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ORIGINAL ARTICLE

Cold-stored platelet hemostatic capacity is maintained for three weeks of storage and associated with taurine metabolism

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Abstract

Background: Platelet (PLT) product transfusion is a life-saving therapy for actively bleeding patients. There is an urgent need to maintain PLT function and extend shelf life to improve outcomes in these patients. Cold-stored PLT (CS-PLT) maintain hemostatic potential better than room temperature–stored PLT (RT-PLT). However, whether function in long-term CS-PLT is maintained under physiological flow regimes and/or determined by cold-induced metabolic changes is unknown.

Objectives: This study aimed to (i) compare the function of RT-PLT and CS-PLT under physiological flow conditions, (ii) determine whether CS-PLT maintain function after 3 weeks of storage, and (iii) identify metabolic pathways associated with the CS-PLT lesion.

Methods: We performed phenotypic and functional assessments of RT- and CS-PLT (22 °C and 4 °C storage, respectively; $N = 10$ unique donors) at storage days 0, 5, and/or 21 via metabolomics, flow cytometry, aggregation, thrombin generation, viscoelastic testing, and a microfluidic assay to measure primary hemostatic function.

Results: Day 21 4 °C PLT formed an occlusive thrombus under arterial shear at a similar rate to day 5 22 °C PLT. Day 21 4 °C PLTs had enhanced thrombin generation capacity compared with day 0 PLT and maintained functionality comparable to day RT-PLT across all assays performed. Key metrics from microfluidic assessment, flow cytometry, thrombin generation, and aggregation were associated with 4 °C storage, and metabolites involved in taurine and purine metabolism significantly correlated with these metrics. Taurine supplementation of PLT during storage improved hemostatic function under flow.

Conclusion: CS-PLT stored for 3 weeks maintain hemostatic activity, and storage-induced phenotype and function are associated with taurine and purine metabolism.

KEYWORDS
blood platelets, hemostasis, metabolism, microfluidics, platelet function tests

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1 | INTRODUCTION

Cold-stored platelets (CS-PLT) have better maintained hemostatic function compared to storage at room temperature *in vitro* [1] and have demonstrated short-term *in vivo* efficacy [2]. Hence, there has been a resurgence in interest in their use during hemostatic resuscitation of patients with active bleeding, as witnessed by many clinical trials currently underway (NCT05220787, NCT04834414, NCT04667468, NCT05820126, and NCT04726410). CS-PLT preserved hemostatic function has been demonstrated *in vitro*, in some experiments up to 21 days of storage at 1 to 6 °C [3–7], primarily via measurement by clinical platelet function assays [8,9]. However, due to their stagnant/static nature, these commercially and clinically available assays do not account for physiological flow, an important regulator of platelet function *in vivo* [10,11]. Moreover, while these assays exist to characterize platelet function, it is unclear how assay endpoints translate into meaningful clinical therapeutic value in the context of managing bleeding patients. Finally, how metrics generated by each of these assays may be interrelated with respect to stored PLT function is poorly defined.

In the US, CS-PLT (either whole blood–derived or apheresis products) are licensed for use within 72 hours of collection (CFR 640.24(d)). Seven blood collection centers in the US and the US Army, Navy, and Air Force Blood Programs have received a variance from the Food and Drug Administration (FDA) for storage out to 14 days when standard PLT are not available or practical for actively bleeding patients. In June 2023, the FDA published guidance allowing for alternative procedures to produce 14-day CS-PLT streamlining the process for their production and use [12]. US providers are able to use low-titer type O whole blood (LTOWB) stored for up to 3 to 5 weeks with refrigeration depending on the manufacturing method and storage solution, and *in vitro* data demonstrate that platelets in 3-week-old LTOWB maintain hemostatic function [13–15]. The majority of CS-PLT literature has established hemostatic function out to 14 days of cold storage [16,17], with recent reports on function of CS-PLT stored for 21 days (3 weeks) [3–7].

Metabolic activity is crucial for key aspects of platelet hemostatic function [18,19], yet this activity declines during traditional room temperature (RT) PLT storage [20,21], leading to reduced function over time. Cold storage has been shown to alter platelet metabolic activity, such as reducing glucose consumption [22,23], but how cold storage–induced metabolic changes directly impact specific aspects of platelet hemostatic function remains to be delineated. Recently, Zhao et al. reported univariate correlations of metabolites with metrics of PLT phenotype and function in whole-blood–derived PLT stored at 4 °C out to 14 days [22]. They found significant correlation between high energy nucleosides and clot formation in viscoelastic testing, and between L-carnitine and PLT aggregation. Moreover, there are inherent differences (eg, flow profiles, residence times, and biomaterial exposures) in various PLT manufacturing procedures (ie, apheresis vs whole blood–derived), which promote distinct biochemical environments and alter functionality [24]. Thus, if and how the relationship between metabolism and hemostatic function changes with respect to apheresis collection and alternative storage methods remains to be determined. Moreover, whether metabolic pathways associated with traditional hemostatic measurements (eg, aggregation) are similarly related to platelet hemostatic function in the setting of physiologically relevant flow regimes remains unknown and is a priority for the field of transfusion medicine, as indicated in the recent state of science sponsored by the National Heart, Lung, and Blood Institutes [25].

To this end, a comprehensive evaluation of multiple metrics of CS-PLT phenotype, metabolism, and function is necessary to improve our understanding of stored platelet biology. The objectives of this study were to (i) establish that CS-PLT stored out to 3 weeks maintained hemostatic function, (ii) identify a functional signature/profile associated with CS-PLT, and (iii) determine if key metabolic pathways were associated with this CS-PLT hemostatic profile. Ultimately, this will define potential areas for metabolic intervention to improve stored platelet hemostatic function.

2 | MATERIALS AND METHODS

2.1 | Platelet collection and storage

Single donor apheresis (Trima Accel; Terumo BCT, Inc) double collection platelet units (PLT) stored in plasma from 10 healthy donors were provided by ImpactLife (formerly Mississippi Valley Regional Blood Center) on day of collection (Washington University in St. Louis IRB #201710149). These donations were collected in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Splitting and storage details are outlined in the Supplementary material. Paired PLT were stored at 22 (± 2 °C) or 4 (± 2 °C) for up to 21 days, and sampled at baseline, day 5, or day 21 for assessment. Both 22 °C and 4 °C products were stored with continuous agitation to reduce the number of differing variables between study groups, as this primary goal of this study was to identify features specific to cold storage. For all downstream assessment, samples were analyzed without further dilution to understand the full intrinsic hemostatic capacity of the products.

2.2 | PLT phenotyping

PLT samples (50 μL) were assayed using the Coulter AcT diff2 Hematology Analyzer, and data were reported as PLT count (PLT count, × 10^3/μL) and mean PLT volume (MPV, fL). Microparticle content was assayed by dynamic light scattering using the Thrombo-LUX device (LightIntegra Technology) [26] per manufacturer's protocol (details in the Supplementary material). Resting PLT surface receptor phenotyping was performed with the following panel: CD62P, CD328, CD63, CD41/61 (PAC-1), CD42b, and CD49b. Samples were acquired on a Novocyte 3000 and then analyzed using FlowJo v10.2 software (Treestar). Detailed staining methods and antibody clones are listed in the Supplementary material.

2.3 | Platelet function testing

Thrombin generation (TG) was measured in intact product samples (akin to platelet-rich plasma) using a calibrated automated thrombinoscope (CAT; Diagnostica Stago Inc) per manufacturer's protocol. Endogenous thrombin potential ("ETP," nM·minutes), time to thrombin generation (TG lagtime, minutes), maximal thrombin generation (TG peak, nM), and time to peak (TG time to peak, minutes) were reported. Platelet impedance aggregometry induced by ADP, TRAP6 (TRAP), or collagen (COL) was assayed using a Multiplate (DiaPharma Group, Inc) per manufacturer's protocol. Data were reported as area under the aggregation curve (AUC), aggregation (height of the curve), and velocity (maximum slope of the curve). Rotational thromboelastometry (ROTEM; ROTEM delta analyzer [TEM International GmbH]) was used to measure platelet unit–intrinsic clotting ability to both extrinsic and intrinsic pathway activation. Assays were run for 60 minutes per manufacturer's suggested protocol using PLT sample in place of whole blood. Clotting time (CT, seconds), clot formation time (CFT, seconds), alpha angle (α, °), and maximum clot firmness (MCF, mm) were recorded.

2.4 | Microfluidic assay

PLT aliquots were filtered (70 μm) to prevent occlusive embolic artifacts and perfused through the collagen-coated microfluidic chamber via a gravity-induced constant pressure head to allow true occlusion of the chamber. Flow was monitored and phase images of the chamber were recorded in real time at a frame rate of 0.62 s^{-1} until occlusion of the chamber or for 60 minutes. See Supplementary Figure S1 and Supplementary Video S1 for microfluidic assay overview and Supplementary Figure S2 for custom analysis methodology. Metrics extracted include the time it took to occlude the channel (occlusion time, OT, seconds), the % occlusion, occlusion area under the curve (OAUC), occlusion incidence (%), microfluidic (MF) lagtime (seconds), and MF growth (pixels/s). Details concerning microfluidic chamber design, fabrication, and preparation or use in assays are provided in the Supplementary material.

2.5 | Metabolic profiling

PLT samples (250 μL) were prepared and shipped to the University of Colorado Anschutz Medical Campus for metabolomic assessment and analysis. Details regarding sample preparation and normalization are provided in the Supplementary material. Metabolite annotation and peak integration were performed as previously described using Maven [27,28]. Graphs and statistical analyses were prepared with GraphPad Prism 9.3.1 and Metaboanalyst 3.0 [29]. A full list of metabolites measured can be found in Supplementary Table S1. Partial least squares–discriminant analysis (PLS-DA) and hierarchical clustering were used to analyze metabolic profiles of stored PLT products (mixOmics package for R [30]; Morpheus platform, Broad Institute).

2.6 | Statistical analysis

Data were visualized and analyzed using GraphPad Prism software. Data in figures are represented as individual points, with error bars reported as median (IQR), unless otherwise indicated in figure legends. Comparison of a given variable at a specific time point between storage temperatures was performed using a *t*-test (Mann–Whitney test). Significance is denoted by asterisks: $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. Comparison of a given variable at a specific storage temperature over time (ie, baseline [day 0 vs 4 °C day 5 vs 4 °C day 21], [day 0 vs 22 °C day 5 vs 22 °C day 21]) was performed by one-way ANOVA (Kruskal–Wallis test), and if an alpha of 0.05 was achieved, a Dunn's test was performed to account for multiple comparisons. Adjusted *P* values of a given storage day to baseline are denoted by pound symbols: $^{\#}P < .05$; $^{\#\#}P < .01$; $^{\#\#\#}P < .001$. The adjusted *P* values of comparison of day 5 to day 21 at a given storage temperature are denoted by percent symbols: $^{\%}P < .05$; $^{\%\%}P < .01$; $^{\%\%\%}P < .001$. Of note, a paired *t*-test was used to compare 22 °C day 5 to 4 °C day 21 for key metrics of interest. TG curves are presented as mean (± SEM) as the data were parametric. Spearman's correlation coefficients were calculated for phenotypic, functional, and metabolic parameters. Coefficients with a *P* value of $< .05$ were considered significant. Principal component analysis (PCA) was performed using Prism; principal components (PC) were selected using the "Kaiser rule" wherein those PC with eigenvalues of > 1.0 were used for analyses.

3 | RESULTS

We found no significant differences in PLT count over the course of either 4 °C or 22 °C storage (Figure 1A). In contrast, we found that 21 days of 22 °C storage increased the MPV from a baseline median of 7.5 to 8.4 (Figure 1B), which was statistically significantly different from day 21 4 °C counterparts, as well as day 5 22 °C. We assessed the intrinsic ability of stored PLT to form clots under physiologically relevant flow conditions (arterial shear) over a biological surface (Supplementary Figure S1, Supplementary Video S1) using an established stenotic microfluidic model [31]. This model allows for visualization of PLT adherence, aggregation, and clot formation (Supplementary Video S1), thereby providing an ideal platform to measure PLT-intrinsic function in response to cold storage. PLT stored at 4 °C were able to occlude the stenotic channel faster than donor-matched 22 °C–stored counterparts (Figure 2A). OAUC quantification revealed that day 5 22 °C PLT have similar OAUC to day 21 4 °C PLT ($P = .53$; Figure 2B). Moreover, day 21 4 °C PLT occluded the channel as often day 5 22 °C PLT (Figure 2C). To determine which aspect of clot formation/occlusion that 4 °C storage was enhancing or potentiating, we measured the average MF lagtime and growth rates for each PLT (Supplementary Figure S2). While not statistically significant, there was a trend in decreased MF lagtime in 4 °C PLT compared to 22 °C PLT (Figure 2D), yet MF growth rates were similar irrespective of storage temperature (Figure 2E).

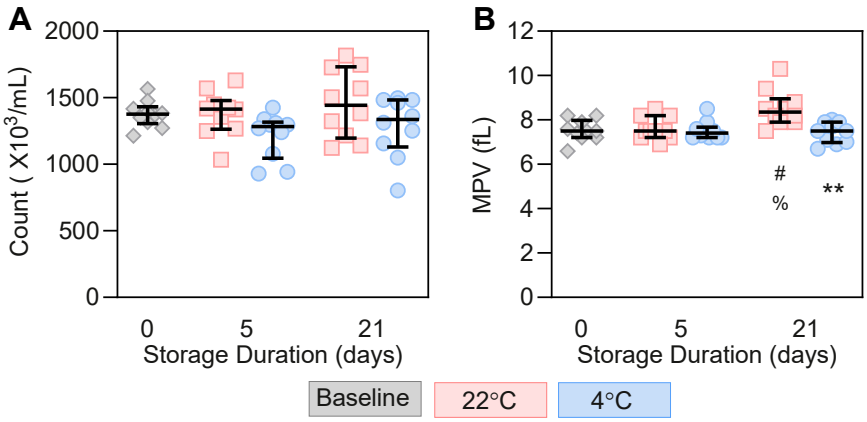

FIGURE 1 Four-degree-Celsius storage maintains count and mean platelet volume out to 21 days of storage. (A) Count reported as $\times 10^3/\mu L$, $N = 10$. (B) MPV reported in femtoliters (fL), $N = 10$. Data are represented as median (IQR), asterisks denote degree of significance when comparing 4 °C to 22 °C at the given time point, pound symbols denote degree of significance when comparing the given group to baseline, and percent symbols denote significance when comparing day 5 to day 21 at a given storage temperature. MPV, mean platelet volume.

To better understand how day 21 4 °C PLT maintained function under physiological flow, we performed a comprehensive series of assessments, including traditional and clinical hemostatic assessments, to identify potential correlates of function under flow. We measured the surface expression of receptors representative of the activation status of platelets and receptors important for initial platelet hemostatic function under physiologically relevant flow conditions (Figure 3). Cold storage increased expression of markers of α-granule mobilization (CD62P, CD328) and activated CD41/61 (GPIIb/IIIa, binds fibrinogen) by day 5 as compared to 22 °C storage, but this difference was lost by day 21 (Figure 3A–C). CD63 (LAMP3, a marker of dense granule mobilization) surface expression remained unchanged at day 5 between the 2 storage temperatures, but by day 21, 22 °C PLT had increased CD63 expression compared to 4 °C PLT (Figure 3D). Finally, there was no difference in CD42b (GPIbα, binds von Willebrand factor [VWF]) and CD49b (ITGA2, binds collagen) expression at day 5 of storage between 4 °C and 22 °C PLT, whereas by day 21 of storage, 4 °C PLT had increased expression of both receptors compared to 22 °C PLT (Figure 3E, F).

We next assayed for changes in specific aspects of platelet function. For aggregation, 4 °C storage better maintained responses to both ADP and collagen agonists at day 5 of storage than 22 °C storage (Figure 4A, B). However, by day 21 of 4 °C storage, PLT were unresponsive to both ADP and collagen yet maintained responses to TRAP (Figure 4C). Prolonged 4 °C storage (day 21) enhanced TG (Figure 4D–G), as shown by higher ETP and peak values compared to day 0. PLT storage is known to modulate MP content [6,32], and platelet-derived MP are known to potentiate thrombin burst [33]; thus, we measured MP content and found increased MP in 4 °C PLT (Figure 4H). Finally, we used viscoelastic testing as a "global" representative of hemostatic function [34] and found that day 21 4 °C PLT responded to both extrinsic and intrinsic activation similar to day 5 donor-matched 22 °C PLT (Supplementary Figure S3), as indicated by preserved CFT and maintenance of ~75% of baseline MCF values.

To determine if there was a unique set of phenotypic and/or functional metrics that was distinctly associated with cold storage, we performed PCA on data generated (metrics listed in Supplementary Table S2) from all 5 study groups: baseline day 0, day 5 4 °C, day 21 4 °C, day 5 22 °C, and day 21 22 °C (Figure 5A). PLT stored for longer periods were readily identified via PCA, with storage duration representing the proportion of variance (38.9%) in PC1 and storage temperature representing the proportion of variance (16.6%) in PC2. To identify metrics of functional PLT that may be associated with storage temperature, we repeated our PCA but restricted our analyses to those study groups with PLT that were functional as identified through our comprehensive functional assessments (Figures 2–4, Supplementary Figure S3): baseline day 0, day 5 4 °C, day 21 4 °C, and day 5 22 °C (Figure 5B). PC2 once again provided variance in accordance with storage temperature, and therefore, we isolated those metrics with strong positive loading vector values in PC2 (Figure 5C). Specifically, the metric with the highest positive loading vector values and greatest contribution to PC2 was OAUC (loading vector: 0.82; 16.5% contribution), the metric that accounts for hemostatic function under arterial shear. Additional metrics were MF growth, CD41/61, CD328, CD62P, ADP_AUC, TG peak, COL_AUC, CD63, MP, CD49b, and CD42b (see Supplementary Table S3).

As platelet hemostatic function is dependent on robust metabolic function and cold storage of platelets modulates metabolite availability—at the very least because of the impact of cold temperatures on enzyme kinetics [22,23]—we next determined whether there were distinct metabolic hubs that characterized the cold-storage phenotype. Mass spectrometry–based metabolomics was performed on all stored PLT samples. PLS-DA revealed distinct clustering of each study group, suggesting a unique metabolic profile at each storage duration and temperature (Figure 6A), with increased variance mostly attributed to storage duration—similar findings to our previous study on CS-PLT metabolism [23]. Of interest, metabolites associated with taurine metabolism had high variable importance in projection (VIP) scores, and specifically, taurine most readily discriminated all 5 study groups (Figure 6B, Supplementary Figure S4). Hierarchical clustering of the top 50 metabolites with significant changes across groups (ANOVA, $P < .05$) revealed many similarities between day 0, day 5 22 °C, day 5 4 °C, and day 21 4 °C, whereas day 21 22 °C had a completely distinct metabolic profile (Figure 6C).

To identify key metabolites/metabolic hubs that were commonly and significantly associated with 4 °C PLT phenotype and function

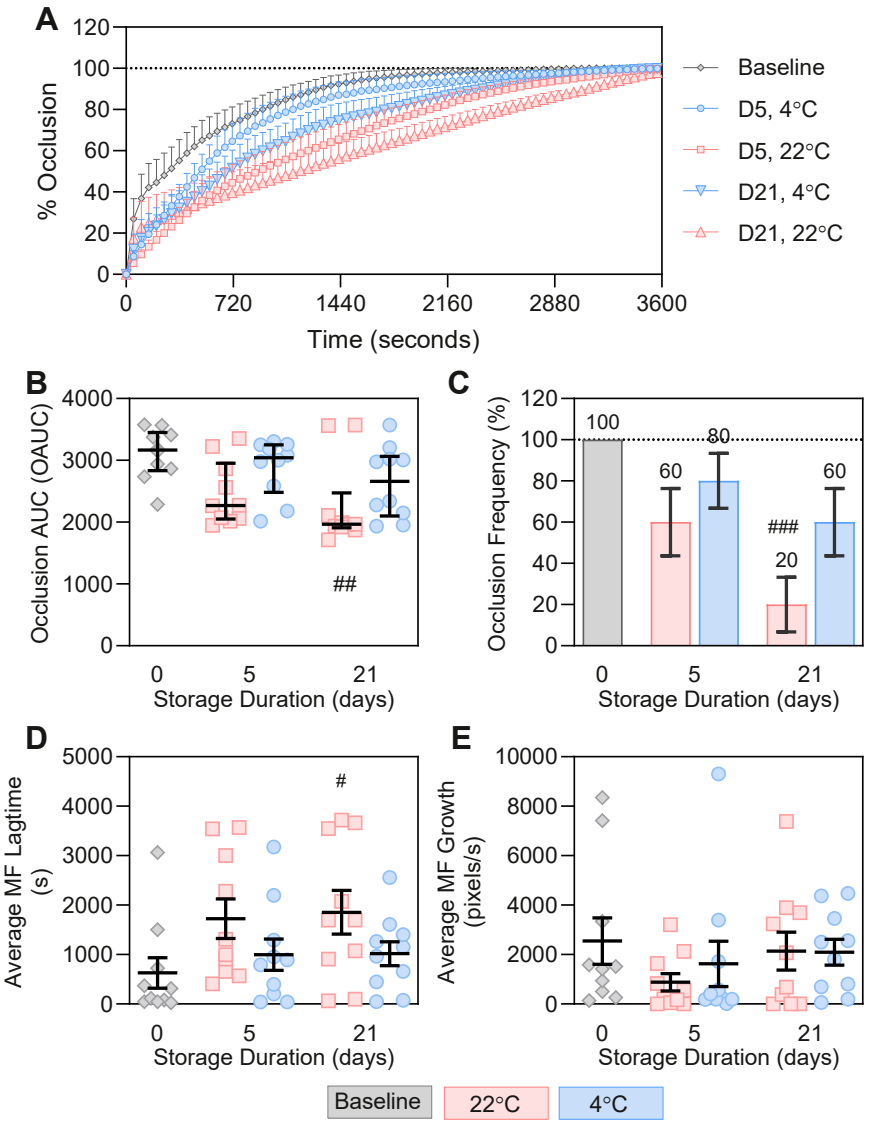

FIGURE 2 Storage of PLT at 4 °C preserves collagen-induced clot formation at high shear as compared to 22 °C storage. (A) Percent occlusion for PLT in each group; data graphed as mean + SEM with the upper error bars plotted, $N = 10$. (B) AUC for each percent occlusion trace—OAUC values. (C) Frequency of PLT that occluded the channel in each group; for example, 6 out of 10 22 °C PLT occluded the channel on day 5 of storage. (D) Average MF lagtime for each PLT. (E) Average MF growth time for each PLT. For panels B, D, and E, $N = 10$ and data graphed as median (IQR); pound symbols denote the degree of significance when comparing the given group to baseline; $^{\#}P < .05$; $^{\#\#}P < .01$. AUC, area under the curve; MF, microfluidic; OAUC, occlusion index area under the curve; PLT, platelet.

metrics, we next performed Spearman correlations of our metabolic data in 4 °C PLTs with the phenotypic and functional metrics we found that were associated with cold storage (as identified in Figure 5C). There were a large number of metabolites that correlated with the expression of key surface receptors involved in ligand engagement—specifically CD49b, CD42b, and CD41/61 (Figures 7A–C), and fewer metabolites that correlated with expression of receptors that are markers of degranulation (Figures 7D–F). Despite the strong loading vector values and contribution of microfluidic variables to PC2 (as identified in Figure 5), both OAUC and MF growth correlated with very few metabolites (Figure 7G, H). For metrics associated with thrombin generation capacity—TG peak and MP—we saw fewer significant correlations (Figure 7I, J) than those were seen with surface receptor expression. Finally, both ADP and COL aggregation responses had correlation with similar profiles of metabolites (Figure 7K, L). Quantification of the top 10 metabolites (5 largest significant positive ρ + 5 largest significant negative ρ) for each metric found 25 metabolites that were associated with ≥2 of the twelve 4 °C–associated metrics (Figure 8A). Notably, taurine and xanthine both correlated with 6 (50%) of 4 °C–associated metrics. Pathway analysis of these 25 metabolites identified enrichment in both purine metabolism and taurine and hypotaurine metabolism (Figure 8B).

4 | DISCUSSION

The data presented herein support that single donor apheresis platelet products stored between 1 °C and 6 °C in plasma out to 3 weeks (day 21 4 °C, CS-PLT) function as effectively as single donor apheresis platelet products stored for 5 days at RT (day 5 22 °C, RT-PLT) when assessed by viscoelastic testing, thrombin generation, and ability to form an occlusive thrombus under conditions of arterial shear. The similar function between briefly stored RT-PLT and long-stored CS-PLT has public health implications. The use of CS-PLT for active bleeding

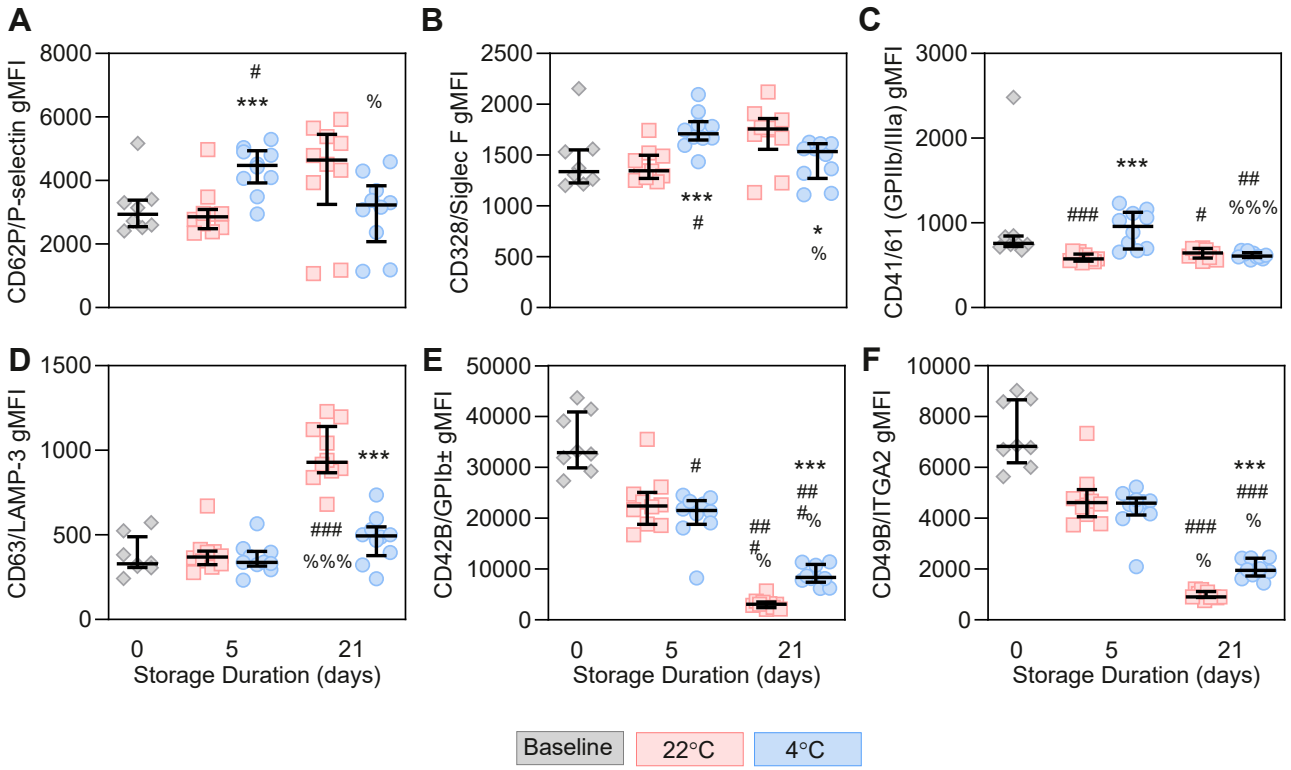

FIGURE 3 Cold storage of PLT increases expression of surface receptors associated with alpha granule mobilization by day 5 of storage. Surface expression of (A) CD62P (P-selectin), (B) CD328 (Siglec F), (C) CD41/61 (GPIIbIIIa; PAC-1), (D) CD63 (LAMP3), (E) CD42B (GPIba), and (F) CD49B (ITGA2) was measured by flow cytometry and gMFI reported. For all panels, data are represented as median (IQR), asterisks denote degree of significance when comparing 4 °C to 22 °C at the given time point, pound symbols denote degree of significance when comparing the given group to baseline, and percent symbols denote significance when comparing day 5 with day 21 at a given storage temperature. gMFI, geometric median fluorescence intensity.

would improve availability of PLT with hemostatic function at health care facilities that cannot afford to keep RT-PLT inventories due to the 5-day shelf life [35]. This could have a significant impact on the high rates of morbidity and mortality for patients with severe bleeding, as PLT transfusion is associated with improved outcomes for patients with traumatic life-threatening bleeding [36]. In addition, the number of PLT transfused in the US for active bleeding vs prophylactic transfusions is nearly equal [37–39], suggesting that the 4-fold increase in shelf life with day 21 CS-PLT has the potential to reduce PLT waste, which is paramount in the current crisis of insufficient PLT availability [40].

Platelet hemostatic function is dependent on both intrinsic factors, such as receptor expression and granule content to sense and respond to the given surroundings, and extrinsic factors, such as the rate of blood flow. Current *in vitro* assays for assessing platelet hemostatic function heavily lean on platelet-intrinsic factors while failing to account for extrinsic factors. Using a microfluidic assay to provide physiological flow regimes, we found that PLT stored out to 3 weeks at 4 °C retained the ability to form an occlusive thrombus at shear rates of ≥2000 s^{-1} on a collagen-coated surface (Figure 2) while expressing greatly reduced levels of key surface receptors involved in primary hemostatic function (CD42b, CD49b, CD41/61; Figure 3) and being unresponsive to aggregation induced by collagen as measured by impedance aggregometry (Figure 4). Of note, day 21 CS-PLT had intact dense granules (Figure 3D), enhanced thrombin generating potential (Figure 4), preserved metabolic function (Figure 6, Supplementary Figure S4), and retained function as assessed by viscoelastic testing (Supplementary Figure S3). These data suggest that assays that account for function via dependence on multiple features may provide a better assessment of stored platelet hemostatic function than those assays that report single metrics or evaluate highly specific features of platelet function. Current PLT licensing [41] requires products to meet 3 specific quality control metrics: platelet yield, volume, and pH—none of which directly speak to the functional quality of platelets. Moreover, aggregometry still is viewed as the gold standard in platelet function testing [42]; our data suggest that aggregometry underrepresents the full hemostatic potential of platelets and fails to capture other important functional components such as aggregation under flow conditions and thrombin egneration to support fibrin polymerization [43].

Measuring platelet surface receptor expression provides an immediate snapshot of functional potential and insight into the activation status of the given platelet. We found that storage duration had a more prominent effect on the expression of surface receptors CD42b and CD49d, whereas storage temperature imparted a greater effect on regulating the expression of receptors stored in alpha granules (CD62P, CD328), and both storage temperature and duration modulated dense granule receptor expression (CD63).

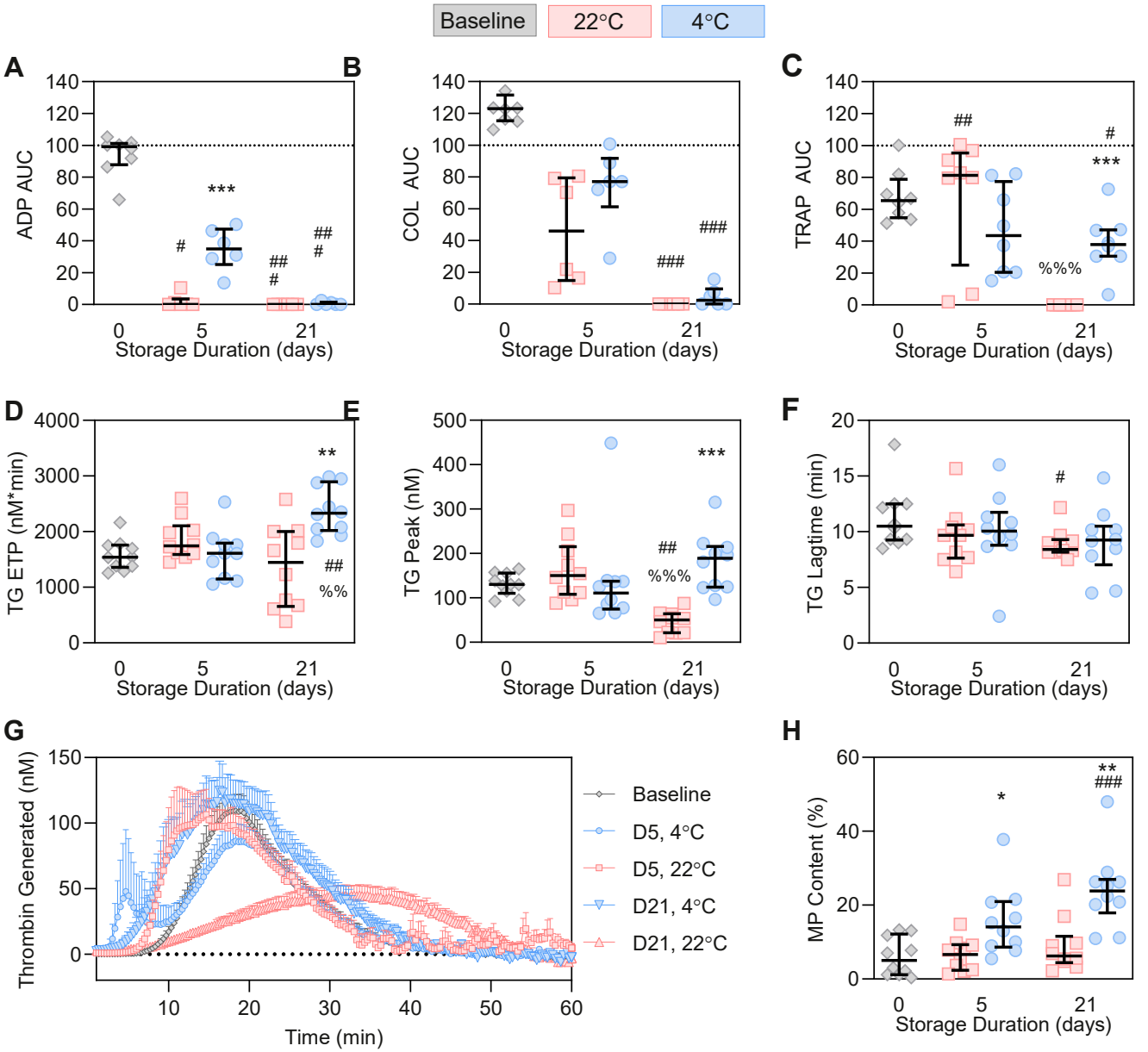

FIGURE 4 Platelet responsiveness to thrombin activation is maintained over 3 weeks of cold storage. Platelet aggregation in response to (A) ADP, (B) COL, and (C) TRAP as measured by AUC. For each storage temperature there were $n = 6$ PLT. (D–H) Thrombin generation in stored PLT measured at baseline, day 5, and day 21. For each storage temperature there were $n = 10$ PLT. (D) Endogenous thrombin potential (TG ETP, nM·minutes), (E) peak thrombin, or highest amount of thrombin generated (TG peak, nM), and (F) TG lagtime (minutes) were recorded. (G) Thrombin generation curves for all platelet products. Data represented as mean + SEM, with the upper error bars plotted. (H) Frequency of particles that are microparticle (MP)–sized in the platelet product bag. For all panels, excluding panel G, data are represented as median (IQR). For all panels, asterisks denote degree of significance when comparing 4 °C with 22 °C at the given time point, pound symbols denote degree of significance when comparing the given group to baseline, and percent symbols denote significance when comparing day 5 with day 21 at a given storage temperature. ADP, adenosine diphosphate; AUC, area under the curve; COL, collagen; MP, microparticle; TRAP, thrombin receptor activating peptide.

CD42b,c/a/d (GPIbα,β/IX/V; VWF binding) and CD49b/CD29 (integrin α2β1; collagen binding, anchoring, and signaling) are 2 key surface receptors that mediate platelet adhesion during primary hemostasis. We hypothesized that microfluidic assessment would be the best functional reflection of the expression of these key receptors; however, we found that CD42b and CD49b levels strongly correlated with impedance aggregation (ADP and COL_AUC) and viscoelastic assay measurements (ExTEM, InTEM CFT and MCF), with little to no correlation with microfluidic assay measurements reflective of adhesion (MF lagtime; Supplementary Figure S5). Moreover, day 21 CS-PLT had greatly reduced expression of both CD42b (GPIbα) and CD49b (integrin α2) compared to baseline values (Figure 3) as measured by flow cytometry, but still maintained the ability to function and form an occlusive clot under

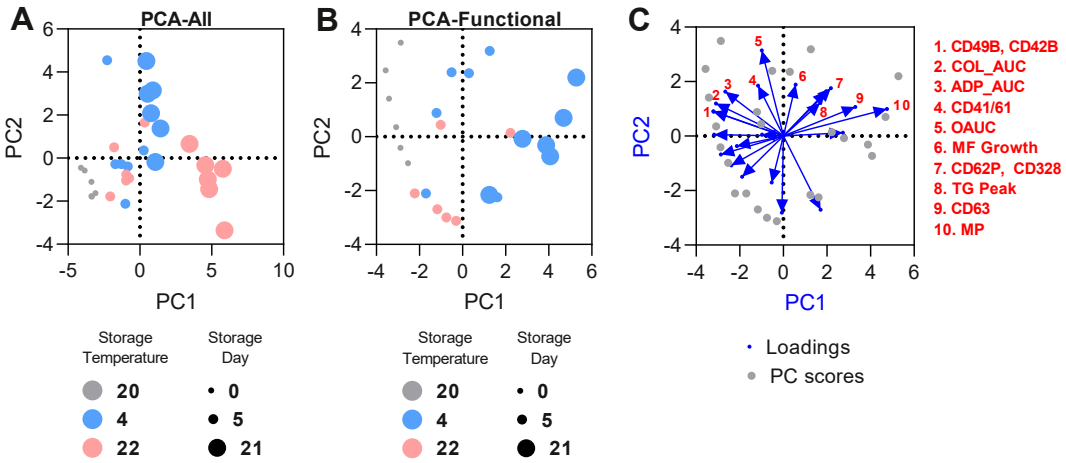

FIGURE 5 PCA of phenotype, function, storage duration, and storage temperature. The list of metrics used for these analyses are detailed in Supplementary Table S2. *n* = 6 per group for all analyses. (A) PCA of metrics collected from all 5 study groups (PCA-all): baseline day 0, day 5 4 °C, day 21 4 °C, day 5 22 °C, and day 21 22 °C. (B) PCA of metrics collected from study groups with responsive functional PLT (PCA-functional): baseline day 0, day 5 4 °C, day 21 4 °C, and day 5 22 °C. (C) Loading vector values and PC scores for PCA-functional. Those metrics with positive PC2 values are denoted with numeric labels and listed to the right of the plot; values available in Supplementary Table S3. PC, principal component; PCA, principal component analysis.

arterial shear conditions (Figure 2) with faster kinetics than day 5 RT-PLT, which have twice the expression of CD42b and CD49b as compared to day 21 CS-PLT. These findings suggest that day 21 CS-PLT may have additional mechanisms induced by cold storage which allow for enhanced clot formation under flow conditions as compared to RT-PLT.

Our comprehensive phenotypic and functional assessment and analysis of stored PLT allowed us to identify metrics that were strongly associated with the cold storage lesion. Via metabolic profiling, we identified that taurine is a key discriminator between study groups. Further analysis of phenotypic and functional metrics in conjunction with metabolic data found that metabolites involved in taurine/hypotaurine metabolism and purine metabolism were significantly associated with specific PLT metrics to include those that most strongly drove the variance in our principal component analyses (OAUC, MF Growth, CD49b, CD42b, CD41/61, CD328, CD62P, ADP and COL AUC, TG peak, and MP). Taken together, these findings suggest that increased taurine metabolism may be associated with the CS-PLT lesion.

The biology underlying the role of taurine in platelet function is both an historic and active area of study. In 1989, Hayes et al. demonstrated that decreased taurine levels were associated with hyperaggregatory platelet function in cats depleted of taurine, whereas in healthy human volunteers supplemented with taurine, there was reduced aggregation [44]. These data and other more recent studies [45] suggest that taurine may play an inhibitory role in modulating endogenous/native platelet function via modulating platelet calcium signaling, yet the exact mechanism remains undefined. Interestingly, taurine has been found to play a prominent role in mitochondrial membrane stabilization in nucleated cells. As platelet hemostatic function is bioenergetically demanding and thus heavily reliant on mitochondrial responsiveness, perhaps the role of taurine in modulating platelet function is mediated in a mitochondrial-specific manner. In this vein, Hegde et al. recently published antioxidant supplementation of CS-PLT with *N*-acetylcysteine reduced storage lesion–associated mitochondrial damage, improving circulation times and reducing bleeding times in a NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (immunodeficient) murine model of transfusion [46]. This suggests that metabolic supplementation strategies of stored PLT which improve mitochondrial function may also improve hemostatic function. Notably, taurine was found to inhibit oxidative stress in red cell concentrate lesions [47], suggesting a non-mitochondrial role for the effects of taurine in stored blood products. Further studies on the role of taurine in modulating stored platelet function both *in vitro* and *in vivo* will provide insight into the effects of taurine on platelet hemostatic function.

In the context of endogenous platelet biology, potentiation of thrombin generation is commonly associated with a procoagulant platelet phenotype [48]. Procoagulant platelets tend to be less effective at aggregation and are metabolically inert due to a loss of mitochondrial membrane integrity and diminished adenosine triphosphate (ATP) [49,50]. Day 21 CS-PLT contain ATP and are metabolically active (Supplementary Figure S4) [23] while simultaneously supporting increased thrombin generation compared to baseline (day 0) and adhering, aggregating, and generating occlusive clot under arterial shear settings. Moreover, the increased MP content in day 21 CS-PLT may also contribute to enhanced thrombin generation and clot formation, as platelet-derived extracellular vesicles (such as MP) are known to potentiate clot formation during hemostatic resuscitation [51]. These findings suggest that CS-PLT have features common to both resting/activated and procoagulant endogenous platelets—a hybrid platelet phenotype primed for active clot formation—and thus the potential ideal therapy for actively bleeding patients. Multiple trials are underway to determine the clinical efficacy and safety of

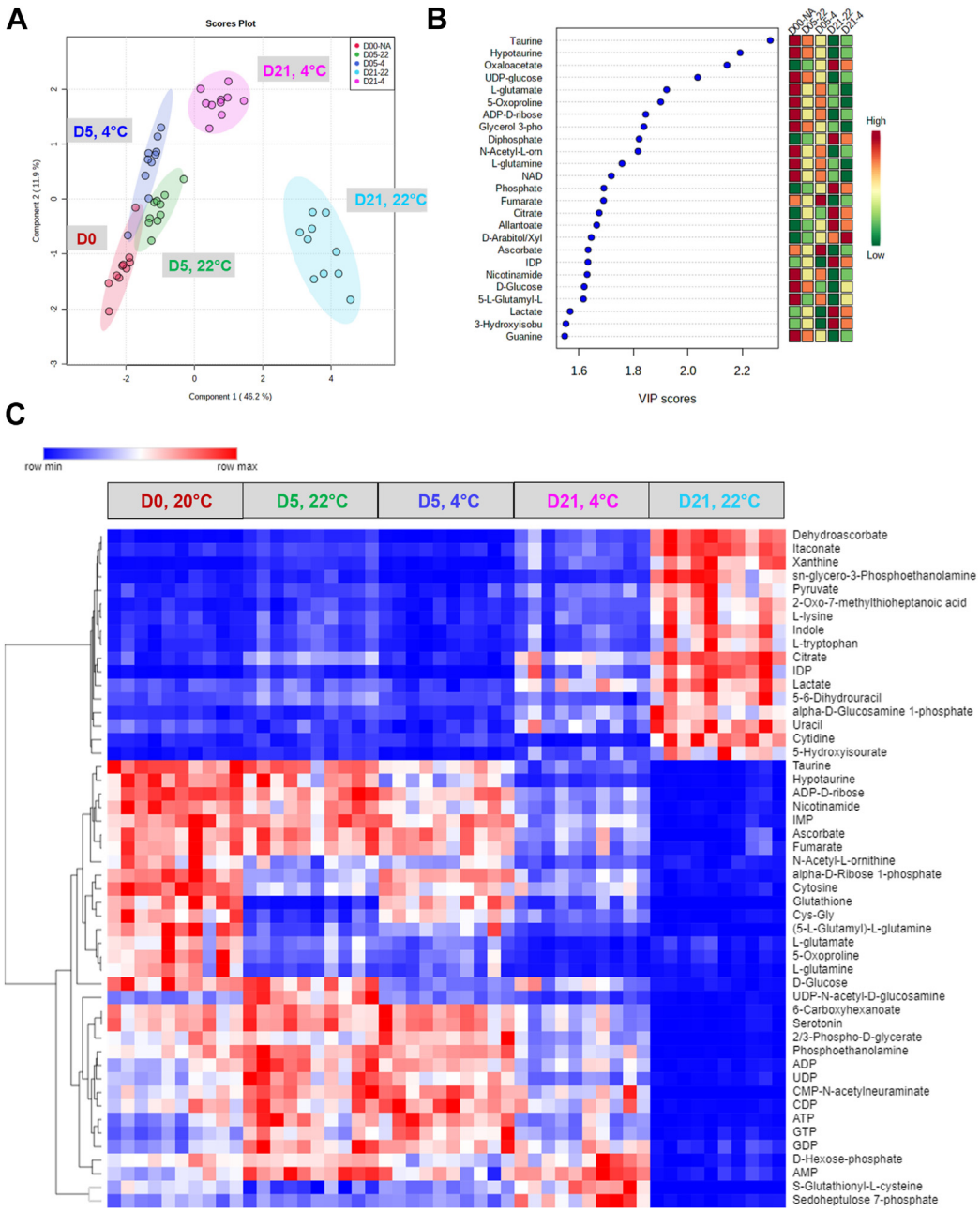

FIGURE 6 Metabolic profiling of stored PLT. (A) PLS-DA of all 5 study groups (baseline day 0, day 5 4 °C, day 21 4 °C, day 5 22 °C, and day 21 22 °C). (B) Variable importance in projection (or VIP) score plot from the PLS-DA. (C) Heat map of top 50 significant metabolites, as determined by ANOVA, using hierarchical clustering. PLS-DA, partial least squares–discriminant analysis; PLT, platelet product.

CS-PLT transfusion in various patient populations (eg, NCT04667468 ["CriSP-HS"], NCT04726410 ["CriSP-TBI"], NCT05220787 ["CHASE"], NCT04834414 ["CHIPS"]). An interesting consideration for these trials will be whether CS-PLT are cleared in bleeding patients similar to clearance rates in healthy donor studies [52,53], especially as longer stored CS-PLT have greatly reduced expression of CD42b (GPIbα), the main receptor by which cold storage–induced desialylation and clustering mediate clearance from circulation [54,55]. An additional consideration is whether the enhanced thrombin generating capacity of longer stored CS-PLT may potentiate clot formation in areas of low shear, such as in deep vein thrombosis. As bleeding patients with traumatic injury can have systemic endotheliopathy, the latter concept will be important in the CriSP-HS and CriSP-TBI trials, which are focused on CS-PLT transfusion in patients with hemorrhagic shock and traumatic brain injury, respectively. Furthermore, our data clearly show that a single assay does not provide an adequate assessment of platelet function. Therefore, more comprehensive approaches to platelet assessment can help identify important metrics or features reflective of hemostatic function, which may then be employed as a collective scoring system to assess function during clinical trials.

Our findings are both corroborated by historic studies concerning CS-PLT [56] and lend new insights into the field of stored platelet

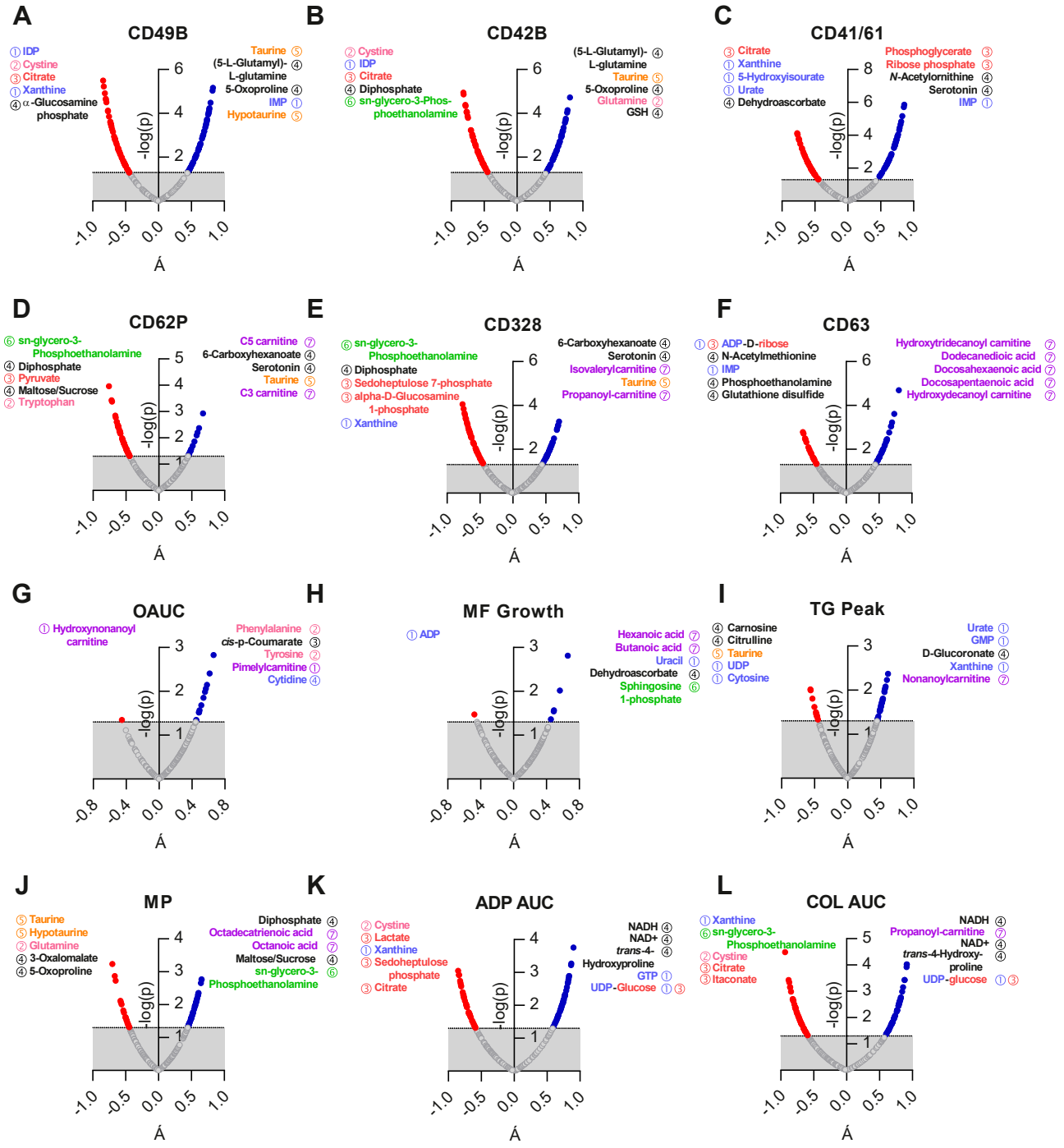

FIGURE 7 Correlation of metabolites and metrics of phenotype and function in 4 °C PLT. Spearman correlation coefficients (ρ) for metabolites correlated to the designated 4 °C PLT metric. Above the dotted line and in color are statistically significant correlations ($P < .05$), whereas below the line in gray are statistically insignificant correlations ($P \geq .05$). The left half/red data points denote metabolites with levels that are inversely correlated to the designated PLT metric, whereas the right half/blue data points denote metabolites with levels that directly correlate to the designated PLT metric. The top 5 significant metabolites for both negative and positive correlations are annotated on each graph and coded by pathway association via number and color: 1, blue—nucleotides; 2, pink—amino acids; 3, red—energy metabolites; 4, black—other; 5, orange—taurine/hypotaurine; 6, green—lipid building blocks; and 7, purple—acylcarnitines and fatty acids. Phenotypic 4 °C PLT metrics are (A) CD49B, (B) CD42B, (C) CD41/61, (D) CD62P, (E) CD328, and (F) CD63. Functional 4 °C PLT metrics are (G) OAUC, (H) MF growth, (I) TG peak, (J) MP, (K) ADP_AUC, and (L) COL_AUC. ADP, adenosine diphosphate; AUC, area under the curve; COL, collagen; MF, microfluidic; MP, microparticle; OAUC, occlusion index area under the curve; PLT, platelet product; TG, thrombin generation.

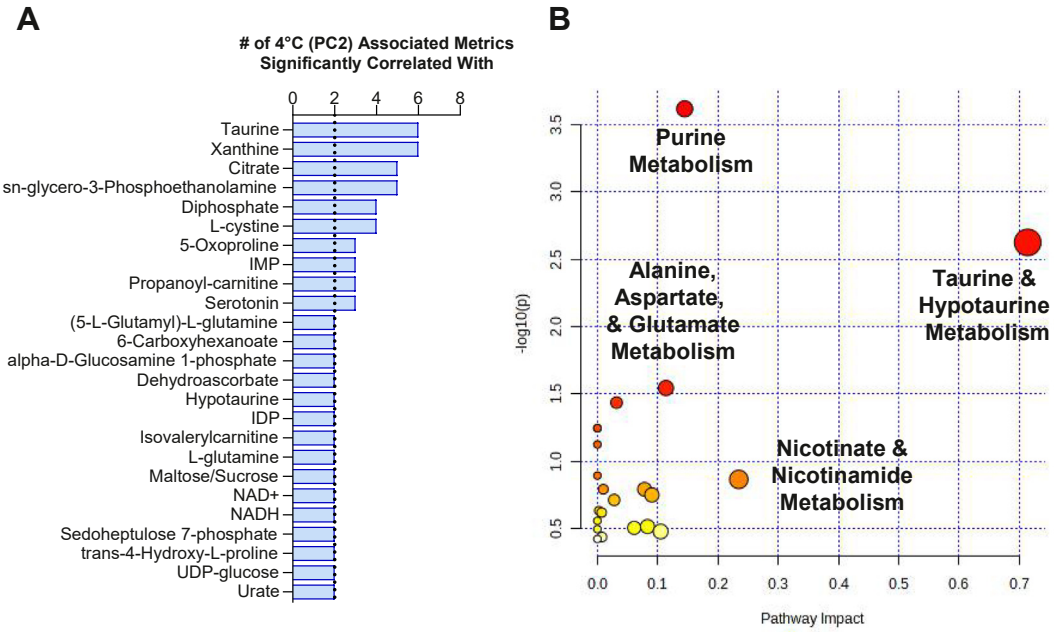

FIGURE 8 Taurine is associated with CS-PLT phenotype and function. (A) Graphical representation of metabolites that were found to repeatedly correlate with cold storage metrics: *y*-axis, specific metabolite, *x*-axis, and number of 4 °C–associated metrics the indicated metabolite was found to be both highly and significantly ($P < .05$) correlated with. (B) MetaboAnalyst plot of these same metabolites, indicating which metabolic pathways are represented by these 25 metabolites.CS-PLT, Cold-stored platelet.

biology; however, there are limitations to these findings. Our data are restricted to a limited number of donors ($N = 10$), a few key time points of the storage lesion, and performed in Trima apheresis PLT stored in plasma. As such, whether our results can be extrapolated to products collected on other systems (ie, Amicus and MCS+ 9000 platforms), whole blood–derived collections (ie, not single donor), or stored in other storage solutions (ie, platelet additive solution [PAS]), remains to be determined. Various PLT collection platforms have each been shown to induce unique phenotypic and functional profiles [24], and outside of the US, whole-blood–derived PLT stored in PAS and plasma are far more common. Therefore, it is worthwhile to determine whether findings in Trima plasma CS-PLT would be similar in PLT collected and stored under alternative conditions. To this end, we found that day 21 Trima CS-PLT stored in PAS are the functional equivalent of day 7 RT-PLT counterparts when forming occlusive thrombus at arterial shear (Supplementary Figure S6). Moreover, with the advent and implementation of pathogen reduction technologies (PRT), understanding the effects of PRT on the cold-storage lesion is an important area of study [57–59]. Finally, our objective was focused on the intrinsic hemostatic function of stored PLT, with the understanding that findings from this study would be used for future work in systems accounting for the complex endogenous physiologies associated with bleeding patients, such as damaged endothelium, activated immune cells, and dysregulated red blood cells.

In summary, platelet function is metabolically demanding, and cold storage of platelets slows metabolic activity, thereby potentially preserving energy stores to increase later functional activity. Our findings support the concept that current commercially available assays that are static in nature most likely under- or overrepresent the hemostatic function of stored platelets, as day 21 CS-PLT form clots under physiologically relevant flow regimes while simultaneously lacking aggregation responses to both ADP and collagen. We also demonstrate that there is a set of platelet hemostatic metrics that are associated with the cold-storage lesion and that taurine metabolism may be an underappreciated biochemical component of stored platelet hemostatic activity.

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AUTHOR CONTRIBUTIONS

S.M.S. and K.A.T. designed the study, performed PLT function and phenotyping assays, analyzed data, prepared figures, and drafted the first version of the manuscript. J.A.R. assisted with study design, performed metabolomics and data analyses, prepared figures, and edited the manuscript. E.P.M., R.M.G.R., K.C.R., and A.C. performed experiments, analyzed data, prepared figures, and edited the manuscript. F.G. performed metabolomics analyses and edited the manuscript. A.D. assisted with study design, performed data analyses, and

edited the manuscript. P.C.S. assisted with study design, reviewed data analyses, edited the manuscript, and provided funding. All authors critically contributed to the finalization of the paper.

DECLARATION OF COMPETING INTERESTS

P.C.S. is a consultant for Cerus Corporation and Haima Therapeutics. All other authors have no competing interests to disclose.

DATA AVAILABILITY

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SUPPLEMENTARY MATERIAL

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