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A platelet RNA biomarker of ticagrelor responsive genes is associated with platelet function and cardiovascular events

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Structured Abstract

Background: Identifying patients with the optimal risk:benefit for ticagrelor is challenging. The aim was to identify ticagrelor-responsive platelet transcripts as biomarkers of platelet function and cardiovascular risk.

Methods: Healthy volunteers (n=58, discovery; n=49, validation) were exposed to 4-weeks of ticagrelor with platelet RNA data, platelet function, and self-reported bleeding measured pre/post ticagrelor. RNA sequencing was used to discover platelet genes affected by ticagrelor and a subset of the most informative were summarized into a composite score and tested for validation. This score was further analyzed (1) in CD34+ megakaryocytes exposed to an P2Y12 inhibitor *in vitro*, (2) with baseline platelet function in healthy controls, (3) in peripheral artery disease patients (n=139) vs. patient controls (n=30) without atherosclerosis and (4) in peripheral artery disease patients for correlation with atherosclerosis severity and risk of incident major adverse cardiovascular and limb events.

Results: Ticagrelor exposure differentially expressed 3409 platelet transcripts. Of these, 111 were prioritized to calculate a Ticagrelor Exposure Signature score which ticagrelor reproducibly

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Disclosures

Co-authors (RAM, GSG, DV) are listed as co-inventors on an invention disclosure related to use of platelet RNA biomarkers to predict bleeding due to platelet P2Y12 inhibitors. This article was prepared while Geoffrey S Ginsburg was employed at Duke University. The opinions expressed in this article are the author's own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

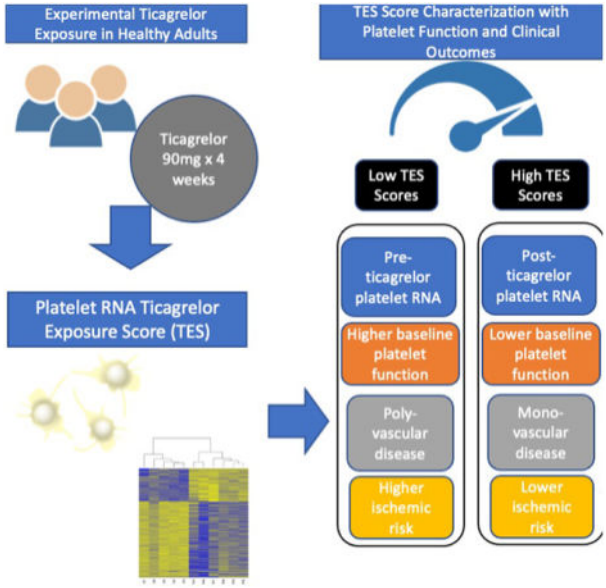
Supplementary Matrial

- Expanded materials and methods
- Tables S1–S7
- Figures S1–S3

increased in discovery and validation cohorts. Ticagrelor's effects on platelets transcripts positively correlated with effects of P2Y12 inhibition in primary megakaryocytes. In healthy controls, higher baseline scores correlated with lower baseline platelet function and with minor bleeding while receiving ticagrelor. In patients, lower scores independently associated with both the presence and extent of atherosclerosis and incident ischemic events.

Conclusions: Ticagrelor responsive platelet transcripts are a biomarker for platelet function and cardiovascular risk and may have clinical utility for selecting patients with optimal risk:benefit for ticagrelor use.

Graphical Abstract



Keywords

Platelet inhibitor; transcriptomics; biomarker; cardiovascular risk; bleeding complications; precision medicine

Introduction

Platelet mediated vascular events such as myocardial infarction (MI), stroke, and limb ischemia are major contributors to global morbidity and mortality. Aspirin alone is increasingly being avoided for primary prevention of MI due to excessive risk of bleeding that outweigh any ischemic benefits.[1] Platelet P2Y12 inhibitors, when added to aspirin, are effective in preventing vascular events following acute coronary syndromes[2 3 4 5] or cerebrovascular events[6] though are associated with an increased risk of bleeding. Ticagrelor is a non-thienopyridine platelet P2Y12 inhibitor and compared with clopidogrel or prasgruel produces more potent and uniform P2Y12 platelet inhibition.[7] Ticagrelor is approved for patients with stable ischemic heart disease with or without diabetes based on randomized trial data.[8] In that trial of patients with stable ischemic heart disease and diabetes, the addition of ticagrelor to aspirin led to a modest reduction in ischemic events, though at the

expense of a clinically significant increase in the risk of bleeding, including intracranial hemorrhage. Other clinical subgroups that may have a more favorable risk:benefit of addition of a P2Y12 inhibitor for prevention of cardiovascular events include those with multiple vascular bed atherosclerosis[9] and prior coronary artery bypass grafting[10]. While these findings represent significant advances, additional approaches are needed to identify additional patient subgroups who are ideal candidates for P2Y12 inhibitors to prevent MI without an excessive risk of bleeding. As has been the approach for statins[11] or proprotein convertase subtilisin/kexin type 9 inhibitors[12] precision medicine approaches can identify patients most likely to benefit from these agents. Such an approach for platelet P2Y12 inhibitors could have clinical utility to identify patients most likely to benefit and/or least likely to be harmed by this effective class of medications and prevent a major adverse cardiovascular event. The objective of this study was to 1) use ticagrelor as a molecular probe to comprehensively characterize the on- and off-target effects of potent and consistent platelet P2Y12 inhibition on circulating platelet RNA 2) to compare these effects with low- and high-dose aspirin and 3) to assess the extent to which these molecular changes contribute to platelet function, and ischemic, and bleeding outcomes.

Methods

All raw and processed RNA sequencing data and platelet function generated from the Discovery volunteer cohort described below are available through the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158765). The corresponding authors will make all other data underlying this article available upon reasonable request.

Overview

An overview of the methods used is depicted in Figure 1. Discovery for ticagrelor-responsive platelet transcripts was carried out using an experimental protocol of aspirin and ticagrelor exposure in healthy volunteers (Figure 1A). Differentially expressed transcripts were prioritized into a Ticagrelor Exposure Signature gene set and summarized into a Score. The Score was tested for validation in an independent healthy volunteer cohort (Figure 1B), an *in vitro* P2Y12 inhibitor exposure in primary megakaryocytes (Figure 1C), platelet function outcomes in healthy volunteers, and for clinical relevance in patients with vascular disease (Figure 1D). The primary effects of aspirin on platelet RNA have been previously described.[13]

Discovery and validation ticagrelor exposure studies in healthy volunteers

We conducted two prospective (Figure 1A and 1B), previously described independent studies in healthy volunteers (NCT05278637).[13] Briefly, we conducted a discovery study where volunteers were randomly exposed to open-label 81mg/day or 325 mg/day aspirin, crossover to the alternate aspirin dose, aspirin washout and ticagrelor 90mg twice daily exposure. Each exposure period lasted four weeks and purified platelet mRNA sequencing (before and after each 4-week exposure) and platelet biomarker analysis (before, 3 hours, and 4 weeks after each exposure) was performed at each time point. Separately, we recruited

a validation cohort of volunteers consisting of only a 4-week ticagrelor 90mg twice daily exposure with identical platelet RNA purification and platelet function analyses.

Inclusion criteria for both cohorts were age ≥ 30 and ≤ 75 and self-reported non-smokers. Key exclusion criteria were a diagnosed bleeding disorder and prescribed daily medications (except oral contraceptives).

Baseline demographic, comorbidity, and concomitant over-the-counter medication data was gathered for each participant. At each visit, phlebotomy for platelet function testing, purified platelet RNA collection, and peripheral blood cell counts was performed using sodium citrate tubes. Self-reported adverse events were collected at each visit and those attributed to ticagrelor based on the drug label were prospectively defined as: shortness of breath, bleeding (hematoma, nosebleed, gastrointestinal, menstrual, subcutaneous, or dermal), and allergic skin reactions (rash or itching).

The Duke University Institutional Review Board approved these study protocols.

In vitro P2Y12 inhibitor exposure validation studies—Primary megakaryocytes were exposed to *in vitro* P2Y12 inhibitor as an additional validation of ticagrelors effects on circulating platelet RNA (Figure 1C). Cluster of differentiation (CD) 34^+ cells were purchased from Fred Hutchinson Cancer Research Center (Seattle, WA), from 4 unique donors were plated in 6-well plates at 2×10^5 cells per mL in StemSpan™ Serum-Free Expansion Medium II (SFEM II, #09655, STEMCELL Technologies) supplemented with 50 ng/mL recombinant human thrombopoietin (#288-TPE-050, R&D Systems), 25 ng/mL recombinant human stem cell factor (#255-SC-050, R&D Systems), 100 U/ml penicillin and 100 μg/ml streptomycin (#15140122, Gibco™) and cultured at 37°C in a 5% CO_2. Cells were passaged into fresh media on days 3, 6 and 9, with the exception that human stem cell factor was removed after day 3. On day 10, cells were labelled with R-Phycoerythrin (Mouse Anti-Human CD42a antibody (#558819, BD Biosciences), isolated with the EasySep™ Release Human R-Phycoerythrin (Positive Selection Kit (#17654, STEMCELL Technologies) and plated on 12-well plates at a concentration of 2×10^5 cells/mL. On day 11, cells were treated with 5 μM $P2Y_{12}$ inhibitor AZD1283 (#27649, Cayman Chemicals) or 0.025% dimethyl sulfoxide (vehicle control) for 24 hours. The samples were lysed in QIAzol® Lysis Reagent (#79306, QIAGEN) and total RNA isolated using Direct-zol RNA MicroPrep columns (#R2062, Zymo Research). RNA quality and quantity were determined with a Bioanalyzer 2100 (Agilent Technologies). Sequencing libraries were barcoded and prepared using the Clontech SMART-Seq HT with Nxt HT kit (Takara Bio USA), and libraries sequenced single end on an Illumina NovaSeq 6000. FASTQ files from RNA-sequencing were analyzed in R studio and differential expression analysis was performed via DESeq2.

Platelet function testing and calculations of a platelet function score

Platelet function testing by light transmittance aggregometry and Platelet Function Analyzer 100 before and after each platelet inhibitor exposure in the healthy volunteer cohorts (Figures 1A and 1C) was performed in the Duke Hemostasis and Thrombosis Core Laboratory as previously described.[14] Briefly, platelet rich plasma was tested for light

transmittance aggregometry using epinephrine (0.5, 1, and 10 uM, 12 minutes), adenosine diphosphate (1, 5, and 10 uM, 6 minutes), and collagen (2 and 5 ug/ml, 6 minutes) and the area under the aggregation curve was recorded. In addition, the Platelet Function Analyzer 100 closure time (seconds) was measured using a collagen/epinephrine cartridge. We have previously described[13, 14] the development and validation of an approach using principal components analysis to summarize the behavior of this panel of platelet function assays into a platelet function score.

Platelet purification, RNA isolation, RNA sequencing in the discovery cohort of healthy volunteers

Methods for generating messenger ribonucleic acid (RNA) sequencing data from purified platelets have been previously described.[13] Briefly, a leukocyte depletion procedure was used to isolate platelets from platelet rich plasma derived from ~ 40ml of fresh whole blood collected in sodium citrate tubes. Samples with sufficient quantity and quality RNA went on to poly(A) messenger RNA capture and construction of stranded mRNA-seq libraries from total RNA and sequenced on a HiSeq 4000 Illumina sequencing platform.

Targeted gene expression analyses in validation cohort of healthy volunteers

Targeted gene expression of selected transcripts identified using RNAseq was performed in the validation cohort using Nanostring technology[15] as previously described[13]. Nanostring profiling of the messenger RNA samples was processed using the R bioconductor package Nanostring QC Pro (v1.8). Digital counts were standardized using positive control probe-based scaling factors, background hybridization levels for each probe and lane were established using both negative control probes and water-only samples, and samples were normalized using platelet-specific housekeeping genes (N = 7) for which the mean count was greater than the mean counts of the negative control probes (mean log2 counts > 5) and expression was stable (quartile coefficient of dispersion < 0.2) in the Nanostring expression data.

Overview of patient cohorts

Two separate cohorts of patients with available platelet RNA were recruited from New York University Langone Health (Figure 1D). The New York University Grossman School of Medicine Institutional Review Board approved the protocol for these cohorts.

Environmental exposures and vascular function in Type 2 Diabetes (EMERALD) Cohort—Methods for this cohort have been published.[16 17] Adults without known cardiovascular disease (CVD) across the glycemic spectrum (with diabetes, glycated hemoglobin ≥ 6.5%, prediabetes, glycated hemoglobin 5.7–6.4%, or normal glucose control) in the 6 months prior to enrollment were recruited from outpatient offices for a study of environmental exposures on non-invasive vascular function. Inclusion criteria included age 18 years; no prior cardiovascular, cerebrovascular or peripheral arterial disease (PAD) and no active tobacco smoking in the past year. Key exclusion criteria include autoimmune, rheumatologic, or inflammatory disease; known active cancer; pregnancy; anemia; chronic kidney disease (creatinine clearance <30 ml/min).

Platelet Activity & Cardiovascular Events Following Vascular Surgery (PACE) Cohort—Methods have been previously described.[18] Briefly, adults age >= 21 years with symptomatic PAD who were referred for lower extremity revascularization. Key inclusion criteria were severe PAD, defined as lower extremity ulceration, gangrene, pain at rest, or a resting ankle brachial index of < 0.6. Key exclusion criteria were non-steroidal anti-inflammatory drug use within 72 hours of the procedure, thrombocytopenia or thrombocytosis, renal failure (creatinine clearance <30ml/min or on dialysis), co-morbid inflammatory disease, cancer, or active infection, severe anemia (hemoglobin < 8 g/dL), or known history of hemorrhagic diathesis.

Peripheral blood for both EMERALD and PACE cohorts was obtained prior to revascularization procedure (in PACE) into sodium citrate tubes. Platelets were isolated by CD45 negative selection and samples with sufficient leukocyte depletion were used for subsequent platelet mRNA sequencing analysis as previously described.[19] Within the PAD cohort, patients were followed for major adverse cardiac and limb events (MACLE) defined as a composite of death, myocardial infarction, stroke, major amputation, or acute limb ischemia leading to revascularization.

Statistical analyses

Healty volunteer discovery cohort—Statistical analyses of platelet RNA sequencing data in the discovery cohort have been previously described.[13] Because the level of leukocyte contamination can greatly influence differential expression, we assessed if *CD45*/*ITGA2B* expression was associated with ticagrelor exposure and found none (p = 0.42). Therefore, no adjustments for the level of leukocyte contamination were made in the primary analysis. Gene expression was modeled as a function of treatment drug, controlling for sex, starting RNA concentration, flow cell, and repeated measures from individual participants. Repeated measures from the same patient were adjusted for by using subject id as a blocking variable and estimating intra-patient correlation and incorporating this into the model covariance matrix. Voom weights[20] were estimated and used in the linear model fit using the empirical bayes method. Generalized linear hypothesis testing (i.e., contrasts) was used to test differences between specific treatment drugs or combinations of treatment drugs. A false discovery rate of less than 5% to determine statistical significance. Analyses were conducted in the statistical program R using packages limma and edgeR.

To prioritize the list of differentially expressed genes to a manageable number amenable for validation we used three criteria described in the Supplemental. Genes that met all three criteria were selected for validation. Briefly these three criteria were:

1. Genes that had average expression (log2 counts per million) > 2 in at least one of the treatment groups (untreated or post treated).
2. Differential expression analysis using three alternative quantification and/or normalization starting points was conducted. Differentially expressed genes with evidence of treatment response in any of these three analyses were prioritized for replication.
3. To fulfill the third criterion, one of the following had to be met:

a. Differentially expressed genes that also appeared in regularized logistic regression multi-gene model of drug exposure.

b. Differentially expressed genes that also were among the top genes within a weighted gene co-expression network analysis module that was differently expressed at an false discovery rate of 5%. Gene ranks within a weighted gene co-expression network analysis module were based on correlation to the module eigengene or connectivity.

Calculation of a Ticagrelor Expression Signature Score—The ticagrelor expression signature (TES) score was calculated as the mean difference of up regulated genes and down regulated genes (directionally based on the RNA-seq discovery analysis). The genes included in the TES Score calculation included all 3409 differentially expressed genes or a subset of 111 that met the prioritization criteria above. Because Nanostring is an alternative measure of gene expression compared to RNA sequencing, 27 of the 111 TES genes did not correlate between Nanostring and RNA sequencing in a subset of discovery cohort samples and thus were excluded from calculation of the TES using Nanostring.

Healthy volunteer validation cohort—TES genes measured by Nanostring gene expression profiling were tested for differential expression was tested using linear mixed effect models, modeling log2 normalized gene expression as a function of ticagrelor treatment and sex, with a participant-level random intercept. Significance of the treatment effect was assessed using a likelihood ratio test of nested models. A TES Score in the validation cohort was constructed similar to the discovery cohort using Nanostring data and was tested for association with treatment using the same mixed effect model as used for the individual genes. The linear relationship between the baseline platelet function score and the TES Score was assessed using 3 models - 1) a linear mixed effect model using both on and off treatment samples while controlling for sex, treatment, and participant level repeated measures, 2) a pre-treatment linear fixed effect model with sex as covariate, and 3) a post-treatment linear fixed effect model, also adjusting for sex.

Meta-analyses—Parameter estimates from the TES associations with platelet function measures, platelet count, and bleeding events were combined across the discovery and validation cohorts using meta-analysis. Parameter estimates were weighted using the inverse variance weights and summed to generate the combined estimates and standard errors, using the R package meta. Both the fixed effect and random effect model for between study variances were used. Significance was assessed using standard normal assumptions for the Z score derived from the combined estimates and standard errors. Similarly, gene-level associations from the discovery and validation studies were combined using the same approach.

Patient cohorts—Briefly, FASTQ files from RNA-sequencing were processed using the Seq-N-Slides pipeline.[21] Reads were aligned to the human genome 38 using Spliced Transcripts Alignment to a Reference v2.6.1.[22] Reads were quantified using featureCounts v1.6.3.[23] Read quality was assessed using FASTQC v0.11.7[24] and fastqscreen.[25] TES scores were calculated as described above by taking the difference between the average value of all

upregulated genes and the average value of all downregulated genes. Univariate comparisons of TES values between groups was performed using Wilcoxon non-parametric rank sum tests. Multivariate analyses were done using generalize linear models with TES and age, sex, race, smoking, diabetes, hypertension, hyperlipidemia, statin use, and antiplatelet (aspirin or clopidogrel) use as covariates. Figures were created using ggplot2.[26]. All analyses and plotting were done in R.

Results

Ticagrelor Exposure and Platelet biomarker outcomes

A summary of the experimental protocol and data analysis for the discovery and validation healthy volunteer cohort studies is summarized in Figure 1A and 1B, respectively. The numbers of patients participating in each cohort is in ST1. In the discovery cohort, 58 participants had a mean age of 43 years, were 67% female, 44% white, and 43% black (ST2) and completed ticagrelor exposure. In the validation cohort, 49 participants had mean age of 43 years and were 58% female, 55% white, and 33% black (ST2) and completed ticagrelor exposure. As expected, a 180 mg ticagrelor loading dose significantly reduced platelet function as measured using a previously described[14] composite platelet function score (PFS) in both the discovery (Figures 1E) and validation cohorts (Figure 1F), as well as each individual assay of platelet function (ST3 and ST4) in both cohorts. After 4 weeks of twice daily dosing with 90 mg ticagrelor, platelet function remained robustly inhibited using the PFS metric (Figures 1E and 1F) as well as using individual measures of platelet function (ST3 and ST4).

Discovery and validation of a ticagrelor exposure signature (TES) gene set and score

In the discovery cohort, there were 14333 transcripts available for statistical analysis after quality control across 312 samples (baseline, post-aspirin, aspirin washout, and post-ticagrelor). At a false discovery rate of 5% ticagrelor exposure was associated with the upregulation of 1820 genes and the down regulation of 1589 genes (Figure 2A). We used a set of prioritization criteria (SF1) based on robustness of association and minimizing correlated transcripts to reduce the set of differentially expressed genes to a smaller number for validation while retaining the biological effects of ticagrelor on global platelet gene expression. After applying these criteria, 111 genes (ST5) remained and were used to define a Ticagrelor Exposure Signature (TES) gene set. Next, the expression levels of each gene within the TES gene set were summarized into a score summarizing the aggregate effects of ticagrelor on these genes. To confirm that the subset of 111 genes captures similar information present in the full 3409 gene set, we correlated the scores using both sets of genes and found that a score calculated on the subset of 111 genes was strongly correlated ($r = 0.92$, $p < 0.001$) with one calculated on all 3402 genes (SF2). In the discovery cohort of healthy volunteers, the TES score increased in response to ticagrelor and was unaffected by low- or high-dose aspirin exposure (Figure 2B).

To independently validate ticagrelor's effects on individual platelet gene expression, we generated a custom transcriptomic assay and measured the expression of the TES genes in an independently recruited validation cohort (n = 49, Figure 1B) and calculated TES scores

on available TES genes as in the discovery cohort. In the validation cohort, we found that ticagrelor significantly increased the TES score (beta = 0.48, standard error = 0.08, p < 0.001, Figure 2C).

Last, we used an *in vitro* exposure of primary megakaryocytes (platelet precursors) to a P2Y12 inhibitor to further validate our findings (Figure 1C). The *in vitro* effect of exposure to P2Y12 inhibition in megakaryocytes on individual genes from the TES was significantly correlated with the effect of *in vivo* ticagrelor exposure on circulating platelet TES genes in healthy volunteers (r = 0.52, p <0.001, Figure 2D).

Therefore, TES represents a group of genes affected by P2Y12 inhibition – but not aspirin

Ticagrelor therapy alters the expression of platelet gene pathways associated with aggregation and granule release

To explore the biologic networks perturbed by ticagrelor exposure, we used weighted gene co-expression network analysis to identify sets of co-expressed genes (or “modules” each named a different color) within the RNA sequencing dataset in an unsupervised manner. Of the 30 unique modules identified, the aggregate expression of 21 was associated (false discovery rate < 5%) with ticagrelor exposure (ST6). We used Gene Ontology to annotate each of the differentially expressed modules with known biological pathways and 11 of 21 had significant Gene Ontology annotations (Figure 3). We identified modules - and by extension the co-expressed genes underlying each module - annotated to endosomes, secretory granules, mitochondrial components, and ribosomes as being affected by ticagrelor exposure. Therefore, ticagrelor exposure affects specific networks of co-expressed platelet genes that characterize platelet secretory or adhesion functions.

Ticagrelor responsive genes and platelet biomarker measurements

To evaluate whether the effect of ticagrelor on platelet genes correlates with platelet activity we compared the TES score with a composite PFS, measured before and after ticagrelor exposure in the combined discovery and validation cohorts. At baseline (i.e., in the absence of antiplatelet therapy exposure) we observed an inverse association between the TES score and the PFS (meta-analysis beta = −0.6, 95% confidence interval: [−1.16, −0.04], p = 0.04, Figure 4A). In contrast, after 4 weeks of ticagrelor exposure, TES was no longer associated with on-treatment platelet activity (meta-analysis p = 0.74, data not shown). While no individual component of PFS was significantly associated with TES scores, their directions of association were all consistent with platelet activity: lower TES scores reflect higher levels of baseline platelet activity (Figure 4B).

To further analyze the inverse relationship between TES scores and baseline platelet function we next analyzed individual TES genes. First, we found that among the TES genes with the strongest positive correlations with baseline platelet function (beta coefficient > 1), 19/26 (73%) were down regulated by ticagrelor. In contrast, among the TES genes with the strongest negative correlation with baseline platelet function (beta coefficient < −1), 7/8 (88%) were up regulated by ticagrelor exposure. Second, for all TES genes, we plotted the magnitude of effect of ticagrelor on regulating that gene in platelets vs. the correlation of that gene with baseline platelet function and found a significant, inverse correlation

(correlation = −0.39, p < 0.001, Figure 4C). This inverse correlation suggests that TES genes that are positively correlated with baseline platelet function tend to be down-regulated by ticagrelor and vice versa.

Thus, using TES scores or individual TES gene expression, lower levels of baseline platelet activity are associated with greater ticagrelor-like effects in circulating platelet messenger RNA.

TES Score correlations with cardiovascular risk in patients and bleeding in healthy participants

Based on the effects of ticagrelor on platelet messenger RNA and platelet function, we next aimed to assess the relationship between the TES score and clinical outcomes, including self-reported bleeding and vascular disease. Patients with cardiovascular risk factors but without established vascular disease (n=30), and patients with established peripheral artery disease (PAD, n = 139) had platelet RNA sequencing performed (Figure 1D) and TES scores calculated. Patients with PAD were older, more likely male, and had more cardiovascular risk factors than those without PAD. (ST7) Antiplatelet therapy was common in the PAD cohort with aspirin used by 86% and clopidogrel (another P2Y12 inhibitor) was used by 37%.

Consistent with the known hyperreactive platelet phenotype in PAD[18, 27], patients with PAD had a significantly lower TES score compared to patients with cardiovascular risk factors but without established vascular disease (Figure 5A, p<0.001), which persisted after adjustment for covariates including antiplatelet therapy (adjusted effect −1.4, standard error = 0.4, p = 0.001). Among patients with PAD, patients with polyvascular disease (i.e., PAD and coronary artery disease) had lower TES scores than those with PAD alone (Figure 5B, adjusted p-value < 0.001). Patients with PAD were followed longitudinally, and during a median follow up of 285 days (IQR: 103–780 days), 69 (49%) patients experienced a major adverse cardiovascular or limb event (MACLE). At the beginning of follow up, TES scores were lower in those patients that went on to develop MACLE compared to those who did not (Figure 5C), which persisted after covariate adjustment, including coronary artery disease (adjusted p-value = 0.006). Thus, a lower TES was independently associated with PAD, polyvascular disease, and incident MACLE.

Based on the association of higher TES scores with ticagrelor exposure and decreased platelet activity at baseline, we explored the association between TES and self-reported bleeding outcomes. Self-reported, minor bleeding was recorded in a small number of participants in the discovery (N = 6/58) cohort during ticagrelor exposure. We stratified TES scores by bleeding and by visit and found that in participants who reported minor bleeding during ticagrelor exposure had higher TES levels at baseline and after ticagrelor exposure compared to non-bleeders (Figure 4D). There was no difference in the magnitude of change in TES between those who did vs. did not report bleeding (p = 0.4, data not shown). Instead, this difference in bleeding was driven primarily by a difference in TES scores at baseline (p-value = 0.06 for difference in TES levels at baseline between bleeders vs. non-bleeders). Therefore, participants more likely to experience minor bleeding during ticagrelor exposure tended to have higher baseline TES scores prior to ticagrelor exposure.

Discussion

Platelet P2Y12 inhibitors such as ticagrelor are widely used to prevent platelet-mediated thrombotic events such as myocardial infarction and stroke. We used genome wide profiling of platelet mRNA before and after ticagrelor exposure to comprehensively characterize the platelet transcriptional response to ticagrelor exposure. We tested the hypothesis that ticagrelor's effects on platelet gene expression are associated with platelet biomarkers including platelet function, vascular risk, and bleeding. We identified a unique set of platelet transcripts that reproducibly change in response to ticagrelor exposure that we summarized as the TES score. We found that ticagrelor (but not aspirin) raises TES scores, and higher TES scores are associated with lower levels of baseline platelet function. Consistently, lower TES scores are associated with vascular risk, and higher baseline TES scores are associated with minor bleeding during ticagrelor exposure. In summary, through a global transcriptional analysis of ticagrelor's effects on platelets, we propose a novel gene signature with potential clinical utility as a biomarker for identifying patients who may benefit the most and be harmed the least by ticagrelor therapy.

Platelets are anucleate cells and therefore are incapable of *de novo* transcription. Instead, platelets are endowed with a repertoire of messenger RNA during thrombopoiesis as part of a regulated process. Our study was not designed to elucidate the mechanism by which ticagrelor alters platelet messenger RNA levels. However, because we found correlation between the *in vitro* effects of P2Y12 inhibition on megakaryocytes and the *in vivo* effects of ticagrelor on platelets (Figure 2D), we hypothesize that megakaryocytic P2Y12 activity leads to changes in circulating platelet messenger RNA levels proportional to the level of global platelet function. This hypothesis is supported by the associations with higher TES levels and decreased baseline platelet function or with vascular risk in patients who were not treated with ticagrelor. We chose ticagrelor for this proof-of-concept series of studies due to its more potent P2Y12 inhibition compared to prasugrel and clopidogrel and our desire to minimized variable levels of P2Y12 inhibition so that we could more readily capture the downstream changes in platelet RNA. We acknowledge, however, that clopidogrel is the most commonly prescribed P2Y12 inhibitor, especially for indications outside of acute coronary syndromes. Given the reproducibility of the findings in the current study with ticagrelor, future work can now move to expansion to clopidogrel and prasgurel through cross-sectional analyses of patients prescribed and adherent to these therapies compared with controls. Although we do not have experimental data surrounding effects of alternative P2Y12 inhibitors on TES gene expression, a small number of patients with PAD were prescribed clopidogrel in our study where we found a trend towards increased TES Scores compared to those not prescribed antiplatelet therapy (Cohen's $d = 0.6$, $p = 0.2$). Therefore, TES gene expression may be sensitive to alternative P2Y12 inhibitors beyond ticagrelor which may have direct effects at the level of megakaryocytes.

We identified that patients with PAD, multivascular disease, and patients with incident MACLE events had lower TES levels independent of potential confounding risk factors. None of these patients were exposed to ticagrelor at the time of platelet messenger RNA collection. Therefore, the finding of lower TES scores with vascular risk can be interpreted as potential to treat such patients with ticagrelor to reduce their vascular risk. Platelet

hyperreactivity is a well-known risk factor for atherothrombotic events[28] and is associated with vascular disease[29]. The inverse correlation with ticagrelor effects on platelet transcripts and platelet function (Figure 4A and 4C) suggests that the finding of lower TES scores in those with vascular disease may be mediated by platelet hyperreactivity in these patients. These findings raise the hypothesis that there may be other patients without PAD who may have similarly low TES Scores (and thus similarly elevated vascular risk) who may derive greater than expected benefit from ticagrelor therapy to prevent their first atherothrombotic event. Given the well-known challenges of measuring platelet function in outpatient clinical settings, a platelet RNA-based biomarker may have more translational potential for risk stratification and guiding P2Y12 therapies. For example, platelet RNA biomarkers could be generated from platelet rich plasma which unlike leukodepleted platelets used in this study could be generated by standard centrifugation in routine laboratories. By stabilizing RNAases at the time of processing, samples could be shipped to centralized facilities where automated instruments for RNA extraction and quantification of a reduced number of TES genes (e.g. <50) and calculation of a TES score is all feasible. Because decisions regarding chronic P2Y12 inhibitor therapy are made in the outpatient setting, rapid turnaround is not required which would facilitate batching results for more efficient testing. Future interventional trials enriching for patients with low TES scores using such an approach, or assessment of genetically mediated lower TES scores and vascular risk will be needed as part of future research.

The main barrier to expanded use of P2Y12 inhibitors is bleeding complications, in particular those that can be fatal such as intracranial bleeding. Excessive platelet inhibition is a well-known predictor of bleeding complications in patients exposed to P2Y12 inhibitors.[30] In an exploratory analysis of the few, minor bleeding events that occurred during a 4-week ticagrelor exposure we found that these participants had higher TES levels throughout our study, particularly at baseline, compared to those who did not report bleeding (Figure 5D). The inverse correlation of ticagrelor effects on platelet messenger RNA and baseline platelet function (Figure 4A) is consistent with the associations with bleeding. Future studies will be needed to extend these preliminary findings to clinically significant, major bleeding complications and to the entire class of P2Y12 inhibitors. If confirmed, they may be useful in identifying patients in whom P2Y12 inhibitors should be avoided because of a heightened risk of bleeding.

Despite the strengths of our study: prospective assessment of drug effects in an experimental model, independent validation, and robust assessment of gene expression changes, there are limitations. First, our trial did not include a placebo control. We believe the independent validation in a *de novo* cohort that was exposed to ticagrelor and the lack of effects during aspirin exposure in the discovery cohort address this limitation. Second, we acknowledge that clopidogrel is the most commonly prescribed platelet P2Y12 inhibitor and that future work will be needed to understand the extent to which the effects of ticagrelor extend to other platelet P2Y12 inhibitors. Third, residual confounding due to unmeasured covariates may explain the associations between TES and vascular risk. Last, the associations between TES Scores and bleeding were exploratory and will require additional validation in larger datasets.

In summary, by using ticagrelor as a molecular probe to characterize the response to this antiplatelet agent, we identified a set of platelet genes that change in response to ticagrelor exposure. The effect of this drug in circulating platelets was associated with platelet function, vascular risk, and potentially bleeding. Together, these findings lay the foundation for a precision medicine approach to identifying patients who may benefit the most or be harmed by the addition of ticagrelor to prevent their first atherothrombotic events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RNA	Ribonucleic acid
PACE	Platelet Activity & Cardiovascular Events Following Vascular Surgery
EMERALD	Environmental exposures and vascular function in Type 2 Diabetes Cohort
CD	Cluster of differentiation
TES	Ticagrelor expression signature
PFS	Platelet function score
MACLE	Major adverse cardiovascular or limb event

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Highlights

- Using ticagrelor as a molecular probe in healthy volunteers we identify a “signature” of ticagrelor’s effects on platelet RNA
- This signature of P2Y12 platelet inhibition correlates with baseline platelet function, *in vitro* effects on megakaryocytes, atherosclerosis burden, and incident atherothrombotic events.
- These findings lay the foundation for a novel approach of individualizing which patients should receive platelet P2Y12 inhibitors using platelet RNA biomarkers

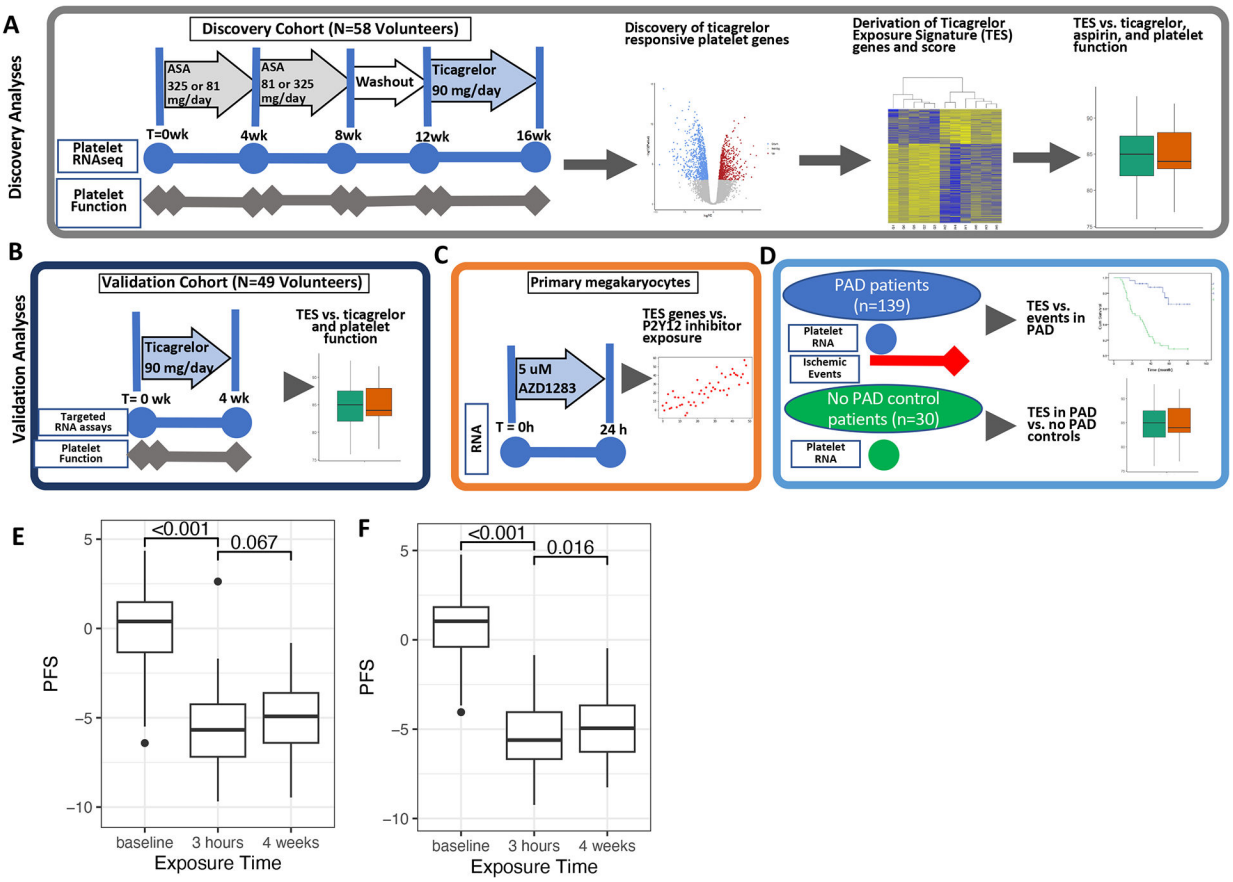

Figure 1. Overview of experimental design and effects of ticagrelor on platelet biomarkers.

Panel A: Overview of exposures in discovery. The discovery cohort began with a baseline visit, randomization to either low- or high-dose aspirin for 4 weeks each, cross over to the other aspirin dose for 4 weeks, aspirin washout, and 4 weeks of ticagrelor exposure. Platelet RNA was collected before/after each exposure and platelet function testing was performed before each exposure as well as 3 hours after the initial and final doses of antiplatelet therapy. Panel B: The validation cohort completed a baseline visit and a single follow up visit after 4 weeks of daily ticagrelor. Panel C: Primary megakaryocytes were exposed to an *in vitro* P2Y12 inhibitor AZD1283 for 24 hours. Panel D: Patients with peripheral vascular disease (PAD) or those with cardiovascular risk factors had platelet RNA collected and compared. PAD patients were also followed for major adverse cardiovascular and limb ischemic events. Panel E and F: An aggregate measure of platelet function (platelet function score [PFS], y-axis) at baseline, 3 hours after witnessed, 180mg ticagrelor loading dose, and after 3 hours after witnessed final 90mg dose of ticagrelor in discovery cohort (Panel E) and validation cohort (Panel F), with t-test p-values liner mixed effect exposure time parameter estimates denoted at the top.

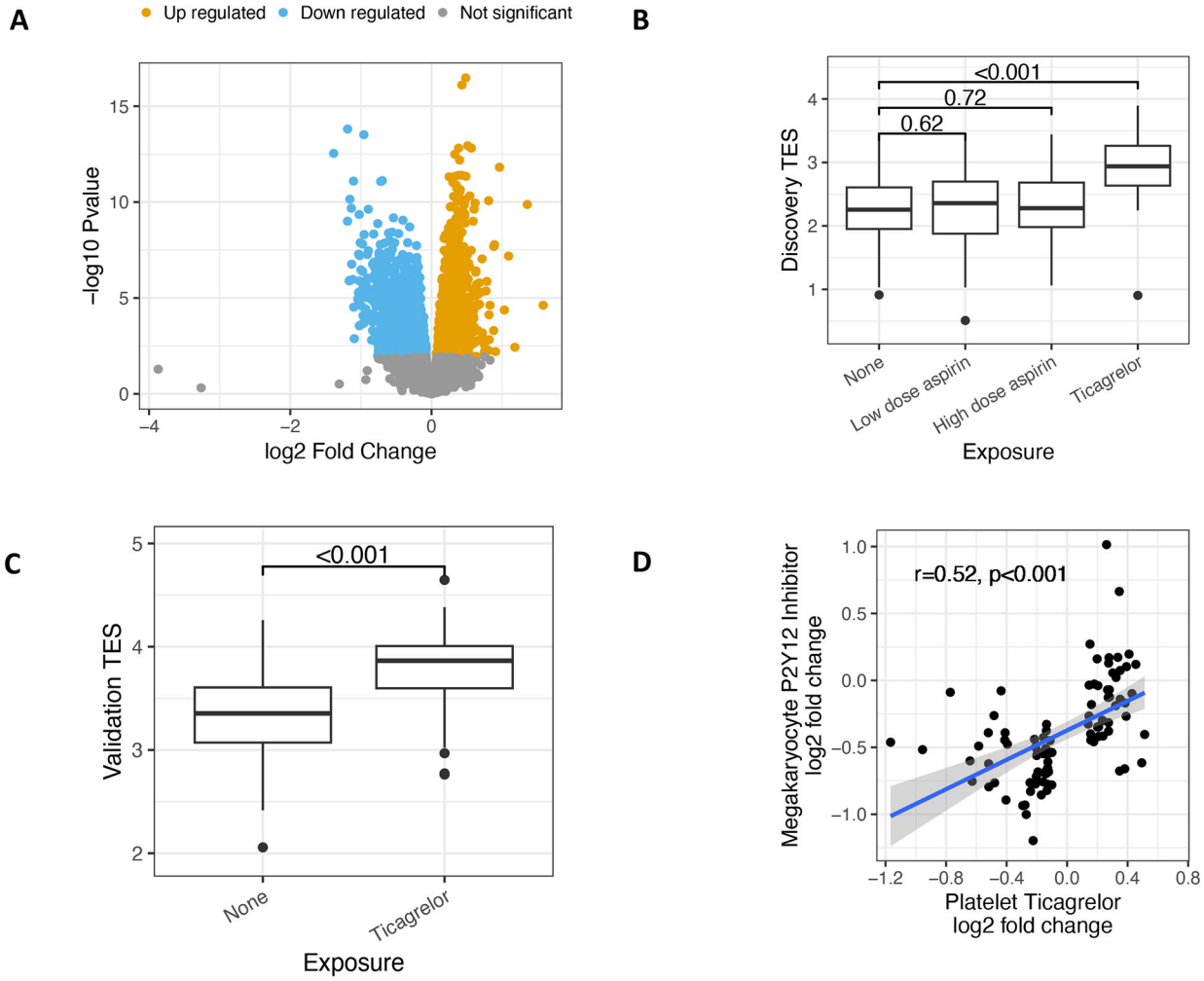

Figure 2. Generation and validation of ticagrelor exposure signature (TES) genes.

Panel A: Volcano plot of 14333 platelet transcripts tested for differential expression with ticagrelor using empirical Bayesian linear regression implemented in R::Bioconductor::limma. Unadjusted p-values are presented on the y-axis, and genes are color coded based on directionality of the association and meeting the experiment-wide false discovery rate threshold of <0.05.. Panel B: Box-whisker plots of TES levels based on 111 genes in Discovery cohort in absence of drug exposure, after low- and high dose aspirin, and ticagrelor exposures. Panel C: TES score based on 84 TES genes measured using Nanostring in Validation cohort. Panel D: The log fold change of *in vitro* exposure to P2Y12i inhibitor AZD1283 in CD34+ derived megakaryocytes (y-axis) for TES genes is plotted against the log-fold change for TES genes in platelets after a 4-week daily ticagrelor exposure in volunteers (x-axis). Unadjusted p-values generated from general linear mixed effect modeling are presented in panels B and C. Spearman correlation coefficient and p-value presented in panel D. “

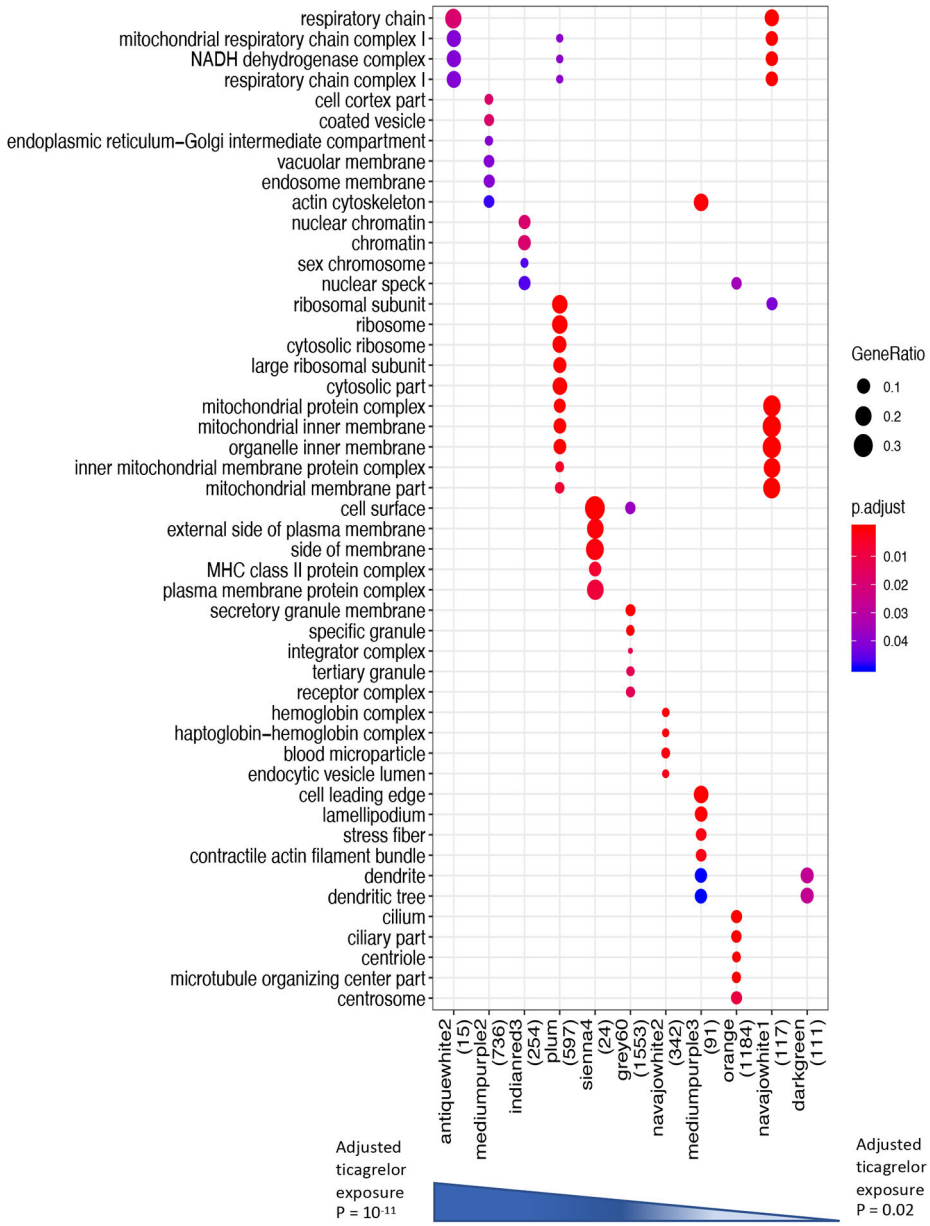

Figure 3. Pathways analysis of differentially expressed genes by ticagrelor.

Gene Ontology analysis of weighted gene co-expression network modules that change in response to ticagrelor exposure. Column names represent individual modules or networks (each named a different color, number of genes in each module in parentheses) and are listed in descending order of strength of association between the module eigengene value and ticagrelor exposure.

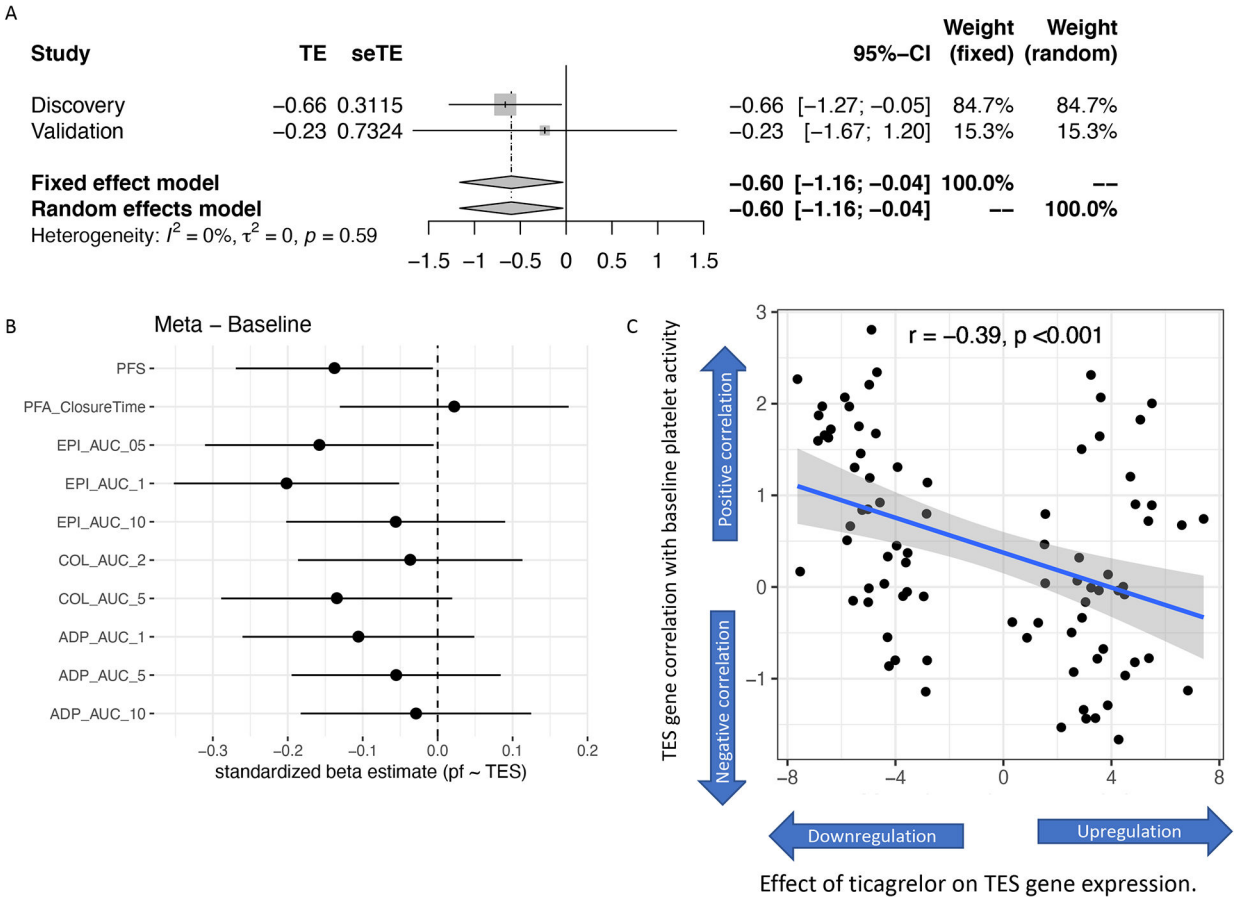

Figure 4. Ticagrelor exposure signature score associations with baseline platelet function measures.

Panel A: Forest plot of association between TES scores measured in Discovery and Validation cohort with the aggregate measure of platelet function, platelet function score, and combined using meta-analysis. Panel B: Forest plot of meta-analysis beta estimates for association between TES score and individual measures of baseline platelet function and the aggregate PFS. Panel C: The effect of regressing each TES gene in baseline platelet RNA from healthy volunteers on baseline platelet function score (y-axis) is plotted against the effect of ticagrelor on each TES genes in healthy volunteers (x-axis). The correlation coefficient and p-value is plotted for the correlation of these effects.

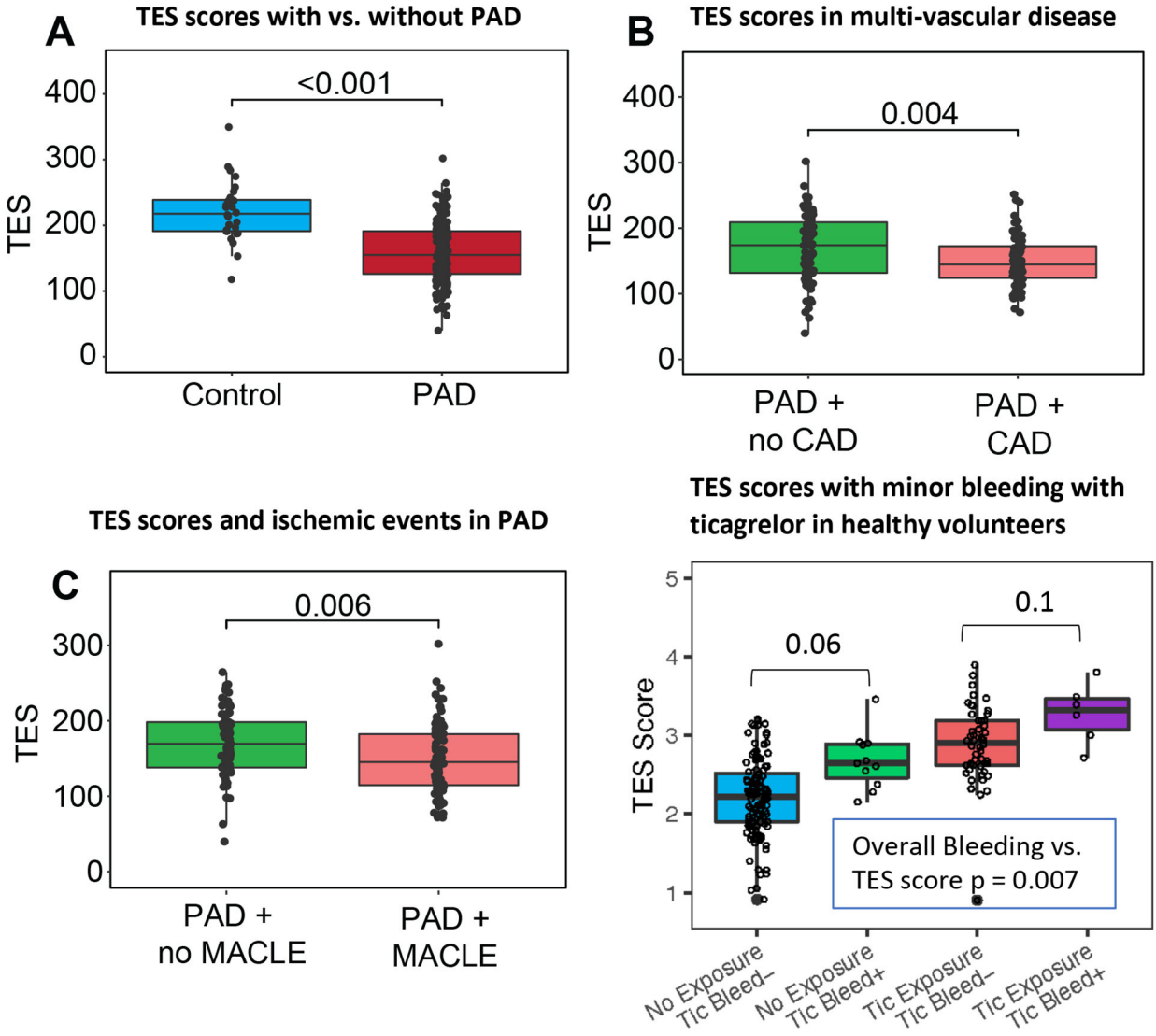

Figure 5. Ticagrelor exposure signature (TES) score associations with cardiovascular risk and minor bleeding.

Panel A: Box-whisker plot of TES scores (y-axis) in patients with peripheral arterial disease (PAD) vs. control patients with vascular risk factors and unadjusted p-value. Panel B: Box-whisker plots of TES scores (y-axis) in patients with PAD with vs. without concomitant coronary artery disease (CAD) and unadjusted p-value. Panel C: Box-whisker plots of TES scores (y-axis) in control patients with vascular risk factors, PAD with vs. without incident major adverse cardiovascular or limb events (MACLE) and unadjusted p-values. Panel D: Box-whisker plot of TES scores (y-axis) in healthy patients at baseline ('No Exposure') and after a 4-week ticagrelor exposure ('Tic Exposure') stratified by those who did not (TicBleed-) vs. did (TicBleed+) report minor bleeding during ticagrelor exposure and mixed-effects p-value comparing TES scores in those who did vs. did not report bleeding. Wilcoxon rank sum tests were used to compare TES scores in Panels A, B, and C. A linear

mixed effect model was used to test the association between bleeding outcomes and TES scores at each exposure in Panel D.