Received: 21 August 2024 | Revised: 23 May 2025 | Accepted: 6 June 2025

https://doi.org/10.1016/j.jtha.2025.06.009

ORIGINAL ARTICLE

Acute kidney injury after cardiac surgery is associated with platelet activation

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Funding information
British Heart Foundation (RG/13/6/29947), (CH/12/1/29419) to G.J.M., M.W., T.K., and H.A.; Leicester and Bristol National Institute for Health Research Cardiovascular Biomedical Research Units.

Abstract

Background: Postcardiac surgery acute kidney injury (AKI) is common and associated with high mortality and morbidity. Its pathogenesis remains unclear.

Objectives: To determine if platelet activation, extracellular vesicles (EVs), and microRNA levels are associated with postoperative AKI.

Methods: Plasma samples from 95 microRNA in Post CArdiac Surgery study patients were collected before, immediately after, and 6 to 12, 24, and 48 hours after surgery. Platelet responsiveness was assessed using a Multiplate aggregometer. Flow cytometry was used to measure platelet and leukocyte activation and for EV derivation. EV size and concentration were analyzed using NanoSight. Circulating biomarkers were measured using immunoassays, and microRNA was analyzed using TaqMan arrays and validated by quantitative real-time polymerase chain reaction.

Results: AKI occurred in 57% of patients. Platelet-derived EVs increased 24 hours after surgery in AKI patients. Platelets were desensitized to adenosine diphosphate at 6 to 12 hours, independent of aspirin or P2Y12 antagonist use. AKI patients had more activated platelets at 6 to 12 hours, more platelet–granulocyte aggregates before and at 6 to 12 and 24 hours after surgery, and higher soluble ICAM1 levels before and 48 hours after surgery. TaqMan arrays showed miR-668 was downregulated before and miR-92a-1, -920, -518a-3p, -133b, and -1262 were upregulated after surgery in AKI patients. Quantitative real-time polymerase chain reaction confirmed miR-1262 upregulation. Multivariate analysis showed that granulocyte-platelet aggregates were independently associated with AKI before and at 6 to 12 and 24 hours after surgery. Activated glycoprotein IIb/IIIa and adenosine diphosphate were associated with AKI at 6 to 12 and 24 hours and soluble ICAM1 at 48 hours.

Manuscript handled by: Zhi-Cheng Jing

Final decision: Zhi-Cheng Jing, 6 June 2025

Naomi Brown and Nikol Sullo contributed equally to this study.

Conclusions: AKI is associated with platelet activation, suggesting alternative platelet inhibition may offer renoprotection. Larger studies are needed to validate these findings.

KEYWORDS
acute kidney injury, cell-derived microparticles, microRNAs, surgical procedure, blood platelets

1 | INTRODUCTION

Acute kidney injury (AKI) is a common complication of cardiac surgery that is associated with increased morbidity and mortality [1] and prolonged hospital stays and increases healthcare costs [2]. AKI is defined clinically by an acute decrease in glomerular filtration rate as determined by changes in serum creatinine concentrations and urine output. The mechanisms that underlie AKI are poorly understood, but inflammation and activation of the vascular endothelium and platelets are thought to contribute [3].

Leukocytes and platelets play important roles in AKI pathophysiology, and their activation could serve as an AKI biomarker. Leukocytes contribute to the inflammatory response observed in AKI by releasing proinflammatory cytokines and reactive oxygen species. Platelet activation and aggregation within the renal microvasculature can lead to capillary occlusion, impairing blood flow and worsening tissue injury [4]. In inflammatory states, activated platelets bind monocytes and neutrophils through interaction of P-selectin with P-selectin glycoprotein ligand 1. Chemokines and cytokines released by platelets further modulate leukocyte activity, triggering neutrophil extracellular trap formation and enhancing the inflammatory response [5,6].

Extracellular vesicles (EVs) are small, membrane-bound particles secreted by cells into the extracellular space and are involved in the transfer of proteins and genetic information (messenger RNA and microRNA) specific to their cellular origin. microRNAs (miRNAs) are short, noncoding RNA oligonucleotides actively or passively (eg, dead cells) secreted either within EVs or complexed with proteins (AGO-2 or NPM1 [7]) or high-density lipoproteins [8,9]. Several miRNAs have been associated with the pathogenesis of AKI [10,11], and plasma miRNAs have been proposed as novel biomarkers for AKI in both adult [12] and pediatric [13] populations. Hence, changes in the concentration of EVs and miRNAs during pathological processes can both promote pathological changes and serve as an indicator for affected tissues or cells [14].

In AKI, platelet-derived EVs that carry procoagulant factors, such as tissue factor and P-selectin, can contribute to microvascular thrombosis within the kidney [15]. Platelet-derived EVs can transfer inflammatory mediators and miRNAs, such as miR-223, that enhance the inflammatory response and leukocyte recruitment to the site of injury, leading to further tissue damage [16]. The interaction between platelet EVs and endothelial cells can also disrupt endothelial integrity, increasing vascular permeability and promoting injury [17,18]. Therefore, platelets and platelet-derived EVs could play a causal role in AKI and be potential therapeutic targets.

We hypothesized that platelet activation, circulating extracellular vesicles (EVs) derived from different cell types, and miRNA levels are associated with postoperative AKI. To test this hypothesis, we performed longitudinal measurements, from baseline to 48 hours, of platelet, leukocyte, and endothelial activation simultaneously with assessments of EVs and miRNAs in plasma from adults undergoing cardiac surgery.

2 | METHODS

2.1 | Study design and setting

An observational case-control study to identify the role of EV and EV derived MicroRNA in Post CArdiac Surgery AKI was designed as a prospective, single-center observational study, approved by East Midlands–Leicester South Research Ethics Committee (REC reference 13/EM/0383), and conducted between December 11, 2014 and May 30, 2017 in Glenfield General Hospital in Leicester, UK. The protocol was registered at ClinicalTrials.gov as NCT02315183 (https://clinicaltrials.gov/study/NCT02315183).

2.2 | Study cohort

The study prospectively recruited 95 consecutive adult cardiac surgery patients (>16 years old) undergoing coronary artery bypass grafting or valve surgery with moderately hypothermic cardiopulmonary bypass (CPB, 32-34 °C) blood cardioplegia. All patients received 300 to 400 units of heparin during the CPB in addition to 10 000 units of heparin in the CPB pump. Patients at high risk for postcardiac surgery AKI were identified using a modified risk score [19]. Exclusion criteria included preexisting inflammatory state (sepsis undergoing treatment, AKI within 5 days, chronic inflammatory disease, or congestive heart failure), emergency or salvage procedure, ejection fraction <30%, critical preoperative state (stage 3 AKI or requiring ionotropes, ventilation, or intraaortic balloon pump), or pregnancy.

2.3 | Blood sampling, storage, and bias

Blood was collected preoperatively, on return to the intensive care unit (ICU), and 6 to 12, 24, 48, 72, and 96 hours postoperatively. The total volume collected from each patient was 104.8 mL collected in clotting, EDTA, citrate, and hirudin tubes (S-Monovette; Sarstedt) using venous lines, inserted as part of standard care during anesthetic induction (perioperative samples) or by venipuncture. All anticoagulated samples were processed within 2 hours of collection, and plasma (centrifuged twice at 1500 × *g*) was frozen in single-use aliquots at −80 °C for subsequent analysis. Samples were identified by a trial acronym and the patient's study ID. Routine measurements, including serum creatinine, were performed in National Health Service laboratories by personnel who were unaware that the participant was in a trial. For all other analyses, laboratory staff were blinded to the AKI status of patients.

2.4 | Leukocyte and platelet activation

Flow cytometry (Cyan ADP; Beckman Coulter) was used to measure leukocyte and platelet activation in citrated blood samples. For platelets, 5 μL of whole blood was incubated at room temperature for 25 minutes with 1:20 dilutions of either fluorescein isothiocyanate-coupled PAC-1 (BD Biosciences), or CD62P (Abcam) together with PE-coupled CD41 (Affymetrix) antibodies in phosphate-buffered saline in a total volume of 100 μL. Red blood cells and leukocytes were gated out in forward and side scatter. For leukocyte analysis, red cells in 100 μL of whole blood were lysed with FACS Lysing Solution (BD Biosciences) for 10 minutes at room temperature. Leukocytes, gated in forward and side scatter, were labeled with fluorescein isothiocyanate-coupled CD64, CD11b, CD16, CD14, and PE-coupled CD163 (Affymetrix) antibodies at 1:20 dilution for 25 minutes at room temperature.

2.5 | Soluble biomarker analysis

Levels of soluble circulating biomarkers CXCL1, IL-6, IL-8, IL-10, and soluble ICAM1 (sICAM1) were measured by multiplex assays on MAGPIX analyzer (BioTechne).

2.6 | EV analysis

EV analysis was performed directly in minimally processed hirudinized plasma samples without isolation or enrichment procedures. Hirudinized blood samples were centrifuged twice at 1500 × *g* for 15 minutes at room temperature to obtain platelet-poor plasma, following the protocol used in our previous work. EV concentration and size distribution in citrated plasma samples were estimated using NanoSight NS500 nanoparticle tracking device (Malvern Instruments). The flow cytometry analysis of EVs was performed using fluorescently labeled annexin V or antibodies against CD235a, CD41, CD144, CD142, CD14, CD16, CD284, and CD3. Fluorescently labeled isotype control immunoglobulins G were used as controls. All antibodies and isotype controls were from ThermoFisher. Plasma samples (20 μL) were incubated with antibodies at 1:20 dilution in annexin V binding buffer (Thermo Fisher) for 25 minutes at room temperature in a total volume of 100 μL. The samples were analyzed by flow cytometry (Cyan ADP; Beckman Coulter). EV-specific gates (0.1-1 μm) were determined using polystyrene particles (Spherotech). Percentage of EVs positive for specific antibodies was adjusted for isotype controls and analyzed as described in the statistical analysis section. Our EV methodology is in agreement with MISEV2023 guidelines [20].

2.7 | miRNA analysis

RNA from EVs was isolated from citrated plasma using exoRNeasy kit (Qiagen) according to the manufacturer's instructions. RNA (100 ng) was reverse transcribed using the Taq-Man Multiplex RT set for TaqMan Array Human MicroRNA Panel v2.0 (Applied Biosystems) and Veriti thermal cyclers. Preamplified complementary DNA from each pool was analyzed using TaqMan Array Human MicroRNA Card A and B v2.0 (Applied Biosystems). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out on an Applied BioSystems 7900HT thermocycler. All measurements were performed in triplicate. Any assays in which the cycling threshold (Ct) value was <20 were removed from the analysis, and ΔCt (difference in cycling threshold) values were calculated (ΔCt = miRNA Ct − reference miRNA Ct) using reference control U6 snRNA-001973 present in duplicates on each miRNA array. Undetermined assays were assigned the maximum cycling value. Data were normalized across arrays using quantile normalization. Differential expression analysis was performed with *limma* R package [21].

Selected differentially expressed miRNAs were analyzed using rotor-gene (Qiagen) qRT-PCR instruments in samples from all patients using TaqMan Advanced miRNA assays for hsa-miR-17, hsa-miR-223, hsa-miR-486, hsa-miR-668, hsa-miRs-92a-1, hsa-miRs-920, hsa-miRs-518a-3p, hsa-miRs-133b, and hsa-miRs-1262 (ThermoFisher) according to the manufacturer's recommendations. The data was standardized against levels of hsa-miR-17 and hsa-miR-486, which showed constant average expression levels across all time points and patient groups (Supplementary Figure S1).

2.8 | Statistical analysis

Statistical analysis was performed using R Studio Version 4.3.1 [22], and plots prepared with *ggplot* R package [23]. Data normality was assessed using the Shapiro–Wilk test, with normally distributed variables expressed as mean (± SD) and nonnormally distributed variables expressed as median (IQR). Analyses were performed between patients with AKI and without AKI. Comparisons of miRNA analyzed with PCR arrays and EV size were adjusted for multiple comparisons using Benjamini–Hochberg method. Multivariate logistic models were

fitted with *glm* function, and receiver operating characteristic (ROC) curves were fitted using *pROC* package. Results are reported as odds ratios (ORs) with 95% CI. Density plots were generated using *density* function, which calculates the distribution of a numeric variable using kernel density estimates.

3 | RESULTS

3.1 | Participants

Screening of 230 cardiac surgery patients identified 150 who were eligible for the study. Of these, 53 declined consent and 2 became ineligible after giving consent. Ninety-five patients completed the study (Figure 1A). Fifty-four of these patients (57%) developed post-cardiac surgery AKI, determined according to the Kidney Disease: Improving Global Outcomes definition [24], with the majority (n = 45, 47.4%) exhibiting stage 1, 2 patients developing stage 2 (2.1%), and 7 patients developing stage 3 (7.4%) AKI (Figure 1A). Patient baseline demographics and intraoperative characteristics, shown in Table, were not different between the groups. The mean age was 72.8 (non-AKI, range 54-84) and 70.6 (AKI, range 50-84), with 85% in both groups being male. As expected, levels of serum creatinine were significantly higher in AKI patients 6 to 96 hours after surgery (Figure 1B; Table 1; Supplementary Table S1).

The AKI patients had longer post-surgery ventilation times (AKI: 13, range 10-17 hours vs non-AKI: 9, range 7-14 hours, P = .001) and longer hospital stays (AKI: 10, range 7-13 days vs non-AKI: 8, range 6-9 days, P = .017). Thirty-eight patients had coronary artery bypass graft (CABG) surgery (AKI: 19 [35.2%] vs non-AKI: 19 [46.3%]), 34 patients had valve surgery (AKI: 19 [35.2%] vs non-AKI: 15 [36.6%]), and 23 had both types or another type of surgery (AKI: 16 [29.6%] vs non-AKI: 7 [17.1%]). We did not observe a statistically significant association between the type of surgery (CABG, valve, or combined/other procedures) and the development of AKI (P = .324).

Three patients developed infections after surgery (sternal wound infection, lower respiratory tract infection, and urinary tract infection). There was no difference in the frequency of infections between AKI groups. We also did not observe any difference in postoperative bleeding (Table).

3.2 | Platelet response

Aspirin (75 mg/d) was given to 61 (64.2%) patients before surgery (32 [61.5%] AKI and 29 [74.4%] non-AKI). P2Y12 antagonists (clopidogrel or ticagrelor) were stopped 5 days before surgery. There was no significant difference in antiplatelet administration between AKI groups (Table).

The platelet response was analyzed using a Multiplate aggregometer using adenosine diphosphate (ADP), arachidonic acid (ASPI), and thrombin receptor–activating peptide (TRAP) assays. Aspirin was the only drug that could affect platelet response during the study because patients received it daily. As expected, taking aspirin resulted in a lower area under the curve (AUC) in the ASPI test, which was, and remained, below the cutoff value for aspirin responsiveness for the majority (78%-88%) of aspirinated patients (Figure 2A). There was no statistical difference in the ASPI AUC in the numbers of aspirin high responders between AKI and non-AKI groups (Figure 2B) or in responses to ASPI (Figure 2C; Supplementary Table S1) or any time point.

The range of responses to ADP was broad (Figure 2D) and generally, the ADP AUC was reduced after surgery. There was an overall trend for the AKI patients to have a lower ADP response at every time point, although the statistical difference between the groups was only observed at 6 to 12 hours after surgery (Figure 2D; Supplementary Table S1). The TRAP test, which is insensitive to aspirin, indicated that the platelets showed the highest levels of aggregation 6 to 12 hours after surgery, which then fell over the subsequent 48 hours to levels that were lower than the responses before surgery. There was no difference in TRAP response between AKI groups (Figure 2E; Supplementary Table S1).

3.3 | Thrombo-inflammatory cellular markers

Activation of blood cells implicated in the pathogenesis of AKI was measured using flow cytometry. These included markers of leukocyte activation (CD11b), monocyte activation (CD64 and CD163), and platelet activation (CD62P and activated glycoprotein [GP] IIb/IIIa), as well as circulating platelet–monocyte and platelet–granulocyte aggregates (CD14/CD41 and CD16/CD41, respectively). Due to technical limitations during sample collection, we were not able to analyze up to 34.4% of all patients (Figure 1A). However, the numbers of patients with and without AKI whose samples were missing were comparable with the numbers in the full cohort (missing samples: AKI = 18 [18.8%], no-AKI = 15 [15.6%]) and not statistically significantly different (chi-squared test with full cohort: χ^2 = 0.00064808, df = 1, P = .9797).

Significantly higher levels of platelet activation (activated GPIIb/IIIa detected with PAC-1 antibody) (Figure 3A; Supplementary Table S1) were seen in the AKI patients 6 to 12 hours after surgery. P-selectin expression (CD41/CD62P), a marker of platelet degranulation, was no higher in the AKI patients and did not change over time, but higher levels of granulocyte-platelet aggregates (a consequence of P-selectin expression on platelets) were seen in the AKI patients both before surgery and 6 to 12 and 24 hours afterward (Figure 3B; Supplementary Table S1). There was no difference in platelet count between AKI groups (Figure 3C).

Analysis of soluble biomarkers of inflammation included CXCL1, sICAM1, IL-6, IL-8, and IL-10. Of these, only sICAM1 was altered and was significantly higher before surgery and 48 hours afterward in the patients who developed AKI (Figure 3D; Supplementary Table S1). Although there was no statistical difference in aspirin administration between the AKI groups before surgery, patients who received aspirin had significantly lower sICAM1 levels (1.27 μg/mL, IQR: 0.77-1.72 vs 1.62 μg/mL, IQR: 0.96-2.71, P = .046),

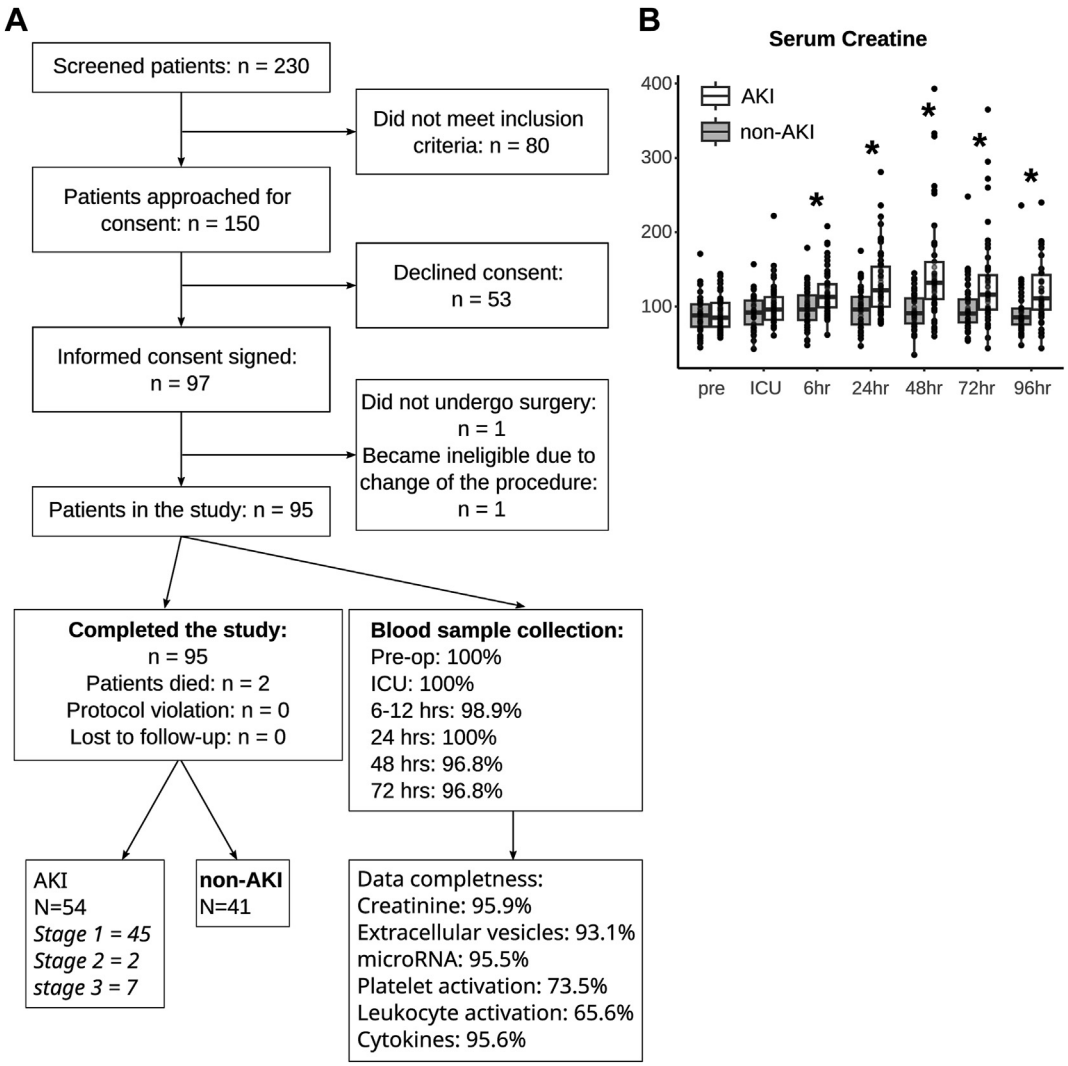

FIGURE 1 (A) CONSORT diagram. (B) Levels of serum creatinine. Asterisks indicate a significant difference between no-AKI and AKI ($P < .05$). AKI, acute kidney injury; ICU, intensive care unit.

3.4 | Extracellular vesicles

EV concentrations ranged from 5.46×10^8 to 14.56×10^8 with no overall difference between AKI and non-AKI patients (Figure 4A; Supplementary Table S1) at any time point. Particle sizes also did not differ between groups (Figure 4B). Derivation analysis identified EVs from monocytes (CD14), endothelial cells (CD144 and CD62E), granulocytes (CD16), red cells (CD235a), myeloid cells (CD284), T cells (CD3), and platelets (CD41). We also assayed EVs positive for annexin V and tissue factor (CD142), which would indicate a pro-thrombotic phenotype. The most abundant EVs were of myeloid origin (CD284-positive; 8.77%-15.88%) followed by annexin V-positive EVs (8.198%-13.44%). The least abundant were EVs positive for CD41 (0.05%-0.255%). However, only platelet-derived EVs (CD41-positive) were expressed at significantly higher levels in patients with AKI 24 hours after surgery (0.095%, IQR: 0.05-0.185 vs 0.05%, IQR: 0.02-0.18, $P = .028$; Figure 4C; Supplementary Table S1).

3.5 | miRNAs

To identify AKI-related miRNAs, 95 samples were pooled into 24 batches at each of 3 time points (preoperative, ICU, and 24 hours after surgery) and analyzed with qRT-PCR arrays for 754 human miRNA species. miR-668 was significantly lower in the AKI patients before surgery, while miR-92a-1, -920, -518a-3p, -133b, and -1262 were higher in the samples from the AKI patients immediately after surgery (in ICU; Figure 4D; Supplementary Table S2). Expression of these miRNAs was then verified by qRT-PCR in each of 95 samples at 3 time points. Levels of miR-1262 were significantly higher immediately after surgery (ICU) in AKI patients, which was the result of lower expression levels in the non-AKI group. Levels of miR-133b were significantly lower in AKI patients before surgery (Figure 4E; Supplementary Table S1) but not after surgery. Levels of miR-920 and mir-92a-1 were not different in AKI patients. Expression of miR-668 and miR-518a-3p were below detection level in most samples.

TABLE Baseline and intraoperative characteristics.

Characteristic	No-AKI (*n* = 41)	AKI (*n* = 54)	*P*
Diabetes	20 (48.8%)	31 (57.4%)	.416
Chronic kidney disease	9 (22.0%)	11 (20.4%)	.852
Age (y)	70.6 (6.9)	72.8 (7.6)	.151
Male (n)	35 (85.4%)	46 (85.2%)	.980
Caucasian	41 (100.0%)	52 (96.3%)	.504
Body mass index (kg/m^2)	31.7 (5.2)	31.4 (6.1)	.745
Surgery type			
Coronary artery bypass graft	19 (46.3%)	19 (35.2%)	.324
Valve	15 (36.6%)	19 (35.2%)	
Both/others	7 (17.1%)	16 (29.6%)	
Medical history			
Myocardial infarction	7 (17.1%)	10 (18.5%)	.856
CVA/TIA	5 (12.2%)	8 (14.8%)	.713
Chronic pulmonary disease	1 (2.4%)	2 (3.7%)	.999
Previous cardiac surgery	2 (4.9%)	4 (7.4%)	.696
Smoker			
Current	12 (30.0%)	22 (42.3%)	.478
Ex-smoker	18 (45.0%)	19 (36.5%)	
Never	10 (25.0%)	11 (21.2%)	
NYHA functional classification			
Class I	5 (12.2%)	8 (14.8%)	.491
Class II	29 (70.7%)	32 (59.3%)	
Class III and IV	7 (17.1%)	14 (25.9%)	
CCS angina grade			
Asymptomatic	11 (27.5%)	24 (44.4%)	.110
Class I	10 (25.0%)	11 (20.4%)	
Class II	16 (40.0%)	19 (35.2%)	
Class III	3 (7.5%)	0 (0.0%)	
Left ventricular ejection fraction			
Good (>49%)	24 (63.2%)	35 (67.3%)	.682
Fair (30-49%)	14 (36.8%)	17 (32.7%)	
Left main stem disease	4 (10.3%)	9 (17.3%)	.342
Extent of coronary disease			
Normal	6 (15.4%)	11 (21.2%)	.287
Single-vessel disease	11 (28.2%)	9 (17.3%)	
2-vessel disease	8 (20.5%)	6 (11.5%)	
3-vessel disease	14 (35.9%)	26 (50.0%)	
Antiplatelet drugs			
Aspirin (preoperative)	29 (74.4%)	32 (61.5%)	.288
P2Y12 inhibitors (stopped 5 d before surgery)	5 (12.8)	7 (13.5)	1
Aspirin (1 d after surgery)	34 (87.2%)	40 (76.9%)	.332
P2Y12 inhibitors (1 d after surgery)	7 (17.9%)	6 (11.5)	.574
Dual platelet therapy (1 d after surgery)	7 (17.9%)	6 (11.5)	.574
Serum troponin I (ng/L)	20 (15-26)	15 (15-27)	.627
Serum creatinine (μmol/L)	88 (73-103)	85 (73-105)	.848
Heart rate (bpm)	68.9 (12.4)	66.4 (11.7)	.328
Mean arterial pressure (mmHg)	98.7 (11.9)	97.5 (14.4)	.674
Hemaglobin (g/dL)	13.3 (1.7)	12.9 (1.4)	.246
Hematocrit (%)	39.1 (4.7)	38.3 (4.0)	.418
White blood cells (× 10^9/L)	7.9 (1.8)	8.1 (1.9)	.623
Platelets (× 10^9/L)	225.6 (76.8)	214.9 (50.9)	.457
eGFR (mL/min/1.73 m^2)	82.4 (30.1)	78.2 (22.4)	.453
Albumin (g/L)	40.5 (2.3)	40.6 (3.6)	.825
Bilirubin (μmol/L)	13.1 (5.0)	13.1 (6.5)	.992
Aspartate aminotransferase (IU/L)	23.0 (4.8)	25.1 (7.1)	.107
Alanine aminotransferase (IU/L)	24.1 (11.0)	24.8 (9.9)	.760
Alkaline phosphatase (IU/L)	80.1 (26.5)	83.1 (31.9)	.634
γ-glutamyl transferase (IU/L)	24 (18-34)	32 (19 -58)	.086
CBP time (min)	93 (82-109)	106 (82-139)	.117
Cross-clamp time (min)	55 (46-77)	65 (47-97)	.259
Ventilation time (h)	**9 (7-14)**	**13 (10-17)**	**.001**

(Continues)

(Continues)

TABLE (Continued)

Characteristic	No-AKI (*n* = 41)	AKI (*n* = 54)	*P*
ICU stay (h)	27 (25-53)	48 (25-93)	.080
Hospital length of stay (d)	**8 (6-9)**	**10 (7-13)**	**.017**
Bleeding			
Yes	38	50	1
No	3	3	
Intraoperative transfusion			
Patients receiving red blood cells	7	10	1
Patients not receiving red blood cells	34	44	
Units of RBC transfused	0 (0-4)	0 (0-2)	.844
Patients receiving platelets	4	10	.261
Patients not receiving platelets	37	44	
Units of platelets transfused	0 (0-3)	0 (0-2)	
Patients receiving FFP	4	10	.696
Patients not receiving FFP	37	44	
Units of FFP transfused	0 (0-3)	0 (0-2)	.234

Tests between groups were conducted by exact test for categorical variables and *t*-test or nonparametric Kruskal–Wallis test for continuous variables. Data are presented as *n* (%) for categorical variables and mean (SD) or median (IQR) for continuous variables.

Bold values indicate a significant difference between AKI and non-AKI.

AKI, acute kidney injury; CCS, Canadian Cardiovascular Society; CCS, Canadian Cardiovascular Society; CVA, cerebrovascular accident; eGFR, estimated glomerular filtration rate; FFP, fresh frozen plasma; ICU, intensive care unit; NYHA, New York Heart Association; TIA, transient ischemic attack.

Missing data: smoking staus (*n* = 3), CCS angina grade (*n* = 1), left ventricular ejection fraction (*n* = 5), left main stem disease (*n* = 4), extent of coronary disease (*n* = 4), antiplatelet drugs (*n* = 4)

3.6 | Diagnostic utility of the tested variables

We performed a correlation analysis of all significant variables and most likely confounders for AKI, age, diabetes, and baseline kidney function followed by multivariate logistic models to test the variables' association with AKI. The discriminatory ability of the significant variables was tested by fitting ROC curves.

Before surgery, levels of granulocyte-platelet aggregates (CD16/CD41) correlated significantly with sICAM1 and miR-133b, and sICAM1 also correlated with patients' age (Figure 5A). We fitted logistic models together with age, diabetes, and baseline serum creatinine levels. Granulocyte-platelet aggregates were tested separately from sICAM1 and miR-133b to avoid multicollinearity. Levels of platelet–granulocyte aggregates were significantly associated with an increased risk of AKI. Each unit increase was linked to a 4% rise in the odds of developing AKI (OR = 1.04, 95% CI: 1.01-1.07, *P* = .01). We found no association between AKI and sICAM1 and miR-133b (Supplementary Table S3). ROC curves fitted for granulocyte-platelet aggregates, age, and diabetes indicated that these variables have moderate discriminative power for AKI (AUC: 78.5%, 95% CI: 67.1%-90.0%; Figure 5B).

At ICU, levels of miR-1262 correlated significantly with baseline serum creatinine levels (Figure 5C). However, miR-1262 was not associated with AKI (Supplementary Table S3).

The analysis of significant variables at the 6 to 12 hour time point did not detect any significant correlations (Figure 5D). Therefore, levels of activated GPIIb/IIIa, Multiplate ADP, and platelet–granulocyte aggregates were included in the multivariate logistic model. Higher expression of activated GPIIb/IIIa (OR: 1.03, 95% CI: 1.00-1.06, *P* = .04), CD16/CD41 (OR: 1.06, 95% CI: 1.01-1.11, *P* = .02), age (OR: 1.11 per year, 95% CI: 1.01-1.23; *P* = .03), and diabetes (OR: 4.81, 95% CI: 1.15-20.02, *P* = .03) were independently associated with AKI. ADP showed a statistically significant inverse association (*P* = .01), while serum creatinine at baseline was not significantly associated (*P* = .56; Supplementary Table S4). An ROC curve was fitted for activated GPIIb/IIIa, ADP, platelet–granulocyte aggregates, age, and diabetes and resulted in AUC: 83.1% (95% CI: 72.9%-93.3%), which indicated good discriminative ability between patients with and without AKI (Figure 5E).

At 24 hours, CD41-positive EVs correlated significantly with platelet–granulocyte aggregates and age (Figure 5F). Therefore, we tested the platelet-related parameters separately in multivariate logistic models. Supplementary Table S4 shows that only platelet–granulocyte aggregates were independently associated with AKI (OR: 1.03, 95% CI: 1.00-1.06, *P* = .03). Diabetes showed a trend toward significance (OR: 2.71; 95% CI: 0.88-8.36, *P* = .08), while age and baseline serum creatinine were not significantly associated with the outcome (*P* = .14 and *P* = .68, respectively, Supplementary Table S3). The CD41-positive EVs were not associated significantly with AKI. Therefore, we fitted a ROC curve only for platelet–granulocyte aggregates. It offered a modest discriminative ability between AKI and non-AKI patients with AUC: 67.5% (54.1%-80.9%; Figure 5G).

At the 48-hour time point, sICAM1 correlated significantly with patient age (Figure 5H). In a multivariate logistic model, sICAM1 was significantly associated with the outcome (*P* = .01), although the OR was reported as 1.00 with a 95% CI of 1.00 to 1.00, suggesting a statistically significant but numerically negligible effect. Age, diabetes, and baseline serum creatinine were not significantly associated with the outcome (*P* > .05 for all, Supplementary Table S4). The AKI discriminatory potential of sICAM1 at 48 hours after surgery was modest with AUC: 69.9% (58.8%-81.0%; Figure 5I; Supplementary Table S3).

3.7 | Sensitivity analyses

Given that most patients were receiving aspirin, we restricted the analysis to patients on aspirin only. The results of this analysis were

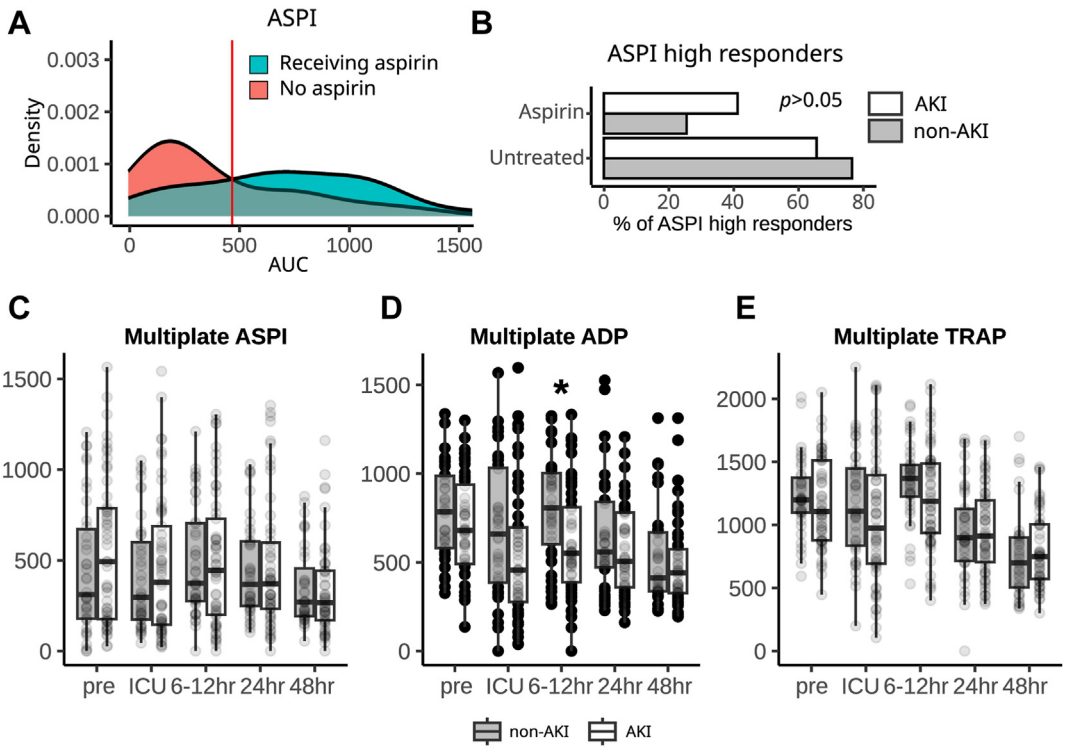

FIGURE 2 (A) Density plot of the area under the curve (AUC) values acquired with Multiplate ASPI test. The vertical red line indicates the position of a cutoff value for ASPI high responders (AUC = 473). (B) Fraction of ASPI high responders in people with AKI or those without. The analysis was performed for aspirin-treated and untreated patients. (C) Box plot of AUC data acquired using Multiplate ADP assay. Asterisks indicate a significant difference between AKI and non-AKI groups ($P < .05$). Open and gray boxes in B and D represent data from patients with and without AKI, respectively. AKI, acute kidney injury; AUC, area under the curve; ICU, intensive care unit.

very similar to those of all patients. Patients with AKI had higher levels of serum creatinine 6 or 96 hours after surgery, platelet-derived EVs before surgery and at 24 hours after surgery, miR-1262 levels at ICU, platelet–leukocyte aggregates before and 6 to 12 hours after surgery, and sICAM1 at 48 hours after surgery. Multiplate ADP AUC was lower at 6 to 12 hours after surgery in patients with AKI. However, we did not observe significant differences for activated GPIIb/IIIa and hsa-miR-133b, platelet–leukocyte aggregates 24 hours after surgery, and sICAM1 before surgery (Supplementary Table S4).

The grade of AKI is often associated with increased inflammation [25]; therefore, we analyzed the data without patients who developed AKI stages 2 and 3. This results of this analysis were nearly identical to the analysis in all patients. The differences included significantly elevated platelet–leukocyte aggregates at 48 hours, significantly elevated activated monocytes (CD163/CD14), and no significant difference in platelet activation at 6 to 12 hours (Supplementary Table S5).

4 | DISCUSSION

Longitudinal analysis of cellular activation, inflammatory response, and levels of circulating EVs and miRNA showed a significant elevation of platelet activation, as demonstrated by platelets with activated GPIIb/IIIa and by platelet–granulocyte aggregates, in cardiac surgery patients who develop AKI. Levels of platelet–granulocyte aggregates (CD16/CD41) were significantly higher before surgery and at 6 to 12 hours and 24 hours after surgery in the AKI group. This group also had increased levels of platelets with activated GP IIb/IIIa (PAC-1 positive) and platelet-derived EVs at 6 to 12 and 24 hours post-surgery, respectively, alongside increased ADP insensitivity 6 to 12 hours after surgery. Moreover, circulating sICAM1 levels were higher, and levels of plasma miR-133b were lower at baseline in the AKI group. Both correlated with levels of platelet–granulocyte aggregates at baseline. miR-1262 was most differentially expressed in the AKI group immediately after surgery and correlated with preoperative sICAM1 levels.

The results demonstrate that platelet activation, resulting in platelet–leukocyte aggregation, is associated with AKI after cardiac surgery. Platelets have been shown to have an important role in experimental AKI, where platelet–granulocyte aggregates facilitate intraglomerular neutrophil recruitment and ROS production [26]. In rodents, platelets guide neutrophils to areas with reduced endothelial integrity such as glomeruli [3], whose glycocalyx is disrupted during AKI [27]. In the current study, sICAM1 and platelet–leukocyte aggregates were positively correlated before surgery, suggesting that a proinflammatory state could prime the patient's susceptibility to renal injury. We did not observe any difference between AKI groups immediately after surgery (ICU time point), and the data shows that platelet activation was lowest at this time point. The lack of difference

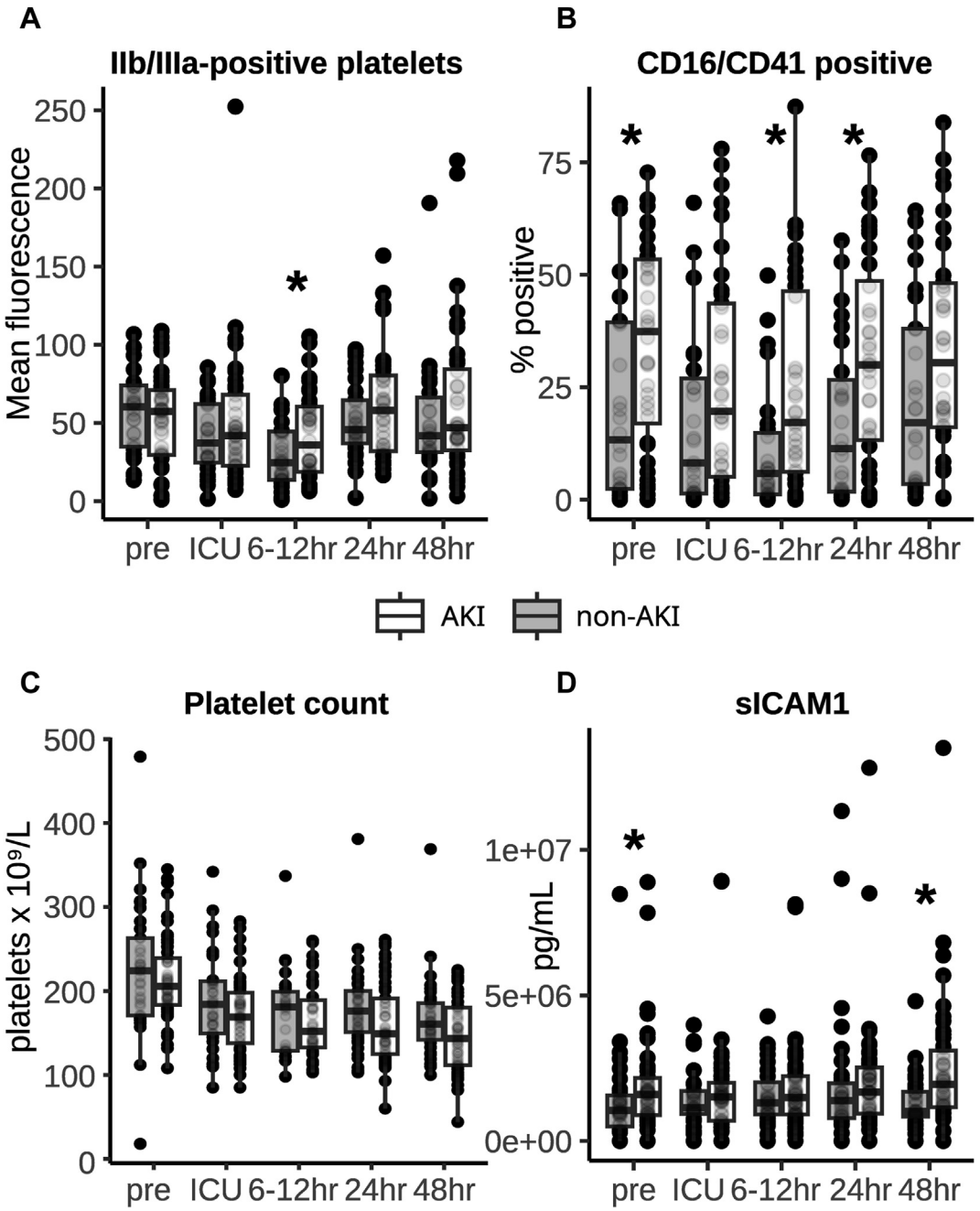

FIGURE 3 (A) Box plot of mean expression values for IIb/IIIa detected with PAC-1 antibody in flow cytometry. (B) Box plot of a fraction of events positive for CD16 and CD41. (C) Box plot of sICAM1 concentrations in citrated plasma acquired with a MAGPIX device. Asterisks indicate a significant difference between AKI and non-AKI groups ($P < .05$). Open and gray boxes in A–C represent data from patients with and without AKI, respectively. AKI, acute kidney injury; ICU, intensive care unit.

could be attributed to several factors, including hemodilution, hypothermia, administration of heparin followed by protamine, and exposure to artificial surfaces of the CPB circuit [28]. These factors could have various effects on platelets, including decreasing their activation (hypothermia); further activation due to, eg, contact with artificial surfaces; and sequestration of activated platelets and platelet-leucocyte aggregates in the lung [29]. After the CPB, platelet activation increased earlier in patients who developed AKI. That is particularly evident in the platelet–leukocyte aggregates data, whose levels increased faster in AKI patients at 6 to 12 and 24 hours after surgery. It is generally acknowledged that platelet–leukocyte aggregates are a much more sensitive way of measuring platelet degranulation than measuring platelet surface P-selectin expression, as originally proposed by Michelson et al. [30]. We believe this increased rate may be to higher levels of platelet activation before surgery, although this needs to be further investigated. We also observed higher levels of activated GPIIb/IIIa and desensitization to ADP 6 to 12 hours after surgery in AKI patients. The activation of platelets is generally a very

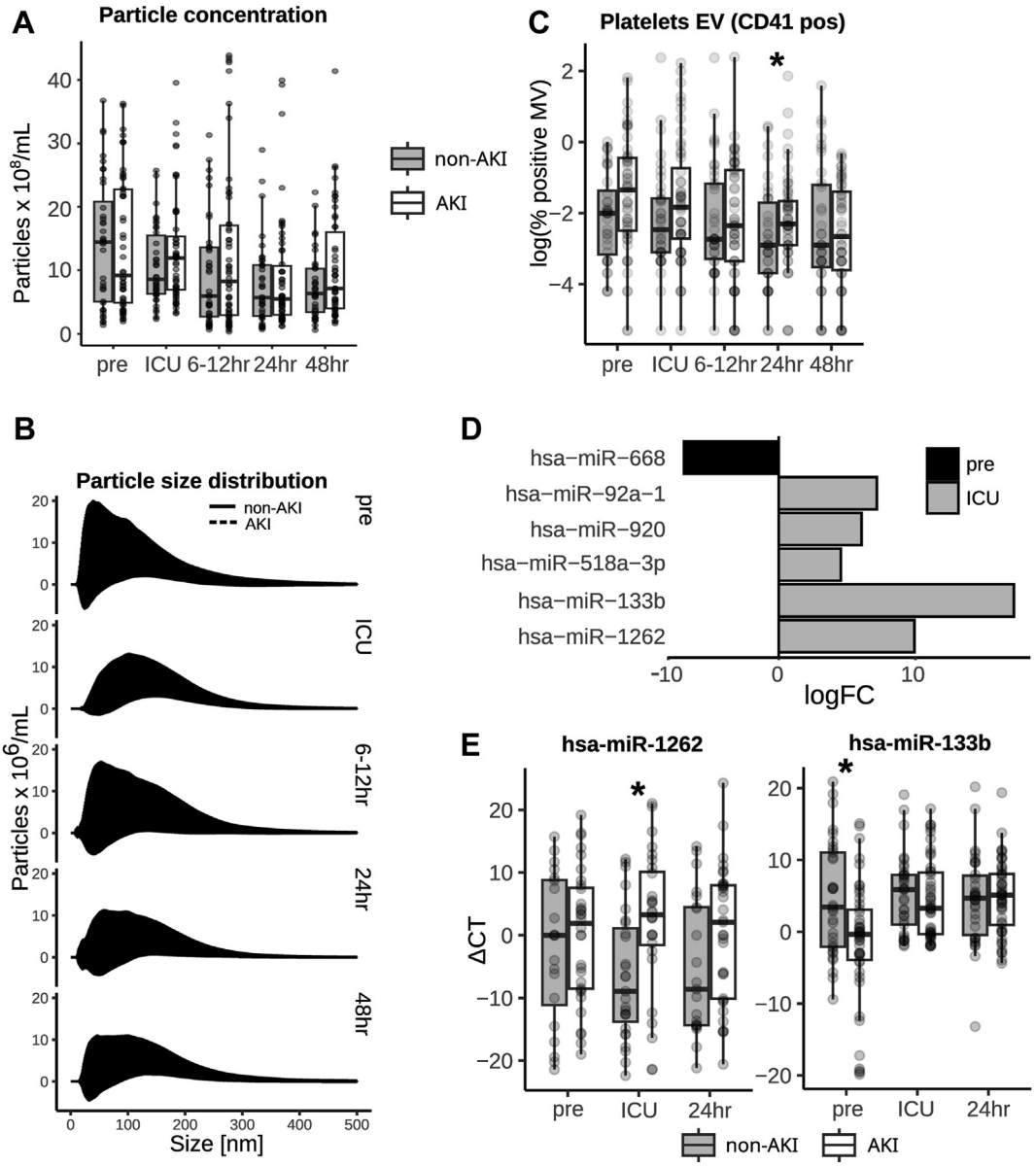

FIGURE 4 (A) Total particle concentrations in citrated plasma. (B) Mean size distribution of particle concentrations (± SD). (C) Box plot of EVs positive for platelet marker CD41. (D) Bar plot showing the log fold difference in miRNA identified by qRT-PCR arrays between AKI and non-AKI. Only miRNA with adjusted $P < .05$ are shown (see Supplementary Table S2 for details). (E) Box plot of validation results for miR-133b and miR-1262. Asterisks indicate a significant difference between AKI and non-AKI groups ($P < .05$). Open and gray boxes in A, C, and E represent data from patients with and without AKI, respectively. AKI, acute kidney injury; EV, extracellular vesicle; qRT-PCR, quantitative real-time polymerase chain reaction.

rapid process, so the higher levels of activated and desensitized platelets in the AKI group point to the fact that the AKI patients have platelets that are more prone to activation.

By 48 hours after surgery, there were no differences in platelet activation between the groups, which may reflect better antiplatelet medication in all patients. AKI, as detected by rising serum creatinine, is typically seen at 48 hours, implying that kidney damage occurs sometime after the causative events. This is supported by the elevated level of sICAM1 at 48 hours, which is likely a delayed response to platelet activation requiring gene transcription. It is well established that activated platelets binding to leucocytes and endothelial cells are potent inducers of ICAM1 expression [31,32].

The observed desensitization of platelets to ADP at 6 to 12 hours after surgery is not a consequence of the action of P2Y12 antagonists, which were not administered until 24 hours after surgery. It could be explained by the release of ADP in vivo from activated platelets, damaged red cells, or other tissues [33]. Alternatively, the response to ADP could be explained by the fact that platelet aggregation is transiently increased in response to heparin [34,35]. We also show that aspirin did not affect the observed changes in platelet function in the

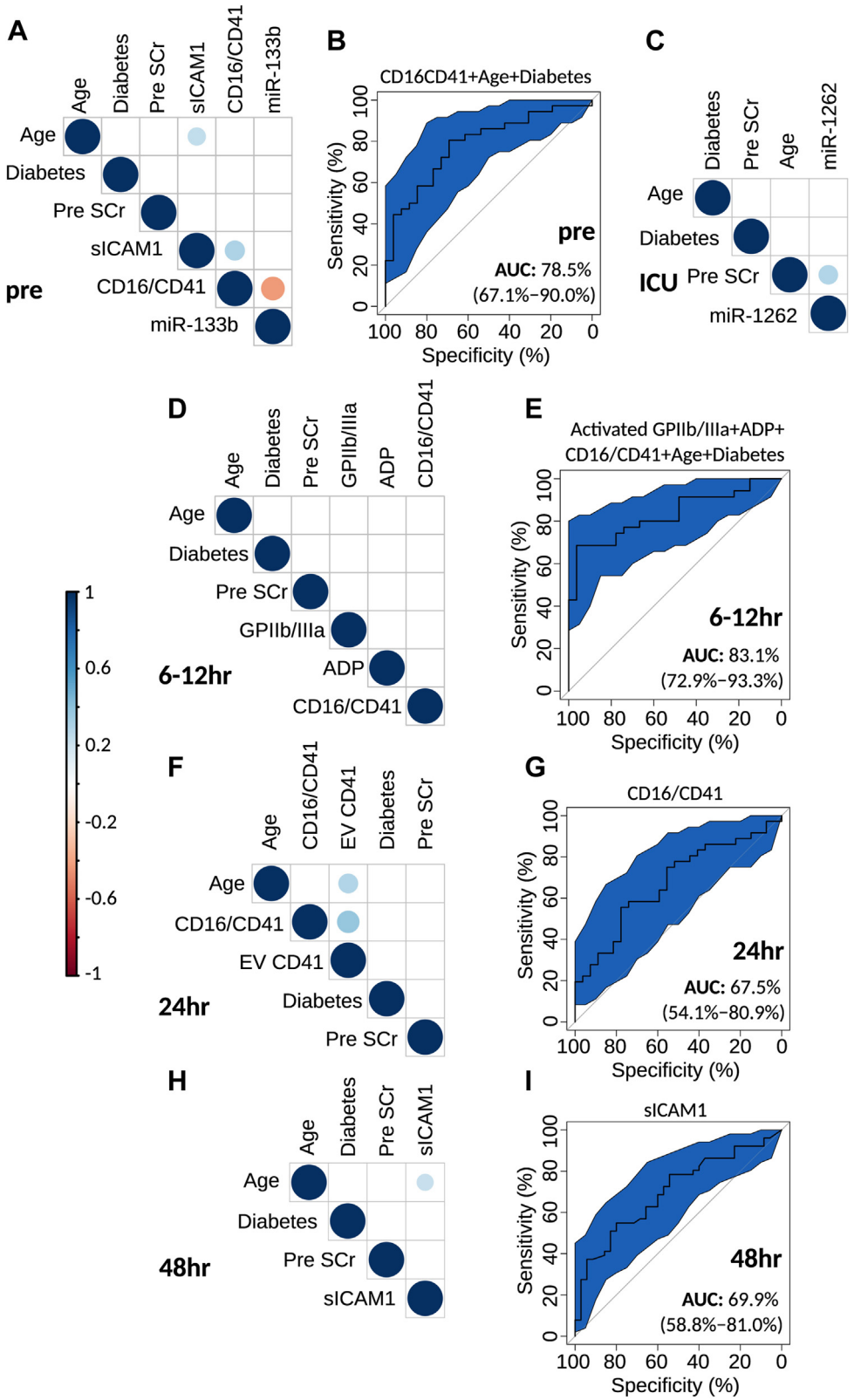

FIGURE 5 (A, C, D, F, H) Correlations (Spearman) between significant variables at each time point and AKI risk factors: age, diabetes, and preoperative serum creatinine. Blue circles indicate significant positive and red circles negative correlation. (B, E, G, I) Receiver operating characteristic curve for variables with significant association with AKI (Supplementary Table S4). AKI, acute kidney injury; AUC, area under the curve; ICU, intensive care unit.

AKI group. No trial, to our knowledge, has evaluated the renoprotective effect of antiplatelet agents in cardiac surgery [36,37]. Although multiple randomized controlled trials have compared the effects of different antiplatelet agents on vein graft patency rates, arterial thromboses, and bleeding post-surgery [38], none of these trials reported AKI event rates. Indirect evidence for a renoprotective effect of platelet inhibition comes from small trials of nitric oxide donors or prostacyclin [39–41], which have known antiplatelet effects and improve creatinine clearance after cardiac surgery. The increased levels of platelet–granulocyte aggregates in AKI patients before and after surgery brings to light the interaction between P-selectin and P-selectin glycoprotein ligand-1, which facilitate leukocyte rolling and firm adhesion of activated leucocytes to the endothelium at sites of vascular injury or inflammation and promote the release of proinflammatory cytokines and reactive oxygen species, thereby amplifying the inflammatory response [42,43]. Together, our results support the reevaluation of existing trials or new trials of novel antiplatelet agents or blockers of P-selectin. These include drugs like crizanlizumab recently approved for sickle-cell disease [44] or inclacumab, which is currently in the development phase.

Previous clinical studies also have implicated platelet activation in postcardiac surgery AKI. Yang *et al.* [45] found that patients who developed AKI had significantly higher levels of circulating platelet–leukocyte aggregates before and within the first 20 hours after surgery and that elevated preoperative platelet–leukocyte aggregates were associated with increased AKI risk [45]. The authors observed elevated platelet activation was not attenuated by the CPB as in our study. However, the study enrolled only patients with rheumatic valve disease, which is associated with higher inflammation levels. It strengthens our hypothesis that preoperative inflammation is responsible for the observed changes in platelet aggregation with leukocytes.

Ma et al. [46] reported that patients with AKI had increased concentrations of platelet-derived EVs (microparticles) following CPB. In that study, both platelet- and endothelial-derived microvesicle levels were higher in the AKI group than in non-AKI patients 3 days after surgery, and elevated microparticle counts (especially endothelial-derived) were independent predictors of subsequent AKI. The authors measured EVs before surgery and 12 hours and 3 days afterward. Unfortunately, only the first 2 time points match our study, and additional work would be needed to evaluate the changes in platelet EVs beyond 48 hours. The support for changes in platelets in AKI patients also comes from indirect indices, including mean platelet volume (a surrogate of platelet activation [47]) and postoperative thrombocytopenia (reflecting platelet activation [48]) are associated with a higher risk of developing AKI. In our study, platelet counts also decreased after surgery; however, we did not observe any differences in the patients with AKI compared with the non-AKI group. However, a study by Kertai et al. [48] enrolled 4217 participants, which provides much greater power to detect smaller differences.

miR-1262 has been associated with mesangial proliferative glomerulonephritis [49]; however, it remains to be tested whether its correlation with sICAM1 is functionally related. There is also little known about the role of miR-133b, which correlated negatively with platelet–granulocyte aggregates. It has been shown to regulate kidney fibrosis by inhibiting connective tissue growth factor [50] and was previously associated with diabetic nephropathy, in which its serum levels increase [51], but no previous studies have associated miR-133b with either platelets or granulocytes. There are conflicting reports regarding miR-133 (unspecified strand or isoform), which was found enriched in human platelets by Diehl et al. [52] but not by Ambrose et al. [53].

As in any other study of AKI biomarkers, the major limitation is the use of serum creatinine to define renal injury [54]. More recent biomarkers, such as TIMP-2 and IGFBP7, have been shown to be useful diagnostic tools for AKI. However, they are not discriminatory in the cardiac surgery cohort [11]. Another limitation is the use of pooled samples for the initial identification of AKI-associated miRNAs. This was dictated by the budgetary limitations, and analysis of all patients, or a selected cohort of patients with well-defined clinical characteristics, could potentially result in the identification of more relevant miRNAs. Sample pooling can also be behind the discrepancies in miR-133b expression between discovery and validation.

Due to the small sample volumes, we were not able to perform all elements of EV characterization recommended by MISEV2023 to validate the EV identity [20], eg, we did not purify EVs to confirm their identity with an alternative method. Furthermore, NanoSight, used to estimate particle concentration, does not discriminate between cell-derived, membrane-bound particles and other particulates in plasma such as lipoproteins. Therefore, we were not able to conclude whether the increases in specific particle sizes are associated with platelet-derived EVs. Another limitation is the small size of the study and high levels of missing data for flow cytometry analysis of platelet and leukocyte activation. The missing data were due primarily to sample availability and technical limitations during sample collection, not to any systematic differences between AKI and non-AKI groups. We cannot be sure that a larger sample or one with different eligibility criteria would not provide a different result. Furthermore, we acknowledge that the possibility of random missingness introducing bias cannot be excluded. Such random variation could affect the statistical power to detect differences and may influence the robustness of associations.

A systematic review by Hu et al. [55] identified differences in AKI incidence between patient groups undergoing different surgery types, such that CABG surgery had a lower incidence than valve surgery or other types [55]. While these trends have been observed in broader patient populations, individual studies may not always detect statistically significant differences. The small sample size could also explain the lack of significant difference between some of the tested biomarkers, particularly inflammatory cytokines. Another factor that could explain why some biomarkers in our study did not show statistically significant differences could be the fact that our inclusion and exclusion criteria selected a relatively homogeneous group of elective surgery patients, possibly reducing biological variability but also

attenuating differences in some markers. For example, the exclusion of patients with preexisting inflammatory state could potentially limit the role of inflammation in AKI pathogenesis.

In conclusion, AKI is associated with higher levels of platelet–leukocyte aggregates before surgery and a faster rate of platelet activation within the first 24 hours after surgery. Our data suggests that platelet inhibition, particularly P-selectin, could offer renoprotection. However, larger studies are needed to validate these findings.

AUTHOR CONTRIBUTIONS

G.J.M., A.H.G., and M.J.W. designed the study. M.J.W. and N.B. wrote the manuscript with critical input from G.J.M. and A.H.G. L.J.-D., T.K., and H.A. managed the conduct of the study. N.S., B.E.-H., S.S. and K.T. collected samples and performed wet lab experiments. N.B., N.S., F.Y.L., and M.J.W. performed statistical analyses.

DECLARATION OF COMPETING INTEREST

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2025.06.009.