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Original Article

Platelet Function is Preserved After Moderate Cardiopulmonary Bypass Times But Transiently Impaired After Protamine

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Objectives: Previous studies have described impaired platelet function after cardiopulmonary bypass (CPB). Whether this is still valid in contemporary cardiac surgery is unclear. This study aimed to quantify changes in function and number of platelets during CPB in a present-day cardiac surgery cohort.

Design: Prospective, controlled clinical study.

Setting: A single-center university hospital.

Participants: Thirty-nine patients scheduled for coronary artery bypass graft surgery with CPB.

Interventions: Platelet function and numbers were measured at 6 timepoints in 39 patients during and after coronary artery bypass graft surgery; at baseline before anesthesia, at the end of CPB, after protamine administration, at intensive care unit (ICU) arrival, 3 hours after ICU arrival, and on the morning after surgery.

Measurements and Main Results: Platelet function was assessed with impedance aggregometry and flow cytometry. Platelet numbers are expressed as actual concentration and as numbers corrected for dilution using hemoglobin as a reference marker. There was no consistent impairment of platelet function during CPB with either impedance aggregometry or flow cytometry. After protamine administration, a decrease in platelet function was seen with impedance aggregometry and for some markers of activation with flow cytometry. Platelet function was restored 3 hours after arrival in the ICU. During CPB (85.0 ± 21 min), the number of circulating platelets corrected for dilution increased from $1.73 \pm 0.42 \times 10^9$/g to $1.91 \pm 0.51 \times 10^9$/g ($p < 0.001$).

Conclusions: During cardiac surgery with moderate CPB times, platelet function was not impaired, and no consumption of circulating platelets could be detected. Administration of protamine transiently affected platelet function.

Key Words: platelet function; flow cytometry; impedance aggegometry; protamine; cardiopulmonary bypass; cardiac surgery

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CARDIAC SURGERY is associated with an increased risk of perioperative blood loss and the need for transfusion of blood products.[1] Cardiopulmonary bypass (CPB) interacts with the patient complexly, eliciting an inflammatory response and activating coagulation. It also is reported to affect platelet function and consume circulating platelets.[2] These interactions and heparin administration increase the risk of bleeding during and after surgery and contribute to mortality.[3] How and when different systems associated with hemostasis are affected during the perioperative process is intricate and not fully described or understood. The general viewpoint has been that platelets are affected by CPB times and that this may account for some of the coagulopathy associated with cardiac surgery.[4-8]

Significant surgical and technical improvements have been introduced in cardiac surgery using CPB in recent decades. Technical innovations, such as improved membrane oxygenators and the incorporation of new biocompatible materials, have reduced the damage to blood constituents during CPB.[9] It is, therefore, reasonable to reevaluate the effects of CPB and surgical procedures on platelet function in present-day cardiac surgery procedures.

The understanding of platelet function also has deepened with new methods of analysis, improvements in analysis techniques, and handling when studying platelets both in vitro and in vivo. Several methods have been used, such as light-transmission aggregometry,[10,11] bleeding time,[11] flow cytometry,[12,13] and impedance aggregometry.[14-16] No consensus exists on which method best describes the clinically relevant aspects of platelet function in cardiac surgery.

It has been documented previously that protamine affects platelet function[17,18]; thus, it is important to distinguish the effect of CPB from the effect of protamine *per se*. In some previous studies on platelet function during CPB, sampling was done after protamine administration; in some, it was unclear when sampling was done in relation to protamine administration.[14,16,19-21] It is, therefore, imperative in studies describing platelet function and consumption in relation to CPB to clearly state whether sampling after CPB was done before or after administering protamine.

This study aimed to describe, in more detail, the changes in platelet function and numbers after CPB and protamine administration during contemporary cardiac surgery by using flow cytometry with a modern protocol, together with impedance aggregometry.

Methods

Study Population

The regional ethics review board approved the study. After giving oral and written consent, 40 patients undergoing coronary artery bypass graft (CABG) surgery using CPB were included.

Exclusion criteria were emergency surgery, inclusion in interfering studies, prior diagnosis of bleeding disorders, such as coagulopathy or platelet dysfunction, or treatment with P2Y12-receptor antagonists not terminated at least 5 days before surgery.

Blood Sampling

Blood was drawn from the radial artery after inserting a 20-gauge arterial cannula (Becton Dickinson and Company, Franklin Lakes, NJ), combined with a Saf-T Holder device (Smiths Medical ASD, London, Great Britain). Ethylenediaminetetraacetic acid tubes (Becton Dickinson and Company) were used for cell counting, and hirudin tubes (Roche Diagnostics Rotkreuz, Switzerland) were used for impedance aggregometry and flow cytometry. Blood was collected immediately after insertion of lines before anesthesia induction (Baseline), immediately after decannulation after CPB (end CPB), 5 minutes after reversal of heparin with protamine (Protamine), on arrival in the intensive care unit (ICU), 3 hours after arrival in the ICU (ICU 3 hours) and the morning after surgery (ICU 1 day). In a subset of 23 patients, the authors also collected blood 30 minutes after CPB started. Because of logistical reasons, it was impossible to use flow cytometry at this time. Immediately after blood sampling, 100 μL of blood from each hirudin tube also was transferred to one Eppendorf tube, with 34 μL formaldehyde 4% for fixation for analysis of circulating platelets, exposing the activation marker P-selectin, and also for analysis of the percentage of leukocytes in conjugates with platelets in vivo.

Clinical Management

Anesthesia was induced with 2-to-6 mg/kg of sodium pentothal and 0.3-to-0.5 mg of fentanyl. Muscle relaxation was achieved with 50 mg of rocuronium. Anesthesia was maintained with isoflurane before, during, and after CPB. Supplemental fentanyl was given on demand, according to the attending anesthesiologist. After leaving the operating room, anesthesia was maintained with propofol until the patient was extubated in the ICU. A heart-lung machine (LivaNova Sorin Stockert S5, LivaNova, London, United Kingdom) with reservoir (Inspire HVR N/S, LivaNova), tubing (Sorin IN00325H, LivaNova), and membrane oxygenator (Inspire 8F M, LivaNova) primed with 1,400 mL of Ringer's acetate, 200 mL of mannitol 150 mg/mL (Fresenius Kabi AB, Uppsala, Sweden), and 5000 U of heparin (LEO Pharma, Malmö Sweden) was used for CPB. In 31 patients, a roller pump (Sorin S5 Roller Pump, LivaNova, London) was used, and in 8 patients, a centrifugal pump (CP5, LivaNova) was used. The initial heparin dose was calculated using the Hepcon hemostasis management system (HMS Plus, Medtronic, Minneapolis, MN) to achieve an activated clotting time of 480 seconds, according to instructions from the manufacturer. If needed, a supplemental dose was given to achieve an activated clotting time of >480 seconds before starting CPB. Additional doses of heparin were given during bypass to keep a therapeutic activated clotting time level (>480 seconds). Antifibrinolytic treatment was given according to the attending surgeon, with 2 g of tranexamic acid (Pfizer AB, Sollentuna, Sweden) given to all but 4

patients after heparin administration before the start of CPB. Two patients also received an additional 2 g of tranexamic acid after CPB. When given post-CPB, platelet aggregation sampling was done before tranexamic acid administration. During CPB, the patient's temperature was kept between 36°C and 37°C.

After CPB, heparin anticoagulation was reversed with protamine sulfate. The protamine dose was calculated using the HMS Plus according to instructions from the manufacturer. The dose was split into an initial dose, given immediately after decannulation, and a supplemental dose corresponding to the heparin in the blood remaining in the heart–lung machine and infused back into the patient after decannulation. Before leaving the operating room, the HMS Plus was used to detect residual heparin. Residual heparin was reversed with an additional third dose of protamine if needed. All anesthetics, heparin, and protamine treatment were given according to clinical routine, and the attending physician was blinded to the aggregometry and flow cytometry results. Transfusion of blood products was given according to the clinical preference of the attending physician. Analysis of hemoglobin concentration and platelet count was performed by the Department of Clinical Chemistry at the hospital. A dilution correction was made using hemoglobin as a marker to assess the actual number of circulating platelets. The concentration of platelets was divided by the hemoglobin concentration and is henceforth called "dilution-corrected platelet numbers".

Flow Cytometry

Flow cytometry was performed using a flow cytometry protocol enabling simultaneous usage of multiple activators and markers to study platelet function.[22] In short, agonists used to activate the platelets were adenosine diphosphate (ADP; final concentration 5 μM), specific peptides activating the thrombin receptors PAR1 (same as TRAP-6 used in Multiplate, final concentration 10 μM), and PAR4 (final concentration 100μM). As a GPVI agonist, the authors used cross-linked collagen-related peptide (CRP-XL[23]; final concentration of 2 μg/mL). To differentiate between primary activation from thrombin receptors and secondary activation from endogenously released ADP, the authors used apyrase, a final concentration of 0.2 U/mL, to degrade ADP released from activated platelets.

Markers used for platelet function evaluation were PAC-1 (binds activated fibrinogen receptor [GPIIb/IIIa]), anti-P-selectin (CD62P, used as a marker for platelet alpha granule release), anti-LAMP-1 (a marker for platelet lysosome release), annexin V-V450 (binds phosphatidylserine), and the mitochondrial dye $DilC_1(5)$ and its positive control CCCP (that disrupt the mitochondrial membrane integrity).

Tubes also were prepared to evaluate P-selectin exposure on circulating platelets and the percentage of circulating leukocyte-platelet conjugates from the fixed blood samples. Platelets were identified by anti-GPIIb (CD41, binds the fibrinogen receptor regardless of activation), and anti-P-selectin was used to evaluate P-selectin expression.

The staining and activation of platelets were started 60-to-120 minutes after blood sampling, and platelets were activated for 10 minutes before dilution and immediate analysis by flow cytometry.

Flow cytometry data were collected with a Gallios (Beckman Coulter, Inc, Brea, CA) flow cytometer using the ultra-wide angle of detection (submicron particle setting) for the forward scatter, and a fluorescence threshold on FL3 (CD41-ECD) to maximize detection of small particles, as previously described.[24] Data were processed and analyzed with Kaluza Analysis Software (Beckman Coulter) and Microsoft Excel (Microsoft Corporation, Redmond, WA).

Flow cytometry data describing platelet activation can be expressed in 2 different ways: percentage positive platelets or median fluorescence intensity (MFI). Median fluorescence intensity is the more discriminative way to express flow cytometry data if the activation is high.[25] Therefore, MFI was used in this study when the median percentage of positive platelets exceeded 90%.

Because previous studies have shown that platelets only transform to a procoagulant state with phosphatidylserine exposure and mitochondrial membrane disruption when strongly activated,[24,26] the authors present only results for annexin V and $DilC_1(5)$ after exposure to CRP-XL in high concentration and in combination with a PAR-activator.

Impedance Aggregometry

Platelet aggregability was analyzed by the coagulation laboratory at the university hospital using the Multiplate platelet function analyzer (Verum Diagnostica GmbH, Munich, Germany) according to the manufacturer's instructions, with ADP (ADP-test) and thrombin receptor-activating peptide 6 (TRAP-test) as agonists. Results were presented as the area under the curve in units (U). The authors used simple linear regression to evaluate the effect of platelet concentration on aggregation. The authors calculated an expected aggregative response using the slope from the regression line and the change in platelet concentration between baseline and 30 minutes on CPB. The authors then compared the expected response with the measured response using Student's *t*-test.

Statistics

Statistical analysis and graphs were made using GraphPad Prism 9.3.1 (GraphPad Software, La Jolla, CA). Data are presented as mean and standard deviation, 95% CIs or median and IQR, as appropriate. Repeated measures analysis of variance or mixed-effects model (depending on missing values) and Šídák's multiple comparisons test were used. In the longitudinal data, all time points were compared with the baseline. In addition, the timepoint after protamine (Protamine) also was compared to the timepoint immediately before protamine administration (end CPB). For correlation analysis, Pearson's test or simple linear regression was used. Expected and actual aggregative response at 30 minutes of CPB was compared with Student's *t*-test. A p value of < 0.05 was considered

statistically significant. When studying platelet function, all results after receiving platelet concentrates were excluded. When studying platelet numbers and concentration, all results were excluded after receiving platelet or erythrocyte concentrates.

Results

The authors included 40 patients who were scheduled for CABG surgery. One patient received tirofiban up until 8 hours before surgery, and because platelet function was affected at baseline, the patient was excluded, resulting in 39 patients in this analysis. Two were women. All patients received 75 mg of acetylsalicylic acid daily until the day before surgery. The mean CPB time was 85.0 ± 21 minutes (range, 42-138 min), and the mean postoperative bleeding was 683 ± 320 mL (range, 190-2090 mL). The mean heparin dose was 30,721 ± 6,340 U, and the mean dose per body weight was 348 ± 101 U/kg. The mean total protamine dose was 161 ± 45 mg, and the protamine:heparin ratio was 0.539 ± 0.14 mg/100 U. For patient characteristics, see Table 1. Four patients received one platelet concentrate each after arrival in the ICU. All results after receiving platelets were excluded. Six patients received 12 erythrocyte concentrates (one during surgery and 5 after arrival in the ICU). Results regarding platelet numbers in relation to hemoglobin after erythrocyte concentrates had been given were excluded from further analysis.

Flow Cytometry

When using ADP as an activator and the binding of PAC-1 as a marker of activation of the fibrinogen receptor, there was an increase in activation ability from baseline to the end of CPB (Fig 1, A). Mean MFI increased from 2.17 ± 1.57 to 2.55 ± 1.59 (p = 0.001). After administration of protamine, the ability for activation of the fibrinogen receptor decreased to 2.01 ± 1.29 (p < 0.001). Activation ability then continuously increased to 2.38 ± 1.38 in the morning the day after surgery. With the collagen peptide CRP-XL as an activator (Fig 1, B), there was no difference in activation ability between baseline and end of CPB. After protamine, the binding of PAC-1 decreased from 1.44 ± 1.4 to 0.95 ± 0.85 (MFI, p < 0.001). The activation ability after that increased to 1.10 ± 0.88 3 hours after arriving in the ICU.

A similar pattern was seen when using the thrombin-receptor agonists PAR1-AP or PAR4-AP as activators (Fig 1, C and D). Similar to the results with ADP, the ability for activation of the fibrinogen receptor was unchanged at the end of CPB compared with baseline but decreased after protamine. With PAR1-AP as an activator, PAC-1 MFI decreased after protamine from 1.03 ± 0.56 to 0.72 ± 0.30 (p < 0.001), and with PAR4-AP from 2.60 ± 1.3 to 1.81 ± 0.97 (p < 0.001). Both thereafter increased and peaked 3 hours after arrival in the ICU.

Exposure of P-selectin as a marker of alpha-granule release showed varying results depending on which agonist was used (Fig 2). With ADP (Fig 2, A) as an agonist, the ability for exposure of P-selectin MFI increased between baseline and end of CPB from 2.47 ± 0.72 to 2.81 ± 0.74 (p = 0.003). The response continued to be higher than baseline until the day after surgery. With PAR1-AP as an agonist (Fig 2, C), there was a decrease in P-selectin exposure after stimulation at the end of CPB from 4.16 ± 2.5 to 3.35 ± 2.5 (p = 0.007). The ability for exposure of P-selectin then decreased further after protamine and arrival in the ICU, whereafter it increased and peaked 3 hours after arrival in the ICU. When using the collagen peptide CRP-XL or PAR4-AP as activators, no significant differences were found for exposure of P-selectin (Fig 2, B and D).

As with exposure to P-selectin, exposure to LAMP-1 (as a marker of lysosomal exocytosis) showed different findings depending on the agonist used (Fig 3). With ADP as an activator (Fig 3, A), the authors found a steady increase in the percentage of platelets able to expose LAMP-1, which peaked 3 hours after arrival in the ICU. With PAR1-AP (Fig 3, C) as an activator, the authors found a decrease after baseline with the lowest value after protamine. When the authors used the collagen peptide CRP-XL (Figure 3, B) or PAR4-AP (Fig 3, D) as agonists, they found no differences in the ability for exposure of LAMP-1 at the different sampling points.

To differentiate between the activation directly through PAR1 and the secondary activation by endogenously released ADP from activated platelets, the authors used apyrase to degrade ADP in the samples activated with PAR1-AP. In this way, the authors could study the ADP-independent response from PAR1-AP. With all 3 markers of platelet activation (PAC-1, P-selectin, and LAMP-1), all responses were markedly lower, and the statistically significant differences disappeared except for the decrease in PAC-1 binding ability after protamine (Fig 1, C, Figure 2, C, and Figure 3, C). When the authors subtracted the ADP-independent response from the total response, they found a decrease in the ADP-dependent response during CPB for P-selectin and LAMP-1 and after

Table 1
Patient Characteristics

	Values
Age (y)	66.7 ± 9.3
Sex (Male/Female)	37/2
Body mass index (kg/m^2)	28.3 ± 3.8
Operative time (min)	184 ± 36
CPB time (min)	85.0 ± 21
Cross-clamp time (min)	53.7 ± 17
Heparin dosing (units)	30,721 ± 6,340
Protamine dosing (mg)	161 ± 45
Preoperative bleeding (mL)	631 ± 185
Postoperative bleeding, first 3 h (mL)	207 ± 101
Total postoperative bleeding (mL)	683 ± 324
Total bleeding (mL)	1,328 ± 437
Reoperation	2 (5.1%)
Patients receiving erythrocyte concentrate transfusion	6 (15.3%)
Patients receiving platelet transfusion	4 (10.3%)
Plasma fibrinogen before surgery (g/L)	3.5 ± 0.8
Plasma fibrinogen after surgery (g/L)	2.4 ± 0.9

NOTE. Values are presented as No. (%) or mean ± SD.
Abbreviation: CPB, cardiopulmonary bypass.

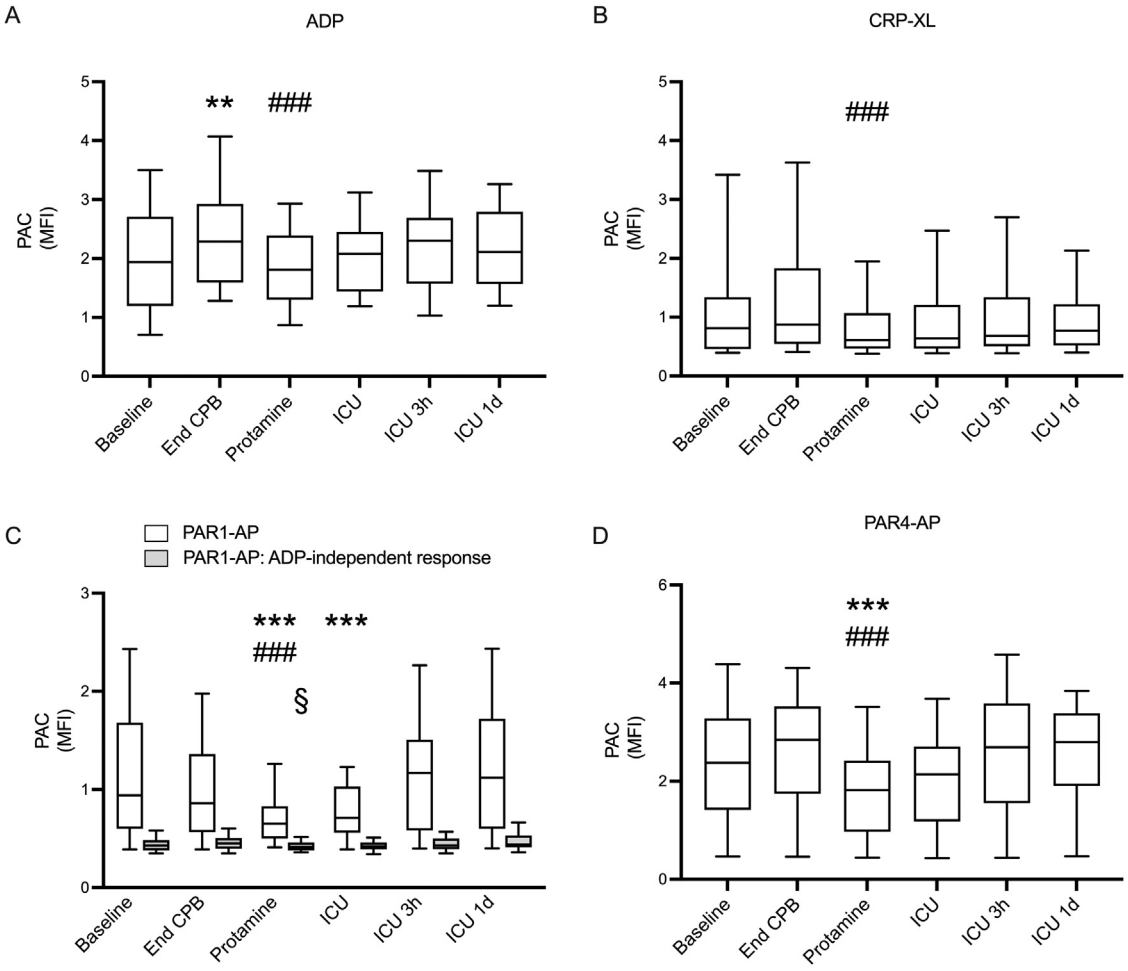

Fig 1. Activation of the fibrinogen receptor on platelets after stimulation. Binding of PAC-1 was used as indicator of activation of the fibrinogen receptor. (A) Adenosine diphosphate 5 μM was used as an activator. (B) Collagen-related peptide 0.15 μg/mL was used as an activator. (C) PAR1-AP 10 μM was used as an activator. (D) PAR4-AP 100 μM was used as an activator. The gray boxes in (C) had apyrase combined with PAR1-AP to degrade endogenously released adenosine diphosphate. Results are presented as median fluorescence intensity. The box indicates quartiles with the line as median and whiskers the 10 to 90 percentile range. ADP, adenosine diphosphate; CPB, cardiopulmonary bypass; CRP-XL, collagen-related peptide; ICU, intensive care unit; MFI, median fluorescence intensity. $^{**}p < 0.01$. $^{***}p < 0.001$ compared with baseline. $^{\#\#\#}p < 0.001$ when compared with end of cardiopulmonary bypass using mixed-effects analysis with Šídák's multiple comparisons test. $^{\S}p < 0.05$ compared with end of cardiopulmonary bypass for apyrase-treated samples.

protamine for all 3 markers (PAC-1, P-selectin, and LAMP-1). Data are shown in Supplemental Figure S1.

Finally, the authors investigated platelet ability to activate to a procoagulant state, with binding of annexin V as a marker of exposure of phosphatidylserine, and $DilC_1(5)$ as a marker of intact mitochondrial membranes. The authors used the collagen peptide CRP-XL alone in a high concentration or with either PAR1-AP or PAR4-AP. The differences the authors found were small and inconsistent. Data are shown in Supplemental Figure S2.

The authors also fixed samples immediately after sampling to measure in vivo activation with exposure of P-selectin in circulating platelets. These immediately-fixed samples would reflect the activation level of the circulating platelets at sampling time. Before anesthesia, 7.6 ± 3.4% of the circulating platelets were exposed P-selectin. No differences in the percentage of P-selectin-positive platelets at the different sampling points were observed (Fig 4). The mean percentage of monocyte-platelet conjugates at baseline was 30.2 ± 27%. During CPB, the percentage increased to 40.2 ± 19% ($p = 0.05$) and then increased further 5 minutes after protamine to 45.8 ± 23 ($p < 0.001$ compared with baseline). The percentage of monocyte-platelet conjugates then decreased to 22.2 ± 15% on arrival in the ICU. The percentage of neutrophil- and lymphocyte-platelet conjugates decreased during CPB (neutrophils from 18.6 ± 20% at baseline to 9.91 ± 9.8% at the end of CPB, $p < 0.001$ and lymphocytes from 7.97 ± 5.3% to 5.49 ± 2.3%, $p = 0.007$).

There was no correlation between time on CPB and impairment in platelet activation markers at the end of CPB. In contrast, the authors found a weak correlation between longer CPB duration and increased ability for exposure to P-selectin when activated by PAR4-AP (R^2 0.13, $p = 0.04$).

Impedance Aggregometry

Platelet function measured with impedance aggregometry, using ADP and TRAP (PAR1-AP) as agonists, was unchanged

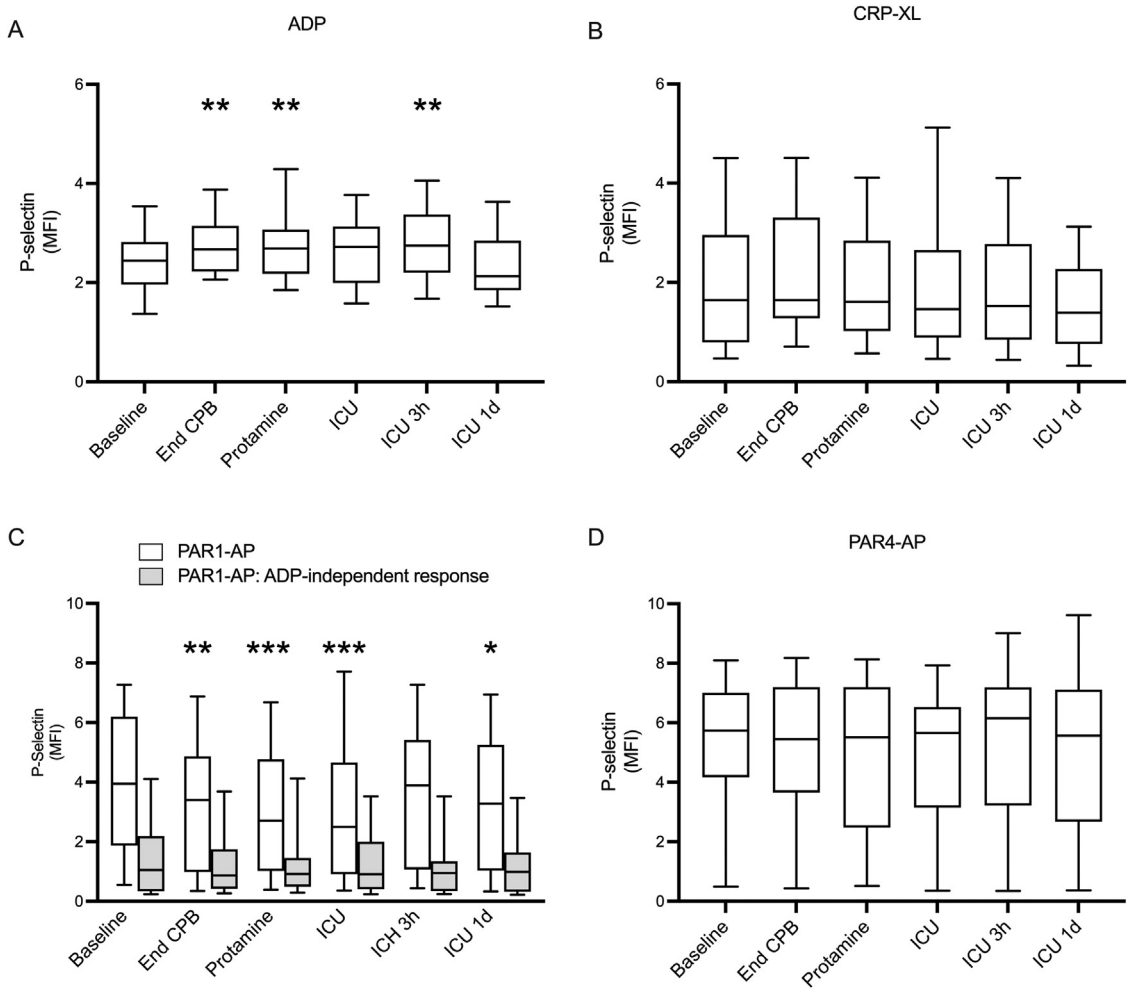

Fig 2. P-selectin exposure on platelets after activation. Exposure of P-selectin was used as an indicator of release of alpha granule. (A) Adenosine diphosphate 5 μM was used as an activator. (B) Collagen-related peptide 0.15 μg/mL was used as an activator. (C) PAR1-AP 10 μM was used as an activator. (D) PAR4-AP 100 μM was used as an activator. The gray boxes in (C) had apyrase combined with PAR1-AP to degrade endogenously released adenosine diphosphate. Results are presented as median fluorescence intensity. The box indicates quartiles with the line as median and whiskers the 10 to 90 percentile range. ADP, adenosine diphosphate; CPB, cardiopulmonary bypass; CRP-XL, collagen-related peptide; ICU, intensive care unit; MFI, median fluorescence intensity. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared with baseline using mixed-effects analysis with Šídák's multiple comparisons test.

at the end of CPB compared with baseline. After protamine, the aggregative response decreased. The platelet function returned to baseline levels at arrival in the ICU using the TRAP-test, and 3 hours after arrival to the ICU using the ADP-test (Fig 5, A and B).

In the subset of 23 patients from whom the authors collected blood 30 minutes after the start of CPB, they found a reduced platelet aggregation when activated with ADP at 30 minutes of CPB compared with baseline (from baseline 77.1 ± 26 U to 49.3 ± 25 U, $p < 0.001$). This decrease in platelet function coincided with the decrease in platelet concentration among these patients at that time (from baseline 227 ± 48 to 161 ± 32 × 10^9/L, $p < 0.001$). The authors used simple linear regression to evaluate the effect of platelet concentration on aggregation. At baseline, the authors found a weak correlation between aggregation and platelet concentration for both ADP ($R^2 = 0.35$; $p < 0.001$) and TRAP ($R^2 = 0.16$; $p < 0.001$) as agonists. The authors then calculated an expected aggregative response at 30 minutes of CPB in relation to the lower platelet concentration at that time point. There was no difference between the expected aggregative response and measured aggregative response using ADP (expected aggregative response 53.6 ± 30 U, measured aggregative response 49.3 ± 25 U, $p = 0.61$) or TRAP (expected aggregative response 92.7 ± 32 U, measured aggregative response 95.2 ± 32 U, $p = 0.80$), suggesting that the hemodilution could explain the reduction in aggregation at 30 minutes of CPB. Using impedance aggregometry, there was no correlation between CPB times and aggregometry data.

Platelet Numbers

Platelet concentration before anesthesia was 227 ± 48.3 × 10^9/L. At the end of CPB, it had decreased to 179 ± 38 × 10^9/L ($p < 0.001$). Platelet concentration then continued to be below baseline (Fig 6, A).

The authors calculated a corrected number of platelets by dividing by the actual hemoglobin concentration to correct for

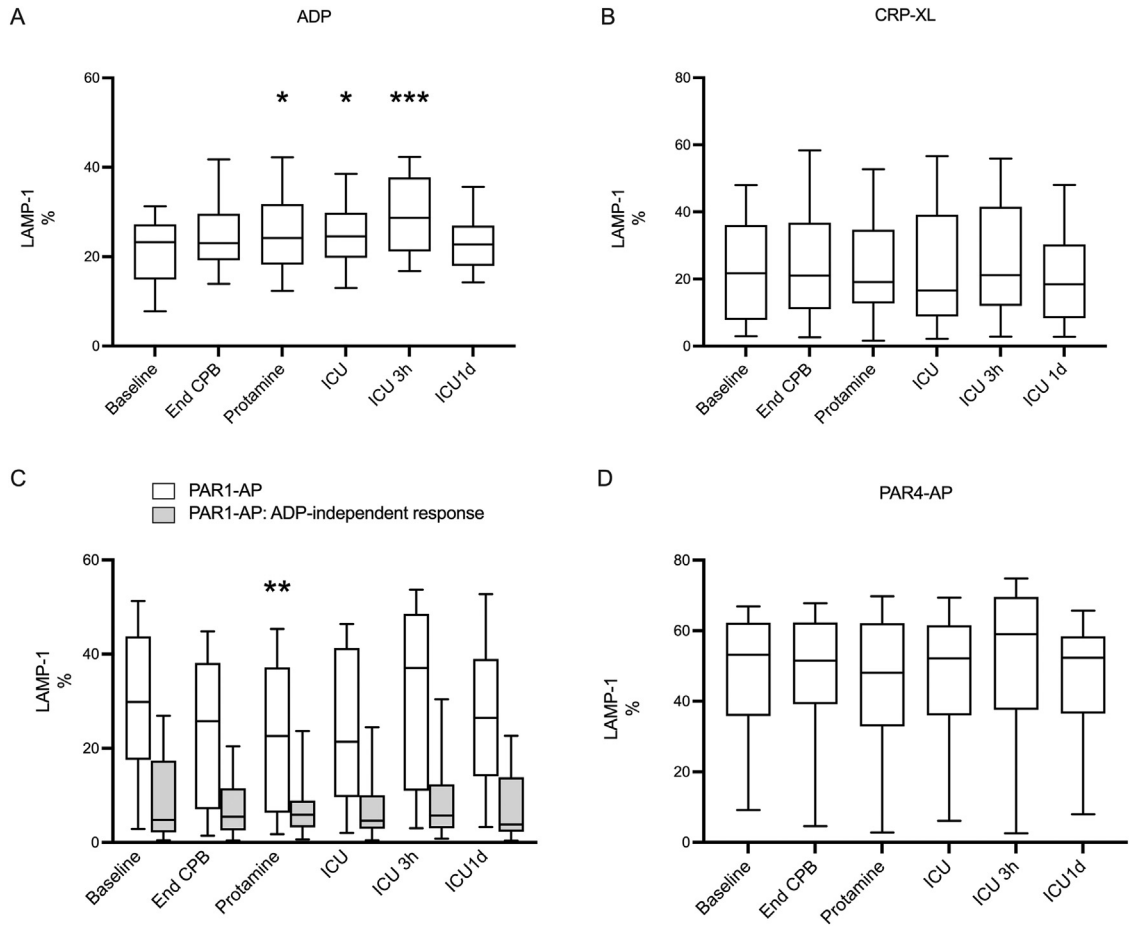

Fig 3. Lysosomal release in platelets. Exposure of LAMP-1 was used as an indicator of lysosomal exocytosis. Activators used were in (A) adenosine diphosphate 5 μM, (B) collagen-related peptide 0.15 μg/mL, (C) PAR1-AP 10 μM, and (D) PAR4-AP 100 μM. The gray boxes in (C) had apyrase combined with PAR1-AP to degrade endogenously released adenosine diphosphate. Results are presented as percentage of positive platelets (%). The box indicates quartiles with the line as median and whiskers the 10 to 90 percentile range. ADP, adenosine diphosphate; CPB, cardiopulmonary bypass; CRP-XL, collagen-related peptide; ICU, intensive care unit. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared with baseline using mixed-effects analysis with Šídák's multiple comparisons test.

the dilution that occurs at the start of CPB. When correcting for dilution, the authors did not see any decrease in dilution-corrected platelet numbers during CPB. Instead, dilution-corrected platelet numbers had increased at the end of CPB from $1.73 \pm 0.43 \times 10^9$/g hemoglobin (Hb) at baseline to $1.90 \pm 0.49 \times 10^9$/g Hb ($p < 0.001$) (Fig 6, B). After protamine, there was a reduction in dilution-corrected platelet numbers to $1.75 \pm 0.51 \times 10^9$/g Hb ($p = 0.0012$), whereafter platelets increased again and peaked in the morning the day after surgery at $1.83 \pm 0.46 \times 10^9$/g Hb ($p < 0.001$ compared with baseline).

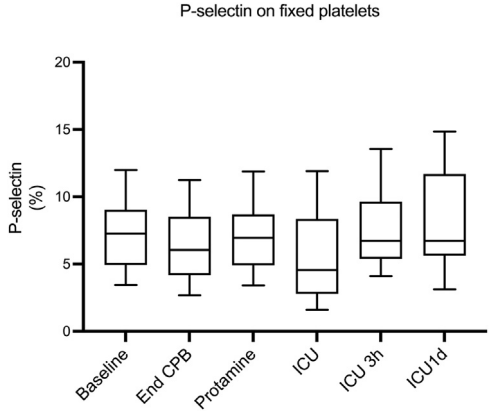

Fig 4. Exposure of P-selectin in circulating platelets immediately fixed after sampling measured with flow cytometry. Results are presented as percentage of positive platelets (%). The box indicates quartiles with the line as median and whiskers the 10 to 90 percentile range. CPB, cardiopulmonary bypass; ICU, intensive care unit.

Discussion

In this longitudinal study of platelet function during CABG surgery, platelets had a preserved ability to respond to agonist stimulation ex vivo at the end of CPB compared with baseline. Thus, no impairment of platelet function after CPB was seen. There were not any signs of in vivo activation in circulating platelets. Data confirmed that protamine administration was followed by a reduction in dilution-corrected platelet numbers and a transient impairment of platelet reactivity to exogenous agonists, as previously described.[17,18] There were no signs of platelet consumption during CPB, but rather preserved numbers of platelets when corrected for dilution.

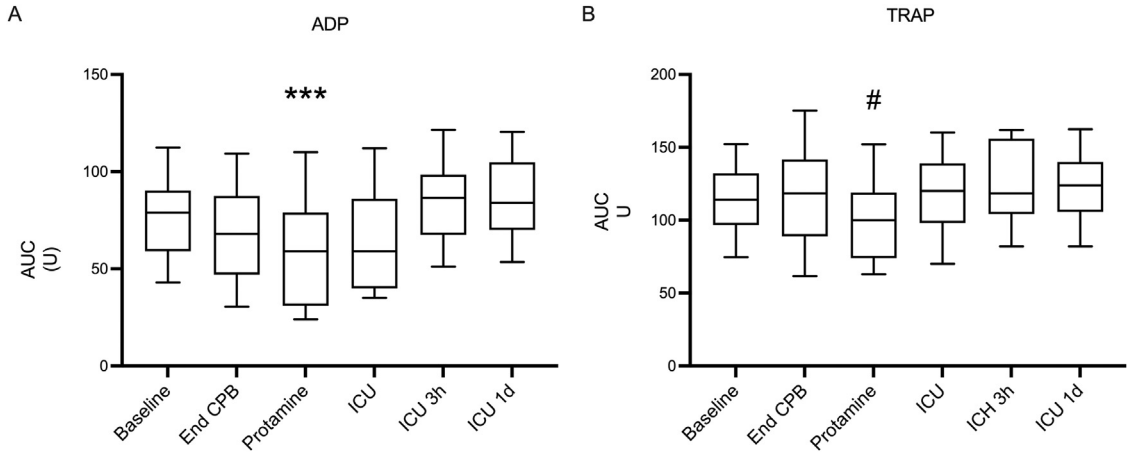

Fig 5. (A) Adenosine diphosphate 6.5 μM and (B) PAR1-AP (TRAP) 32 μM was used as an activator. Results are shown as area under the curve in units. The box indicates quartiles with the line as median and whiskers the 10 to 90 percentile range. AUC, area under the curve; CPB, cardiopulmonary bypass; ICU, intensive care unit. ***$p < 0.001$ compared with baseline. $^{\#}p < 0.05$ compared with end of cardiopulmonary bypass, using mixed-effects analysis with Šídák's correction for multiple comparisons.

The authors used flow cytometry and impedance aggregometry to study the effect of CPB on platelet function. Flow cytometry enables the simultaneous study of many different aspects of platelet activation. The authors measured activation of the fibrinogen receptor as a marker of aggregation, the release of alpha granules and lysosomes as markers of secretion, and, lastly, exposure of phosphatidylserine and rupture of the mitochondrial membrane as markers of platelet transition to a procoagulant state. The authors found no consistent results indicating impaired platelet function after CPB compared with baseline. This absence of deterioration in platelet function during CPB contrasted with previous studies.[10-14,16,27-30] The authors also found no increase in circulating platelets exposing P-selectin during CPB. Rinder et al.[13] fixed blood immediately after sampling, in a similar manner as done herein, to study the percentage of circulating platelets expressing P-selectin as a marker of prior in vivo activation. They described an increase from 7% before CPB to around 30% when CPB was terminated. In the present study, the percentage of circulating platelets exposing P-selectin at baseline was around 7%, but in contrast to Rinder, it did not increase during CPB. In another study by Rinder et al.,[31] an increase of monocyte-platelet-conjugates from 18% to 44%, a slight increase in neutrophil-platelet-conjugates, and a decrease in lymphocyte-platelet-conjugates, was demonstrated. In the present study, the percentage of monocyte-platelet-conjugates increased from 30% at baseline to 40% at the end of CPB. The authors' neutrophil-platelet-conjugates and lymphocyte-platelet-conjugates decreased during CPB. It is hard to interpret the authors' results in relation to Rinder et al. because their baselines differed. This could be due to different methods, and the absolute values depend partly on how the gate is set to identify positive conjugates (the standardized method for gating the authors use was relatively strict). The lesser increase in monocyte-platelet-conjugates and the absence of an increase of circulating platelets expressing P-selectin could indicate that modern normothermic CPB causes less platelet activation than older techniques.

The authors found similar dynamics with impedance aggregometry using ADP and TRAP as activators. After 30

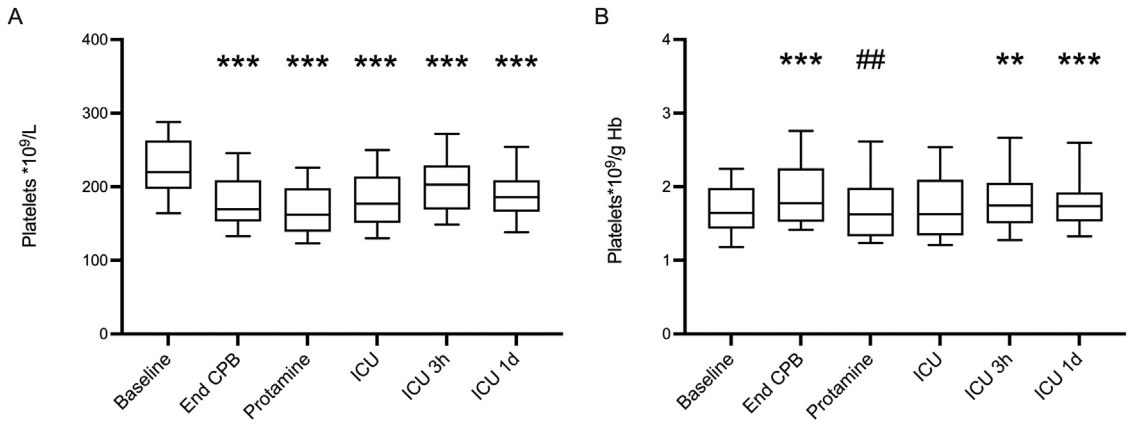

Fig 6. Platelet count and corrected numbers during cardiac surgery. (A) Platelet concentration at different timepoints during and after cardiac surgery. (B) Platelet numbers are corrected for dilution by dividing platelet concentration by hemoglobin concentration at the same timepoint. The box indicates quartiles with the line as median and whiskers the 10 to 90 percentile range. CPB, cardiopulmonary bypass; ICU, intensive care unit. **$p < 0.01$ and ***$p < 0.001$ compared with baseline. $^{\#\#}p < 0.01$ compared with end of cardiopulmonary bypass, using mixed-effects analysis with Šídák's correction for multiple comparisons.

minutes on CPB, there was a decline in aggregability compared with the baseline. However, the aggregability reduction was in the same order as what could be expected from the concomitant reduction in platelet concentration at 30 minutes of CPB. This could indicate that the aggregability reduction was mainly due to hemodilution and not an impairment of platelet function *per se*. This was supported by studies showing a decrease in aggregability with lower platelet counts using impedance aggregometry.[32-35] Hanke et al. presented a reduction in ADP-aggregative response after a reduction in platelet concentration comparable to the authors' data. At the end of CPB, the authors found no difference in aggregability compared with the baseline. Considering the difference in platelet concentration, this could imply a possible increase in the platelet's individual response to activation, which corresponded to the increase in response the authors found with flow cytometry between baseline and the end of CPB using ADP or CRP-XL as activators and PAC-1 as a marker of activation of the fibrinogen receptor.

After protamine administration, the platelet response to activation decreased, as seen in previous studies.[17,18,36] Olsson et al.[18] reported a decrease in platelet response to stimulation by almost 50% compared with only 15% in the authors' data. A probable reason for this discrepancy was that less protamine was used in the present study (160.9 ± 45.2 mg compared with 402 ± 72 mg in the study by Olsson et al.).[18] Platelet function, in the present study, returned to baseline values at arrival to the ICU, and aggregability using TRAP exceeded the baseline values 3 hours after arrival to the ICU.

In 2 recent studies[14,16] investigating platelet function with aggregometry after CPB, the sampling of platelets after CPB was done after protamine administration. The reduction in platelet aggregation reported in these studies exceeded the authors', but matches the finding by Olsson et al.[18] They also used the same protamine:heparin ratio (1:1) as Olsson et al. A dose-dependent impairment of platelet function after protamine exposure is supported by in vitro data,[17] in which protamine in lower concentrations had a stimulating effect on platelet aggregation with activation of the fibrinogen receptor, but at higher concentrations inhibited secondary activation of thrombin receptors. The mechanism behind this dual, concentration-dependent effect on platelet activation is unclear. A possible mechanism could be electrical interaction with surface charges, as discussed by Tanaka et al.,[37] or interaction with surface receptors. Another explanation could be that protamine affects the platelet's ability to promote and increase platelet activation by releasing endogenous activating substances such as ADP.

To differentiate whether the impairment from protamine depends on an alternation in the direct response to receptor interaction or is an effect on the response to activation from the endogenously released ADP,[38] the authors performed tests combining PAR1-AP with apyrase. Apyrase degrades ADP and removes the secondary activation from ADP released from dense granules in activated platelets. Without the released ADP, the impairing effect of protamine was blunted. This indicated that part of the negative effect of protamine on platelet activation is mediated through alteration in the release and/or response to endogenous ADP. Considering the direct stimulating effect of protamine previously described,[17] one possible explanation could be that protamine induces a partial release of ADP, resulting in a diminished ADP response to further activation. The authors found a similar decrease in the stimulated release of alpha- and dense granules after CPB in the calculated ADP-dependent part of the PAR1-AP response, indicating that CPB may induce the release of endogenous ADP and, therefore, impair the endogenous ADP activation.

During CPB, the platelet concentration decreased by 21% and dropped another 5% after protamine administration. Considering the hemodilution caused by the CPB priming volume and using hemoglobin concentration as a marker of dilution, there was no decline but rather a small increase in dilution-corrected platelet numbers during CPB. This was in contrast to many authors who described the consumption of platelets during CPB.[10,13,27]

The decline in platelet concentration of 21% during CPB in the present study was lower than in previous data. Zilla et al.,[10] Van Poucke et al.,[14] and 2 reviews on coagulopathy after CPB[7,8] all described a decline in platelet count of 40% or more during CPB. The factors behind this decrease were probably hemodilution due to the CPB priming volume and the use of cardioplegia. In the authors' data, dilution-corrected platelet numbers decreased after protamine administration. Many studies have sampled blood after protamine administration[39,40] or did not specify whether the sample was drawn before or after the administration of protamine.[41] Consumption of platelets after protamine administration could contribute to the reduction in platelet count reported if the sampling of the platelets after CPB was done after the protamine administration instead of before.

To determine if CPB results in a true decrease in the number of platelets, the dilution effect must be corrected. The authors found no decrease in the number of platelets after correcting for the dilution. Zilla et al.[10] and Rinder et al.[13] corrected their platelet count for hemodilution (Zilla et al. by calculating the dilution from the fluids given and the patient blood volume, and Rinder et al. by the change in hematocrit) but found a decrease during CPB.

With modern perfusion circuits and pumps, it is possible to circulate and oxygenate blood outside the body without profound impairment of platelet function. A study on extracorporeal membrane oxygenation treatment reported a difference in platelet function on day 1 compared with healthy controls, but no further impairment between days 1 and 3.[42] This could support the hypothesis that the difference between the authors' findings and previous studies regarding less impairment of platelet function during CPB may be explained by improved perfusion circuits and techniques.[7,43,44]

Less activation and less consumption could explain why platelets in this study did not decrease in the same way as observed in previous studies. Because the authors did not directly measure platelet turnover, only the number of circulating platelets, they cannot exclude an exchange with a noncirculating pool of platelets. Platelets recruited from a

noncirculating pool could mask the consumption of platelets during CPB. In the authors' data, platelet numbers increased at the end of CPB before protamine administration. One explanation could be the autotransfusion of noncirculating platelets from the spleen. The spleen is said to hold as much as a third of the total amount of platelets in an exchangeable and recruitable pool.[45-47]

Limitations

This was a small study of CABG patients with relatively short normothermic CPB times. The data should, therefore, be interpreted with caution regarding other situations with longer CPB times, complex cardiac surgery, and the use of hypothermia. The authors did not, however, find any correlation in their material between CPB times and effects on platelet function. Another limitation was that the methods used did not cover all aspects of platelet function or possible effects on primary hemostasis. The authors did not study platelet turnover and, thus, could not determine if an influx of platelets from a spleen pool occurred and affected their numbers and characteristics. Nevertheless, platelet turnover in the data was comparable to other studies of platelets during CPB. Also, this study did not explain the mechanisms behind the effect of protamine on platelet function.

Conclusions

In this longitudinal study of CABG patients, the data could not support prior studies describing a major impairment of platelet function after CPB. There was no detectable platelet activation during CPB, and platelet ability to be activated after stimulation with exogenous agonists was preserved. With flow cytometry, the authors could confirm and further characterize previous in vitro and ex vivo impedance aggregometry findings of impairment of platelet function by protamine exposure. Corrected for dilution, the number of circulating platelets was increased at the end of CPB.

Conflicts of Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1053/j.jvca.2023.03.013.

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