COLLECTION, PRODUCTION AND STORAGE OF BLOOD COMPONENTS

Brief report

Platelets from blood diversion pouches (DPs) are a suitable alternative for functional, bioenergetic, and metabolomic analyses

Orlando Esparza[1], Giovanny Hernandez[1], Guadalupe Rojas-Sanchez[1], Jorge Calzada-Martinez[1], Travis Nemkov[2], Marguerite Kelher[3], Kathleen Kelly[4], Christopher C. Silliman[1,3,5], Melkon DomBourian[4], Larry J. Dumont[5], Angelo D'Alessandro[3], Pavel Davizon-Castillo[1,6]

[1]*Department of Pediatrics Hematology/ Oncology and Bone Marrow Transplantation, University of Colorado Anschutz Medical Campus, Aurora CO, United States of America;* [2]*Department of Surgery, University of Colorado Anschutz Medical Campus, Aurora CO, United States of America;* [3]*Department of Biochemistry and Molecular Genetics, University of Colorado Anschutz Medical Campus, Aurora CO, United States of America;* [4]*Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora CO, United States of America;* [5]*Vitalant Research Institute, Denver CO, United States of America;* [6]*Hemophilia and Thrombosis Center, University of Colorado School of Medicine, Aurora CO, United States of America*

Background - The collection of the first blood flow into a diversion pouch (DP) has become widely adopted in blood donation systems to reduce whole-blood unit contamination from skin bacteria. The strict control of pre-analytical variables, such as blood collection and proper anticoagulant selection, is critical to diminish experimental variability when studying different aspects of platelet biology. We hypothesize that the functional, mitochondrial, and metabolomic profiles of platelets isolated from the DP are not different from the ones isolated from standard venipuncture (VP), thus representing a suitable collection method of platelets for experimental purposes.

Materials and methods - Whole blood from the blood DP or VP was collected. Platelets were subsequently isolated and washed following standard protocols. Platelet function was assessed by flow cytometry, light transmission aggregometry, clot retraction, and under flow conditions using the total thrombus formation analyzer (T-TAS). Mitochondrial function and the platelet metabolome profiles were determined by the Seahorse extracellular flux analyzer (Agilent, Santa Clara, CA, USA) and ultra-high-pressure liquid chromatography-mass spectrometry metabolomics, respectively.

Results - Platelets isolated from VP and the DP have similar functional, mitochondrial, and metabolic profiles with no significant differences between both groups at baseline and upon activation by any of the assays mentioned above.

Discussion - The findings of our study support the use of platelets from the DP for performing functional and metabolic studies on platelets from a wide range of blood donors. The DP may serve as an alternative blood collection method to standard VP, allowing the study of diverse aspects of platelet biology, such as age, sex, race, and ethnicity, in many eligible individuals for blood donation.

Keywords: *platelet biology, pre-analytical variables, platelet activation, platelet bioenergetics, platelet metabolomics.*

Arrived: 8 December 2022
Revision accepted: 15 March 2023
Correspondence: Pavel Davizon-Castillo
e-mail: pavel.davizon-castillo@cuanschutz.edu

INTRODUCTION

The study of platelets requires careful control of pre-analytical variables, including patient demographics, anticoagulant selection, needle size, blood collection method, processing time, transportation, and storage[1-8]. This is critical to minimize platelet priming or activation throughout the collection and isolation steps. In light of this, certain aspects of blood collection like the type of vacuum, needle size, collection method (venipuncture or central line), and post-collection practices (transport method and time or sample processing) must be controlled to diminish variability and ensure accurate and reproducible results. Common sample collection errors include using incorrect anticoagulants (citrate-based tubes are gold-standard for platelet analyses), incomplete filling of the tube, inadequate or vigorous mixing of the tube, and traumatic collections (e.g., extended use of tourniquet)[9-11].

Platelets are a critical blood component used to treat or prevent bleeding in multiple conditions. Platelet transfusions are a critical therapeutic intervention in trauma, surgery, hematologic diseases (such as cancer, bone marrow failure, and platelet dysfunction), and chemotherapy-induced thrombocytopenia. One limitation to advancing the knowledge in platelet biology is the access to blood for platelet isolation from large numbers of healthy individuals to investigate basic questions of platelet physiology or to serve as control groups (age and sex-matched) against platelets from patients with diverse pathologies. Therefore, obtaining blood for platelet isolation from eligible healthy individuals that participate in blood donation is an alternative to circumvent this limitation in a high throughput and cost-effective manner. This way, platelet products can serve two purposes, therapeutic transfusion, and basic research investigations.

The use of a diversion pouch (DP) in blood collection systems has been widely implemented across blood donor centers to reduce bacterial contamination of whole-blood units[12-14]. The DP is located immediately after the access needle at a Y-junction in closed collection systems and captures any skin bacteria still found in the first few millimeters of blood collected[15]. After approximately 40 mL of blood has been collected in the DP, the sequestered blood is isolated from the primary collection container, often by heat seal, to maintain sterility. DP blood is then utilized for routine donor testing, and the remaining volume is subsequently discarded as biological waste. If suitable, collecting blood from the DP may provide a convenient method to obtain platelets for functional studies from many individuals.

We hypothesize that the functional, bioenergetic, and metabolomic profiles of platelets isolated from DPs are not significantly different from the ones isolated by standard venipuncture (VP). Metabolomic and bioenergetic assays allow us to understand the metabolic regulation of platelets in health and disease. Differences in these parameters will provide specific signatures associated with the onset or progression of chronic and degenerative diseases, for example.

MATERIALS AND METHODS

Healthy volunteer blood collection

To compare VP and DP samples among healthy volunteers, blood was collected from healthy adult (24-72 years) donors. This study was approved by the Colorado Multiple Institutional Review Board (COMIRB 00-004 and 10-0477). All donors gave voluntary and informed consent to allow the use of samples for research.

Platelet preparation

Whole blood from sex- and age-matched healthy volunteers was collected via standard VP or from the DP. Healthy volunteers underwent phlebotomy with a 19-gauge needle. To prevent sample clotting, whole blood from the DP was collected into citrate tubes within 1 minute of initial venipuncture. Whole blood from the DP was collected directly into a 2.7 mL, 3.2% buffered sodium citrate tube (Cat. # BD 363083) via a Vacutainer Luer-Lok connection (Cat. # BD 364902) that is attached to the DP. VP whole blood collection was performed according to standard protocol into a 2.7 mL, 3.2% buffered sodium citrate tube. Platelets were isolated as follows. The whole blood was centrifugated. Platelet-rich plasma (PRP) was separated and incubated with PIG_2 (1.5 µg/mL) for 3 minutes, then centrifuged. Isolated platelets were resuspended in Tyrode's buffer (pH 7.3) as previously described[16].

Light transmission aggregometry

Light transmission aggregometry (LTA) of washed platelets at 200,000 platelets per microliter was performed in a PAP-8E aggregometer PAP-8E (Bio/Data Corporation, Horsham, PA, USA). Aggregations were induced with

thrombin (0.1 IU/mL) or convulxin (2 µg/mL), and the change in light transmission was monitored over eight minutes at 37°C. Maximum aggregation was calculated as a percentage of the difference between washed platelets and Tyrode's buffer, as previously described[17].

Flow cytometry

Flow cytometry analysis of platelet activation was determined using thrombin (0.075 IU/mL) or ADP (1.25 µM). The activation profiles at baseline (unstimulated) and 5 minutes after stimulation with thrombin or ADP were measured. Platelet activation was determined using the activation-specific antibody against the αIIbβ3 integrin (PAC-1, BD Biosciences [Franklin Lakes, NJ, USA], 340507) and anti-P-selectin antibody (Biolegend [San Diego, CA, USA], 148304). Samples were acquired in a Cytoflex (Beckman Coulter, Brea, CA, USA). Flow cytometry data were analyzed using FlowJo V10 software [Ashland, OR, USA], as previously described[17].

Clot retraction

Clot retraction of platelet-rich plasma (PRP) at 300,000 platelets per microliter was performed in a 10 mL glass tube. Glass tubes were filled with 745 µL of Tyrode's buffer and 5 µL of red blood cell (RBC). 200 µL of PRP was gently added to the glass tube. Thrombin (1 U/mL final concentration) was subsequently added, and the tube was gently mixed. The reaction was incubated at room temperature for 60 minutes. Clot retraction was assessed by the weight of the extruded serum as previously reported[18].

Total thrombus formation (T-TAS)

Total thrombus formation was performed with the Total Thrombus Analysis System (T-TAS, Kanagawa, Japan) using the AR-chip (a flow chamber coated with collagen and tissue thromboplastin). Briefly, 480 µL of citrated-whole blood was mixed with 20 µL of 0.3M calcium solution containing 1.25 mg/mL corn trypsin inhibitor. The assay was immediately performed after the addition of calcium solution. Total thrombus formation was assessed by occlusion time (OT; measured in seconds), which is the time for complete occlusion of the capillary by the thrombus[19].

Bioenergetics

Platelet mitochondrial bioenergetics was measured using the Seahorse XFe24 bioenergetic analyzer (Seahorse Bioscience, North Billerica, MA, USA). Briefly, platelets were isolated and washed as described above. Platelets (2.2×10^6/well) were seeded in XF DMEM media (containing 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate with pH 7.4). The mitochondrial stress test was performed using oligomycin (1.5 µM), carbonyl-cyanide-p-trifluoromethoxyphenylhydrazone (FCCP [1.0 µM]) and rotenone/antimycin A (0.5 µM) as previously described[17]. Mitochondrial function parameters were calculated according to the manufacturer.

Metabolomics

Sample processing and metabolomic analysis were performed as previously described[17,20].

Statistics

Statistical analyses were performed using the unpaired 2-tailed Student *t* test (GraphPad [San Diego, CA, USA] Software v9.1.2). Data expressed as mean plus or minus standard error of the mean (SEM). Significance was determined at $p<0.05$.

RESULTS

A total of 75 sex- and age-matched subjects (38 VP and 37 DP group) were included in this study. Flow cytometry (No.=16), LTA (No.=10), clot retraction (No.=7), total thrombus formation (No.=8), bioenergetics (No.=22), and metabolomics (No.=12) analyses were performed. Subjects were distributed among the two groups (VP and DP) for each specific assay.

Platelets from diversion pouch and venipuncture have similar functional profiles

The platelet functional profile was interrogated using LTA, clot retraction, total thrombus formation, and flow cytometry. Percent platelet aggregation was measured after activation with thrombin and convulxin. No differences were noted in platelet aggregation between the VP and DP groups with both agonists (p=0.54 and p=0.65, respectively) (**Figure 1A**). Clot retraction was measured after activation with thrombin and an incubation period of 60 minutes. No differences were noted in the weight of the extruded serum between the VP and DP groups (p=0.89) (**Figure 1B**). Total thrombus formation was measured with a microfluidics flow chamber (T-TAS analyzer) coated with collagen and tissue thromboplastin. No differences were measured in the OT between the VP and DP groups (p=0.54) (**Figure 1C**). We subsequently measured platelet activation under static conditions using flow cytometry. No differences were identified regarding the activation

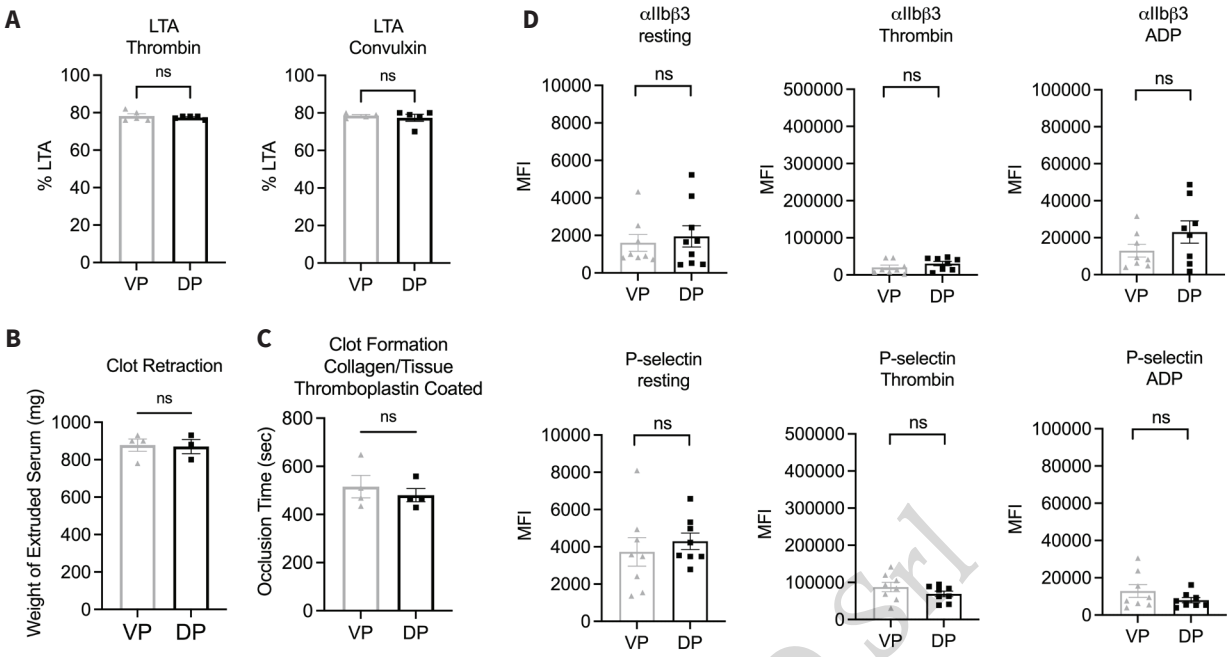

Figure 1 - The functional profiles of platelets isolated by venipuncture (VP) or from diversion pouches (DPs) are similar
(**A**) Light transmission aggregometry as measured by the change in light transmission after activation with thrombin and convulxin. (**B**) Clot retraction was measured by the weight of extruded serum one hour after activation with thrombin. (**C**) Total thrombus formation was measured in seconds on a collagen and tissue thromboplastin-coated flow chamber. (**D**) Platelet activation profile by flow cytometry at baseline (not activated) and upon activation with thrombin and ADP.

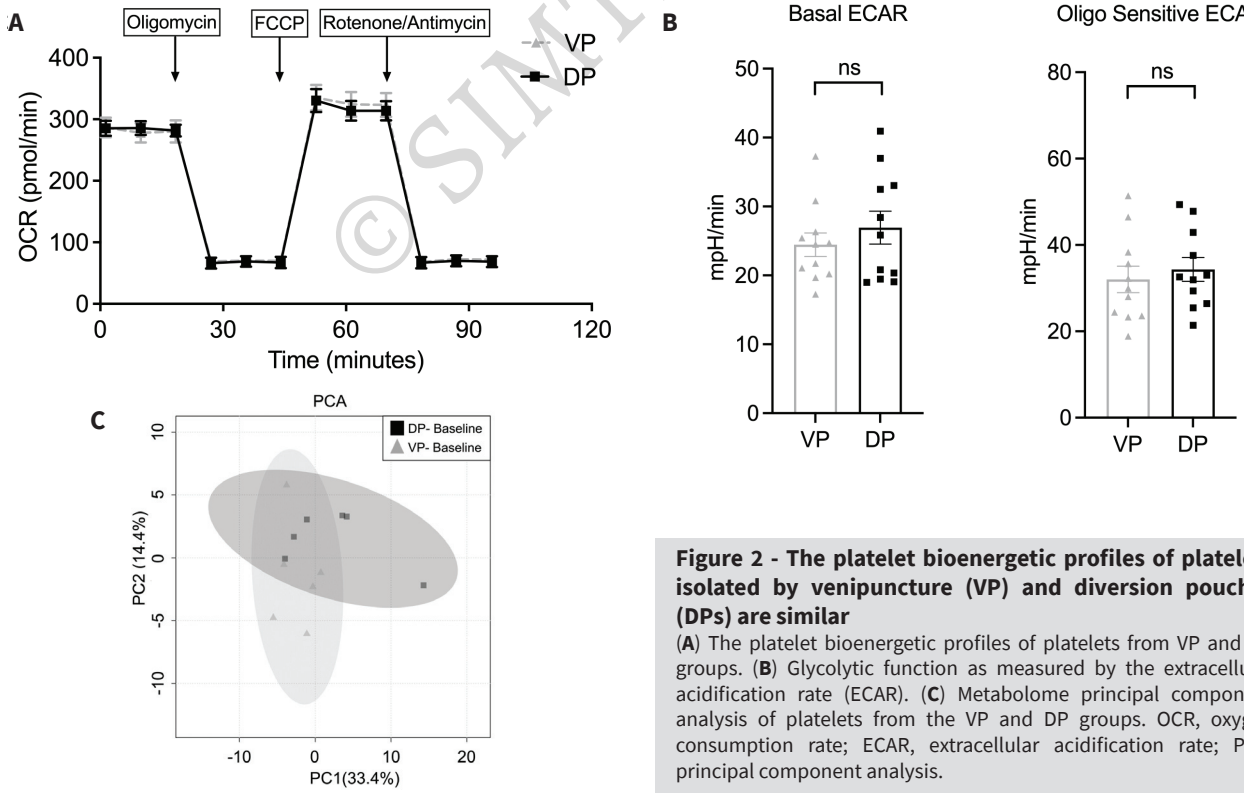

Figure 2 - The platelet bioenergetic profiles of platelets isolated by venipuncture (VP) and diversion pouches (DPs) are similar
(**A**) The platelet bioenergetic profiles of platelets from VP and DP groups. (**B**) Glycolytic function as measured by the extracellular acidification rate (ECAR). (**C**) Metabolome principal component analysis of platelets from the VP and DP groups. OCR, oxygen consumption rate; ECAR, extracellular acidification rate; PCA, principal component analysis.

of the αIIbβ3 integrin between the VP and DP groups at baseline (resting conditions, p=0.65) or upon activation with thrombin (p=0.24) and ADP (p=0.17). Similarly, platelet degranulation, as measured by P-selecting surface marker expression, was not different between groups under resting conditions (p=0.53), activation with thrombin (p=0.23), and activation with ADP (p=0.53) (**Figure 1D**). We detected platelet degranulation directly by measuring P-selectin exposure at baseline and after activation using flow cytometry. Although not measured directly, dense granule content is likely not different between VP and DP, as platelet aggregation studies and total thrombus formation under flow (T-TAS) were not different between them. These results suggest alpha and dense granules similarly contributed to clot formation in both VP and DP groups. If dense granule content or exposure differed between groups, we would have expected to find differences in platelet aggregation profiles (disaggregation) or prolonged occlusion times.

The bioenergetic and metabolomic profiles of platelets from DPs and VPs are comparable

The mitochondrial stress test was performed to determine if there were any differences in mitochondrial bioenergetics of platelets isolated from VP and DP. No differences were detected in platelet mitochondrial bioenergetics (oxygen consumption rate [OCR] and extracellular acidification rate [ECAR]) between the VP and DP groups (**Figure 2A**, **2B**). To further interrogate metabolic profiles, ultra-high-performance liquid chromatography-tandem mass spectrometer (UHPLC-MS/MS) based metabolomics was performed. Principal component analysis revealed no differences between the VP and DP groups (**Figure 2C**).

DISCUSSION

The control of pre-analytical variables in the study of platelet biology is essential to ensure accurate and reproducible results. Collection of whole blood into citrated tubes must be carefully performed to avoid inadvertent activation of platelets or the coagulation system. To ensure that whole blood from DPs is a suitable alternative for the study of platelet biology, we have provided an in-depth analysis of functional, bioenergetic, and metabolomic analyses between whole blood from standard VP and DP.

There are no significant differences in the functional profile of platelets isolated from VP or DP. Although the DP lacks an anticoagulant, this did not impact platelet aggregometry or platelet surface markers of activation (e.g., αIIbβ3 and P-selectin) when blood is quickly transferred from the DP into citrate-containing collection tubes within one minute of initial venipuncture using Vacutainer Luer-Lok connection. We suspect that if whole blood from the DP is not immediately collected, albeit slowly, this could lead to spurious platelet activation, as this has been documented to occur in samples that are inappropriately collected[9-11].

In addition, there were no significant differences in the mitochondrial bioenergetics and metabolomic profiles of platelets isolated from VP and DP, and no significant differences were noted in the key parameters of mitochondrial respiration (e.g., basal respiration, maximal respiration, spare respiratory capacity, proton leak, non-mitochondrial oxygen consumption, and ATP-linked respiration). In platelets, over 90% of the ECAR can be ascribed to glycolysis. Our findings indicate that platelets from the VP and DP groups had similar basal ECAR and oligo-sensitive ECAR[21]. Our data shows that isolating platelets from the DP (collected within one minute from initiating the blood collection) does not impact their basal glycolysis or glycolytic capacity (i.e., the cell's ability to increase glycolysis after ATP synthase is inhibited).

Further interrogation of metabolic pathways *via* UHPLC-MS/MS based metabolomics revealed no significant differences between the two groups. Principal component analysis is an unsupervised analysis that shows sample clustering based on metabolic profiles. The analysis here shows that samples from the two groups overlap, with no clear and significant separation between the two groups, suggesting that the groups are overall indistinguishable based on metabolic phenotypes alone. Thus, our findings suggest that collecting whole blood from the DP has no impact on platelet mitochondrial respiration, glycolysis, or metabolic pathways involved in ATP production.

CONCLUSIONS

The presented data validate the use of platelets isolated from the DP as a suitable alternative for investigating the functional and bioenergetic-metabolome characteristics of platelets. This process requires tight coordination between the blood banking team to collect blood into

anticoagulated tubes within one minute of initiating the blood donation to avoid *in vitro* activation of platelets or the coagulation system. The widespread use of DPs across blood centers provides a unique opportunity. It opens the door to collecting platelets from large groups of healthy (eligible for blood donation) individuals as controls for comparison to platelets from patients with diverse pathologies. In addition, platelets isolated from DPs can be employed to investigate the impact of critical variables that modify platelet function, such as age, race, ethnicity, and anthropometric variables.

ACKNOWLEDGMENTS

This work was supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health under the award number K99HL156058-01 and the Hemophilia and Thrombosis Center from the University of Colorado School of Medicine.

SOURCES OF SUPPORT

This work was supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health under the award number K99HL156058-01 and the Hemophilia and Thrombosis Center from the University of Colorado School of Medicine.

AUTHORSHIP CONTRIBUTIONS

OE planned, performed experiments, analyzed data, and wrote the first draft article. GH performed experiments and analyzed data. GR and JC performed data analysis and wrote the article. TN and AD'A performed and analyzed metabolomics assays. MK, KK, CCS, MDB, and LJD recruited, performed sampling, and provided intellectual input. PD-C planned, analyzed, wrote the article, and provided financial support.

DISCLOSURE OF CONFLICT OF INTEREST

Though unrelated to the contents of this manuscripts, the Authors declare that AD'A and TN are founders of Omix Technologies Inc. AD'A is also a consultant for Altis Biosciences LLC., Rubius Inc. and Forma Inc. and Hemanext Inc. All the other Authors disclose no conflicts of interest relevant to this study.

REFERENCES

1. Favaloro EJ, Lippi G, Adcock DM. Preanalytical and postanalytical variables: The leading causes of diagnostic error in hemostasis? Semin Thromb Hemost 2008; 34: 612-634. doi: 10.1055/s-0028-1104540.
2. Harrison P, Mackie I, Mumford A, Briggs C, Liesner R, Winter M, et al. British Committee for Standards in H.. Guidelines for the laboratory investigation of heritable disorders of platelet function. Br J Haematol 2011; 155: 30-44. doi: 10.1111/j.1365-2141.2011.08793.x.
3. Sharp MK, Mohammad SF. Scaling of hemolysis in needles and catheters. Ann Biomed Eng 1998; 26: 788-797. doi: 10.1114/1.65.
4. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Picheth G, Guidi GC. The effective reduction of tourniquet application time after minor modification of the clsi h03-a6 blood collection procedure. Biochem Med (Zagreb) 2013; 23: 308-315. doi: 10.11613/bm.2013.037.
5. Mackie I, Cooper P, Lawrie A, Kitchen S, Gray E, Laffan M. Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. Int J Lab Hematol 2013; 35: 1-13. doi: 10.1111/ijlh.12004.
6. Gp41. Collection of diagnostic venous blood specimens. Clinical Laboratory Standards Institute. Available at: https://clsi.org/standards/products/general-laboratory/documents/gp41/ 2017 Accessed on 15/01/2022.
7. H21-a5. Collection, transport, and processing of blood specimens for testing plasma-based coagulation assays and molecular hemostasis assays. Clinical Laboratory Standards Institute. Available at: https://clsi.org/standards/products/hematology/documents/h21/. Accessed on 15/01/2022.
8. Cattaneo M, Cerletti C, Harrison P, Hayward CP, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: A consensus of the working party from the platelet physiology subcommittee of ssc/isth. J Thromb Haemost 2013; 11: 1183-1189. doi: 10.1111/jth.12231.
9. Magnette A, Chatelain M, Chatelain B, Ten Cate H, Mullier F. Pre-analytical issues in the haemostasis laboratory: Guidance for the clinical laboratories. Thromb J 2016; 14:49. doi: 10.1186/s12959-016-0123-z.
10. Adcock DM, Favaloro EJ, Lippi G. Critical pre-examination variables in the hemostasis laboratory and their quality indicators. Clin Biochem 2016; 49: 1315-1320. doi: 10.1016/j.clinbiochem.2016.08.022.
11. Lippi G, Salvagno GL, Montagnana M, Lima-Oliveira G, Guidi GC, Favaloro EJ. Quality standards for sample collection in coagulation testing. Semin Thromb Hemost 2012; 38: 565-575. doi: 10.1055/s-0032-1315961.
12. Lee CK, Wong HK, Ho PL, Tsoi WC, Lee KY, Tsui GT, et al. Significant bacterial contamination risk reduction with the use of diversion pouch. Transfus Med 2012; 22: 404-408. doi: 10.1111/j.1365-3148.2012.01194.x.
13. de Korte D, Marcelis JH, Verhoeven AJ, Soeterboek AM. Diversion of first blood volume results in a reduction of bacterial contamination for whole-blood collections. Vox Sang 2002; 83: 13-16. doi: 10.1046/j.1423-0410.2002.00189.x.
14. Liumbruno GM, Catalano L, Piccinini V, Pupella S, Grazzini G. Reduction of the risk of bacterial contamination of blood components through diversion of the first part of the donation of blood and blood components. Blood Transfus 2009; 7: 86-93. doi: 10.2450/2008.0026-08.
15. McDonald CP. Interventions implemented to reduce the risk of transmission of bacteria by transfusion in the english national blood service. Transfus Med Hemother 2011; 38: 255-258. doi: 10.1159/000330474.
16. Zhou Z, Gushiken FC, Bolgiano D, Salsbery BJ, Aghakasiri N, Jing N, et al. Signal transducer and activator of transcription 3 (stat3) regulates collagen-induced platelet aggregation independently of its transcription factor activity. Circulation 2013; 127: 476-485. doi: 10.1161/CIRCULATIONAHA.112.132126.
17. Davizon-Castillo P, McMahon B, Aguila S, Bark D, Ashworth K, Allawzi A, et al. Tnf-α-driven inflammation and mitochondrial dysfunction define the platelet hyperreactivity of aging. Blood 2019; 14: 727-740. doi: 10.1182/blood.2019000200.
18. Tucker KL, Sage T, Gibbins JM. Clot retraction. Methods Mol Biol 2012; 788: 101-107. doi: 10.1007/978-1-61779-307-3_8.
19. Al Ghaithi R, Mori J, Nagy Z, Maclachlan A, Hardy L, et al. Evaluation of the total thrombus-formation system (t-tas): Application to human and mouse blood analysis. Platelets 2019; 30: 893-900. doi: 10.1080/09537104.2018.1535704.
20. Nemkov T, Hansen KC, D'Alessandro A. A three-minute method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways. Rapid Commun Mass Spectrom 2017; 31: 663-673. doi: 10.1002/rcm.7834.
21. Kramer PA, Ravi S, Chacko B, Johnson MS, Darley-Usmar VM. A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: Implications for their use as bioenergetic biomarkers. Redox Biol 2014; 2: 206-210. doi: 10.1016/j.redox.2013.12.026.