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ORIGINAL ARTICLE

Plasma levels of complement components C5 and C9 are associated with thrombin generation

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Abstract

Background: The thrombin generation assay (TGA) evaluates the potential of plasma to generate thrombin over time, providing a global picture of an individual's hemostatic balance.

Objectives: This study aimed to identify novel biological determinants of thrombin generation using a multiomics approach.

Methods: Associations between TGA parameters and plasma levels of 377 antibodies targeting 236 candidate proteins for cardiovascular risk were tested using multiple linear regression analysis in 770 individuals with venous thrombosis from the Marseille Thrombosis Association (MARTHA) study. Proteins associated with at least 3 TGA parameters were selected for validation in an independent population of 536 healthy individuals (Etablissement Français du Sang Alpes-Méditerranée [EFS-AM]). Proteins with strongest associations in both groups underwent additional genetic analyses and *in vitro* experiments.

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Results: Eighteen proteins were associated ($P < 1.33 \times 10^{-4}$) with at least 3 TGA parameters in MARTHA, among which 13 demonstrated a similar pattern of associations in EFS-AM. Complement proteins C5 and C9 had the strongest associations in both groups. *Ex vivo* supplementation of platelet-poor plasma with purified C9 protein had a significant dose-dependent effect on TGA parameters. No effect was observed with purified C5.Several single nucleotide polymorphisms associated with C5 and C9 plasma levels were identified, with the strongest association for the C5 missense variant rs17611, which was associated with a decrease in C5 levels, endogenous thrombin potential, and peak in MARTHA. No association of this variant with TGA parameters was observed in EFS-AM.

Conclusion: This study identified complement proteins C5 and C9 as potential determinants of thrombin generation. Further studies are warranted to establish causality and elucidate the underlying mechanisms.

KEYWORDS

complement C5, complement C9, proteomics, thrombin, venous thromboembolism

1 | INTRODUCTION

Thrombin, also known as "activated factor (F)II," is the key enzyme of coagulation, responsible for the conversion of fibrinogen to fibrin to form a clot upon vascular injury. Furthermore, thrombin modulates many other processes crucial for hemostatic balance, such as the activation of platelets [1], FV, FVIII, FXI, and FXIII, [2,3] protein C [4], and the thrombin activatable fibrinolysis inhibitor [5]. In addition to its well-established role in hemostasis, thrombin also plays an important role in different processes beyond coagulation, such as inflammation [6], neurodevelopment [7], atherosclerosis [8], angiogenesis [9], and tumor progression [10], among many others [11]. It is therefore not surprising that the quantification of thrombin generation has drawn increasing research attention over the years. The thrombin generation assay (TGA) is a global coagulation assay that was developed to study the potential of plasma to generate thrombin over time following *in vitro* activation of coagulation by adding tissue factor (TF), phospholipids, and calcium. Through continuous measurement of thrombin formation and inhibition, the TGA provides a global picture of the plasma hemostatic balance of an individual [12,13]. The TGA results can be output as a thrombogram or thrombin generation curve using specialized computer software, from which 4 parameters are generally derived: the lag time, the time needed to generate the first traces of thrombin upon triggering coagulation; the peak height, which represents the maximum concentration of thrombin reached in the assay; the time to peak, the time needed to reach the maximum concentration of thrombin; and the endogenous thrombin potential (ETP) or area under the curve, which represents the total amount of active thrombin formed during the assay.

There is growing evidence that the parameters of the TGA curve are useful markers to assess bleeding [14,15] or thrombotic risk [16–18] and to monitor the effects of anticoagulant therapy [19,20].

Since the introduction of the TGA, several studies have been conducted to explore the main determinants of thrombin generation [21–23]. These studies show that, in the absence of added thrombomodulin or activated protein C, the plasma levels of FII (prothrombin), antithrombin, and fibrinogen are the main determinants of thrombin generation. However, most of these studies have primarily focused on the traditional coagulation factors and inhibitors. In this study, we aimed to identify novel biological determinants of thrombin generation by integrating extensive antibody-based affinity proteomics, *in vitro* functional studies, and genomics data.

2 | METHODS

2.1 | Study participants and methods

For this study, data from the *Marseille Thrombosis Association* (MARTHA) project were analyzed. MARTHA is a hospital-based cohort of over 1500 unrelated individuals who had a consultation visit at the Thrombophilia Center of La Timone Hospital (Marseille, France) initially between 1994 and 2005 and further extended over the 2010-2012 period. All patients had a history of a first venous thromboembolic (VTE) event documented by venography, Doppler ultrasound, angiography, and/or ventilation/perfusion lung scan. They were all free of any chronic conditions and free of any well-characterized major genetic risk factors, including antithrombin, protein C or protein S deficiency, homozygosity for FV Leiden or FII 20210A, and lupus anticoagulant. Venous blood samples were obtained from each patient, and plasma samples were prepared and stored at −80 °C at the certified biological center of the *Assistance Publique des Hopitaux de Marseille* for further analysis. In-depth

information regarding all methodological aspects of the MARTHA project can be found elsewhere [24–27].

The present study relied on a subset of 770 MARTHA patients who were not on anticoagulant therapy at the time of plasma sampling, who had been measured for TGA, and for whom targeted plasma proteomic profiling (see dedicated section below) had been performed.

2.2 | External validation cohort

Main proteins-TGA associations detected in MARTHA subjects were tested for validation in an independent population of 536 healthy blood donors with no personal history of cardiovascular disease, including VTE, recruited between February and December 2015 at the Etablissement Français du Sang Alpes-Méditerranée (EFS-AM) [28].

Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Ethics approval was obtained from the "Departement santé de la direction générale de la recherche et de l'innovation du ministère" (projects DC: 2008-880 and 09.576).

2.3 | Thrombin generation measurements

Thrombin generation was measured in platelet-poor plasma (PPP) using the calibrated automated thrombography method [12,13], as previously described [24]. Briefly, 80 μL of PPP was mixed in 96-well microtiter plates with 20 μL of PPP-Reagent (Thrombinoscope BV) containing a mixture of TF and phospholipids, yielding final concentrations of 5 pM and 4 μM, respectively. After incubation for 10 minutes at 37 °C, thrombin generation was initiated by the automated addition of 20 μL FluCaKit mixture (Stago) containing the fluorescent substrate Z-Gly-Gly-Arg-AMC and calcium ion buffer. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems), and thrombin generation curves were calculated using the Thrombinoscope software (Stago). Four thrombin generation parameters were derived from the curves: lag time (minutes), ETP (nM.minutes), peak height (nM), and time to peak (minutes).

2.4 | Plasma proteomic profiling

The plasma protein profiles were generated using antibodies coupled to color-coded magnetic beads and analyzed on a Luminex system (FlexMAP 3D instrument, Luminex Corporation), as previously described [29–31]. In brief, the multiplex antibody suspension bead array was created by covalent binding of 339 antibodies from the Human Protein Atlas (HPA), along with 13 antibodies from commercial providers and 25 monoclonal BioSystems International antibodies (BioSystems International Kft). For simplicity, we will refer to all these antibodies as "HPA." These antibodies targeted a total of 236 unique candidate proteins. These proteins were selected for (1) their known roles in the coagulation/fibrinolysis cascade and/or intermediate traits relevant to thrombosis, (2) their specific expression in endothelial cells (a key cell type involved in thrombosis physiopathology), or (3) encoded by genes identified in large-scale association studies to associate with several cardiovascular disease-linked biological pathways (eg, platelet function, renal function, or inflammation). The relative amount of each protein complex was expressed as median fluorescence intensity obtained by read-out on a FlexMAP 3D instrument (Luminex Corporation).

A detailed list of the antibodies used and the specific proteins they targeted is provided in Supplementary Table S1.

2.5 | Target verification

To verify C5 and C9 as the targets of the HPAs found associated with TGA parameters, the plasma levels of C5 and C9 were measured in 40 randomly selected MARTHA samples using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol (Abcam).

2.6 | *In vitro* functional studies on identified C5 and C9 complement proteins

To add further experimental support to the associations pertaining to C5 and C9 plasma levels observed in our cohorts, a series of *in vitro* experiments were performed using calibrated automated thrombography, as described above.

First, 10 μL of protein dilution of human purified C5 or C9 protein (Quidel) prepared at increasing concentrations (0, 3.75, 7.5, 15, 30, and 60 μg/mL) were added to 70 μL PPP from healthy volunteers ($n = 7$). PPP was then mixed with 20 μL of PPP-Reagent (Stago) containing a mixture of TF (5 pM) and phospholipids (4 μM) and incubated for 10 minutes at 37 °C. Thrombin generation was initiated by the automated addition of 20 μL FluCaKit (Stago) and measured and analyzed as described above.

To confirm that the observed effect was specifically caused by C9, thrombin generation was also measured in PPP from healthy volunteers ($n = 3$) supplemented either with denatured C9 protein (30 μg/mL) that was heat-inactivated at 95 °C for 10 minutes, or with albumin (30 μg/mL) as controls.

To further explore the potential influence of platelets on the effect of C9, thrombin generation was assessed by supplementing platelet-rich plasma (PRP) with C9 in parallel with PPP using the same triggering reagent. This approach aimed to maintain equivalent concentrations of TF and phospholipids in both plasmas, with the only variable being the presence of platelets in PRP.

To investigate the potential involvement of C5 cleavage and consequent terminal complement activation and membrane attack complex (MAC or C5b-9) formation in the observed effect, thrombin generation was assessed in PPP supplemented with C9 protein, preincubated with 100 μg/mL of eculizumab (Alexion), a potent inhibitor of C5 cleavage by C5 convertase [32–34].

2.7 | Statistical analysis

Associations of each HPA plasma level with each of the 4 TGA parameters were tested using multiple linear regression analysis. Analyses were adjusted for age, sex, and internal controls of the proteomics assay [30]. To assess the potential influence of inflammation, analyses were further adjusted for FVIII. Prior to the analysis, lag time and time to peak were log-transformed to reduce skewness in their distribution.

A Bonferroni-adjusted P value of 1.33×10^{-4} (.05 divided by the number of targeting antibodies, P = .05/377) was used to declare statistical significance while correcting for multiple testing. Significant associations were then further validated in the EFS-AM cohort using the same statistical framework.

Spearman correlation was used to assess correlations between TGA parameters, while Pearson correlation was used to assess the correlation between levels of C5 and C9 measured by affinity proteomics and ELISA.

2.8 | Genetic analysis

To get additional support for the observed HPA-TGA associations, we investigated whether single nucleotide polymorphisms (SNPs) reported to associate with plasma levels of proteins expected to be targeted by the identified HPAs also associate with TGA parameters in MARTHA and EFS-AM samples. For this, we interrogated the genome-wide association results of the Fenland study [35] (n = 10 708) to determine whether our candidate proteins had been measured using the Somalogic platform and whether their plasma levels were under the influence of genetic polymorphisms at the corresponding loci (ie, *cis* genetic effects). Haplotype tagging *cis* SNPs with genome-wide significant association ($P < 5 \times 10^{-8}$) with C5 or C9 levels in the Fenland study were then selected for further association testing with C5, C9, and TGA parameters in MARTHA and healthy donors. As previously described [36,37], MARTHA samples were genotyped with high-density Illumina arrays, and imputation of the SNPs was performed on the 1000 Genomes Haplotypes—Phase 3 version 5 using Minimac 4 imputation software [38]. In the EFS-AM cohort, allele-specific TaqMan polymerase chain reaction (Applied Biosystems, Thermo Fisher Scientific) was used to genotype selected SNPs. Genotype associations were conducted using linear regression analyses adjusted for age and sex separately in MARTHA patients and healthy donors.

Unless otherwise specified, all analyses were carried out using the statistical software package R, version 3.5.0 (R Foundation for Statistical Computing), and SAS statistical software version 9.4 (SAS Institute).

3 | RESULTS

3.1 | Sample characteristics

The baseline characteristics of the studied populations are summarized in Table 1. The proportion of females in MARTHA was nearly 2-fold that of males, whereas there was a higher proportion of males in healthy donors. The mean age was slightly higher in MARTHA (46 years vs 40 years), and body mass index was the same for both populations (25 kg/m^2). Regarding the history of VTE in MARTHA, the majority of subjects (83%) experienced isolated deep vein thrombosis, whereas only a small proportion (6%) had an isolated pulmonary embolism event, and 11% had a deep vein thrombosis associated pulmonary embolism event.

TABLE 1 Main clinical characteristics of the studied populations.

Variables	MARTHA (n = 770) Mean ± SD[a]	EFS-AM (n = 536) Mean ± SD[a]
Gender and biometry		
Sex (male/female)	237/533	356/180
Age at sampling (y)	46 ± 15	40 ± 13
Body mass index (kg/m^2)	25 ± 5	25 ± 3
Type of thrombosis		
DVT	640	-
PE	47	-
DVT + PE	83	-
Thrombin generation parameters		
ETP (nM.min)	1746 ± 360	1577 ± 334
Peak (nM)	327 ± 70	258 ± 68
Lag time (min)	3.2 ± 0.9	2.7 ± 0.4
Time to peak (min)	5.9 ± 1.2	6.1 ± 1.2

DVT, deep vein thrombosis; EFS-AM, Etablissement Français du Sang Alpes-Méditerranée; ETP, endogenous thrombin potential; MARTHA, Marseille Thrombosis Association; PE, pulmonary embolism.
[a] Count data are shown for categorical variables, other reported values are mean ± SD.

The correlations between TGA parameters in both populations are summarized in Figure 1. Strong correlations were found between lag time and time to peak, as well as ETP and peak height. A weak correlation was observed between peak height and lag time, while a moderate inverse correlation was identified between ETP and time to peak in MARTHA and EFS-AM. ETP and lag time, and peak height and time to peak showed no correlation or weak inverse correlation for MARTHA and EFS-AM, respectively.

3.2 | Association between proteomics and thrombin generation parameters

Main results of the association analyses between HPA and TGA parameters in MARTHA are summarized in Figure 2. These include the HPA-TGA associations that reached the predefined statistical threshold of $P < 1.33 \times 10^{-4}$, comprising 74 HPAs targeting 63 proteins. Of these, 23 proteins were associated with ETP, 24 proteins

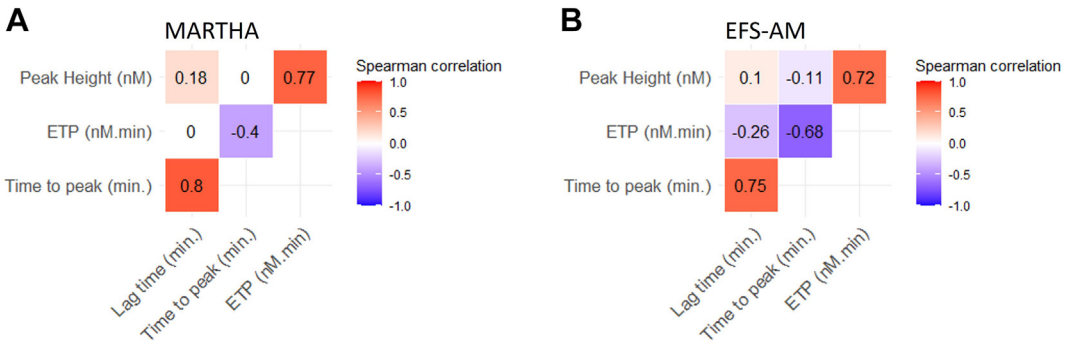

FIGURE 1 Correlation matrix. Spearman correlations between thrombin generation parameters in the 2 studied populations, Marseille Thrombosis Association (MARTHA; A) and Etablissement Français du Sang Alpes-Méditerranée (EFS-AM; B). ETP, endogenous thrombin potential.

with peak height, 54 proteins with lag time, and 16 with time to peak. Eighteen proteins were associated with at least 3 TGA parameters in MARTHA subjects, among which 13 demonstrated a similar pattern of associations in healthy individuals, as summarized in Figure 3. Details of these associations can be found in Supplementary Table S2. This list of 13 candidates includes proteins that are important players in the coagulation/fibrinolysis system, such as FVIII, protein S, and plasminogen. More interestingly, besides the classical coagulation factors, the strongest associations observed both in MARTHA and EFS-AM were for C5 and C9, 2 proteins belonging to the complement system. These 2 proteins are also strongly correlated in MARTHA and EFS-AM, with correlation coefficients ranging from 0.53 to 0.91 and all correlations significant at $P < 10^{-4}$ (Supplementary Table S3). Despite being acute-phase proteins and positively correlated with FVIII (Supplementary Table S4), C5 and C9 remained significantly associated with TGA parameters even after adjusting the model for FVIII as a proxy for general inflammation (Supplementary Tables S5 and S6). We thus decided to further follow-up with C5 and C9 proteins.

3.3 | Target verification

To verify that C5 and C9 are specifically bound to their targeting antibodies in the affinity proteomic assay, we measured the plasma levels of C5 and C9 proteins by ELISA in 40 randomly selected plasma samples of the MARTHA subset. Pearson correlations showed that median fluorescence intensity values from the proteomics assay were well correlated with ELISA measurements of C5 and C9 plasma levels, providing support that C5 (Bsi0765, Bsi0732, and HPA075945) and C9 (Bsi0270, Bsi1404, and HPA070709) targeting antibodies specifically bound and measured C5 and C9 from plasma (all correlation coefficients lying between .43 and .62, maximum P value = .0062), as shown in Supplementary Figure S1.

3.4 | *In vitro* functional studies

In order to investigate the effects of complement components C5 and C9 on thrombin generation *in vitro*, we measured thrombin generation in PPP from healthy volunteers after supplementation with increasing concentrations of purified human complement protein C5 or C9. As shown in Figure 4A, supplementation with C5 protein had no effect on TGA parameters. However, C9 supplementation had a significant effect on TGA parameters, as observed in Figure 4B, reducing the lag time and time to peak while increasing the ETP and peak in a dose-dependent manner.

To confirm that the effects of C9 on TGA parameters were specific, the purity of the C9 protein was verified by gel electrophoresis followed by silver staining, which showed a single band with the expected molecular weight (Supplementary Figure S2). Moreover, we measured thrombin generation in PPP from healthy volunteers supplemented with C9 and with C9 protein that was denatured by heat-inactivation, as well as albumin, as controls. There was no effect on thrombin generation in PPP supplemented with denatured C9 or albumin, confirming the specificity of the effect by C9 (Supplementary Figure S3).

Furthermore, since C9 is a component of the MAC, we wanted to explore if the observed effect of C9 on thrombin generation could be mediated by a potential interaction with residual platelets (membranes). To test this hypothesis, we measured thrombin generation after supplementation with C9 protein in both PPP and PRP. The effect of C9 on thrombin generation was not significantly different in the presence of platelets compared with PPP (Supplementary Figure S4).

To further investigate whether the effect of C9 was mediated by C5 cleavage and consequent MAC formation, we assessed thrombin generation after supplementation with C9 in PPP preincubated with eculizumab, which inhibits C5 cleavage by C5 convertase. The effect of C9 on thrombin generation parameters was not significantly different in the presence or absence of eculizumab (Supplementary Figure S5).

3.5 | Genetic analysis

From the Fenland study, we identified 4 cis protein-associated SNPs (pSNPs) for C9 plasma levels (rs17468519, rs2910958, rs696766, and rs700239, $P < 2.22 \times 10^{-8}$) and 8 cis pSNPs in strong linkage

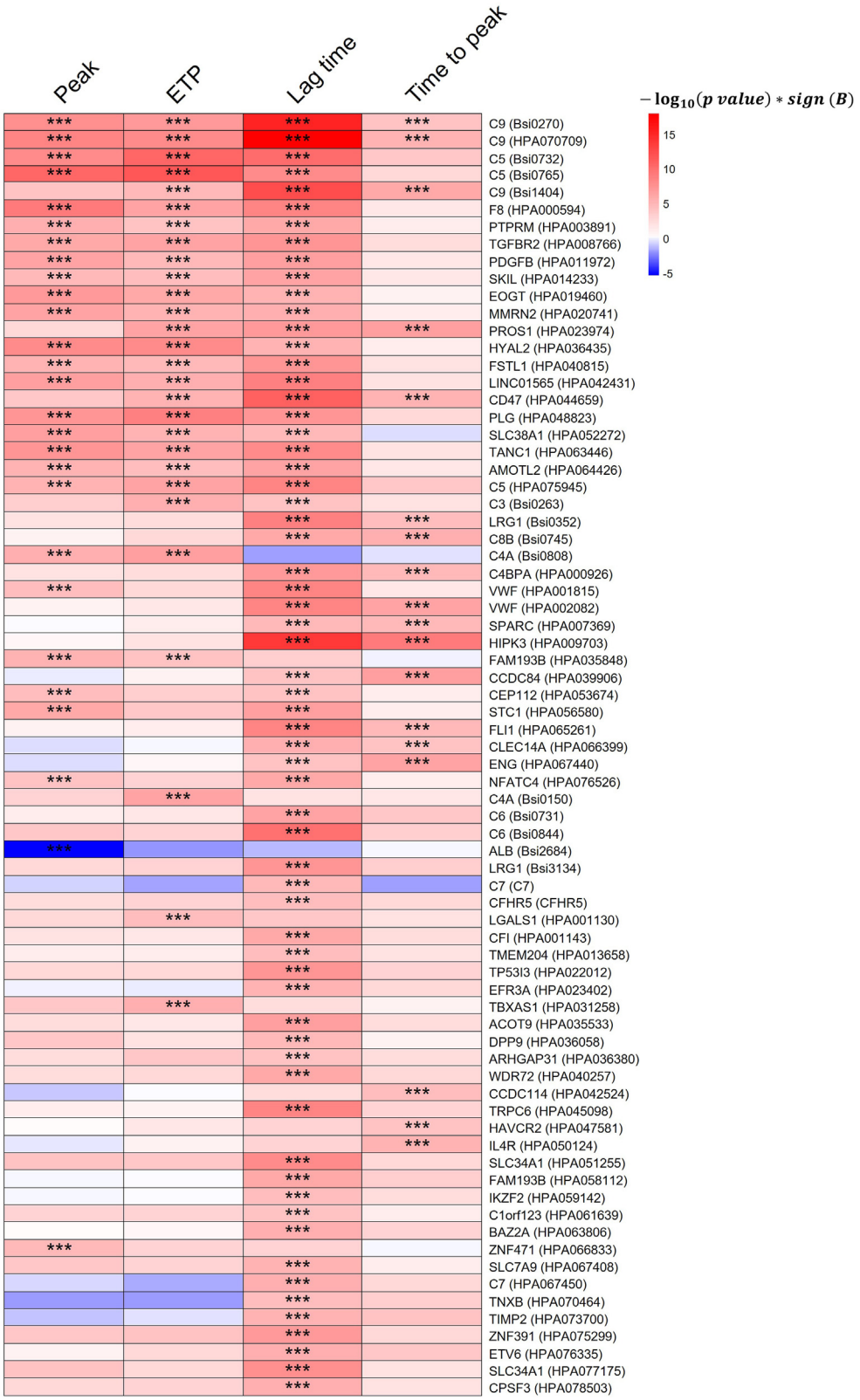

FIGURE 2 Summary of associations between thrombin generation assay parameters and targeted proteins. Heatmap depicting significant associations (***) between Human Protein Atlas targeting antibodies and thrombin generation parameters by using a multiple linear regression model in Marseille Thrombosis Association patients. Color represents the sign of the effect (β) to distinguish between positive (red) and negative (blue) associations, and color intensity represents the strength of the association by *P* value. ***p value $< 1.33 \times 10^{-4}$.

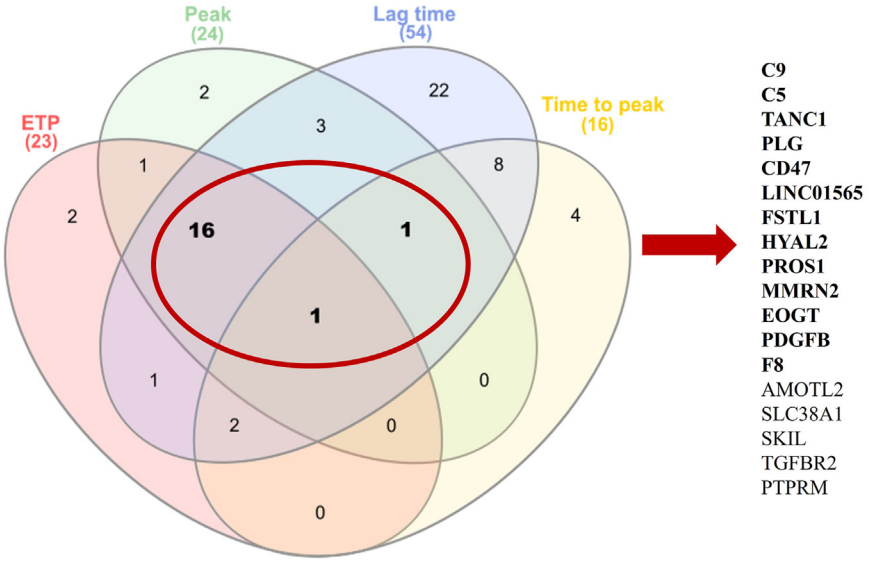

FIGURE 3 Selection of candidate proteins. Venn diagram depicting the 18 proteins that were significantly associated with at least 3 thrombin generation assay parameters in the Marseille Thrombosis Association, among which 13 (in bold) were replicated in healthy individuals.

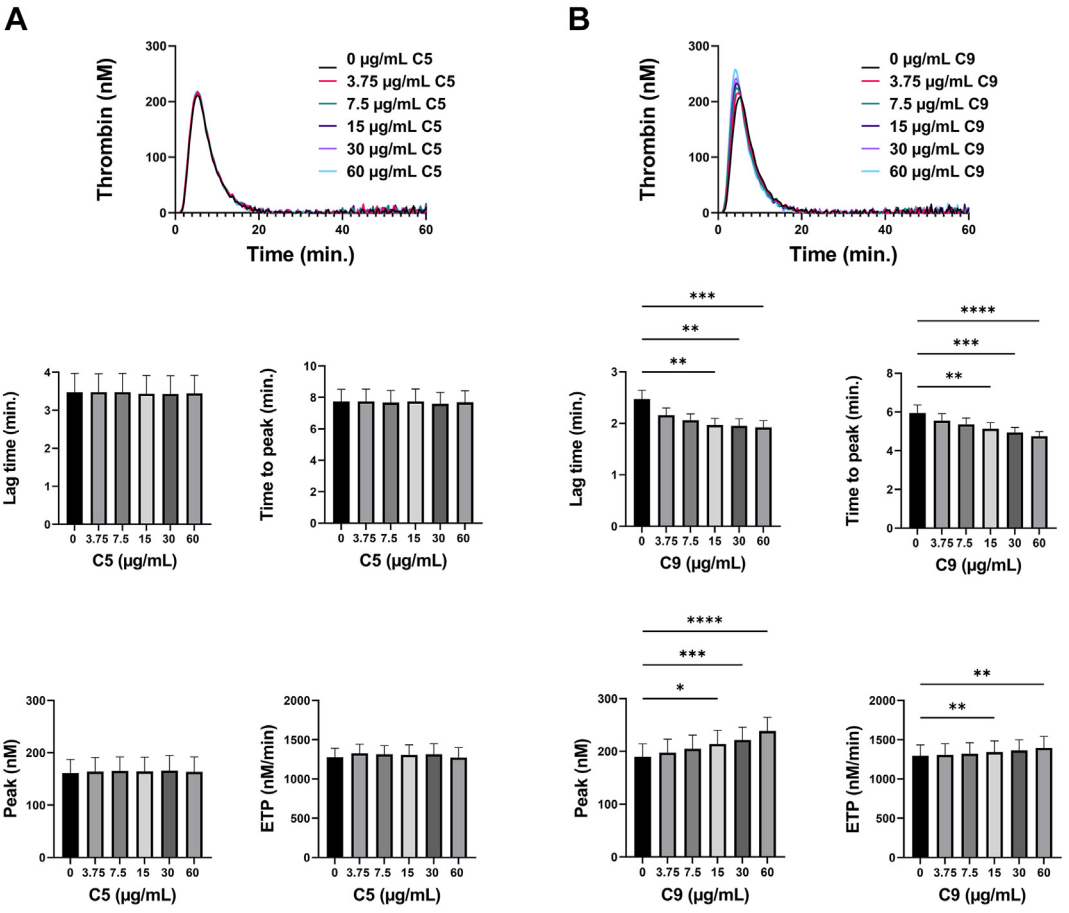

FIGURE 4 Effect of C5 and C9 supplementation on thrombin generation. Representative thrombin generation curves and thrombin generation parameters from measurements performed in platelet-poor plasma from healthy volunteers (n = 7) supplemented with increasing concentrations (0, 3.75, 7.5, 15, 30, and 60 µg/mL) of purified human complement component C5 (A) and C9 (B). Data are represented as mean ± SEM and analyzed by Friedman test with Dunn's post hoc. *P < .05, **P < .01, ***P < .001, ****P < .0001. ETP, endogenous thrombin potential.

TABLE 2 Association of *C5* rs17611 with C5-targeting Human Protein Atlas and thrombin generation assay parameters in Marseille Thrombosis Association.

	rs17611			
	C/C *n* = 220	C/T *n* = 382	T/T *n* = 144	
Variables	Mean (SD)	Mean (SD)	Mean (SD)	*P*[a]
C5_Bsi0765	15 384 (2142)	14 456 (2323)	12 995 (1987)	9.67×10^{-31}
ETP	1786 (348)	1724 (340)	1713 (421)	.04
Peak	333 (68)	325 (66)	317 (80)	.03
Lag time	3.20 (0.94)	3.25 (1.03)	3.24 (0.72)	.73
Time to peak	5.87 (1.31)	5.89 (1.26)	5.95 (1.18)	.63

C, major allele of the rs17611; ETP, endogenous thrombin potential; T, minor allele of the rs17611.

[a] Genotype association test (2 degrees of freedom) adjusted for age and sex. Analyses on Bsi0765 were further adjusted for Human Protein Atlas internal controls.

disequilibrium (rs1035029, rs11314681, rs112832843, rs17220750, rs17611, rs3764912, rs7864675, and rs9408926, $P < 1.66 \times 10^{-16}$) for plasma C5 levels. Strongest statistical associations were observed for the rs696766 pSNP ($P = 1.04 \times 10^{-25}$) in the *C9* gene and rs1035029 pSNP ($P = 2.94 \times 10^{-49}$) in the *C5* gene (Supplementary Tables S7 and S8).

In MARTHA ($n = 1386$), we observed a marginal association ($P = .006$) of *C9* rs700239 with C9 targeting HPAs. By contrast, highly significant associations (P as low as 10^{-29}) were observed between selected *C5* pSNPs and C5 targeting HPA Bsi0765. The strongest association was observed for the missense variant rs17611 ($P = 3.88 \times 10^{-29}$), which was selected for further analyses. Associations of selected cis pSNPs with HPAs targeting C9 and C5 in MARTHA are summarized in Supplementary Tables S9 and S10.

In the subset of MARTHA patients with measured TGA parameters, the *C5* rs17611 T allele was associated with a decrease in C5 levels as measured by Bsi0765 ($P = 9.67 \times 10^{-31}$) and with a decrease in ETP ($P = .04$) and peak ($P = .03$; Table 2). While the association of rs17611 with C5 targeting antibody Bsi0765 was replicated in healthy donors ($P = 2.96 \times 10^{-25}$), no association of this variant with TGA parameters was observed (Table 3). *C9* rs700239 was marginally associated with C9 levels as measured by HPA070709 ($P = .04$) but without association with any TGA parameters in MARTHA (Supplementary Table S11).

In addition, we assessed the association between the *C5* rs17611 and the risk of venous thrombosis in 71 111 patients and 1 051 782 controls of European ancestry from the International Network of VENous Thromboembolism Clinical Research Networks (INVENT) consortium meta-GWAS (Genome wide association studies) analysis [39]. No association ($P = .40$) was observed without any evidence of heterogeneity between cohorts ($P = .45$).

4 | DISCUSSION

Our study aimed to identify novel biological determinants of thrombin generation by integrating antibody-based affinity proteomics, *in vitro* functional studies, and genomics data. We identified 18 proteins significantly associated with at least 3 of the studied thrombin generation parameters in the MARTHA population. We successfully replicated 13 of these protein-TGA associations in an independent population of healthy individuals. This list of 13 candidates includes players in the coagulation/fibrinolysis system, such as FVIII, protein S, and plasminogen, which provides additional validation for our approach. More interestingly, besides the classical coagulation factors, the most robust associations observed in both MARTHA and EFS-AM were for C5 and C9, 2 proteins belonging to the complement system. Although both C5 and C9 are acute-phase proteins, their associations with TGA parameters remained significant even after adjusting the model for FVIII as a proxy for general inflammation. We here demonstrate an association between C5 and C9 plasma levels and thrombin generation, a known intermediate phenotype of VTE [16,17,40]. Several studies have previously highlighted the role of C5 in thrombotic mechanisms [41–43]. Recently, Skjeflo et al. [41] demonstrated the association between plasma C5 and risk of future VTE in a nested case-control study. Our findings suggest that this association with VTE risk may be mediated, at least in part, by changes in thrombin generation associated with C5 levels.

The potential causal relationship between C5, C9, and thrombin generation lies in the strong interplay between the complement and coagulation systems. The complement system, in addition to its well-established role in inflammation, is closely linked to the coagulation system [11,44]. The relationship between the 2 systems is complex and multifaceted. The complement system can directly enhance blood clotting properties and intensify an inflammatory response, thereby potentiating coagulation [45]. Conversely, coagulation factors can also affect the complement system. Notably, complement component C5 plays a key role in these interactions. Its proteolytic cleavage results in the release of the anaphylatoxin C5a and of a second fragment (C5b) that promotes the formation of the MAC, both of which play critical roles in thrombosis. C5a can contribute to blood thrombogenicity by upregulating TF and plasminogen activator inhibitor-1 expression on various cell types [46], while the MAC incorporates into the cellular membrane of platelets, exposing a procoagulant surface that enhances clotting [47]. The MAC also induces the release of microparticles bearing TF on their surface and affects the procoagulant properties of

TABLE 3 Association of *C5* rs17611 with C5-targeting Human Protein Atlas and thrombin generation assay parameters in the Etablissement Français du Sang Alpes-Méditerranée.

	rs17611			
	C/C *n* = 147	C/T *n* = 239	T/T *n* = 93	
Variables	Mean (SD)	Mean (SD)	Mean (SD)	*P*[a]
C5_Bsi0765	14 142 (2082)	12 784 (2006)	11 703 (1897)	2.96×10^{-25}
ETP	1585 (348)	1558 (330)	1569 (390)	.87
Peak	257 (65)	256 (68)	318 (80)	.45
Lag time	2.66 (0.39)	2.65 (0.46)	2.66 (0.42)	.42
Time to peak	6.11 (1.12)	6.08 (1.24)	6.07 (1.18)	.20

C, major allele of the rs17611; ETP, endogenous thrombin potential; T, minor allele of the rs17611.

[a] Genotype association test (2 degrees of freedom) adjusted for age and sex. Analyses on Bsi0765 were further adjusted for Human Protein Atlas internal controls.

the endothelium [48,49], which ultimately increases thrombin generation.

To investigate the potential causality of these associations, we used 2 complementary approaches: *in vitro* studies and genetic studies. In our *in vitro* experiments, we observed that supplementation of PPP of healthy individuals with C5 did not exert any direct effect on *in vitro* thrombin generation. However, this does not necessarily exclude the role of C5, as the correlation between C5 levels and TGA parameters observed in the population studies may be a consequence of *in vivo* complement activation. In fact, it has been reported that C5 is a positive determinant of the activity of the classical and alternative complement pathways [50]. Ongoing complement activation *in vivo* might cause the release of procoagulant components from platelets and/or endothelial cells [44], thus inducing a hypercoagulable state that, following blood withdrawal and plasma preparation, can be detected by measuring thrombin generation *in vitro*. Although speculative, this mechanism could account for the association between C5 and thrombin generation parameters observed in the population studies despite failure of added C5 to influence thrombin generation in PPP *in vitro*. In this respect, C5 could be compared with vitamin K antagonists or female hormones. The unquestionable effects of warfarin or estrogen on thrombin generation are readily detectable in population studies but cannot be replicated by simply adding these compounds directly to plasma *in vitro* because they are mediated by biological processes that take place *in vivo*. In contrast, supplementation of normal PPP with C9 had a significant effect on TGA parameters, reducing the lag time and time to peak while increasing the ETP and peak in a dose-dependent manner. These results suggest that C9 might have a direct role in thrombin generation, possibly through interaction with coagulation factors or other plasma proteins.

Interestingly, the effect of C9 on *in vitro* thrombin generation was not significantly different in the presence of platelets, which supports the idea that the observed effect of C9 may be independent of the interaction with platelets upon C5 cleavage and subsequent MAC formation. Furthermore, even in the presence of eculizumab (inhibitor of C5 cleavage by C5 convertase [51]), we still observed the effect of C9 on thrombin generation, providing evidence that C9 may influence thrombin generation through a distinct pathway or mechanism that is not reliant on traditional MAC formation.

Through genetic analysis, we identified SNPs associated with C5- and C9-targeting HPAs. We observed a marginal association of *C9* rs700239 with C9 targeting HPAs, which we successfully replicated in EFS-AM. However, we did not observe any association between this variant and TGA parameters. This could be attributed to the sample size, which might not have been large enough to reach statistical significance.

In contrast, we observed highly significant associations between selected *C5* SNPs and C5-targeting HPAs. Among these associations, the strongest one was observed for the missense variant rs17611 and C5-targeting HPA Bsi0765. The *C5* rs17611 T allele was associated with a decrease in Bsi0765 levels as well as with a decrease in ETP and peak. Interestingly, while the association of rs17611 with C5 targeting antibody Bsi0765 was replicated in healthy donors, we did not find any association of this variant with TGA parameters in this healthy population. This finding suggests that there might be some disease-specific or interacting factors at play influencing the association between this genetic variant and thrombin generation. This SNP encodes an amino acid substitution that makes C5 more susceptible to cleavage by neutrophil elastase [52]. We then analyzed GWAS data from the INVENT consortium and found no association between this SNP and the risk of VTE. Further investigation is needed to fully understand the underlying mechanisms and how they may differ between healthy individuals and those with a history of VTE.

Regarding the strengths of our study, it is noteworthy to mention that our study offers a unique and significant contribution as it is the first, to the best of our knowledge, to investigate determinants of thrombin generation through the integration of a multifaceted approach in a large population with results replicated in an independent population. In comparison with previous studies, we provided a more extensive profile of plasma proteins involved in thrombin generation, with more than 200 proteins included in the analysis. To the best of our knowledge, such a comprehensive analysis has not been conducted before.

Despite the strengths of our study, certain limitations should be acknowledged. One of the limitations is the use of targeted

proteomics compared with untargeted proteomics. The choice of targeted proteomics restricted us to a predetermined set of proteins included in the proteomics study, potentially overlooking other relevant proteins that might influence thrombin generation. The inherent selection bias in targeted proteomics limits the comprehensiveness of our analysis and may restrict the identification of novel proteins associated with thrombin generation. Furthermore, since our focus was on identifying new determinants of thrombin generation, key determinants such as prothrombin, antithrombin, and fibrinogen were not included in the panel. This prevented us from further exploring the impact of these factors on the observed associations.

Another limitation pertains to the selection of a population with a history of VTE. Although this population allowed us to explore potential associations in a context relevant to thrombosis, it may restrict the generalizability of our findings to a broader population. Nevertheless, we were able to address this concern by replicating the main proteomic associations in an independent population of healthy individuals. This replication strengthens the robustness of our results, suggesting that the associations between complement proteins and thrombin generation are not exclusive to individuals with a history of VTE.

The criteria used to select proteins associated with at least 3 thrombin generation parameters in MARTHA may be considered somewhat arbitrary. Although this approach allowed us to identify a subset of proteins with consistent associations, it is possible that other proteins with significant effects on thrombin generation were missed by our criteria.

Lastly, our study design does not allow a direct comparison between cases and controls, as the healthy population used as the replication cohort is not matched in a traditional "cases-controls" setting. Future studies incorporating matched controls would provide a more comprehensive understanding of the associations between complement proteins and thrombin generation in both pathologic and healthy states.

In conclusion, our study reveals an association between complement proteins C5 and C9 and *in vitro* thrombin generation. Whether this association is due to a direct effect on coagulation reactions, as might be the case for C9, or rather reflects procoagulant changes in plasma composition due to ongoing complement activation *in vivo* remains to be elucidated. In any case, our data consolidate the strong interplay between coagulation and complement cascades and provide some new indications on how complement can affect the risk of thrombosis. Further investigations are warranted to determine the precise mechanisms by which C5 and C9 impact thrombin generation, which may provide valuable insights into the pathophysiology of various diseases associated with complement dysregulation and thrombotic disorders, potentially paving the way for the development of novel therapeutic strategies that target both systems.

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AUTHOR CONTRIBUTIONS

Conception and design: L.G., D.A.T., P.E.M., and S.D. Data collection: R.V.D., D.A.T., J.F.D., P.E.M., M.I.K., A.B., M.G., J.O., M.J.I., N.S., and E.K. Statistical analysis: R.V.D., L.G., P.E.M., D.A.T., A.P.R., G.M., and M.G. Interpretation of data: R.V.D., L.G., D.A.T., P.E.M., S.D., E.C., and J.N. Draft of manuscript: R.V.D., L.G., P.E.M., and D.A.T. Review and approval of the final version: all authors.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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