Genome Sequencing Unveils a New Regulatory Landscape of Platelet Reactivity

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Abstract

Exaggerated platelet aggregation at the site of vascular injury is the underlying pathophysiology of thrombotic diseases. Here, we conduct the largest whole genome sequencing (WGS) effort to uncover the genetic determinants of platelet aggregation. Leveraging 3,855 NHLBI Trans-Omics for Precision Medicine (TOPMed) individuals deeply phenotyped for platelet aggregation, we identify 18 loci using single-variant approaches. This includes the novel RGS18 locus encoding a myeloerythroid lineage-specific regulator of G-protein signaling that co-localizes with eQTL signatures for RGS18 expression in platelets. A gene-based approach focusing on deleterious coding variants identifies the SVEP1 gene, previously shown to be associated with coronary artery disease, as a novel determinant of platelet aggregation. Finally, in an integrative approach leveraging epigenetic data on megakaryocytes, we find strong association between rare variants mapping to a super enhancer region for PEAR1. This is a novel finding implicating the importance of rare variants with regulatory potential in a previously documented GWAS-identified locus.

Platelet aggregation at the site of atherosclerotic vascular injury is the underlying pathophysiology of myocardial infarction and stroke¹. Identification of the factors that contribute to increased platelet reactivity is critical to understanding the biology of arterial thrombus formation and in the development of novel treatment strategies for patients at risk for pathologic vascular thrombosis. Here, we conduct the largest whole genome sequencing (WGS) effort to uncover the genetic determinants of platelet aggregation by leveraging 3,855 NHLBI Trans-Omics for Precision Medicine (TOPMed) participants deeply phenotyped for platelet aggregation. Using single-variant approaches, we identify 16 independent loci including the novel *RGS18* locus, which encodes a myeloerythroid lineage-specific regulator of G-protein signaling that co-localizes with expression quantitative trait loci (eQTL) signatures for *RGS18* expression in platelets. Additionally, a gene-based approach focusing on deleterious coding variants identifies the *SVEP1* gene, a known contributor of coronary artery disease risk, as a novel determinant of platelet aggregation. Finally, in an integrative approach leveraging epigenetic data on megakaryocytes (MK), we observe strong association between rare variants mapping to a super-enhancer region for *PEAR1* and platelet aggregation. Together, these results unveil novel loci contributing to platelet reactivity, provide insights regarding the mechanism(s) by which genetic variation may influence cardiovascular disease risk, and underscore the importance of utilizing rare variant and regulatory approaches to better understand loci contributing to complex phenotypes.

Previous studies have shown that platelet aggregation in response to agonists is highly heritable²⁻⁶ and prior genome-wide association studies (GWAS) have identified 7 common variants for platelet aggregation in response to different agonists.⁷⁻⁹ Using a WGS approach, we aimed to i) refine previously identified GWAS loci; ii) identify novel loci; iii) examine the burden of deleterious coding variants; and iv) evaluate rare non-coding variants within MK-specific super-enhancer regions for 19 harmonized phenotypic measures of platelet aggregation in response to different doses of adenosine diphosphate (ADP), epinephrine, and collagen (**Supplementary Table 1**).

Genome-wide single variant tests for association were performed on ~28 million variants in 3,125 European Americans (EA) and 730 African Americans (AA) (**Supplementary Table 2**) from the Framingham Heart Study (FHS), Older Order Amish Study (OOA), and the Genetic Study of Atherosclerosis Risk (GeneSTAR). There was no evidence of test statistic inflation using a mega-analysis approach combining all samples (**Supplementary Figure 1**). We identified 101 variants associated with platelet aggregation in response to ADP, epinephrine, or collagen ($P\text{-value} < 5 \times 10^{-8}$, **Figure 1**). Using iterative conditional analyses, genome-wide significant variants were refined down to 16 independent loci (**Table 1**). With the exception of two variants (rs12041331 and chr17:21960955) all of these loci were uniquely associated with platelet aggregation in response to one agonist, and most of the identified loci were novel (**Table 1**, **Supplementary Figure 2**). Replication of discovery findings was performed in up to 2,009

additional samples imputed to the TOPMed reference panel that were not in the discovery analyses (**Supplementary Table 3a**), and extended to the Caerphilly Prospective Study [CaPS] (N=1,183) for ADP and collagen-induced platelet aggregation phenotypes^{8,10} (**Supplementary Table 4**).

Given that all of the 16 loci identified using single-variant approaches are located in non-coding regions of the genome, we tested for co-localization between these regions and eQTL data available through RNA sequencing of platelets in 180 European Americans from GeneSTAR (**Supplementary Table 5**). We found that sentinel variants in the *PEAR1* and *RGS18* loci were eQTLs for *PEAR1* and *RGS18*, respectively. Furthermore, co-localization signatures for *PEAR1* and *RGS18* were identified using Bayesian approaches (**Figure 2**). Previous work demonstrated a single, common (~14% MAF) intronic peak variant in *PEAR1* (rs12041331) is associated with platelet phenotypes using GWAS, regional sequencing, and exonic approaches^{8,11–13}. The minor allele of rs12041331 is linked to decreased *PEAR1* platelet protein levels^{13,14}, potentially through alteration of a methylation site in MKs¹⁵. In addition, the role of this gene in platelet signaling is supported by mechanistic studies^{16,17}. Our co-localization results with platelet eQTLs are consistent with a model that a single, common causal variant explains the platelet reactivity signal. While results of previous association studies on *PEAR1* variants and coronary disease are mixed^{18–21}, UK BioBank²² data supports an association (**Supplementary Table 6**).

In the novel *RGS18* region, several variants were replicated using independent samples imputed with the TOPMed panel (**Supplementary Table 3**), and independent evidence was also found for ADP and collagen aggregation phenotypes in the CaPS study (**Supplementary Table 4**). Overlaying the associated variants with platelet eQTLs and megakaryocytic epigenome features, there are several potential candidate variants (**Supplementary Table 7**). Consistent with our human results, independent *Rgs18*^{-/-} mouse studies suggest *Rgs18* inhibits pre-agonist stimulated platelet reactivity, with knockouts exhibiting exaggerated platelet reactivity to multiple agonist pathways, decreased bleeding times, and increased arterial occlusion^{23,24}. This is attributed to a loss of inhibition of multiple G-protein coupled receptor signaling pathways in platelets²⁵. Notably, variants in *RGS18* identified in our study are also associated with altered risk for cerebral infarction and subarachnoid hemorrhage in the UK BioBank (**Supplementary Table 6**).

SKAT²⁶ gene-based tests using a MAF threshold of 0.05 were conducted for deleterious variants mapping to 17,774 protein-coding genes (**Supplementary Table 8, Supplementary Figure 3**) with significant findings after Bonferroni correction for *SVEP1* (ADP-induced platelet aggregation, P-value=2.6x10⁻⁶), *BCO1* (epinephrine-induced platelet aggregation, P=8.9x10⁻⁷), *NELFA* (collagen-induced platelet aggregation, P=1.7x10⁻⁶) and *IDH3A* (collagen-induced platelet aggregation, P-value=2.6x10⁻⁶). Using a *leave-one-out* analysis, we observed that these associations were driven mainly by single or limited sets of rare variants (**Supplementary Figure 4, Supplementary Table 9**). For example, the *SVEP1* association with ADP-induced platelet

aggregation was solely driven by a nonsynonymous variant (Gly229Arg) in the second exon (rs61751937, MAF 0.028, P-value= 5.8×10^{-6}). This variant alters a highly conserved residue located in the protein's VWFA domain (**Figure 3a-c**). The finding replicated in the WGS imputed cohorts (P-value =0.004) and CaPS (P-value=0.008) demonstrating an association with increased ADP-induced platelet reactivity. Interestingly, a different variant in *SVEP1*, rs111245230, is associated with hypertension and increased cardiovascular risk in an exome study²⁷. This variant is in linkage equilibrium ($r^2=0.0002$; $D'=0.01$) with our peak variant and not associated with ADP-induced platelet aggregation (P=0.06). Both variants are modestly associated with CVD outcomes in the UK BioBank (**Supplementary Table 10**). Collectively, these results suggest that *SVEP1* may have previously unappreciated and multifactorial roles in contributing to CVD. Homozygous *Svep1*^{-/-} mice die from edema, and heterozygous mice, as well as zebrafish, experience arterial and lymphatic vessel malformations²⁸⁻³⁰. Consistent with previous investigations³¹, RNAseq data in a subset of GeneSTAR participants do not indicate expression of *SVEP1* in platelets. The protein is strongly conserved (**Figure 3c**) and could potentially affect platelet function and CVD through several mechanisms including cell-cell adhesion, cell differentiation, and functions in bone marrow niches³².

To investigate the role of genetic variation on regulatory importance in the context of super-enhancers, we aggregated rare non-coding variants across a set of 1,065 published MK-specific super-enhancers (**Supplementary Figure 5**)³³. We found rare non-coding variants in a super-enhancer at the *PEAR1* locus was significantly associated with ADP- (P= 2.4×10^{-8}), epinephrine- (P-value= 1.1×10^{-7}) and collagen- (P-value= 2.7×10^{-5}) induced platelet aggregation. We observed, in marked contrast to our gene-based coding variant analyses, that the association signal in the *PEAR1* super-enhancer is driven by multiple rare variants in the region (**Supplementary Figure 6**).

In this first WGS study of platelet aggregation, we identify and replicate several novel loci contributing to trait variation. Among the 16 identified independent variants, 7 were associated with wide range of blood-related traits including CVD outcomes in a phenome-wide association study using UK Biobank data (**Supplementary Table 6**). While population genetic and genomic studies remain limited in size for platelet function traits, our results indicate the potential for novel discoveries made through sequencing approaches in deeply phenotyped individuals which may ultimately relate to CVD risk and treatment efficacy. This is underlined by data supporting the hypothesis that hyper-reactive platelets may predict future thromboses in both healthy, untreated individuals³⁴ and those who have already experienced thrombosis^{35,36}, as well as the fact that antiplatelet therapy remains standard of care in secondary prevention of atherothrombotic diseases such as myocardial infarction and stroke.

Figure 1: Genome-wide association study results for platelet aggregation in response to epinephrine, ADP and collagen in 3,855 TOPMed participants. P-values presented are a summary across all individual phenotypes for the single agonist (i.e. the minimum P-value for the variants from 8, 7 and 4 individual phenotypes for epinephrine, ADP, and collagen, respectively) (**Supplementary Table 1**). Loci passing genome wide significance (5×10^{-8}) are marked by red dots. Locus names represent the nearest (for novel) or previously annotated (for prior) gene. The blue horizontal line indicates a P-value threshold of 1×10^{-5} corresponding to suggestive significance threshold and the red line indicates a P-value threshold of 5×10^{-8} , corresponding to genome-wide significance.

Figure 2: Co-localization of WGS association signal and platelet eQTL signatures. Top panel shows co-localization between *PEAR1* platelet eQTL signatures and WGS association evidence for platelet aggregation in response to Epi_low1 (see **Supplementary Table 1**) on Chr1:156899922 and bottom panel is between *RGS18* platelet eQTL signatures with WGS association evidence for platelet aggregation in response to Epi_low 5 (see **Supplementary Table 1**) on Chr1:156899922. Region of co-localization is zoomed to peak SNV +/- 25kb, strength of the eQTL evidence for the gene is shown as a heat scale in red, SNVs that were not included in eQTL analysis are shown in gray. Annotations of regulatory elements include ENCODE-aggregated cell model data for both open chromatin (DNase, Red) as well as transcription factor binding clusters (TFBS, Blue) and cell type-specific megakaryocyte distal enhancer elements (Enhancers, Green).

Figure 3a-c: Association of aggregated rare deleterious coding variants in *SVEP1* and ADP-induced platelet aggregation. **3a.** Using a *leave-one-out* approach, we identified a rare coding variant (rs61751937) that explains most of the association. First orange dot represents the overall gene based test including 64 variants. Subsequent orange dots represent the $-\text{Log}_{10}(\text{P-value})$ of the gene based test when the specific labeled variant was left out, and blue bars represent minor allele frequency (MAF) of specific variant being left out. **3b.** Schematic protein structure of SVEP1. rs61751937 substitutes glycine for arginine at position 229. Another variant in *SVEP1* has been associated with coronary artery disease which substitutes glycine for aspartic acid at position 2702. **3c.** Using UniProt, a total of 98 orthologs were identified for the largest human SVEP1 protein isoform and aligned. Alignments were visualized in MAFFT (v.7, <https://mafft.cbrc.jp/alignment/server/>, Katoh et al. 2017)³⁷ with ClustalW coloring. Both amino acids 229G and 2702D are highly conserved across diverse species, as well as their surrounding protein domains.

Table 1: Sixteen loci identified through single variant approaches for genome-wide association for platelet aggregation in response to epinephrine, ADP and collagen in 3,855 TOPMed participants. P-values presented are a summary across all individual phenotypes for the single agonist (i.e. the minimum P-value for the SNV from 8, 7 and 4 individual phenotypes for epinephrine, ADP, and collagen, respectively) (see **Supplementary Table 1**).

Known vs Novel	hg38	rsID	ref/alt	Nearest Gene	MAF	ADP		Epinephrine		Collagen	
						P-value	Beta	P-value	Beta	P-value	Beta
Novel	Chr1:192194880	rs1175170	G/C	RGS18,RGS21	0.442	7.86E-06	0.123	1.96E-09	0.154	2.37E-02	-0.056
Known	Chr1:156899922	rs12041331	G/A	PEAR1	0.148	7.61E-17	-0.329	2.30E-18	-0.357	7.57E-17	0.317
Novel	Chr1:20567949	rs12137738	A/T	FAM43B,CDA	0.077	0.0002	0.219	1.04E-08	0.305	0.017	-0.107
Novel	Chr1:67128641	rs142001088	C/T	C1orf141	0.018	0.0281	0.203	2.51E-03	0.275	9.25E-09	-0.502
Novel	Chr5:19109993	rs112157462	T/C	LINC02223,CDH18	0.022	1.64E-02	-0.281	5.90E-03	-0.229	1.18E-08	0.457
Novel	Chr6:121921871	rs58250884	A/G	GJA1,HSF2	0.087	1.60E-03	-0.153	2.22E-08	-0.272	2.34E-02	0.105
Novel	Chr9:28873884	rs185159562	T/A	LINGO2	0.005	3.87E-08	-0.987	5.10E-03	-0.987	1.45E-01	0.243
Novel	Chr10:75490891	rs138028657	A/G	LRMDA	0.006	0.6443	0.072	0.6144	-0.1017	1.52E-08	-0.858
Known	Chr10:111139289	rs7097060	T/A	ADRA2A,GPAM	0.137	5.45E-01	0.028	6.68E-12	0.251	6.73E-01	-0.014
Novel	Chr11:92185065	rs183146849	A/T	DISC1FP1,FAT3	0.012	3.10E-08	-0.702	1.33E-02	-0.375	4.38E-01	-0.080
Novel	Chr12:132589485	rs140148392	G/A	FBRSL1,LRCL1	0.009	1.51E-01	0.253	8.00E-03	0.137	2.88E-08	0.668
Novel	Chr13:96912429	rs61974290	A/G	HS6ST3,LINC00359	0.057	9.57E-02	0.088	3.36E-09	-0.399	9.40E-03	0.133
Novel	Chr17:21960955	.	A/T	KCNJ18,UBBP4	0.276	2.87E-08	0.167	7.73E-19	0.259	1.80E-03	-0.089
Novel	Chr17:16451482	rs575524466	G/A	LRRC75A-AS1	0.003	1.90E-01	-0.315	2.60E-03	-0.462	3.12E-08	1.168
Novel	Chr18:29059923	rs138845468	TAAATA/T	CDH2,MIR302F	0.082	6.90E-02	0.087	1.21E-01	0.086	3.22E-08	-0.250
Novel	Chr20:50142397	rs542707094	CTG/C	TMEM189,TMEM189-UBE2V1	0.003	3.16E-02	-0.485	3.05E-02	-0.486	3.52E-08	1.193

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