HHS Public Access

Author manuscript

Thromb Haemost. Author manuscript; available in PMC 2019 March 21.

Published in final edited form as:

Thromb Haemost. 2018 September ; 118(9): 1572–1585. doi:10.1055/s-0038-1667199.

Genome-wide association study links receptor tyrosine kinase inhibitor Sprouty2 to thrombocytopenia after coronary artery bypass surgery

Jörn A. Karhausen, MD[1,*], **Wenjing Qi, PhD**[2,*], **Alan M. Smeltz, MD**[1], **Yi-Ju Li, PhD**[2,3], **Svati H. Shah, MD, MHS**[3,4], **William E. Kraus, MD**[3,4], **Joseph P. Mathew, MD, MHSc**[1], **Mihai V. Podgoreanu, MD**[1,*], **Miklos D. Kertai, MD, PhD**[1,*], and **Duke Perioperative Genetics and Safety Outcomes (PEGASUS) Investigative Team**

[1]Department of Anesthesiology, Duke Perioperative Genomics Program, Duke University Medical Center, Durham, NC 27710, USA

[2]Department of Biostatistics and Bioinformatics, Duke University Medical Center, Durham, NC 27710, USA

[3]Molecular Physiology Institute, Duke University Medical Center, Durham, NC 27710, USA

[4]Division of Cardiology, Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Introduction: Thrombocytopenia after cardiac surgery independently predicts stroke, acute kidney injury, and death. To understand underlying risks and mechanisms, we analyzed genetic variations associated with thrombocytopenia in patients undergoing coronary artery bypass grafting (CABG) surgery.

Methods: Study subjects underwent isolated on-pump CABG surgery at Duke University Medical Center. Postoperative thrombocytopenia was defined as platelet count < 100×10^9/L. Using a logistic regression model adjusted for clinical risk factors, we performed a genome-wide association study in a discovery cohort (n=860) and validated significant findings in a replication cohort (n=296). Protein expression was assessed in isolated platelets by immunoblot.

Results: 63 SNPs met a priori discovery thresholds for replication, but only 1 (rs9574547), in the intergenic region upstream of sprouty 2 (*SPRY2*), met nominal significance in the replication cohort. The minor allele of rs9574547 was associated with a lower risk for thrombocytopenia (discovery cohort, OR 0.45, 95% CI 0.30–0.67, P = 9.76×10^{-5}) with the overall association confirmed by meta-analysis (meta-P = 7.88×10^{-6}). Immunoblotting demonstrated expression of

Corresponding author: Jörn A. Karhausen, MD, Assistant Professor, Department of Anesthesiology, Duke University Medical Center, DUMC 3094, Durham, NC 27710; 2301 Erwin Road, 5693 HAFS Bldg, Durham NC 27710. Tel: 919.681.6752. Fax: 919.681.8994. jorn.karhausen@duke.edu.

*The first 2 and last 2 authors contributed equally to this work.

Conflict of Interest Disclosure

The authors report no conflicts of interests related to the content of the manuscript.

SPRY2 and its dynamic regulation during platelet activation. Treatment with a functional SPRY2 peptide blunted platelet ERK phosphorylation after agonist stimulation.

Conclusions: We identified the association of a genetic polymorphism in the intergenic region of *SPRY2* with a decreased incidence of thrombocytopenia after CABG surgery. Because *SPRY2* – an endogenous receptor tyrosine kinase inhibitor – is present in platelets and modulates essential signaling pathways, these findings support a role for *SPRY2* as a novel modulator of platelet responses after cardiac surgery.

INTRODUCTION

Coronary artery bypass grafting (CABG) surgery with cardiopulmonary bypass (CPB) stimulates powerful inflammatory and tissue-injurious responses that can lead to significant organ damage, including acute kidney, neurocognitive, and lung injury, and cause considerable morbidity and mortality.(1) However, the mechanisms that drive tissue injury in this setting are not well defined. This deficit is a major obstacle to the development of effective preventive and treatment strategies.

We recently reported that, similar to observations in critically ill patients,(2) postoperative thrombocytopenia (i.e., a minimum in-hospital platelet value of $< 100 \times 10^9$/L) is associated with acute kidney injury, stroke, and increased risk for mortality after CABG surgery.(3, 4) Indeed, cardiac surgery is associated with dramatic acute changes in platelet function that manifest both perioperatively as platelet dysfunction and postoperatively as platelet hyper-reactivity.(5)

Platelets have emerged as important and ubiquitously present regulators of systemic and local inflammation with powerful influences on endothelial responses, neutrophil recruitment, and associated distant organ injury.(6, 7) Observations of organ-protective effects of perioperative anti-platelet therapy in cardiac surgery patients,(8, 9) and the association of thrombocytopenia with postoperative thrombophilia (evidence by the increased incidence of ischemic stroke) in our earlier study (4), strongly suggest that platelet activation and resultant consumption lead to the reduction of circulating platelet numbers. In this setting, end-organ damage could be mediated through small vessel occlusion and/or platelet-dependent microvascular inflammation and would therefore attribute platelets an integral role within the pathophysiology of perioperative organ injury. The increasing appretiation of platelets as inflammatory cells with important implications for perioperative organ injury therefore calls for a substantial re-evaluation of current anticoagulation therapies. However, since risk for bleeding is also associated with perioperative anti-platelet therapy, there is a need for a better understanding of the etiology of thrombocytopenia after CABG surgery and importantly, its link to the development of adverse outcomes.

Genetic association studies are powerful tools for identifying disease associated genes and to discover previously unidentified pathways that contribute to certain phenotypes. Many of these studies have demonstrated that genetic variants play a substantial role in altering platelet function.(10–15) Such variants, or single-nucleotide polymorphisms (SNPs), have been reported in genes that regulate key surface receptors and reactive granule constituents.(10, 12) More recently, however, SNPs have been found in non-coding regions associated

with enhancer elements or promoters in megakaryocytes.(11) In contrast to these large-scale, community-based cohort studies, a few largely candidate gene studies have searched for genetic variants that influence acute platelet responses to cardiac surgery and cardiopulmonary bypass, and have identified gene polymorphisms that are associated with perioperative bleeding (16, 17) and risk for postoperative cardiac injury.(18, 19)

Since currently known clinical and procedural risk factors (3, 4, 8) do not adequately account for variability in the occurrence of postoperative thrombocytopenia, we hypothesized that within a multifactorial etiology, genetic variations play a significant role. To test this hypothesis and to learn more about relevant platelet regulatory pathways, we conducted a genome-wide association study (GWAS) aimed at identifying common genetic variants associated with postoperative thrombocytopenia in the setting of CABG surgery.

MATERIALS AND METHODS

We designed this study and reported our findings according to the “Strengthening the Reporting of Genetic Association Studies” (STREGA) recommendations.(20) Two independent cohorts of patients who underwent CABG surgery at the Duke Heart Center at Duke University Medical Center, Durham, North Carolina, were used for initial common variant discovery by GWAS and replication analysis of top candidate SNPs. Each of the parent studies was approved by the Institutional Review Board at Duke University Medical Center, and all subjects provided written informed consent.

Our discovery cohort was comprised of a 1,004 patient subset from the Perioperative Genetics and Safety Outcomes Study (PEGASUS), comprising prospectively enrolled patients who underwent isolated non-emergent CABG surgery with cardiopulmonary bypass (CPB) between 1997 and 2006.(21) For patients who had more than one cardiac surgery during that period, only data from the first surgery were included. Of the original 1,004 study subjects, 860 patients met our inclusion criteria of self-reported European ancestry and complete phenotypic and genotypic data.

Our replication cohort was comprised of patients in the CATHeterization GENetics (CATHGEN) study who underwent cardiac catheterization between 2001 and 2010 to evaluate ischemic heart disease, and who subsequently underwent CABG surgery with CPB between 2006 and 2010. Of the 475 patients reviewed, 296 without concurrent valve surgery met our inclusion criteria.

Intraoperative anesthetic, perfusion, cardioprotective, and transfusion management was standardized, as described previously.(21, 22) Briefly, general anesthesia was maintained with a combination of fentanyl and isoflurane. Perfusion support consisted of nonpulsatile CPB (30°C- 32°C), crystalloid prime, pump flow rates > 2.4 L/min per m^2, cold blood cardioplegia, α-stat blood-gas management, activated clotting times > 450 seconds maintained with heparin, ε-aminocaproic acid infusion administered routinely, and serial hematocrits maintained at > 0.18. During the study period, the decision for intraoperative and postoperative blood and blood product transfusion was guided by laboratory testing

consistent with the recommendations of the American Society of Anesthesiologists in "*Practice Guidelines for Blood Component Therapy*". (23)

Clinical risk factors, data collection and end-point definition

Patient and procedural characteristics for both cohorts were recorded and collected using the Duke Information System for Cardiovascular Care, an integral part of the Duke Databank for Cardiovascular Disease. The clinical risk factors for postoperative thrombocytopenia included patient characteristics, preoperative cardiovascular medication use, CPB and aortic cross-clamp times, insertion of intra-aortic balloon pump (IABP), intraoperative and/or postoperative blood or blood product transfusions, and platelet counts. Per institutional practice, preoperative antiplatelet therapy with aspirin was maintained until the day before surgery; clopidogrel was discontinued at least 7 days before surgery; and warfarin was discontinued 4 days before surgery and "bridged" with intravenous heparin infusion.(3)

Also per institutional practice, platelet counts for both the discovery and replication cohorts were measured in the Duke Clinical Pathology Laboratory on a pocH 100i automated hematology analyzer (Sysmex, Kobe, Japan) at baseline (preoperative) and daily postoperatively until postoperative day 10 or at discharge, whichever came first. Postoperative thrombocytopenia as a qualitative endophenotype was the primary outcome of the study. It was ascertained by using selecting subjects whose postoperative minimum (nadir) platelet values were $< 100 \times 10^9$/L (moderate to severe thrombocytopenia group), and comparing to subjects whose postoperative minimum (nadir) platelet values were $> 150 \times 10^9$/L (normal postoperative platelet count group). Further, since a continuous outcome is known to be generaly more informative than a dichotomous outcome, we also used minimum (nadir) postoperative platelet values as a quantitative trait of thrombocytopenia (secondary outcome of the study), to enhance the power of our study to detect potential associations between genetic risk variants and postoperative platelet count.

Genotyping and quality controls

Genotyping platforms and quality controls (QCs) for genotype and sample exclusion have been described previously.(21) Briefly, all of the 1,004 samples in the PEGASUS cohort (discovery samples) were genotyped at the Duke Genomic Analysis Facility using the Illumina Human610-Quad BeadChip (Illumina, Inc, San Diego, CA, USA). Human genome build 37 (GRCh37/hg19) from February 2009 was used for the reference of ranges of genes and locations of SNPs. Various QC criteria were applied to ensure genotype quality. Markers with a GenCall (http://support.illumina.com/downloads/gencall_software.html) score ≤ 0.15 or call frequency < 98% were excluded. Samples with a call rate < 98% or sex specification errors were also excluded in this initial QC. At the sample level, we used PLINK software to check cryptic relatedness and duplications.(24) For a pair of samples with an identity-by-descent value > 0.1875 (between second- and third-degree relative), one sample was excluded from further analysis. Population structure was investigated using the EigenSoft program(25) to generate 15 principal components (PCs) and multiple PC plots to identify any obvious outliers that deviated from the main cluster and hence, should be excluded. As expected, we found no PCs that were associated with postoperative thrombocytopenia. Consequently, no PCs were included in the final association analysis models. At this stage,

the QC'ed genotype dataset consisted of 960 study subjects with 561,091 markers. However, 100 of these patients had not only CABG sugeries, and therefore were excluded from further analysis. Thus, the final discovery dataset (PEGASUS cohort) consisted of 860 patients of European descent with both genotype and phenotype data available.

All CATHGEN samples were genotyped at the Duke Genomic Analysis Facility using the Illumina OMNI 1-Quad BeadChip (Illumina, Inc, San Diego, CA, USA), and were subject to the same marker and sample QC criteria as described above for PEGASUS. GRCh37/hg19 was also used for reference for CATHGEN samples. For the replication dataset, we selected a subset of 296 patients out of 475 subjects from the CATHGEN cohort based on availability of genotype, and patient and procedural characteristics.

Demographic and clinical characteristics, as well as comparisons between the 2 cohorts are shown in Supplementary Table 1. In the discovery cohort, the primary outcome – moderate to severe postoperative thrombocytopenia – was observed in 176 out of 860 (20%) patients (postoperative minimum [nadir] platelet value $< 100 \times 10^9$/L), while 268 out of 860 (31%) patients had a normal postoperative platelet count (postoperative minimum [nadir] platelet value $> 150 \times 10^9$/L). In the replication cohort, 76 out of 296 (26%) patients developed moderate to severe postoperative thrombocytopenia, and 70 out of 296 (24%) patients had a normal postoperative platelet count. Only genotyped SNPs identified in the discovery cohort were tested in the CATHGEN cohort for replication purposes. Since different genotype platforms were used for the discovery and replication samples, we used imputed markers in the replication cohort to maximize the shared SNPs between the 2 cohorts. We also imputed markers for the purpose of fine mapping in the genetic regions of interest in the discovery cohort. To impute untyped SNPs, we used the IMPUTE2 program,(26) and the post-QCed PEGASUS genotype cohort (960 samples with 561,091 markers) and phased haplotypes from the 1,000 genome CEU reference panel.

Ex vivo platelet analysis

Whole blood (40 mL) was collected in acid-citrate-dextrose sodium citrate (1:9 ACD v/v) from healthy individuals under a protocol approved by the Institutional Review Board at Duke University Medical Center. Blood was centrifuged at 120 ×*g* for 8 minutes to isolate plateletrich plasma (PRP). Platelets were isolated from the PRP by spinning at 650×g and washed in buffer containing 36 mM citric acid, 5mM glucose, 5mM KCl, 1mM $MgCl_2$, 103 mM NaCl, 2 mM $CaCl_2$, 3.5 g/L bovine serum albumin, and resuspended at a concentration of 1E+07/ml in standard Tyrodes buffer. During entire isolation, platelet activation was prevented by addition of 1 μM prostaglandin E_1 and 0.2 U/mL apyrase (Millipore Sigma, Burlington, MA). In a subset of experiments, the washed platelets were passed through a neutrophil reduction filter (Pall Corporation, Port Washington, NY, USA) to eliminate contamination of the platelet population.

Final platelet populations were then immediately harvested (resting), or activated for 2, 5, 15 or 30 minutes using thrombin at concentrations as outlined (Millipore Sigma), 60ng/ml Convulxin (Cayman Chemical, Ann Arbor, MI) or a mixture of 100μM ADP (Bio/Data, Horsham, PA) and 100μM epinephrine (Millipore Sigma). At each activation time-point, a

platelet aliquot was fixed for 10 minutes in 1% formalin, and the remaining platelets were lysed in their reaction buffer by addition of SDS to a final concentration of 2%.

The fixed platelets were used to document the level of platelet activation at the time-points examined by western blotting. For this, platelets were resuspended in PBS, stained with anti-CD62-APC antibody (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed immediately on a FACScalibur Flow cytometer. Results were based on the analysis of 150.000 events/sample. Isotype control BD-treated (BD Biosciences) samples were used as negative controls.

Western blotting was performed after determining and normalizing the protein content in the lysates (DC protein assay, BioRad, Hercules, CA, USA). After adding 4× Laemmli buffer (BioRad) and boiling, samples were separated by SDS-PAGE on 12% gradient gels and transferred to PVDF membranes (BioRad). Membranes were blocked using Tris/HCl-buffered salt solution supplemented with 0.1% Tween 20 and 5% skim milk powder, and then incubated with primary antibodies overnight at 4°C. Primary antibodies were anti-Sprouty-2 (Abcam, Cambridge, MA, USA) and anti-CD45 (Thermo Fisher Scientific, Waltham, MA, USA), anti-ERK1/2 (Cell Signalling Technology, Danvers, MA), anti-p-ERK (Santa Cruz Biotechnology, Dallas, TX) and anti-Phospho-Tyrosine (Cell Signaling). After washing, membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase conjugates (BioRad), and targets were visualized using Supersignal West Pico Luminol Enhancer solution (Thermo Fisher). Membranes were then stripped and re-probed with a mouse monoclonal anti-β-actin antibody (Millipore Sigma) as loading control.

For phosphatase treatement, activated platelets were lysed in buffer containing 10 mM TRIS, 10 mM NaCl and 1% NP40-LB and aliquoted to control treatment, or treatment with calve alkaline phosphatase, or lambda phosphatase (both Millipore Sigma). Reactions were stopped by adding 1% SDS and boiling for 10minutes.

SPRY2 octapeptides were custom synthesized by peptide 2.0 (Chantilly, VA) based on protein sequences published by Hanafusa et al.(27). To make peptides cell permeable, we added a c-terminal HIV-tat sequence as previously published (28). The final sequences were YGRKKRRQRRRTNEYTEGP-NH2 for the control peptide and YGRKKRRQRRRTNE(pY)TEGP-NH2 for the phosphorylated peptide. Peptides were solubilized as 50mM stock solutions in DMSO. Washed platelets were treated with control on phosphorylated peptide at a final concentration of 50μM for 20minutes in presence of apyrase and PGE1 as outlined above, then spun down and resuspended in Tyrodes buffer.

After this treatment, platelets remained in resting state as documented by lack of CD62 externalization and the lack of binding of Oregon488-labeled, soluble fibrinogen (Thermo Fisher). However, platelet remained functionally intact as ascertained after platelet reconstitution in platelet poor plasma and agonist-induced platelet aggregation using the PAP-8E Platelet Aggregation Profiler (Bio/Data) (Supplementary Figure 1).

Statistical Analysis

Descriptive statistics of clinical variables are presented as frequency and percentage for categorical variables and mean ± SD for continuous variables. Univariable and multivariable logistic regression models were applied to evaluate the association between demographic, clinical and procedural characteristics with moderate to severe postoperative thrombocytopenia (minimum [nadir] platelet values < 100×10^9/L). To derive the final multivariable logistic regression model containing variables with P values < 0.05, univariable associations with a P value < 0.15 were evaluated using a backward stepwise technique and the Schwarz-Bayes criterion.(29) Analyses of demographic and clinical and procedural characteristics were conducted using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA).

Genetic association analyses were performed using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) for all genotyped markers in the discovery cohort. At the data analysis stage, additional QC criteria excluded markers that were significantly deviated from the Hardy-Weinberg equilibrium ($P < 10^{-6}$) or had a minor allele frequency (MAF) < 2%. For each of the SNPs, allelic associations with postoperative thrombocytopenia, (defined as a qualitative trait [primary outcome analysis] or as a quantitative trait [secondary outcome analysis]), were assessed using multivariable logistic regression analyses or multivariable linear regression analyses as appropriate, adjusted for the same set of clinical variables identified as part of the final multivariable logistic regression model containing demographic, clinical, and procedural characteristics. These association testsassumed an additive inheritance model (homozygote major allele *versus* heterozygote *versus* homozygote minor allele). Markers with $P < 5 \times 10^{-8}$ were considered to be genome-wide significant, which is the most commonly accepted significance threshold for GWAS. In addition to this stringent criterion, a relaxed significance threshold of $P < 1 \times 10^{-4}$ was applied when choosing SNPs for replication in the CATHGEN cohort.(22) Similar to the discovery cohort, 41 genotyped markers were tested using PLINK in the replication cohort. For 22 imputed markers in the discovery cohort, SNPTEST (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html) was used to conduct genetic association analyses based on genotype dosages. The same multivariable logistic regression model adjusted for the final set of demographic, clinical and procedural characteristics as in the discovery cohort was applied to the replication cohort. To assess the overall effect of candidate SNPs, we conducted a meta-analysis as implemented in METAL (http://www.sph.umich.edu/csg/abecasis/metal).

The final candidate SNP(s) were prioritized based on 1) meeting nominal significance in the replication cohort, 2) showing the effect in the same direction for postoperative thrombocytopenia (defined as a qualitative trait) in the replication cohort as in the discovery cohort, and 3) reaching statistical significance in the meta-analysis. Marker density for the top candidate gene(s) or region(s) was increased by using imputed markers within the gene or region in the discovery cohort to examine the association pattern within the region. SNPTEST was again used to conduct genetic association analyses based on genotype dosages for imputed markers. Markers with info measure (measure of the observed statistical information associated with the allele frequency estimate) below 0.4 were excluded from the analyses. Regional association plots of genome-wide association results

within the region of interest were generated using LocusZoom.(30) Finally, we used genome-wide compex trait analysis (http://cnsgenomics.com/software/gcta/) to estimate the extent of variance in the primary outcome (moderate to severe postoperative thrombocytopenia as a qualitative trait) that was attributed to candidate SNPs.

RESULTS

For our study, the primary outcome – moderate to severe postoperative thrombocytopenia was present in 176 patients in the discovery dataset and 76 patients in the replication dataset. Demographic, clinical, and procedural characteristics of the patients in these 2 datasets, stratified according to moderate to severe postoperative thrombocytopenia or normal postoperative platelet count, are shown in Table 1. The overall mean age was 63±11 years in the discovery dataset and 61±11 in the replication dataset. Both datasets had higher proportions of male patients (340 [76.6%] in the discovery cohort and 100 [68.5%] in the replication cohort). The mean±SD postoperative minimum (nadir) platelet counts was similar between the 2 datasets (145±60 versus 131±59×10^9/L). In the discovery dataset, patients with moderate to severe postoperative thrombocytopenia had a significantly lower mean postoperative minimum (nadir) platelet count compared to controls with normal postoperative platelet count (81×10^9/L [±16] vs 187×10^9/L [±37], $P < 0.0001$). Similarly, patients in the replication dataset with moderate to severe postoperative thrombocytopenia had a significantly lower mean postoperative minimum (nadir) platelet count compared to controls with normal postoperative platelet count (79×10^9/L [±15] vs 187×10^9/L [±29], $P < 0.0001$).

Univariable predictors of moderate to severe postoperative thrombocytopenia are shown in online Appendix Supplementary Table 2. Several demographic, clinical, and procedural characteristics were significantly associated with increased risk for moderate to severe postoperative thrombocytopenia. According to our multivariable analysis, age, duration of cardiopulmonary bypass, and use of blood products intraoperatively and/or within 2 days postoperatively remained independent risk factors for moderate to severe postoperative thrombocytopenia (online Appendix Supplementary Table 2), and were subsequently incorporated as covariates in multivariable logistic regression models to adjust the SNP associations with postoperative thrombocytopenia.

After applying our initial QCs, 561,091 genotyped markers were available for analysis. Of these, 3 SNPs were excluded due to deviation from Hardy-Weinberg equilibrium, and an additional 36,022 SNPs were excluded due to MAF < 0.02. The remaining 525,066 markers were tested in the 444 subjects in the discovery dataset for association with the primary outcome of moderate to severe postoperative thrombocytopenia. GWAS results in the discovery cohort are depicted using a Manhattan plot (online Appendix Supplementary Figure 2). None of the SNPs reached genome-wide significance. However, 53 SNPs met the a priori defined discovery threshold of $P < 1 \times 10^{-4}$ and were then analyzed in the replication dataset (online Appendix Supplementary Table 3).

Of the 63 SNPs analyzed in the replication dataset (Table 2), 22 SNPs had imputed genotypes due to differences between the 2 BeadChips used in these 2 datasets. We

identified 1 SNP, rs9574547, with nominal significance ($P < 0.05$) in the replication dataset (Table 2). In both datasets, the minor allele of rs9574547 in the intergenic region between *LOC729479* and Sprouty receptor tyrosine kinase (RTK) Signaling Antagonist 2 (*SPRY2*) was associated with a decreased incidence of moderate to severe postoperative thrombocytopenia (discovery dataset: OR, 0.45; 95% CI, 0.30–0.67; $P = 9.76 \times 10^{-5}$; and replication dataset: OR, 0.47; 95% CI, 0.24–0.92; $P = 0.03$). The meta-analysis of both cohorts by METAL showed that rs9574547 remained significantly associated with decreased risk for moderate to severe postoperative thrombocytopenia (meta-$P = 7.88 \times 10^{-6}$; Table 2).

Using a total of 1353 imputed markers (info ≥ 0.4), we increased the marker density in the *LOC729479* to *SPRY2* intergenic region on chromosome 13 in order to identify SNPs most strongly associated with moderate to severe postoperative thrombocytopenia and potentially close to the causal variants in this gene: 80585271–80915086 (in the *LOC729479* to *SPRY2* region). The relative location of the genotyped SNP rs9574547 in the *LOC729479* I *SPRY2* region, local LD and recombination patterns are shown in Figure 1.

Finally, we performed genome-wide complex trait analyses to estimate the proportion of variance in moderate to severe postoperative thrombocytopenia explained by rs9574547. The results indicated that rs9574547 explained 3.41% of attributable variance in the risk of developing moderate to severe postoperative thrombocytopenia after CABG surgery.

To further study the association of identified SNPs with postoperative thrombocytopenia, defined as a quantitative thrombocytopenia trait, we conducted multivariable linear regression analyses adjusted for the same set of demographic, clinical, and procedural variables as for the qualitative trait in the discovery (N = 860) and replication (N = 296) cohorts. The results showed significant association of the minor allele of rs9574547 in the *LOC729479* I *SPRY2* region with higher postoperative minimum platelet count in individual patients (for every additional minor allele of rs9574547, individual postoperative minimum platelet counts were estimated to increase by 10.8±2.5; $P = 1.62 \times 10^{-5}$ in the discovery cohort, and by 10.2±3.8, $P = 0.0086$ in the replication cohort, Supplementary Figure 3).

We also studied changes in platelet counts throughout the perioperative period in patients with and without postoperative thrombocytopenia. Our analysis revealed some notable differences in pre-operative platelet counts between patients in the moderate to severe postoperative thrombocytopenia group vs. those in the normal postoperative platelet count group (Figure 2). This finding led us further to explore whether there was an association between rs9574547 and preoperative platelet count. The result of our univariable analysis indicated, that indeed, there was a significant association between rs9574547 and preoperative platelet count defined as a continuous variable ($P = 0.004$). Subsequently we performed a multivariable linear regression analysis to study whether the association between rs9574547 and postoperative thrombocytopenia was independent from preoperative platelet count, and our results indicated that the association between rs9574547 and postoperative thrombocytopenia remained statistically significant ($P = 0.0056$).

Ex vivo platelet analysis

In order to explore potential mechanisms behind the association of post-operative thrombocytopenia and the identified SNP, we next explored the possibility that SPRY2 is involved in regulating platelet reactivity. However, SPRY2 expression has not yet been documented in platelets, therefore we first measured SPRY2 protein expression in isolated washed platelets before and after agonist stimulation. As expected, agonist treatment rapidly led to platelet activation as evidenced by surface expression of CD62 in FACS analysis (Figure 3A, online Appendix Supplementary Figure 4). Robust SPRY2 protein expression was observed under baseline conditions as a single protein band. Following platelet activation either via the protease-activated receptor pathway (PAR) using thrombin, via the Glycoprotein VI receptor pathway (GPVI) using the collagen receptor agonist convulxin or by co-stimulation of the purinergic P2Y12 and alpha(2A)-adrenergic receptor using epinephrine and ADP, however, a second higher-molecular-weight band appeared, and the initial lower-molecular-weight SPRY2 signal diminished (Figure 3B, online Appendix Supplementary Figure 4). Overall SPRY2 protein levels did not change upon platelet activation, as quantified by densitometric measurements of the combined SPRY2 signal (data not shown). To verify purity, unstimulated platelets were passed through a leukocyte depletion filter, which led to some baseline platelet activation but supported our above observations. In addition, reprobing of blots for the pan-leukocyte epitope CD45 showed no indication for leukocyte contamination.

A crucial determinant of SPRY2 protein activity in cellular responses to growth factors is phosphorylation of its Tyr 55 residue,(27) and we next examined whether tyrosine phosphorylation may account for some of the change in the SPRY2 banding pattern after platelet activation. As shown in Figure 4A, we observed an increased phospho-tyrosine band in the SPRY2 region by western blotting. In addition, phosphatase-treatment of activated platelet lysate abolished the higher molecular weight band of SPRY2, together indicating that in the course of platelet activation, SPRY2 is modified by phosphorylation (Figure 4B).

Phosphorylation is essential for SPRY2-regulator functions of the MAPK pathway (27). Because MAPK pathway is also activated during platelet stimulation (Figure 4C), we next explored whether SPRY2 may have similar effects in platelet MAPK signaling using a previously published phospho-SPRY2 octapeptide (27) that we made cell permeable through addition of a HIV-tat sequence. In accordance with above report which had studied the effect of octapeptide microinjections on fibroblast responses to growth factor treatment (27), pretreatment of platelets with the phosphorylated SPRY2 peptide but not with the non-phosphorylated control peptide resulted in blunted phosphorylation of ERK after platelet stimulation (Figure 4D).

Together, our ex-vivo data therefore provides primary evidence that SPRY2 protein is present in circulating platelets and suggests that differential regulation of the SPRY2 protein during platelet activation may serve to dynamically modulate platelet functions.

DISCUSSION

Using a GWAS approach, we identified a novel susceptibility locus at 13q31 (rs9574547), associated with moderate to severe postoperative thrombocytopenia after CABG surgery with CPB. Patients carrying one or both of the minor alleles of this SNP were at decreased risk for moderate to severe postoperative thrombocytopenia. Notably, our findings suggested an independent association even after adjusting for clinical and surgery-related variables known to be associated with risk for postoperative thrombocytopenia. Corroborating our genome-wide association findings, we demonstrated for the first time that *SPRY2* is expressed in resting platelets and provide evidence that SPRY2 may modify ERK phosphorylation in ex vivo platelet activation. These findings add to mounting data that implicate genetic variations in platelet responsiveness and postoperative thrombocytopenia.(13, 31, 32) Importantly, we previously reported that a reduction in platelet count after cardiac surgery is linked to increased risk for acute kidney injury and mortality, and appears to occur in a state of platelet hyper-responsiveness, as evidenced by increased risk for stroke in these patients.(3, 4) Thus, understanding the role of *SPRY2* in regulating platelet function may provide the key to defining the currently unresolved pathophysiology of postoperative thrombocytopenia after cardiac surgery, and may lead to novel pharmacologic targets to prevent postoperative thrombocytopenia and subsequent complications after CABG surgery.

The 13q31.1 locus identified here is in the intergenic region bounded by *LOC729479* (a hypothetical gene) and the *SPRY2* gene (chr13q31.1; lowest meta-$P = 7.9 \times 10^{-6}$). Of these 2 genes, the protein encoded by *SPRY2* serves as a crucial regulator of receptor tyrosine kinase (RTK) signaling. Initially identified in *Drosophila*, SPRY is an endogenous inhibitor of growth factor signaling (mammalian *SPRY2* and the single *dSpry* isoform found in *Drosophila* play similar roles reviewed in (33)) that interferes with the assembly of essential adaptor protein complexes downstream of the RTK receptor.(27) As such, *SPRY2* functions in a classic feedback loop of RTK activity.

RTKs are a family of cell surface receptors that regulate key cellular processes including proliferation and differentiation, cell survival, metabolism, migration and cell cycle control.(34) Although the function of RTK signaling has been most extensively examined in the context of effects on growth factors involved in proliferation and development, a critical influence on platelet activation has also been established. Most prominent is the collagen receptor glycoprotein VI (GPVI), which is constitutively associated with an RTK, ie, the Immune Tyrosine Activation Motif (ITAM)-bearing Fc receptor g-chain.(35) Another example is the Tyro3/Axl/Mer (TAM) receptor tyrosine kinase family, which plays important roles in hemostasis and inflammation, e.g. by interacting with vitamin K-dependent protein Gas6.(36) Platelet activation via these receptors enables responses that are distinct from G-protein coupled receptor (GPCR) engagement, i.e. after thrombin binding (35) and appear to allow very finely tuned and graded platelet responses. For example, ITAM signaling, but not GPCR signaling, controls vascular integrity during inflammation,(37) and a deficiency in TAM receptor signaling protects against thrombosis but does not carry a bleeding phenotype.(38)

Thus, it is not surprising that, while RTK inhibitors in certain anti-cancer agents elicit significant platelet function abnormalities,(39) tyrosine kinases are also increasingly recognized as potential targets for novel anti-platelet agents.(40) The concept of such drugs is based on the notion that they may have the capacity to control the rheostat of platelet-inhibitory and platelet-activating factors, in constrast to currently approved drugs that cause profound platelet inhibition and therefore, increase the risk for bleeding complications. Although many aspects of the tyrosine kinase signaling pathway in platelets have not yet been explored, our finding of a susceptibility locus for moderate to severe postoperative thrombocytopenia in the intergenic region upstream of the *SPRY2* gene, may be useful in the search for regulators of tyrosine kinases in platelets.

Importantly, the role of SPRY2 has not been investigated in the context of platelet function, yet its major binding partners are strongly implicated in platelet regulation. For example, SPRY2 impedes interaction between adaptor protein growth-factor receptor-bound protein 2 (Grb2) and RTKs, thereby acting as an endogenous RTK inhibitor.(27) Importantly, Grb2 regulates collagen receptor signaling in platelets and thus, also serves as an important modulator of hemostasis and thrombosis.(41) Based on these reports, it would be expected that the endogenous RTK inhibitor SPRY2 functions in a platelet inhibitory way and thus leads to a similar platelet phenotype as Grb2 knock-out,(41) or RTK inhibitors used in anti-cancer therapy.(39)

Although SPRY2 has been indirectly associated with megakaryocytic differentiation,(42) its expression in platelets has not been documented. This presented a major obstacle to assessing the relevance of our GWAS finding to possible platelet function abnormalities. Our finding that indeed, SPRY2 is expressed in platelets and further, that it appears to be post-translationally modified after platelet activation, provides important support for further investigation of SPRY2 as a modulator of platelet function. SPRY2 functions are tightly regulated by post-translational modification including phosphorylation (27) and ubiquitination and proteasomal degradation. (43, 44) To provide first insights how SPRY2 might function in platelets, we adapted an approach previously used by Hanafusa et al. (27). Importantly, evidence of SPRY2 tyrosine phosphorylation following platelet activation suggested that SPRY2 may function in a similar fashion as noted by these authors when examining SPRY2 as a modulator of fibroblast growth factor responses. This work had employed microinjection of a SPRY2 octapeptide to mimic SPRY2 function and had shown that a peptide phosphorylated at the tyrosine equivalent to Tyrosine 55 in full length SPRY2 blunted growth factor induced ERK phosphorylation. Using a HIV-tat strategy to render the peptide cell permeable, we were indeed able to modify ERK responses following platelet activation, providing primary evidence that SPRY2 serves to modulate platelet responses. However, further work will be needed to fully define the functional role of SPRY2 within the complex context of MAPK signaling in platelet physiology. As such, the ERK pathway regulates store-mediated calcium entry in platelets, (45) and is an important modulator of platelet integrin αIIbβ3 activation.(46) In addition, ERK plays distinct roles, dependent on the context. For example, agonist-induced MAPK activation regulates early but transient platelet granule secretion, while integrin-mediated MAPK activation facilitates late and sustained responses such as clot retraction. (47)

Limitations

In the present study, we used an unbiased GWAS approach to identify genetic predictors of moderate to severe postoperative thrombocytopenia after CABG surgery; however, several limitations remain. First, because the identified SNP is a tagging marker in the intergenic region of *LOC729479* I *SPRY2*, the observed associations could be due to changes in regulation of gene expression or high linkage disequilibrium with the true unidentified causal SNPs. Indeed, intergenic regions can be transcribed, and the resulting long non-coding RNAs are known to perform several different functions ranging from regulation of epigenetic modifications and gene expression, to acting as scaffolds for protein signaling complexes.(48) Therefore, future research to identify causal intergenic SNP(s) and to decipher their relationship to epigenetic modifications and/or expression of their neighboring genes, is important.

Second, based on current sample size and an incidence of moderate to severe postoperative thrombocytopenia of 39.6%, an allele frequency of 0.22 as our top SNP (rs9574547), and a complete linkage disequilibrium between SNP and causal variant, our power calculations show that our study has 73.4% power to detect a genotypic relative risk of 0.61, which is equivalent to an OR of 0.44. Although we used a relatively large cohort of cardiac surgery patients, our study is powered to detect only common variants with relatively large effects. Thus, we did not examine the possibility of rare genetic variants that influence a pronounced clinical phenotype.

Third, a number of nongenetic clinical and procedure-related factors are potentially associated with a higher risk for postoperative thrombocytopenia.(3, 4) However, given our study design and the relative size of the discovery and replication cohorts, we were not able to investigate effects of these factors on postoperative thrombocytopenia.

Fourth, of the 176 patients in the discovery cohort and the 76 patients in the replication cohort with moderate to severe postoperative thrombocytopenia, none developed heparin-induced thrombocytopenia (HIT). However, per institutional protocol, routine testing for postoperative HIT is left to the discretion of the intensive care unit team. Nevertheless, as indicated by our previous studies,(3, 4) early-onset and persistent thrombocytopenia in CABG surgery patients is seldom caused by postoperative HIT and thus it is unlikely that we may have missed a diagnosis of of postoperative HIT.

Fifth, our finding that SPRY2 modulates platelet ERK signaling and the critical influence of RTK signaling on platelet activation supports the intriguing hypothesis that the observed variation noted upstream of the *SPRY2* gene modulates platelet function. However, the association of the identified SNP also with pre-operative platelet counts could suggest that variants of SPRY2 may take effect on platelet generation or maturation, a situation that may be further unmasked by perioperative platelet loss and consumption. Indeed, a work by Yang et al. (49) suggests that *SPRY1* may negatively regulate hematopoiesis. However, while a role of *SPRY2* in thrombopoiesis cannot currenly be excluded, we verified the association of rs9574547 with post-operative platelet count independent of the pre-operative platelet status by multivariable regression analysis. In addition, it is important to note that in our cohort – patients undergoing CABG surgery – pre-operative platelet counts cannot be taken as a true

baseline/resting status. In fact, significant ongoing platelet activation has been observed in these patients and linked to the underlying vasculopathy (50–52) and to co-existing diseases such as hypertension(53) or diabetes mellitus(54). In addition, we screened existing literature and available databases such as UK Biobank and the GRASP database and found no report linking either *SPRY2* itself or *rs9574547* to platelet counts outside of the surgical context. As a consequence, differences in pre- and post-operative platelet counts may both reflect variations of platelet response programs after stimulation.

Similarly, we cannot currently exclude that in absence of any platelet anomaly, endothelial or inflammatory SPRY2 targets drive the decline of platelet count after cardiac surgery. Our ex vivo platelet analysis clearly demonstrated that SPRY2 is present in platelets and regulated following activation. Therefore, our study provides the first evidence of SPRY2 involvement in modulating platelet functions, but more work is needed to define not only the role of SPRY2 role but also of rs9574547 allele-specific differences for platelet responses and outcomes in cardiac surgery.

Finally, all of our study subjects were of European descent and thus, our findings cannot be generalized to other ethnic groups.

In summary, we conducted a comprehensively designed GWAS in a cohort of patients at risk for moderate to severe postoperative thrombocytopenia after CABG surgery, and identified and characterized a novel intergenic susceptibility locus for moderate to severe postoperative thrombocytopenia after CABG surgery with CPB. The development of post-operative thrombocytopenia is associated with a significant risk of experiencing adverse outcomes such as stroke,(4) AKI and increased mortality.(3) Currently, it remains unknown what causes reduction of platelet numbers after cardiac surgery and how such thrombocytopenia is linked to the development of adverse outcomes. As a consequence, our data suggesting that modulated expression of the endogenous tyrosine kinase inhibitor SPRY2 is protective against the development of thrombocytopenia opens new opportunities to better understand the underlying pathophysiology and to develop pharmacological approaches to prevent post-operative thrombocytopenia. Notably, the ongoing development of anti-platelet agents that target the tyrosine kinase pathway may provide novel therapeutic options to limit the extent of perioperative platelet activation and the associated end-organ injury and mortality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Jasmine Fowler, BS for technical support and Dr. Gowthami M. Arepally MD (Division of Hematology at Duke University Medical Center) for critical discussions and help with functional platelet testing. The members of the Duke Perioperative Genetics and Safety Outcomes (PEGASUS) Investigative Team are: Cooter M, Daneshmand M, Funk B, Gaca JG, Ghadimi K, Ginsburg GS, Glower DD, Haney J, Hauser E, Karhausen J, Kertai MD, Laskowitz DT, Li YJ, Lodge AJ, Mathew JP, Milano CA, Moretti EW, Newman MF, Quinones QJ, Podgoreanu MV, Schroder J, Smith MP, Smith PK, Stafford-Smith M, Swaminathan M, Waldron NH, Welsby IJ

Sources of Funding

Funding for this study was provided in part by: 1) the Duke Anesthesiology Developing Research Excellence in Anesthesia Management (DREAM) Award (to Dr Kertai); National Institutes of Health grants 1R56HL126891-01 (to Dr Karhausen), HL075273 and HL092071 (to Dr Podgoreanu), HL096978, HL108280, and HL109971 (to Dr Mathew), HL095987 (to Dr Shah), and HL101621 (to Dr Kraus); American Heart Association grants 15SDG25080046 (to Dr Karhausen), 9951185U (to Dr Mathew), and 0120492U (to Dr Podgoreanu); and a Duke School of Medicine Health Scholar award (to Dr Karhausen). Logistics support was provided by CATHGEN. The authors are solely responsible for the design and conduct of this study, all study analyses, and drafting and editing of the manuscript and its final contents.

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Extra table:

What is known on this topic:

Thrombocytopenia after cardiac surgery is an independent predictor of stroke, acute kidney injury, and death; however, baseline and clinical patient characteristics do not adequately account for the occurrence of postoperative thrombocytopenia.

What this paper adds:

The study identified a genetic polymorphism in the intergenic region upstream of *sprouty 2* (*SPRY2)* that is associated with decreased risk for developing postoperative thrombocytopenia after CABG surgery. This study also shows that *SPRY2, which acts as an* endogenous receptor tyrosine kinase inhibitor, is expressed in platelets and modulates ERK signaling during platelet activation. These findings support a role for *SPRY2* in the modulation of platelet responses after cardiac surgery.

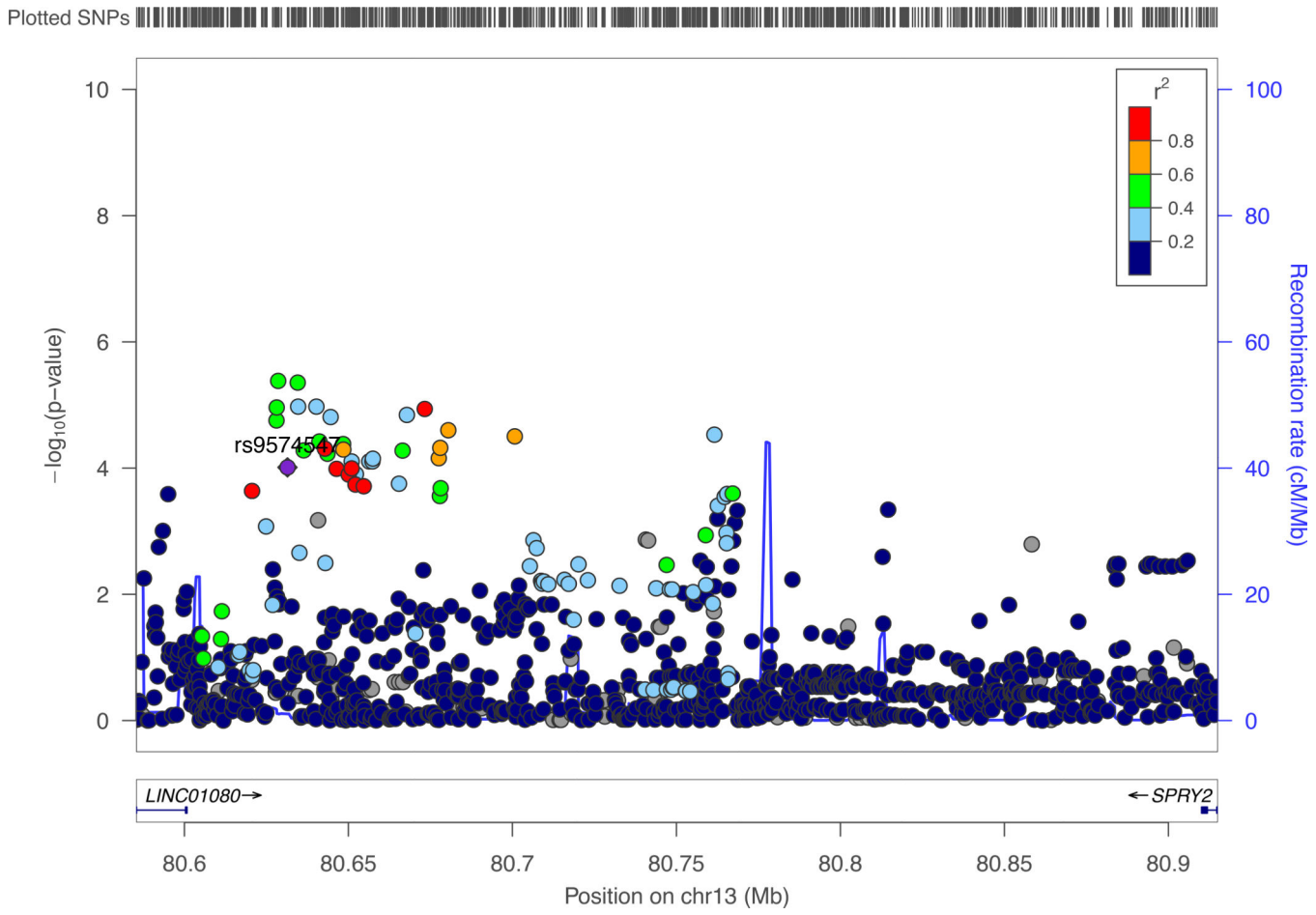

Figure 1.

LocusZoom plot of the *LOC729479 I SPRY2* region based on the discovery cohort. The left x-axis is the $-\log_{10}$ (p-value) of the tests of association and the right x-axis is the recombination rate (cM/Mb). The identified SNP, rs9574547 is plotted as the reference SNP color-coded as a purple diamond. Surrounding SNPs are plotted as circles color-coded by strength of linkage disequilibrium (LD) measured as r^2, where red represents a complete or very strong LD, and blue represents a weak LD or independent variants. LD was calculated based on 1000 Genomes EUR genome build (November 2014).

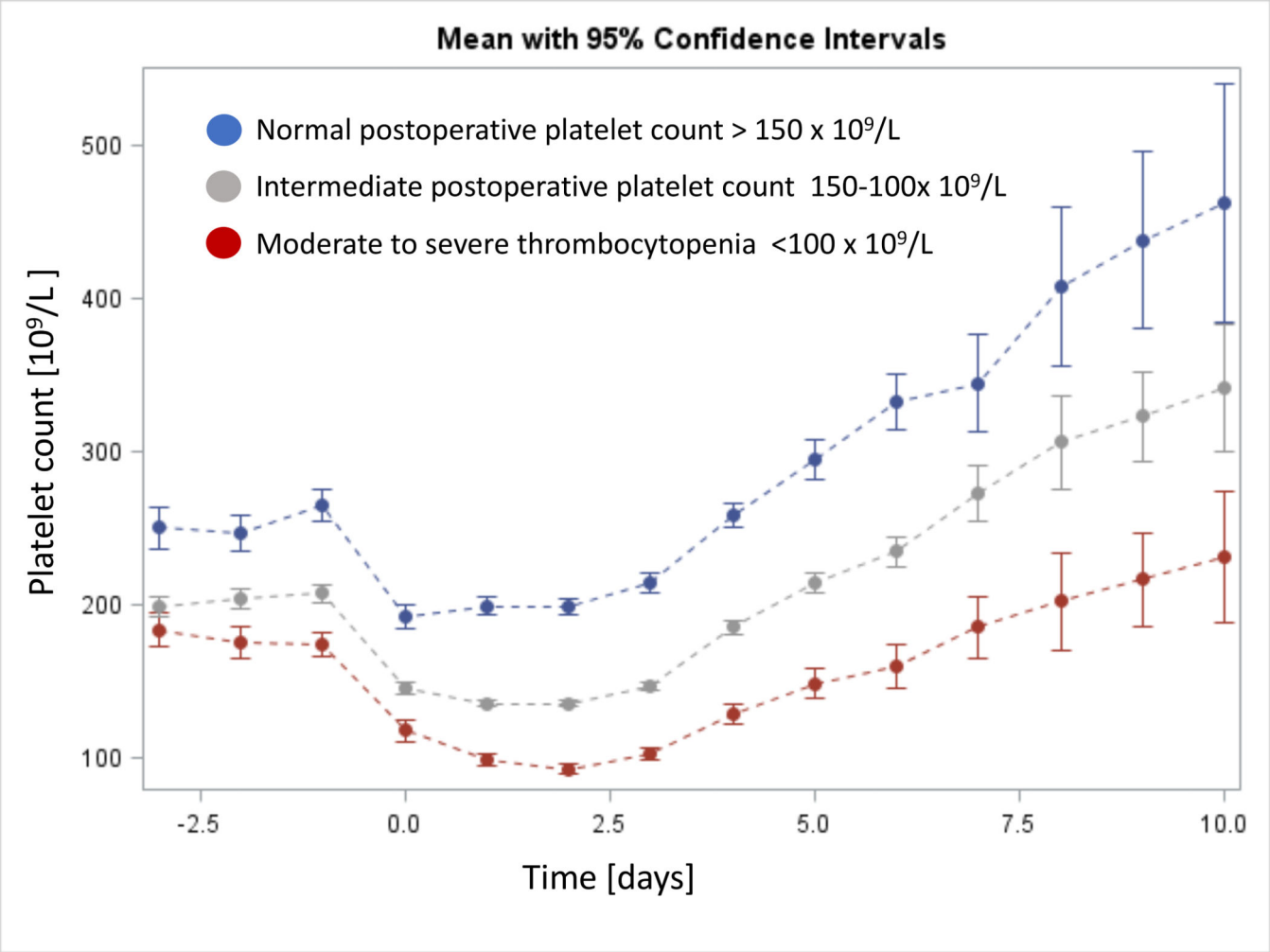

Figure 2:

Development of platelet counts in the perioperative period. Platelet counts (in platelets × 10^9/L) were plotted during the perioperative time period (day −3 to day +10 relative to the day of surgery) for the three groups defined by their nadir postoperative platelet count as noted in the legend. Values are shown as mean with 95% conficence intervals.

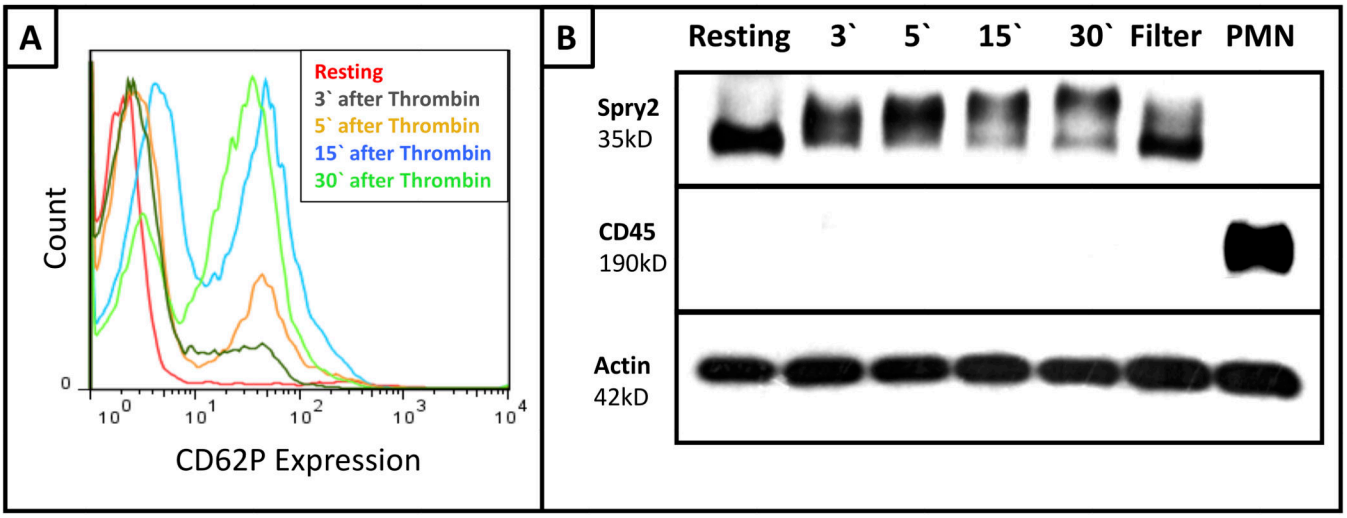

Figure 3:

Sprouty 2 is expressed in human platelets. Human platelets from healthy volunteers were isolated and activated with thrombin for indicated time points [in minutes]. **A)** Flow cytometry was performed from aliquots of samples before platelet lysis and western blotting and surface expression of the platelet activation marker CD62P was measured. **B)** Platelet proteins were separated by gel electrophoresis and probed using anti-Sprouty 2 Antibody. Leukocyte contamination was excluded by passing a subset of unstimulated platelets through a leukocyte reduction filter (“Filter”) and by probing for the leukocyte marker CD45 (isolated polymorphnuclear cells (“PMN”) were used to document positive CD45-staining). Beta-actin served as protein loading control. Protein size is denoted in kilo Dalton (kD). Images are representative of n=5 experiments from distinct volunteers.

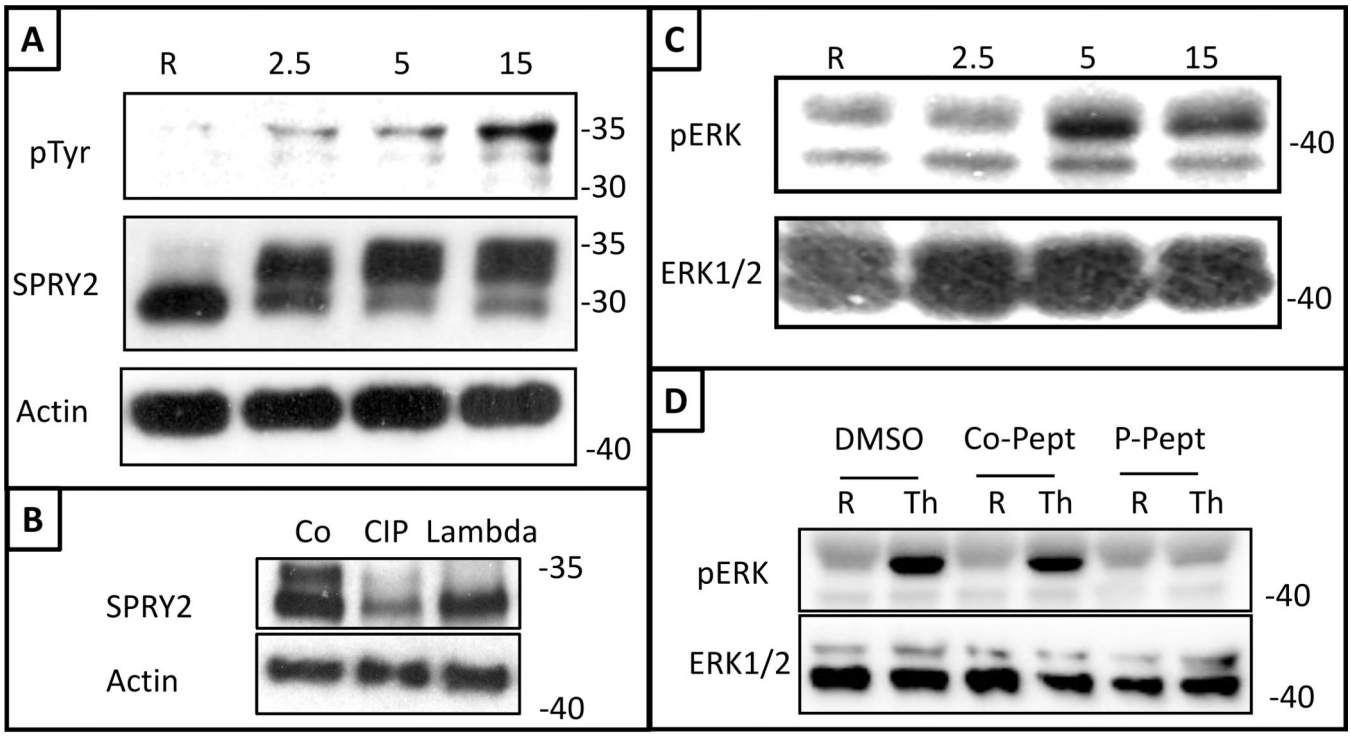

Figure 4: Sprouty 2 modulates ERK phosphorylation pathway during platelet activation.

A) Western blot analysis of platelet lysates following activation with 0.2U/ml thrombin for indicated timepoints probed for phospho-tyrosine (p-Tyr), Sprouty 2 (SPRY2) and b-actin. **B)** Platelet lysate from platelets activated for 5 minutes with 0.2U/ml thrombin was treated with enzyme buffer (co), calf intestinal alkaline phosphatase (CIP) or lambda phosphatase. Western blot was labeled using anti-Sprouty2 (SPRY2) or anti- β-actin antibodies. **C)** Western blot analysis of platelet lysates following activation with 0.2U/ml thrombin for indicated timepoints probed for ERK1/2 (ERK) or phospho-ERK (p-ERK). **D)** Platelet were pre-treated with vehicle (DMSO) or with 50μM of a Sprouty2-octapeptide either in its unphosphorylated (control: co-Pept) or phosphorylated form (P-Pept), exposed to 0.2 U/ml thrombin for 5minutes and then lysed. Western blots were probed for ERK1/2 (ERK) or phospho-ERK (p-ERK). Molecular weight is indicated in kilodalton. Images are representative of 3 independent experiments.

Thromb Haemost. Author manuscript; available in PMC 2019 March 21.

Table 1.

Demographic, and clinical and procedural characteristics of discovery and replication datasets based on presence of moderate to severe postoperative thrombocytopenia

	DISCOVERY DATASET (N=444)			REPLICATION DATASET (N=146)		
Predictor	**Patients with nadir platelet > 150×10^9/L (n=268)**	**Patients with nadir platelet <100×10^9/L (n=176)**	**P-value***	**Patients with nadir platelet >150×10^9/L (n=70)**	**Patients with nadir platelet <100×10^9/L (n=76)**	**P-value***
Demographics						
Age, y	60.86±10.26	66.93±9.82	< 0.0001	56.10±10.82	65.37±9.16	< 0.0001#
Female sex	62 (23.13)	42 (23.86)	0.859	16 (22.86)	30 (39.47)	0.031
Laboratory test result						
Preoperative creatinine, mg/dL	1.0±0.23	1.28±1.40	0.0001	1.06±0.39	1.08±0.33	0.669
Minimum postoperative platelet count, 10^9/L	187.40±36.73	80.52±15.62	< 0.0001	186.74±29.25	78.82±15.16	< 0.0001
Left ventricular function			0.0107			0.399
Normal	178 (66.42)	93 (52.84)		36 (51.43)	42 (55.26)	
Moderate dysfunction	81 (30.22)	71 (40.34)		30 (42.86)	26 (34.21)	
Severe dysfunction	9 (3.36)	12 (6.82)		4 (5.71)	8 (10.53)	
Preoperative medications						
Acetylsalicylic acid	164 (65.60)	111 (69.38)	0.428	41 (62.12)	57 (80.28)	0.019
Angiotensin-converting enzyme inhibitors	134 (50.0)	105 (59.66)	0.046	45 (64.29)	46 (60.53)	0.640
Beta-receptor blockers	206 (76.87)	145 (82.39)	0.162	56 (80.0)	70 (92.11)	0.034
Calcium-channel blockers	52 (20.97)	25 (15.63)	0.178	10 (15.15)	12 (17.39)	0.725
Diuretics	53 (21.37)	44 (27.50)	0.156	16 (24.24)	21 (29.58)	0.482
Nitrates	92 (37.10)	62 (38.75)	0.737	17 (25.76)	25 (35.21)	0.231
Statins	162 (60.45)	123 (69.89)	0.042	47 (67.14)	51 (67.11)	0.996
Intraoperative characteristics						
Duration of cardiopulmonary bypass per minutes	108.29±30.34	131.10±49.44	< 0.0001	110.51±35.06	132.54±47.47	0.002
Duration of aortic-cross clamp per minutes	60.79±21.74	73.82±34.19	< 0.0001	57.46±21.88	72.49±27.95	0.0004#
Intraoperative insertion of intra-aortic balloon pump	5 (1.87)	16 (9.09)	0.0005	4 (5.71)	15 (19.74)	0.014#
Blood product use intraoperatively and postoperative within 2 days of surgery	67 (25.28)	109 (62.64)	< 0.0001	27 (38.57)	51 (67.11)	0.0006

Continuous variables are presented as means ± standard deviation, and categorical variables as number (%).

* Comparisons were made using 2 sample tests to test the differences in demographic, clinical, and procedural characteristics between subjects with and without moderate to severe postoperative thrombocytopenia, separately for discovery and replication datasets.

P-values were derived from the Wilcoxon rank sum tests or 2 sample t tests (#) for continuous variables, and Chi-Square tests or Fisher exact tests (#) for categorical variables, as appropriate.

Table 2.

Logistic regression analysis of *SPRY2* as a predictor of moderate to severe postoperative thrombocytopenia (nadir platelet < 100 ×10^9/L) in the discovery and replication datasets

							DISCOVERY DATASET				REPLICATION DATASET				COMBINED
							MAF				MAF				
CHR	SNP	BP	Gene symbol	Gene location	MiA	MaA	Pt with nadir platelet > 150×10^9/L	Pt with nadir platelet < 100×10^9/L	OR	*P* value	Pt with nadir platelet > 150×10^9/L	Pt with nadir platelet < 100×10^9/L	OR	*P* value	Meta-P
13	rs9574546	80628138	LOC729479 | SPRY2	INTERGENIC	A	C	0.42	0.28	0.45 (0.32–0.64)	1.10×10^{-5}	0.41	0.36	0.85 (0.48–1.51)	0.58	5.44×10^{-5}
13	**rs9574547**	**80631417**	**LOC729479 | SPRY2**	**INTERGENIC**	**A**	**C**	**0.28**	**0.17**	**0.45 (0.30–0.67)**	**9.76×10^{-5}**	**0.27**	**0.19**	**0.47 (0.24–0.92)**	**0.03**	**7.88×10^{-6}**
13	rs4885692	80640222	LOC729479 | SPRY2	INTERGENIC	T	C	0.49	0.38	0.46 (0.32–0.65)	1.06×10^{-5}	0.43	0.41	1.17 (0.67–2.05)	0.58	5.68×10^{-5}
13	rs9318669	80643643	LOC729479 | SPRY2	INTERGENIC	T	G	0.43	0.29	0.49 (0.35–0.70)	5.94×10^{-5}	0.41	0.32	0.74 (0.41–1.33)	0.31	7.01×10^{-5}
13	rs12431307	80644618	LOC729479 | SPRY2	INTERGENIC	A	G	0.49	0.38	0.47 (0.33–0.66)	1.55×10^{-5}	0.47	0.44	0.84 (0.47–1.50)	0.55	5.73×10^{-5}

BP, base pair; Chr, chromosome; MaA; major allele; MAF, minor allele frequency; MiA, minor allele; OR, adjusted odds ratio; Pt, patients; SNP, single-nucleotide polymorphism

*Intergenic region is expressed with "|" between 2 franking genes.

≠Adjusted for age, duration of cardiopulmonary bypass time, and use of blood or blood products intraoperatively and/or within 2 days postoperatively.