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Platelet Transfusions and Mortality in Necrotizing Enterocolitis

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Abstract

BACKGROUND: Prior studies have suggested an association between platelet transfusions (PTX) and worse outcomes among infants with necrotizing enterocolitis (NEC), potentially mediated by pro-inflammatory factors released by platelets. However, the effects of storage on platelet pro-inflammatory factor release and the confounding role of illness severity on NEC outcomes have not been determined.

STUDY DESIGN AND METHODS: First, Neuropeptide Y (NPY, a potent splanchnic vasoconstrictor released by platelets) was measured by ELISA in fresh frozen plasma (FFP) and in the supernatant of leukoreduced apheresis-derived PLTs (LR-A-PLTs) at different times during storage. Next, we evaluated the relationship between PTX rates and death in a multicenter cohort of very low birth weight (VLBW) infants with NEC, adjusting for illness severity.

RESULTS: NPY levels increased over time in the supernatant of LR-A-PLTs, and were 4.4-fold and 8.9-fold higher than in FFP on days 2 and 3 of storage, respectively (P<0.001). Among 598 VLBW infants, 44 developed NEC. In unadjusted analysis, PTX rate was 30.3 (95% CI 11.5–80.1) per 100 infant-days among infants who died, compared to 6.0 (95% CI 3.2–11.2) among survivors (incidence rate ratio [IRR] 5.1; 95% CI 1.6–16.2; P=0.006). In multivariable analysis, there was no association between PTX rate and mortality (IRR 3.0; 95% CI 0.6–15.0; P=0.18), although estimation was imprecise.

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CONCLUSION: Pro-inflammatory mediators accumulate in platelet suspensions during storage. Although PTX rates were not associated with increased mortality among infants with NEC in our study, our estimates suggest the potential for such an association that needs evaluation in larger studies.

Keywords

Chemokine; inflammation; necrotizing enterocolitis; neonate; platelet

INTRODUCTION

Necrotizing enterocolitis (NEC) is a devastating disease that predominantly affects premature infants. Although the incidence of NEC is decreasing[1], concomitantly with improvements in neonatal intensive care, its mortality has not changed substantially[2]. Risk factors such as prematurity, formula feeding, and bacterial colonization, among others, are thought to trigger the activation of pro-inflammatory signaling, ultimately leading to bowel necrosis[3]. More recently, severe anemia has been identified as a significant risk factor for NEC, although the underlying mechanisms remain unclear[4].

NEC is one of the main causes of severe neonatal thrombocytopenia[5,6]. Although there is no correlation between the severity of the thrombocytopenia and the incidence of major bleeding[5,6], infants with NEC-associated severe thrombocytopenia (platelet counts $<60 \times 10^9$/L) had 2-times more bleeding events per day than infants with severe thrombocytopenia not-related to NEC[7]. In a prospective observational study conducted in the UK, NEC and gestational age were the strongest predictors for an increased number of bleeding events[7], although the majority of those were minor. For these reasons, despite the complete absence of data from prospective randomized trials, infants with NEC-associated moderate to severe thrombocytopenia are frequently treated with platelet transfusions in an attempt to diminish the occurrence or the severity of hemorrhages[8,9].

Paradoxically, the number of platelet transfusions (PTX) in infants with NEC has been shown to correlate with increased morbidity (short bowel syndrome and/or cholestasis) but not mortality[10]. These investigators hypothesized that substances such as cytokines, chemokines or bioactive lipids generated during platelet storage may amplify the inflammatory bowel injury[10,11]. Given that platelets transfused to neonates are typically leukoreduced and therefore WBC-poor, only the platelet-derived bioactive substances likely play a role in NEC.

Pro-inflammatory cytokines involved in the pathogenesis of NEC (TNF-α, IL-1, IL-6, IL-8)[11] show increasing levels during platelet storage, but this finding has been predominantly attributed to WBCs[12–15], which are present in very low numbers in the leukoreduced products transfused to neonates. Platelets, however, also contain multiple cytokines, including CD40 ligand, regulated on activation normal T expressed and secreted (RANTES), TGF-beta 1 and PF4, among others. These chemokines are released from platelets during storage, and leukoreduction does not prevent their accumulation[12,16–18]. Platelet activating factor (PAF) has also been implicated as a key mediator of intestinal inflammation and necrosis in models of NEC[19,20]. Neuropeptide Y (NPY) is a potent

vasoconstrictor[21,22] responsible for regional splanchnic vasoconstriction during stress. Importantly, NPY is synthesized in megakaryocytes and stored in platelets, with increased platelet levels found under different stress conditions[23–25]. Although an increase in NPY levels during platelet storage could potentially have an aggravating influence on NEC, and could contribute to the association between PTX and NEC-related morbidity, NPY levels have never been evaluated in stored leukoreduced apheresis-derived PLTs (LR-A-PLTs).

In the present study, we first determined whether NPY, a potential novel contributing factor to the pathophysiology of NEC, accumulates in LR-A-PLTs during storage. Next, we evaluated the frequency and characteristics of PTX administered to a cohort of neonates with NEC Bell Stage 2 or greater, who were part of a large prospective multicenter observational cohort of VLBW infants. In this cohort, we tested the hypothesis that PTX rates after onset of NEC would be higher among infants who died compared to those who survived, after accounting for two major confounding factors, birth weight and illness severity.

MATERIAL AND METHODS

Preparation of platelet components for analysis

Leukoreduced apheresis-derived PLTs (LR-A-PLTs) were obtained from the American Red Cross Blood Services, Southern Region (Douglasville, GA, USA); the storage container used for platelets was Baxter PL2410 (Fenwal, Inc., Lake Zurich, IL, USA). No donor collection information was available. The LR-A-PLTs were placed on an agitator (Helmer, Inc., Noblesville, IN) and stored at 22 ± 2ºC. Samples were obtained through sterile couplers from LR-A-PLT units right before being released for transfusion, on day 2 (n=5), day 3 (n=11) and day 4/5 (n=4) of storage. Two individual LR-A-PLTs were additionally serially sampled on days 3, 4 and 5. At each time point, 3 ml were aseptically removed from each unit and processed as described. Samples were centrifuged to pellet the cells, after which the supernatants were removed and stored in aliquots at 70ºC until analysis. Fresh frozen plasma (FFP) samples from healthy donors were used as controls (n=10).

Platelet Assays

Levels of NPY were measured using a competitive enzyme immunoassay (EIA)-extraction-free absorbance assay from Peninsula Laboratories (San Carlos, CA, USA), according to manufacturer's instructions. As internal control for the degree of platelet activation and degranulation during storage, we measured the levels of another platelet-derived chemokine, platelet factor 4 (PF4), which is known to increase steadily during storage[26]. PF4 was measured by a commercially available EIA following the manufacturer instructions (Stago Systems, Parsippany, NJ, USA).

Cohort design

To estimate PTX rates among infants with NEC, we performed a secondary, retrospective analysis of a prospective, multi-center, observational birth cohort study that investigated the transfusion-transmission of cytomegalovirus [4,27,28]. This cohort consisted of infants cared for in 5 hospitals in Atlanta, Georgia, with initial enrollment at three birthing hospitals and follow-up of any infants referred to two free-standing Children's hospital NICUs. The

inclusion criteria for the parent study included: 1) Birth weight ≤ 1500 grams; and 2) Postnatal age ≤ 5 days. Exclusion criteria included: 1) Not expected to survive for more than 5–7 days; 2) Severe congenital anomaly; 3) Transfusion prior to admission; or 4) Maternal refusal. This study was approved by the Institutional Review Boards and/or Research Oversight Committees at all participating centers. For the present NEC study, enrolled infants who developed NEC Bell Stage 2 or greater were identified and their records reviewed.

Definitions

Data, including all blood products administered and all diagnoses of NEC, were collected in standardized case report forms. NEC was defined as Bell Stage 2 or greater according to established criteria, as previously described[4]. Onset of NEC was defined as the time of the first clinical signs of NEC (e.g. emesis, bloody stool, etc.). Surgical NEC was defined as the receipt of exploratory laparotomy or peritoneal drain placement. Illness severity at the onset of NEC was quantitatively assessed using the SNAP-II score, a validated measure of neonatal severity of illness[29].

Statistical analysis

One-way analysis of variance was used to compare NPY (natural log, base e) and PF4 by storage day. A test for linear trend across storage time was performed using a linear orthogonal polynomial contrast[30]. Pairwise comparisons between FFP and LR-A-PLTs at each storage day were made by t-tests. After one-way analysis of variance, log NPY mean and its 95% confidence interval (CI) were back transformed to the original scale and reported as the geometric mean with 95% CI. Similarly, back transformation of the difference between the mean of log transformed NPY for FFP and LR-A-PLTs yielded the ratio of the geometric means. Confidence intervals for the geometric mean ratio were computed by back transforming the 95% confidence bounds for the mean difference. A geometric mean ratio of 3.0 suggests the NPY in one group (LR-A-PLTs) is 3 times that of another group (FFP). NPY levels on days 3 and 4/5 from the same LR-A-PLT unit (serial sampling from 2 units, A and B) were analyzed with a repeated-measures model and reported as the mean ± the standard error of the mean. Prior to implementation of simple linear regression to quantify the relationship between NPY (outcome) and PF4 levels (predictor) in platelet supernatants, all assumptions were assessed.

For epidemiologic analyses, PTX rates per 100 infants-days for surviving and non-surviving infants with NEC were estimated and compared by performing a generalized estimating equations (GEE) Poisson regression analysis of the transfusion counts implemented with the SAS GENMOD procedure (version 9.4; SAS Institute, Cary, NC). To calculate PTX rates, the number of platelet transfusions after onset of NEC were included in the numerator and days of hospitalization after onset of NEC (through the study follow-up period) were included in the denominator. For reference, transfusion rates for red cells, FFP, and cryoprecipitate in the same cohort were also reported and calculated using a similar approach. The incidence rate ratio (IRR) was used to compare the incidence rate in non-survivors to that of survivors. Univariable results are presented as the IRR and its 95% confidence interval (CI). The model-based estimates are unbiased with unbalanced and

missing data, so long as the missing data are non-informative (missing completely at random, MCAR). To address confounding by indication for platelet transfusion, the regression model for the primary analysis was adjusted for both birth weight and illness severity at the onset of NEC (SNAP-II score). Deaths on the day of NEC onset were excluded, as transfusion rates could not be estimated (< 1 d at risk). Multivariable results are reported as the adjusted IRR and the 95% CI for non-survivors relative to survivors after adjustment for other factors in the final model. The same method described above for the univariable analysis of surviving and non-surviving infants with NEC was used to estimate and compare transfusion rates of blood components after onset of surgical NEC or medical NEC.

RESULTS

Platelet assays

NPY and PF4 levels during storage.—As hypothesized, these studies demonstrated that NPY levels increased significantly during the storage of platelet concentrates under standard blood banking conditions. NPY levels were 4.4-fold (geometric mean (GM): 60 pg/mL; geometric mean ratio (GMR): 4.4; 95% CI: 2.4–8.1), 8.9-fold (GM 120 pg/mL and GMR 8.9; 95% CI: 5.5–14.4) and 7.1-fold (GM 96 pg/mL and GMR 7.1; 95% CI:3.7–13.7) higher in Day 2, Day 3 and Day 4/5 LR-A-PLTs, respectively, compared with control plasma (GM 13.5 pg/mL: 95% CI: 9.4–19.4) (p<0.001 for all comparisons) (Fig 1A). Similarly, serial sampling in two individual LR-A-PLT on days 3 and 4/5 confirmed that NPY levels increased over time in each individual unit (Fig 1B).

We also found a significant time-dependent accumulation of PF4 during platelet storage, compared with the control plasma units (Fig 2A). Consistent with the hypothesis that both chemokines (NPY and PF4) are released due to platelet degranulation during storage, we found a positive correlation between NPY and PF4 levels in platelet supernatants (p<0.001) (Fig 2B). In fact, the NPY pattern of accumulation was very similar to that of PF4 (Figs 1A and 2A, test for linear trend P <0.001). The fact that NPY and PF4, the two cytokines selected in our study, are derived exclusively from platelets excluded the possibility of contaminating leukocytes as a source.

Platelet transfusion rates among infants with NEC

We identified 44 infants who developed NEC among a cohort of 598 VLBW infants enrolled in the parent study. Among the 44 infants with NEC, the mean (SD) gestational age was 26.6 (2.3) weeks and mean birth weight was 820 (250) grams (Table 1). In the 24 and 48 hours after onset of NEC symptoms, 12% and 26% of affected infants, respectively, received one or more PTX (Table 2). In contrast, 79% of infants with NEC received an RBC transfusion within the 48 hours following the onset of NEC symptoms. Transfusion rates of all blood products were higher among infants with surgical NEC compared to medical NEC, with PTX rates being 12 times higher (incidence rate ratio (IRR) 12; 95% CI 4.1–34) among the 14 (36%) infants with surgical NEC compared to 25 infants with medical NEC (Figure 3 and Supplemental Table 1). Consistent with transfusion threshold data from a prior survey of PTX practices among North American neonatologists[8], PTX in our study were given to

neonates with a mean platelet count of 70 × 10^9/L (95% CI: 53–87 × 10^9/L; range: 6–210 × 10^9/L) (Figure 4).

Assessment of relationship between platelet transfusion and mortality

A total of 13 (30%) infants with NEC died before hospital discharge (Table 1). The PTX rate among infants with NEC who died was higher than among those who survived: 30.3 (95% CI 11.5–80.1) vs. 6.0 (95% CI 3.2–11.2) transfusions per 100 infant days; IRR 5.1; 95% CI 1.6–16.2; P=0.006. After adjusting for birth weight and illness severity using the SNAP II score, however, the PTX rates were no longer significantly different (16.8 vs. 5.6 transfusions per 100 infant days; adjusted IRR 3.0; 95% CI 0.6–15; P=0.18). As birth weight was not significantly associated with mortality after onset of NEC in the multivariable model, we also performed a model only adjusting for illness severity. In this model with adjustment for illness severity at onset of NEC using the SNAP II score (P=0.0004 for association with mortality), the PTX rates were significantly different among infants who died vs. survived (22.5 vs. 5.7 transfusions per 100 infant days; adjusted IRR 3.9; 95% CI 1.1–15.0).

DISCUSSION

In this study, we showed for the first time a substantial time-dependent increase in NPY levels in the supernatant of LR-A-PLTs, stored under normal blood banking conditions. NPY, found in human platelets[24,25], is a linear peptide containing 36 amino acid residues, closely related to peptide YY and pancreatic polypeptide (PP)[31]. In addition to its well-known vasoconstrictor effects[22], NPY enhances neutrophil adhesion to endothelial cells, induces histamine release from mast cells, and stimulates macrophage adhesion, chemotaxis, phagocytosis and superoxide anion production[32]. NPY (as well as peptide YY) also has a profound inhibitory effect on gastric emptying and acid secretion, gut motility, and exocrine pancreatic secretion[33]. Thus, at least theoretically, a rise in the NPY plasma levels of preterm neonates with NEC could have an aggravating influence on intestinal ischemia and inflammation.

NPY levels have not been measured in preterm infants. However, in full-term cord blood and in the blood of infants and children, NPY levels have been previously reported to be below the limit of detection (50 pg/mL) in the majority of patients, with a few subjects having concentrations slightly above that[34]. Since our apheresis derived PLTs are usually transfused on day 3 of storage to preterm neonates with a circulating plasma volume of approximately 80 ml/Kg, a 15 ml/Kg PTX containing ~120 pg/ml of NPY would provide an NPY “load” of 1,800 pg/Kg, which would be predicted to increase the plasma NPY levels by approximately 23 pg/mL. Given the low plasma levels found in healthy infants, this might be a significant increase, particularly if multiple platelet transfusions are given to the same patient, as is often the case in neonates with NEC. However, little is known about the *in vivo* effects of transfused chemokines[35] and, therefore, the clinical significance of the transfused NPY in NEC is unclear. Nevertheless, our study provides proof of principle that platelet-derived active substances, such as NPY and PF4, are released and accumulate in platelet

concentrates during storage, which may contribute to or exacerbate the inflammatory gut injury among infants with NEC.

To further test this hypothesis, we then evaluated the relationship between PTXs and mortality in a cohort of infants with NEC. As hypothesized, our epidemiological analyses showed that PTX rates were higher among infants with NEC who died, compared to those who survived. However, the association between PTX rate and death diminished after adjustment for illness severity at the onset of NEC, and was no longer significant. Therefore, our findings suggest that higher PTX rates among infants who died could be confounded by an increased severity of NEC. However, given that the point estimate for the PTX rate ratio was 3 times greater among infants who died compared to survivors, even after adjustment for illness severity and birth weight, it is also possible that we were underpowered to detect a difference in mortality among groups. Thus, further studies are needed to understand if platelet transfusions causally contribute to poorer outcomes among infants with NEC, or are simply a marker of illness severity. A previous single center cohort study[10] found that infants who died with NEC, compared to those who survived, received a larger number (8 vs. 2) and total volume (170 ml vs. 65 ml) of platelet transfusions, although neither difference was statistically significant. Previous studies have shown that infants with NEC are among the highest users of PTXs in the neonatal intensive care unit [36]. Given that NEC is an inflammatory condition[37] and PAF has been shown to be an important factor in the pathogenesis[19,38], we believe that infants with NEC are an important cohort to study in order to understand the potential inflammatory effects of PTXs.

We should acknowledge the strengths and limitations of our study. We were able to estimate the onset of NEC with good accuracy and were able to follow infants who were transferred for surgery, which allowed us to estimate the transfusion burden through discharge without censoring of outcomes. In addition, we ascertained PTX rates, rather than just number of transfusions, which better accounts for the time at risk and allows for a more robust measure of the intensity of PTXs. Without accounting for time at risk, as was performed in this study, patients who survive could have a relatively longer follow-up period to observe PTX compared to patients who died and this could bias the relationship between PTX and mortality among infants with NEC. Although we had a relatively large multicenter cohort to study, the low incidence of NEC may have yielded too few patients to detect a significant association between PTX rate and mortality. In addition, we were unable to evaluate the relationship between PTX and short-gut syndrome among survivors given our follow-up ending at 90 postnatal days, which is too soon to confidently ascertain the diagnosis of short-gut syndrome in some infants with NEC. Finally, we recognize the potential confounding effects of other unmeasured variables, such as the presence of cytokines and bioactive substances in red cell transfusions,[39,40] which nearly all infants in our cohort received.

Based on the findings of the PlaNeT-2 trial in which neonates (including 16% with NEC) randomized to a higher platelet transfusion threshold had a significantly higher rate of death or major bleeding,[41] we recommend the use of restrictive platelet thresholds of 25×10^9/L for transfusion to patients with NEC. In addition, larger, well-powered cohort studies are needed to better understand the role of PTX on relevant outcomes among infants with NEC,

and particularly the link between donor platelet cytokines, storage time, and neonatal outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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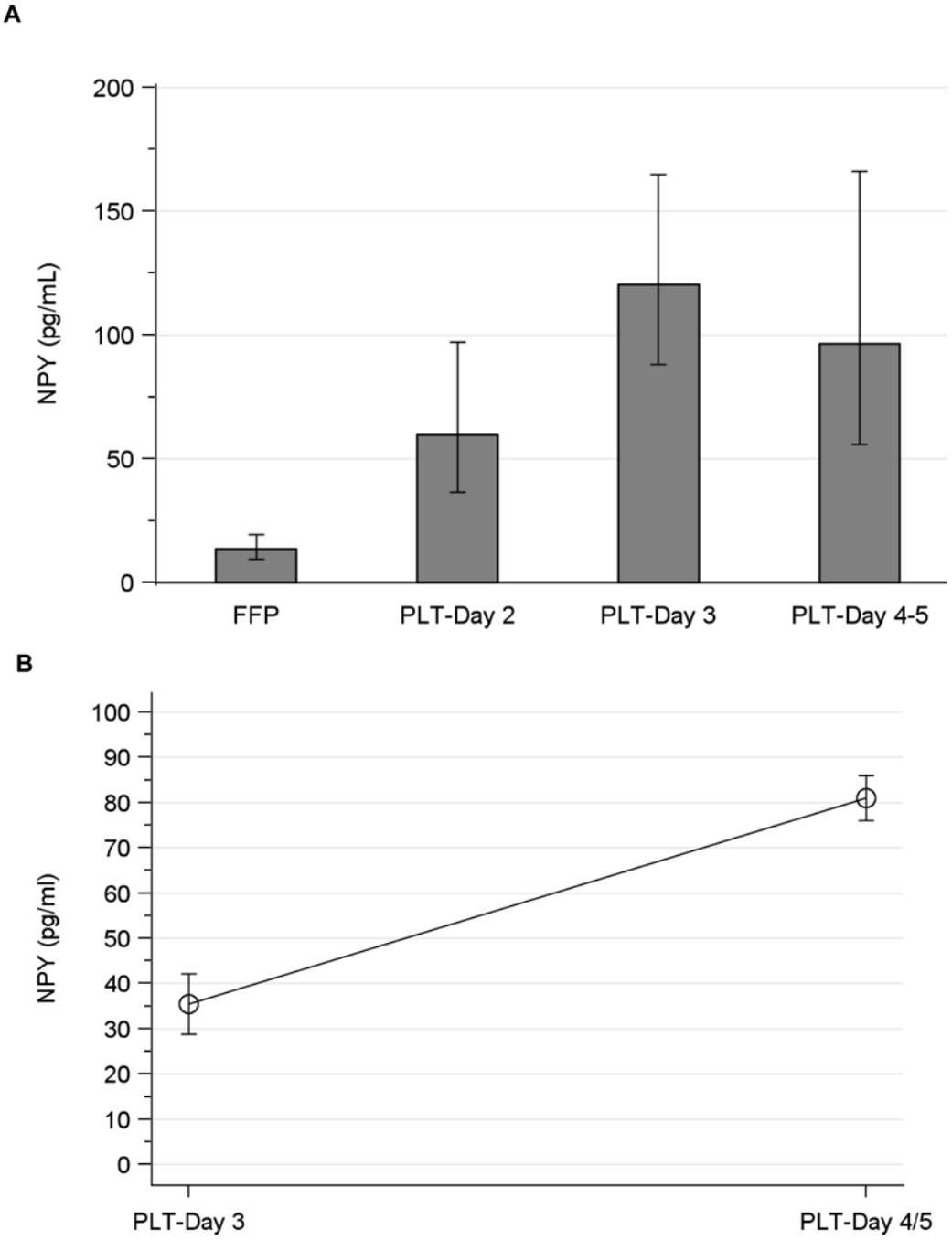

Figure 1. NPY levels increase in LR-A-PLTs during storage.

A. NPY levels were measured in individual LR-A-PLTs right before release for transfusion. Columns and error bars represent the geometric mean and 95% confidence interval of 5 (Day 2), 11 (Day 3) and 4 (Day 4/5) samples per time point; test for linear trend, $p < 0.001$.

B. NPY levels were serially measured in two LR-A-PLT units on days 3 and 4/5 of storage; $p = 0.02$ for increase over time).

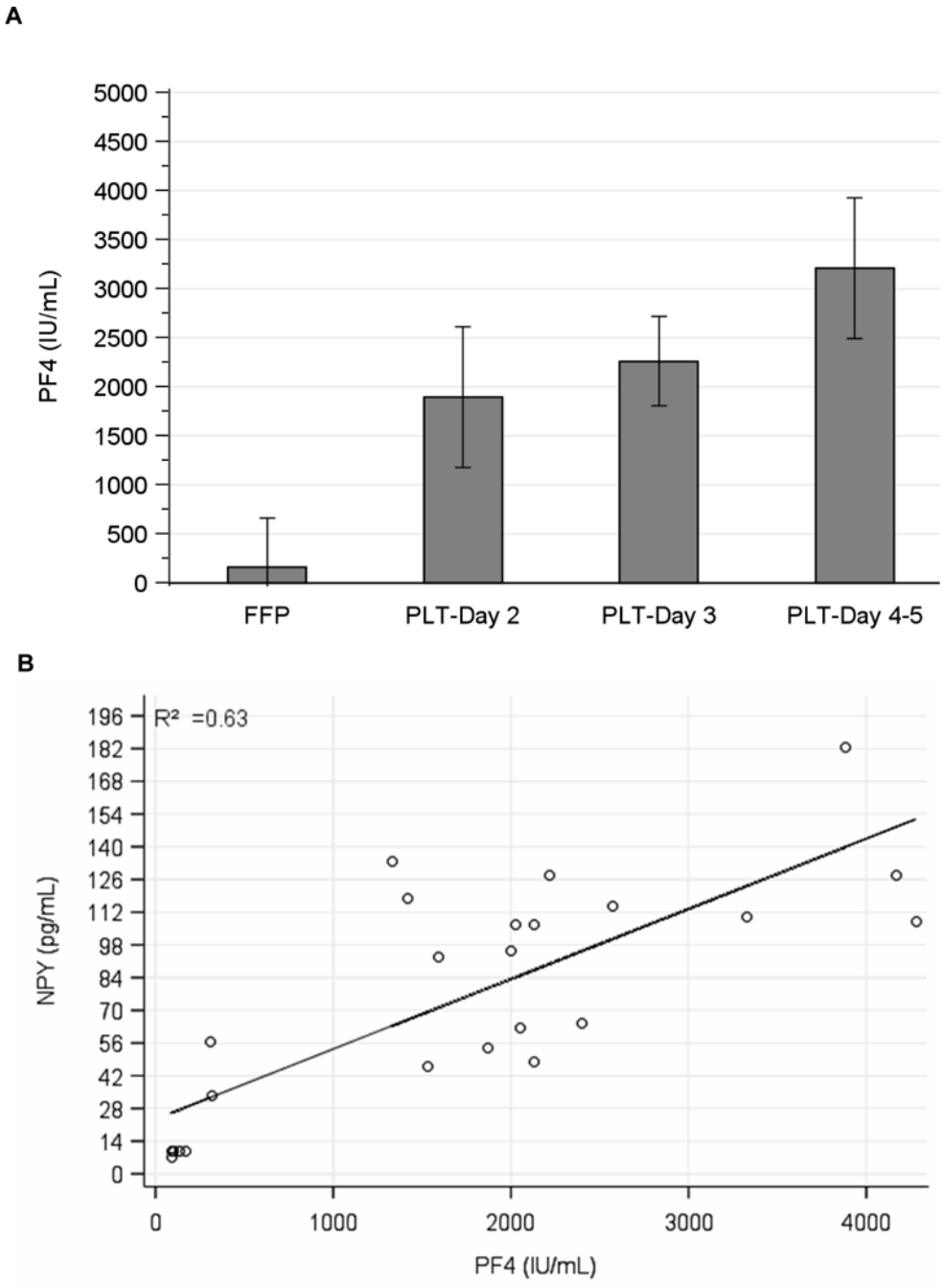

Figure 2. PF4 levels increase in LR-A-PLTs during storage.

A. Columns and error bars represent the mean and 95% confidence interval of 5 (Day 2), 11 (Day 3) and 4 (Day 4/5) samples per time point; test for linear trend, $p < 0.001$. **B.** Association between NPY (y-axis) and PF4 (x-axis) protein levels from 8 samples of fresh frozen plasma (FFP) and 17 samples of platelet (PLT) with both NPY and PF4 levels ($p < 0.001$ by linear regression, $R^2 = 0.63$).

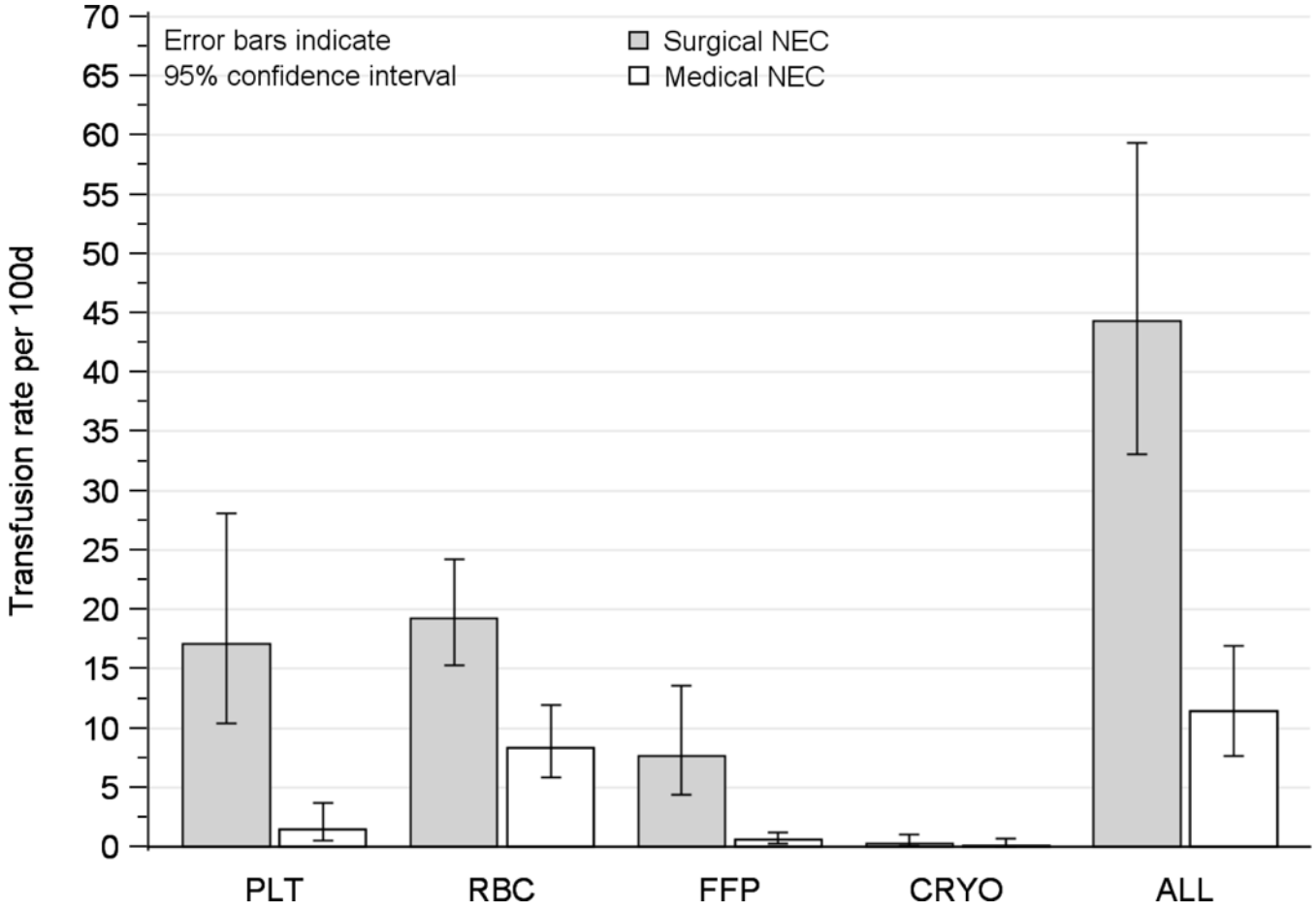

Figure 3. Transfusion rates after NEC.

Figure depicts the rates of transfusion of blood components after onset of medical and surgical NEC. Platelet transfusion rates were higher among 14 infants with surgical NEC, compared to 25 infants with medical NEC (IRR 12; 95% CI 4.1–34). Infants who died on the day of NEC onset not included. Abbreviations: NEC, necrotizing enterocolitis; IRR, incidence rate ratio; CI, confidence interval; RBC, Red blood cell; PLT, platelet; FFP, fresh frozen plasma; CRYO, cryoprecipitate; ALL, any blood component.

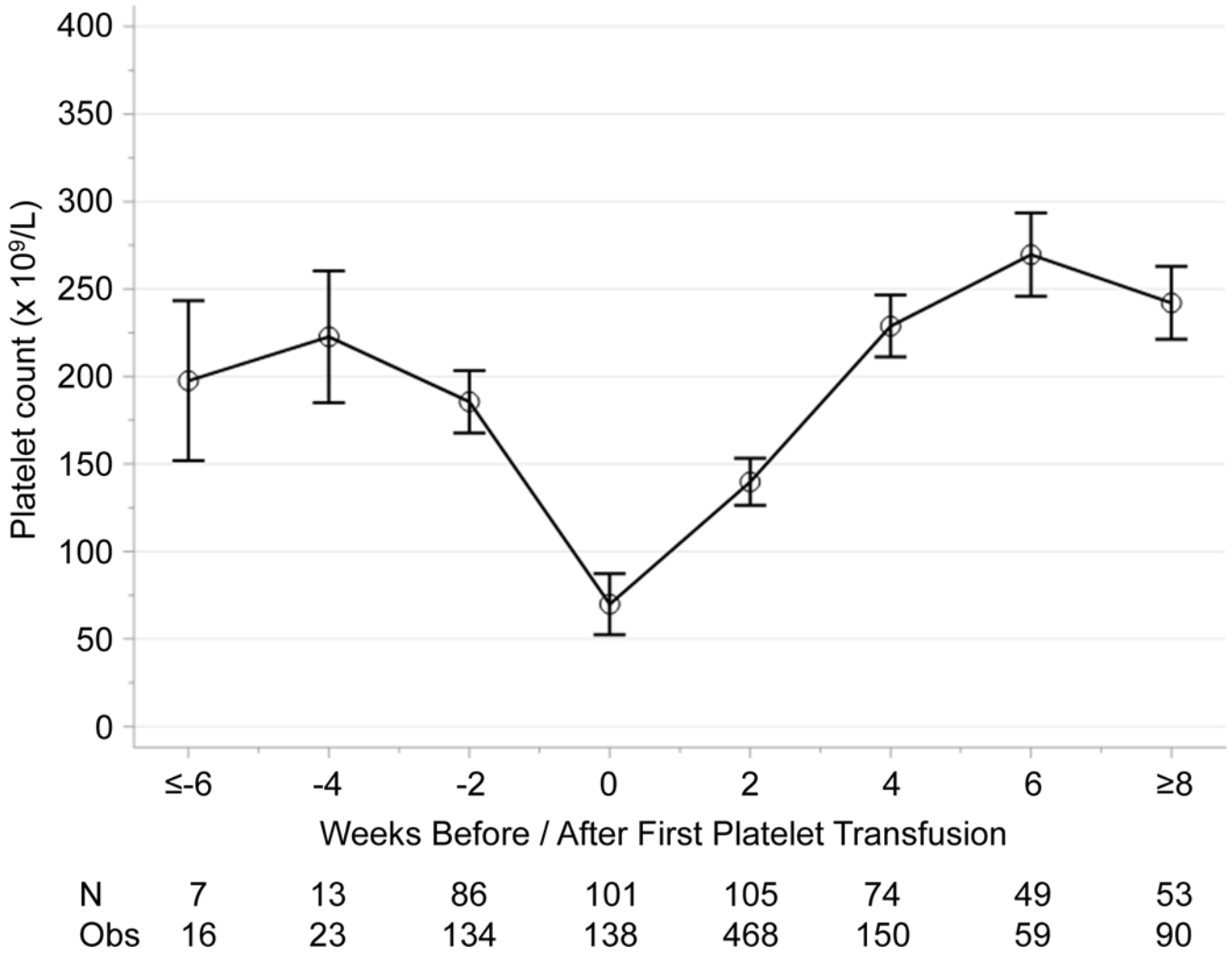

Figure 4. Mean platelet count at first transfusion.

Figure shows platelet trajectory and count at timing of first platelet transfusion to reflect general platelet transfusion practices at centers involved in this study (108 infants and 1078 platelet count measurements). Model-based means and 95% confidence intervals were estimated from repeated measures model.

Table 1.

Characteristics of infants with NEC

Characteristics	N=44
Gestational age in weeks, mean ± SD	26.6 ± 2.3
Birth weight in grams, mean ± SD	820 ± 250
Male gender	20 (46%)
Race	
Black	31 (71%)
White	11 (25%)
Other	2 (5%)
Singleton birth	34 (77%)
Small for gestational age	14 (32%)
Receipt of antenatal steroids	33 (75%)
Death	13 (30%)
Patent ductus arteriosus	17 (39%)
Intraventricular hemorrhage (grade 2 or greater)	9 (21%)
Ever fed breast milk	43 (98%)

Data are presented as n, (%) unless indicated otherwise.
Abbreviations: NEC, necrotizing enterocolitis; SD, standard deviation.

Table 2.

Frequency of transfusions in the immediate post-NEC period

Type of blood product	Receipt within 24 hours after NEC onset, number (%) of infants	Receipt within 48 hours after NEC onset, number (%) of infants
Red blood cell	28 (67%)	33 (79%)
Platelet	5 (12%)	11 (26%)
Fresh frozen plasma	8 (19%)	11 (26%)
Cryoprecipitate	0	1 (2%)
Any of above	31 (74%)	34 (81%)