Biochemical, molecular and clinical aspects of coagulation factor VII and its role in hemostasis and thrombosis

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ABSTRACT

Activated factor VII (FVIIa), the first protease of clotting, expresses its physiological procoagulant potential only after complexing with tissue factor (TF) exposed to blood. Deep knowledge of the FVIIa-TF complex and *F7* gene helps to understand the Janus-faced clinical findings associated to low or elevated FVII activity (FVIIc). Congenital FVII deficiency, the most frequent among the recessively inherited bleeding disorders, is caused by heterogeneous mutations in the *F7* gene. Complete FVII deficiency causes perinatal lethality. A wide range of bleeding symptoms, from life-threatening intracranial hemorrhage to mild mucosal bleeding, is observed in patients with apparently modest differences in FVIIc levels. Though clinically relevant FVIIc threshold levels are still uncertain, effective management, including prophylaxis, has been devised, substantially improving the quality of life of patients. The exposure of TF in diseased arteries fostered investigation on the role of FVII in cardiovascular disease. FVIIc levels were found to be predictors of cardiovascular death and to be markedly associated to *F7* gene variation. These genotype-phenotype relationships are among the most extensively investigated in humans. Genome-wide analyses extended association to numerous loci that, together with *F7*, explain >50% of FVII level plasma variance. However, the ability of *F7* variation to predict thrombosis was not consistently evidenced in the numerous population studies. Main aims of this review are to highlight i) the biological and clinical information that distinguishes FVII deficiency from the other clotting disorders and ii) the impact exerted by genetically predicted FVII level variation on bleeding as well as on the thrombotic states.

Introduction

Blood coagulation is initiated by the formation of a complex between tissue factor (TF), a single-pass transmembrane glycoprotein, and activated factor VII (FVIIa), a serine protease highly dependent for its procoagulant activity on TF.[1-4] The absence of either of these components is incompatible with life.[5] However, small amounts of these proteins, interacting in the FVIIa-TF complex, are sufficient to initiate a number of reactions[6] on membrane surfaces.[4] The FVIIa-TF complex might also possess non-hemostatic, signaling properties.[7]

Detailed knowledge of the physiological and biochemical properties of FVIIa has enabled its pharmacological application as recombinant FVIIa (rFVIIa), a landmark in the management of bleeding disorders.[8] Genetic[9] and clinical studies have defined the heterogeneous molecular basis[10-12] of FVII deficiency[13] and could lay the foundations for gene therapy.

Extensive plasma studies and genetic investigations[14] have defined the importance of the *F7* gene variation in determining the large FVII variance in plasma concentration,[15] with implications in predisposition to thrombosis[16-18] in both individuals and the population as a whole.

A comprehensive review on FVII is complex because of the wealth of information available both in the field of hemostasis and thrombosis (Figure 1). The main aim of this review is to provide an integrated and balanced perspective of the bio-

logical and clinical information currently available. That is, the relationship between the reduced levels of FVII/bleeding tendency and high levels of FVII/ risk of cardiovascular disease. We will particularly focus on distinctive features of the FVII protein and *F7* gene (Table 1) as well as on open issues (Table 2).

Genetics and biochemical aspects

Expression of the *F7* gene and FVII protein is shown in Figure 2. Key biochemical and genetic findings are summarized on a historical time line in Figure 1A, and some of the key unanswered questions are summarized in Table 2. The *F7* gene[9] (12.8 KB) is located on chromosome 13q34, 2 KB apart from the homologous *F10* gene, which suggests evolution by duplication. In addition to *F10*, the *F7* gene structure and coding sequence[19] displays a noticeable homology also with *F9* (factor IX, FIX) and *PROC* (protein C).

Mutations that disrupt promoter activity in severe FVII deficiency[20,21] highlight the importance of the transcription factors SP1 and HNF4α, but do not sustain the androgen-dependent rescue observed for mutations in the *F9* promoter in hemophilia B. The evolutionary history of the *F7* promoter region, which contains frequent polymorphisms[22,23] modulating FVII expression (see dedicated paragraph), may differ in human populations.

The *F7* gene gives rise to three mRNA transcripts[12] through *FVII* mRNA alternative splicing. Whereas two transcripts (NM_019616.4 and NM_000131.4) encode an identical mature and circulating FVII protein, the third (NM_001267554.1) lacks the amino terminal domains and is of unknown physiological significance.

The FVII protein and FVIIa-TF complex

A scheme of FVII activation, activity and inhibition is shown in Figure 3. Whereas the mature circulating FVII protein (50 KDa) sequence[19] is composed of 406 residues, the mRNA (NM_019616.4) encodes 60 additional aminoacids, the pre-propeptide sequences which drive FVII biosynthesis/secretion and are removed by intracellular proteolysis (Figure 2). The first numbering (1-406 residues) is currently used in protein studies, and the second one (1-466 codons) in molecular genetics.
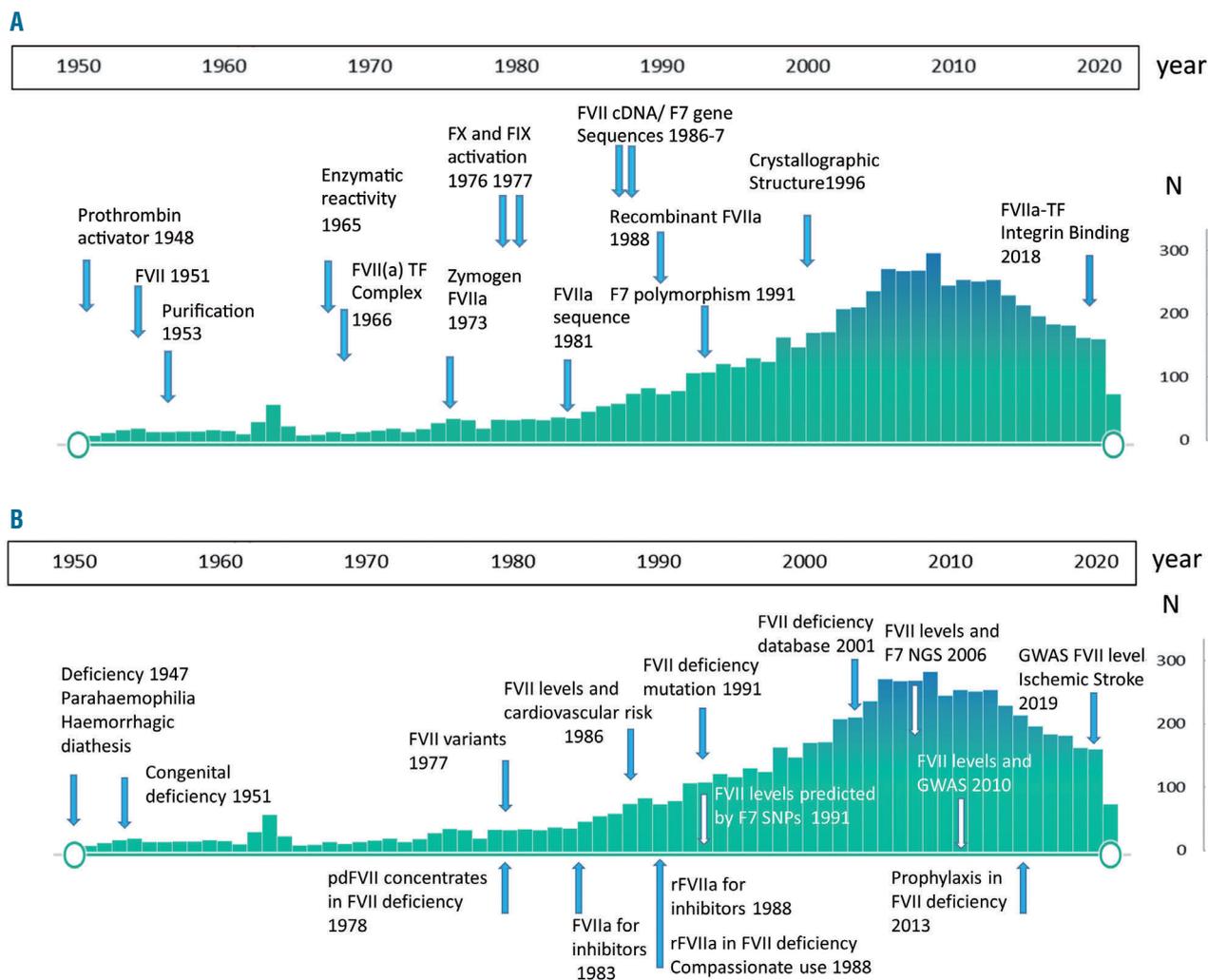
Figure 1. Publications over 70 years and some key achievements related to coagulation factor VII. (A) Coagulation factor VII (FVII) biochemistry and *F7* genetics. (B) FVII deficiency, FVII level associated cardiovascular disease, FVII levels and *F7* gene: N: number of publications reported in Pubmed. Only some of the discoveries are referenced in the text due to space constraints. A complete list is available on request. GWAS: Genome Wide Analysis Study; NGS: next-generation sequencing.

FVII zymogen circulates in plasma at low concentration (500 ng/mL, 5nM) and the extracellular proteolytic cleavage[1] of a minute portion (about 1%, 0.1 nM) of FVII giving rise to FVIIa, occurs between residues Arg152-Ile153, (Figure 2)[2] producing the light and heavy chains. The origin of FVIIa in plasma is still debated. Substantially decreased levels of plasma FVIIa in individuals with congenital FIX deficiency suggest that the generation of FVIIa is dependent on an activation product of FIX. Recently, it has been proposed that different forms of activated FIX (FIXα and FIXβ) participate in a reciprocal activation mechanisms of FVII(a) and FIX(a) (white and red-curved arrows, Figure 3, left panel) that would not require a protein cofactor.[24]

The FVIIa heavy chain contains the domain characterized by the (chymotrypsin) serine protease family catalytic triad. The light chain contains the calcium binding, vitamin K-dependent domain (gamma-carboxyl-glutamic, GLA) and two epidermal growth factor-like domains, essential for the interaction with membranes and other proteins[2] of the coagulation cascade.

Considerable variation in FVIIa plasma concentrations between individuals has been reported (Figure 4),[25] exceeding one order of magnitude. Differently from other serine protease of the coagulation cascade, FVIIa displays a plasma half–life (2-3 hours) remarkably close to that of the FVII zymogen, which might be explained by the "zymogen-like" properties of FVIIa that have been weakened by mutagenesis, thus increasing its activity.[26]

FVIIa interacts with TF, a membrane receptor exposed following vascular lesion (extrinsic pathway). The FVIIa-TF complex,[1] essentially conserved in all jawed vertebrates,[27] activates both FIX and factor X (FX)[28] (Figure

3) on the platelet surface in the presence of Ca^{2+}, leading to the generation of thrombin and the subsequent deposition of fibrin.

The crystal structure of the complex between the active-site-inhibited FVIIa and the cleaved, soluble extracellular domain of TF (sTF) revealed the details of the contoured embrace of FVIIa and sTF domains and the extensive intermolecular contacts.[29] Neutron and X-ray scattering experiments[30] suggested that FVIIa in solution has an elongated domain structure with significant flexibility, which allows rapid interaction with sTF over a large surface area to form the high-affinity complex (dissociation constant, KD 2–5 nM).

The FVIIa-TF complex is highly dependent on specific lipids for physiological activity.[2] Activated phospholipid membranes host both TF, an integral membrane protein, and FVIIa, which binds membranes through its GLA domain. Externalization of phosphatidylserine to the outer membrane leaflet and allosteric TF disulphide bond exchanges (decryption on membranes)[4] make TF the FVIIa-activating cofactor. The FVIIa-TF complex formation shapes the FVIIa catalytic site by allosteric interactions and increases its activity up to 10^6–fold. As a matter of fact, circulating FVIIa is the active portion of the total FVII mass only after high-affinity binding with TF. These FVIIa-TF complex-specific molecular events that provide FVIIa with its physiological activity are believed to represent the true initiation of the extrinsic pathway (Table 1).

The physiological negative control of the FVIIa-TF complex (Figure 3) occurs through the reversible inhibition by tissue factor pathway inhibitor (TFPI),[31] mediated by the TFPI Kunitz domain 1 and with protein S as cofactor.[32] An
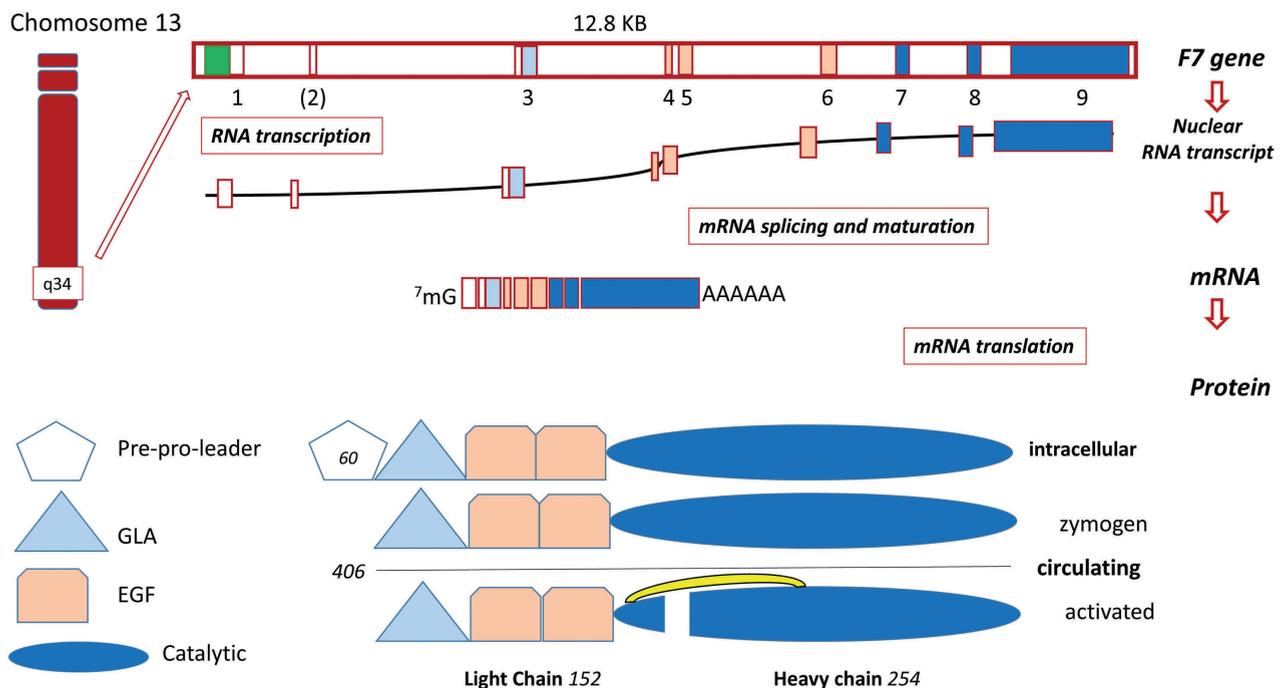

Figure 2. Schematic diagram of the *F7* gene and factor VII protein expression. Upper part: Exons are numbered (1-9) and colored in accordance with the encoded protein domains (lower part). Exon 2 is in parenthesis because it is not included in the most abundant mature mRNA. FVII: factor VII; *F7*: factor VII gene; KB: kilobase. In addition to the complete nuclear transcript the most represented FVII mRNA is indicated. Lower part: Protein domains are indicated with different colors. GLA, triangle, γ-carboxyglutamic acid-containing domain. EGF, trapezium, epidermal growth factor-like domain. Green box, promoter. The complete intracellular protein, and the circulating forms are depicted. The numbers of residues in the pre-pro-leader sequence (n=60), in the circulating forms (n=406) and in the light (n=152) and heavy (n=254) chains are indicated. The interchain disulphide bridge is also depicted (yellow bracket).

apparently redundant and irreversible inhibition of FVIIa is also exerted by the serpin antithrombin (AT),[33] and a substantial portion of FVIIa may be cleared through this relatively stable complex (FVIIa-AT),[34] which circulates in plasma at a concentration similar to that of FVIIa. FVII and FVIIa also bind the protein C receptor on endothelial cells (EPCR) with relevant affinity, but at present the pathophysiological significance of this interaction is unclear.

Congenital factor VII deficiency

Definition, prevalence and epidemiology

FVII deficiency was first described as a bleeding disorder by Alexander and colleagues[13] and represents a model disease to understand the pathophysiology of recessively inherited coagulation deficiencies. Relevant findings on FVII deficiency are summarized on a historical time scale in Figure 1B, and some of the open issues are summarized in Table 2. Congenital FVII deficiency (OMIM 227500, ORPHA 327) is defined as a bleeding disorder associated with FVII coagulant activity below 50% of normal. However, this threshold includes asymptomatic heterozygotes for causative mutations and may comprise individuals homozygous for frequent FVII lowering single nucleotide polymorphisms (SNP).[35] These subgroups substantially contribute to the belief that FVII deficiency is a mild bleeding disorder.[36]
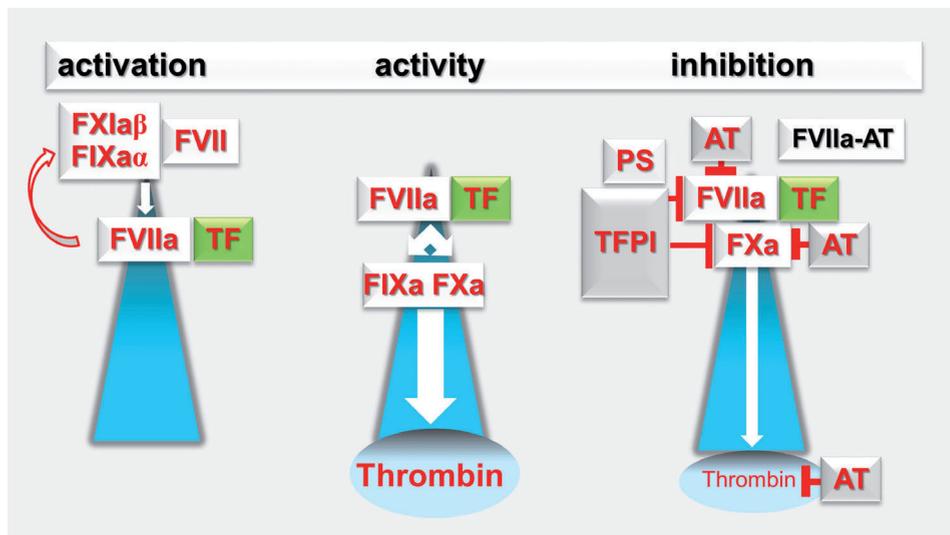

Figure 3. Schema showing factor VII activation, activity and inhibition (see text for specific information and references). TF: tissue factor; FIXaα: FIXa cleaved only after Arg226; FIXaβ: FIX cleaved after Arg191 and Arg226 (ref. [24]); AT: antithrombin; PS: protein S.
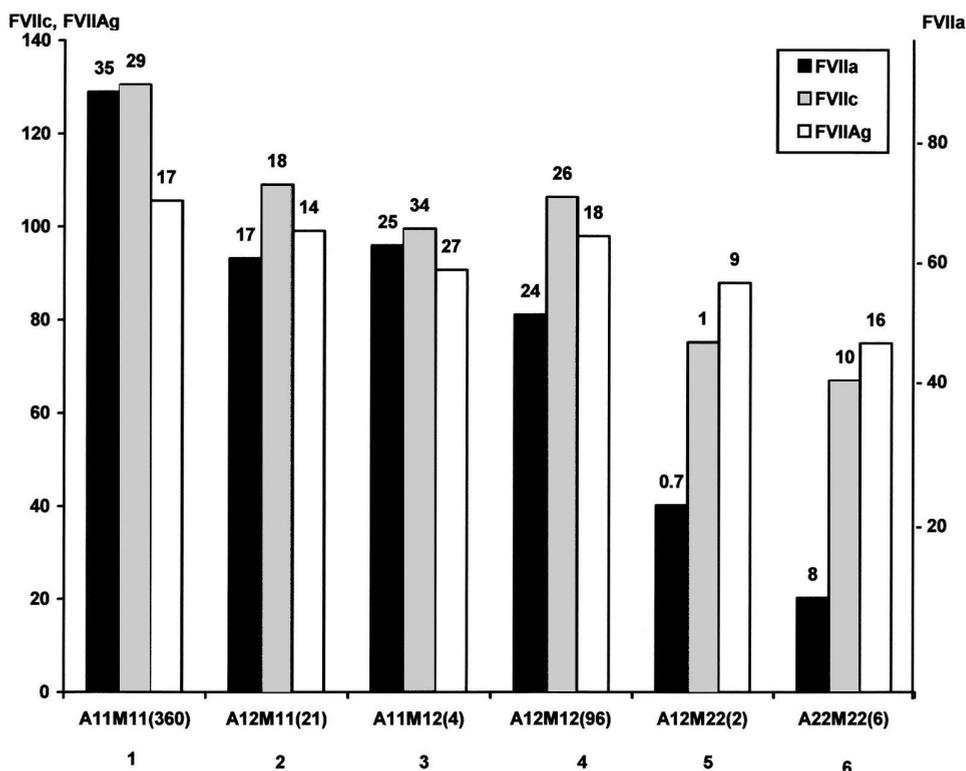
Figure 4. *F7* genotype driven differences in FVIIa, FVIIc and FVIIAg in the European population. Mean values of FVIIa (mU/mL), FVIIc (% of PNP), and FVIIAg (% of PNP) in genotype groups determined by the promoter (5'F7 ins del) and missense 353(413)Arg/Gln polymorphisms. Standard deviation is reported above each column. The number of subjects is reported in parentheses. Significant differences, groups 1–6 (*P*<0.001): FVIIc: 1 *vs.* 2, 4, 6; 4 *vs.* 6; FVIIa: 1 *vs.* 4, 6; 2 *vs.* 6; 4 *vs.* 6; and FVIIag 1 *vs.* 4, 6. Adapted from "Contribution of factor VII genotype to activated FVII levels. Differences in genotype frequencies between Northern and Southern European Populations".[107] FVII: factor VII; FVIIc: FVII activity; FVIIAg: FVII antigen; PNP: pooled normal plasma; ins: insertion; del: deletion

FVII deficiency is believed to be very rare (one case in 500,000 individuals).[36,37] The World Federation of Hemophilia[38] estimates around 5,000 FVII-deficient individuals in the countries covered by this organization. However, the Italian Registry of Congenital Rare Bleeding Disorders[39] (RBD) reported 1,023 cases with FVII deficiency among the 2,178 patients registered. This figure, similar to that reported by the UK registry (n=1.373),[38] suggests a prevalence only slightly lower than that of hemophilia B, at least in Italy (FVII deficiency, 1:59,000; haemophilia B, 1:67,000 individuals). Accordingly, FVII deficiency accounts for the most frequent RBD in most registries (36-49%).[37,39] These data support the concept (Table 1) that FVII deficiency, including mild, moderate and severe forms, might be much more prevalent than previously reported. Large differences in prevalence, with variation reaching one order of magnitude, have been reported in those geographical areas where consanguineous marriages are frequent.

Clinical picture

The bleeding tendency in FVII deficiency varies from forms more severe than those seen in the hemophilias[40] to very mild forms or asymptomatic cases. A simple classification of the clinical severity was proposed:[10] i) severe forms (central nervous system [CNS] and/or gastrointestinal [GI] bleeding and/or hemarthrosis; ii) moderate forms (three or more symptoms other than CNS and/or GI bleeding and/or hemarthrosis); iii) mild forms (one or two symptoms, mostly muco-cutaneous, and other than CNS and/or GI and/or hemarthrosis).

In the International *F7* Registry and the Seven Treatment Evaluation Registry (STER)[41] that include 787 cases, 12% of individuals had a severe to very severe phenotype and three fourth of them bled within the first year of age (Table 4). About 20% of individuals with FVII deficiency suffer from a bleeding disorder that can be life-threatening and/or be a major handicap. On the other hand, the most frequently observed patient cohort (>50%) is characterized by muco-cutaneous types of bleeding: epistaxis (two thirds), gum bleeding (one third), easy bruising (one third), and menorrhagia (70% of the females). In general, the bleeding tendency remains the same during the lifetime of the patient with the first bleeding event being a strong and independent predictor of the risk for the subsequent bleeding phenotype.[41] Co-inheritance of factor V Leiden enhances thrombin formation and is associated with a mild bleeding phenotype.[42]

Levels of FVIIc higher than 26%[11,41,43] may have a nil or minimal clinical relevance in terms of spontaneous bleeding and subjects with FVIIc levels <10% who remain asymptomatic most of their lives are not rare in being diagnosed during family studies or hemostatic screenings.[10,11,35]

Table 1. Distinctive features of coagulation factor VII (FVII), activated FVII , FVII deficiency, FVII levels and cardiovascular disease.

FVII	FVII (FVIIa)	FVII deficiency	FVII levels
Very low plasma concentrations (0.5 μg/mL)	First protease in the coagulation cascade. Lowest catalytic activity without cofactor (TF).	Highest prevalence among autosomally recessive rare bleeding disorders	High variability in human populations High number of environmental determinants
Short half-life 2-4 hours Stable in plasma	Half-life of FVIIa and FVII similar (2-3 hours) rFVIIa for replacement therapy	Complete deficiency results in perinatal mortality High incidence of intracranial bleeding (severe form)	High proportion of variance explained by *F7* gene variation The genotype/phenotype relationship is one of the most extensively investigated among human genes
Conserved in all jawed vertebrates	*F7* polymorphisms are strong predictors of FVIIa levels	Wide variation of bleeding phenotype in relation to modest FVII level variations	Frequent FVII level- lowering alleles with wide differences in human populations
High similarity with factor IX, factor X and Protein C	Very low amounts of FVIIa sufficient to initiate coagulation «Stable» FVIIa-AT complex	Homozygous FVII-lowering alleles mimic Mendelian heterozygous deficiency	High/low levels associated with predisposition/ protection towards/from cardiovascular disease

FVII: factor VII; FVIIa: activated FVII; rFVIIa: recombinant FVIIa; TF: tissue factor; AT: serpin antithrombin; *F7*: factor VII gene.

Table 2. Some open issues in factor VII (FVII) protein and *F7* genetics, FVII deficiency, FVII levels and cardiovascular disease.

FVII / *F7*	FVII deficiency	*F7* gene variation cardiovascular disease
Mechanisms underlying FVIIa levels	Residual FVII concentration predicting the most severe symptoms is still puzzling. Residual FVII concentration preventing spontaneous bleeding is difficult to define.	Population features causing large differences in the prediction of cardiovascular disease risk by *F7* genotypes
Decryption of TF for FVIIa binding and catalytic activity	Short plasma half life of FVII/rFVIIa for therapeutic purposes. Discrepancy between rFVIIa pharmacokinetic and pharmacodynamic data	Impact of FVII-lowering alleles in the protection from cardiovascular disease
Physiological balance between FVIIa inhibitors	Impact of FVII-lowering alleles in increasing the clinical penetrance of pathological *F7* gene lesions	Impact of FVII-increasing alleles in the predisposition toward specific cardiovascular disease forms

FVII: factor VII; FVIIa: activated FVII; rFVIIa: recombinant FVIIa; TF: tissue factor; *F7*: factor VII gene.

However, the therapeutic thresholds conferring protection from traumatic or post-operative bleeds are still a matter of debate.[44,45] Surgical bleeding has been reported in 15-24% of cases[42,45] with FVIIc <7%.[42] Bleeds in the first post-operative day[46] are particularly frequent if the bleeding disorder was not diagnosed before, and are mostly reported during orthopedic procedures,[46] a major hemostatic challenge. Presenting symptoms as post-circumcision bleeds and hemorrhage from the umbilical stump frequently herald a severe disease form.

Diagnosis

The spectrum of bleeding symptoms is wide, involves both males and females, can start after birth and may occur at any age. The most important predictors of bleeding risk, familial and personal histories, can be silent as observed in diseases with recessive inheritance. The relevance of the gynecological and obstetric histories are discussed in a dedicated paragraph.

The laboratory workup[47] performed after a bleeding episode or during a family screening includes routine screening assays (prothrombin time [PT], activated partial thromboplastin time [aPTT]), followed by FVIIc measurement, which is necessary to confirm the diagnosis. Evaluation of FVIIc is determined with a one-stage assay adding diluted plasma samples to FVII-deficient plasma and thromboplastin as the source of TF. The accuracy of FVIIc assays is related to the sensitivity of the thromboplastin reagent and to the quality of the FVII-deficient plasma and of the calibrators. The most suitable activators are the human placenta-derived and the recombinant preparations,[47] that are also recommended to monitor the treatment with rFVIIa.[25] New chromogenic or fluorogenic assays for FVIIa are available for research purposes and to monitor treatment with rFVIIa.[48]

The sensitivity of the FVIIc assay to levels below 2% may be poor, with negative implications for the accurate definition of the relationship between FVII levels and bleeding phenotypes (Table 1), as well as between genotype and phenotype. A low assay sensitivity may explain some inconsistencies such as the paradoxical observation of asymptomatic cases with low FVII levels. Furthermore, severe bleeding is sporadically observed in individuals with moderately reduced levels of FVII. The combination of the low assay sensitivity with the minimal amount of FVIIa-TF complex needed to trigger coagulation does not permit one to efficiently differentiate clinical phenotypes. As a consequence, in FVII deficiency a variety of bleeding phenotypes are observed in the presence of apparently modest differences in FVIIc levels (Table 1). In summary, the clotting tests do not always predict the degree of bleeding tendency in the single individual.

The presence of the FVII protein (FVII antigen [FVIIag]) can be determined by enzyme-linked immunosorbent assays or immune-turbidimetric assays, using monoclonal epitope-specific antibodies.[47] The FVIIag assay[47] does not predict the bleeding tendency, but does allow one to distinguish between type I (quantitative defects, with decrease of both FVIIc and FVIIAg), and type II defects (qualitative defects, with low FVIIc and normal or reduced levels of FVIIAg), and may help to clarify the different mutational mechanisms.

Molecular genetics

The key findings are summarized on a historical time scale in Figure 1B, and the main findings in individuals with FVII deficiency are shown in Table 3. The functional characterization of protein variants in plasma was carried out in seminal studies[49] well before the genetic characterization of FVII deficiencies. The first *F7* gene mutations in FVII deficiency were reported in the UK[50] and Italian populations, and more than 1,000 genetic diagnoses in several countries are reported in the International Registry on FVII deficiency (IRF7)[10] and the Greifswald Registry.[11] Mutations and residual FVII levels have been presented in databases.[51] The recent EHAD database[12] (http://www.umd.be/F7/W_F7/index.html) includes 221 unique variants identified in 728 individuals, of great help for the evaluation of *F7* mutations found during genetic counseling and diagnosis, including prenatal diagnosis.[5]

F7 mutations have been detected by sequencing coding, splicing and in promoter regions in the vast majority of patients. High throughput genomic sequencing will help in the case no mutations are found.[52] Cases with severe symptoms are virtually all homozygous or doubly heterozygous for *F7* mutations. Symptomatic mutations impairing vitamin K metabolism, and thus posttranslational modification of GLA residues, have been found to cause combined vitamin K-dependent clotting factor deficiency that includes an abnormal FVII biosynthesis and low FVIIc levels.

As reported in registries[11] and databases,[12] single nucleotide variants are the most common type of gene defect (around 90%), and include a large proportion (between two thirds and three quarters) of missense mutations.

Numerous missense mutations have been found in the homozygous condition, related to geographical, ethnic and mutational pattern features. Clusters of homozygotes often indicate genetic isolates or ethnic customs favoring consanguinity (see below).

Since they have been well presented in databases,[12] we shall briefly comment on a few of them.

The severe Gln100(160)Arg[53,54] and the moderate/mild Ala294(354)Val[55,56] are prevalent in Northern Europe. The frameshift mutation in the last carboxyl terminal codons (Pro404(464)Hfs) is very prevalent in cis with the Ala294(354)Val missense change and causes a 28 residues-elongated FVII molecule. This moderate to severe and combined variant is frequent in Central Europe and especially in Slovakia.[10] The mild Gly331(391)Ser mutation is prevalent in the gulf of Naples, Italy, as well as in other countries.[57] Other mild/asymptomatic changes, the Arg304(364)Gln[50], Cys310(370)Phe/Ser and Thr359(419)Met substitutions, have been found in several populations, suggesting recurrent nucleotide changes at a CpG site. The frequent Arg304(364)Gln is also characterized by discrepant FVII activity findings depending on the thromboplastin reagent.[49]

Splicing mutations are not rare,[52] and the homozygous splicing mutation IVS7+5 g>a, relatively frequent in Italy, has been used for cellular[58,59] and animal[60] models of gene therapy.

F7 deletions are also not rare and together with duplications, insertions and indel rearrangements represent about 10% of all genetic lesions.[12] However, large and extended deletions have not been described in homozygous individuals. Differently from hemophilia A and B, individuals with complete FVII deficiency might die perinatally or

Table 3. Overview of molecular genetics findings in factor VII deficiency.

Molecular genetics of FVII deficiency	
Genetic diagnosis	Sanger sequencing of exons and splicing junctions in >1,000 individuals with FVII deficiency Next-generation sequencing in a few individuals Phenotypic assays do not define specific genetic defects* No highly frequent gene lesions Large heterogeneity of genetic causes (n>300 different mutations) Geographical/ethnic clustering of identical-by-descent mutations (n>15) Several recurrent mutations in different populations
Mutation zygosity and disease severity	Severe deficiencies caused by compound heterozygous/homozygous mutations Increased prevalence of homozygotes in genetic isolates/consanguineous marriages Otherwise asymptomatic heterozygous variants associated with frequent FVII-lowering single nucleotide polymorphism in mild deficiencies
Mutation type frequency	Missense>>Small deletions>Splicing>Nonsense>Large deletions (not reported in homozygosity)
Recombinant expression	Numerous variants expressed after site directed mutagenesis A few purified protein variants Translational read through over premature termination codons Gain-of-function premature termination codon

*The asymptomatic Arg364Gln is detectable by using different thromboplastins[59]. Small deletions include also insertions and indels. FVII: factor VII.

Table 4. Management of factor VII deficiency using recombinant factor VII (synopsis of the STER study data).

Clinical Context	Treatments (n)	Treatment days Median, range	Total dose of rFVIIa Median, range (µg/kg of body weight)	Outcome
Spontaneous bleeding	79	1 (1-14)	60 (10-3,600)	3 rebleeding 1 inhibitor
Major surgery^	24	3.5 (1-16)	31.9 (12-120)	3 bleeding (orthopaedic surgery)
Minor surgery and invasive procedures	29	1 (1-4)	20 (7.2-510)	1 inhibitor
Prophylaxis§	31 18 (3/week) 13 (2/week)	3/week or 2/week	30 (3 per week) 24 (3 per week)	Partly effective in one case in each of the two arms§

The data express data analysis, not recommendations; ^ Mariani *et al.* 2011; §Napolitano *et al.* 2013 (no statistical difference between arms). FVII: factor VII; rFVII: recombinant FVII; STER: Seven Treatment Evaluation Registry.

shortly after birth due to severe hemorrhage.[5] Complete FVII deficiency causes perinatal mortality in "knockout" mice, in which the presence of a trace amount of FVII (0.7%) seems sufficient for survival with symptoms.[61] Several cases with heterozygous combined FVII and FX deficiency, due to large deletions within the terminal end of chromosome 13, have been reported.[62]

Recombinant expression after site-directed mutagenesis has been performed for a number of mutations detected in patients, which permits one to investigate the molecular bases of the deficiency. Recombinant studies of the rare and potentially null homozygous nonsense mutations support the notion that gain-of-function and translational readthrough over premature stop codons may prevent truly null conditions and may contribute to the unexpectedly variable bleeding phenotype in individuals homozygous for *F7* nonsense changes.[63] Whereas life-threatening symptoms have been associated with the p.Ser52(112)X, moderate bleeding was observed in p.Cys82 (132)X homozygotes. The Arg402(462)X change permits the secretion of a small amount of protein with gain-of-function features, causing a mild phenotype paradoxically associated with a "null" mutation.[64]

Chimeric fluorescent FVII molecules have also been constructed to investigate intracellular processing of variants, and chemical chaperones have been used to stimu-

late their secretion.[54] The FVII protein has been purified and biochemically characterized for very few variants, among these the frequent Arg304(364)Gln[50] and Ala294(354)Val[55] changes, as well as the severe Gln160Arg[33] and the activation sequence Val154(214)Gly[65] changes. These laborious experiments provided strong support for the understanding of the mild or severe coagulation phenotypes *in vivo* and for FVII altered expression and properties.

The homozygous conditions for polymorphisms predicting lower FVII levels might not be pathogenic but mimic Mendelian heterozygous FVII deficiency[35] (Table 1), which frequently leads to the request for clotting defect diagnosis in pre-surgical screenings, and generates confusion in estimating the incidence of inherited FVII deficiency (see dedicated section). In light of their effects on lowering FVII plasma levels, these variants might contribute to pathogenicity of co-inherited variants.[52] As a matter of fact the frequencies of the FVII-lowering alleles were significantly higher[51] in the FVII-deficient individuals tested than in controls, suggesting that the presence of these alleles may have increased, through increased clinical significance (Table 2), the likelihood of identifying "causative" *F7* gene lesions. Genotyping of these frequent variants would be especially useful for the interpretation of mild and moderate FVII deficiency.

Replacement therapy and prophylaxis

Replacement therapy (RT) options are determined by: the rarity of the disorder; the availability and supply of products and the economic and geographical factors. These include: i) recombinant FVIIa (rFVIIa), ii) plasma-derived FVII (pdFVII), iii) fresh frozen plasma (FFP) and prothrombin complex concentrates (PCC). In the STER prospective trial (comprising 312 RT) most therapies were carried out with rFVIIa (78%), the remaining with FFP (10%), pdFVII concentrates (10%%) and PCC (2%).

Based on the modest catalytic activity of FVIIa in the absence of TF, the therapeutic/prophylactic use of FVIIa and rFVIIa (initially proposed for bleeding diathesis other than FVII deficiency) represents a therapeutic milestone[8] and distinguishes replacement therapy in FVII deficiency from that in the other coagulation defects (Table 1). Although in FVII deficiency rFVIIa is employed (Figure 1A) at doses (Table 3) much lower than those needed for patients with FVIII inhibitors, supraphysiological FVIIa concentrations are also produced in plasma of FVII deficient subjects.

It is still a matter of debate whether rFVIIa acts through the binding with TF, provided by microparticles shed into the circulation following diverse stimuli, or binds at high concentration[66] to anionic phospholipids exposed on activated platelets, thus directly activating FX to FXa. FXa would in turn generate thrombin, bypassing the tenase complex. It is tempting to speculate that the physiological FVIIa-TF function (Figure 3) prevails at the rFVIIa doses used in FVII deficiency and in the presence of normal FVIII levels. rFVIIa easily diffuses into the extravascular spaces where it could be retained for extended time periods. As supported by pharmacokinetic studies,[67] rFVIIa prolongs its pharmacological effects at low concentration, in accordance with the hypothesis of physiological binding to TF.

rFVIIa has a very good safety-to-efficacy ratio.[46] One-day therapy with 'intermediate' doses of rFVIIa can be effective and safe for the treatment of most of spontaneous bleeds as well as for minor surgery and invasive procedures (Table 3). Replacement with rFVIIa is also effective to prevent bleeding in major surgical procedures.[46]

The large volume of the infusions and the limited availability make pd-FVII concentrates less appealing. Currently, the average pd-FVII dosages used are 15–20 IU/kg for mucosal bleeding, and 30–40 IU/kg for severe or life-threatening hemorrhages.

Although FFP is easily available in developing countries, its effectiveness is limited owing to the high risk of fluid overload and the consequent need for slow infusions.

In the case of mild mucosal bleeds (epistaxis, mild menorrhagia) tranexamic acid and hormones are currently considered.

Anti-fibrinolytic agents are contraindicated in hematuria, and may trigger thrombosis in association to PCC.

Prophylaxis is warranted for patients with the most severe bleeding picture and should be prescribed from childhood or soon after the first bleeding event.[45,68] Effective prophylaxis schedules have been reported for rFVIIa[68,69] and pdFVII concentrates.[70]

Treatment complications

Inhibitors to FVII are a rare (1-2%)[71] complication that occurs mainly in severely deficient patients, particularly in children younger than 1 year on prophylaxis. We observed only high responders (>5 BU Bethesda Unit)[71], and in the presence of a high-titer inhibitor to FVII treatment becomes a problem.[71]

At variance with the homologous deficiency of FIX (*F9*, HB), complete homozygous gene deletions that predispose to FIX inhibitors have never been detected in FVII deficiency (Table 1),[5,12] and FVII inhibitors have never been shown to be complicated by allergic reactions.

Paradoxically, a dozen of cases with FVII deficiency and thrombosis have been reported. Surgical interventions and/or replacement therapies had a close temporal relationship with thrombotic episodes, but apparently spontaneous events were also reported.[68,72] Different replacement therapies were associated with the thrombotic events: PCC (three cases), rFVIIa (three cases), pdFVII (two cases), FFP (one case), no replacement (three cases).[72] This suggests that FVII deficiency does not seem to offer protection from strong thrombosis risk factors such as surgery/high dose radiotherapy.

Women with factor VII deficiency

Autosomally transmitted RBD occur as frequently in women as in men, but women may experience more bleeding than men because gynecological and obstetric challenges to hemostasis add an important burden to the background bleeding related to the hemostatic defect. Menstruation and ovulation are associated with an increased risk of bleeding, as are pregnancy and delivery.[73] While in normal and heterozygous women FVII plasma levels rise during pregnancy, no such increase is observed in women with a severe homozygous deficiency. As a consequence, symptomatic women with FVII deficiency may continue to bleed during pregnancy and postpartum. In a large study (234 women with FVII deficiency),[74] menorrhagia during the reproductive age occurred in half of the cases, and in 12% represented the first bleed. Further, frequent gynecological problems, such as uterine fibroids, were diagnosed earlier because of the hemostatic defect. Although FVIIc was an important predictor of gynecological bleeding, other determinants including endocrine pathologies may also play a role.

For severe menorrhagia, management has been moved from hemostatic agents like tranexamic acid, oral contraceptives and intra-uterine devices, to replacement therapy and prophylaxis with effective single- or multiple-dose schedules.[68,73,74] This will hopefully change clinical practice that, until recently, included surgical approaches such as endometrial ablation or hysterectomy.

Therapeutics in development

The first attempts to improve rFVIIa activity through mutagenesis, or its half-life in plasma by glycoPEGylation, have increased FVII antigenicity. Longer-acting FVIIa has been tested in different recombinant preparations, which are currently in clinical trials (reviewed in Menegatti *et al.*)[75]: i) addition of a c-terminal peptide[76] potentially suitable for subcutaneous administration that led to prolonged pharmacodynamics effects, ii) an Fc receptor-fused rFVIIa that displayed a 5-fold longer plasma half-life and iii) an albumin-fused rFVIIa molecules that showed improved features compared to rFVIIa (2- to 3-fold longer half-life and 4- to 8-fold lower clearance) in patients with congenital FVII deficiency.[77] Recently, an engineered albu-

min-fused rFVIIa molecule showed enhanced transcellular transport upon intranasal delivery and extended plasma half-life in transgenic mice.[78]

The development of non-substitutive therapy for the hemophilias, such as the use of anti-antithrombin RNA interference or of aptamer and monoclonal antibodies directed against TFPI, have raised expectations for improvement of the quality of life in individuals with FVII deficiency.[75]

Based on adeno-associated viral-mediated expression of FVII[79], gene therapy has produced sustained correction of severe FVII deficiency in dogs. In addition, gene therapy using FVIIa, inserted into the same viral vector, could also be used to treat congenital FVII deficiency with lower vector doses, which increases the chance of efficient and long term expression of the transgene. Small engineered RNA have also been used as an approach of personalized gene therapy to correct a human FVII splicing mutation in mice.[59]

Factor VII levels, *F7* genotypes and cardiovascular disease

Several findings have created interest and still lead to an intense investigation on the role of FVII in cardiovascular disease (Figure 1B). Exposure of TF to blood in (coronary) artery disease, and especially plaque rupture, may favor FVIIa-TF complex formation. Further, lipids are important components of atherosclerosis and determinants of FVII activation and activity as well.[79,80] Additional links between high FVII levels and risk for thrombosis have been suggested[81] by finding that the TF-FVIIa-Xa complex activates FVIII before coagulation amplification and that heparanase increases generation of FXa by the FVIIa-TF-complex.[82] Furthermore, the FVIIa-TF complex is believed to mediate non-hemostatic functions in diverse biological processes, such as angiogenesis, inflammation, atherosclerosis and vascular and cardiac remodeling, by activating signaling pathways (reviewed in D'Alessandro *et al.*)[84] through FVIIa-integrin binding and PAR2 cleavage.[85]

The *F7* promoter may respond to a number of metabolic components, and FVIIc levels are associated with several environmental factors linked to atherosclerosis i.e., body mass index, dietary fat intake, plasma lipids and particularly triglyceride concentration.

Age- and sex-related variations in FVII levels add complexity to the investigation of clinical correlates. FVII levels were found to increase with age and to be significantly lower in women than men at younger ages. Postmenopausal women displayed the highest levels, except when undergoing hormone replacement therapy.[86] This conundrum of environmental factors interacts with the *F7* gene variation.

Factor VII levels and F7/genome wide genotypes

FVII levels show ample variation in the normal population and have a substantial heritable component, also in relation to polymorphisms[15] (Figure 4). Missense,[87] repeat number variation,[58] insertion/deletion[22] and SNP[23] were found to be associated with FVIIc, FVIIa and FVIIag levels (Figure 4) through epigenetic, transcription, biosynthesis- or stability-mediated mechanisms. Whereas twin studies[88] estimated about 60% of genetic-associated level of variance in plasma, the *F7* locus variation accounted for up to 40% of variance. Genotype effects, perhaps stronger on FVIIa than on FVIIag (Figure 4),[15] might modulate the response to environmental stimuli and the sex-dependent regulation.[89] Serum phospholipids were found to be strong and *F7* genotype-associated FVIIa determinants.[90]

The number of *F7* single SNP was substantially increased by highthroughput *F7* gene sequencing, which enabled very informative *F7* population studies.[91] Genome-wide association studies (GWAS) confirmed[14,92] the strong association between FVII levels and *F7* gene variation.[93] Importantly, GWAS have detected a number of genomic regions associated with FVII levels (*GCKR, ADH4, MS4A6A, PROCR, APOA5, HNF4A, REEP3-JMJD1C, JAZF1-AS1, MLXIPL* and *XXYLT1*),[14,92] that could explain an additional one fifth of the FVII variance in plasma levels and are also, in part, associated with lipids which in turn modulate FVII activity. These gene-based association scan initiatives have been established in several populations.[94] Overall, this picture defines one of the most extensively investigated relationship between genotypes and multiple quantitative phenotypes (FVIIc, FVIIag and FVIIa) (Table 1).

Levels, genotypes and cardiovascular disease

The Northwick Park Heart Study investigators were the first to report that high FVII levels were predictors of death due to coronary disease,[16] and a number of studies confirmed this observation in different populations by also evaluating FVIIa levels.[95]

Recent investigations on the level of the FVIIa-AT complex, which may reflect levels of FVIIa as well its interaction with TF, indicated that higher complex concentrations were associated with increased mortality in the Cardiovascular Health Study.[96] In patients with stable coronary artery disease (Verona Heart Study) higher FVIIa-AT complex levels were associated with increased cardiovascular mortality and increased thrombin and FXa generation,[97] particularly in the coagulation initiation phase.[98] However, in small groups of patients with unstable angina, acute myocardial infarction[99] or post-infarction[80] FVIIa levels were not higher than in controls. High plasma FVIIag levels were associated with failure of thrombolytic therapy in patients with myocardial infarction.[100] Elevated levels of FVII have not been consistently associated with venous thromboembolism.[101]

The influence of *F7* genotypes on the hemostatic balance and on the susceptibility to cardiovascular disease has been extensively investigated in several large cohorts of patients in relation to both myocardial infarction and stroke. *F7* polymorphisms with an opposite effect on FVIIa levels may positively or negatively modulate the risk of MI in males with advanced coronary artery disease,[18] and some FVII genotypes may protect against myocardial infarction[17] by affecting transcription levels and reducing protein functional activity. The modulation of stroke risk in atrial fibrillation by *F7* genotypes may follow a similar scheme[102] and recently, in a meta-analysis of several GWAS[14] variations in FVII-related genes and FVIIc levels were associated with a risk of the incidence of ischemic stroke in the general population. The physiological effects of FVII lowering alleles may represent a natural model for anticoagulation; in fact *F7* polymorphisms were shown to play a role in determining the initial response to warfarin[103] and influencing the risk of thrombosis in patients with essential thrombocythemia.[104]

Large haplotype studies confirmed that the *F7* gene strongly influences FVII levels, but associations with coronary artery disease and FVII level were inconsistent.[105] Variable genetic, environmental and atherogenic risk factors in different populations may explain this discrepancy (Table 2). The frequencies of *F7* genotypes predicting lower FVII levels are higher in Caucasians and much lower in Chinese and Malays populations. On the other hand, the distribution of these F7 genotypes covaries across populations in Europe with the rate of myocardial infarction mortality, being higher in countries at low risk.[106]

The most recent meta-analysis of large studies[14] appears to be consistent with positive and potentially clinically important causal effects of FVIIc levels, both on coronary artery disease and venous thromboembolism. However, several open issues remain (Table 2) and the usefulness of FVIIc and FVIIa evaluation in patients with cardiovascular risks is still unclear.

Conclusive remarks

In summary, in the 70 years since FVII investigation started, research has been particularly intense (Figure 1), and provided excellent examples at the basic science and translational levels. The multidisciplinary approaches have spanned structural biology, biochemistry, recombinant protein biotechnology, molecular and population genetics, pharmacology, clinics and epidemiology, and encompassed various areas of hematology, hemostasis and thrombosis.

The large number of studies on FVII have not only served to substantially improve our knowledge on FVII biology and related phenotypes and to improve the quality of life of the individuals with FVII deficiency, but also to train three generations of scientists in different fields.

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