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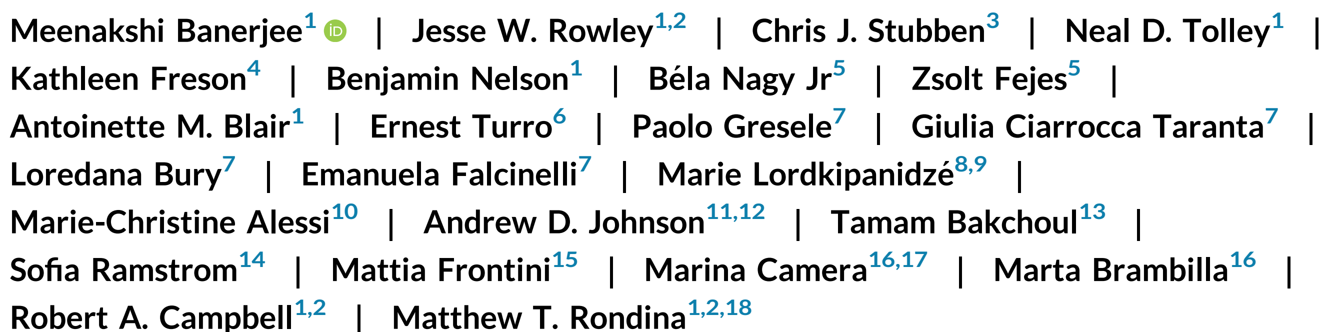
ISTH SSC COMMUNICATION

Prospective, international, multisite comparison of platelet isolation techniques for genome-wide transcriptomics: communication from the SSC of the ISTH



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Abstract

Genome-wide platelet transcriptomics is increasingly used to uncover new aspects of platelet biology and as a diagnostic and prognostic tool. Nevertheless, platelet isolation methods for transcriptomic studies are not standardized, introducing challenges for cross-study comparisons, data integration, and replication. In this prospective multicenter study, called "Standardizing Platelet Transcriptomics for Discovery, Diagnostics,

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and Therapeutics in the Thrombosis and Hemostasis Community (STRIDE)" by the International Society on Thrombosis and Haemostasis Scientific and Standardization Committees, we assessed how 3 of the most commonly used platelet isolation protocols influence metrics from next-generation bulk RNA sequencing and functional assays. Compared with washing alone, more stringent removal of leukocytes by anti-CD45 beads or PALL filters resulted in a sufficient quantity of RNA for next-generation sequencing and similar quality of RNA sequencing metrics. Importantly, stringent removal of leukocytes resulted in the lower relative expression of known leukocyte-specific genes and the higher relative expression of known platelet-specific genes. The results were consistent across enrolling sites, suggesting that the techniques are transferrable and reproducible. Moreover, all 3 isolation techniques did not influence basal platelet reactivity, but agonist-induced integrin $\alpha IIb\beta_3$ activation is reduced by anti-CD45 bead isolation compared with washing alone. In conclusion, the isolation technique chosen influences genome-wide transcriptional and functional assays in platelets. These results should help the research community make informed choices about platelet isolation techniques in their own platelet studies.

KEYWORDS
leukocytes, next-generation RNA-seq, platelets, platelet transcriptomics

1 | INTRODUCTION

Platelets are abundant, anucleated, circulating blood cells known for their roles in hemostasis and thrombosis. Emerging studies highlight that platelets have essential functions spanning immune, inflammatory, and thrombotic continuums [1,2]. Although they lack a nucleus, platelets have a rich and complex transcriptional repertoire consisting of thousands of messenger RNAs (mRNAs), microRNAs, long noncoding RNAs, and other types of transcripts [3–8]. Platelets also harbor alternative structural features of RNA that diversify the platelet transcriptome and proteome, and are known to alter platelet function, including alternative start and stop sites, exon skipping, intron retention, and circular RNAs [7,9–11]. Moreover, it has recently been shown that the platelet transcriptome is not immutable [12]. Rather, the platelet transcriptome changes dynamically in response to systemic inflammation (eg, sepsis) [13], invading pathogens (eg, HIV, dengue, and SARS-CoV-2) [14–16], cancer [17], myocardial infarction [18,19], or other stressors (eg, obesity and weight loss [20,21] and intralipid infusion [22]). Comparative analyses of the platelet transcriptome show marked differences in differential expression of genes between platelets from older and younger healthy donors [23,24]. Platelets are able to synthesize proteins de novo, both basally and in response to *ex vivo* agonists [10,25,26]. This diverse and dynamic transcriptome allows platelets to synthesize new proteins and modulate their actions to participate in mediating host responses. Moreover, the expression of mRNA, long noncoding RNA, and circular RNA in platelets also changes during platelet storage [27]. With the advance of techniques such as next-generation RNA sequencing (RNA-seq), platelet transcriptomics is increasingly being used to discover novel aspects of platelet biology, as diagnostic and prognostic markers, and for therapeutic development efforts [28–33].

Despite the widespread interest in platelet transcriptomics, methods for platelet isolation are not standardized. For example, some procedures use washed platelets, while others employ magnetic bead depletion techniques [13,29]. The choice of procedure can influence several experimental variables, which may ultimately affect the

quantitative characterization of the platelet transcriptome [34]. These variables include the degree of retention of residual leukocytes and red blood cells (RBCs) and the quality and quantity of platelet RNA [35,36]. These preanalytical variables are important to identify and control, yet rigorous studies comparing these techniques in platelet next-generation sequencing studies are absent in the field. These and other differences may limit the comparability, reproducibility, and generalizability of platelet transcriptomic studies. We, therefore, performed a prospective study comparing the performance of commonly used platelet isolation protocols on gene expression metrics from next-generation bulk RNA-seq and functional assays. This study is called the Standardizing Platelet Transcriptomics for Discovery, Diagnostics, and Therapeutics in the Thrombosis and Hemostasis Community (STRIDE) by the International Society on Thrombosis and Haemostasis Scientific and Standardization Committees. Our prespecified primary outcome was the expression of *PTPRC*, encoding for the leukocyte-specific marker CD45, between isolation techniques.

2 | METHODS

2.1 | Participant recruitment

Fifty-three healthy male and female participants were recruited from 4 enrollment sites starting in January 2021 till January 2022: (site 1) the University of Utah in Salt Lake City, Utah, United States (*n* = 22); (site 2) the Centro Cardiologico Monzino, Instituto di Ricovero e Cura a Carattere Scientifico (IRCCS) in Milan, Italy (*n* = 11); (site 3) the Università degli Studi di Perugia in Perugia, Italy (*n* = 10); and (site 4) the University of Debrecen in Debrecen, Hungary (*n* = 10). Two additional sites were approved for participant recruitment, but due to COVID-19–related research restrictions, they were unable to recruit participants for this study. As a result, more participants were recruited at the University of Utah to achieve our prespecified enrollment target of at least 50 participants.

Participants were eligible for inclusion in the study if they were healthy and were not taking any prescription medications or over-the-counter medications potentially affecting platelets (eg, nonsteroidal anti-inflammatory drugs, aspirin, and ibuprofen, or other medications known to affect platelet functions). Participants were excluded if they had diabetes mellitus, had hyperlipidemia (defined as total cholesterol >200 mg/dL or triglycerides >150 mg/dL), had a diagnosis of hypertension, had any heart or lung disease, had a history of any thrombotic event (including coronary artery disease, stroke, transient ischemic attack, or venous thrombosis), had any known inherited or acquired platelet or bleeding disorder, had a diagnosis of any cancer or treatment for cancer within the preceding 6 months, had any blood transfusion within the preceding 30 days, had any acute or chronic liver or kidney disease, used tobacco, had any illness, reported hospitalization or had undergone surgery within the preceding 60 days, had donated blood or plasma within the preceding 6 weeks, had been vaccinated against SARS-CoV-2, or had received a positive COVID-19 diagnostic test result within the preceding 8 weeks of blood collection for this study. At the start of the study in January 2021, COVID-19 vaccines were not yet widely available, so participants enrolled in this study prior to the rollout of the vaccines were all unvaccinated. As study enrollment progressed and COVID-19 vaccines became available, we included COVID-19 vaccination as an exclusion criterion to ensure consistency among enrolled study participants. Women who were pregnant or breastfeeding or had delivered a child within the preceding 6 weeks were excluded from the study. This study was approved by each enrollment center's ethics committee (University of Utah institutional review board [IRB] number 0011947, Università degli Studi di Perugia protocol number 2019-20, Centro Cardiologico Monzino IRCCS protocol number R1309/20-CCM1377, University of Debrecen protocol number 32568-3/2020/EÜIG), and all participants gave written informed consent.

After obtaining informed consent, demographic information was collected from each participant, including age at the time of study enrollment, self-identified ethnicity/race, and self-identified biological sex which was either male or female.

2.2 | Blood collection

Standardized venipuncture kits were sent to each enrolling site for whole blood collection. Supplementary Figure S2 lists the components of these venipuncture kits. Whole blood was drawn using the same equipment and procedure for all samples obtained. Briefly, the antecubital vein in resting subjects was sterilized, visualized, and then accessed using a BD vacutainer UltraTouch Push Button blood collection set with a 21-gauge needle. The first 3 mL of whole blood was drawn into a BD purple top vacutainer tube containing EDTA and was used for whole blood–based cell counting and flow cytometry, where available. If cell counting or flow cytometry was unavailable, this 3 mL of blood was discarded. Next, whole blood was drawn into 6 BD yellow top vacutainer tubes containing acid citrate dextrose solution A, which is a standard anticoagulant used for blood draws that inhibits platelet aggregation. Increased platelet aggregation could lead to platelet clumping and reduced platelet yield for RNA-based studies. Each tube holds a maximum of 8.5 mL of whole blood; therefore, ∼50 mL of whole blood was drawn from each participant.

2.3 | Platelet isolation

The primary objective of the STRIDE study was to test 2 commonly used platelet isolation protocols: (1) a washed platelet isolation protocol (called "washed platelets") and (2) a leukocyte-depleted protocol using CD45 magnetic beads (called "leukocyte-depleted platelets" [LDPs]). During our study, 1 enrolling site had the capacity to test a third protocol using PALL filtration (developed by Pall Corporation), and results from this single site are included in this report (called "PALL-filtered platelets") [37]. Platelets isolated using each technique were obtained from the same starting whole blood sample (∼50 mL) from the same participant (Figure 1 and Supplementary Figure S1).

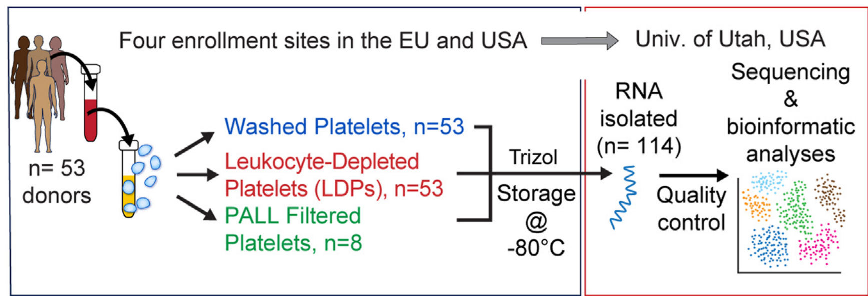

FIGURE 1 Schematic of the overall study design. Blood was drawn, and platelets were isolated from 53 healthy participants recruited from 3 European Union (EU) sites and 1 site in the United States. Once isolated, platelets were lysed in TRIzol, an RNA-preserving buffer, and platelet lysates were frozen at −80 °C. Following the completion of participant recruitment, samples were sent on dry ice to the University of Utah, where platelet RNA was isolated and sequenced on an Illumina platform. LDP, leukocyte-depleted platelet.

Whole blood in acid citrate dextrose tubes was initially centrifuged (spin 1: 100× *g* for 20 minutes, at room temperature [RT], with no brake). The platelet-rich plasma (PRP) was then carefully transferred to a 15-mL Falcon tube (Corning), mixed with 2-μL/mL prostaglandin E_1 (PGE_1), and centrifuged (spin 2: 500× *g*, 20 minutes at RT with no brake). The top platelet-poor plasma layer was then carefully removed without disturbing the lower cell pellet, resuspended in 6-mL PIPES saline glucose (PSG) buffer (prewarmed at 37 °C) containing 2 μL/mL PGE_1, and mixed by gently inverting several times. Next, this resuspended platelet solution in PSG containing 2-μL/mL PGE_1 was split equally into 2 volumes: one 3-mL fraction was used for isolating washed platelets, while the other 3-mL fraction was used for leukocyte depletion to isolate LDPs.

A small volume (30-100 μL) was set aside to count platelet numbers using Sysmex XN-10 or Hemavet 950 from the isolated washed platelets. The rest of the platelet-containing PSG suspension was centrifuged one final time (1500× *g*, 10 minutes at RT with no brake) to pellet the platelets. The supernatant was carefully discarded, and 1 mL of TRIzol (Thermo Fisher Scientific, catalog number 15596026) per 1 × 10^9 platelets was added. Platelets were lysed in TRIzol by vigorous pipetting to ensure no pellet was visible. Samples were incubated at RT for 5 minutes. The tube was labeled with the date and deidentified sample identifier (ID), and stored at −80°C until RNA was isolated.

For LDPs, Invitrogen CD45 Dynabeads were prepared by first suspending them in 2 mL of phosphate buffered saline (PBS)/10-mM citrate buffer (pH 7.4) at a ratio of 50 μL CD45 beads per 2-mL PRP volume collected after spin 1 (Supplementary Figure S2) and then magnetized (~2 minutes) on the DynaMag-15 Magnet column (Thermo Fisher Scientific). Once the beads are magnetized, they will adhere to the side of the Falcon tube (these are visible as a streak of brown beads on the side of the tube). The supernatant was discarded, leaving the magnetized beads adherent to the side of the Falcon tube. To this Falcon tube containing the magnetized CD45 Dynabeads, the leftover 3 mL of platelet-containing PSG fraction was added, and the tube was gently rotated for 20 minutes at RT, allowing the mixing of the platelets in PSG with the CD45 Dynabeads. The tube containing the CD45 Dynabead/platelets in the PSG mixture was then magnetized a second time by placing it on the DynaMag-15 Magnet column for 2 minutes. This enables separation via negative selection where CD45+ cells are bound to CD45 Dynabeads (bead-bound cells) adherent to the side of the tube. In contrast, the CD45-nonbound cells (eg, platelets) do not adhere and thus remain in solution. The solution containing CD45-nonbound cells was then transferred to a fresh 15 mL Falcon tube. A small volume (30-100 μL) was set aside to count platelet numbers using Sysmex XN-10 or Hemavet 950. The remaining volume was mixed with 2-μL/mL PGE_1 and then centrifuged one final time (1500× *g*, 10 minutes at RT with no brake) to pellet the platelets. The supernatant was carefully discarded, and 1 mL of TRIzol per 1 × 10^9 platelets was added. Platelets were then lysed in TRIzol by vigorous pipetting to ensure that no pellet was visible. Samples were incubated at RT for 5 minutes. The tube was labeled with date and deidentified sample ID, and stored at −80 °C until RNA was isolated.

For isolation of PALL-filtered platelets, which was performed only at 1 of the 4 sites, a third of the PRP collected after spin 1 was mixed with 2 μL/mL of PGE_1 and passed through a leukocyte-depletion filter (PALL RC100DJ, Pall Corporation) at RT for 5 minutes and collected in a fresh 15-mL Falcon tube (Supplementary Figure S2). A small volume (30-100 μL) was set aside to count platelet numbers using Sysmex XN-10 or Hemavet 950 (Drew Scientific). Platelets were then lysed in TRIzol (1 mL per 1 × 10^9 platelets) by vigorous pipetting to ensure that no pellet was visible. Samples were incubated at RT for 5 minutes. The tube was labeled with date and deidentified sample ID and then stored at −80 °C. Samples were batch-shipped on dry ice from each enrolling site to the University of Utah, where they were stored at −80 °C until RNA was isolated.

2.4 | Cell counting

Platelet counts and mean platelet volume (MPV) were counted at all sites for each sample collected by a hematologic analyzer (Sysmex XN-10 or Hemavet 950). Additionally, 2 enrolling sites measured the number of white blood cells (WBCs), RBCs, and reticulocytes and the immature platelet fraction (IPF) using a hematologic analyzer (Sysmex XN-10).

2.5 | Platelet RNA isolation

RNA was isolated from all 114 samples by a single investigator at the University of Utah. Washed platelets, LDPs, and PALL-filtered platelet pellets were thawed at RT or following a short exposure (<5 minutes) to

37 °C. RNA was isolated using the standard TRIzol protocol (Thermo Fisher Scientific, catalog number 15596018) by adding 20 μg of glycogen (Sigma, catalog number 10901393001) at the isopropanol precipitation step. Following a 75% ethanol wash, RNA pellets were allowed to air-dry. RNA pellets were next suspended in nuclease-free water. Any potential contaminating DNA was digested for 25 minutes at 37 °C using Turbo DNA Free Kit (Thermo Fisher Scientific, catalog number AM1907), and the final RNA was cleaned and eluted in 26 μL of nuclease-free water using Zymo RNA Clean & Concentrate kit (catalog number Zymo R1017) following the product instructions. RNA concentration was measured using a NanoDrop (Thermo Fisher Scientific) and recorded for each sample. All RNA was stored at −80 °C until library preparation and sequencing at the University of Utah High Throughput Genomics Core. All samples were thawed at the same time to prepare stranded mRNA libraries. Sequencing was done for all the samples at the same time in batches of 20 randomized samples.

2.6 | Next-generation RNA-seq

RNA was submitted to the University of Utah High Throughput Genomics Core for quality assessment and RNA library preparation. Intact poly(A) RNA was purified from total RNA samples (150 ng) with oligo(dT) magnetic beads. RNA concentration was evaluated using a NanoDrop. Purified libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay (catalog numbers 5067-5582 and 5067-5583). Stranded mRNA-seq libraries were prepared using the Illumina TruSeq Stranded mRNA Library Preparation Kit (catalog numbers RS-122-2101, RS-122-2102) with polyA selection. The molarity of adapter-modified molecules was defined by quantitative polymerase chain reaction using the Kapa Biosystems Kapa Library Quant Kit (catalog number KK4824). Individual libraries were normalized to 5 nM, and equal volumes were pooled in preparation for sequencing. RNA was sequenced by an Illumina NovaSeq 6000 sequencer using v1.5 reagents and an S4 flow cell kit to produce 150 × 150 base pair paired-end reads.

The human GRCh38 genome and gene annotation files were downloaded from Ensembl Release 104, and a reference database was created using the RNA-seq aligner STAR version 2.7.9a with splice junctions optimized for 150 base pair reads [38,39]. Optical duplicates were removed from the paired-end FASTQ files using Clumpify v38.34 and reads were trimmed of adapter sequences using Cutadapt 1.16 [40]. The trimmed reads were aligned to the reference database using STAR in 2-pass mode to output a BAM file sorted by coordinates. Mapped reads were assigned to annotated genes using featureCounts version 1.6.3 [39]. The output files from Cutadapt, FastQC, FastQ Screen, Picard CollectRnaSeqMetrics, STAR, and featureCounts were summarized using the MultiQC modular tool (v1.12) to check for sample outliers and determine general statistics (eg, reads sequenced, guanine-cytosine [GC] content, RNA biotypes, mapped reads, and Phred scores) [38–40]. Sequencing data are deposited and publicly available (GSE262073).

Differentially expressed genes at a 5% false discovery rate were identified using the DESeq2 software (version 1.36.0) and hciR package on GitHub for each isolation technique across all 4 sites [38]. First, site and method were combined into a single status group, and pairwise comparisons of interest (eg, Site 1_Washed vs Site 2_Washed) were performed to compare each method across different sites. Second, methods at each site were analyzed separately with an additional patient (ie, donor) variable in the design formula to control for individual patient effects. Finally, to test if the washed vs bead (or washed vs PALL filter or bead vs PALL filter) method differs across sites, we followed the "Group-specific condition effects, individuals nested within groups" section in the DESeq2 software to test interactions while controlling for patient effects.

The variance stabilizing transform (VST) values from DESeq2 were used for the principal component analysis (PCA) and visualizations [41]. The VST values are similar to $\log_2$-normalized counts, except that the variance in low-count genes is reduced. In addition, "removeBatchEffect" in the limma package was used to remove sex-related effects on principal component 2 in the PCA plots. The VSTs were also used in a linear mixed effects model to identify genes where a large fraction of variation in gene expression is explained by biological factors like site, method, patient, sex, age, or *PTPRC* expression or technical factors like RNA quality (RNA integrity [RIN] scores) and RNA concentration. Analyses for genome-wide comparisons were performed in R and ggplot2.

2.7 | Platelet functional studies

Two enrolling sites performed flow cytometry for PAC-1 binding to activated integrin $\alpha IIb\beta_3$. PAC-1 is a pentameric IgM κ-immunoglobulin which detects an epitope on integrin $\alpha IIb\beta_3$ in activated platelets. At one site (site 2), PAC-1 binding to activated integrin $\alpha IIb\beta_3$ was analyzed by flow cytometry in resting (untreated) platelets isolated from washed platelets, LDPs, or PALL-filtered platelets. Briefly, 1×10^6 platelets from each isolation technique were labeled with 20 μL per flow tube of PAC-1 antibody (Fluorescein isothiocyanate [FITC]-conjugated mouse PAC-1, BD Bioscience, catalog number 340507) (t = 30 minutes in the dark at RT). A total of 10 000 platelets per sample was then acquired with a Gallios flow cytometer (Beckman Coulter), and data were analyzed using Kaluza analysis software v1.5 (Beckman Coulter). At another site (site 3), PAC-1 binding to activated integrin $\alpha IIb\beta_3$ on agonist-activated platelets was measured using flow cytometry as follows: 5 μL of either washed platelets or LDPs were diluted in 100 μL of PBS, incubated with agonists adenosine diphosphate (ADP) (10 μM) or protease activated receptor (PAR)-1–activating peptide (Ser-Phe-Leu-Leu-Arg-Asn [SFLLRN], also called thrombin receptor activating peptide [TRAP] [20 μM]; t = 15 minutes for both at RT) or left untreated/resting, and labeled with 20 μL per flow tube of PAC-1 antibody (FITC-conjugated mouse PAC-1, BD Bioscience, catalog number 340507; t = 30 minutes in the dark at RT). Samples were then diluted with 1 mL of PBS, pH 7.4, and 10 000 platelets per sample were analyzed in a CytoFLEX flow cytometer (Coulter Corporation).

3 | RESULTS

The platelet isolation technique influences RNA yield and quantity for transcriptomics

A total of 53 healthy participants from 4 enrolling sites across the European Union and United States were recruited with near-equal representation of male and female sex (Figure 1 and Table). The majority of participants self-identified as Caucasian, while 8% of participants self-identified as Hispanic. From the 53 participants, a total of 114 samples (100% of platelet preparations) were sequenced by the University of Utah Sequencing Core. As shown in Figure 1, there were 53 washed platelet RNA samples, 53 LDP platelet RNA samples, and 8 PALL-filtered platelet RNA samples that underwent sequencing.

We first compared platelet yield (ie, the percentage of platelets recovered) from the 3 isolation techniques. Platelet yield was highest in washed platelet preparations and lower but comparable in both LDP and PALL-filtered platelet preparations relative to corresponding whole blood samples (Figure 2A). The concentration and amount of total RNA obtained after each isolation technique, as measured by NanoDrop, were significantly lower in LDPs and PALL-filtered platelets than in washed platelets (Figure 2B, C). The mean total RNA yield per platelet was 0.053 fg per platelet in washed platelets, 0.023 fg per platelet in LDPs, and 0.015 fg per platelet in PALL-filtered platelets. Nevertheless, both LDPs and PALL-filtered platelets contained sufficient RNA for sequencing. The MPV was similar in washed platelets and LDPs (Supplementary Figure S3A), while the IPF was lower in LDPs (Supplementary Figure S3B). The MPV and IPF were not measured in the small number of PALL-filtered platelet preparations (n = 8).

All 114 platelet transcriptomes were examined for sequencing quality using the MultiQC modular tool (v1.12), including the proportion of bases that are either guanine or cytosine; proportion of unique, multi-, or uncertain mapped reads assigned; the distribution of bases in the different genomic regions (coding, untranslated, intronic, intergenic, or ribosomal); and the distribution of the mean base call quality scores (Phred scores). As shown in Figure 3A–D, sequencing quality was comparable between all 3 isolation techniques and the proportion of RNA biotypes corresponding to ribosomal RNA and mRNA, genes the reads were aligned to. As shown in Figure 3A–D and Supplementary Figure S4, A–Dsequencing quality was comparable between all 3 isolation techniques. Interestingly, LDPs and PALL-filtered platelets had fewer unmapped reads and a higher proportion of ribosomal RNA than washed platelets (Figure 3B, C and Supplementary Figure S4A). Sequencing results from all 114 platelet preparations were used for transcriptomic analyses.

Next, we used PCAs to examine the major components of variation across platelet isolation techniques. LDP and washed platelet samples generally separated according to isolation technique independent of enrollment site along the first principal component, which accounted for the majority of variation when samples were analyzed before or after adjustment for genes on the sex chromosomes

TABLE Demographics of the study participants.

Demographic variables	Number	Percentage
Age (y), mean ± SD	38 ± 11	
Sex		
Male	25	47
Female	28	54
Self-identified race/ethnicity		
Caucasian	43	83
Hispanic	5	8
Asian	4	7
American Indian/Alaskan Native	1	2

(Figure 4A, B). Multivariate analyses examining potential confounding variables revealed that our primary outcome of *PTPRC* expression, which encodes for the leukocyte-specific CD45 protein, was the most prominent factor among these variables contributing to the transcriptome-wide variance (Figure 4C).

3.2 | The degree of leukocyte retention is associated with the relative expression of platelet- and leukocyte-specific genes

Residual WBC counts were also measured in platelet preparations at 1 enrolling site (site 2) by both Sysmex and flow cytometry. In both techniques, residual WBC counts were significantly higher in washed platelets than in LDPs or PALL-filtered platelets (Figure 5A, B). While our protocols did not use CD235a glycophorin bead depletion or RBC lysing techniques to remove RBCs, residual RBCs and reticulocytes were nearly undetectable in both washed platelets and LDPs as measured by a single site (site 1, Supplementary Figure S5A, B). Residual RBCs and reticulocytes were not measured in PALL-filtered platelet preparations.

In agreement with higher residual WBCs in washed platelet preparations, the relative RNA expression of *PTPRC* was significantly higher in washed platelets than in either LDPs or PALL-filtered platelets (Figure 5C). In contrast, the relative RNA expression of the canonical platelet gene *ITGA2B*, which encodes for integrin αIIb protein, was significantly higher in LDPs and PALL-filtered platelets relative to washed platelets (Figure 5D). As we and others have found before, the expression of *ITGA2B* significantly and negatively correlated with the expression of *PTPRC* (Figure 5E) [3,13].

We also compared the relative expression of other canonical leukocyte- and platelet-related transcripts across the 3 isolation techniques. Consistent with our findings with *PTPRC*, the expression of other leukocyte-related transcripts was significantly higher in washed platelets than LDPs or PALL-filtered platelets (Supplementary

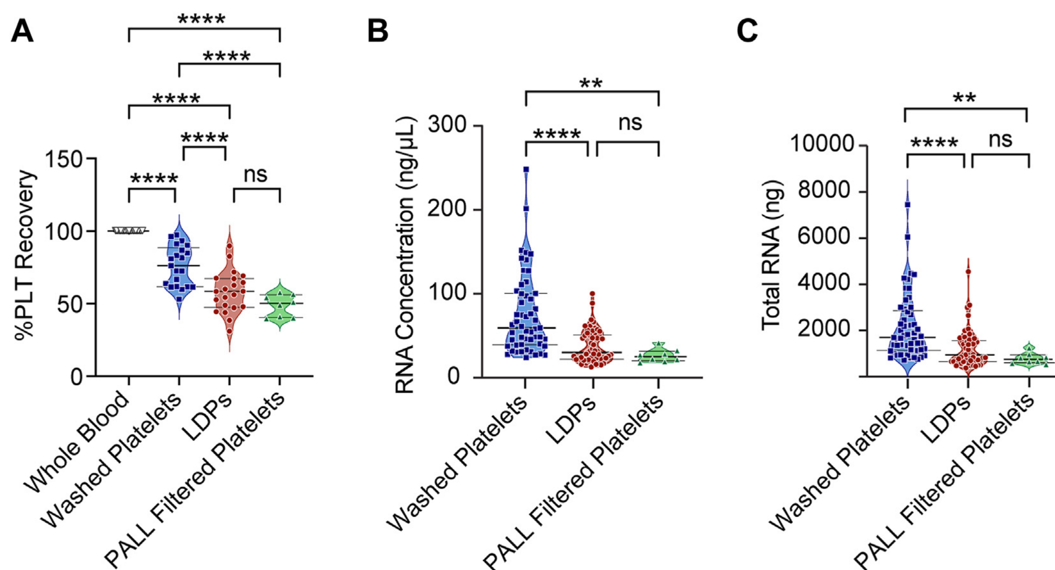

FIGURE 2 Platelet isolation technique influences RNA yield. (A) Percentage of platelets (PLTs) recovered from whole blood and each isolation technique, (B) PLT RNA concentration, (C) total PLT RNA in PLT isolations prepared by washing, leukocyte-depleted PLTs (LDPs), or PALL filtration. Statistical significance was determined using 1-way analysis of variance using Tukey's multiple comparisons test. Violin plots show median plus IQR. ns, not significant. **$P < .01$; ****$P < .0001$.

Figure S6A–C and Supplementary Tables S1 and S2). In contrast, the expression of platelet-related transcripts was generally lower in washed platelets than in LDPs or PALL-filtered platelets (Supplementary Figure S6D–F and Supplementary Tables S1 and S2). When analyzed by biological sex and stratified by isolation technique, the expression of leukocyte- and platelet-related transcripts was similar between male and female participants (Supplementary Figure S7A–H). The relative expression of hemoglobin-related transcripts was lower in washed platelets than in LDPs, also without any sex-specific patterns (Supplementary Figure S8A–F and Supplementary Table S3).

3.3 | The platelet isolation technique influences agonist-induced platelet reactivity

Whether or not the choice of isolation technique influences acute platelet functional responses needs to be better established. Two enrollment sites (sites 2 and 3) probed for platelet functional responses by measuring $\alpha IIb\beta_3$ activation (as measured by PAC-1 antibody binding, which detects an epitope on the activated platelet integrin $\alpha IIb\beta_3$) by flow cytometry. As shown in Figure 6A, basal (or resting) integrin $\alpha IIb\beta_3$ activation (as measured by PAC-1 binding) was minimal and comparable between the 3 different isolation techniques. As shown in Figure 6B, when measured at another site (site 3), basal (or resting) PAC-1 binding was also consistently low and similar between washed platelets and LDPs. In response to protease activated receptor (PAR)-1–activating peptide (SFLLRN, also called thrombin receptor activating peptide [TRAP]) activation, however, PAC-1 binding was significantly lower in LDPs than in washed platelets (Figure 6B). PAC-1 binding did not differ between LDPs and washed platelets following activation with ADP (Figure 6B). This suggests that leukocyte bead depletion during isolation may affect agonist-induced functional responses in platelets.

4 | DISCUSSION

In this prospective, international, multicenter, head-to-head study, we compared platelet RNA quality and quantity, genome-wide and targeted transcriptional expression, and functional responses across common approaches for isolating human platelets from whole blood. Our prespecified primary outcome was the expression of *PTPRC*, encoding for the leukocyte-specific marker CD45, between isolation techniques.

All platelet isolation techniques yielded sufficient RNA in quantity and quality, adequately suitable for next-generation sequencing. We did find that platelet RNA yield was lowest in LDPs using anti-CD45 Dynabeads. We speculate that platelets endogenously adherent to leukocytes, which express CD45, may be removed with the use of anti-CD45 Dynabeads. If true, this may be a cause for the lower platelet RNA yields with CD45 depletion. Nevertheless, RIN scores were comparable across all 114 samples derived from the 3 isolation techniques (average RIN scores of 5.5 for washed platelets, 4.9 for LDPs, and 5.4 for PALL-filtered platelets) and sufficient to perform next-generation sequencing. Perhaps expectedly, we found that the relative expression of *PTPRC* was highest when a series of centrifugation and washes were used to isolate platelets and lowest when either CD45 magnetic beads (eg, LDPs) or a PALL filter were used to deplete leukocytes further. High expression of *PTPRC* in washed platelet preparations was accompanied by higher numbers of residual leukocytes and higher expression of other canonical leukocyte genes, including *SELPLG*, *ITGB2*, and *CD3D*. We also observed that reads

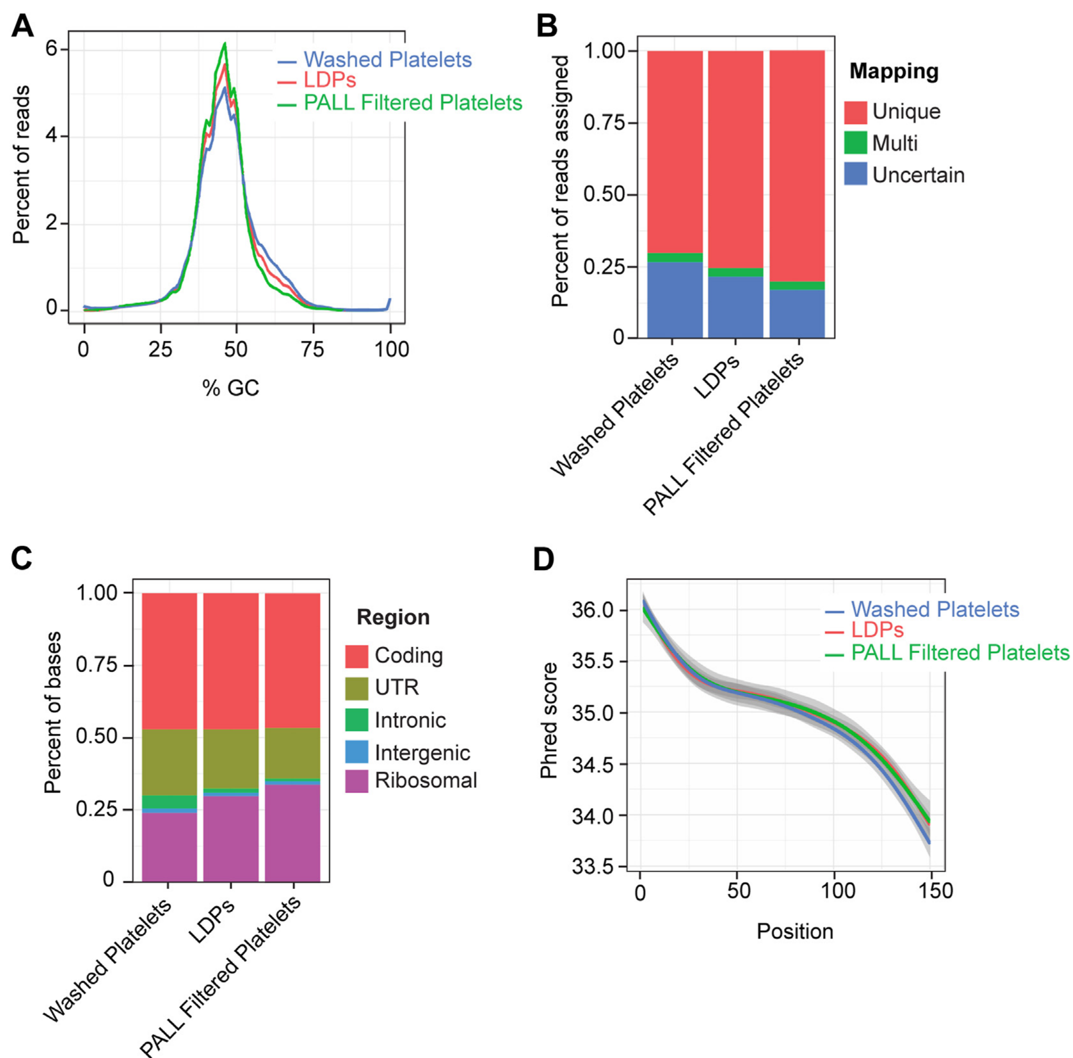

FIGURE 3 RNA sequencing metrics were similar across platelet isolation techniques. (A) Histogram of guanine-cytosine (GC) content, (B) RNA read mapping, (C) base assignment (UTR), and (D) histogram of Phred scores in platelet isolations prepared by washing, leukocyte-depleted platelets (LDPs), or PALL filtration. UTR, untranslated region.

mapping to intronic regions were greatest in washed platelet preparations and lower in both LDPs and PALL-filtered platelets. Platelets generally have fewer introns [34,35,42], and therefore, are consistent with our findings that the relative expression of leukocyte genes was highest in washed platelet preparations.

In contrast, the relative expression of platelet-related genes (eg, *ITGA2B*, *GP9*, *PF4*, *GP1BA*, and others) was generally higher in LDPs or PALL-filtered platelets than in washed platelets. The expression of select leukocyte- and platelet-related transcripts was comparable between LDPs and PALL-filtered platelets, consistent with genome-wide comparisons by PCA. Significantly higher expression of *PTPRC* in washed platelet preparations, compared with LDPs, was consistent across all 4 enrolling sites. This suggests that teams across the globe could readily and reproducibly implement techniques for obtaining LDPs.

Our results are concordant with an independent study examining the influence of 2 platelet isolation protocols on the platelet transcriptome by microarray analysis in a single donor [43]. While there were some differences between the 2 protocols tested in the study by Chebbo et al. [43] and in our study, both studies were similar in that they primarily examined how the use of anti-CD45 beads in platelet isolation protocols influenced the purity of the platelet preparation. Both studies also found that compared to washing alone, the use of anti-CD45 beads to obtain LDPs was associated with reduced numbers of leukocytes, reduced relative expression of leukocyte-related transcripts, and increased relative expression of platelet-related transcripts.

These findings of increased relative expression of leukocyte transcripts and decreased relative expression of platelet transcripts in platelets isolated by washing alone can be reasonably attributed to the residual leukocytes remaining in preparations [36]. In platelets prepared by centrifugation and washing alone (eg, without the use of anti-CD45 beads), the degree of WBC contamination was 309 ± 350

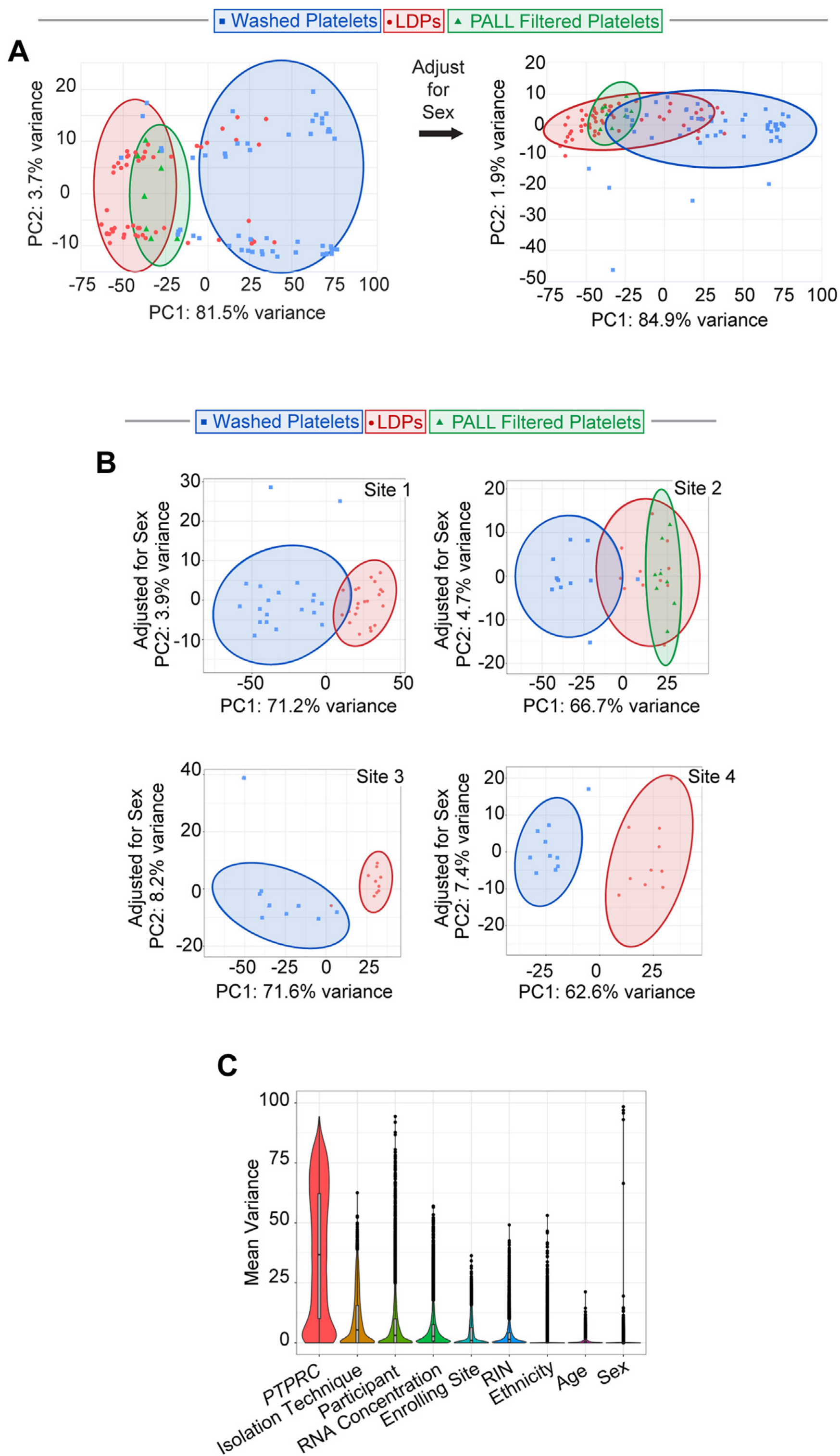

FIGURE 4 *PTPRC* expression is associated with the highest variance in the platelet transcriptome. (A) Principal component (PC) analyses of all 114 platelet RNA samples from 4 enrollment sites based on isolation technique. The left PC plot is before adjustment for biological sex, while the right PC plot is after adjustment for biological sex. (B) PC analyses were separated by individual enrolling site after adjustment for biological sex. Note that site 2 also isolated platelets by PALL filtration (green). (C) Multivariate analysis with violin plots shows the fraction of mean-variance within each sample attributable to each variable. The thick horizontal line indicates the median with the IQR. Each dot represents an RNA sequencing dataset that lies outside the IQR. RIN, RNA integrity. LDP, leukocyte-depleted platelet.

leukocytes per 10^9 platelets for washed platelets, 10 ± 6.3 leukocytes for LDPs, and <5 for PALL-filtered platelets as measured by Sysmex and flow cytometry.

Leukocytes contain an estimated 1000 times more RNA than platelets [6,44]. Therefore, when genome-wide RNA expression is measured on platelets prepared without more stringent leukocyte removal strategies and the sequencing data subsequently normalized through bioinformatic pipelines, the expression of leukocyte genes will be overrepresented (and thus relatively increased or upregulated). In contrast, the relative expression of platelet genes will be underrepresented and, therefore, relatively decreased or downregulated. We postulate that the method of isolating platelets may influence the

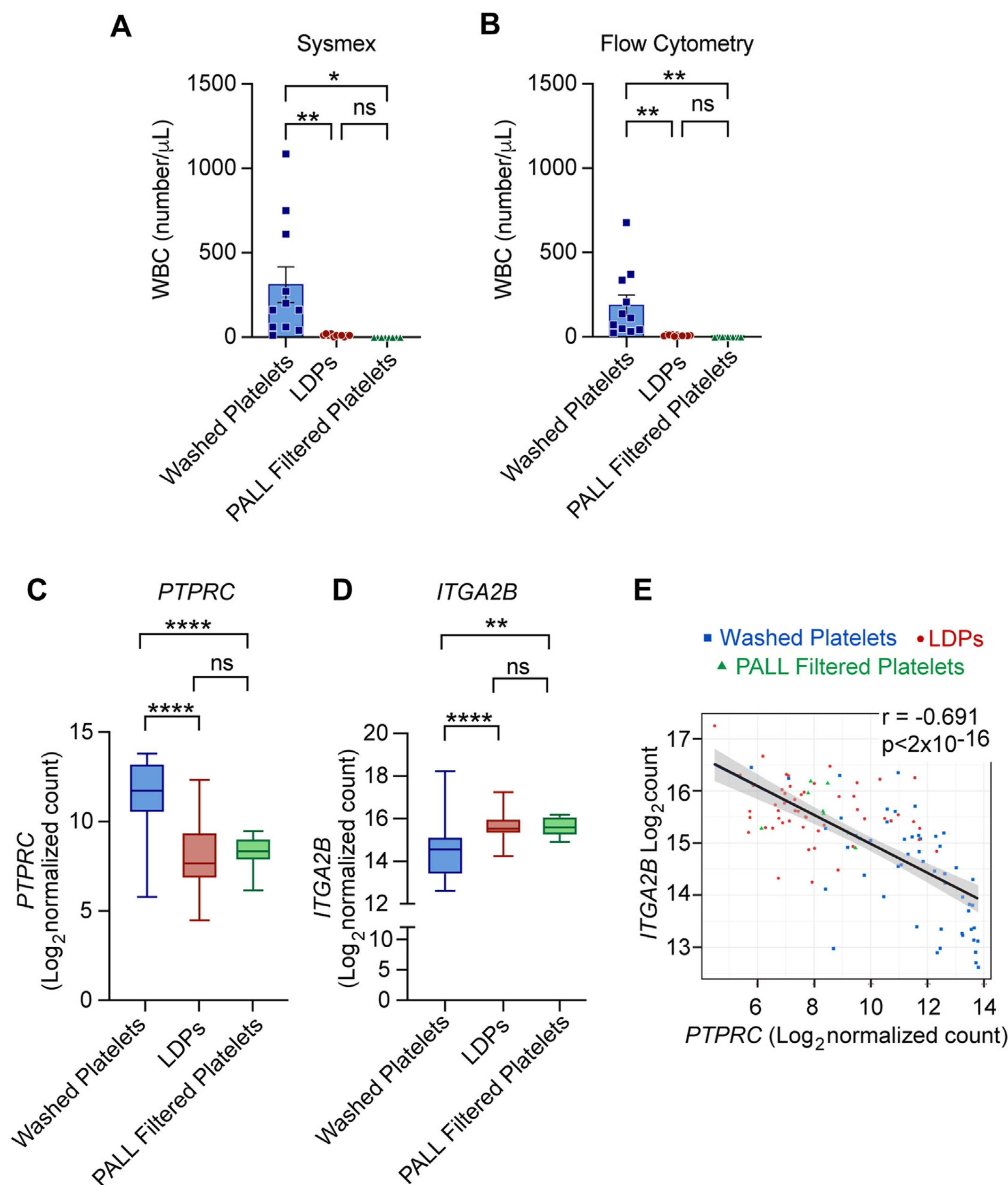

FIGURE 5 The number of residual leukocytes and the relative expression of the leukocyte transcript *PTPRC* and the platelet transcript *ITGA2B* are influenced by platelet isolation technique. Residual leukocyte counts, plotted as mean ± SEM, were measured by (A) Sysmex or (B) flow cytometry in washed platelets, leukocyte-depleted platelets (LDPs), and PALL-filtered platelets. (C,D) Normalized RNA expression was plotted as a median with IQR of (C) *PTPRC* and (D) *ITGA2B* in washed platelets, LDPs, and PALL-filtered platelets. (E) Correlation between *PTPRC* and *ITGA2B* expression in platelet preparations. Statistical significance was determined using 1-way analysis of variance using Tukey's multiple comparisons test and Pearson's correlation). ns, not significant; WBC, white blood cell. $^{*}P < .05$; $^{**}P < .01$; $^{****}P < .0001$.

output of differential gene expression analyses. Bioinformatic strategies correcting for high or discordant levels of *PTPRC* expression in comparative transcriptomics may help minimize this confounding, and we are currently working on developing these techniques. On the other hand, RBCs being anucleate like platelets lack typical transcriptomes with their overall RNA content being relatively low [45]. However, they are enriched in globin mRNAs, which is consistent with our findings and indicative of low-grade RBC contamination in our platelet isolates (Supplementary Figure S8) [45].

Isolation techniques did not influence resting or basal integrin $\alpha IIb\beta_3$ activation, suggesting that a more rigorous isolation technique does not artifactually induce platelet activation as measured by this single assay. However, integrin $\alpha IIb\beta_3$ activation to a widely used platelet agonist, PAR-1–activating peptide, was significantly lower in LDPs than in washed platelets. Whether or not this extends to other functional assays, such as aggregation, spreading, and clot retraction, is unknown. Nevertheless, these findings may have implications for investigative teams wishing to perform functional studies on platelets

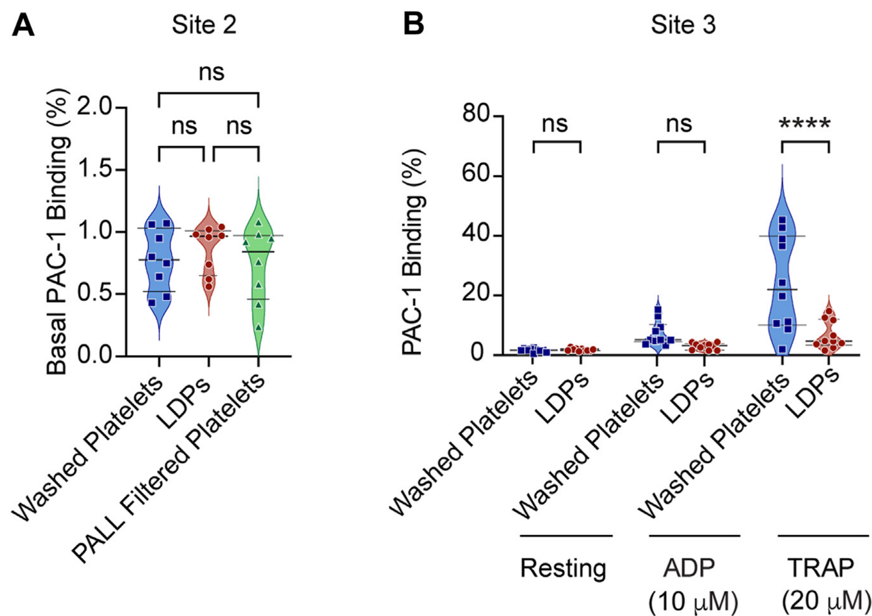

FIGURE 6 Platelet isolation technique influences integrin αIIbβ3 activation. (A) Basal integrin $\alpha IIb\beta_3$ activation as assessed by PAC-1 antibody (which detects an epitope on the activated platelet integrin $\alpha IIb\beta_3$) binding measured by flow cytometry on washed platelets, leukocyte-depleted platelets (LDPs), or PALL-filtered platelets at site 2. (B) Integrin αIIbβ3 activation as assessed by PAC-1 antibody binding in unstimulated (resting), adenosine diphosphate (ADP)-stimulated, or PAR-1–activating peptide (Ser-Phe-Leu-Leu-Arg-Asn [SFLLRN], also called thrombin receptor activating peptide [TRAP])–stimulated washed platelets and LDPs at site 3. Statistical significance was determined using 1-way analysis of variance using Tukey's multiple comparisons test. Violin plots show median plus IQR. ns, not significant. ****$P < .0001$.

while isolating platelets for RNA analyses [46]. In these settings, we suggest removing an aliquot of PRP before incubation with CD45 magnetic beads (or a PALL filter) for any planned functional studies.

We acknowledge that there are limitations to our study. There was relatively limited diversity in ancestry among our participants. Nevertheless, we have no reason to believe that our findings would not apply across various ancestries. Our study participants were all healthy and generally younger, limiting our ability to generalize to diseased states where platelets and leukocytes are activated and to settings of older biological age. To the best of our knowledge, head-to-head comparisons of platelet isolation techniques, as done in the current study, are yet to be performed in disease settings. Whether or not the isolation technique influences residual leukocyte numbers and *PTPRC* expression during disease is unknown. Still, it can be expected that the isolation of platelets without leukocyte contaminants will be more difficult in patients with severe (macro)thrombocytopenia. In those cases, a positive selection of platelets using CD41 beads may be considered to circumvent low platelet yields. Moreover, in individuals with genetic variants affecting both platelets and RBCs (eg, inherited dominant negative growth factor independence 1B variants) [47,48], depleting both leukocyte (using CD45) and RBC (using CD235) beads may be considered to improve platelet yields for adequate RNA analyses. We also acknowledge that not all enrolling sites performed functional assays and leukocyte counts. Enrollment of participants in this study occurred during the COVID-19 pandemic, when research restrictions were limiting, and not all enrolling sites had the equipment to perform every assay. We also were unable to assess directly platelet–leukocyte interactions at baseline or upon activation. Finally, this study did not compare results of directly pelleting PRP and isolating and sequencing RNA, where relative platelet counts might be much higher. Still, WBC contamination might be hypothesized to be an issue similar to washed platelets.

5 | CONCLUSIONS

In conclusion, isolation approaches influence genome-wide and targeted transcriptional analyses in platelets. Leukocyte depletion is readily achievable for laboratories but may affect platelet responses in functional assays. For studies of platelet bulk transcriptomics, we suggest that investigative teams consider the relative advantages and disadvantages of these platelet isolation techniques to determine their preferred method of platelet isolation.

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AUTHOR CONTRIBUTIONS

M.B., J.W.R., C.J.S., N.D.T., K.F., B.N., B.N. Jr., Z.F., A.M.B., E.T., P.G., G.C.T., L.B., E.F., M.L., M.-C.A., A.D.J., T.B., S.R., M.F., M.C., M.B., R.A.C., and M.T.R. contributed to study design, study completion, and/or data acquisition and analyses. M.B., J.W.R., M.T.R., E.T., P.G., M.L., M.-C.A., A.D.J., S.R., M.F., M.C., and R.A.C. contributed to manuscript preparation. All authors read and approved the final manuscript.

DECLARATION OF COMPETING INTERESTS

M.T.R. reports patents pending or issued on using platelet transcriptomics. All other authors have no conflicts to declare.

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SUPPLEMENTARY MATERIAL

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