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THROMBOSIS AND HEMOSTASIS

A genome-wide association study identifies new loci for factor VII and implicates factor VII in ischemic stroke etiology

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KEY POINTS

- We identify 2 new genetic loci associated with FVII activity and highlight *REEP3* and *JAZF1* as potential underlying causal genes.
- We provide evidence for a causal effect of FVII activity on the risk of IS.

Factor VII (FVII) is an important component of the coagulation cascade. Few genetic loci regulating FVII activity and/or levels have been discovered to date. We conducted a meta-analysis of 9 genome-wide association studies of plasma FVII levels (7 FVII activity and 2 FVII antigen) among 27 495 participants of European and African ancestry. Each study performed ancestry-specific association analyses. Inverse variance weighted meta-analysis was performed within each ancestry group and then combined for a *trans*-ancestry meta-analysis. Our primary analysis included the 7 studies that measured FVII activity, and a secondary analysis included all 9 studies. We provided functional genomic validation for newly identified significant loci by silencing candidate genes in a human liver cell line (HuH7) using small-interfering RNA and then measuring *F7* messenger RNA and FVII protein expression. Lastly, we used meta-analysis results to perform Mendelian randomization analysis to estimate

the causal effect of FVII activity on coronary artery disease, ischemic stroke (IS), and venous thromboembolism. We identified 2 novel (*REEP3* and *JAZF1-AS1*) and 6 known loci associated with FVII activity, explaining 19.0% of the phenotypic variance. Adding FVII antigen data to the meta-analysis did not result in the discovery of further loci. Silencing *REEP3* in HuH7 cells upregulated FVII, whereas silencing *JAZF1* downregulated FVII. Mendelian randomization analyses suggest that FVII activity has a positive causal effect on the risk of IS. Variants at *REEP3* and *JAZF1* contribute to FVII activity by regulating *F7* expression levels. FVII activity appears to contribute to the etiology of IS in the general population. (*Blood*. 2019;133(9):967-977)

Introduction

As the initiator of the extrinsic coagulation pathway, coagulation factor VII (FVII) plays a central role in fibrin formation. Like many coagulation factors, FVII is produced as an inactivate zymogen, and is activated through proteolytic cleavage by other coagulation factors: mainly by FX, and also by thrombin, FIX, and FXII. Once activated FVII binds to tissue factor, its activity greatly increases. The complex of activated FVII and tissue factor activates FX and FIX, which ultimately leads to conversion of prothrombin to thrombin, which converts fibrinogen into fibrin. Plasma levels of FVII are associated with several clinical outcomes. For example, FVII deficiency is a rare bleeding disorder associated with hemorrhagic complications,[1] whereas elevated levels of FVII have been associated in some studies with arterial thrombosis and venous thromboembolism (VTE).[2-5]

FVII activity and levels have a substantial heritable component, with estimates of the heritability of FVII activity ranging from 40% to 52%.[6,7] The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium previously conducted genome-wide association studies (GWASs) with data on over 2 million common single-nucleotide polymorphisms (SNPs) in 15 795 European-ancestry participants, identifying 4 new candidate genes for FVII in addition to the protein-coding locus, *F7*.[8,9] The fact that lead variants at these known loci explain only 7.7% of the variance in FVII suggests that further heritability remains to be uncovered.[8]

To discover additional genetic variants associated with FVII, we performed an expanded GWAS with data on over 10 million common and low-frequency SNPs and insertion-deletions in 27 495 participants across 9 studies, including 3420 African American participants. Gene silencing in a human liver cell line was used to validate the genomic function of significantly associated loci. Lastly, we performed Mendelian randomization analyses to estimate the causal effects of FVII activity on atherosclerotic and thrombotic diseases, including coronary artery disease (CAD), ischemic stroke (IS), and VTE.

Methods

Study design and participating cohorts

This study was organized within the CHARGE Consortium Hemostasis Working Group.[9] Nine studies were included: the Atherosclerosis Risk in Communities (ARIC) study,[10] the Cardiovascular Health Study (CHS),[11] the Coronary Artery Risk Development In young Adults (CARDIA) study,[12] the Genetic Analysis for Idiopathic Thrombophilia 2 (GAIT2) study,[13] the Framingham Heart Study (FHS),[14] the LUdwigshafen RIsk and Cardiovascular Health (LURIC) study,[15] the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study,[16] the Precocious Coronary Artery DISease (PROCARDIS) study,[17] and the Rotterdam Study (RS).[18] Descriptions and ancestry composition of participating cohorts are found in supplemental Methods and supplemental Table 1 (available on the *Blood* Web site). All studies were approved by appropriate research ethics committees and all respondents signed informed consent prior to participation. Seven studies (ARIC, CHS, CARDIA, GAIT2, LURIC, MEGA, RS) including 23 434 participants measured FVII activity (percentage or IU/mL × 100) and 2 studies (FHS, PROCARDIS) including 4061 participants measured FVII antigen (percentage or IU/mL × 100). CHS, FHS, RS, and a subset of ARIC were also included in the discovery analysis for the previous FVII GWAS,[8] whereas PROCARDIS and a second subset of ARIC were included in the replication. CARDIA, GAIT2, LURIC, and MEGA were not included in the previous GWAS.

Genotyping and imputation

All participating cohorts performed genome-wide genotyping using commercial platforms available from Illumina or Affymetrix. Each study performed standard preimputation quality control checks and imputed autosomal and X-chromosome variants from the 1000 Genomes Project (1000G) phase 1 version 3 reference panel using available imputation methods.[19-22] Genotyping, preimputation quality control, and imputation procedures are described in supplemental Table 2.

Cohort-specific association analyses

Natural log-transformed FVII was analyzed within each cohort. Participants with values 3 standard deviations (SDs) above or below the population mean were removed prior to cohort-level analysis and any individuals on anticoagulant therapy were also excluded. Ancestry-stratified, study-specific regression analyses using an additive genetic model were performed between genome-wide 1000G imputed variant dosages and phenotype levels, adjusted for age, sex, ancestry-informative principal components, and study-specific variables, such as center. Analyses of the X-chromosome were stratified by sex, with variants in males coded as 0/2. The covariates used in each of the studies are shown in supplemental Table 2. Quality control assessment of ancestry-specific result files from each study was conducted prior to meta-analysis using the EasyQC software package.[23] Quality control procedures are further described in supplemental Methods.

Trans-ancestry meta-analysis

The discovery *trans*-ancestry meta-analysis was conducted in 2 steps. First, the METAL program was used to perform ancestry-specific inverse-variance weighted meta-analysis.[24] We then used the same method to meta-analyze the ancestry-specific results. As suggested by Huang et al,[25] we adopted a genome-wide significance threshold of $P < 2.5 \times 10^{-8}$. Compared with

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the traditional genome-wide significance threshold of 5×10^{-8}, this stricter threshold additionally corrects for the low-frequency variants that were not included in the initial generation of GWASs.[26] Finally, a locus was defined as ±1 Mb from the variant with the lowest *P* value.

To reduce heterogeneity, the primary *trans*-ancestry meta-analysis included the 7 studies that measured FVII activity, and not the 2 studies (FHS and PROCARDIS) that measured FVII antigen. In a secondary meta-analysis, we added results from the 2 studies that measured FVII antigen.

Postdiscovery analyses

Newly identified loci were validated and characterized by using small interfering RNA (siRNA) to silence candidate genes in human liver HuH7 cells, and measuring F7 messenger RNA (mRNA) levels and release of FVII protein levels. We based the design of these functional validation experiments on 2 assumptions: (1) that functional mechanisms underlying the genetic associations at new loci involve the candidate genes we selected and (2) that the functional mechanisms underlying the genetic associations involve FVII synthesis or release, as opposed to FVII activation or clearance. The methods used in these experiments are described in detail in supplemental Methods. As a positive control, we also silenced the *F7* gene itself and measured the effect on *F7* mRNA levels.

To identify additional independent signals at the associated loci, an approximate method implemented in the Genome-wide Complex Trait Analysis (GCTA) software was used for conditional and joint analysis using meta-analysis summary statistics from the *trans*-ancestry meta-analysis of FVII activity.[27,28] Further details on the conditional analysis are provided in supplemental Methods.

Mendelian randomization is an approach that uses genetic variants associated with an exposure as an instrument to examine the causal effect of the exposure on an outcome. Although direct examination of the exposure-outcome association might be impeded by confounding or reverse causation, genetic instruments are less likely to be affected by these issues, allowing for causal inference. Mendelian randomization analyses were used to investigate the potential causal effect of FVII activity on CAD, IS, and VTE. We used 2-sample methods that rely on summary statistics (β coefficients with standard errors from GWASs).[29] We used summary statistics from the *trans*-ancestry meta-analysis of FVII activity, and obtained summary statistics for CAD from the CARDIoGRAMplusC4D Consortium (http://www.cardiogramplusc4d.org/data-downloads/),[30] summary statistics for IS from the MEGASTROKE Consortium,[31] and summary statistics for VTE from the International Network against VENous Thrombosis (INVENT) Consortium.[32] The methods used to perform Mendelian randomization can be found in supplemental Methods. In brief, we used 4 techniques to obtain causal-effect estimates based on the lead variants at the genome-wide significant loci: (1) inverse-variance weighted meta-analysis (primary analysis), (2) MR-Egger,[33] (3) weighted median estimator,[34] and (4) restriction of the analysis to the lead variant at the *F7* locus. Given that the lead variant at the *F7* locus is located in the gene that encodes the FVII protein, it is less likely to have clinical effects through pathways that are not directly mediated by FVII.[35]

Results

Baseline characteristics

In total, 20 014 European-ancestry and 3420 African-ancestry subjects from 7 studies were included in the meta-analysis of FVII activity and an additional 4061 European-ancestry subjects were included in the combined meta-analysis of FVII activity and antigen. Baseline characteristics of participants in each of the studies included in the GWAS are shown in supplemental Table 1. The mean age across the studies was 57.2 years, and 52.2% of the participants were women.

Trans-ancestry meta-analysis

After quality control, 10 044 948 variants across the autosomal and X chromosomes were examined in the *trans*-ancestry meta-analysis of FVII activity. Of these variants, 9 316 598 were SNPs and 728 350 were insertions-deletions. The genomic inflation factors that were used to apply genomic control correction to each of the included studies were all <1.05 and are shown in supplemental Table 2. A QQ plot and Manhattan plot are shown in supplemental Figures 1 and 2, respectively.

Genome-wide significant results are presented in Table 1. Briefly, 1637 variants located in 8 loci exceeded the genome-wide significance level of $P < 2.5 \times 10^{-8}$. Among the associated regions, loci containing *F7*, *PROCR*, *GCKR*, *MS4A6A*, *ADH4*, and *TSKU* represented previously described loci (supplemental Figures 3),[8] whereas 2 loci were novel: *REEP3* and *JAZF1-AS1*. The most significant variant at the *REEP3* locus was an intronic variant, rs10761784 (β = 0.013; $P = 6.7 \times 10^{-10}$) in *REEP3* (supplemental Figure 4). At the second novel locus, the lead variant, rs498475, was located within the antisense RNA *JAZF1-AS1* (β = 0.012; $P = 1.5 \times 10^{-8}$; supplemental Figure 4). Lead variants at *PROCR* and *GCKR* were identical to previously reported lead variants, whereas the lead variants at the remaining known loci differed from previously reported lead variants (supplemental Table 3). Forest plots showing study-specific results for known and novel loci are provided in supplemental Figure 5. In addition to these genome-wide significant findings, variants at a further 15 loci were suggestively associated ($P < 2.5 \times 10^{-6}$) with FVII activity (supplemental Table 4).

Ancestry-specific results for FVII activity are presented in supplemental Table 5. The European-specific meta-analysis identified genome-wide significant associations at 7 of 8 loci (all but *JAZF1-AS1*). On the other hand, the African-specific meta-analysis only identified a genome-wide significant association at the *F7* locus itself. Ancestry-specific Manhattan plots are shown in supplemental Figures 6 and 7, whereas ancestry-specific regional plots are shown in supplemental Figure 8. A comparison of the ancestry-specific betas (effect sizes) of the 8 genome-wide significant lead variants from the *trans*-ancestry analysis is shown in supplemental Figure 9. Overall, effect sizes were very similar across the 2 ancestry groups.

No additional genome-wide significant loci emerged when adding data from 2 additional studies in the combined analysis of FVII activity and antigen, but variants at the *TSKU* and *JAZF1-AS1* loci were no longer genome-wide significant (supplemental Table 6). A QQ plot and Manhattan plot for the combined analysis of FVII activity and antigen are shown in supplemental Figures 10 and 11, respectively. The lead variants at *TSKU* and

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Table 1. Lead variants at loci associated with FVII activity when excluding studies that measured FVII antigen from the *trans*-ancestry meta-analysis

Variant rsID	Chr:Pos*	Alleles†	Freq‡	Freq EA§	Freq AA‖	β	SE	*P*	Variance explained, %	Closest gene	Annotation	Discovery status
rs569557	13:113769917	G/A	0.88	0.88	0.88	0.157	0.003	6.4×10^{-600}	13.9	*F7*	Intronic	Known
rs867186	20:33764554	G/A	0.10	0.11	0.09	0.057	0.003	3.3×10^{-64}	1.6	*PROCR*	Missense	Known
rs1260326	2:27730940	T/C	0.39	0.41	0.15	0.024	0.002	2.3×10^{-30}	0.7	*GCKR*	Missense	Known
rs7935829	11:59942815	G/A	0.39	0.40	0.21	0.018	0.002	6.3×10^{-18}	0.4	*MS4A6A*	Intronic	Known
rs6532796	4:100042242	G/A	0.70	0.69	0.83	0.016	0.002	2.6×10^{-13}	0.3	*ADH4*	Downstream	Known
rs1149616	11:76498369	T/C	0.17	0.17	0.16	0.017	0.003	1.7×10^{-10}	0.2	*TSKU*	Intronic	Known
rs10761784	10:65308750	A/T	0.53	0.51	0.67	0.013	0.002	6.7×10^{-10}	0.2	*REEP3*	Intronic	Novel
rs498475	7:28256240	G/A	0.42	0.38	0.74	0.012	0.002	1.5×10^{-8}	0.2	*JAZF1-AS1*	ncRNA	Novel

The variance explained shown in this table was calculated using the sample size weighted mean variance of log-transformed FVII and the betas and frequencies from the *trans*-ancestry meta-analysis summary statistics.

AA, African ancestry; Chr, chromosome; EA, European ancestry; Freq, frequency; ncRNA, noncoding RNA; Pos, position; rsID, reference SNP cluster ID; SE, standard error.

*The Chr:Pos column shows the chromosome and position (build 37).

†The Alleles column shows the FVII-increasing allele/FVII-decreasing allele.

‡The Freq column shows the frequency of the FVII-increasing allele.

§The Freq EA column shows the frequency specifically in participants of European ancestry.

‖The Freq AA column shows the frequency specifically in participants of African ancestry.

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Table 2. Conditional analysis results for FVII activity using the *trans*-ancestry meta-analysis results

rsID	Chr:Pos*	Alleles†	Freq‡	β§	*P*§	Joint β‖	Joint *P*‖	Variance explained, %¶
F7								
rs117989138	13:113697671	A/G	0.02	0.086	3.6×10^{-22}	0.081	8.7×10^{-20}	0.6
rs36086577	13:113728498	C/A	0.87	0.035	2.2×10^{-19}	0.031	7.5×10^{-15}	0.6
rs71446935	13:113734376	A/G	0.31	0.035	5.5×10^{-38}	0.032	5.1×10^{-31}	1.2
rs1046205	13:113752057	A/T	0.79	0.121	3.9×10^{-573}	0.121	$<1.0 \times 10^{-320}$	12.1
PROCR								
rs6119569	20:33672371	G/A	0.78	0.022	8.8×10^{-17}	0.019	3.9×10^{-13}	0.3
rs867186	20:33764554	G/A	0.10	0.057	3.3×10^{-64}	0.055	8.1×10^{-59}	1.4

The variance explained shown in this table was calculated using the sample size weighted mean variance of log-transformed FVII and the betas and frequencies from the *trans*-ancestry meta-analysis summary statistics.

Abbreviations are explained in Table 1.

*The Chr:Pos column shows the chromosome and position (build 37).

†The Alleles column shows the FVII-increasing allele/FVII-decreasing allele.

‡The Freq column shows the frequency of the FVII-increasing allele.

§The β and *P* columns are based on the association of each variant in isolation.

‖The Joint β and Joint *P* columns are based on the association of each variant test conditioned on the other variants.

¶The Variance Explained column is based on the joint analysis.

JAZF1-AS1 had opposing-effect directions on FVII activity and antigen, but lead variants at the remaining 6 loci had relatively similar effects on FVII activity and antigen (supplemental Figure 12). The variance in FVII activity explained by the lead variants at the 8 significant loci was 17.6%. The variance explained by each of the lead variants individually is shown in Table 1.

Conditional analysis

Conditional analysis identified 4 independent signals at the *F7* locus as well as 2 independent signals at the *PROCR* locus. The conditional analysis of the *trans*-ancestry meta-analysis of FVII activity is shown in Table 2. Among the independently associated variants at the *F7* locus was a low-frequency variant (minor allele frequency = 0.02) with the second largest effect size discovered in GWASs of FVII thus far (joint β = 0.08; joint $P = 8.7 \times 10^{-20}$). By considering these independent signals, the variance in FVII activity explained by the *F7* locus increased from 13.9% to 15.2%, whereas the variance explained by the *PROCR* locus increased from 1.6% to 1.8%. The total variance explained therefore increased from 17.6% to 19.0%.

Functional validation of novel loci

The s47939 and s37271 silencing siRNAs both suppressed mRNA expression of their target gene, *REEP3*, by 88% compared with the scramble siRNA (negative control). Experiments were repeated 3 times with consistent results, showing that silencing of *REEP3* resulted in upregulation of *F7* mRNA ($P = .0001$ for s47939; $P > .05$ for s37271; Figure 1) and a corresponding increase in FVII protein levels ($P = 9.1 \times 10^{-5}$ for s47939; $P = .0003$ for s37271; Figure 1).

At the *JAZF1-AS1* locus, we targeted the *JAZF1* gene for silencing rather than the antisense RNA in which the lead variant was located. The s225897 silencer reduced mRNA expression of its target gene, *JAZF1*, by 68%, whereas the s48121 silencer reduced *JAZF1* mRNA expression by 75%. As shown in Figure 1, silencing of *JAZF1* resulted in modest downregulation of *F7* mRNA ($P = .02$ for s225897; $P < 2 \times 10^{-6}$ for s48121) and a corresponding decrease in FVII protein expression: silencing experiments showed no effect on FVII protein in the media of cells silenced with s225897, but a significant decrease upon silencing with s48121 ($P = 1.1 \times 10^{-6}$).

As a positive control, we also silenced the *F7* gene itself, which led to an 88% reduction in *F7* mRNA levels (supplemental Figure 13). This demonstrates that our gene-silencing system was able to effectively assess differences in FVII expression.

Mendelian randomization

Figure 2 contains forest plots showing causal-effect estimates of FVII activity on (A) CAD, (B) IS, and (C) VTE. Causal effect estimates are given as odds ratios (ORs) per 1 unit higher natural log-transformed FVII activity (percentage or IU/mL × 100). The variant at the *PROCR* locus, rs867186, was removed from all analyses due to outlying causal -effect estimates for CAD, IS, and VTE. No heterogeneity in the single-variant causal-effect estimates was observed among the remaining variants for each outcome ($P_{heterogeneity} > .05$).

All outcomes had positive point estimates for the causal effect across all main and sensitivity analyses. A significant causal effect of FVII activity on IS was detected using the inverse-variance weighted approach (OR = 1.37; 95% confidence interval [CI] = 1.14-1.65). Given that the SD of natural-log-transformed FVII activity ranged from 0.18 to 0.26 across our studies, the causal-effect estimate corresponds to an approximate OR of 1.06 to 1.09 per SD change in natural log-transformed FVII activity. Results were consistent across sensitivity analyses, including the use of MR-Egger, the weighted median estimator, and restriction of the analysis to the rs569557 variant at the *F7* locus. These findings indicate that pleiotropy is unlikely to have biased the causal estimate. Causal-effect estimates for CAD (OR = 1.14; 95% CI = 0.97-1.34) and VTE (OR = 1.22; 95% CI = 0.80-1.85) using the inverse-variance weighted approach were more modest and had wider CIs. Nevertheless, the magnitude of these

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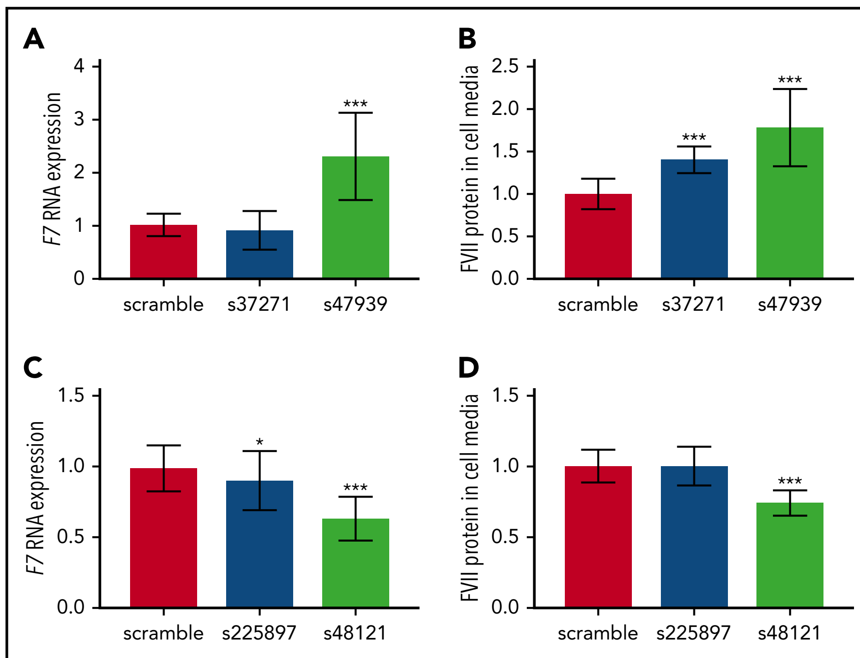

Figure 1. Gene silencing in HuH7 cells. (A) *F7* RNA expression after silencing *REEP3*, (B) FVII protein levels in cell media after silencing *REEP3*, (C) *F7* RNA expression after silencing *JAZF1*, and (D) FVII protein levels in cell media after silencing *JAZF1*. *P = .01-.05; ***$P < .001$. Error bars indicate ±1 SD.

effect estimates was consistent across sensitivity analyses, including when the rs569557 variant at the *F7* locus was examined in isolation (OR_{CAD} = 1.14; 95% CI = 0.96-1.36; OR_{VTE} = 1.31; 95% CI = 0.84-2.07).

Discussion

In this GWAS of circulating FVII levels, we identified the 6 previously known FVII loci as well as 2 new loci: *REEP3* and *JAZF1-AS1*. In total, the 8 loci associated with FVII activity explained 19.0% of the variance. For each new discovery, we showed functional impact in vitro of candidate genes on *F7* mRNA and FVII protein expression: *REEP3* gene silencing in liver cells increased *F7* mRNA and FVII protein expression, whereas *JAZF1* gene silencing decreased *F7* mRNA and FVII protein expression. Mendelian randomization analyses suggest that FVII activity has a positive causal effect on the risk of IS.

Annotation of associated loci

REEP3 encodes receptor accessory protein 3. Although this protein has not been widely studied, there is evidence that an absence of this protein leads to defects in mitosis and a proliferation of intranuclear membranes derived from the nuclear envelope.[36] The locus containing *REEP3* has been previously associated to several other coagulation phenotypes, namely circulating fibrinogen levels,[37,38] mean platelet volume,[39,40] and platelet aggregation,[41] as well as to liver enzyme concentrations.[42,43] For many of these phenotypes, the gene that was reported is not *REEP3* but *JMJD1C*, with missense variants localized in the *JMJD1C* being associated with mean platelet volume.[39] Functional studies in zebrafish indicate that *JMJD1C* has a major role in hematopoiesis.[44] Although we did not examine the consequences of *JMJD1C* silencing on *F7* expression and FVII release, our experiments implicate *REEP3* as a causal gene for FVII. These results are consistent with tissue-specific pleiotropic effects at this locus, with *JMJD1C* being involved in hematopoiesis and *REEP3* being of functional relevance in the liver, although further research is needed to confirm this hypothesis.

JAZF1-AS1 is a noncoding antisense RNA that may regulate the adjacent *JAZF1* gene, which encodes a transcriptional repressor. We targeted *JAZF1* in our gene-silencing experiments instead of *JAZF1-AS1* because many antisense noncoding RNAs regulate the protein-coding gene that they are closest to. Silencing of *JAZF1* in liver cells resulted in lower *F7* mRNA and FVII protein expression, suggesting that the mechanism underlying the genetic association is likely to involve FVII levels. However, variants at the *JAZF1-AS1* locus were associated with FVII activity, and their effect on FVII was attenuated when we included studies that measured FVII antigen. A possible explanation is that variants and this locus have an additional effect on FVII activity that is independent of FVII protein levels.

Apart from *REEP3* and *JAZF1-AS1*, we identified 6 known loci: *F7*, *PROCR*, *GCKR*, *MS4A6A*, *ADH4*, and *TSKU*. The results of this study may aid in the identification of causal variants at these loci. For example, lead variants in *PROCR* and *GCKR* were both missense variants leading to amino acid substitutions (Ser219Gly in *PROCR* and Pro446Leu in *GCKR*). These variants were also the lead variants in their respective loci in the previous GWAS of FVII,[8] and have been associated with other hemostatic phenotypes.[45,46] In contrast, the lead variants that we identified at the *F7*, *MS4A6A*, *ADH4*, and *TSKU* loci differ from those published in the previous GWAS and may be in stronger linkage disequilibrium with the true causal variant.[8]

We also identified 15 additional loci that were suggestively associated with FVII activity ($P < 2.5 \times 10^{-6}$). These loci harbor several notable candidate genes, including *MLXIPL*, *HNF4A*, and *XXYLT1*. The first 2 genes have been previously found to be associated with FVII using a candidate gene design.[47] *XXYLT1* encodes xyloside xylosyltransferase 1, which elongates *O*-linked glycans by adding the second xylose to *O*-glucose-modified residues in the epidermal growth factor repeats of proteins. FVII has epidermal growth factor repeats that are known to be *O*-linked glycosylated.[48]

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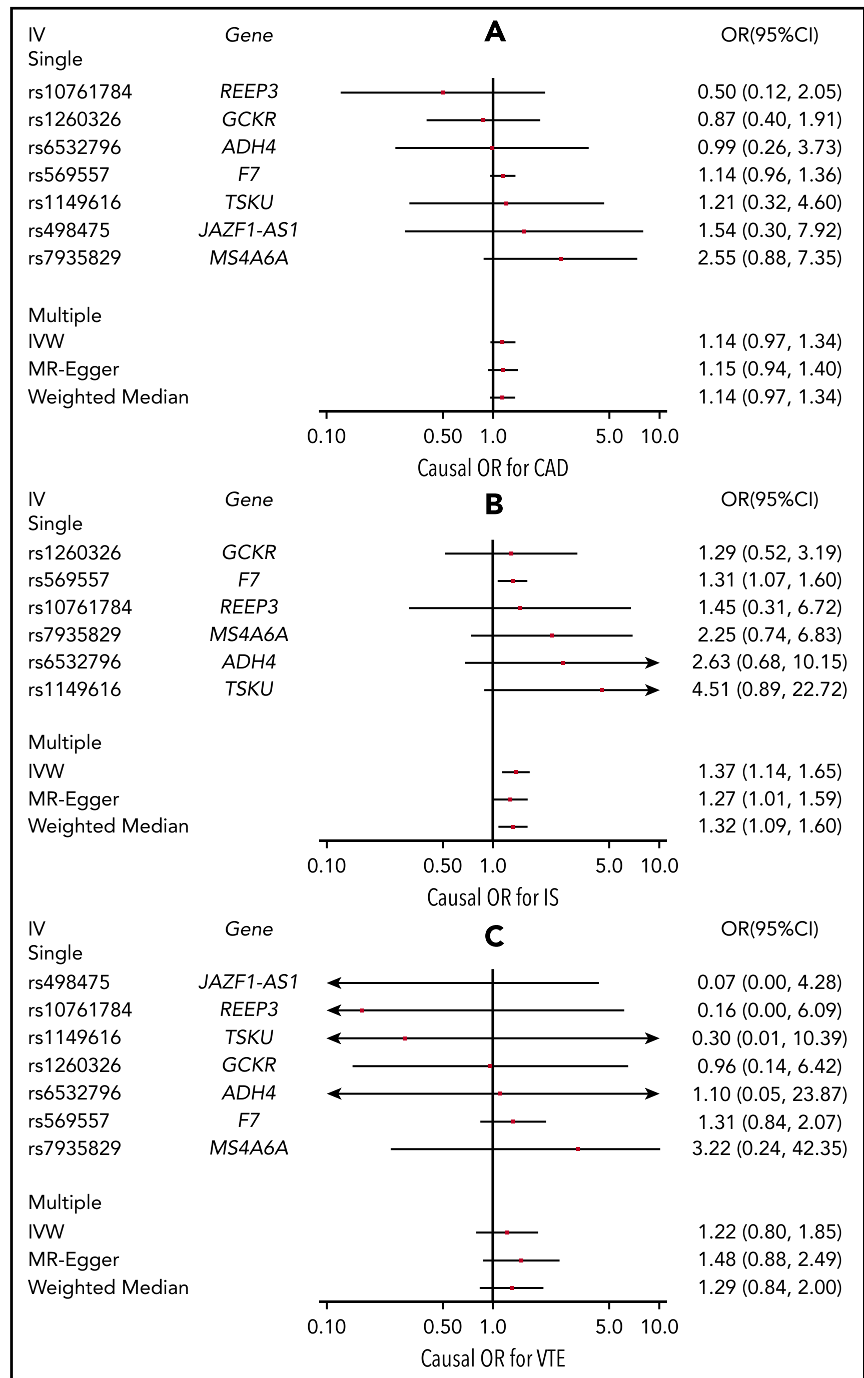
Figure 2. Causal-effect estimates. Causal-effect estimates of FVII activity on CAD (A), IS (B), and VTE (C) using Mendelian randomization. Causal-effect estimates are shown as ORs and 95% CIs per 1 unit higher natural log-transformed FVII activity (percentage or IU/ml × 100) FVII activity. Causal estimates based on single variant ("Single" in plot title) instrumental variables (IVs) are shown, as well as causal estimates based on the combination of these variants ("Multiple" in plot title) using inverse variance weighted (IVW) meta-analysis, MR-Egger, and weighted median estimation.



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Mendelian randomization

Using the genetic-association results generated in this study, we performed Mendelian randomization analyses to estimate the causal effect of FVII activity on CAD, IS, and VTE. These analyses suggest that lifetime elevations in FVII activity influence the risk of IS in the general population. These results warrant further etiological research on the role of FVII in IS, as well as translational research on potential clinical applications involving FVII. Lifetime differences in FVII activity driven by genetic variants may have greater effects on outcomes than transient modifications made later in life. Therefore, even if a causal effect is confirmed, clinical research is needed to quantify the effect of specific interventions targeting FVII.[49,50] Potential clinical applications that should be investigated include the reduction of FVII activity through lifestyle or pharmaceutical interventions for the prevention of IS. Our results also provide independent support for the restriction of off-label use of recombinant FVII, which trials have found to increase the risk of arterial thrombosis.[51-53]

For CAD and VTE, the point estimates of the causal effects were smaller than for IS and the CIs were wider. Although not statistically significant, these results do not exclude the possibility of additional causal effects of FVII activity on CAD and VTE. In fact, when using the rs569557 variant at the *F7* locus in isolation as an instrumental variable, the point estimate of the causal effect on VTE was similar to, but less precise than, the estimate of the

causal effect on IS. Lower statistical power for the Mendelian randomization analysis of VTE may explain the lack of a statistically significant causal effect of FVII activity on this outcome: the GWAS from which we obtained the effect of the variants on IS was composed of 60 341 cases and 454 450 controls,[31] whereas the GWAS on VTE consisted of 7507 cases and 52 632 controls.[32] Our results are thus consistent with positive and potentially clinically important causal effects of FVII activity on CAD and VTE. Further Mendelian randomization studies with increased sample sizes are warranted given the wide CIs.

Strengths and limitations

Our GWAS included 27 495 participants, a 74% increase in sample size as compared with the largest previous GWAS of FVII levels.[8] Other strengths include insights into causal effects and disease etiology generated by functional validation of newly identified loci, through silencing candidate genes in human liver cell lines, and the use of Mendelian randomization. However, these approaches also have limitations. In the gene-silencing experiments, we silenced a single gene at each locus. Because an effect of FVII levels was observed in both cases, we did not pursue further experiments involving other genes at these loci. As such, we cannot exclude the possibility that other genes at these loci also influence FVII. However, our results provide a basis for the additional research that will be required to fully uncover the functional mechanisms linking these loci to FVII. Mendelian randomization analyses can be potentially biased by pleiotropic effects. To minimize the impact of pleiotropy on our results, we validated observed causal effects using MR-Egger, weighted median estimation, and restricting the Mendelian randomization analysis to the rs569557 variant at the *F7* locus, each of which estimate causal effects using independent methodologies robust to bias from pleiotropy.[33,34] The estimates of the causal effect of FVII activity on IS, CAD, and VTE were consistent across these sensitivity analyses. However, there is a degree of sample overlap between our GWAS of FVII and the GWAS of IS, CAD, and VTE, which may bias the effect estimates away from the null.[54] At the same time, differences in the ancestral composition of the samples used to generate summary statistics for FVII activity, CAD, IS, and VTE may bias the effect estimates toward the null. Finally, we imputed genotypes using the 1000 Genomes Project reference panel. Compared with previous genetic-association studies on FVII, we therefore have improved coverage and more accurate determination of variants across the allele frequency spectrum.[55] Nevertheless, the coverage of low-frequency and rare variants could be improved even further through the use of whole-genome sequencing instead of imputation genotypes. Thus, sequencing-based studies might identify further associations missed by our study, especially those involving rare variants.

In conclusion, this study identifies 2 novel loci associated with FVII activity, and functional studies suggest that *REEP3* and *JAZF1* are causal genes within these loci. Mendelian randomization analyses indicate that FVII activity is causally involved in the development of IS, while not excluding similar causal effects on CAD and VTE.

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Authorship

Contribution: P.S.d.V., M.S.-L., J.E.H., J.M., C.S., A.D.J., A.C.M., C.J.O., and N.L.S. contributed to the study design and conception, and to the drafting of the manuscript; M.S.-L., H.G.d.H., A.M.-P., M.P.M.d.M., M. Frånberg, M.E.K., F.R., J.M.S., W.T., G.H.T., A.G.U., A.v.H.V., S.S., E.B., A.-K.G., M.K.I., S.J.K., B.M.P., A.P.R., M.S., K.D.T., M. Fornage, A.H., W.M., F.R.R., J.C.S., A.D.J., A.C.M., and C.J.O. contributed to data collection and processing; P.S.d.V., M.S.-L., J.E.H., J.M., C.S., N.P., T.M.B., H.G.d.H., G.E.D., J.D.E., A.M.-P., C.K.W.-C., J.A.B., M.-H.C., D.G., M.E.K., N.M.D., B.M., A.D., and N.L.S. contributed to data analysis and interpretation; M.S.-L. conducted the gene-silencing experiments; and all authors critically reviewed and approved the manuscript.

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Footnotes

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