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ORIGINAL ARTICLE

Factor V Leiden-independent activated protein C resistance: Communication from the plasma coagulation inhibitors subcommittee of the International Society on Thrombosis and Haemostasis Scientific and Standardisation Committee

Gary W. Moore[1,2] | Elisabetta Castoldi[3] | Jun Teruya[4,5,6] | Eriko Morishita[7] | Dorothy M. Adcock[8]

[1]Department of Haematology, Specialist Haemostasis Unit, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

[2]Faculty of Science and Technology, Middlesex University, London, UK

[3]Department of Biochemistry, CARIM, Maastricht University, Maastricht, the Netherlands

[4]Department of Pathology & Immunology, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas, USA

[5]Department of Pediatrics, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas, USA

[6]Department of Medicine, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas, USA

[7]Department of Hematology, Kanazawa University Hospital, Kanazawa, Japan

[8]Laboratory Corporation of America Holdings, Burlington, North Carolina, USA

Correspondence
Gary W. Moore, Department of Haematology, Specialist Haemostasis Unit, Cambridge University Hospitals NHS Foundation Trust, Hills Road, Cambridge CB2 0QQ, UK.
Email: gary.moore@addenbrookes.nhs.uk

Abstract

Activated protein C resistance (APC-R) due to the single-nucleotide polymorphism factor V Leiden (FVL) is the most common cause of hereditary thrombophilia. It is found predominantly in Caucasians and is uncommon or absent in other populations. Although FVL is responsible for >90% of cases of hereditary APC-R, a number of other *F5* variants that also confer various degrees of APC-R and thrombotic risk have been described. Acquired APC-R due to increased levels of coagulation factors, reduced levels of inhibitors, or the presence of autoantibodies occurs in a variety of conditions and is an independent risk factor for thrombosis. It is common for thrombophilia screening protocols to restrict assessment for APC-R to demonstrating the presence or absence of FVL. The aim of this Scientific and Standardisation Committee communication is to detail the causes of FVL-independent APC-R to widen the diagnostic net, particularly in situations in which *in vitro* APC-R is encountered in the absence of FVL. Predilution clotting assays are not FVL specific and are used to detect clinically significant *F5* variants conferring APC-R, whereas different forms of acquired APC-R are preferentially detected using the classical activated partial thromboplastin time-based APC-R assay without predilution and/or endogenous thrombin potential APC-R assays. Resource-specific recommendations are given to guide the detection of FVL-independent APC-R.

KEYWORDS
acquired, activated protein C resistance, coagulation assays, inherited, thrombosis

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Gary W. Moore and Elisabetta Castoldi contributed equally to this study.

1 | INTRODUCTION

Activated protein C (APC), with its cofactors protein S (PS) and factor V (FV), functions as a natural anticoagulant through proteolytic cleavage of activated factor V (FVa) and activated factor VIII (FVIIIa), thereby inactivating the cofactors of the prothrombinase and intrinsic tenase complexes, respectively [1]. Functional abnormalities of the protein C (PC) pathway, manifesting *in vitro* as a reduced anticoagulant response of the plasma to APC, are associated with an increased risk of venous thromboembolism (VTE) arising from resistance to the actions of APC [2].

The factor V Leiden (FVL) single-nucleotide polymorphism (1691G>A) leads to a single amino acid change (Arg506Gln) that abolishes the predominant APC cleavage site in FVa and confers activated protein C resistance (APC-R) [3]. Specifically, this cleavage site is required for efficient inactivation of FVa by APC and for the expression of the APC cofactor activity of native FV, in synergy with PS, which results in FVIIIa inactivation [1]. Predominantly found in Caucasians, FVL thrombophilia is the most common hereditary thrombophilia, accounting for ~20% of unselected patients with first VTE, up to 50% of those with familial thrombosis, and >90% of those with hereditary APC-R [1,4]. Consequently, diagnostic hemostasis and thrombosis facilities tend to restrict testing for APC-R to the detection of FVL, some even bypassing phenotypic assays and directly performing DNA analyses [4–6].

The recent recommendations by the International Society on Thrombosis and Haemostasis Scientific and Standardisation Committee (ISTH-SSC) for clinical laboratory testing for APC-R concentrated on FVL detection, recommending screening using phenotypic assays and confirmation using DNA analyses, and only briefly considered hereditary and acquired FVL-independent APC-R [7]. The present ISTH-SSC communication is intended as an adjunct to that publication to detail FVL-independent APC-R and recommend approaches for testing. FVL-independent APC-R has been reported to occur in 10% to 15% of the general population [8,9] and to be associated with an increased risk of VTE [10,11].

2 | NON-FVL APC-R

The occurrence of hereditary APC-R in the absence of FVL suggests the existence of additional genetic causes of APC-R, particularly in ethnic groups in which FVL is (virtually) absent. Although the classical activated partial thromboplastin time-based activated protein C resistance (CAPC-R) test can detect the effects of mutations that increase the levels of factor VIII (FVIII) (eg, FVIII Padua [12]) or factor II (FII) (eg, *F2* G20210A [13]), *F5* (encoding FV) remains the main genetic determinant of the APC-R phenotype as measured using the activated partial thromboplastin time-based assay [14].

FVa plays a central role in the PC pathway because it is both a substrate and cofactor of APC [15]. APC inactivates membrane-bound FVa via proteolytic cleavage of the heavy chain at positions Arg^{306}, Arg^{506}, and Arg^{679} [16,17]. Cleavage at Arg^{506} usually occurs first but causes only partial loss of cofactor activity. Complete FVa inactivation requires lipid-dependent cleavage at Arg^{306}, which is ~20-fold slower but greatly stimulated by the APC cofactor PS [18,19]. On the other hand, FV enhances the proteolytic inactivation of membrane-bound FVIIIa by the APC-PS complex [20] by stimulating cleavage of FVIIIa at both Arg^{336} and Arg^{562} [21]. The APC cofactor activity of FV requires APC-mediated cleavage of FV at Arg^{506} [22] and an intact junction between the B domain (C-terminal portion) and the light chain of FV [23].

Both the roles of FV in the PC pathway (ie, FVa as a substrate and FV as a cofactor of APC) are probed using the CAPC-R assay [15], and predilution of sample plasma in factor V-deficient plasma (FVDP) in the modified activated protein C resistance (MAPC-R) assay increases the sensitivity and specificity for FV defects conferring APC-R. An *F5* mutation may be suspected whenever the MAPC-R assay yields positive results at repeated testing and the genetic test for FVL yields negative results. Notable exceptions are rare cases of liver or bone marrow transplantation between individuals with different FVL genotypes [24], wherein a genotype or phenotype discrepancy arises, because the circulating FV assessed in the functional assay originates from the liver, whereas the genomic DNA used for FVL genotyping is usually isolated from bone marrow–derived blood cells. If necessary, a patient's own genotype can be assessed using DNA isolated from a buccal swab.

In the past 25 years, several *F5* missense mutations and polymorphisms different from FVL have been shown to be associated with APC-R in various populations (Table). These variants may affect an APC cleavage site directly, make one of these sites less accessible to APC-mediated cleavage, or interfere with the binding of FVa to negatively charged phospholipids, which is required for both FVa inactivation and the APC cofactor activity of FV. Moreover, they may reduce the expression or secretion of FV, thereby decreasing the availability of FV as an APC cofactor and lowering the level of circulating tissue factor pathway inhibitor-α (TFPIα) [25].

Two different mutations affecting the Arg^{306} APC cleavage site have been reported. FV-Arg306Thr (FV Cambridge) was first described in a British patient with VTE and APC-R in the absence of FVL [26] and later sporadically encountered in other patients and controls, all of Caucasian descent [27–29]. FV-Arg306Gly (FV Hong Kong) was originally discovered in Hong Kong Chinese [30], among whom, 4.5% of blood donors are carriers [31]; however, it is also

Essentials

- Factor V Leiden thrombophilia is the most common hereditary thrombophilia.
- Some rare *F5* variants are associated with activated protein C resistance (APC-R) and thrombosis.
- Acquired APC-R, with thrombotic risk, occurs in a variety of conditions.
- Appropriate assay selection is required to detect different causes of APC-R.

TABLE Non–factor V Leiden causes of activated protein C resistance.

Non-FVL APC-R	Thrombotic risk	Preferred phenotypic assays for routine APC-R diagnostics	Further information
Hereditary			
FV Cambridge	Little or none	Predilution clotting assay	Not all heterozygotes experience thrombosis
FV Hong Kong	Little or none	Predilution clotting assay	May not manifest in phenotypic clotting assays
FV Liverpool	Thrombotic risk	Predilution clotting assay	Heterozygotes are asymptomatic; thrombotic risk when FV level is reduced
FV-Met443Leu, FV-Glu461Gln, FV-Gly493Glu	Unclear	Predilution clotting assay	Further functional studies required
FV Bonn	Thrombotic risk	Predilution clotting assay	Heterozygosity is a thrombotic risk
FV-Glu666Asp	Weak thrombotic risk	Predilution clotting assay	Not all subjects with phenotypic APC-R experience thrombosis
FV Nara	Thrombotic risk	Predilution clotting assay	Heterozygotes are asymptomatic and have intermediate APC-R ratios; homozygosity associated with reduced FV and TFPIα
FV Kanazawa	Thrombotic risk	Predilution clotting assay	Heterozygotes are asymptomatic; thrombotic risk when FV level is reduced (mild APC-R)
FV Besançon	Thrombotic risk	Predilution clotting assay or ETP-based APC-R[a]	Heterozygotes are asymptomatic; homozygosity associated with reduced levels of FV and TFPIα; marked APC-R, especially with PRP in ETP-based assay
FV (H)R2	Only in combination with FVL	Predilution clotting assay	Phenotype more severe than that for FVL heterozygosity alone
FV-Arg485Lys	Little or none	Predilution clotting assay	Conflicting reports of the presence or absence of APC-R and of clinical significance
Acquired			
Elevated FVIII	Thrombotic risk	CAPC-R	APC-R does not manifest in other assays
Elevated prothrombin	Thrombotic risk	CAPC-R	APC-R can manifest in ETP and theoretically in the RVV-X assay without predilution
Reduced FPS	Thrombotic risk	ETP-based APC-R	APC-R can manifest in CAPC-R if FPS <20%
Reduced TFPIα	Thrombotic risk	ETP-based APC-R	APC-R does not manifest in clotting assays
Hormone-induced (OC, pregnancy, HRT)	Thrombotic risk	ETP-based APC-R	APC-R due to elevated FVIII levels can manifest in CAPC-R
Solid tumors	Thrombotic risk	CAPC-R	APC-R does not manifest in other assays
Hematologic malignancies	Thrombotic risk	ETP-based APC-R	APC-R can manifest in CAPC-R if FPS <20%
Antiphospholipid syndrome	Thrombotic risk	CAPC-R or ETP-based APC-R	APC-R in ETP associated with thrombosis; potent LA-induced APC-R may manifest in predilution clotting assays

(Continues)

TABLE (Continued)

Non-FVL APC-R	Thrombotic risk	Preferred phenotypic assays for routine APC-R diagnostics	Further information
Other autoantibodies (PC, PS, FV)	Thrombotic risk	CAPC-R or ETP-based APC-R	Potent antibody–induced APC-R may manifest in predilution clotting assays

APC-R, activated protein C resistance; CAPC-R, classic activated protein C resistance assay; ETP, endogenous thrombin potential assay; FPS, free protein S; FX, factor X; FXa, activated factor X; FV, factor V; FVIII, factor VIII; FVL, factor V Leiden; HRT, hormone replacement therapy; LA, lupus anticoagulant; OC, oral contraceptive; PC, protein C; PRP, platelet-rich plasma; PS, protein S; RVV, Russell's viper venom; TFPIα, tissue factor pathway inhibitor α.
[a] It is likely that the markedly reduced FV activity in a homozygous patient would compromise CAPC-R and predilution APC-R assays. Suitable predilution clotting assays include those based on activated partial thromboplastin time, *Crotalus viridis helleri* FX activator, exogenous FXa, FX activator from RVV-X, and a combination of the FV activator from RVV-V and noscarin.

present at a very low frequency in other ethnic groups [27,28,32,33]. Recombinant FV Cambridge and FV Hong Kong are functionally indistinguishable and show incomplete APC-catalyzed FVa inactivation, which is improved by PS, and slightly decreased APC cofactor activity in FVIIIa inactivation [34]. This translates to mild APC-R in reconstituted FV-deficient plasma [34,35]. The contribution of these mutations to the risk of VTE seems modest, if any at all [36].

The FV-Ile359Thr (FV Liverpool) mutation was identified in the pseudohomozygous condition in 2 Caucasian brothers with recurrent VTE in their teens and mild APC-R [37]. This amino acid substitution induces glycosylation of Asn^{357} in the heavy chain of FV, which, in turn, hinders the APC-mediated cleavage of FVa at Arg^{306} and abolishes the APC cofactor activity of FV [38]. In the 2 brothers, the procoagulant properties of FV Liverpool were probably exacerbated by FV deficiency on the counterpart allele (through a pseudohomozygous APC-R mechanism [39], see subsequent sections). In fact, the probands' mother, who carried only FV Liverpool, had neither APC-R nor a history of VTE despite 4 pregnancies.

Sequencing of *F5* exon 10 in Chilean Amerindians with VTE and APC-R in the absence of FVL revealed 3 private variants (FV-Met443Leu, FV-Glu461Gln, and FV-Gly493Glu) that were absent in 100 healthy subjects from the same population [40]. The association of these variants with APC-R needs to be confirmed in a larger cohort and/or using functional studies of the recombinant mutants.

FV-Ala512Val (FV Bonn) was identified as the underlying cause of moderate APC-R (comparable with that of FVL heterozygotes in the CAPC-R test) in 6 apparently unrelated Caucasian patients with VTE or (recurrent) abortions and in 2 family members [41]. Characterization of the recombinant mutant showed that FV Bonn interferes with both FVa inactivation and the APC cofactor activity of FV because of reduced APC-mediated cleavage at the nearby Arg^{506} site [41]. FV Bonn also demonstrates more procoagulant activity in the absence of APC, possibly because of increased affinity for activated factor X (FXa), as suggested by its central location in a major FXa-interaction surface [42].

The FV-Glu666Asp mutation was associated with APC-R in a Chinese thrombophilic family, which was attributed to interference with cleavage of FVa at the nearby Arg^{679} site [42]. The mutation was absent in 50 healthy controls [43] and 163 unrelated patients who underwent thrombophilia screening [44].

A 13-year-old Japanese boy with recurrent VTE, low level of FV activity (FV:C 10%), and pronounced APC-R was found to be homozygous for the FV-Trp1920Arg (FV Nara) mutation [45]. His heterozygous parents had only mild APC-R and no history of VTE. Recombinant FV Nara showed markedly delayed FVa inactivation, especially in the presence of PS, and no APC cofactor activity in FVIIIa inactivation [45]. In contrast to the previous APC-R mutations, FV Nara is located in the light chain of FV (C1 domain), which mediates the binding of FV to phospholipids. This interaction is necessary for all functions of FVa, including the assembly of the FVa-inactivating complex, in which membrane-bound FVa and PS synergistically promote the binding of APC to phospholipids [46]. Defective interactions of FVa Nara with phospholipids and/or PS account for its decreased susceptibility to APC-mediated FVa inactivation [46] and might explain its lack of APC cofactor activity.

A 42-year-old Japanese man with recurrent deep vein thrombosis was compound heterozygous for Tyr1961Cys (FV Kanazawa) and Asn1982_Ser1983del and had reduced FV activity and antigen levels (6% and 38%, respectively). His brother, with the same variants, also developed recurrent deep vein thrombosis; however, their parents, carrying either variant in the heterozygous state, had no history of VTE. The FV antigen and activity levels of recombinant FV-Tyr1961Cys (rFV-Y1961C) were ~80% and 30%, respectively, of those of wild-type FV. The patient's plasma exhibited weaker APC-R than heterozygous FVL, and rFV-Y1961C was less APC resistant than recombinant FVL. Tyr^{1961} is located in the same C1 domain as FV Nara, and the binding capacity of rFV-Y1961C to phospholipids was reduced. Therefore, FV Kanazawa is similar to FV Nara in terms of symptoms and mutation sites and may result in impaired inactivation of membrane-bound FVa by APC (E. Morishita; unpublished observations).

A similar mechanism of APC-R is thought to apply to FV-Ala2086Asp (FV Besançon), another missense mutation in the light chain of FV (C2 domain), which was identified in the homozygous state in a 37-year-old Moroccan patient with a history of recurrent VTE since the age of 19 years [47]. Homozygosity for FV Besançon was associated with FV deficiency (FV activity, 3%) and pronounced APC-R, especially in the endogenous thrombin potential (ETP)-based assay performed in platelet-rich plasma (whereas thrombin generation in platelet-poor plasma suggested a hypocoagulable state). Functional

characterization of FV Besançon in highly diluted plasma from the patient provided indirect evidence for reduced phospholipid affinity and showed delayed FVa inactivation in the presence of PS and complete loss of the APC cofactor activity of FV in FVIIIa inactivation [47]. However, heterozygosity for the *F2* G20210A mutation and a low TFPIα level may have also contributed to the patient's APC-R phenotype.

The FV (H)R2 haplotype, tagged by His1299Arg and Asp2194Gly polymorphisms, is overrepresented (especially in the homozygous state) among individuals with APC-R in the absence of FVL [48,49]. Moreover, it is enriched in FVL carriers with particularly marked APC-R [48,50]. FV R2 carriership predicts slightly decreased FV levels [50] and a relative increase in the level of a FV glycosylation isoform (FV1) that has reduced affinity for phospholipids [51]. Both the changes contribute to impaired APC cofactor activity in FVIIIa inactivation, which is the main mechanism of FV R2–associated APC-R [51]. The FV R2 haplotype has a worldwide distribution, with an incidence of ~10% in most populations [48,49]. Its associated risk of VTE is mild, if any [52].

FV-Arg485Lys, another common variant with a wide geographic distribution [53], was initially reported as a neutral polymorphism [54]. Later studies have suggested its association with APC-R [55], VTE [56], coronary artery disease [55], and pre-eclampsia [57,58] in different ethnic groups. However, these findings are not consistent among the studies and need to be confirmed.

Finally, it is worth noting that the coinheritance of an APC-R mutation on 1 *F5* allele and a FV deficiency mutation on the other *F5* allele results in enhanced APC-R and risk of VTE. This rare condition, known as "pseudo-homozygous APC-R" [39], has been best studied for FVL but also applies to other *F5* mutations that confer APC-R. Pseudohomozygous individuals are genotypically heterozygous but functionally homozygous for APC-R mutations because the counterpart (non-APC-R) allele is not expressed. As a consequence of low FV levels, the APC cofactor activity of FV and the TFPIα level are also reduced, increasing the APC-R and the risk of VTE well beyond those in conventional heterozygotes with a normal counterpart allele [59,60].

3 | ACQUIRED APC-R

Acquired APC-R represents an independent risk factor for VTE and can increase thrombotic risk in patients with hereditary thrombophilia [1,8]. A variety of mechanisms can lead to acquired APC-R, such as increased levels of coagulation factors and reduced qualitative and quantitative levels of naturally occurring inhibitors, or it can be antibody mediated. Consequently, the detection of acquired APC-R is assay dependent [61,62].

3.1 | Elevated coagulation factor levels

The ABO blood group and, hence, von Willebrand factor, have a marked effect on the plasma levels of FVIII, with higher levels of von Willebrand factor and FVIII being encountered in non-O groups (highest in the AB group) [63]. Other contributors to higher FVIII levels include increasing age, sex (higher in women), ethnicity, stress, exercise, surgery, pregnancy, and other causes of an acute-phase response [63]. Recently, a gain-of-function duplication in the FVIII gene, designated as FVIII Padua, was shown to be associated with markedly elevated FVIII levels and severe thrombophilia [12]. Persistently elevated plasma levels of FVIII (>150 IU/dL) are a prevalent, dose-dependent risk factor for VTE and recurrence [63,64], which is associated with increased thrombin generation and acquired APC-R [8,65].

Elevated FVIII levels are a common cause of acquired APC-R when assessed using the CAPC-R assay, exhibiting an inverse relationship between plasma levels of FVIII and APC-R ratios [5,61,63,66]. The MAPC-R assay is insensitive to elevated FVIII levels because dilution in FVDP normalizes the levels. Other assays, such as those that employ snake venom coagulation activators, bypass the intrinsic pathway and are innately unaffected by FVIII levels [5,61]. ETP assays modified to detect APC-R are generally insensitive to APC-R caused by elevated FVIII levels, likely related to the concentration of tissue factor (TF) in the assays [61,67].

A similar situation exists for elevated FII levels, whether due to *F2* G20210A or other causes, in that it is associated with increased thrombin generation and acquired APC-R when assessed using the CAPC-R assay [1,13,68]. There is an inverse relationship between FII levels and CAPC-R assay ratios, and ratios low enough to indicate APC-R have been reported in subjects with a FII activity of >110 IU/dL [69], which may be reagent dependent [61]. Predilution in FVDP normalizes the FII levels, rendering the MAPC-R assay and other predilution assays insensitive to the APC-R of elevated FII. Although PS and TFPIα are the main determinants of ETP-based APC-R assays [61], these assays are, nonetheless, able to detect APC-R associated with elevated FII levels [70]. As determined using the ETP-based APC-R assay, FII-induced APC-R is modulated by PS levels. The mechanism of acquired APC-R arises from dose-dependent inhibition of APC-catalyzed proteolysis of FVa by FII [71].

Both activated factor IX and FXa exhibit protective effects on their cofactors FVIIIa and FVa from APC-mediated proteolysis, and elevated levels of both the factors are associated with an increased risk of VTE, although elevated factor X levels are not a risk factor when the levels of other vitamin K–dependent factors are taken into account [18,19,72–74]. Thus, elevated levels may cause APC-R through increased cofactor protection [1], although no studies have been performed to assess the detection of APC-R due to elevated levels of factor IX and factor X in routinely employed assays.

3.2 | Reduced levels of naturally occurring inhibitors

Hereditary and acquired quantitative and functional deficiencies of free PS can lead to APC-R through reduced APC cofactor activity in the inactivation of FVa and FVIIIa, and also from increased protection of FVIIIa by FXa, which is otherwise abrogated by normal PS levels

[1,62,72]. If due to reduced levels of free PS alone, the acquired APC-R will manifest in the CAPC-R assay only when the free PS levels are <20%, and again, dilution in FVDP normalizes these levels, rendering other such APC-R assays insensitive to reduced levels of free PS [5].

Although FII and FVIII are the main determinants of the CAPC-R assay, PS and TFPIα are the main determinants of ETP APC-R assays [61]. TFPIα, in conjunction with PS as the cofactor, inhibits only slow-onset FXa and thrombin generation, such as that in the initiation phase of coagulation, but is too slow to effectively inhibit fast generation of FXa in ETP assays that employ high TF concentrations [75]. In the high-TF-concentration ETP-based APC-R assay, reduced PS levels have a marked effect because the anticoagulant effect of APC is >95% PS dependent. At normal functional levels of TFPIα and PS, the TFPIα-PS system further reduces FXa formation once the APC-PS system sufficiently retards thrombin generation, whereas reduced TFPIα levels lead to increased FXa formation, thereby facilitating the protective effect of FXa on FVa proteolysis by APC [75]. Moreover, TFPIα has been shown to interact directly with FV [25,76] and inhibit its cleavage at Arg^{1545} [77], thereby delaying full FV activation [77], regulating prothrombinase activity [76], and preserving the APC cofactor activity of FV in FVIIIa inactivation [23]. Impairment of these additional functions of TFPIα might also contribute to increased APC-R at low TFPIα levels.

3.3 | Hormone-induced APC-R

Acquired APC-R through the effects of hormones can be encountered with the use of oral contraceptives (OCs), during pregnancy, and with postmenopausal hormone replacement therapy (HRT) [1]. Estrogen-containing OCs and HRT medications are associated with altered hemostatic balance and a dose- and preparation-dependent increase in the risk of VTE [78,79]. Although there are changes in the levels of several coagulation factors and inhibitors, marked reductions in TFPIα and PS levels are the main contributors to hormone-induced APC-R [1,61,78,80]. Consequently, ETP-based APC-R assays should be used to detect the acquired APC-R of OC and HRT [1,61,81,82]. A recently described TF-activated fibrin clot kinetic assay, FibWave, exhibited sensitivity comparable with that of a standardized ETP-based APC-R assay for the detection of OC-associated APC-R and differentiation of resistance levels [83]. Further standardization of methods and confirmation of results are needed to establish the suitability of the assay for routine clinical use.

Although similar hemostatic alterations are encountered during pregnancy, the marked elevation in FVIII levels and reduction in PS levels can permit acquired APC-R to also manifest in the CAPC-R assay [84]. There are conflicting reports about whether acquired APC-R during pregnancy is a risk factor for VTE [85,86], and an association with recurrent fetal loss has been reported [87,88].

3.4 | Cancer-associated APC-R

Acquired APC-R is common in patients with cancer and is associated with VTE; however, it is also found in patients with cancer and without thrombosis [1,89]. Acquired APC-R in solid tumors is due mainly to elevated levels of procoagulant factors, such as FVIII, and can be detected using the CAPC-R assay [89]. Conversely, acquired APC-R encountered in patients with hematologic malignancies is correlated with decreased levels of PS and TFPIα, requiring ETP-based APC-R assays for detection [90]. Some chemotherapeutic agents may also contribute to the occurrence of APC-R [91].

3.5 | Autoantibodies and acquired APC-R

Acquired APC-R has been described in patients with autoantibodies directed to targets in the PC system, such as PC itself, PS, and FV, and can be accompanied by a severe thrombotic phenotype [92–94]. Such antibodies often occur in association with antiphospholipid syndrome and systemic lupus erythematosus but can also occur in isolation. APC-R induced by these antibodies may manifest in clotting-based assays and/or ETP-based APC-R assays.

An additional consideration for patients with antiphospholipid antibodies is that lupus anticoagulants (LA), anti-β2 glycoprotein I (aβ2GPI) and anti-phosphatidyl serine/prothrombin (aPS/PT) antibodies can also induce clinically significant acquired APC-R [95,96]. LAs are a particular analytical challenge because they can interfere with baseline clotting times of the CAPC-R assay and, in predilution assays, if the LA is sufficiently potent to overcome the dilution, unless the assay is innately LA insensitive [5,95,97]. However, the results are still reliable because LA-induced acquired APC-R, indicated by a reduced ratio, is evidenced by a reduction in clotting time with the addition of APC [5,95,97]. Acquired APC-R due to LAs, anti-β2 glycoprotein I, and antiphosphatidyl serine/prothrombin can manifest in ETP-based APC-R assays.

3.6 | Liver transplantation and acquired APC-R

The transplantation of an FVL liver to a non-FVL recipient gives rise to the intriguing concept of acquired hereditary APC-R, which can lead to thrombosis in some cases [98]. A direct genetic analysis for FVL using DNA from recipient leukocytes or a buccal swab will not detect the phenotypic alteration, necessitating APC-R assessment using functional assays. Hypercoagulability is a feature that occurs in the first weeks after liver transplantation, and the associated elevation in FVIII levels has been linked to acquired APC-R [99]. Thus, transplantation of a non-FVL liver to a non-FVL recipient could, at first, reveal APC-R in the CAPC-R assay, which is not reflected in the MAPC-R assay or an FVIII-independent assay. If APC-R is detected in the latter assays, it is suggestive of an FVL liver; however, they should be checked again once any acute-phase response has subsided.

4 | WHICH ASSAYS TO USE AND WHEN

In line with recent recommendations on thrombophilia testing, indiscriminate searches for hereditary causes of non-FVL APC-R are discouraged [100–102]. In practice, where those recommendations

are followed, attempts to detect non-FVL variants of *F5* conferring APC-R will only be initiated upon finding a positive APC-R assay but absent FVL in patients already deemed appropriate for testing. As depicted in the Table, there are a number of *F5* mutations different from FVL that can cause APC-R, and there are various causes of acquired APC-R. One must consider which assay to perform first in a practical setting, functional APC-R or genotypic FVL assay. A single APC-R assay is likely less costly than FVL assays. However, as with many other functional coagulation tests, the CAPC-R assay is affected by many preanalytical variables such as changes related to acute thrombosis, the use of anticoagulants, or the presence of an LA. Other APC-R assays, particularly those that employ sample predilution, are less affected by these issues compared with the CAPC-R assay, with the degree of interference being dependent on assay design and reagent composition [7]. For instance, predilution in FVDP corrects for factor deficiencies and elevations, including the effects of vitamin K antagonists, and many reagents contain heparin neutralizers. Dilution can also reduce interference by LAs, and some assays are designed to be anyway insensitive to, or are unaffected by, LAs. Users should be aware of interferences affecting functional APC-R assays in local use, the details of which are given in the ISTH-SSC recommendations on clinical laboratory testing for APC-R [7]. Predilution APC-R assays can approach a sensitivity and specificity of 100% for *F5* mutations that confer APC-R, provided that interferences are robustly excluded; however, the sensitivity to acquired APC-R is markedly reduced. Therefore, repeat testing of APC-R is not uncommon, whereas repeating a molecular assay for FVL is only rarely indicated [103], although it can be more analytically complex and, thus, not available at all centers.

The frequency of hereditary causes of non-FVL APC-R is far lower than FVL. Consequently, many institutions bypass APC-R assays and include FVL in their first-tier thrombophilia workup. However, genotyping for other rare *F5* mutations may be performed as third-tier workup because it cannot be performed even in most reference clinical laboratories. Negative FVL and positive predilution APC-R assay results can be employed as indicators of progression to genotyping for rare *F5* mutations that confer APC-R in appropriate patients, ideally after the exclusion of acquired APC-R, with the recognition that concomitant acquired APC-R and non-FVL hereditary APC-R are not beyond possibility. Although acquired APC-R is considered to be an independent risk factor, the risk factors for thrombophilia that lead to acquired APC-R can be detected using other assays such as FVIII, PC, PS, or LA assays. The clinical conditions that can cause APC-R, such as cancer or estrogen use, can be assessed using other diagnostic procedures or by history taking. To date, there is no convincing evidence to show that the presence of APC-R in patients with antiphospholipid syndrome should change clinical management in terms of the strength or duration of anticoagulant therapy. The development of APC-R is not necessarily ubiquitous in conditions associated with acquired APC-R. Testing using appropriate assays can confirm the presence of APC-R as a potential contributor to any thrombotic events and, in some situations, function as a risk stratifier [96,104]. Some practitioners employ the CAPC-R assay in tandem with a predilution assay to detect some forms of acquired APC-R during first-tier screening, in which a reduced CAPC-R ratio but normal ratio using the predilution assay suggests acquired APC-R [7,97]. The European Medicines Agency recommends use of ETP-based APC-R assays to investigate APC-R in the development of steroid contraceptives in women [105]. The use of ETP-based APC-R assays after 1 cycle of hormonal treatment has been suggested to identify women who are oversensitive to combined OCs, with the potential to alter prescribing decisions [106]. An ETP-based APC-R assay has been validated for clinical investigation of steroid contraceptives in women, although further clinical studies are required before it can be recommended as a tool for clinical management decisions [107,108]. As stated previously, after liver or bone marrow transplantation, an APC-R assay should be performed because FVL assays are performed using DNA from leukocytes, not the liver, which is where circulating FV is synthesized.

FVL is more common in Caucasians, particularly in Europe, and absent in the Southeast Asian, Japanese, and African populations [109], such that patient ancestry can dictate whether to use APC-R or FVL assays at the first instance. Native populations, such as those of the Americas, Australia, and Greenland, also lack FVL unless admixture with the White population has occurred.

When determining the best assay options, several factors need to be considered: in-house laboratory or reference laboratory capabilities for APC-R and FVL testing, patient population, and volume of patients with thrombosis. A preanalysis of simple cost comparisons between APC-R and FVL assays is difficult. Thrombophilia workup guidelines should be in place based on local resources, expertise, and patient demographics. Screening using functional APC-R assays fits well where relatively large numbers are being tested and where expertise and resources favor coagulation-based assays, although positive test results require follow-up using genotypic FVL assays, which may involve sample referral to reference centers. First-tier FVL testing circumvents preliminary screening and provides direct confirmation of the presence or absence of the most common hereditary causes of APC-R but risks missing other causes of APC-R if functional APC-R assays are not available for appropriate patients.

5 | CONCLUSIONS

As shown in the Figure, APC-R is a complex phenotype. In the evaluation of thrombophilia, consideration should be given to acquired causes of APC-R and hereditary APC-R in the absence of FVL.

5.1 | Key recommendations

- Guidelines for thrombophilia workup need to be developed depending on the patient population, capacity of the hospital or institution, and available resources.
- If there is a strong suspicion of hereditary thrombophilia based on positive APC-R and negative FVL assay results, molecular workup for rare, non-FVL *F5* mutations may be warranted.

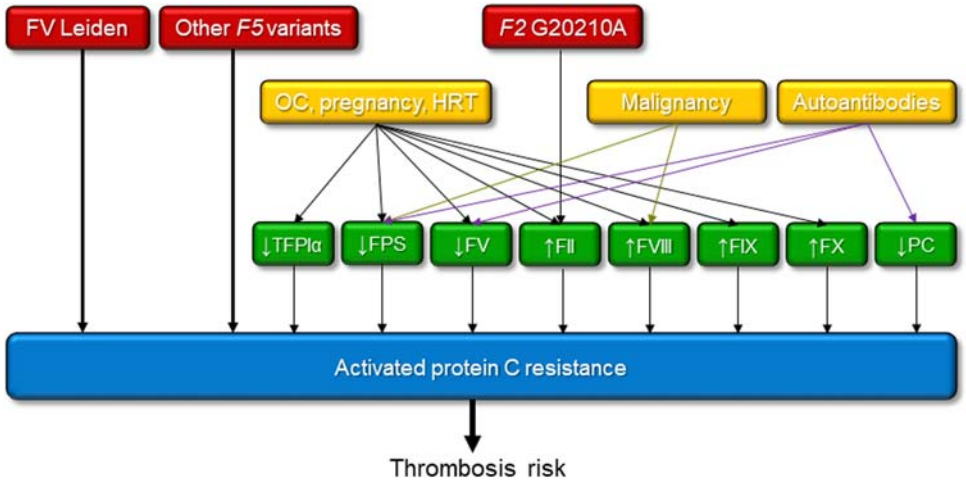

FIGURE Activated protein C resistance (APC-R) as a complex phenotype (modified from [1]). Factor V Leiden is responsible for >90% of cases of hereditary APC-R; however, other *F5* variants that confer variable degrees of APC-R and thrombotic risk exist. Acquired alterations to the levels of coagulation factors and naturally occurring inhibitors or the presence of autoantibodies can also give rise to APC-R, which is detectable using clotting-based and/or endogenous thrombin potential-based assays. APC-R, activated protein C resistance; FII, factor II; FV, factor V; FVIII, factor VIII; FIX, factor IX; FX, factor X; FPS, free protein S; HRT, hormone replacement therapy; OC, oral contraceptive; PC, protein C; TFPIα, tissue factor pathway inhibitor α.

- Although not readily available in most laboratories, ETP-based APC-R and CAPC-R assays may assist in the evaluation of acquired APC-R.
- If assessment for APC-R is indicated after bone marrow or liver transplantation, a functional APC-R assay needs to be performed.

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AUTHOR CONTRIBUTIONS

J.T. and G.W.M. conceived the idea of the manuscript. E.C. wrote the section regarding non–factor V Leiden hereditary activated protein C resistance. E.M. added information on factor V Kanazawa. G.W.M. wrote the section about acquired activated protein C resistance. J.T. and G.W.M. wrote the section about assay use. D.M.A. supplied additional information and edited the manuscript. All authors critically reviewed and revised the manuscript and approved the final version of the paper.

DECLARATION OF COMPETING INTEREST

G.W.M. reports consultancy fees from Technoclone. All other authors declare no competing interests related to this work.

ORCID

Gary W. Moore https://orcid.org/0000-0002-2987-281X
Elisabetta Castoldi https://orcid.org/0000-0002-3268-0552
Jun Teruya https://orcid.org/0000-0002-6124-803X
Eriko Morishita https://orcid.org/0000-0001-9116-3363
Dorothy M. Adcock https://orcid.org/0000-0002-0182-6272

REFERENCES

[1] Castoldi E, Rosing J. APC resistance: biological basis and acquired influences. *J Thromb Haemost*. 2010;8:445–53.
[2] Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci U S A*. 1993;90:1004–8.
[3] Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*. 1994;369:64–7.
[4] Kadauke S, Khor B, Van Cott EM. Activated protein C resistance testing for factor V Leiden. *Am J Hematol*. 2014;89:1147–50.
[5] Perez Botero J, Majerus JA, Strege AK, Johnson RD, Chen D, Pruthi RK. Diagnostic testing approaches for activated protein C resistance and factor V Leiden: a comparison of institutional and national provider practices. *Am J Clin Pathol*. 2017;147:604–10.
[6] Linnemann B, Hart C. Laboratory diagnostics in thrombophilia. *Hamostaseologie*. 2019;39:49–61.
[7] Moore GW, Van Cott EM, Cutler JA, Mitchell MJ, Adcock DM. Subcommittee on Plasma Coagulation Inhibitors. Recommendations for clinical laboratory testing of activated protein C resistance; communication from the SSC of the ISTH. *J Thromb Haemost*. 2019;17:1555–61.
[8] Tosetto A, Simioni M, Madeo D, Rodeghiero F. Intraindividual consistency of the activated protein C resistance phenotype. *Br J Haematol*. 2004;126:405–9.
[9] Taralunga C, Gueguen R, Visvikis S, Regnault V, Sass C, Siest G, Lecompte T, Wahl D. Phenotypic sensitivity to activated protein C in healthy families: importance of genetic components and environmental factors. *Br J Haematol*. 2004;126:392–7.
[10] de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. *Blood*. 1999;93:1271–6.
[11] Rodeghiero F, Tosetto A. Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. *Ann Intern Med*. 1999;130:643–50.
[12] Simioni P, Cagnin S, Sartorello F, Sales G, Pagani L, Bulato C, Gavasso S, Nuzzo F, Chemello F, Radu CM, Tormene D, Spiezia L,

Hackeng TM, Campello E, Castoldi E. Partial *F8* gene duplication (factor VIII Padua) associated with high factor VIII levels and familial thrombophilia. *Blood*. 2021;137:2383–93.

[13] Castaman G, Tosetto A, Simioni M, Ruggeri M, Madeo D, Rodeghiero F. Phenotypic APC resistance in carriers of the A20210 prothrombin mutation is associated with an increased risk of venous thrombosis. *Thromb Haemost*. 2001;86:804–8.

[14] Gaunt TR, Lowe GD, Lawlor DA, Casas JP, Day IN. A gene-centric analysis of activated partial thromboplastin time and activated protein C resistance using the HumanCVD focused genotyping array. *Eur J Hum Genet*. 2013;21:779–83.

[15] Castoldi E, Brugge JM, Nicolaes GA, Girelli D, Tans G, Rosing J. Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. *Blood*. 2004;103:4173–9.

[16] Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem*. 1994;269:31869–80.

[17] Nicolaes GA, Tans G, Thomassen MC, Hemker HC, Pabinger I, Váradi K, Schwarz HP, Rosing J. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. *J Biol Chem*. 1995;270:21158–66.

[18] Rosing J, Hoekema L, Nicolaes GA, Thomassen MC, Hemker HC, Váradi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. *J Biol Chem*. 1995;270:27852–8.

[19] Norstrøm EA, Tran S, Steen M, Dahlbäck B. Effects of factor Xa and protein S on the individual activated protein C-mediated cleavages of coagulation factor Va. *J Biol Chem*. 2006;281:31486–94.

[20] Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in the degradation of factor VIIIa. *J Biol Chem*. 1994;269:18735–8.

[21] Gale AJ, Cramer TJ, Rozenshteyn D, Cruz JR. Detailed mechanisms of the inactivation of factor VIIIa by activated protein C in the presence of its cofactors, protein S and factor V. *J Biol Chem*. 2008;283:16355–62.

[22] Thorelli E, Kaufman RJ, Dahlbäck B. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. *Blood*. 1999;93:2552–8.

[23] Thorelli E, Kaufman RJ, Dahlbäck B. The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V. *J Biol Chem*. 1998;273:16140–5.

[24] Parker J, Pagliuca A, Kitiyakara T, Whitehead M, Heaton N, O'Grady J, Arya R. Discrepancy between phenotype and genotype on screening for factor V Leiden after transplantation. *Blood*. 2001;97:2525–6.

[25] Duckers C, Simioni P, Spiezia L, Radu C, Gavasso S, Rosing J, Castoldi E. Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency. *Blood*. 2008;112:3615–23.

[26] Williamson D, Brown K, Luddington R, Baglin C, Baglin T. Factor V Cambridge: a new mutation (Arg^{306}->Thr) associated with resistance to activated protein C. *Blood*. 1998;91:1140–4.

[27] Franco RF, Maffei FH, Lourenco D, Morelli V, Thomazini IA, Piccinato CE, Tavella MH, Zago MA. Factor V Arg^{306}->Thr (factor V Cambridge) and factor V Arg^{306}->Gly mutations in venous thrombotic disease. *Br J Haematol*. 1998;103:888–90.

[28] Franco RF, Elion J, Tavella MH, Santos SE, Zago MA. The prevalence of factor V Arg^{306}->Thr (factor V Cambridge) and factor V Arg^{306}->Gly mutations in different human populations. *Thromb Haemost*. 1999;81:312–3.

[29] Le Cam-Duchez V, Chretien MH, Saugier-Veber P, Borg JY. Factor V Cambridge mutation and activated protein C resistance assays. *Thromb Haemost*. 2006;95:581–3.

[30] Chan WP, Lee CK, Kwong YL, Lam CK, Liang R. A novel mutation of Arg306 of factor V gene in Hong Kong Chinese. *Blood*. 1998;91:1135–9.

[31] Liang R, Lee CK, Wat MS, Kwong YL, Lam CK, Liu HW. Clinical significance of Arg306 mutations of factor V gene. *Blood*. 1998;92:2599–600.

[32] Ruiz-Argüelles GJ, López-Martínez B, Valdés-Tapia P, Gómez-Rangel JD, Reyes-Núñez V, Garcés-Eisele J. Primary thrombophilia in Mexico. V. A comprehensive prospective study indicates that most cases are multifactorial. *Am J Hematol*. 2005;78:21–6.

[33] Dölek B, Eraslan S, Eroğlu S, Kesim BE, Ulutin T, Yalçiner A, Laleli YR, Gözükirmizi N. Molecular analysis of factor V Leiden, factor V Hong Kong, factor II G20210A, methylenetetrahydrofolate reductase C677T, and A1298C mutations related to Turkish thrombosis patients. *Clin Appl Thromb Hemost*. 2007;13:435–8.

[34] Norstrøm E, Thorelli E, Dahlbäck B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. *Blood*. 2002;100:524–30.

[35] Van der Neut Kolfschoten M, Dirven RJ, Tans G, Rosing J, Vos HL, Bertina RM. The activated protein C (APC)-resistant phenotype of APC cleavage site mutants of recombinant factor V in a reconstituted plasma model. *Blood Coagul Fibrinolysis*. 2002;13:207–15.

[36] Cheng ZP, Tang L, Liu H, Zeng W, Wang QY, Wu YY, Hu B, Hu Y. Lack of association between factor V Hong Kong and venous thrombosis in the Chinese population. *Thromb Res*. 2015;135:415–6.

[37] Mumford AD, McVey JH, Morse CV, Gomez K, Steen M, Norstrom EA, Tuddenham EG, Dahlback B, Bolton-Maggs PH. Factor V I359T: a novel mutation associated with thrombosis and resistance to activated protein C. *Br J Haematol*. 2003;123:496–501.

[38] Steen M, Norstrøm EA, Tholander AL, Bolton-Maggs PH, Mumford A, McVey JH, Tuddenham EG, Dahlbäck B. Functional characterization of factor V-Ile359Thr: a novel mutation associated with thrombosis. *Blood*. 2004;103:3381–7.

[39] Simioni P, Scudeller A, Radossi P, Gavasso S, Girolami B, Tormene D, Girolami A. "Pseudo homozygous" activated protein C resistance due to double heterozygous factor V defects (factor V Leiden mutation and type I quantitative factor V defect) associated with thrombosis: report of two cases belonging to two unrelated kindreds. *Thromb Haemost*. 1996;75:422–6.

[40] Guzmán N, Larama G, Ávila A, Salazar LA. Three novel variants in the coagulation factor V gene associated with deep venous thrombosis in Chilean patients with Amerindian ethnic background. *Clin Chim Acta*. 2015;444:24–8.

[41] Pezeshkpoor B, Castoldi E, Mahler A, Hanel D, Müller J, Hamedani NS, Biswas A, Oldenburg J, Pavlova A. Identification and functional characterization of a novel F5 mutation (Ala512Val, FVB onn) associated with activated protein C resistance. *J Thromb Haemost*. 2016;14:1353–63.

[42] Steen M, Tran S, Autin L, Villoutreix BO, Tholander AL, Dahlbäck B. Mapping of the factor Xa binding site on factor Va by site-directed mutagenesis. *J Biol Chem*. 2008;283:20805–12.

[43] Cai H, Hua B, Fan L, Wang Q, Wang S, Zhao Y. A novel mutation (G2172->C) in the factor V gene in a Chinese family with hereditary activated protein C resistance. *Thromb Res*. 2010;125:545–8.

[44] Xin-Guang C, Yong-Qiang Z, Shu-Jie W, Lian-Kai F, Hua-Cong C. Prevalence of the factor V E666D mutation and its correlation with activated protein C resistance in the Chinese population. *Clin Appl Thromb Hemost*. 2015;21:480–3.

[45] Nogami K, Shinozawa K, Ogiwara K, Matsumoto T, Amano K, Fukutake K, Shima M. Novel FV mutation (W1920R, FVNara) associated with serious deep vein thrombosis and more potent APC resistance relative to FVLeiden. *Blood*. 2014;123:2420–8.

[46] Gierula M, Salles-Crawley II, Santamaria S, Teraz-Orosz A, Crawley JTB, Lane DA, Ahnström J. The roles of factor Va and protein S in formation of the activated protein C/protein S/factor Va inactivation complex. *J Thromb Haemost*. 2019;17:2056–68.

[47] Castoldi E, Hézard N, Mourey G, Wichapong K, Poggi M, Ibrahim-Kosta M, Thomassen M, Fournel A, Hayward CPM, Alessi MC, Hackeng TM, Rosing J, Morange PE. Severe thrombophilia in a factor V-deficient patient homozygous for the Ala2086Asp mutation (FV Besançon). *J Thromb Haemost*. 2021;19:1186–99.

[48] Bernardi F, Faioni EM, Castoldi E, Lunghi B, Castaman G, Sacchi E, Mannucci PM. A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. *Blood*. 1997;90:1552–7.

[49] Okada H, Toyoda Y, Takagi A, Saito H, Kojima T, Yamazaki T. Activated protein C resistance in the Japanese population due to homozygosity for the factor V R2 haplotype. *Int J Hematol*. 2010;91:549–50.

[50] de Visser MC, Guasch JF, Kamphuisen PW, Vos HL, Rosendaal FR, Bertina RM. The HR2 haplotype of factor V: effects on factor V levels, normalized activated protein C sensitivity ratios and the risk of venous thrombosis. *Thromb Haemost*. 2000;83:577–82.

[51] Hoekema L, Castoldi E, Tans G, Girelli D, Gemmati D, Bernardi F, Rosing J. Functional properties of factor V and factor Va encoded by the R2-gene. *Thromb Haemost*. 2001;85:75–81.

[52] Castaman G, Faioni EM, Tosetto A, Bernardi F. The factor V HR2 haplotype and the risk of venous thrombosis: a meta-analysis. *Haematologica*. 2003;88:1182–9.

[53] Helley D, Besmond C, Ducrocq R, da Silva F, Guillin MC, Bezeaud A, Elion J. Polymorphism in exon 10 of the human coagulation factor V gene in a population at risk for sickle cell disease. *Hum Genet*. 1997;100:245–8.

[54] Gandrille S, Greengard JS, Alhenc-Gelas M, Juhan-Vague I, Abgrall JF, Jude B, Griffin JH, Aiach M. Incidence of activated protein C resistance caused by the ARG 506 GLN mutation in factor V in 113 unrelated symptomatic protein C-deficient patients. *Blood*. 1995;86:219–24.

[55] Le W, Yu J, Lu L, Tao R, You B, Cai X, Cao W, Huang W, He R, Chen Z, Gong L. Arg485Lys polymorphism of factor V increases the risk of coronary artery disease in a Chinese population. *Chin Med J (Engl)*. 2000;113:963–6.

[56] Hiyoshi M, Arnutti P, Prayoonwiwat W, Nathalang O, Suwanasophon C, Kokaseam R, Hashimoto S, Takubo T, Tagawa S, Fukui M, Tatsumi N. A polymorphism nt 1628G–>A (R485K) in exon 10 of the coagulation factor V gene may be a risk factor for thrombosis in the indigenous Thai population. *Thromb Haemost*. 1998;80:705–6.

[57] Watanabe H, Hamada H, Yamakawa-Kobayashi K, Yoshikawa H, Arinami T. Evidence for an association of the R485K polymorphism in the coagulation factor V gene with severe preeclampsia from screening 35 polymorphisms in 27 candidate genes. *Thromb Haemost*. 2001;86:1594–5.

[58] Faisel F, Romppanen EL, Hiltunen M, Helisalmi S, Laasanen J, Punnonen K, Salonen JT, Heinonen S. Susceptibility to pre-eclampsia in Finnish women is associated with R485K polymorphism in the factor V gene, not with Leiden mutation. *Eur J Hum Genet*. 2004;12:187–91.

[59] Simioni P, Castoldi E, Lunghi B, Tormene D, Rosing J, Bernardi F. An underestimated combination of opposites resulting in enhanced thrombotic tendency. *Blood*. 2005;106:2363–5.

[60] Duckers C, Simioni P, Tormene D, Carraro S, Rosing J, Castoldi E. Factor V Leiden pseudo-homozygotes have a more pronounced hypercoagulable state than factor V Leiden homozygotes. *J Thromb Haemost*. 2011;9:864–7.

[61] de Visser MC, van Hylckama Vlieg A, Tans G, Rosing J, Dahm AE, Sandset PM, Rosendaal FR, Bertina RM. Determinants of the APTT- and ETP-based APC sensitivity tests. *J Thromb Haemost*. 2005;3:1488–94.

[62] Clark P, Walker ID. The phenomenon known as acquired activated protein C resistance. *Br J Haematol*. 2001;115:767–73.

[63] Jenkins PV, Rawley O, Smith OP, O'Donnell JS. Elevated factor VIII levels and risk of venous thrombosis. *Br J Haematol*. 2012;157:653–63.

[64] Timp JF, Lijfering WM, Flinterman LE, van Hylckama Vlieg A, le Cessie S, Rosendaal FR, Cannegieter SC. Predictive value of factor VIII levels for recurrent venous thrombosis: results from the MEGA follow-up study. *J Thromb Haemost*. 2015;13:1823–32.

[65] O'Donnell J, Mumford AD, Manning RA, Laffan MA. Marked elevation of thrombin generation in patients with elevated FVIII:C and venous thromboembolism. *Br J Haematol*. 2001;115:687–91.

[66] Graf LL, Welsh CH, Qamar Z, Marlar RA. Activated protein C resistance assay detects thrombotic risk factors other than factor V Leiden. *Am J Clin Pathol*. 2003;119:52–60.

[67] Rietveld IM, Schreuder M, Reitsma PH, Bos MHA. Elevated coagulation factor levels affect the tissue factor-threshold in thrombin generation. *Thromb Res*. 2018;172:104–9.

[68] Lavigne-Lissalde G, Sanchez C, Castelli C, Alonso S, Mazoyer E, Bal Dit Sollier C, Drouet L, Juhan-Vague I, Gris JC, Alessi MC, Morange PE. Prothrombin G20210A carriers the genetic mutation and a history of venous thrombosis contributes to thrombin generation independently of factor II plasma levels. *J Thromb Haemost*. 2010;8:942–9.

[69] Tripodi A, Chantarangkul V, Mannucci PM. Hyperprothrombinemia may result in acquired activated protein C reistance. *Blood*. 2000;96:3295–6.

[70] Castoldi E, Simioni P, Tormene D, Thomassen MC, Spiezia L, Gavasso S, Rosing J. Differential effects of high prothrombin levels on thrombin generation depending on the cause of the hyperprothrombinemia. *J Thromb Haemost*. 2007;5:971–9.

[71] Yegneswaran S, Nguyen PM, Gale AJ, Griffin JH. Prothrombin amino terminal region helps protect coagulation factor Va from proteolytic inactivation by activated protein C. *Thromb Haemost*. 2009;101:55–61.

[72] O'Brien LM, Mastri M, Fay PJ. Regulation of factor VIIIa by human activated protein C and protein S: inactivation of cofactor in the intrinsic factor Xase. *Blood*. 2000;95:1714–20.

[73] Vlieg AV, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. *Blood*. 2000;95:3678–82.

[74] de Visser MC, Poort SR, Vos HL, Rosendaal FR, Bertina RM. Factor X levels, polymorphisms in the promoter region of factor X, and the risk of venous thrombosis. *Thromb Haemost*. 2001;85:1011–7.

[75] Hackeng TM, Maurissen LF, Castoldi E, Rosing J. Regulation of TFPI function by protein S. *J Thromb Haemost*. 2009;7:165–8.

[76] Wood JP, Bunce MW, Maroney SA, Tracy PB, Camire RM, Mast AE. Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation. *Proc Natl Acad Sci U S A*. 2013;110:17838–43.

[77] van Doorn P, Rosing J, Wielders SJ, Hackeng TM, Castoldi E. The C-terminus of tissue factor pathway inhibitor-alpha inhibits factor V activation by protecting the Arg1545 cleavage site. *J Thromb Haemost*. 2017;15:140–9.

[78] Gialeraki A, Valsami S, Pittaras T, Panayiotakopoulos G, Politou M. Oral contraceptives and HRT risk of thrombosis. *Clin Appl Thromb Hemost*. 2018;24:217–25.

[79] Kemmeren JM, Algra A, Meijers JC, Tans G, Bouma BN, Curvers J, Rosing J, Grobbee DE. Effect of second- and third-generation oral contraceptives on the protein C system in the absence or presence of the factor VLeiden mutation: a randomized trial. *Blood*. 2004;103:927–33.

[80] Tchaikovski SN, Rosing J. Mechanisms of estrogen-induced venous thromboembolism. *Thromb Res*. 2010;126:5–11.

[81] Curvers J, Thomassen MC, Nicolaes GA, Van Oerle R, Hamulyak K, Hemker HC, Tans G, Rosing J. Acquired APC resistance and oral contraceptives: differences between two functional tests. *Br J Haematol*. 1999;105:88–94.

[82] Morimont L, Donis N, Bouvy C, Mullier F, Dogne JM, Douxfils J. Laboratory testing for the evaluation of phenotypic activated protein C resistance. *Semin Thromb Hemost*. 2022;48:680–9.

[83] Evrard J, Morimont L, Didembourg M, Haguet H, Siriez R, Dogne JM, Douxfils J. Assessment of acquired activated protein C resistance with the FibWave and comparison with the ETP-based APC resistance. *Int J Lab Hematol*. 2021;43:802–12.

[84] Clark P, Brennand J, Conkie JA, McCall F, Greer IA, Walker ID. Activated protein C sensitivity, protein C, protein S and coagulation in normal pregnancy. *Thromb Haemost*. 1998;79:1166–70.

[85] Zotz RB, Gerhardt A, Kluft C, Scharf RE. Venous thromboembolism during pregnancy is not associated with persistent elevated activated protein C (APC) sensitivity ratio based on the endogenous thrombin potential. *Thromb Haemost*. 2005;93:306–10.

[86] Bergrem A, Dahm AE, Jacobsen AF, Mowinckel MC, Sandvik L, Sandset PM. Resistance to activated protein C is a risk factor for pregnancy-related venous thrombosis in the absence of the F5 rs6025 (factor V Leiden) polymorphism. *Br J Haematol*. 2011;154:241–7.

[87] Rai R, Shlebak A, Cohen H, Backos M, Holmes Z, Marriott K, Regan L. Factor V Leiden and acquired activated protein C resistance among 1000 women with recurrent miscarriage. *Hum Reprod*. 2001;16:961–5.

[88] Sharma A, Singh N, Mahapatra M, Ranjan R, Kishor K, Saxena R. Factors contributing to APC-resistance in women with recurrent spontaneous miscarriages: Indian perspective. *Blood Cells Mol Dis*. 2015;55:213–5.

[89] Sarig G, Michaeli Y, Lanir N, Brenner B, Haim N. Mechanisms for acquired activated protein C resistance in cancer patients. *J Thromb Haemost*. 2005;3:589–90.

[90] Negaard HF, Iversen PO, Ostenstad B, Mowinckel MC, Sandset PM. Increased acquired activated protein C resistance in unselected patients with hematological malignancies. *J Thromb Haemost*. 2008;6:1482–7.

[91] Roselli M, Ferroni P, Riondino S, Mariotti S, Laudisi A, Vergati M, Cavaliere F, Palmirotta R, Guadagni F. Impact of chemotherapy on activated protein C-dependent thrombin generation—association with VTE occurrence. *Int J Cancer*. 2013;133:1253–8.

[92] Ramirez GA, Mackie I, Nallamilli S, Pires T, Moll R, Pericleous C, Isenberg DA, Cohen H, Efthymiou M. Anti-protein C antibodies and acquired protein C resistance in SLE: novel markers for thromboembolic events and disease activity? *Rheumatology (Oxford)*. 2021;60:1376–86.

[93] Nojima J, Iwatani Y, Ichihara K, Tsuneoka H, Ishikawa T, Yanagihara M, Takano T, Hidaka Y. Acquired activated protein C resistance is associated with IgG antibodies to protein S in patients with systemic lupus erythematosus. *Thromb Res*. 2009;124:127–31.

[94] Kalafatis M, Simioni P, Tormene D, Beck DO, Luni S, Girolami A. Isolation and characterization of an antifactor V antibody causing activated protein C resistance from a patient with severe thrombotic manifestations. *Blood*. 2002;99:3985–92.

[95] Saenz AJ, Johnson NV, Van Cott EM. Acquired activated protein C resistance caused by lupus anticoagulants. *Am J Clin Pathol*. 2011;136:344–9.

[96] Pontara E, Cattini MG, Cheng C, Bison E, Denas G, Pengo V. Insight into the hypercoagulable state of high-risk thrombotic APS patients: contribution of aβ2GPI and aPS/PT antibodies. *J Thromb Haemost*. 2021;19:805–13.

[97] Moore GW, Chege E, Culhane AP, Hunt BJ. Maximising the diagnostic potential of APTT-based screening assays for activated protein C resistance. *Int J Lab Hematol*. 2015;37:844–52.

[98] Solano C, Self MJ, Cobcroft RG. Liver transplant acquired activated protein C resistance presenting with deep vein thrombosis 4 years after transplant. *Blood Coagul Fibrinolysis*. 2001;12:325–6.

[99] Ayala R, Martinez-Lopez J, Cedena T, Bustelos R, Jimenez C, Moreno E, Ribera C. Recipient and donor thrombophilia and the risk of portal venous thrombosis and hepatic artery thrombosis in liver recipients. *BMC Gastroenterol*. 2011;11:1–9.

[100] Stevens SM, Woller SC, Bauer KA, Kasthuri R, Cushman M, Streiff M, Lim W, Douketis JD. Guidance for the evaluation and treatment of hereditary and acquired thrombophilia. *J Thromb Thrombolysis*. 2016;41:154–64.

[101] Connors JM. Thrombophilia testing and venous thrombosis. *N Engl J Med*. 2017;377:1177–87.

[102] Arachchillage DJ, Mackillop L, Chandratheva A, Motawani J, MacCallum P, Laffan M. Thrombophilia testing: a British Society for Haematology guideline. *Br J Haematol*. 2022;198:443–58.

[103] Jennings I, Goodeve A, Theophilus B, Hill M, Cumming A, Kitchen S, Walker ID, Perry D. Confirmation of genetic testing results in haemostasis and thrombosis—survey of current practice in the field. *Haemophilia*. 2016;22:e239–41.

[104] Arachchillage DR, Efthymiou M, Mackie IJ, Lawrie AS, Machin SJ, Cohen H. Anti-protein C antibodies are associated with resistance to endogenous protein C activation and a severe thrombotic phenotype in antiphospholipid syndrome. *J Thromb Haemost*. 2014;12:1801–9.

[105] European Medicines Agency. Guideline on clinical investigation of steroid contraceptives in women. https://www.ema.europa.eu/documents/scientific-guideline/guideline-clinical-investigation-steroid-contraceptiveswomen_en.pdf, 2005 [accessed July 7, 2022]

[106] Morimont L, Haguet H, Dogne JM, Gaspard U, Douxfils J. Combined oral contraceptives and venous thromboembolism: review and perspective to mitigate the risk. *Front Endocrinol*. 2021;12:769187.

[107] Douxfils J, Morimont L, Delvigne AS, Devel P, Masereel B, Haguet H, Bouvy C, Dogne JM. Validation and standardization of the ETP-based activated protein C resistance test for the clinical investigation of steroid contraceptives in women: an unmet clinical and regulatory need. *Clin Chem Lab Med*. 2020;58:294–305.

[108] Morimont L, Didembourg M, Haguet H, Modaffari E, Tillier M, Bouvy C, Lebreton A, Dogne JM, Douxfils J. Interlaboratory variability of activated protein C resistance using the ETP-based APC resistance assay. *Res Pract Thromb Haemost*. 2021;5:e12612.

[109] Kujovich JL. Factor V Leiden thrombophilia. *Genet Med*. 2011;13:1–16.