figure 3). A clear discrimination was thus obtained between plasmas from patients carrying the R506Q mutation and normals. Furthermore, the discrimination was globally acceptable (but not perfect) between heterozygotes and homozygotes when using directly the FV-L concentration. In presence of doubts, establishing the FV-L/FV activity or FV-L/FV: Antigen ratios can help in confirming the classification as explained below.

Comparison of FV-L with FV: clotting activity and FV: Antigen:

Seventy-three plasma samples from patients with the FV-L mutation (38 heterozygous, untreated, 19 heterozygous, with VKA therapy, 16 homozygous) and 7 normal samples from EFS, with a FV-L < 5% were further analyzed for Factor V clotting activity and FV: Antigen (Figures 3 and 4). FV-L concentration for normal plasmas is usually expected < 5%. The expected FV-L concentration in plasmas from patients with the R506Q mutation is for heterozygotes usually between 25 and 75%, and for homozygotes > 75%.

FV-L to FV: clotting activity or to FV: Antigen ratios were between 0.4 and 1.0 for heterozygous plasma samples, and > 1.0 for homozygous samples (Table 3 and Figure 4), including those with less than 75% FV-L concentration and a similar Factor V clotting activity presented in Figure 3. These ratios are expected to be of about 0.5 for heterozygotes and 1.00 for homozygotes. The higher ratios obtained for some patients are due to the use of plasma samples stored frozen for a long time, and for which some loss of FV activity occurred, whilst FV-L is more resistant. These FV-L/FV: Antigen ratios are closer to the expected ones, as FV: Antigen is better preserved during storage.

Figure 3: Comparison of FV-L concentration with FV Clotting Activity (left panel) or with FV Antigen concentration (right panel) for normals, FV-L heterozygous patients untreated, or VKA treated, or homozygous patients.

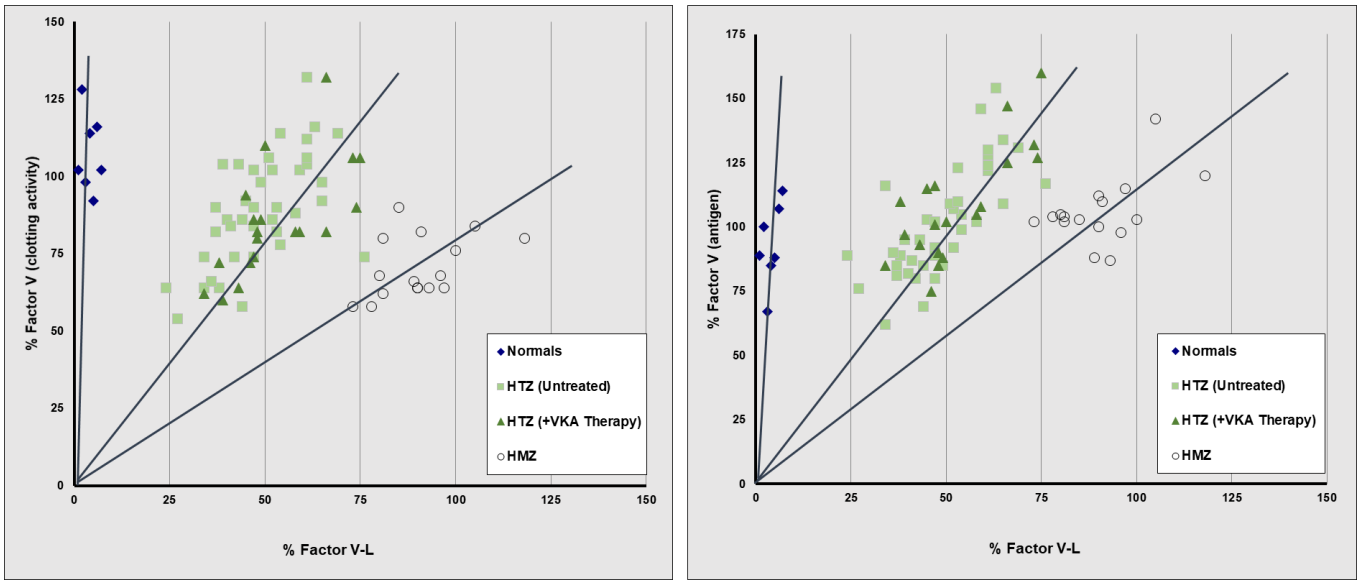
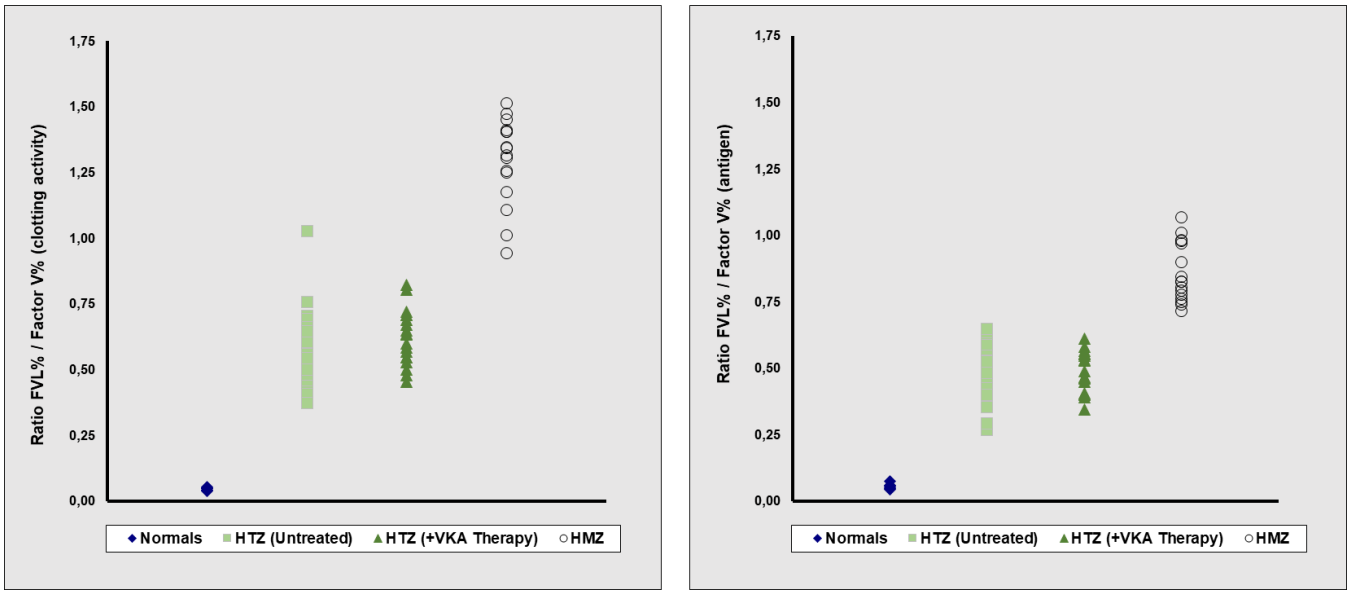


Table 3: Mean values and ranges of FV-L (%), FV:Clotting (%), FV:Antigen (%), and their respective ratios measured on citrated plasmas form normal individuals, heterozygous untreated or VKA treated and homozygous patients carrying the R506Q mutation.

	Assays and measures				
Samples	FV-L (%)	FV: Clotting (%)	FV: Antigen (%)	FV-L/FV: Clotting ratio	FV-L/FV: Antigen ratio
Normals (n=7)	<5	93 (67-114)	107 (92-128)	<0.05	<0.05
Heterozygous, untreated (n=39)	49 (24-76)	89 (54-132)	102 (62-154)	0.55 (0.38-1.03)	0.48 (0.27-0.65)
Heterozygous, VKA therapy (n=19)	53 (34-75)	85 (60-132)	108 (75-160)	0.62 (0.45-0.82)	0.49 (0.35-0.61)
Homozygous (n=16)	90 (73-118)	71 (58-90)	106 (87-142)	1.30 (0.94-1.52)	0.86 (0.72-1.07)

Figure 4: FV-L / FV: Clotting or FV-L / FV: Antigenic ratios for the normal group, untreated or VKA treated heterozygous or homozygous patients carrying the R506Q mutation.



Stability:

Reagent stability after reconstitution was of 24 hours at 2-8°C, 12 hours at room temperature (RT), 1 month frozen at -20°C, or 17 hours onboard of CS-series instruments. Associated calibrators/controls were stable for 24 hours at 2-8°C, 8 hours at RT, or 24 hours onboard of CS-series, thus covering a daily working period in routine laboratory practice.

Discussion:

FV mutations and thrombosis risk:

Many mutations have been identified on FV, some of them without any impact on its activities but associated to FV variations in blood concentration [32], and others with a decreased APC dependent inhibition [21, 33-36]. The most frequent mutation, which has a unique origin, is the R506Q one, and it is located on one of the APC cleaving sites on FV, at position 506 [12, 18, 19]. FV-L is resistant to the APC-PS cleavage, especially in its activated form, and when bound to

phospholipids. In addition, APC-PS can cleave FV to other sites, at amino acids 306 and 609 for a complete inhibition of FVa. However, in presence of the R506Q mutation, APC-PS cannot cleave FV at this position 506. Cleavage of the other FV sites is then delayed, and this induces the long-term survival of FVa-L in blood circulation. In vitro investigations have shown that in defined conditions normal FVa is fully inhibited after 5 min incubation with APC-PS-Phospholipids and Ca++, but 50% to 70% FVa-L is still present after 10 minutes incubation, and still > 10% after 1 hour [16]. FVa-L is then inactivated with a 10-fold lower rate than normal FVa [16, 17].

The FV-L / R506Q mutation is rather abundant in Caucasians (about 5%), with 2 types of allele distribution: a low one in western and Mediterranean Europe (< 2.8%); and a high one on a band in North and Central Europe, from Scandinavia to Greece, with a pocket of low allele distribution in Balkans [21, 37-39]. This mutation is also present in North Africa and some Middle East countries, such as Lebanon. In North and South America, its presence reflects the immigration history. In countries where the R506Q mutation is present, most of the unprovoked spontaneous familial thrombosis are associated with this defect. There is an odds ratio of 6 to 7 for occurrence of thrombosis in heterozygous patients, and this odds ratio is up to 80 for homozygous [20]. Thrombotic disease is often venous, but it can also be arterial [22-24]. Following a first thromboembolic event in individuals carrying the FV-L mutation, the recurrence risk is high, and an anticoagulant prophylactic therapy is usually instaured. In the absence of thrombosis, the need for a preventive anticoagulation is discussed [25].

APC-R, FV-L and risk for thrombosis:

Diagnosis of individuals carrying the R506Q mutation is then very important and useful for understanding the diathesis favoring the development of thrombotic diseases. It is yet of higher usefulness for investigating patients with unprovoked thrombotic events, along with the measurement of other anticoagulant factors, such as Antithrombin, PC, FPS, and eventually Plasminogen [3, 4]. Whilst AT, PC, FPS deficiencies contribute to about 15% of total unprovoked thrombosis, especially in younger patients, APC-R and the FV-L mutation are involved in about 60 % of these events [24]. Introduction of the APC-R clotting assay, performed with or without APC, or its subsequent improved versions, using a dilution in FV deficient plasma, have contributed to characterize most of the concerned patients, and to investigate affected families [14, 30]. With the discovery of the R506Q mutation, as the cause for APC-R, molecular biology tools were developed, and it became possible to genetically characterize the concerned individuals [12]. The molecular biology tool for the R506Q mutation appeared to be more accurate and reliable, and became rapidly the gold standard for that diagnosis, even if it was first of limited implementation in laboratories, and if it required a more complex laboratory methodology than performing the combination of 2 clotting assays (with or without APC), and calculating the clotting time ratios. Furthermore, the very first APTT based APC-R assay had many interferences of anticoagulant therapies, presence of coagulation inhibitors, coagulation factor deficiencies or LA.

A variant method was introduced later and is based on the use of activators extracted from snake venom for activating FX and FV. This assay also uses the ratio of clotting times performed with or without APC [40]. Specificity and accuracy of APC-R assays were improved by testing patient's plasma diluted in FV deficient plasma [30]. In current practice, the APC-R clotting assays allow classifying tested individuals as normals, heterozygous or homozygous, depending on the ratio obtained [29, 41]. With the former versions of APC-R clotting assays, normals had a ratio > 2.00, heterozygous between 1.50 and 2.00, and homozygous < 1.50, although the cut-off values need to be adjusted and validated in

each laboratory. In addition, there is some overlapping between ratios obtained for homozygous and heterozygous. Furthermore, some heterozygous patients can generate ratios close to the cut-off value of the normal range, or slightly above, i.e. 2.00 in the example cited. Normals can also have ratios close to this cut-off value or slightly below. There is then a significant number of unconclusive assays. These variations are explained by the characteristics of the tested plasmas or their eventual activation grade, occurring during plasma preparation and storage. The rate of inaccurate diagnosis is then not negligeable.

Using the Coatest® APC-R kit with the ACL-Top instrument, the cut-off value for the normal range was in our hands of 2.39. FV-L and APC-R assays are well documented in recent reports [29, 41]. Patients tested with the qualitative assays, using either molecular biology or the APC-R method, are classed in 3 groups: normal, heterozygous, or homozygous, and not according to the actual FVa-L concentration present. The understanding of mechanisms favoring development of thrombotic events in patients with the R506Q mutation shows that the risk is directly dependent on the FV and FV-L concentration, and to the amount of FVa-L resistant to the APC-PS inactivation, and not on the genetic status [26]. FV and FV-L concentrations present important variations in individuals [9, 26, 27]. Therefore, FVa-L concentrations can present high variations among patients heterozygous or homozygous for the R506Q mutation, and these concentrations are expected to be directly associated with the thrombotic risk. Measuring the real concentration of FV-L can allow to individualize the risk analysis for thromboembolic events, in affected patients.

Laboratory usefulness of the quantitative FV-L assay:

In this article, we describe a quantitative assay, Hemoclot™ Quanti V-L, for the measurement of FV-L clotting activity. The assay is designed with purified factors, which allows an excellent standardization of assay conditions. First, the 1:20 diluted tested sample is mixed with a clotting factors' mixture including human fibrinogen, human FII, human PS, and human APC, all extracted from a pool of human citrated plasma pouches, and highly purified. Of importance is the absence of activated factors in these protein preparations. Concentrations are constant and in excess and correspond to those present in a normal plasma pool, excepted for APC, which is used at the high concentration of 15 µg/ml, which allows a fast and complete inhibition of normal FV. Clotting is initiated by a mixture of FXa and phospholipids, then Ca++. In these conditions, all normal FV, including plasma concentrations up to 200 %, is readily inactivated, whilst FV-L is resistant to that inactivation. The clotting time obtained is then a direct relationship of the FV-L concentration in tested sample. This method is accurate, reliable, and insensitive to heparin or VKA therapy, coagulation factor deficiencies, or presence of antiphospholipid antibodies and LA. Direct Oral anticoagulants targeted to FXa, can impact the assay, by prolonging the clotting time.

The assay limitations concern those samples with a very low FV concentration, as present in the rare FV congenital or acquired deficiencies (severe hepatic diseases). However, there is only a risk of false diagnosis, if FV is < 20%, i.e. a FV-L < 10 % if tested patients are heterozygous. This very rare risk can be controlled by testing the FV clotting activity or the FV: Antigen and comparing them with the FV-L concentration. The ratio obtained is < 0.05 in normals, of about 0.50 in heterozygous, and of about 1.00 in homozygous patients. Some concern can occur when testing is performed on citrated plasma samples, stored frozen for a long time, as this is frequently the case when performing clinical or research studies. FV is a labile factor, especially if samples have been slightly activated during plasma preparation, processing, and storage. FV activities are then decreased, but in a much lesser extend for FV: Antigen. This can impact

the FV and FV-L concentrations reported, especially when testing patients' libraries progressively constituted over time. When performed on fresh samples, or freshly frozen and thawed plasmas, the method offers its best performances, with clear FV-L to FV: Clotting Activity or to FV: Antigen ratios < 0.10 (and in practice < 0.05) for normals, of about 0.50 for heterozygous and of about 1.00 for homozygous.

In a first study, we had the opportunity to compare the FV-L concentrations obtained with the Hemoclot™ Quanti V-L assay, to the APC-R ratios measured with the Coatest® APC-R method. With the lot and the ACL-Top instrument application used, the cut-off value between normals and FV-L patients was of 2.39, i.e. normals had a ratio > 2.39, and patients with the R506Q mutation had ratios < 2.39. In practice all tested patients carrying the R506Q mutation presented a ratio < 2.30 with the APC-R method, and the FV-L concentrations measured with Hemoclot™ Quanti VL had a mean value of 46.1 % and were always > 25%. There was a full consistency between both types of assays, and the 63 patients tested for having FV-L presented all an APC-R ratio below 2.30, whilst all normals tested had a ratio > 2.39, with FV-L concentrations < 0.05%.

In another study, FV-L clotting activity was measured on a library of patients carrying the R506Q mutation, and kindly supplied by Dr Leroy-Matheron from Henri Mondor University Hospital in Créteil (France). All these patients were duly characterized with molecular biology, and for being homozygous or heterozygous. A subset of patients was under current VKA therapy. A group of normal plasmas was tested as controls. All patients presented a FV: Clotting Activity from 60% up to 155 %, in line with usually reported FV distribution range [27]. FV-L ranged from 25% up to 75 % in heterozygous, and from 74% up to 118% in homozygous patients, with a slight overlapping between the high concentrations for heterozygous and the low ones for homozygous. We can anticipate that the thrombotic risk is then very similar for those patients in the overlapping zone, whether heterozygous or homozygous, as they share a same level of FV-L.

At this time, we had no opportunity to investigate the association between FV-L concentrations with the occurrence of thrombosis in affected patients, when adjusted for all the other thrombotic risk factors and physiological variables. This study would be of high value, and support the individualization of the thrombotic risk associated to the R506Q mutation, with the actual concentration of FV-L. However, a published study totally supports this concept by using a subrogate endpoint. Presence of micro-particles (MPs), now globally named extracellular vesicles (EVs), is closely associated to thromboembolic risk. MPs are the consequence and a contributing cause of disease development and evolution. The major MPs source in blood circulation is released from platelets. In an elegant study, Lincz and coworkers demonstrated that the amount of FV-L correlated with the history of thrombosis and the concentration of MPs exposing CD41a [26]. This analysis supports the concept developed and presented in this article.

Conclusion:

This new quantitative assay allows measuring any mutated FV resistant to the action of the APC-PS-Phospholipids-Ca++ complex. In most cases, it concerns FV-L, resulting from the R506Q single mutation. This latter has been selected during evolution, for its beneficial effects in protecting individuals against bleeding, inflammation, fertility, and pregnancy risks, at a time where blood loss was one of the most frequent life-threatening events [42]. However, a few other FV mutations, which induce a prolonged survival of FVa, have been reported [24, 34-36, 43]. These mutations remain very rare, and we had no opportunity to evaluate the behavior of these various mutated FV in that Hemoclot™ Quanti VL assay. Nevertheless, the method presented in this

report, is not fully specific for FV-L itself, but it measures any FVa resistant to APC cleavage and inactivation. There is then a high probability that other FV variants than FV-L are also detected, when they present a FV resistant activity, as it was reported in the recent ISTH review article on assays available for testing the APC-R [29]. As most of patients with FV-L mutations are well-documented, the clotting assays practice is decreasing over time, and the systematic testing is now discussed [41]. Molecular biology is then a definite diagnosis for patients with FV mutations. However, the introduction of the quantitative assay, restimulates the interest for FV-L laboratory assays by personalizing the thrombotic risk associated with this mutated factor. Clinical studies, analyzing the risk between the quantitative amount of FV-L and occurrence of thrombosis, are required for documenting this association.

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Abbreviations:
APA: Anti-Phospholipid-Antibodies
APC: Activated Protein C
APC-R: activated Protein C resistance
APTT: Activated Partial Thromboplastin Time
AT: Antithrombin
Ca++: Calcium
CT: clotting times
DOACs: Direct Oral Anticoagulants
EFS: Etablissement Français du Sang (French Blood Bank)
EVs: extracellular vesicles
FII: Prothrombin
FV: Factor V
FVIIIa: activated Factor VIII
FVa (FVa-L): activated Factor V (activated Factor V Leiden).
FX (FXa): Factor X (activated Factor X)
FPS: Free Protein S
FV-L: Factor V Leiden
LA: Lupus Anticoagulant
LMWH: Low Molecular Weight Heparin
MPs: micro-particles
NIBSC: National Institute for Biological Standards and Controls
PBI: Precision Biologics Inc.
PC: Protein C
PS: Protein S
SSC/ISTH: Secondary Coagulation Standard / International Society for Thrombosis and Hemostasis
TED: Thrombo-Embolic Disease
TFPI: Tissue Factor Pathway Inhibitor
TM: Thrombomodulin
UFH: Unfractionated Heparin
VKA: Vitamin K antagonists
WHO: World Health Organization

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