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Platelet reactivity in young children undergoing congenital heart disease surgery: a NITRIC randomized clinical trial substudy

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Abstract

Introduction The international NITRIC trial studied the hypothesis that nitric oxide (NO) applied into the cardiopulmonary bypass (CPB) oxygenator in infants would improve recovery after heart surgery. In a substudy, we evaluated the effect of NO applied into the CPB oxygenator on the (re)activity of platelets measured as fibrinogen binding (platelet aggregation) and P-selectin expression (platelet degranulation) in young children.

Methods Platelet activity (without agonist exposure) and reactivity (after stimulation by an agonist) was studied in a single center substudy of the NITRIC trial, a multicenter, randomized trial that studied administration of 20 parts per million (ppm) NO during CPB in children younger than 2 years. Blood was collected at 4 time points (T1- T4); before CPB, after CPB start, before and after weaning. Flow cytometry-based platelet activity and reactivity in the presence of 5 agonists was tested. Differences on P-selectin expression and fibrinogen binding (median fluorescence intensity (MFI)) were analyzed with mixed effect modelling (MEM).

Results Blood samples were obtained in 22 patients allocated to NO and 20 controls. Platelet counts dropped after T1 due to the hemodilution of blood in all patients ($p < 0.001$). Beta coefficients for NO allocation derived from the MEM models on fibrinogen binding and P-selectin expression were small (standardized beta coefficients on fibrinogen binding were 0.07[0.03, 0.11] and on P-selectin expression 0.05[0.03, 0.08]) and non-significant. CPB duration did not affect platelet reactivity (standardized beta coefficients 0.09[0.02, 0.12] with $p > 0.27$) in any of the MEMs.

Conclusion 20 ppm NO administration in the sweep gas of the CPB oxygenator did not affect platelet reactivity in young children undergoing heart surgery. Interestingly, duration of CPB exposure also did not have an effect on platelet (re)activity.

Trial registration ANZCTR, ACTRN12617000821392. Registered 5 June 2017, https://anzctr.org.au/

Keywords Nitric oxide, Platelet activation, Platelet function tests, Heart defects congenital, Infant, Cardiopulmonary bypass

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Introduction

Cardiopulmonary bypass (CPB) facilitates congenital heart disease (CHD) surgery in the majority of children who need repair or palliation in infancy. However, CPB which requires systemic heparinization and subsequent protamine reversal also triggers a widespread endothelial and inflammatory system response adversely affecting the coagulation system [1–4]. Bleeding following cardiac surgery is accentuated by dilutional coagulopathy, platelet activation on the artificial CPB surface, and platelet/coagulation factor consumption. In vivo, platelets circulate in an inactive state as spontaneous activation of platelets is inhibited by several endothelial cell-derived molecules, including nitric oxide (NO) [5–7]. NO interacts with a haem group of the enzyme soluble guanylyl cyclase, present in platelets. This interaction allows soluble guanylyl cyclase to catalyze the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G. Protein kinase G phosphorylates a variety of proteins that lead to inhibition of calcium release thereby inhibiting platelet activation [7].

The international NITRIC randomized controlled trial, the largest RCT conducted in congenital heart disease, was designed to study the hypothesis that NO applied into the cardiopulmonary bypass oxygenator in infants would improve their recovery [8, 9]. The trial did not find outcome differences between patients receiving NO at 20 ppm while on CPB compared to controls receiving standard CPB [8], neither for the primary outcome (ventilator free days) nor for any of several secondary outcomes [8]. We also hypothesized that NO administered in the oxygenator might temporarily inhibit platelet activation during CPB, favoring the availability of functional platelets post CPB and this might reduce bleeding after weaning from CPB. In this preplanned single center substudy, we evaluated the effect of 20 ppm NO administered into the sweep gas of the CPB oxygenator on platelet activity and reactivity (after stimulation with agonists) based on flow cytometry measured P-selectin expression and fibrinogen binding in young children during CHD surgery.

Materials and methods

The conduct of the international NITRIC (Nitric Oxide During Cardiopulmonary Bypass to Improve Recovery in Infants with Congenital Heart Defects) trial has been described in detail in the original report and protocol [8–10]. In summary, it was a multicenter, randomized, double-blind trial that compared the administration of 20 parts per million (ppm) NO in the sweep gas of the CPB oxygenator with standard care in children younger than 2 years. Persistently elevated pulmonary vascular resistance, chronic ventilator dependency, severe preoperative shock and sepsis, acute respiratory distress syndrome, and methemoglobinemia and those after cardiac arrest receiving extracorporeal life support were exclusion reasons. This preplanned substudy was conducted as a single center sub-investigation at the University Medical Center Utrecht, the Netherlands in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The protocol was reviewed and approved by the Medical Research Ethics Committee Utrecht "metc.Utrecht" (NL64083.04.18 / METC 18–645/ Date: 6 February 2019). Written informed consent for participation in the NITRIC trial and collection of additional blood samples for platelet assessments in this substudy was obtained from parents or guardians prior to surgery.

Randomization and CPB

Eligible and consented participants were randomized by clinical perfusionists prior to the start of bypass as per the NITRIC trial protocol. Participants were randomized to receive standard care (control group) or nitric oxide administration (NO group) using block randomization. In the NO group, NO was applied into the sweep gas of the CPB oxygenator from initiation until weaning from bypass with a concentration of 20 ppm. In the control group, NO was not applied during CPB. The NO delivery system was covered, and NO delivery rates were only visible to the perfusionists. All patients received perioperative care according to our clinical CHD surgery standards including 1 mg/kg dexamethasone administration during induction of anesthesia. The LivaNova S5 heart lung machine was used with the following tubing and oxygenators: Baby FX05 with Xcoating (amphiphilic polymer) (Terumo Corporation, Tokyo, Japan)(< 8 kg), Quadrox Pediatric with SOFTLINE coating (Getinge, Hirrlingen, Germany) (8–25 kg) and PH.I.S.I.O. (phosphorylcholine) coated tubing (LivaNova, London, UK). CPB prime was composed with red blood cell concentrate, ringer solution, albumin, heparin, sodium-bicarbonate, mannitol 15%, and in infants < 5 kg Omniplasma (a pooled plasma product) was routinely used. Prior to cannulation, heparin was titrated after an initial bolus of 400 IU/kg towards an activated coagulation time (ACT) of > 440 s. Following separation from CPB, modified ultrafiltration (MUF) was used to partly reverse hemodilution, whereafter protamine was administered to reverse heparinization. Baseline characteristics to describe age at day of surgery [3, 11], cardiac anomalies, surgical population (Basic Aristotle for surgical complexity (1.5–15), with duration of CPB and aortic cross clamping, and the use of deep hypothermic cardiac arrest of selective cerebral perfusion) and concurrent diseases were extracted from the trial's case

report form (eCRF) and supplemented with manually collected possible explanatory variables: arterial oxygen saturation prior to surgery, antiplatelet drug use (yes/no), peak flow velocity measured on the most recent transthoracic echo (TTE) prior to surgery [12], and minimal core temperature on CPB [13].

Blood sampling

All blood was collected and stored in heparin tubes (17 IU per ml) on four different time points for each patient: (T = 1) after the start of surgery before CPB, (T = 2) after the start of CPB, (T = 3) before weaning from the CPB, and (T = 4) which was after weaning from CPB, MUF, administration of protamine, and before any platelet transfusions were started. Sampling was done from the patient's arterial blood pressure cannula at T = 1 and 4, and from the arterial CPB circuit at T = 2 and 3. Samples were rested for at least one hour and hereafter incubated at 37˚C with the reagents, fixed with a formaldehyde solution and stored at 4˚C. Platelet counts and mean platelet volume (MPV) tests were performed from a separately obtained tube at our regular laboratory testing facility.

Flow cytrometric assessment of platelet reactivity

Platelet activity (without exposure to an agonist) and reactivity (after exposure to an agonist) was assessed with a flow cytometry-based platelet activation test that quantifies both platelet degranulation (P-selectin expression) and platelet aggregation (fibrinogen binding to integrin αIIbβ3). The handling of whole blood and the flow cytometry technique has been performed according to previously described techniques [14–16]. These tests are feasible in small children as only a limited amount (< 50 μL) of blood was necessary compared to conventional platelet function assessments (light transmission aggregometry). We analyzed platelet reactivity in the presence or absence of five agonists: peptides PAR1-AP and PAR4-AP to activate the thrombin/ protease activated receptors 1 and 4 (PAR1 and PAR4), adenosine diphosphate (ADP) to activate its receptor P2Y12, cross-linked collagen related peptide (CRP-xL) to activate Glycoprotein-VI (GPVI), and U46619, which is a thromboxane A2 mimetic. (Re)activity of platelets (P-selectin expression and fibrinogen binding) is expressed in median fluorescence intensity (MFI). Laboratory staff members were unaware of the treatment allocation during processing and analyzing platelet reactivity.

Sample size calculation

For this single center substudy, we did not perform a preplanned sample size calculation as we intended to obtain platelet samples in all NITRIC trial participants in Utrecht. Due to covid-19 laboratory regulations we were not able to process and obtain samples in 40 patients. A post-hoc sample size calculation for this convenience sample was done based on an unstimulated platelet activity difference at timepoint 3 between the allocation groups. For a relevant fibrinogen binding activity difference between groups of 200 MFI (800–600) with a SD of 300 MFI, power of 0.8 and alpha of 0.05, we would have needed 19.5 subjects in each group (*pwr* package 1.3–0). A very conservative approach for a mixed effect model analysis taking into account different timepoints, and thus we assume that 20 patients per allocation group is an adequate sample size.

Statistical analysis

Baseline characteristics were compared between the allocation groups with t-test or Mann–Whitney U test for continuous variables depending on the distribution. The Fisher exact test was used to assess differences between the allocation groups for categorical data. A two-way repeated measures ANOVA was performed to evaluate the platelet count over time between the allocation groups. Boxplots were used to display platelet counts over time points 1–4 between the allocation groups. In scatterplots with P-selectin expression on the x-axes and fibrinogen binding on the y-axis we depicted the influences of the separate agonists and the distribution between allocation groups. A two-way repeated measures ANOVA was performed for fibrinogen and P-selectin expression separately to assess differences between allocation groups.

We used mixed effect modelling (MEM) to evaluate the effect of NO administration during CPB on the activity and reactivity of platelets over four time points (T1-4). MEM allows for modelling the platelet response (the activity level reflected as MFI) to fixed effects (NO administration, CPB duration, time) while allowing for larger variation on patient level (random effects). As fixed effects we included time as a factor, treatment allocation group (as interaction code: "Allocation: NO"), duration of CPB and the interaction between time and treatment allocation. The fixed effect "Allocation: NO" gives insight in baseline differences between the two allocation groups as on T1 none of the participants had received nitric oxide. To evaluate whether nitric oxide administration during CPB introduced differences in platelet activity we have to look at the interaction between time (T2,3,4) and treatment allocation (as interaction code: "time [2,3,4]: allo"). We extracted coefficients from the MEMs and report standardized beta coefficients (partially scaled coefficients) and *p*-values with Satterthwaite's *p*-value approximation to be able to present the results in a compact manner. A *p*-value of < 0.05 was considered

statistically significant. Analyses were performed using R (version R 4.2.3 GUI 1.79, *lme4* package 1.1–33). Hereafter, also a one-way ANOVA was used to assess differences over time in the complete study cohort without taking into account the allocation group.

Post hoc in vitro NO experiments

A small in vitro experiment was performed with healthy (adult) fresh donor platelets to test the ability of the flow cytometry-based platelet activation test to detect inhibitory effects of NO. Healthy donor platelets were obtained via the Mini Donor Service, a blood donation facility for research purposes for which all adult donors have provided written informed consent. Platelet samples were fresh and incubated with S-nitroso-N-acetyl-DL-penicillamine (SNAP) as a nitric oxide donor in two concentrations (10 μM and 100 μM SNAP) and dimethylsulfoxide (DMSO) as control (0.01% and 0.1% DSMO). Hereafter, flow cytometric assessment of platelet reactivity to agonists were performed at 1, 20, 40, 60, 120 and 180 min.

Results

Platelet samples for flow-cytometry testing of platelet reactivity were obtained in 42 children who participated in the NITRIC trial at the University Medical Center Utrecht. In the other participants, additional blood samples were not drawn as we were unable to process these samples during the covid-19 pandemic laboratory restrictions. Baseline characteristics for the two allocation groups are summarized in Table 1. NO-recipients more often had a syndrome diagnosis, especially trisomy 21 reported. Surgical Aristotle scores, duration of CPB, and minimum core temperatures during CPB were comparable between groups. Two patients in the control- group

Table 1 Baseline characteristics of young children undergoing congenital heart disease surgery with cardiopulmonary bypass receiving nitric oxide (NO) in the oxygenator or standard care

	NO- recipients (*N* = 22)	Controls (*N* = 20)	*p*-Value
Age in days *[median (IQR)]*	68 [10–144]	83 [11–180]	0.31
Gender (m) *[%]*	15 (68%)	13 (65%)	0.75
Weight (kg)	4 [3–5.7]	5 [3–6.3]	0.25
Gestational age (w) *[median (IQR)]*	38 [37–40]	38.5 [37–39]	0.76
Birth weight (kg)	3.0 (0.7)	3.2 (0.8)	0.48
Syndrome diagnosis *(T21; other)*	6 (4;2)	1(1;0)	0.10
Cardiac physiology *(univentricular)*	3 (14%)	3 (15%)	1
Presurgical cyanotic CHD *(SpO2 ≤ 85%)*	3 (14%)	3 (15%)	1
Cardiac lesions and repairs			
VSD/ASD closure	8 (36%)	5 (25%)	
Aortic arch repair ± VSD	5 (23%)	4 (20%)	
AVSD repair	4 (18%)	1 (5%)	
TGA/Arterial switch	2 (9%)	1 (5%)	
TOF repair	0	3 (15%)	
Norwood/IAA/Truncus	3 (14%)	3 (15%)	
Other[a]	0	3 (15%)	
Surgical Aristotle score[b]	9 [6–10]	8 [6.75–10]	0.83
Antiplatelet drug (aspirin) use	0	2 (10%)	0.22
Peak velocity (m/s) on TTE[c] *[median (IQR)]*	2.9 [1.9–4.0]	3.4 [2.7–5.0]	0.21
CPB time (min) *[median (IQR)]*	112 [66–160]	115 [85–144]	0.84
Aortic Cross Clamp (AoX) time (min) *[median (IQR)]*	54 [43–93]	77 [65–93]	0.19
Selective Cerebral Perfusion (SCP)[d]	8 (36%)	5 (25%)	0.51
Deep hypothermic cardiac arrest (DHCA)[d]	2 (9%)	2 (10%)	1
Minimum temperature on bypass (°C) [median (IQR]	27.5 [17.5–29.0]	27.8 [17.6–29.0]	0.95

Abbreviations: *IQR* interquartile range, *T21* Trisomy 21, *CHD* congenital heart disease, *VSD* ventricular septal defect, *ASD* atrial septal defect, *AVSD* atrioventricular septal defect, *TGA* transposition of the great arteries, *TOF* tetralogy of Fallot, *IAA* interrupted aortic arch, *TTE* transthoracic echography, *CPB* cardiopulmonary bypass

[a] Other surgeries performed: Aortopulmonary shunt placement, Ross aortic valve replacement, Total cavopulmonary connection with tricuspid valve repair

[b] Surgical Simple Adjusted Aristotle Score ranging from 1.5–15 points

[c] Maximum peak velocity measured during pre-operative echocardiography at the pulmonary valve (17 patients), descending aorta (8 patients), aortic valve (5 patients), ventricular septal defect (4 patients), tricuspid valve (3 patients), left (2 patients) and right (2 patients) pulmonary artery

[d] Minimum core temperature (nasal) was < 20 degrees Celsius

were on antiplatelet drugs (aspirin) pre-operatively and administration was continued until surgery. However, both patients were in the upper quartile of platelet (re) activity in all tests during four timepoints, indicating that the effect of aspirin on platelet (re)activity was limited. Mean baseline platelet counts did not differ between controls (285×10^9/L) and NO-recipients (267×10^9/L) $p=0.68$. In Fig. 1 the platelet counts are plotted with an expected drop after timepoint 1 due to the hemodilution of blood with CPB prime ($p<0.001$). Platelet counts were above 100×10^9/L in the majority (83%) of patients at T2.

Platelet (re)activity and nitric oxide

Results of the vitro experiments with platelets from 3 healthy donors are plotted in the supplementary material (Suppl Fig. 2). The flow cytometry-based platelet activation tests are able to detect inhibitory effects of NO and the effect duration is dose dependent. In the NITRIC participants, we have plotted average platelet (re)activity over time for fibrinogen binding and P-selectin expression to observe the trend over time for NO-recipients and controls (Fig. 2). Scatterplots for all agonists (PAR1-AP, PAR4-AP, ADP, CRP-xL, and U46619) can be found in the supplementary material (Suppl Fig. 1) and supplemented ANOVA analyses for all conditions are provided in Suppl. Table 1. The plots do not display an evident NO effect on platelet (re)activity on T2-4 except for pre-existing baseline differences.

We have listed coefficients, standardized beta coefficients and p-values obtained from our MEMs for respectively fibrinogen binding and P-selectin expression in Tables 2 and 3. A patient intercept was included as random effect. A random slope did not improve the model based on Akaike information criterion. In all models the fixed intercept of the model is important. The coefficients for "Allocation: NO" provide insight in baseline differences between the treatment allocation groups on platelet reactivity. At baseline (T1) NO-recipients reacted significantly different to PAR4-AP on fibrinogen binding and to PAR1-AP on P-selectin expression. However, these baseline differences were already present before any exposure to the treatment allocation (NO administration). To interpret whether NO administration has

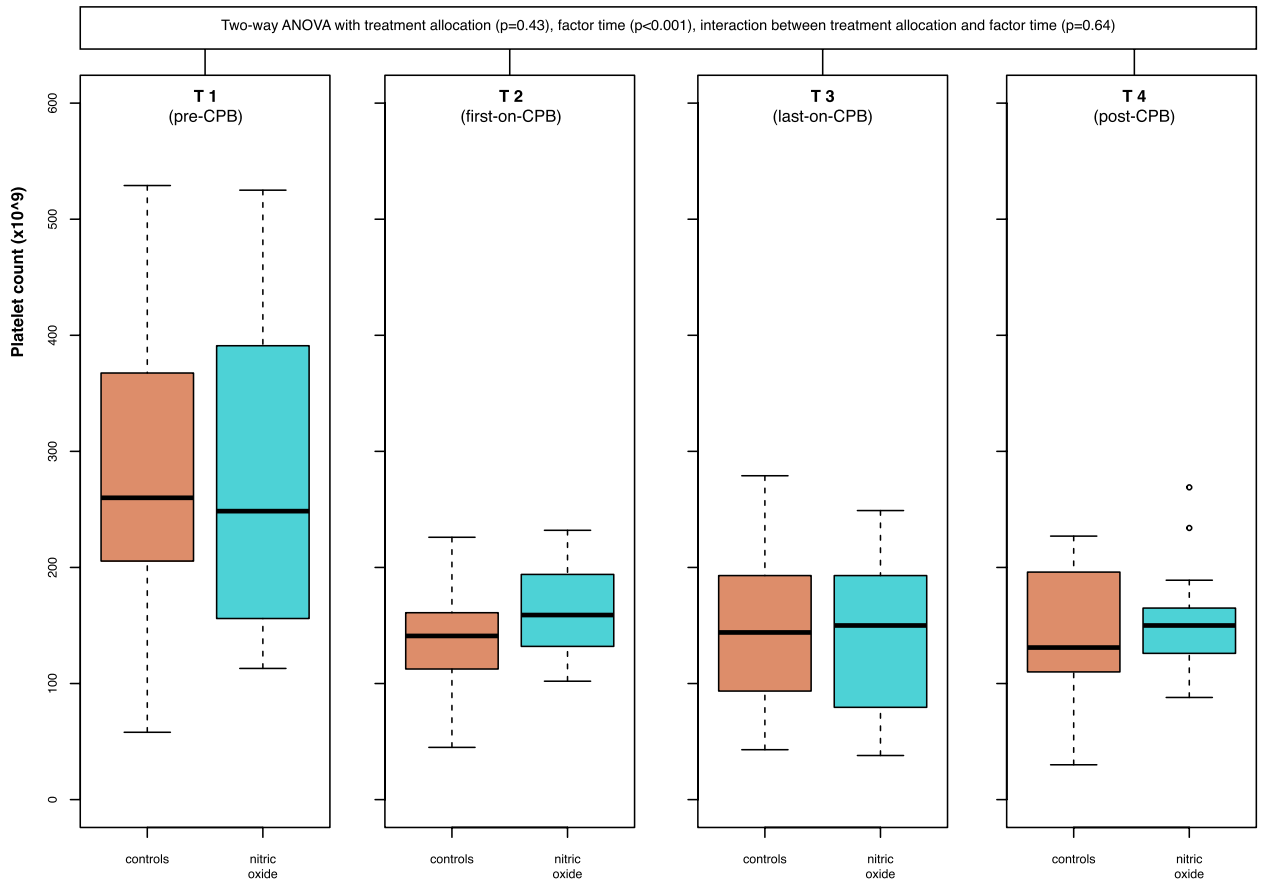

Fig. 1 Platelet count in young children during congenital heart disease surgery with cardiopulmonary bypass per treatment allocation (standard care and nitrix oxide administration in the oxygenator of the CPB)

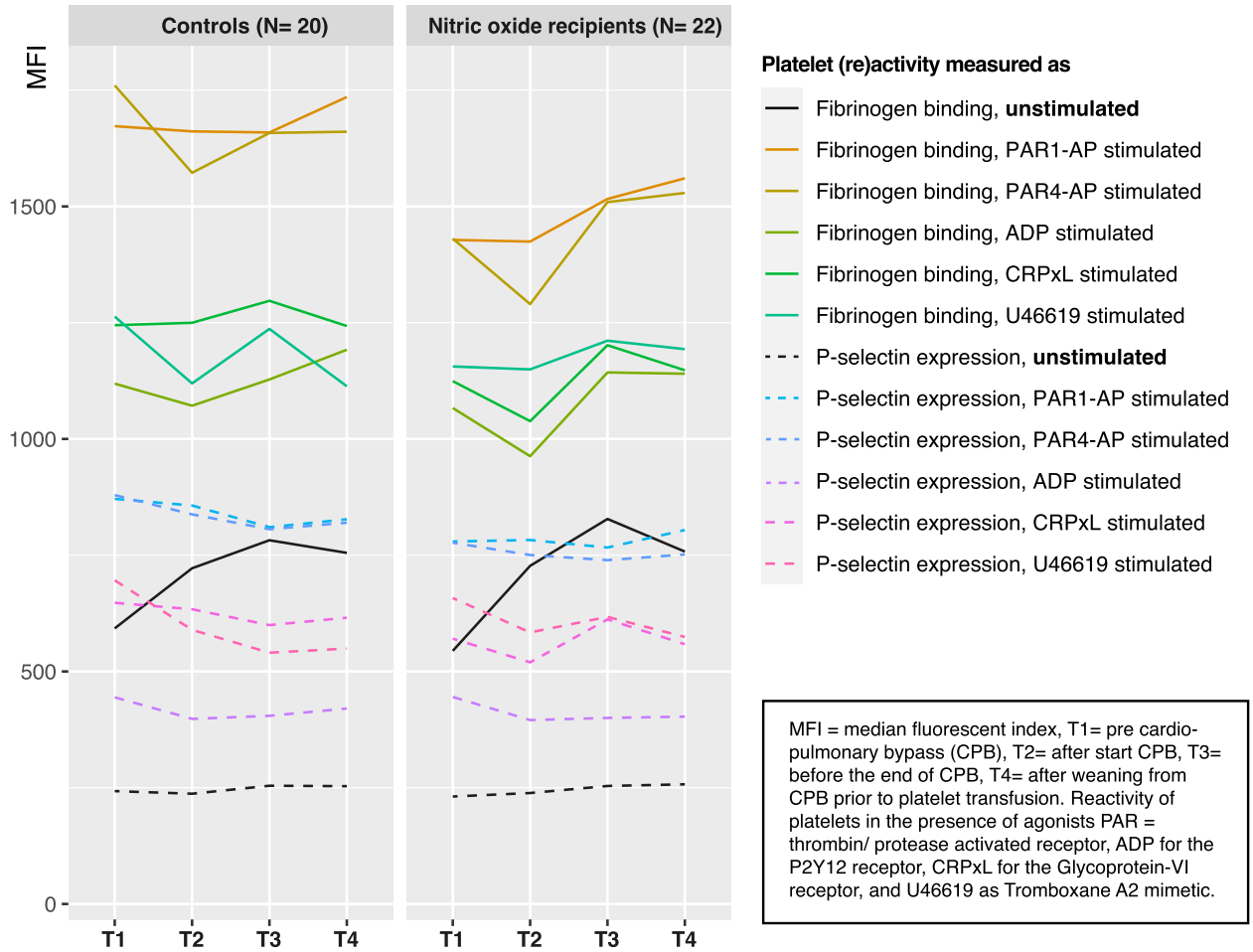

Fig. 2 Average platelet (re)activity during congenital heart disease surgery with cardiopulmonary bypass in children < 2 years old per treatment allocation (controls vs. 20 ppm nitric oxide administration in the oxygenator of the CPB)

a noteworthy effect on platelet (re)activity, we need the interaction between time and treatment allocation (time [2,3,4]: allo). These coefficients are indicated by the light orange bar in Tables 2 and 3. Platelet (re)activity between the treatment groups during timepoints T2-4 were not different, beta coefficients were small, also when corrected for potential confounders as CPB duration and maximum peak flow velocity not statistically different.

Cardiopulmonary bypass and platelet reactivity

Unstimulated platelet activity (platelet activity in absence of an agonist) increased for fibrinogen binding during surgery. The MEM demonstrates a significant increase of 129,190,162 MFI for timepoints T2,3,4 compared to T1 (Table 2) and without a noteworthy difference between the NO-recipients and controls (Table 2). This increased activity in unstimulated platelets over time was not seen for P-selectin expression (Table 3). In Fig. 3 boxplots without allocation for treatment are plotted. Absolute MFI values for unstimulated platelets and relative MFI values for stimulated platelets are plotted for the complete cohort (omitting treatment allocation). The relative values are calculated as the MFI measurement after stimulation divided by the unstimulated value at the at same timepoint, show for fibrinogen binding an inverse effect. In all MEM models, for both fibrinogen binding as P-selectin expression, the intercept, i.e. platelet activity and reactivity at T1, was highly significant and prognostic for the responsiveness of platelets later on. None of the models found a relevant nor significant effect of CPB duration on platelet activity and reactivity. Platelet responsiveness (fibrinogen binding) was induced by maximum peak flow velocity on pre-operative echocardiography in PAR1-AP and PAR4-AP stimulated platelets.

Table 2 Platelet reactivity (Fibrinogen binding (MFI)) during CHD surgery in young children: results from mixed effect models with unstimulated platelets and activators. Satterthwaite's *p*-value approximation

	Unstimulated			***PAR1-AP***			***PAR4-AP***			***ADP***			***CRP-xL***			***U46619***		
Fixed Effects	***Est.***	***Std. Est***	***p***	***Est.***	***Std. Est***	***p***	***Est.***	***Std. Est***	***p***	***Est.***	***Std. Est***	***p***	***Est.***	***Std. Est***	***P***	***Est.***	***Std. Est***	***P***
(Intercept)	460	0	**<.01**	1393	0	**<.01**	1359	0	**<.01**	956	0	**<.01**	1053	0	**<.01**	1048	0	**<.01**
time 2	129	.18	**.02**	-11	-.01	.88	-188	-.17	**.05**	-47	-.06	.46	5	.01	.94	-144	-.15	.09
time 3	190	.28	**<.01**	-1.7	-.00	.98	-101	-.09	.28	7	.01	.91	52	.06	.45	-32	-.03	.71
time 4	162	.23	**<.01**	63	.06	.43	-100	-.09	.29	73	.09	.26	-2	-.00	.98	-150	-.16	.08
Allocation : NO	-19	-.03	.84	-227	-.27	.07	-296	-.31	**.04**	-19	-.03	.85	-82	-.10	.51	-111	-.14	.37
CPB (min)	.4	.07	.58	.1	.01	.91	1.1	.13	.28	.1	.02	.90	.7	.10	.47	.88	.12	.33
Peak velocity (m/s)	24	.10	.34	76	.23	**.03**	77	.21	**.05**	43	.17	.14	30	.10	.38	32	.10	.36
time [2] : allo	72	.07	.3	94	.07	.41	104	.07	.43	-32	-.03	.73	-78	-.06	.43	165	.13	.17
time [3] : allo	108	.12	.16	112	.09	.32	201	.14	.13	82	.08	.37	29	.02	.76	104	.09	.39
time [4] : allo	51	.06	.50	98	.07	.38	211	.15	.11	3	.00	.98	16	.01	.87	189	.16	.11

Formula: Fibrinogen binding (MFI) ~ time point + allocation + time: allocation + CPB duration + peak velocity + (1|subject)

MFI median fluorescent intensity, *NO* nitric oxide administration, *CPB* cardiopulmonary bypass, *allo* NO-recipients, *PAR1-AP/ PAR4-AP* thrombin receptor peptides 1 and 4, *ADP* P2Y receptor stimulator, *CRP-xL* collagen related peptide, *U46618* thromboxane A2 receptor peptide

Table 3 Platelet reactivity (P-selectin expression (MFI)) during CHD surgery in young children: results from mixed effect models with unstimulated platelets and activators. Satterthwaite's *p*-value calculation method

	Unstimulated			***PAR1-AP***			***PAR4-AP***			***ADP***			***CRP-xL***			***U46619***		
Fixed Effects	*Est.*	*Std. Est*	*p*	*Est.*	*Std. Est*	*p*	*Est.*	*Std. Est*	*p*	*Est.*	*Std. Est*	*p*	*Est.*	*Std. Est*	*P*	*Est.*	*Std. Est*	*p*
(Intercept)	206	0	**<.01**	898	0	**<.01**	795	**0**	**<.01**	435	0	**<.01**	589	0	**<.01**	660	0	**<.01**
time 2	-6	-.03	.58	-14	-.03	.71	-41	-.08	.34	-46	-.20	**<.01**	-14	-.03	.70	-107	-.25	**.01**
time 3	8	.04	.43	-57	-.13	.15	-74	-.14	.10	-43	-.19	**<.01**	-49	-.10	.19	-164	-.35	**<.01**
time 4	11	.05	.30	-44	-.10	.26	-59	-.12	.17	-24	-.11	.11	-32	-.07	.38	-147	-.31	**<.01**
Allocation: NO	0	-.00	.99	-115	-.32	**.04**	-119	-.26	.08	7	.03	.83	-68	-.17	.29	-53	-.13	.41
CPB (min)	0.2	.08	.57	.1	.02	.87	0.6	.14	.27	-.1	-.06	.65	.3	.08	.54	.5	.12	.33
Peak velocity (m/s)	5.6	.07	.39	-10	-.07	.52	4.8	.03	.67	6.6	.09	.41	6.0	.04	.74	-5.4	-.03	.76
time [2] : allo	14	.05	.35	24	.04	.66	26	.04	.67	-3	-.01	.87	-48	-.08	.35	33	.05	.57
time [3] : allo	15	.05	.31	56	.10	.31	48	.07	.42	-.3	-.00	.99	91	.15	.08	136	.22	**.02**
time [4] : allo	14	.05	.33	87	.16	.11	48	.07	.43	-16	-.05	.45	20	.03	.69	57	.08	.33

Formula: P selectin expression (MFI) ~ time point + allocation + time: allocation + CPB duration + peak velocity + (1|subject)

MFI median fluorescent intensity, *NO* nitric oxide administration, *CPB* cardiopulmonary bypass, *allo* NO-recipients, *PAR1-AP/ PAR4-AP* thrombin receptor peptides 1 and 4, *ADP* P2Y receptor stimulator, *CRP-xL* collagen related peptide, *U46618* thromboxane A2 receptor peptide

Bleeding, blood product administration and clinical outcomes

The chest tube output in the first 48 h after surgery was not different between the two allocation groups. For NO-recipients chest tube output was on average 6.8 ml·kg [IQR 5.3–13.9] and standard care 6.5 ml·kg [IQR 4.8–12.3] ($p=0.87$) per 24 h. Platelets were transfused in 10 (45%) NO-recipients and 9 (45%) controls after bypass. And in both groups 3 patients received an additional red blood cell transfusion during the first 48 h after surgery. NO-recipients and controls did not differ with regard to length of PICU stay ($p=0.71$), length of hospital stay ($p=0.96$), and ventilator free days ($p=0.55$). All children were alive at 28 days after surgery.

Discussion

In this study we evaluated platelet activity and reactivity in young children undergoing congenital heart disease surgery on CPB as part of the NITRIC study [8]. We did not detect an effect of 20 ppm NO administration in the sweep gas of the CPB oxygenator on unstimulated platelet activity, measured as P-selectin expression and fibrinogen binding, nor on the platelet reactivity after administration of five different agonists on three different timepoints. Furthermore, we detected only a slight increase in unstimulated fibrinogen binding of platelets during CPB and directly after separation from CPB, while stimulation of the thrombin/protease activated receptors 1 and 4, P2Y receptor, Glycoprotein-VI (GPVI) receptor,

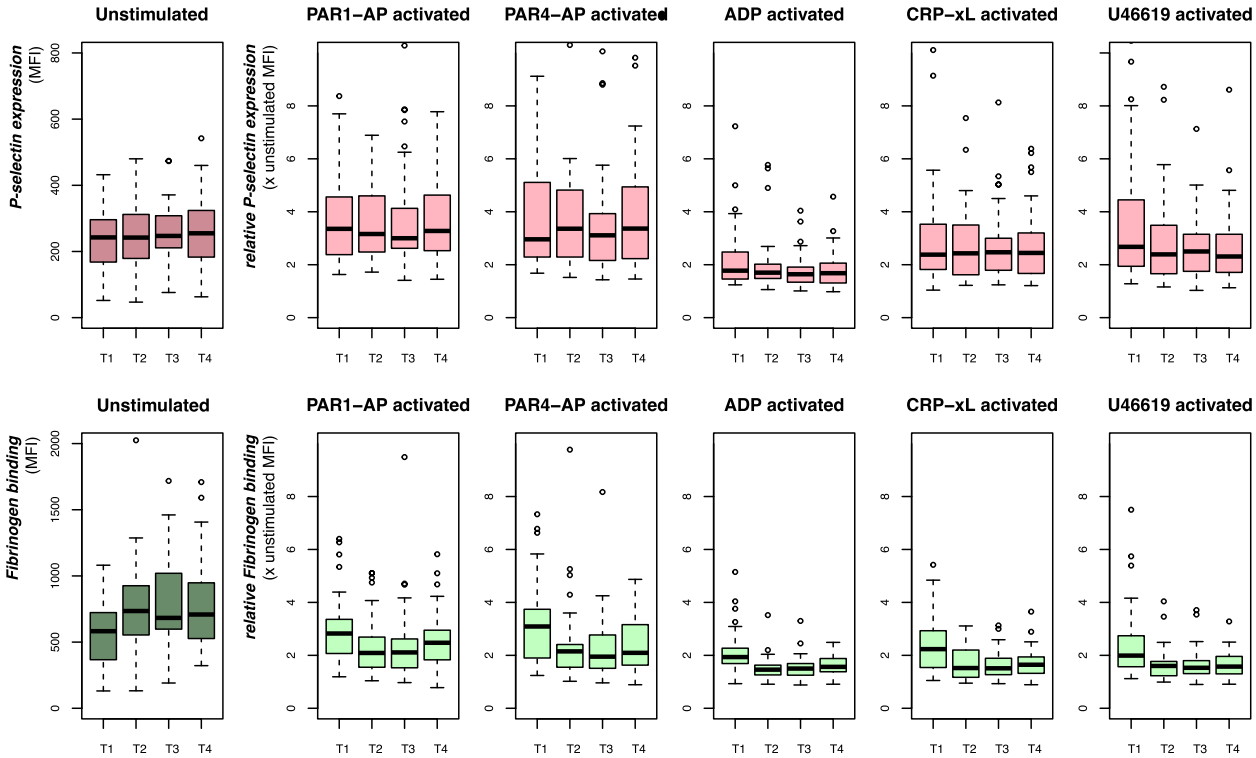

Fig. 3 Platelet activity depicted as P-selectin expression and fibrinogen binding in stimulated relative to unstimulated platelets in a cohort of young children during cardiopulmonary bypass surgery from pre-bypass (T1) to post-bypass (T4)

or the thromboxane A2 receptor, did not alter platelet reactivity measured as fibrinogen binding and P-selectin expression over time. This is a new finding and in contrast with earlier observations. In our study, duration of CPB exposure did not affect platelet reactivity.

The absent effect of 20 ppm NO administration in sweep gas flow to the oxygenator during CPB in CHD surgery in young children on platelet (re)activity is in accordance with the 2020 publication of Niebler et al. [17]. This randomized pilot trial with 40 young children aged < 1 year did not find a significant effect of 20 ppm NO on P-selectin expression, neither on unstimulated platelets nor after thrombin receptor activating protein, U46619, and collagen related peptide (CRP) stimulation. In our study, we analyzed the effect of 20 ppm NO administration on both P-selectin expression and fibrinogen binding and used a mixed effect model to account for CPB duration and patient specific random effects. We did not find differences between NO-recipients and controls on platelet (re)activity. However, NO is a known inhibitor of platelet activation as we confirmed in our in vitro experiments with healthy donor platelets that this effect is dose dependent [5, 6]. A higher incubation dose of NO donor SNAP resulted in a prolonged inhibitory effect on platelet reactivity. We are unable to translate the NO dose from SNAP in the in vitro experiments to the gaseous NO administration dose of 20 ppm administered in trial participants. However, our in vitro experiments showed that the flow cytometry-based platelet activation tests were able to detect NO induced inhibitory effects on platelets. Therefore, it is likely that the dose, administration of 20 ppm NO in the sweep gas flow to the oxygenator during CPB, is too low to have an inhibitory effect on platelet activation, responsiveness and consumption. NO administration was also not associated with bleeding and transfusion of blood products.

Our analysis pointed at a surprising observation: The course of platelet reactivity over time was not impacted substantially by CPB time. The most important factor on platelet activation and responsiveness were the baseline values, platelet (re)activity on T1 before the start of CPB. While CPB had the expected dilutional effects on blood and reduced platelet counts, its influence on platelet responsiveness was negligible and non-significant. This is in contrast with earlier findings. CPB has been frequently reported to have detrimental effects on coagulation and platelet function [1–4, 13]. A recent study of Zwifelhofer et al. 2020 reported on P-selectin, GPVI expression in neonates on cardiopulmonary bypass for similar congenital heart surgeries found differences

between unstimulated and stimulated P-selectin expression in platelets over time. [18]. The study did not evaluate the effect on fibrinogen binding. In the Zwifelhofer study platelet reactivity was not associated with bleeding complications [18]. Bleeding complications were not associated with CPB duration. In our study, we did not find a clear effect of CPB, and CPB duration on P-selectin expression. While, fibrinogen binding in unstimulated platelets increased, this did not significantly affect their ability to respond to the five agonists. P-selectin expression did not differ over time, not in unstimulated platelets, nor after administration of agonists. A potential explanation for this observation could be the changed CPB disposables, their coatings, prime composition and volume and suction technology improvements over the last decades that reduced detrimental effects on platelet activation and consumption [19, 20].

Limitations

This substudy of the NITRIC trial was executed as a single center study. While a potential disadvantage, our institutional approach is highly standardized with regard to anesthesia, surgery and CPB and thus promotes a good comparison between allocation groups. Furthermore, the sample size was small and heterogenous. Fortunately, the block randomization in the NITRIC trial facilitated a relatively well-balanced sample between NO-recipients and controls. What remains is that the small sample limits the ability to unravel why duration of CPB did not affect platelet (re)activity. This finding therefore demands new scientific evaluation as it is contrary to previous studies that pointed at a large and significant effect of bypass time on platelet function [1–4, 18, 21]. It is an important finding to re-evaluate as the previously found detrimental effects on platelets function likely influenced the readiness of pediatric cardiac anesthesiologists and intensivists to transfuse platelets after pediatric heart surgery. If our study findings that CPB duration no longer affects platelet (re)activity stand, this may change platelets transfusion strategies to depend predominantly on platelet count. The sample size also limits the number of explanatory variables in our mixed effect models. We did not include minimum temperature in the model as it is equally distributed among NO recipients and controls and highly correlated with the duration of CPB. Chronic hypoxemia and age at day of surgery were not included for similar reasons. A second notable limitation of this study is the use of flow cytometry-based platelet activation tests that quantify both platelet degranulation (P-selectin expression) and platelet aggregation (fibrinogen binding to integrin αIIbβ3) in neonates and young children. These tests are used and validated in adults, and currently still lack age-stratified normal values. But these more sensitive platelet function test methods will enable a description of developmental changes in platelets, including phenotype and function. Previous studies in children with CHD have shown remarkable differences between levels of procoagulant and anticoagulant factors with their age-matched controls [22]. Platelet (re)activity differences between CHD- patients and healthy controls are therefore likely. A possible causal relation for reduced platelet function was suggested by chronic hypoxemia [3], shear stress (peak flow velocity prior to surgery) [12] and minimum temperature during bypass [3]. However, evidence that chronic hypoxemia causes deficiencies in platelet adhesion and aggregation is scarce, dated, and related to the severity of polycythemia [3, 23]. We found a relation with peak flow velocity in PAR1-AP and PAR2-AP stimulated platelets. This is relevant when looking at the absolute platelet (re)activity values in our cohort, however, will not influence the comparison between allocation groups and change over time. Although we confirmed that flow cytometry-based platelet activation testing was able to detect NO induced platelet inhibition in vitro, we are unable to translate in vitro dosage to the gaseous NO dose in the CPB of our study participants. Evidently, the gaseous NO exposure of platelets in the trial was subtherapeutic. A third limitation is the inability to study the correlation between platelet reactivity testing and thrombelastography (TEG) or rotational thromboelastometry (ROTEM) testing. TEG and ROTEM have proved their additional value in pediatric heart surgery and TEG is used regularly at our institution [24]. In a reasonable number of patients TEG was performed before weaning from CPB. However, not in all patients and mainly before weaning from CPB. Blood withdrawal for TEG was not synchronized with the study samples. Retrospectively, it would have been informative to study whether platelet (re)activity testing would add information to the existing correlation between platelet count and 'maximum amplitude' (MA) TEG indices in a structured approach that facilitates unbiased comparisons.

Conclusion

Nitric oxide administration (20 ppm) in the CPB oxygenator did not affect platelet activity and reactivity in young children undergoing congenital heart disease surgery. The duration of CPB, while earlier identified as an important risk factor for platelet dysfunction, was not influential for platelet reactivity in this cohort of young children (< 2 years old). The most important factor for platelet functioning during and after CHD surgery were the child's baseline values for P-selectin expression and Fibrinogen binding.

Supplementary Information

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Additional file 1: Supplement Fig. 1. Platelet reactivity depicted as fibrinogen binding (y-axis) and P-selective expression (x-axis) over time (T1-4) in young children during congenital heart disease surgery with cardiopulmonary bypass (CPB). Time is depicted vertically as (T1) before CPB, (T2) after the start of CPB, (T3) before weaning from the CPB, and (T4) after weaning from CPB before any platelet transfusion.

Additional file 2: Supplement Fig. 2. In vitro experiment with healthy (adult) donor platelets. Unravelling the effect of nitric oxide (donated by 10 µM and 100 µM SNAP) incubations on platelet reactivity after PAR1-AP, PAR4-AP, ADP, CRP-xL and U46619 stimulation compared compared to DSMO control in time.

Additional file 3: Supplement Table 1. Two-way ANOVA for effect of nitric oxide delivery (T2-4) on platelet count and platelet (re)activity from Platelet reactivity in young children undergoing congenital heart disease surgery: a NITRIC randomized clinical trial substudy.

Additional file 4.

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Authors' contributions
vanLoon, Korporaal had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: vanLoon, Koomen, Korporaal, Schlapbach, Gibbons, Horton, Butt. Acquisition, analysis, or interpretation of data: vanLoon, Hiensch, Korporaal, vanBelle-vanHaaren, Charlier, Lammers, Koelhuis-Faber, Imhof, Breur, Koomen. Critical review of the manuscript: vanLoon, Korporaal, Koomen, Horton, Schlapbach, Nijman, Breur, Koomen, Butt, vanWijk, vanBelle-vanHaaren, Charlier, Lammers, Koelhuis-Faber, Imhof, Hiensch. Statistical analysis: vanLoon, Korporaal. Administrative, technical, or material support: vanLoon, Hiensch, Hennink, Koomen, Korporaal, Horton, Gibbons, Schlapbach, vanBelle-vanHaaren, Charlier, Lammers, Koelhuis-Faber, Imhof.

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Availability of data and materials
The data and code that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests
None of the authors reported a COI.

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References

1. Guay J, Ruest P, Lortie L (2004) Cardiopulmonary bypass induces significant platelet activation in children undergoing open-heart surgery. Eur J Anaesth 21(12):953–956
2. Straub A, Smolich J, d'Udekem Y, Brizard C, Peter K, Horton S (2010) Activation of platelets in young infants during cardiopulmonary bypass. Thromb Haemostasis 103(02):466–469
3. Eaton MP, Iannoli EM (2011) Coagulation considerations for infants and children undergoing cardiopulmonary bypass. Pediatr Anesth 21(1):31–42
4. Andreasen JB, Hvas A, Ravn HB (2014) Marked changes in platelet count and function following pediatric congenital heart surgery. Pediatr Anesth 24(4):386–392
5. Jin RC, Voetsch B, Loscalzo J (2005) Endogenous mechanisms of inhibition of platelet function. Microcirculation 12(3):247–258
6. Gkaliagkousi E, Ferro A (2011) Nitric oxide signalling in the regulation of cardiovascular and platelet function. Front Biosci 16(1):1873
7. Denninger JW, Marletta MA (1999) Guanylate cyclase and the ·NO/cGMP signaling pathway. Biochimica Et Biophysica Acta Bba - Bioenergetics 1411(2–3):334–350
8. Schlapbach LJ, Gibbons KS, Horton SB, Johnson K, Long DA, Buckley DHF et al (2022) Effect of nitric oxide via cardiopulmonary bypass on ventilator-free days in young children undergoing congenital heart disease surgery. JAMA 328(1):38–47
9. Schlapbach LJ, Horton SB, Long DA, Beca J, Erickson S, Festa M et al (2019) Study protocol: NITric oxide during cardiopulmonary bypass to improve Recovery in Infants with Congenital heart defects (NITRIC trial): a randomised controlled trial. BMJ Open 9(8):e026664
10. Long D, Anderson VA, Crossley L, Sood NT, Charles KR, MacDonald AD et al (2023) Longitudinal cohort study investigating neurodevelopmental and socioemotional outcomes in school-entry aged children after open heart surgery in Australia and New Zealand: the NITRIC follow-up study protocol. BMJ Open 13(8):e075429
11. Helm SVD, McCafferty C, Letunica N, Chau KY, Monagle P, Ignjatovic V (2023) Platelet function in neonates and children. Thromb. Res. 231:236–246
12. Zubair MM, Hohimer AR, Bailly DK, Muralidaran A, Madriago EJ, Zubair MH et al (2015) High flow velocity through congenital cardiac lesions predicts preoperative platelet dysfunction. Ann Thorac Surg 99(4):1379–1385
13. Miller BE, Mochizuki T, Levy JH, Bailey JM, Tosone SR, Tam VKH et al (1997) Predicting and treating coagulopathies after cardiopulmonary bypass in children. Anesthesia Analgesia 85(6):1196–1202
14. Waissi F, Dekker M, Bank IEM, Korporaal SJA, Urbanus RT, Borst GJ et al (2020) Sex differences in flow cytometry–based platelet reactivity in stable outpatients suspected of myocardial ischemia. Res Pract Thrombosis Haemostasis 4(5):879–885
15. Huskens D, Li L, Florin L, de Kesel P, de Laat B, Roest M et al (2020) Flow cytometric analysis of platelet function to improve the recognition of thrombocytopathy. Thromb Res 194:183–189
16. van Asten I, Schutgens REG, Baaij M, Zandstra J, Roest M, Pasterkamp G et al (2018) Validation of flow cytometric analysis of platelet function in

patients with a suspected platelet function defect. J Thromb Haemost 16(4):689–698
17. Niebler RA, Chiang-Ching H, Daley K, Janecke R, Jobe SM, Mitchell ME et al (2020) Nitric oxide added to the sweep gas of the oxygenator during cardiopulmonary bypass in infants: a pilot randomized controlled trial. Artif Organs 45(1):22–28
18. Zwifelhofer N, Bercovitz R, Cole R, Yan K, Simpson P, Moroi A et al (2019) Platelet function changes during neonatal cardiopulmonary bypass surgery: mechanistic basis and lack of correlation with excessive bleeding. Thromb Haemostasis 120(01):094–106
19. Sniecinski RM, Chandler WL (2011) Activation of the hemostatic system during cardiopulmonary bypass. Anesthesia Analg 113(6):1319–1333
20. Budde H, Riggert J, Vormfelde S, Tirilomis T, Friedrich MG (2019) The effect of a novel turbulence-controlled suction system in the prevention of hemolysis and platelet dysfunction in autologous surgery blood. Perfusion 34(1):58–66
21. Williams GD, Bratton SL, Ramamoorthy C (1999) Factors associated with blood loss and blood product transfusions: a multivariate analysis in children after open-heart surgery. Anesthesia Analgesia 89(1):57–64
22. Odegard KC, Zurakowski D, Hornykewycz S, DiNardo JA, Castro RA, Neufeld EJ et al (2007) Evaluation of the coagulation system in children with two-ventricle congenital heart disease. Ann Thorac Surg 83(5):1797–1803
23. Mauer HM, McCue CM, Caul J, Still WJ (1972) Impairment in platelet aggregation in congenital heart disease. Blood 40(2):207–216
24. Scott JP, Niebler RA, Stuth EAE, Newman DK, Tweddell JS, Bercovitz RS et al (2018) Rotational thromboelastometry rapidly predicts thrombocytopenia and hypofibrinogenemia during neonatal cardiopulmonary bypass. World J Pediatric Congenit Hear Surg 9(4):424–433

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