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FGL1 as a modulator of plasma D-dimer levels: exome-wide marker analysis of plasma tPA, PAI-1 and D-dimer

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Abstract

Background: Use of targeted exome-arrays with common, rare variants and functionally enriched variation has led to discovery of new genes contributing to population variation in risk factors. Plasminogen activator-inhibitor 1 (PAI-1), tissue plasminogen activator (tPA), and the plasma product D-dimer are important components of the fibrinolytic system. There have been few large-scale genome-wide or exome-wide studies of PAI-1, tPA and D-dimer.

Objectives: We sought to discover new genetic loci contributing to variation in these traits using an exome-array approach.

Methods: Cohort level analyses and fixed effects meta-analyses of PAI-1 (n = 15,603), tPA (n = 6,876) and D-dimer (n = 19,306) from 12 cohorts of European ancestry with diverse study design were conducted, and included both single variant analyses and gene-based burden testing.

Results: Five variants located in *NME7*, *FGL1* and the fibrinogen locus, all associated with D-dimer levels, achieved genome-wide significance ($P < 5 \times 10^{-8}$). Replication was sought for these 5 variants, as well as 45 well- imputed variants with $P < 1 \times 10^{-4}$ in the discovery using an independent cohort. Replication was observed for 3 out of the 5 significant associations, including a novel and uncommon (0.013 allele frequency) coding variant p.Trp256Leu in *FGL1* (Fibrinogen Like 1) with increased plasma D-dimer levels. Additionally, a candidate gene approach revealed a suggestive association for a coding variant (rs143202684-C) in *SERPINB2*, and suggestive associations with consistent effect in the replication analysis include an intronic variant (rs11057830-A) in *SCARB1* associated with increased D-dimer levels.

Conclusion: This work provides new evidence for a role of *FGL1* in hemostasis.

Keywords

Computational Biology; Exome; Fibrinogen; Fibrinolysis; Genetic Association Study

Introduction

The use of targeted gene arrays with rare variants and functionally-enhanced variation has led to the discovery of new genetic loci contributing to population variation in risk factors including lipids, blood pressure, hematology traits including platelet, red cell and white cell measurements, clotting factors, and platelet aggregation.[1-6] Fibrin D-dimer, tissue plasminogen activator (tPA) and plasminogen activator-inhibitor 1 (PAI-1) are important biomarkers and regulators of hemostasis. Plasma PAI-1 degrades tPA, as well as urinary plasminogen activator, and inhibits the conversion of plasminogen to plasmin, thus inhibiting downstream fibrinolysis. Levels and activity of PAI-1 are causally linked to risk of coronary artery disease (CAD), as demonstrated by Mendelian Randomization analysis.[7] Due to its ability to potently activate fibrinolysis, tPA is an effective treatment when administered soon after stroke events.[8] As the major byproduct of fibrinolysis, plasma

D-dimer level reflects fibrin formation and reactive fibrinolysis. Higher D-dimer is a risk factor for venous thromboembolism (VTE), stroke and coronary artery disease.[9]

Given their importance as biomarkers and regulators of clot formation and degradation, deciphering the genetic architecture of these traits may have clinical relevance and may help improve our understanding of fibrinolytic and clotting mechanisms. However, there are few large-scale, population-based genome-wide or exome-wide studies of plasma levels of PAI-1, tPA and D-dimer. These previous works identified 1p21.3 (upstream of *F3*), 1q24.2 (encompassing *F5* and *NME7*) and 4q32.1 (fibrinogen locus, between *FGG* and *FGA*) associated with D-dimer levels,[12] 7q22.1 (*SERPINE1* promoter and near *MUC3A*) and 11p15.3 (within *ARNTL*) associated with PAI-1 levels,[10] and 6q24.3 (within *STXBP5*), 8p11.21 (*POLB-PLAT* locus) and 12q24.33 (within *STX2*) associated with tPA levels.[11] Here, we leveraged an exome-wide variant array designed to capture an enriched portion of functional and rare variation to find new genetic determinants of PAI-1, tPA, and D-dimer.

Methods

This project derives from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Hemostasis Working group and involves participants from twelve cohorts of European ancestry (ARIC, CHS, FHS, GABC, GeneSTAR, HABC, Inter99, LURIC, MARTHA, MESA, PROCARDIS and SCARF).[13] Plasma levels (ng/mL or IU/mL) of D-dimer were measured in 7 studies (N = 19,306), tPA in 7 studies (N = 6,876) and PAI-1 in 11 studies (N = 15,603). Participants using anticoagulant therapy at the time of phlebotomy were excluded. A description of each cohort is given in Supplementary Table 1 and Supplementary Methods. All studies were approved by their respective institutional review board and participants provided informed consent.

Genotypes were assayed using the Illumina HumanExome Beadchip v1.0 or v1.2 (Illumina, Inc., San Diego, CA) in accordance with the manufacturer's instructions. Single nucleotide polymorphism (SNP) calling and quality control procedures were conducted by each study following a common protocol, which has been described previously.[1,14]

Each study performed statistical analyses independently following a common protocol. Phenotype measurements were log transformed and analyses were adjusted for age, sex, principal components (PCs) derived from genotypes and study-design variables. PCs were selected for adjustment if they were significantly associated with the trait analyzed in an age and sex adjusted model. Sex-stratified analyses were also performed for all cohorts except Inter99, and adjusted for age, PCs and study-specific variables. Both single SNP and multiple SNP (gene level) association analyses were conducted with the seqMeta R library (https://github.com/DavisBrian/seqMeta). The results of individual studies were combined using an inverse variance weighted fixed-effect meta-analysis with seqMeta. Conditional analyses were conducted with GCTA-cojo,[15] and linkage disequilibrium estimation was performed with PLINK[16] in the FHS cohort.

For the single SNP analysis, only variants with a minimal allele count greater than 5 across cohorts were interrogated. A total of 101,541 SNPs were considered for association

with D-dimer levels, 95,138 SNPs with PAI-1 levels and 68,725 SNPs with tPA levels. We used both an agnostic and candidate-gene approach involving genes related to the coagulation pathway referenced by the KEGG pathway hsa04610 (Supplemental Table 2). For the agnostic approach, the threshold for significance was set using the Bonferroni method at $P < 1.88 \times 10^{-7}$ (0.05 / 265,404). A replication step to validate the results was performed in the Caerphilly Prospective Study (CaPS),[17] composed of European males, with genotypes imputed using the HRC 1.1 dataset.[18] Both significant and suggestive ($P < 1 \times 10^{-4}$) associations from the discovery meta-analysis were tested in CaPS with a one-sided hypothesis, with a threshold for significance at nominal p-value (0.05).

However, single variants tests lack power to identify associations of rare variants, which constitute a large part of the Exome chip. To assess the effect of these rare variants, we performed gene-based tests, which allow for each gene to test the joint effect of rare variants contained in each gene. Two distinct methods were applied: Sequence Kernel Association Test (SKAT)[19] and the classical burden test.[20] For both tests, the joint effect of variants with minimal allele frequency (MAF) < 0.05 were considered. Only genes with > 1 SNP were tested. For each trait, about 15,000 genes were considered for these analyses, and the threshold for significance was set at $P < 1.09 \times 10^{-6}$ (0.05 / 45,833).

Results from all single-variant and gene-based analyses are publicly available on the GRASP portal (https://grasp.nhlbi.nih.gov/FullResults.aspx).

Results

Single variant analyses

Manhattan and QQ plots representing the results of the discovery meta-analysis of single-SNP associations are provided for D-dimer (Supplementary Figure 1-2), PAI-1 (Supplementary Figure 3-4), and tPA (Supplementary Figure 5-6). No single variant exceeded the threshold of genome-wide significance for tPA or PAI-1 plasma levels. The single-SNP analysis of D-dimer revealed 5 genome-wide significant associations at 3 distinct regions: *FGL1, NME7* and the fibrinogen coding loci (encompassing *FGG*, *FGA* and *FGB*) (Table 1). At the *FGL1* locus, two missense variants rs2653414-A (p.Trp256Leu, Minor Allele Frequency (MAF) = 0.013, $\beta = 0.21$, $P = 3.93 \times 10^{-11}$) and rs3739406-T (p.Ile72Val, MAF = 0.32, $\beta = 0.05$, $P = 3.71 \times 10^{-9}$) were associated with higher D-dimer levels. The 2 *FGL1* variants were in partial linkage disequilibrium ($r^2 = 0.02$; D' = 1.0), but after conditioning the analysis on rs2653414, the association of rs3739406 with D-dimer levels remained high ($p = 9.40 \times 10^{-7}$), implying independent associations. The phenotypic variance explained by rs2653414 and rs3739406 is 0.23% and 0.18%, respectively. The associations observed at *NME7* (rs16861990-C, MAF = 0.070, $\beta = 0.12$, $P = 1.17 \times 10^{-11}$) and upstream of *FGA* (rs13109457-A, MAF = 0.25, $\beta = 0.05$, $P = 1.24 \times 10^{-7}$) were previously described in a GWAS of plasma D-dimer levels,[12] while the *FGG* missense variant (rs148685782-C, p.Ala108Gly, MAF = 0.004, $\beta = -0.38$, $P = 6.75 \times 10^{-11}$) was previously associated with fibrinogen level.[21]

We then sought to replicate the significant associations from the discovery meta-analysis in CaPS. The results from the replication analysis are presented in Table 1. We observed

a replication for 3 out of the 5 significant associations with D-dimer levels, one at each locus: rs16861990 (β = 0.13, P = 0.001) in *NME7*, rs2653414 (β = 0.23, P = 0.04) in *FGL1* and rs13109457 (β = 0.07, P = 0.001) at the fibrinogen locus, upstream of *FGA*. Additionally, we investigated all suggestive associations ($P < 1 \times 10^{-4}$) with D-dimer, tPA or PAI-1 levels from the discovery analysis in CaPS. Of the 79 variants suggestively associated in the discovery, 45 were available in CaPS. We observed directionally consistent results for 3 associations, one with tPA levels and a missense variant in *MTFR1L* (rs201393961, p.Thr83Met), and 2 with D-dimer levels: an intronic *SCARB1* variant (rs11057830), and rs7681423 upstream of *FGG* (Table 1). These variants all had high imputation quality (RSQ > 0.9), except for the *MTFR1L* variant which had moderate quality (RSQ = 0.68).

All significant and suggestive associations from the discovery analysis as well as the results of the replication analysis are provided in Supplementary Table 3. As D-dimer levels can be related to thrombotic events, we also inspected the association of the novel replicated variant (*FGL1* rs2653414) and other novel variants of interest (*FGL1* rs3739406, *SCARB1* rs11057830) with VTE risk in the INVENT GWAS dataset,[22] but none of these variants were found associated (Supplementary Table 4).

Single variant analyses restricted to candidate genes

We also applied a candidate gene approach to retrieve associations implicating genes involved in the coagulation pathway (as listed in the Supplementary Table 2) that did not meet the exome-wide single SNP significance threshold. This approach revealed 2 missense variants suggestively associated with D-dimer: rs201909029-C (p.Lys178Asn, MAF = 0.007, $\beta = -0.72$, $P = 1.25 \times 10^{-6}$) located in *FGB* at the fibrinogen locus, not previously associated with D-dimer or fibrinogen, and rs143202684-C (p.Gly218Ala, MAF = 0.001, $\beta = -0.41$, $P = 8.10 \times 10^{-5}$) located in *SERPINB2*, which encodes the PAI-2 protein. The poor imputation quality of these two rare variants (RSQ < 0.1) prevented our effort to investigate these suggestive associations further in CaPS.

Gene-based burden analyses

The gene-based analysis revealed 2 genes significantly associated with plasma D-dimer: *FGL1* and *FGG*. The results of these 2 associations were similar for both SKAT and T5 methods, and were mainly driven by the variants associated with D-dimer in the single-SNP analysis (the detail of single variant associations involved in both *FGL1* and *FGG* gene-based tests is given in Supplementary Table 5). For each method and for each trait analyzed, the results of the 3 most significant gene-based associations are presented in Table 2, while the results of all associations with P < 0.0001 are provided in Supplementary Table 6.

Sex-stratified analyses

As previous studies have reported that genetic associations with hemostatic factors can differ between males and females, we conducted sex-stratified analyses of the 3 traits.[23] The single variant analyses yielded 2 significant associations: one between D-dimer levels in women and the *FGG* variant rs148685782, previously identified in the main analysis, and one between tPA levels in women and a rare missense variant in *KIAA1432* (rs143886234-G, p.Pro443Arg, MAF = 0.001, MAC=6, β = 1.53, $P = 3.14 \times 10^{-8}$). However, with only 6

minor alleles total were detected in 3 out of the 6 studies with sex-stratified D-dimer results, so this signal could be a false positive, and we were unable to replicate this signal, as CaPS is only composed of men. We also retrieved the sex specific effects for all associations identified in the main analyses. The associations at *NME7/F5 region*, *FGG*, *FGL1*, *FGA*, *SCARB1* and *SERPINB2* all reached at least nominal significance in both sexes, and no significant difference in effect was observed between sex. The association at *FGB* did not reach nominal significance in men, most likely because of its rare frequency. The detail of these associations is available in Supplementary Table 7.

Gene-based sex-specific analyses also revealed a novel gene, *ENOX2*, associated with D-dimer levels in men, according to the results of the SKAT analysis (Supplementary Table 8). However, after further inspection of the *ENOX2* variants with D-dimer levels in men, only 2 variants with MAF < 0.05 were considered for this test (Supplementary Table 9), and the gene-based association was driven by only one of them: rs200194256 (MAF = 0.0001, MAC = 2, $p = 1.19 \times 10^{-7}$).

FGL1 investigation

As FGL1 possesses a fibrinogen C-terminus domain, we investigated its similarity with the fibrinogen subunits proteins. We observed that the FGL1 C-terminus domain is homologous to the fibrinogen gamma subunit (46% according to Clustal2.1), and the variant whose D-dimer association was replicated in CaPS (rs2653414) is located in a codon encoding a tryptophan amino acid conserved in the fibrinogen subunit (Supplementary Figure 7). The rs2653414 variant has not been previously associated with any phenotype or transcript levels (see annotations in Supplementary Table 3). However, it was recently found associated with decreased levels of the FGL1 protein in serum[24] ($\beta = -1.62$, $P = 4.22 \times 10^{-47}$).

Discussion

In order to discover new functional and rare genetic determinants of plasma tPA, PAI-1 and D-dimer levels, we performed both single- and multi-variant meta-analyses using exome-wide marker genotype data from 12 cohorts. For D-dimer, we identified 3 associations previously observed in genome-wide studies of D-dimer levels or fibrinogen,[12,21] and 2 novel associations of variants in *FGL1*, of which one was replicated in CaPS. The analyses of tPA and PAI-1 levels did not reveal any exome-chip wide significant associations, and overall the sex-stratified analyses did not yield strong evidence supporting different genetic effects in men and women at most of the loci we observed.

FGL1, which encodes the fibrinogen-like 1 protein, is expressed mainly in the liver and can be found circulating in plasma. It has been linked to various biological processes including mitogenic activity in hepatocytes promoting liver growth,[25] acute phase reactant upregulated by IL6 during inflammation,[26] and more recently immunity.[27] A role in coagulation has previously been hypothesized because of its similarity with FGG and FGB C-terminal domains,[28] but was mainly rejected because of its lack of sites necessary for fibrin clot formation. However, subsequent studies reported FGL1 to bind fibrin clots in plasma,[29,30] although it is not known how this occurs. The replicated *FGL1* variant (rs2653414) associated with higher levels of D-dimer has been recently found associated with lower

serum levels of the FGL1 protein in an exome-chip analysis of protein levels,[24] but there is no evidence that transcript levels of FGL1 levels are affected according to eQTL resources such as GTeX (see Supplemental Table 3). This discrepancy could be first explained by an impact of the variant on the protein structure, which could either affect the stability of the protein and reduce its levels, or it could alter the epitope of the protein and affect its detection by the proteomic assay. Additionally, since most eQTL resources are based on GWAS arrays they may lack appropriate coverage for this variant. This conclusion seems consistent with the fact that prior large GWAS studies of D-dimer did not discover an association with *FGL1*. Furthermore, an association of an *FGL1* variant with D-dimer levels was previously observed in an exome study of a Finnish population,[31] where an uncommon insertion causing a frameshift in *FGL1* (rs201941547, p.Asn182fs, MAF = 0.037, $\beta = 0.21$, $P = 6.12 \times 10^{-6}$) was associated with higher D-dimer, but the authors were unable to replicate their results due to a lack of D-dimer phenotype in their replication sample. This frameshift variant most likely implicates a loss of function of FGL1, and the similar increase of D-dimer levels observed in our analysis for a missense variant strongly tied to lower circulating FGL1 levels suggests that impaired FGL1 levels or function may generally result in higher D-dimer. Therefore, while the specific role of this protein in the coagulation process is unclear, the associations of *FGL1* missense variants identified in our study together with the results from the Finnish study[31] provide strong evidence for the implication of this gene in the modulation of D-dimer levels.

Additionally, we observed two suggestive associations with D-dimer which could be of interest if further validated. First, a rare *SERPINB2* missense variant (p.Gly218Ala) was associated with lower D-dimer. However, similar to FGL1, the role of PAI-2 in the coagulation process is not clearly established. Early investigations showed that PAI-2 could act as an inhibitor of urokinase plasminogen activator (uPA) *in vitro* and it was found associated to fibrin clots.[32] More recently a study reported that deep venous thrombosis models of mice lacking *Serpinb2* had increased uPA activity and enhanced venous thrombosis resolution.[33] Second, an intronic *SCARB1* variant was associated with higher D-dimer, which was replicated in CaPS. This gene encodes a scavenger receptor protein of class B, which mediates cholesterol transfer in and out of lipoproteins. This variant was previously associated with risk of coronary artery disease,[34] a condition often having a component of altered fibrinolytic function. Interestingly, *Scarb1*$^{-/-}$ mice had increased risk of venous thrombosis.[35] Furthermore, expressing endothelial Scarb1 protected mice against atherosclerosis, and in an *ApoE4*$^{-/-}$ background decreased aortic lesion size ~24% at 8 months, suggesting roles in lipid metabolism and other biological functions at the level of vessel walls where fibrinolysis also occurs.[36]

Previous genetic analyses of D-dimer, PAI-1 and tPA were conducted on a genome-wide scale using imputed datasets. The use of exome chip data in the present study permitted us to confirm some of these previous findings, and more importantly, it allowed us to focus on new associations involving less common variants that are often absent or poorly imputed in GWAS datasets. However, this also impaired our ability to replicate several associations in CaPS, such as the rare *SERPINB2* variant, and it will be of future interest to replicate these associations in new exome chip or sequencing studies. Furthermore, we observed inter-cohort variability in measurements, due in part to the specificity of

each population studied, and also as a result of the different assays used to measure plasma levels of D-dimer, PAI-1 and tPA by each study. This could reduce our power to detect genetic associations. However, to reduce this variability, measurements were log-transformed, and we systematically verified that the direction of effect for all significant and suggestive associations were concordant across cohorts, which substantiate the validity of these associations. Finally, our study and findings are also limited at this time to European ancestry populations, so it remains to be seen if these loci are observed in other populations.

In conclusion, we were able to replicate a significant association implicating the locus *FGL1* in the modulation of D-dimer levels, and we discovered two suggestive associations of interest at the *SERPINB2* and *SCARB1* loci. Most notably, these results provide additional evidence for a role of *SERPINB2* and *FGL1* in the coagulation system, two genes previously suspected to play a role in hemostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Essentials

- D-dimer, PAI-1 and tPA levels are important biomarkers and regulators of hemostasis
- We performed an Exome-Wide association study of these 3 traits in up to 19,300 individuals
- A novel *FGL1* variant was associated with D-dimer and replicated in an independent cohort
- Our study provides new evidence for a role of FGL1 in hemostasis

Table 1:

Results of the Discovery and replication analyses

				Discovery (meta-analysis)					Replication (CaPS)					
Chr:Position	**dbSNPID**	**Gene**	**EA/ NEA**	**MAF**	**N**	**β**	**SE**	**P[a]**	**MAF**	**β**	**SE**	**P[b]**	**RSQ**	
D-dimer											**(N=1,112)**			
1:169135127	rs16861990	*NME7* (intronic)	C/A	0.070	15733	0.119	0.018	1.17E-11	0.061	0.135	0.044	0.0010	0.98	
8:17726069	rs2653414	*FGL1* (p.Trp256Leu)	A/C	0.013	19306	0.213	0.032	3.93E-11	0.006	0.233	0.133	0.0404	0.97	
4:155533035	rs148685782	*FGG* (p.Ala108Gly)	C/G	0.004	19306	−0.384	0.059	6.75E-11	0.001	−0.112	0.386	0.3854	0.48	
8:17739538	rs3739406	*FGL1* (p.Ile72Val)	T/C	0.325	19306	0.047	0.008	3.71E-09	0.291	0.003	0.023	0.4467	1.00	
4:155514879	rs13109457	3kb 5' of *FGA* (intergenic)	A/G	0.249	18607	0.047	0.009	1.24E-07	0.246	0.072	0.024	0.0012	1.00	
4:155542248	rs7681423	8.3kb 5' of *FGG* (intergenic)	T/C	0.238	18607	0.045	0.009	5.58E-07	0.228	0.072	0.024	0.0013	0.99	*
12:125307053	rs11057830	*SCARB1* (intronic)	A/G	0.158	15733	0.058	0.012	3.62E-06	0.146	0.071	0.030	0.0087	0.98	*
tPA											**(N=1,111)**			
1:26153114	rs201393961	*MTFR1L* (p.Thr83Met)	T/C	0.001	3346	0.676	0.162	3.11E-05	0.001	0.536	0.260	0.0196	0.68	*

EA=Effect Allele; NEA=Non Effect Allele; MAF=Minor Allele Frequency; SE=Standard Error; RSQ=Imputation Quality

[a] In the discovery meta-analysis, the threshold for significant associations was set using the Bonferroni method to 1.88×10^{-7}

[b] In the replication analysis, associations below the nominal p-value were deemed significant under a one-sided hypothesis.

* Suggestive associations from the discovery with same effect direction in CaPS and $p < 0.05$

Table 2:

Most significant results from the gene-based analyses for plasma PAI-1, tPA and D-dimer levels

	T5 (MAF < 0.05)					SKAT (MAF < 0.05)			
Gene	**P**	**β**	**SE**	**Cmaf**	**N_{snp}**	**Gene**	**P**	**Cmaf**	**N_{snp}**
PAI-1 (N=15,063)									
STAT3	9.65E-05	−1.63	0.42	0.0001	3	*STAT3*	5.82E-06	0.0001	3
AKAP11	1.07E-04	−0.08	0.02	0.0451	44	*USP38*	9.89E-06	0.0010	8
KIF1B	3.40E-04	−0.06	0.02	0.0763	24	*GPN3*	2.99E-05	0.0021	3
tPA (N=6,876)									
SH2D6	3.15E-06	−0.73	0.16	0.0009	4	*STX2*	2.96E-05	0.0271	7
CRCP	2.49E-05	−0.49	0.12	0.0011	3	*SH2D6*	3.17E-05	0.0009	4
SGCG	2.91E-05	−0.39	0.09	0.0025	7	*ZBTB41*	4.01E-05	0.0062	9
D-dimer (N=19,306)									
FGG	3.75E-08	−0.19	0.03	0.0120	9	*FGG*	4.47E-09	0.0120	9
FGL1	2.60E-07	0.05	0.01	0.0910	17	*FGL1*	2.86E-08	0.0910	17
EIF2AK3	2.02E-06	−0.22	0.05	0.0069	9	*EIF2AK3*	5.98E-06	0.0069	9

SE=Standard Error; N_{snp}=Number of SNPs used in the gene-based test; Cmaf=Cumulative MAF