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ORIGINAL ARTICLE

A Genome Wide Association Study on plasma FV levels identified *PLXDC2* as a new modifier of the coagulation process

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Abstract

Background: Factor V (FV) is a circulating protein primarily synthesized in the liver, and mainly present in plasma. It is a major component of the coagulation process.

Objective: To detect novel genetic loci participating to the regulation of FV plasma levels.

Methods: We conducted the first Genome Wide Association Study on FV plasma levels in a sample of 510 individuals and replicated the main findings in an independent sample of 1156 individuals.

Results: In addition to genetic variations at the *F5* locus, we identified novel associations at the *PLXDC2* locus, with the lead *PLXDC2* rs927826 polymorphism explaining ~3.7% ($P = 7.5 \times 10^{-15}$ in the combined discovery and replication samples) of the variability of FV plasma levels. In silico transcriptomic analyses in various cell types confirmed that *PLXDC2* expression is positively correlated to *F5* expression. SiRNA experiments in human hepatocellular carcinoma cell line confirmed the role of

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PLXDC2 in modulating factor *F5* gene expression, and revealed further influences on *F2* and *F10* expressions.

Conclusion: Our study identified *PLXDC2* as a new molecular player of the coagulation process.

KEYWORDS

biomarkers, coagulation factor, computational biology, factor V, genetics

1 | INTRODUCTION

Coagulation factor V (FV) plays an important and dual role in the regulation of blood coagulation by exhibiting both pro- and anticoagulant functions (reviewed in[1]). In plasma, single-chain FV expresses anticoagulant activity as a cofactor of both TFPI and activated protein C. In situations where the coagulation system is triggered, FV is converted to a highly effective procoagulant cofactor to activated factor X (FXa), which activates prothrombin to thrombin, which in turn catalyzes fibrin deposition and activates platelets. Although FV deficiency is known to associate with bleeding tendency, it has recently been proposed that FV plasma levels were associated with the risk of venous thrombosis (VT) in an ambiguous pattern. Indeed, in 2377 VT patients and 2943 controls of the MEGA study, individuals with either high (>1.22 IU/mL) or low (<0.57 IU/mL) FV levels were at higher risk of disease.[2]

Until now, only one genetic factor, the HR2 haplotype located in the *F5* gene, has been robustly found associated with FV plasma levels[3] even though it has been hypothesized that FV Leiden genotype could also modulate FV plasma levels.[2,4]

We here report the results of the first genome wide association study (GWAS) aiming to identify new genetic determinants of FV plasma levels.

Essentials

- Little is known about the regulation of Factor V plasma levels.
- A Genome Wide Association Study (GWAS) was performed on Factor V plasma levels.
- Genetic variations at the PLXDC2 gene are associated with plasma levels of Factor V.
- This study identifies a novel player of the coagulation process.

2 | MATERIALS AND METHODS

2.1 | Study description

This work builds on two independent samples of unrelated VT patients of European ancestry recruited at the Thrombophilia center of La Timone Hospital (Marseille, France), the MARseille THrombosis Association (MARTHA) and MARTHA12 cohorts.[5] MARTHA patients were used for the discovery GWAS, whereas MARTHA12 individuals were considered for the replication step. All participants provided written informed consent, and the protocol was approved by the ethics committee of the participating institution.

The MARTHA project has already been extensively described.[6,7] It is composed of unrelated subjects of European origin, with the majority being of French ancestry, consecutively recruited at the Thrombophilia center of La Timone hospital (Marseille, France) between January 1994 and October 2012. All patients had a documented history of VT and are free of well-characterized genetic risk factors including AT (Antithrombin), PC (Protein C), or PS (Protein S) deficiency, homozygosity for FV Leiden or FII G20210A, and lupus anticoagulant. The MARTHA12 study is an independent sample of 1245 VT patients recruited between 2010 and 2012, according to the same criteria as the MARTHA patients.[5,8]

2.2 | Hemostatic traits measurements

FV activity plasma levels (FV:C) were measured using human FV deficient plasma on automated coagulometers (STA® analysers from Diagnostica Stago). Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured in plasma with the use of automated coagulometers. Tests were conducted on the same day as blood collection or within a few weeks (after freezing). PT was recorded as a ratio of the patient's PT to the mean normal PT, and aPTT was recorded in seconds (s). FV:C plasma levels were available in 510 participants of the discovery phase and 1156 participants of the replication. FV antigen plasma levels (FV:Ag) were measured by ELISA on freezing samples using the ZYMUTEST Factor V kit (Hyphen Biomed) in a random sample of 60 patients from the MARTHA study (n = 20 per rs927826 genotypes). Factor II (FII) and Factor X (FX) activity levels in plasma were measured in MARTHA and MATHA12 cohorts by using human FII- or FX-deficient plasma on automated coagulometers in a total of 738 patients with no anti-vitamin K antagonists (VKA) at the time of blood sampling.

2.3 | Genotyping

DNA samples of the MARTHA participants were typed with high-density genotyping arrays and imputed for single nucleotide

TABLE 1 Description of the MARTHA and MARTHA12 cohorts

	Discovery MARTHA N = 510	Replication MARTHA12 N = 1156
Age	47.3 ± 16.0	50.4 ± 15.4
Gender (M/F)	176/334	527/629
Current smoker (%)	24.1	20.2
FV Leiden carriers (%)	15.5	11.0
BMI, kg/m^2	25.34 ± 4.96	26.26 ± 5.06
Vitamin K antagonist users (%)	3.7	28
FV:C plasma levels (IU/mL)	1.068 ± 0.235	1.089 ± 0.217
Activated partial thromboplastin time, s	31.68 ± 3.71	33.61 ± 6.95
Prothrombin time (%)	99.11 ± 14.72	83.64 ± 30.03

Note: Mean ± standard deviation.

polymorphisms (SNPs) available in the 1000G reference as previously described.[5] In MARTHA12, wet-lab genotyping was performed using Taqman assay. Genotyping was done using Taqman 5′ nuclease assay (ThermoFischer Scientific, ref. C___2386120_10 (rs27218, *MAST4*), C___8879235_10 (rs1409338, *PLXDC2*)), following the supplier instructions, on an LC480® Real time PCR Instrument (Roche Life sciences), using dedicated Dual Color Hydrolysis Probe program. Reactions were made in a 10 μL final volume in a 1 × final concentration of Genotyping Master Mix (ThermoFischer Scientific ref. 4371355), with 25 ng of DNA, and 0,1 μL of 20× Genotyping Assay containing probes, nucleotides, and DNA Taq Polymerase.

2.4 | Genetic association analyses

For the GWAS discovery phase, association analyses of imputed SNPs with plasma FV levels were performed using linear regression analysis adjusted for age, sex, and the four first principal components derived from the GWAS data as implemented in the Mach2QTL program.[9] Only SNPs with acceptable imputation quality (r^2 > .5) and minor allele frequency >0.05 were considered. At the replication stage, associations of SNPs with plasma FV levels were assessed using standard linear regression analysis adjusted for age and sex. Haplotype association analyses were conducted using THESIAS software.[10] Results from the discovery and replication studies were meta-analyzed via a fixed-effects model based on the inverse-variance weighting method. A replicated SNP was further tested for association with additional hemostatic traits including PT, aPTT, FII, and FX circulating levels. For these analyses, patients with VKA at the time of blood sampling were excluded.

2.5 | RNA interference mediated PLXDC2 silencing experiments

Small interfering RNA (siRNA) *PLXDC2* gene silencing was conducted in human hepatocytes, a key cell type for *F5* regulation, to validate in vitro the association between *PLXDC2* and *F5* gene expressions. Additional expression of coagulation factors *F2*, *F7*, and *F10* were also measured to assess the specificity of the *PLXDC2* association with *F5* expression.

The human hepatocellular carcinoma cell line Hep3B (American Type Culture Collection) was grown at 37°C in 5% CO2 in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum, 2 mmol/L glutamine and 100 U/mL penicillin/streptomycin. Cells were seeded on 12-well plates at 100 000 cells per well and were transfected 72 hours later with 50 nmol/L control siRNA or an siRNA targeting the *PLXDC2* gene, s39607 (sequence ctacagaagatgataccaa from Thermofisher) using lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions. Twenty-four hours following transfection, total RNA was extracted (NucleoSpin RNA II kit; Macherey-Nagel) and reverse transcribed (high-capacity cDNA reverse transcription kit, Life Technologies) and gene expression was analyzed by real-time qPCR using a LightCycler LC480 (Roche). Primers used for quantification of *PLXDC2* and *F5*, *F2*, *F7*, and *F10* are provided as Supporting Information. Expression of mRNA levels was normalized to human non-POU domain containing, octamer-binding housekeeping gene (NONO), human alanin (ALA) and human heat shock protein 90 kDa alpha (cytosolic) class B member 1 (HSP90AB1). Data (Table S1) were expressed as a fold change in mRNA expression relative to control cells.

The impact of *PLXDC2* knock-down on gene expression was tested via two-way analysis of variance.

3 | RESULTS

FV activity plasma levels (FV:C) were measured using human FV deficient plasma on automated coagulometers. The discovery GWAS was composed of 510 patients with venous thromboembolism (VTE) assessed for 6 264 382 SNPs and main significant findings were tested for replication in an independent sample of 1156 patients (see Materials & Methods). Main clinical and biological characteristics of the studied samples are shown in Table 1.

The Manhattan and Quantile-Quantile plots summarizing the GWAS results are shown in Figures S1-S4. About 70 SNPs achieved genome-wide significance ($P < 5 \times 10^{-8}$), with all SNPs mapping to the *F5* locus on chromosome 1q24.2. The lead SNP, rs72708008, with association P value = 8.43×10^{-12}, was in high linkage disequilibrium (LD) (r^2 > .80) with several SNPs including the rs6027 (p.Asp2222Gly, $P = 1.27 \times 10^{-11}$) known to tag the *F5* HR2 haplotype whose association with FV:C plasma levels has already been established.[11] After conditioning on rs6027, no additional signal reached genome-wide significance (Figure S3 and Table S2), the smallest P value being now $P = 3.05 \times 10^{-7}$ and observed at the *PLXDC2* locus. Because two other

TABLE 2 Association with FV activity plasma levels of lead SNPs at two loci that reached suggestive evidence for association in the discovery GWAS

	MAST4 rs27218[a]				*PLXDC2* rs927826[a,b]			
	CC	CT	TT	MAF	TT	TG	GG	MAF
Discovery								
Mean FV plasma levels ± SD	1.03 ± 0.22 N = 271	1.09 ± 0.23 N = 199	1.23 ± 0.29 N = 40	0.274	1.11 ± 0.22 N = 249	1.04 ± 0.24 N = 224	0.95 ± 0.19 N = 37	0.293
Additive allele effect[c]		+0.075 ± 0.015 $P = 9.02 \times 10^{-7}$				−0.076 ± 0.016 $P = 1.10 \times 10^{-6}$		
Replication								
Mean FV plasma levels ± SD	1.102 ± 0.21 N = 605	1.07 ± 0.22 N = 434	1.101 ± 0.21 N = 89	0.271	1.13 ± 0.22 N = 537	1.05 ± 0.21 N = 461	1.02 ± 0.20 N = 87	0.293
Additive allele effect		−0.019 ± 0.010 $P = .054$				−0.062 ± 0.010 $P = 2.89 \times 10^{-10}$		

Abbreviations: MAF, minor allele frequency; SD, standard deviation.
[a]Imputation quality criterion was .984 and .996 for *MAST4* rs27218 and *PLXDC2* rs927826, respectively, in the discovery GWAS.
[b]In the replication study, rs927826 was substituted by rs1409338 that served as a proxy ($r^2 = .97$) because of some technical issues in wet-lab genotyping the rs927826.
[c]Association testing was adjusted for age, sex, and the four main genetics components in the discovery cohort and on age and sex in the replication study.

SNPs at the *F5* locus, rs6025 (p.Arg534Gln) and rs4524 (p.Lys858Arg), have been extensively studied in relation to VT risk,[5] we further analyzed their joint association with rs6027 with respect to FV:C plasma levels in both the discovery and replication cohorts using haplotype analysis. This analysis revealed that in addition to the strong decreasing effect of the rs6027-C allele ($\beta = -.151 \pm .02$, $P = 1.93 \times 10^{-12}$), the rs4524-C allele was associated with a slight decrease ($\beta = -.033 \pm .01$, $P = .003$) in FV:C plasma levels (Table S3). Altogether, these two *F5* SNPs explained ~7% of the variability of FV:C plasma levels.

Aside the aforementioned *F5* association signal, two other loci with several SNPs in LD exhibited suggestive statistical evidence for association with FV:C plasma levels in the discovery cohort, with *P*-values ranging between ~10^{-6} and 10^{-5} (Figure S1, Table S2). These loci were *MAST4* and *PLXDC2* where lead SNPs, rs27218 (intronic; $P = 9.02 \times 10^{-7}$) and rs927826 (intronic; $P = 1.10 \times 10^{-6}$), respectively, were looked for replication. Although the association of *MAST4* rs27218 did not replicate (Table 2), the association of *PLXDC2* rs927826 with plasma FV:C levels was confirmed ($P = 2.89 \times 10^{-10}$). In both the discovery and replication samples, the rs927826-G allele was associated with decreased FV:C plasma levels, $\beta = -.076 \pm .016$ ($P = 1.10 \times 10^{-6}$) and $\beta = -.062 \pm .010$ ($P = 2.89 \times 10^{-10}$), respectively. When both samples were combined, the overall statistical evidence for association of rs927826 with FV:C levels reached $P = 7.54 \times 10^{-15}$, with no evidence for heterogeneity across the two samples ($P = .458$), and with the rs927826 explaining ~3.5% of FV:C plasma variability. As a reminder, the *PLXDC2* locus was the top locus in the GWAS analysis conditioned on *F5* rs6027 (Table S2) and the rs927826 ranked 4th ($P = 3.13 \times 10^{-7}$) in this conditional GWAS.

To assess whether *PLXDC2* could be a true determinant of FV levels and not a determinant of the clotting assay used to measure FV:C levels, we measured FV antigen (FV:Ag) levels by ELISA in a random sample of 60 patients from the MARTHA study. The ELISA data confirmed the specificity of the association of PLXDC2 genotypes with FV plasma levels as the rs927826-G allele was associated with significant ($P = .028$) decrease of FV:Ag levels (Table 3). Note, in this subsample, the correlation between the two FV measurements was 0.76.

We further examined the correlation between *PLXDC2* and *F5* gene expression in several genome-wide gene expression data from multiple cell lines and tissues.[12–17] In all investigated resources except macrophages, we observed positive correlations between *PLXDC2* and *F5* expressions, the strongest correlation ($r = .45$, $P = 3.94 \times 10^{-14}$) being observed in liver (Figure S5) where FV synthesis mainly occurs (Table 4). We also assessed the correlation of liver *PLXDC2* expression with that of other coagulation genes expressed in the liver (*F2*, *F7* and *F10*). We observed a significant positive, but less strong, correlation with *F2* expression ($r = .17$, $P = .007$) and a very low correlation, if any, with *F7* and *F10* expressions (Table 4).

To strengthen the perspective of a specific effect of the rs927826 variant on FV levels, we tested its association with FII and FX plasma levels in 738 participants. Even though FII and FX were moderately correlated with FV plasma levels ($r = .35$, $P = 4.8 \times 10^{-17}$ and $r = .32$, $P = 4.40 \times 10^{-14}$ for FII and FX, respectively), we did not find any association between rs927826 and both FII ($\beta = -.002 \pm .01$, $P = .83$) and FX ($\beta = .003 \pm .01$, $P = .81$) plasma levels (Table S4).

To follow up on these genetic epidemiological findings, we conducted preliminary in vitro study to assess whether *PLXDC2* gene expression could associate with *F5* gene expression in human liver cells (see Materials and Methods). As shown in Figure 1, knock-down expression of *PLXDC2* using siRNA was associated with a significant decrease liver expression of *F5* ($P = .020$). Concurrently, expressions of other coagulation genes (*F2*, *F7*, and *F10*) were also measured, and we also observed a significant decrease of *F2* ($P = .004$) and *F10* ($P = .0003$) expressions when *PLXDC2* is silenced. *F7* expression remained unchanged ($P = .1$).

TABLE 3 Association of rs927826 genotypes with plasma FV antigen (FV:Ag) and activity (FV:C) in a sample of 60 patients from the MARTHA cohort

rs927826	FV:Ag (IU/mL)	FV:C (IU/mL)
TT (n = 20)	0.647 ± 0.163	1.182 ± 0.271
TG (n = 20)	0.621 ± 0.133	1.204 ± 0.265
GG (n = 20)	0.545 ± 0.140	1.006 ± 0.200
P value[a]	*P* = 0.0286	*P* = 0.0263

Note: Mean ± standard deviation.
[a]Associations were tested using a linear regression model adjusted for age and sex, under the assumption of an additive model.

4 | DISCUSSION

Altogether, these results strongly support the role of *PLXDC2* in the regulation of *F5* gene, and more generally in the coagulation cascade. Interestingly, the *PLXDC2* rs927826 has recently been found associated with activated partial thromboplastin time in a Japanese population, but with no formal replication of the statistical findings or experimental validation.[18] We did not observe such association in our population but did observe an association of rs927826 with prothrombin time (Table S4). This polymorphism is in very strong LD (r^2 > .80) with other *PLXDC2* SNPs (Table S5), all located in intronic regions subject to epigenetic regulation,[19] but only the rs927826 is so far predicted to affect transcription factors' binding.[20]

Little is known about the PLXDC2 gene/protein. It is recognized as a cell surface transmembrane receptor expressed in various tissues (see, e.g., GTEx Portal[21]) without so far clear arguments that it could be directly involved in mRNA regulation. We showed that *PLXDC2* expression correlated with *F5* expression in different cell lines and that *F5* mRNA expression was significantly reduced in the liver in the *PLXDC2* knock-down experiment. Interestingly, the siRNA experiment revealed that the *PLXDC2* gene expression could also modulate the liver expression of *F2* and *F10* genes that might indicate a more generalized effect on coagulation. However, we did not find any association between the rs927826 variant and either FII or FX plasma levels.

Of interest, in a recent plasma proteomic profiling study,[22] the *F5* rs6027 and *PLXDC2* rs927826 variants we found here associated

TABLE 4 Correlations between *PLXDC2* and *F2*/*F5*/*F7*/*F10* gene expressions in six different tissues

	Macrophages[a] N = 684	Whole blood[b] N = 78	Monocytes[c] N = 849	Endothelial cells[d] N = 157	Adipose tissue[e] N = 200	Liver[f] N = 253
F2	NA	−0.27 *P* = 0.02	NA	0.16 *P* = 0.05	NA	0.17 *P* = 0.007
F5	0.03 *P* = .45	0.39 *P* = 0.001	0.17 $P = 5.83 \times 10^{-6}$	0.33 $P = 4.87 \times 10^{-5}$	0.31 $P = 6.32 \times 10^{-6}$	0.45 $P = 3.94 \times 10^{-14}$
F7	−0.02 *P* = 0.56	−0.33 *P* = 0.003	−0.05 *P* = 0.18	−0.08 *P* = 0.31	−0.06 *P* = 0.37	0.03 *P* = 0.61
F10	NA	NA	NA	−0.06 *P* = 0.49	−0.08 *P* = 0.26	0.08 *P* = 0.21

NA: Expressions were not available
Corresponding publication for each study: a[12]; b[13]; c[14]; d[15]; e[16]; f[17]

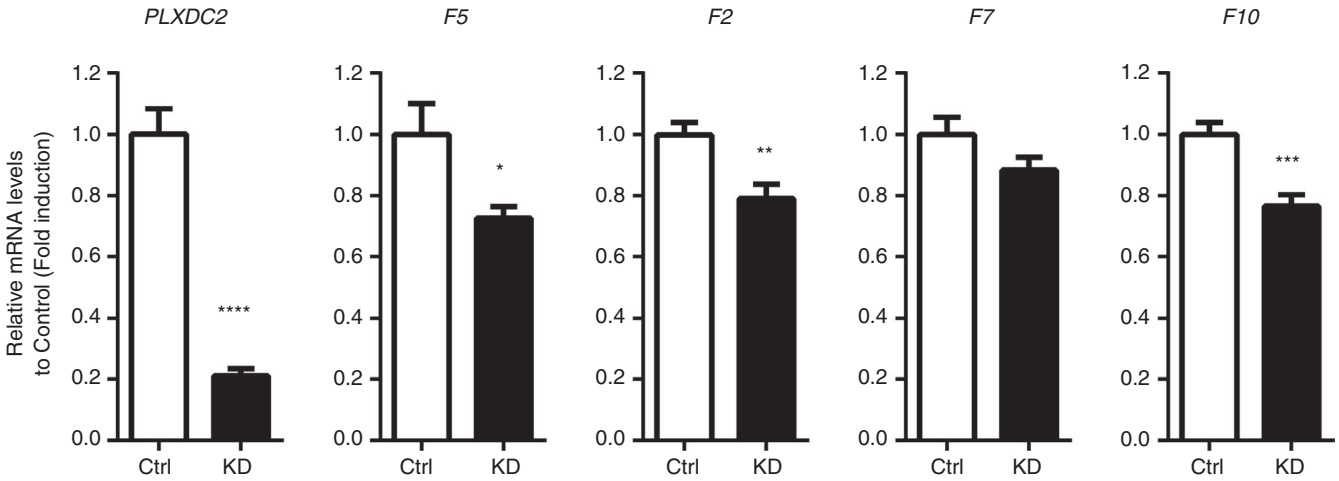

FIGURE 1 Impact of *PLXDC2* gene silencing on *F5* gene expression in liver. Abbreviation: KD = siRNA KnockDown. Quantification of *PLXDC2* and *F5*, *F2*, *F7*, and *F10* mRNA levels in Hep3B cells after 24 hours of treatment with 50 nM siRNA directed to *PLXDC2* gene (n = 9 for both controls and siRNA KD). A 79% decrease ($P < 10^{-8}$) in *PLXDC2* gene expression was followed by a significant decrease in *F5* (−27%, *P* = .02), *F2* (−21%, *P* = .004), and *F10* (−23%, *P* = .0003) gene expression, whereas *F7* expression was not significantly decreased (−12%, *P* = .11)

with plasma FV levels were both found influencing the plasma levels of the same four proteins (CD3E, CDH7, SLC22A16, and TOR1AIP1), such observations adding support for *PLXDC2* and *F5* belonging to a same regulatory pathway.

The strength of our study lies in its novelty. Indeed, there is limited information currently available regarding the regulation of plasma levels of FV. We performed the first GWAS on FV plasma levels and replicated the findings in an independent sample of subjects that limited the risk of spurious findings. Moreover, we increased the specificity of the finding by replicating the observed association between *PLXDC2* rs927826 genotypes and FV activity plasma levels by using an ELISA measuring FV antigen levels in plasma. Several limitations must be acknowledged. First, this study was performed in VT patients, and validation of the observed genetic association deserves to be investigated in healthy individuals to assess whether the observed effect size also holds in non-diseased individuals. Second, the size of our discovery cohort was relatively modest compared with what is currently done in a GWAS context, which has likely hampered our chance to detect additional loci participating to FV regulation. Additional efforts would be needed to measure FV plasma levels in independent cohorts with both available plasma and GWAS data to better characterize the genetic regulation underlying FV plasma variability. With large samples, it would also be interesting to assess the contribution of rare variants which was not possible in the current GWAS study mainly focusing on common polymorphisms. Finally, PLXDC2 is recognized as a cell-surface transmembrane receptor and it is then unclear how it could be involved in the regulation of *F5* gene expression. Moreover, the correlation between *PLXDC2* and *F2* expression in the liver and the significant decrease of *F2* and *F10* gene expressions in the *PLXDC2* knock-down experiment might indicate a more generalized effect on coagulation. Further experimental studies are mandatory to decipher the underlying mechanism.

In conclusion, all these observations point out the existence of a new player in the coagulation cascade whose exact molecular contribution needs to be extensively investigated. Its impact on FV-mediated coagulation related disorders also warrants further investigations as whole blood *PLXDC2* expression levels have been reported to be associated with stroke.[23]

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CONFLICT OF INTEREST

A patent deposit process has been initiated by J-F.D., D-A.T., and P-E.M. The other authors state that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

All statistical analyses were performed by F. Thibord under the supervision of D-A. Trégouët who designed the study together with P.-E. Morange. Wet-lab genotyping was performed by N. Saut, FV:C measurements by M. Ibrahim-Kosta, FV:Ag measurements by L. Goumidi, and experimental works by L. Hardy under the joint supervision of D-A. Trégouët and W. Le Goff. A-S. Pulcrano-Nicolas performed additional bioinformatics analyses. M. Civelek and P. Eriksson provided supplemental datasets for replication. Genetic data acquisition was coordinated by D-A. Trégouët, J-F. Deleuze, and P-E. Morange. Manuscript was drafted by F. Thibord and D-A. Trégouët and further reviewed by all coauthors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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