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Haemostasis*, vol. 20, no. 6, pp. 1331-1349. https://doi.org/10.1111/jth.15698

Digital Object Identifier (DOI):
10.1111/jth.15698

Received: 14 December 2021 | Accepted: 8 March 2022

DOI: 10.1111/jth.15698

jth

ORIGINAL ARTICLE

Multi-phenotype analyses of hemostatic traits with cardiovascular events reveal novel genetic associations

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Funding information
National Heart, Lung, and Blood Institute, Grant/Award Number: HL134894, HL139553 and HL141291; European Social Fund

Abstract

Background: Multi-phenotype analysis of genetically correlated phenotypes can increase the statistical power to detect loci associated with multiple traits, leading to the discovery of novel loci. This is the first study to date to comprehensively analyze the shared genetic effects within different hemostatic traits, and between these and their associated disease outcomes.

Objectives: To discover novel genetic associations by combining summary data of correlated hemostatic traits and disease events.

Methods: Summary statistics from genome wide-association studies (GWAS) from seven hemostatic traits (factor VII [FVII], factor VIII [FVIII], von Willebrand factor [VWF] factor XI [FXI], fibrinogen, tissue plasminogen activator [tPA], plasminogen activator inhibitor 1 [PAI-1]) and three major cardiovascular (CV) events (venous thromboembolism [VTE], coronary artery disease [CAD], ischemic stroke [IS]), were

Manuscript handled by: Sigrid Braekkan

Final decision: Sigrid Braekkan, 8 March 2021

combined in 27 multi-trait combinations using metaUSAT. Genetic correlations between phenotypes were calculated using Linkage Disequilibrium Score Regression (LDSC). Newly associated loci were investigated for colocalization. We considered a significance threshold of 1.85×10^{-9} obtained after applying Bonferroni correction for the number of multi-trait combinations performed ($n = 27$).

Results: Across the 27 multi-trait analyses, we found 4 novel pleiotropic loci (*XXYLT1*, *KNG1*, *SUGP1/MAU2*, *TBL2/MLXIPL*) that were not significant in the original individual datasets, were not described in previous GWAS for the individual traits, and that presented a common associated variant between the studied phenotypes.

Conclusions: The discovery of four novel loci contributes to the understanding of the relationship between hemostasis and CV events and elucidate common genetic factors between these traits.

KEYWORDS
blood coagulation, cardiovascular diseases, genetic pleiotropy, genome-wide association study, hemostasis

1 | INTRODUCTION

Genome-wide association studies (GWAS) have identified dozens of loci underlying the variability of plasma levels for individual hemostatic traits.[1–8] Further, GWAS for venous thromboembolism (VTE),[9,10] coronary artery disease (CAD)[11–13] and ischemic stroke (IS),[11,14] have discovered 34, 169, and 20 genetic risk loci associated with these cardiovascular (CV) events, respectively.

Results from GWAS indicate that several of these hemostatic traits are genetically correlated with each other, sharing genetic loci that regulate their plasma levels.[1,4–8] There are also shared genetic loci between hemostatic traits and CV events, again suggesting common regulators and possibly a causal pathway between the hemostatic trait and the CV event.[4,7–9,12,14] The common regulatory loci between traits—even if the traits are not causally associated with each other—can be used to advance discovery of novel genetic loci common to the traits. This discovery can be accomplished with multi-phenotype methods that incorporate summary statistics from several GWAS, increasing the statistical power to detect loci affecting two or more phenotypes by increasing the effective sample size.[15–17]

In the present study, we used summary statistics of published GWAS from 7 hemostatic traits (FVII, FVIII, VWF, FXI, fibrinogen, PAI-1, tPA), and 3 CV events (VTE, CAD, IS) to calculate their genetic correlations and to conduct multi-phenotype meta-analyses to detect new genetic loci not previously known to be associated with these phenotypes.

Essentials

- Multi-phenotype analysis of genetically correlated phenotypes may lead to novel discoveries.
- Summary statistics of hemostatic traits and cardiovascular events were combined with metaUSAT.
- We identified four novel associations with a shared variant between the studied phenotypes.
- Our results shed light on the relationship between hemostatic traits and cardiovascular events.

2 | METHODS

2.1 | Study design and resources

The setting of the project is the Cohorts of Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium Hemostasis Working Group.[18] We used GWAS summary statistics from seven hemostatic traits (FVII ($N = 27\,495$), FVIII ($N = 32\,610$), VWF ($N = 46\,354$), FXI ($N = 16\,169$), fibrinogen ($N = 120\,246$), PAI-1 ($N = 19\,599$), tPA ($N = 26\,929$)), and three CV events (VTE (N cases = 30 234, N controls = 172 122), CAD (N cases = 172 122, N controls = 566 864), IS (N cases = 60 341, N controls = 454 450)) to perform multi-phenotype analyses. Summary statistics of FVII,[1] FVIII,[4] VWF,[4] VTE,[9] CAD[12,13,19] and IS,[14] come from trans-ethnic analyses, while summary statistics of FXI,[6] fibrinogen,[5] tPA[8] and PAI-1[7] are European ancestry only (additional information, including the sample sizes, detailed ancestry groups, confounders considered and data access URLs, of the phenotypes that have been used, is available in Supplementary Table S1). Summary statistics for FVII, FVIII, VWF, FXI, fibrinogen, tPA, PAI-1, were obtained from the most recent CHARGE meta-analysis data[18] and are available on dbGaP[20] (Appendix A). Data for VTE was obtained from INVENT[9] consortium and is available on request from corresponding authors (Appendix B). Data from IS were obtained from the MEGASTROKE Consortium,[14] and is available at https://www.megastroke.org/ (Appendix C). For CAD, we used METAL to perform an inverse variance weighted meta-analysis between previously combined data

from CARDIoGRAMplusC4D Consortium[19] and UK Biobank datasets[12] (available at https://data.mendeley.com/datasets/2zdd47c94h/1), and the Biobank Japan dataset available at https://humandbs.biosciencedbc.jp/en/hum0014-v21 using METAL. All the included data have been published between December 2012 and October 2020. The overlap of individuals observed in our combinations of phenotypes ranges between 0 and 0.58. The overlap between the CARDIoGRAMplusC4D Consortium and UK Biobank datasets that were combined by other authors, and used in this project, was estimated to be <0.1 %.[12]

2.2 | Study of heritability and genetic correlation

We determined the heritability of each phenotype and genetic correlations (r_g) between all pairs of hemostatic traits, between each hemostatic factor and the CV events and between all pairs of CV events, using linkage disequilibrium (LD) score regression (LDSC).[21] LDSC uses a regression analysis between LD scores and the summary statistics of GWAS to provide an estimate of the shared heritability between phenotypes.[22] We used pre-computed LD scores from the European population of 1000G project.[23] A subset of the European-ancestry summary statistics was used in this step for each trait except for CAD where the European-only meta-analysis was not available. Alleles were merged with the HapMap3 single nucleotide polymorphisms (SNPs) list,[24] to avoid incompatibilities between phenotypes, as recommended by the authors, and missing variants were removed.

For the threshold of statistical significance for each genetic correlation, we applied a Bonferroni correction for multiple comparisons, considering all pairwise genetic correlations calculated (p <.05/45 = .001).

2.3 | Multi-phenotype analysis

We performed multi-phenotype analyses using GWAS summary statistics from different combinations of traits using the metaUSAT R package.[17] metaUSAT is a statistical approach for testing genetic association with one or more phenotypes simultaneously, using only common variants between the phenotypes. metaUSAT allows summary data as input that includes overlapping samples, which can be a source of bias with other methods; further, it does not assume homogeneity of trait effects across studies.[17] Compared to similar methods, metaUSAT performs similarly while requiring less computational time.[25]

In total, we performed 27 multi-phenotype analyses, considering all pairs of hemostatic traits that showed significant genetic correlations (p <.001) (Supplementary Table S2), pairs of combinations between each hemostatic trait and each of the three CV events, and other combinations included based on biological aspects of the analyzed proteins: Fibrinogen-FVII-FXI-tPA were analyzed because all of them are synthesized in the liver —although tPA is mostly produced by endothelial cells, recent studies that focused on the basal plasma tPA activity have also demonstrated the effects of hepatic produced tPA in fibrinolysis—.[26] Secondly, tPA was combined with FVIII and VWF, that are highly correlated, because it is known that these three phenotypes share loci like *STXBP5* that are involved in endothelial exocytosis.[4,8] Finally, the combination of fibrinogen and FVII was included to potentially discover genetic insights to the antithrombin (AT) pathway. It is known that AT deficiency is a strong risk factor for VTE, and that AT inhibits the FVIIa-tissue factor complex's activation of FX.[27,28] Moreover, AT modifies prothrombin's conversion of fibrinogen to fibrin.[29]Given this evidence, we hypothesize that potential common loci that regulate AT, FVII and fibrinogen might arise from this combination. Figure 1 shows all combinations that were analyzed.

For a metaUSAT p-value ($p\text{value}_{\text{multivariate}}$) to be declared statistically significant, it needed to exceed a Bonferroni correction of the traditional GWAS statistical significance threshold to account for multiple testing for 27 multi-trait combinations: $5 \times 10^{-8}/27 = 1.85 \times 10^{-9}$. For those variants with statistically significant metaUSAT p-values, we defined a locus as the genomic region +/− 500 kb around the variant with the lowest p-value and any other variants that were in LD of $r^2 > 0.2$. We used HaploR R package to

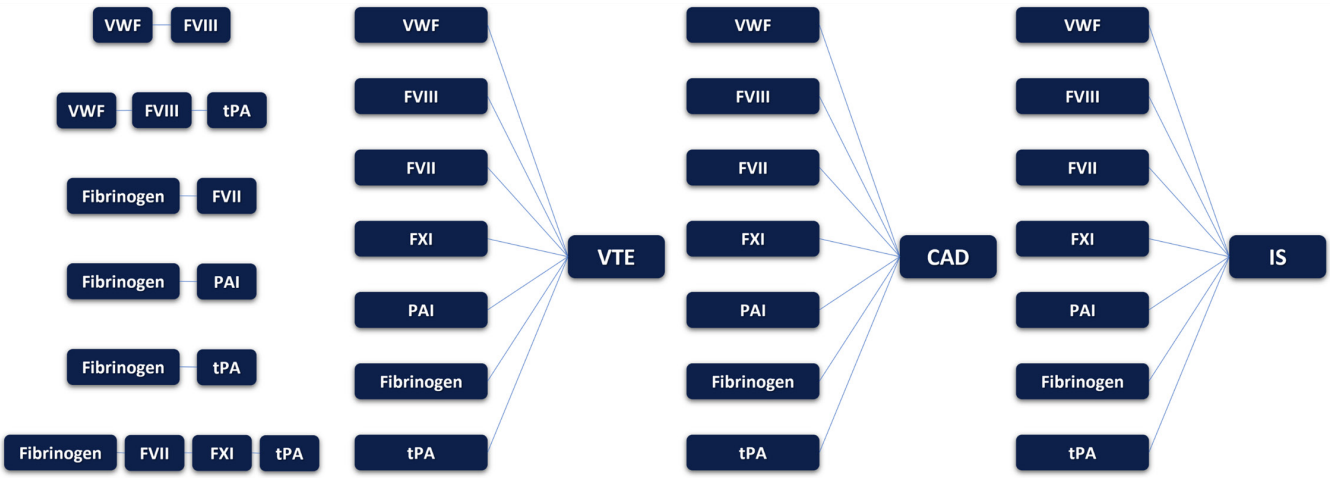

FIGURE 1 Schematic representation of the 27 multi-phenotype combinations

retrieve variants in LD with the lead variant in each locus (the variant with the lowest *p*-value).

In order to identify novel associated loci, we considered the following steps (Figure 2): (1) we identified all loci that were statistically significant in the multi-phenotype analysis ($p\text{-value}_{multivariate} < 1.85 \times 10^{-9}$) (significant loci); (2) among these, we identified all loci with a lead variant that was at least nominally significant for two of the individual datasets used in each combination of phenotypes (lead variant $p\text{-value}_{univariate} < .005$)[30–32] (significant loci driven by more than one phenotype); (3) among the loci from step 2, we then narrowed it down to loci that were new for at least one of the phenotypes used in the combination, defined as loci where no other variant in the locus had a *p*-value lower than 5×10^{-8} ($p\text{-value}_{univariate} < 5 \times 10^{-8}$) in the GWAS we used, and the locus had not been previously detected in another GWAS for the same phenotypes (new loci for one of the phenotypes used in the combination); and (4) among the loci from step 3, we identified loci that were new for all the phenotypes used in the combination (new loci for all the phenotypes used in the combination). We used the GWAS catalog database,[33] (available at https://www.ebi.ac.uk/gwas/docs/file-downloads) to detect loci that were published in previous GWAS. We used HaploReg v4[34] to retrieve previous results and biological annotations from the lead variants.

2.4 | Trait-trait colocalization

For novel loci that were new for all traits, we then performed additional colocalization analysis to look for the existence of common associated variants across multiple traits. We used COLOC[35] for loci associated with pairs of traits, and the R package HyPrColoc[36] for loci associated with more than two traits. We considered windows of +/− 500 kb around the lead variant to define loci. For each locus, COLOC returns posterior probabilities (PP) for 4 different hypotheses (H_n): PP_{H0} (the locus is not associated with any of the traits), PP_{H1}/PP_{H2} (the locus is only associated with one of the traits), PP_{H3} (the locus is associated with both traits but there is no evidence of them sharing a causal variant), PP_{H4} (the locus is associated with both traits and LD patterns suggest the existence of a causal variant). We considered pleiotropic loci those that reached a conditional probability of colocalization (CPC) (PP_{H4} / (PP_{H4} + PP_{H3})) > 0.8, which is defined as the conditional probability of colocalization with one causal variant, assuming the existence of a signal in both traits. To consider pleiotropic loci in multiple traits, we performed colocalization using HyPrColoc, where posterior probabilities of colocalization > 0.7 were required. Regional plots for significant colocalizing loci were done using LocusCompare R package.

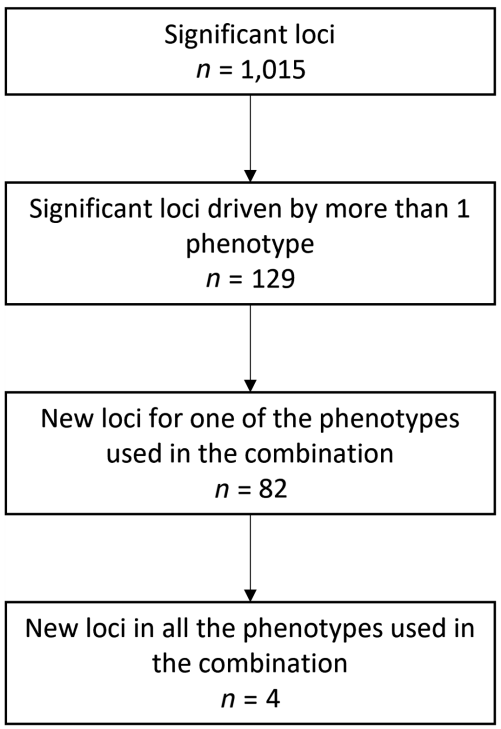

FIGURE 2 Schematic representation of the analysis plan for multi-phenotype analyses

2.5 | Trait-tissues colocalization

In order to prioritize candidate causal genes, we used novel pleiotropic loci identified in previous steps and performed an additional trait-expression colocalization analysis using RNAseq data from the Genotype-Tissue Expression (GTEx) project.[37] First, we identified the lead variants that were significant expression quantitative trait loci (eQTL) and splicing quantitative trait loci (sQTL) for all tissues in GTEx V8 (available at https://www.gtexportal.org/home/datasets). Then, we performed colocalization with HyPrColoc,[36] between the two or more phenotypes and the GTEx eQTL and sQTL results, using the complete GTEx V8 files (available at https://console.cloud.google.com/storage/browser/gtex-resources), in order to identify the functional tissue and elucidate on the biological mechanism causing the associations. We required a probability of colocalization > 0.7 to consider significant colocalization between traits and tissue expression.

We restricted eQTL and sQTL analyses to a subset of GTEx tissues that could be of interest in relation to CV events and hemostatic traits: vascular tissues (artery aorta, artery coronary, artery tibial) lipid metabolism related tissues (adipose subcutaneous, adipose visceral omentum), blood (whole blood) and liver. All loci that showed significant colocalization between at least two traits were analyzed for colocalization with tissue expression in those tissues.

3 | RESULTS

3.1 | Linkage disequilibrium score regression (LDSC)

Genetic correlations were calculated for every pair of phenotypes used, including hemostatic traits and CV events. In total, 45 genetic correlations were calculated, 24 of which presented nominal

significant *p*-values ($p < .05$) and seven were significant after applying multiple testing correction ($p < .001$). Among the seven genetic correlations that were significant, three were between hemostatic traits (VWF-FVIII ($r_g = 0.86$, $p = 1.25 \times 10^{-15}$), fibrinogen-PAI-1 ($r_g = 0.4$, $p = 9.29 \times 10^{-5}$), fibrinogen-tPA ($r_g = 0.28$, $p = 0.001$)) and were used for multi-phenotype analyses, 3 were between a CV event and a hemostatic trait (CAD-fibrinogen ($r_g = 0.19$, $p = 6.6 \times 10^{-6}$), CAD-tPA ($r_g = 0.48$, $p = 4.9 \times 10^{-7}$), CAD-PAI-1 ($r_g = 0.52$, $p = 4.55 \times 10^{-6}$)) and one was between two CV events (CAD-IS ($r_g = 0.5$, $p = 2.23 \times 10^{-22}$)). All genetic correlations are shown in a heatmap in Figure 3 and are available at Supplementary Table S2.

3.2 | Multi-phenotype analysis results

Overall, we performed 27 multi-phenotype analyses: three (FVIII-VWF, fibrinogen-tPA and fibrinogen-PAI-1) based on significant genetic correlations between pairs of phenotypes, three (fibrinogen-FVII-tPA-FXI, fibrinogen-FVII and VWF-FVIII-tPA) due to previously known common regulatory biological pathways, and 21 between combinations of each of the seven hemostatic traits (FVII, FVIII, VWF, FXI, fibrinogen, tPA, PAI-1) and each of the three CV events (VTE, CAD, IS).

The number of significant loci remaining in each step is represented in Figure 2. In total, we found 1 015 significant loci across the 27 multi-phenotype combinations (Supplementary Table S3). Among them, 129 loci were driven by more than one of the phenotypes used in the combination (Supplementary Table S4), and 82 of them were new associations for one of phenotypes of the combination (Supplementary Table S5).

We found four novel associations that were not significant in the original individual datasets and had not been described in previous GWAS of the same traits (Table 1). Additional information on these loci, including the complete COLOC and HyPrColoc results are available at Supplementary Table S6. Figure 4 contains graphic representations of the *p*-values and regional plots for each of these 4 loci.

3.3 | *XXYLT1*

We detected a newly associated locus, with lead variant rs3796159, a 3' UTR variant in *XXYLT1* gene, in the multi-phenotype analysis between VTE and FVII. Significant colocalization analysis (CPC > 0.8) (Figure 4A) in this locus suggested the existence of a common variant as a regulator of both phenotypes, VTE and FVII. Colocalization analysis in tissues indicated that rs3796159 is a significant eQTL for *XXYLT1* in five different tissues (adipose subcutaneous, adipose visceral omentum, artery aorta, artery coronary, artery tibial) and HyPrColoc results (Supplementary Table S7) showed a triple significant colocalization (Posterior probability > 0.7) in adipose subcutaneous, adipose visceral omentum, artery aorta and artery tibial tissues in the *XXLYT1* gene.

3.4 | *KNG1*

Although previously identified in a candidate gene experiment as a risk factor for thrombosis[38] and suggestively associated in other GWAS for VTE,[39] this work represents the first time that the *KNG1* locus, with lead variant rs710446, has been significantly associated to risk of VTE and FVIII in GWAS. rs710446 is a missense variant in the *KNG1* gene that causes an amino acid change at the position 581 (Ile581Thr).[40] Colocalization results between VTE and FVIII (CPC > 0.8) suggest that rs710446 in *KNG1* gene is associated both with FVIII and VTE (Figure 4B). Our results could not provide evidence for this variant being a significant eQTL or sQTL in any of the analyzed tissues which suggest an effect through protein function.

3.5 | *SUGP1/MAU2*

CAD and fibrinogen multi-trait analysis resulted in the identification of a novel association on *SUGP1* gene (lead variant rs10401969, intronic). Colocalization analysis between CAD and fibrinogen (Figure 4C) implicated that there is a shared associated variant at this locus (CPC > 0.8), while the analysis in GTEx tissues (Supplementary Table S7) and colocalization using HyPrColoc indicated a significant triple colocalization between CAD, fibrinogen and the GTEx dataset in blood in *MAU2* gene (Posterior probability > 0.7).

3.6 | *TBL2/MLXIPL*

The multi-phenotype combination of hemostatic proteins that are synthesized in the liver (fibrinogen-FVII-FXI-tPA), revealed a new association on *TBL2* gene (lead variant rs11974409, intronic). Although not reaching the significance threshold for the 4 phenotypes (Posterior probability > 0.8), significant colocalization results (Figure 4D) between FVII and tPA suggest that a shared causal variant regulates both phenotypes in this locus.

rs11974409 variant is an eQTL in adipose subcutaneous, adipose visceral omentum, artery aorta, and whole blood, and an sQTL in adipose subcutaneous and adipose visceral omentum tissues. HyPrColoc analysis results suggested the existence of a common causal variant that regulates FVII, tPA and the expression of three different genes (*AC005089.1*, *MLXIPL*, *BCL7B*) in adipose subcutaneous, adipose visceral omentum and blood tissues (Posterior probability > 0.7) (Supplementary Table S7), and a common causal variant that regulates FVII, tPA and the splicing of *MLXIPL* gene in adipose subcutaneous and adipose visceral omentum tissues (Posterior probability > 0.7) (Supplementary Table S8).

3.7 | *MYRF/TEMEM258/FADS1/FADS2*

Additionally, a novel association on *MYRF/TMEM258/FADS1/FADS2* locus was detected in the multi-phenotype analysis

between IS-VWF. Although just below the limit of significance (p-value$_{multivariate}$ = 1.64 × 10^{-8}) (Supplementary Table S6), *MYRF*/*TMEM8*/FADS1/*FADS2* locus has also been identified in the combinations between VWF and the other two CV events (VTE and CAD), (lead variant rs174528, intronic in *MYRF* gene). This association was reported for VTE in a previous European GWAS[10] and also for CAD,[13] but has not been associated with VWF or IS before. The three colocalization analyses between VWF and the CV events (Supplementary Table S6) in this locus suggest the existence of a variant regulating all traits (CPC > 0.8). HyPrColoc analysis revealed significant colocalization (Posterior probability > 0.7) between VWF, VTE and *FADS1* gene expression in artery tibial and liver tissues (Supplementary Table S7), while also suggested —not significantly— (0.7 > Posterior probability > 0.5) an effect in splicing regulation in adipose visceral omentum in *FADS2* and *FEN1* genes with VTE and CAD (Supplementary Table S8). We were unable to identify triple colocalizations between VWF, IS and gene regulation.

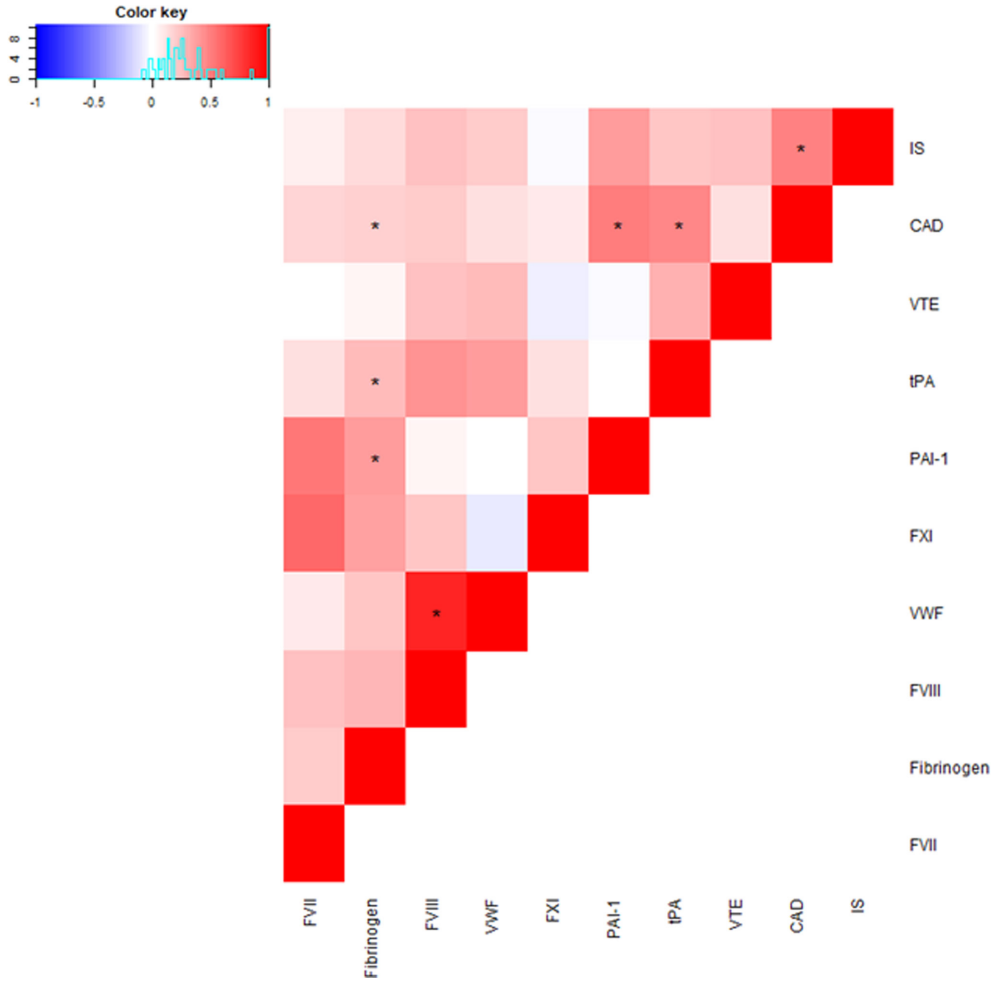

FIGURE 3 Heatmap of the genetic correlations between the two traits used in the multi-phenotype analyses. * Indicates traits are significantly correlated with a p-value <.001.

TABLE 1 Summary of the four novel associations identified in the 27 multi-phenotype analyses that were not significant in the individual datasets and previous GWAS

Marker Name	Traits	Variant	Effect Allele	MAF	Effect 1	Effect 2	Effect 3	Effect 4
3:194790434	VTE-FVII	rs3796159	C	0.2684	0.0605	−0.0112	-	-
3:186459927	VTE-FVIII	rs710446	T	0.4136	−0.0601	−0.012	-	-
19:19407718	CAD-FIBR	rs10401969	T	0.0768	0.0386	−0.0089	-	-
7:72989390	FIBR-FVII-FXI-TPA	rs11974409	A	0.1816	0.0035	0.0154	0.0079	0.023

[a]Posterior probability FVII-tPA.

[b]Conditional probability of colocalization.

4 | DISCUSSION

We performed a multi-phenotype approach using correlated hemostatic traits and three CV events and detected four novel pleiotropic loci that had not been previously described in association with these hemostatic traits or CV events.

Given a common locus between two or more phenotypes, three scenarios are possible: (1) there are different causal variants associated with the different traits, (2) the same variant associates with the different traits separately (horizontal pleiotropy), or (3) the variant associates with one trait, which in turn causes association with another trait (vertical pleiotropy).[41] While our analyses did not allow to differentiate between horizontal and vertical pleiotropy, we have found evidence of common variants in the four new pleiotropic loci, which agrees with the previous notion that pleiotropy is common between variants associated with correlated disease traits.[42] Common genetic regulators, however, do not mean that the associated phenotypes are causally associated. Causal associations between related phenotypes can be explored through Mendelian randomization (MR) methods.

We found a total of 1015 significant loci across all the multi-phenotype combinations. Among these, 129 were driven by more than one phenotype, of which 46 were found in combinations with CAD, 38 with VTE, 28 in combinations between hemostatic traits, and 16 with IS. Finally, among the 82 loci that were new for at least one of the phenotypes used in the combination, 30 were identified in combinations with CAD, 23 with VTE, 15 between hemostatic traits, and 13 with IS.

4.1 | *XXYLT1* and regulation of FVII

XXYLT1 codes for xyloside xylotransferase 1, an enzyme that elongates *O*-linked glycans in the epidermal growth factor (EGF) repeats of *O*-linked glycosylated proteins like FVII.[43] However, the direction of the effect of this variant suggests a decrease in FVII levels for allele C and an increase in the risk of VTE. In addition, MR analyses previously performed between VTE and FVII did not conclusively identify FVII as a cause of VTE,[1] which suggests that the common variant at this locus on xyloside xylotransferase 1 enzyme could be affecting both phenotypes independently, through expression of *XXYLT1* in adipose subcutaneous, adipose visceral omentum, artery aorta or artery tibial tissues. In this direction, it is known that other coagulation factors, like factor IX, are also glycosylated in the EGF repeats.[44] It would be plausible to speculate that *XXYLT1* could affect FVII levels and also other EGF-glycosylated proteins that would eventually modify VTE risk. Therefore, further research in this locus is recommended to fully understand the possible relationship between *XXYLT1*, FVII and VTE, and the possible effect that other hemostatic proteins could have in this association.

4.2 | *KNG1* and risk of VTE through FVIII levels

The protein encoded by this gene, Kininogen-1 (KNG1), is the precursor of two other proteins, obtained through alternative splicing: high-molecular-weight kininogen (HMWK) and low-molecular-weight kininogen (LMWK). Through a process facilitated by Factor XII (FXII), the peptide bradykinin is cleaved from HMWK by the enzyme kallikrein.[45]

There is strong biological evidence that associate *KNG1* gene with the coagulation system.[40,46,47] HMWK, along with FXII and prekallikrein (PK) complex, conform the plasma kallikrein-kinin system (KKS), that plays an important role in human physiology. The activation of KKS components results in the induction of genes and biomolecules that participate in blood coagulation, among other processes.[48,49] Bradykinin, on its turn, is an important molecule involved in vascular permeability and also in mechanism of pain.[45]

We have previously shown[6,50,51] that the lead variant on *KNG1* (rs710446) is also strongly associated to activated partial thromboplastin time (aPTT), prekallirein, FXI and coagulation activity of FVIII in a candidate gene experiment, indicating a pleiotropic effect of this gene on regulating the intrinsic pathway of coagulation,[52] resulting in modified risk of VTE.

Although the association with VTE has been demonstrated in candidate-gene studies,[38] the combination between VTE-FVIII, enhanced the association, suggesting a plausible functional relationship between KNG1 and FVIII that had never been reported in GWAS. This association could be explained by a putative regulation of KNG1 also on FVIII, which would imply an effect of KNG1 on the common pathway of coagulation. Associations of *KNG1* with the entire coagulation cascade, and not just the intrinsic pathway, have been proposed by others.[72] The significant colocalization analysis between VTE and FVIII in this locus aligns with previous evidence

Locus Name	p-value$_{multivariate}$	p-value$_{univariate}$ 1	p-value$_{univariate}$ 2	p-value$_{univariate}$ 3	p-value$_{univariate}$ 4	CPC[b]
XXYLT1	1.78×10^{-9}	6.77×10^{-6}	5.15×10^{-6}	-	-	0.9727
KNG1	4.12×10^{-11}	3.71×10^{-7}	1.1×10^{-6}	-	-	0.9962
SUGP1/MAU2	8×10^{-11}	2.22×10^{-5}	2.99×10^{-7}	-	-	0.9827
TBL2/MLXIPL	3.22×10^{-11}	.0019	1.87×10^{-7}	0.014737	1.71×10^{-5}	0.9896[a]

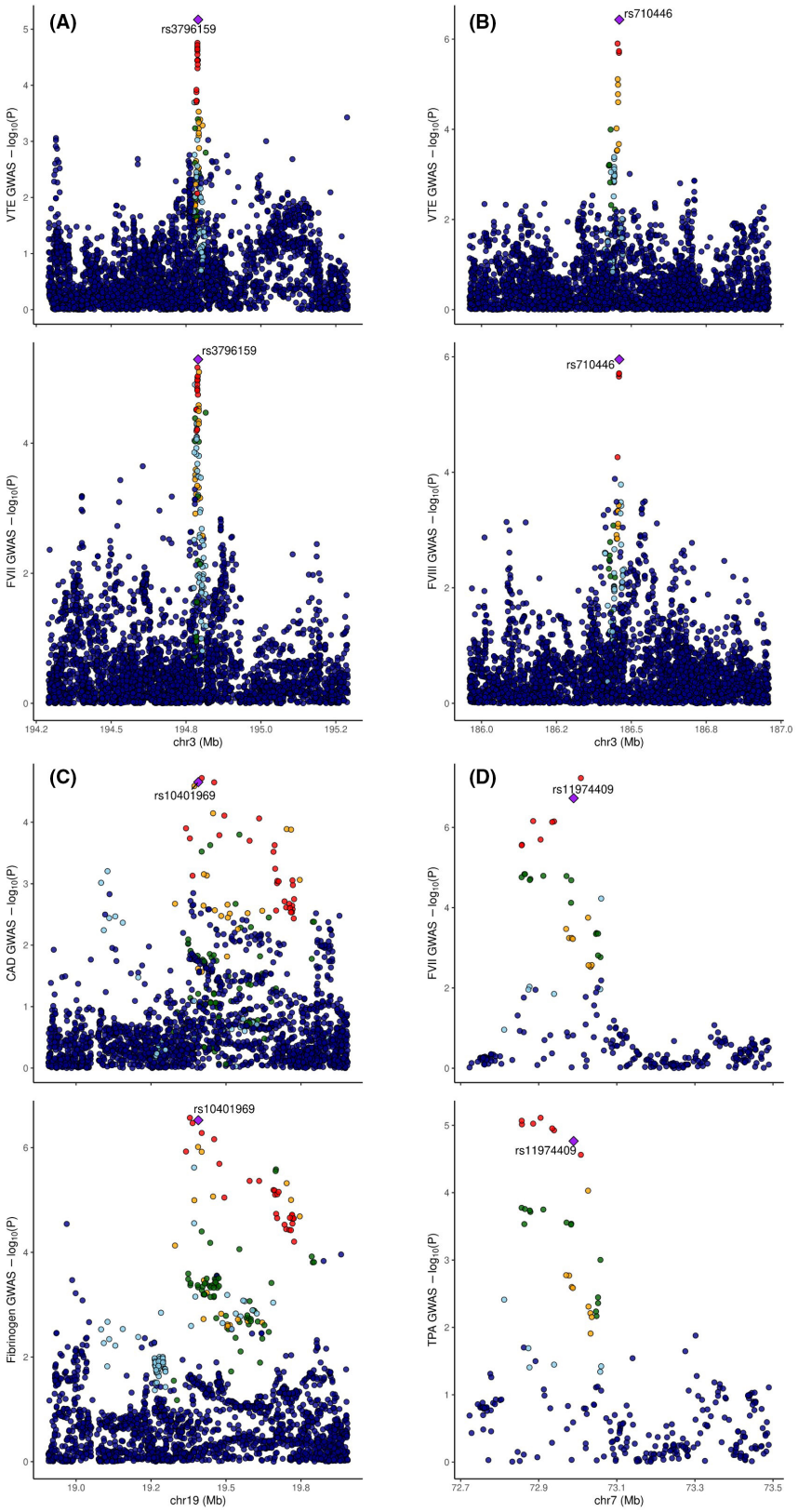

FIGURE 4 A: Regional plots for rs3796159 variant on *XXYLT1* gene on VTE (top) and FVII (bottom). B: Regional plots for rs710446 variant on *KNG1* gene on VTE (top) and FVIII (bottom). C: Regional plots for rs10401969 on *SUGP1* gene on CAD (top) and fibrinogen (bottom). D: Regional plots for rs11974409 on *TBL2* gene on FVII (top) and TPA (bottom).

and suggests that rs710446 affects the regulation of both phenotypes along with other related hemostatic phenotypes.

4.3 | *SUGP1/MAU2* and CAD risk through fibrinogen levels

CAD and fibrinogen multi-trait analysis resulted in the identification of a new pleiotropic locus on *SUGP1/MAU2* genes, with lead variant rs10401969. *SUGP1/MAU2* is a pleiotropic locus that has been associated to lipid's metabolism traits levels (total cholesterol, apoliprotein B, triglycerides), liver related proteins levels (alanine transaminase, asparatate aminotransferase, alanine aminotransferase), blood-related phenotypes (red cell distribution width, mean reticulocyte volume), type 2 diabetes and cirrhosis.[33] This locus has also been associated to CAD in candidate gene studies in Chinese and Caucasian populations but never in GWAS studies.[53,54] *SUGP1* codes for a protein called SURP and G patch domains-containing protein 1 (SUGP1), that it is believed to function in pre-mRNA splicing mechanisms.[55] This is the first time that *SUGP1* is associated with coagulation factors and colocalization results suggest that the common variant at this locus is regulating both, CAD and fibrinogen. The identification of our lead variant, rs10401969, as a significant eQTL for *MAU2* gene in blood, and the identification of this locus in a significant colocalization between CAD, fibrinogen and eQTL data in blood, also suggests the existence of a variant regulating both phenotypes that would take place through the *MAU2* gene expression. *MAU2* codes for MAU2 chromatid cohesion factor homolog and has an important role in loading the cohesion complex to DNA.[56,57] *MAU2* has never been associated to coagulation factor levels.

Fibrinogen levels have been found significantly higher in cases of CAD in epidemiological studies,[58,59] although MR studies have only been able to demonstrate a small causal effect using multi-variant MR approaches.[60,61] Further evidence is needed to clearly elucidate if the effect of this variant on *SUGP1/MAU2* locus on CAD is through fibrinogen levels or if this locus influences both phenotypes in parallel.

4.4 | Liver produced proteins and the *TBL2/MLXIPL* locus

The *TBL2/MLXIPL* locus has been associated with other phenotypes of interest related to lipids metabolism levels (triglycerides, high density lipoprotein, low density lipoprotein) and other liver related proteins levels (alkaline phosphatase, C-reactive protein, gamma glutamyl transferase or alanine aminotransferase),[33] but this is the first time that a variant located on *TBL2* reaches the significance threshold in a GWAS involving hemostatic factors. Also, in a previous candidate gene study for FVII levels, the variant rs7777102, located upstream the *MLXIPL* gene and ~70 kb away rs11974409 ($D' = 0.85$, $R^2 = 0.44$, in 1000G project European population[51]), was found associated to FVII.[51] *TBL2* codes for an endoplasmic reticulum transmembrane protein called transducin (β)-like 2 (TBL2) that, upon ER stress, interacts with PERK (PKR-like ER-resident kinase) and is able to regulate *ATF4* (activation transcription factor 4) translation. It has also been demonstrated that TBL2 has a WD40 (beta-transducin repeat) domain that is essential for the association with mRNA of *ATF4*.[62,63] *MLXIPL* codes for Carbohydrate response element binding protein (ChREBP), a transcription factor highly enriched in the liver with a key role in lipids metabolism. ChREBP has also shown a response for glucose metabolites that change its cellular location and stability and also imply post-translational modifications. ChREBP binds to several proteins that are crucial to induce its nuclear translocation or biding to nuclear receptors.[64] Considering the previous results obtained in a candidate gene studies in FVII,[51] that associated this locus to FVII levels, together with the significant colocalization results between FVII and tPA, and the significant results also of HyPrColoc with several tissues, all suggest that the *TBL2/MLXIPL* locus has a pleiotropic effect on the expression of several hemostatic traits. The colocalization results in tissues suggest *MLXIPL* gene as a good candidate gene, with a common variant in this locus regulating *MLXIPL* expression and splicing in adipose visceral and adipose visceral omentum tissues, FVII levels, and TPA levels at the same time.

4.5 | *MYRF/TMEM258/FADS1/FADS2* and its effect on CV events

MYRF codes for a membrane-associated transcription factor, that participates in the activation of myelin genes and that has been associated to brain development issues.[65,66] Previous GWAS have also associated the genomic region of *MYRF* and *TMEM258* genes to hematologic and lipid metabolism traits.[33] *TMEM258* codes for a protein with two predicted transmembrane domains, with no clear function *in vivo*, that has been associated with endoplasmic reticulum stress when knocked out and as an important regulator of intestinal hemostasis. *TMEM258* has also been described as a potential causal gene of cardiovascular traits and as a regulatory site of abdominal visceral fat.[67,68]

Although not new for VTE and CAD, the identification of the same locus in the multi-phenotype analyses with all 3 CV events and a hemostatic trait supports the idea that the *MYRF/TMEM258/FADS1/FADS2* locus is common regulator. Significant colocalization results between VWF and the cardiovascular outcomes, also support this idea. Our effort to prioritize a causal gene through the colocalization analysis in different tissues, revealed that a common variant at this locus regulates expression of *FADS1* gene in artery tibial and liver and the splicing regulation of *FADS2*, although the colocalization was not significant. *FADS1* and *FADS2* code for members of the fatty acid desaturase gene family that catalyze several steps in the formation of omega-3 and omega-6 fatty acids.[69] The rs174547 variant located on *FADS1*, in high LD with our lead variant rs174528 ($D' = 1$, $R^2 = 0.84$) in the European population of 1000 Genomes project,[70] has been implicated in the risk of suffering multiple CV events, including VTE, CAD and IS,

in a previous MR study.[71] Our results clearly support a regulatory role of this locus on several CV events and suggest the involvement of VWF in the association between *FADS1*/*FADS2* and CV events.

4.6 | Strengths and limitations

This is the first systematic multi-phenotype analysis using summary data for hemostatic traits related to CV events to increase power to detect loci associated with more than one related phenotype. We have leveraged data from the leading consortia worldwide analyzing genetics of hemostatic traits, VTE, CAD, and IS, often providing the largest datasets currently available. We consider this, one of the most major strengths of this work. Moreover, most phenotypes included trans-ethnic population, which give a broader transferability of the results.

We are aware of the existence of several limitations in this work. First, there are notable differences between the sample sizes of the hemostatic traits used, the largest one being fibrinogen (N = 120 246 cases) and the smallest one PAI-1 (N = 19 599), which leads to differences in statistical power between multi-phenotype analyses. Second, we were limited to use summary statistics of mostly European origin to calculate genetic correlations, given the lack of good references in other populations to generate the LD scores, which implies that these results cannot be applied to global populations. LDSC filters out variants with low sample size. For some phenotypes this information was not available, and we used the maximum sample size of the phenotype. This could have created some error. Third, we are also aware that there are limitations associated with the use of GTEx data. This data has limited sample sizes that vary greatly from tissue to tissue. For example, the number of liver samples ($N = 208$) is considerably lower than the samples of tissues such as artery tibial ($N = 584$) or adipose subcutaneous ($N = 581$), which may end up in differences in power to detect associations. The lower numbers of liver samples may have affected our power to detect some of the identified variants as significant eQTL in the liver, and therefore the implication of causal tissues should be interpreted with caution. Finally, we are not providing functional validation of these results. Therefore, further experiments are needed to confirm the implication of the novel suggested loci in disease.

5 | CONCLUSIONS

We have shown that the multi-phenotype analysis of biologically related phenotypes expands discovery of newly associated loci. Using summary GWAS data from hemostasis and CV events, we identified four colocalizing novel loci that were not identified as statistically significant in the source datasets and have not been described in other GWAS of the phenotypes involved. Although our strategy does not allow to unequivocally identify the causal variant or variants at each locus, the colocalization results suggest the existence of common regulatory variants at the newly identified loci.

Some of these loci appear to represent genes that may simultaneously regulate more than one hemostatic trait (horizontal pleiotropy), and some seem to reflect the risk mechanism from a gene to one or more CV events through regulation of a hemostatic factor (vertical pleiotropy),[41] therefore revealing novel biological mechanisms. Both cases of pleiotropy are novel interesting insights that will help understand the pathophysiology of clinical CV events.

ACKNOWLEDGEMENTS

This study is supported in part by the National Heart, Lung, and Blood Institute grants HL134894, HL139553, and HL141291. The views expressed in this manuscript are those of the authors and do not necessarily represent the views of the National Heart, Lung, and Blood Institute; the National Institutes of Health; the Department of Veterans Affairs; the United States Government or the US Department of Health and Human Services. G. Temprano-Sagrera is supported by the *Pla Estratègic de Recerca i Innovació en Salut (PERIS)* grant from the Catalan Department of Health for junior research personnel (SLT017/20/000100). S.M. Damrauer is supported by IK2-CX001780. M. Sabater-Lleal is supported by a *Miguel Servet* contract from the ISCIII Spanish Health Institute (CP17/00142) and co-financed by the European Social Fund.

Data on coronary artery disease has been contributed by CARDIoGRAMplusC4D investigators and have been downloaded from www.CARDIOGRAMPLUSC4D.ORG. We thank the authors P. van der Harst and N. Verwejj who have made available the meta-analysis between CARDIoGRAMplusC4D and UK Biobank data on Mendeley (https://data.mendeley.com/datasets/gbbsrpx6bs/1).

Part of the data used for this research was provided by M. Kubo and is available at the website of the National Bioscience Database Center (NBDC; https://humandbs.biosciencedbc.jp/en/hum0014-v21#cad) of the Japan Science and Technology Agency (JST). We also thank the MEGASTROKE consortium for making the IS data available at https://www.megastroke.org/. The MEGASTROKE project received funding from sources specified at http://www.megastroke.org/acknowledgments.html. Appendix A contains a list of investigators belonging to the CHARGE consortium Hemostasis Working Group that contributed to the hemostatic summary data. Appendix B contains a list of investigators from the INVENT consortium that contributed to the VTE summary data. Appendix C contains a list of investigators from the MEGASTROKE consortium that contributed to the IS summary data.

CONFLICT OF INTEREST

S.M. Damrauer is named as a co-inventor on a government-owned US Patent application related to the use of genetic risk prediction for venous thromboembolic disease filed by the US Department of Veterans Affairs in accordance with Federal regulatory requirements. All other authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

M. Sabater-Lleal, N.L Smith and P.S. de Vries conceived and designed the study and provided access to the data. G. Temprano-Sagrera performed, analyzed, and interpreted the data and drafted the manuscript. C. M. Sitlani, W. P. Bone, and M. Martin-Bornez, analyzed data and contributed to method selection and interpretation. B. F. Voight, A. C. Morrison, S. M. Damrauer, P. S. de Vries N. L. Smith and M. Sabater-Lleal contributed to writing and editing the manuscript. All the authors revised and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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How to cite this article: Temprano-Sagrera G, Sitlani CM, Bone WP, et al. Multi-phenotype analyses of hemostatic traits with cardiovascular events reveal novel genetic associations. *J Thromb Haemost*. 2022;20:1331–1349. doi:10.1111/jth.15698

APPENDIX A

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