Journal of the American College of Cardiology
© 2014 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 63, No. 13, 2014
ISSN 0735-1097/$36.00
http://dx.doi.org/10.1016/j.jacc.2013.11.029

Impact of Red Blood Cell Transfusion on Platelet Aggregation and Inflammatory Response in Anemic Coronary and Noncoronary Patients

The TRANSFUSION-2 Study (Impact of Transfusion of Red Blood Cell on Platelet Activation and Aggregation Studied With Flow Cytometry Use and Light Transmission Aggregometry)

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Objectives This study sought to determine whether red blood cell (RBC) transfusion increases in vivo platelet aggregation and inflammation in coronary and noncoronary patients.

Background RBC transfusion increases in vitro platelet activation and aggregation in healthy volunteers, providing a possible explanation for the increase in recurrent ischemic events and mortality reported after RBC transfusion in patients with acute coronary syndromes (ACS).

Methods Platelet reactivity was measured before and after RBC transfusion in 61 patients (33 with ACS patients and 28 without ACS). Relative changes between baseline and post-transfusion measurements of maximal and residual platelet aggregation were considered with different agonists as well as changes in vasodilator-stimulated phosphoprotein platelet reactivity index and P-selectin expression. Inflammatory and thrombotic biomarkers were also measured before and after transfusion.

Results After RBC transfusion, platelet reactivity was increased when measured using adenosine diphosphate–induced light transmission aggregometry (11.6% relative increase in maximal platelet aggregation, $p = 0.004$; 10.8% increase in residual platelet aggregation, $p = 0.005$) and vasodilator-stimulated phosphoprotein platelet reactivity index (20.7% relative increase, $p = 0.002$), and there was a nonsignificant trend toward an increase in P-selectin expression. Similar results were found with the nonspecific agonist thrombin receptor–activated peptide (relative increases of 11.7% for maximal platelet aggregation, $p = 0.04$, and 12.7% for residual platelet aggregation, $p = 0.02$) but not with collagen or arachidonic acid agonists. There were no significant differences in inflammatory and thrombotic biomarkers before and after transfusion.

Conclusions After RBC transfusion, there is an increase in platelet reactivity, especially with tests measuring the adenosine diphosphate–$P2Y_{12}$ receptor pathway, without significant variations in inflammatory or thrombotic biomarkers. This in vivo effect may account for the excess of ischemic events observed in the context of patients with ACS treated using percutaneous coronary intervention and $P2Y_{12}$ inhibitors. (J Am Coll Cardiol 2014;63:1289–96)

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Abbreviations and Acronyms

AA = arachidonic acid
ACS = acute coronary syndrome(s)
ADP = adenosine diphosphate
BARC = Bleeding Academic Research Consortium
LTA = light transmission aggregometry
MFI = mean fluorescence intensity
MPA = maximal platelet aggregation
PGE_1 = prostaglandin E_1
PRI = platelet reactivity index
RBC = red blood cell
RPA = residual platelet aggregation
VASP = vasodilator-stimulated phosphoprotein

The recent development of potent antiplatelet therapy has led to a decrease in the rate of recurrent ischemic events and mortality despite a constant increase in major bleeding complications and a more liberal use of allogeneic red blood cell (RBC) transfusion (1–3). Bleeding and/or transfusion have been repeatedly associated with an increased risk for adverse outcomes, including ischemic complications, myocardial infarction, and death (4–6).

See page 1297

Making the right decisions for patients with acute coronary syndromes (ACS) presenting with anemia and/or major bleeding events remains a clinical challenge. Indeed, the interruption of effective antithrombotic treatment can lead to fatal thrombosis. RBC transfusion represents another challenging issue, with studies suggesting that the conservative use of RBC transfusion is more favorable in hemodynamically stable patients (7–9).

Transfusion by itself has been shown to be an independent risk factor for recurrent ischemic events and mortality (10–12), although the causal link between bleeding or transfusion and mortality has not been fully elucidated yet. We previously demonstrated that in vitro RBC transfusion increases platelet activation and aggregation in healthy volunteers and can potentially contribute to excess risk and a higher rate of recurrent thrombotic event observed in transfused patients with ACS (13).

To further explore the consequences of RBC transfusion on platelet reactivity and inflammatory response, we conducted the Impact of Transfusion of Red Blood Cell on Platelet Activation and Aggregation Studied With Flow Cytometry Use and Light Transmission Aggregometry (TRANSFUSION-2) study to evaluate our primary hypothesis that patients receiving RBC transfusion would exhibit increased platelet reactivity.

Methods

Study population. The TRANSFUSION-2 study is a cross-sectional observational, prospective study conducted by the Allies in Cardiovascular Trials Initiatives and Organized Networks Group at Institut de Cardiologie, Pitié-Salpêtrière University Hospital (Paris, France). Patients with documented coronary artery disease or without coronary artery disease in whom an allogeneic RBC transfusion was prescribed were enrolled in the study. Inclusion criteria were: 1) age >18 years; 2) stable hemodynamic status; and 3) review of and agreement with the study protocol. Antiplatelet therapy with aspirin, clopidogrel, prasugrel, or ticagrelor was allowed. Exclusion criteria were: 1) previous RBC transfusion in the past 7 days; 2) hemodynamic instability with or without a cardiac assist device; 3) glycoprotein IIb/IIIa inhibitor administration within the past 7 days; 4) septic status at the time of transfusion; 5) use of steroidal and nonsteroidal anti-inflammatory drugs; 6) a low platelet count ($<100 \times 10^6$/l); and 7) concomitant transfusion of platelets. Written informed consent was obtained before participation, and this study was approved by the Pitié-Salpêtrière University Hospital Ethics Committee (Comité de Protection des Personnes Participants à la Recherche Biomédicale). The study was conducted and funded by the Allies in Cardiovascular Trials Initiatives and Organized Networks study group (http://www.action-coeur.org) and performed within the Institut National de la Santé et de la Recherche Médicale unit UMRS 937. A research grant was also obtained from Société Française de Cardiologie and Fédération Française de Cardiologie.

Data collection. All clinical and biological data from patients who provided informed consent were collected into a prospective, Web-based registry, as well as drug intake to evaluate drug-drug interactions. ABO type, rhesus group, number of units of recipient blood, and dates of collection and thawing were also obtained for each transfusion pack. Preparation of RBC packs was done according to French legislation (http://www.dondusang.net), and RBCs were obtained at our institution using the classic principle of centrifugation of total blood using saline-adenine-glucose-mannitol as the additive solution, followed and conserved between 2°C and 6°C for a maximum of 42 days.

lecture fees from AstraZeneca, Daiichi Sankyo, and Eli Lilly. Dr. Abtan has received research grants from Fonds d'Etudes et de Recherche du Corps Médical. Dr. Kerneis has received research grants from Fédération Française de Cardiologie. Dr. Vignalou has received research grants from Servier. Dr. O'Connor has received research grants from Menarini and the European Society of Cardiology. Dr. Collet has received research grants from Bristol-Myers Squibb, Sanofi-Aventis, Eli Lilly, Guerbet Medical, Medtronic, Boston Scientific, Cordis, Stago, Centocor, Fondation de France, Institut National de la Santé et de la Recherche Médicale, Fédération Française de Cardiologie, and Société Française de Cardiologie; consulting fees from Sanofi-Aventis, Eli Lilly, and Bristol-Myers Squibb; and lecture fees from Bristol-Myers Squibb, Sanofi-Aventis, and Eli Lilly. Dr. Montalescot is a consultant to Accumetrics, AstraZeneca, Bayer Healthcare Pharmaceuticals, Biotronik, Bristol-Myers Squibb, Boehringer-Ingelheim, Duke Institute, Eli Lilly, Europa, GlaxoSmithKline, Iroko, Lead-Up, Medtronic, Menarini, Novartis, Pfizer, Roche, Sanofi-Aventis, Springer, The Medicines Company, TIMI Group, WebMD, and Wolters; has received research grants, consulting fees, and lecture fees from Abbott Vascular, Accumetrics, AstraZeneca, Biotronik, Bristol-Myers Squibb, Daiichi Sankyo, Eli Lilly, Féderation Française de Cardiologie, Fondation de France, INSERM, Institut de France, Medtronic, Menarini, Nanospheres, Pfizer, Roche, Sanofi-Aventis, Société Française de Cardiologie, Stentys, and The Medicines Company. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Manuscript received May 2, 2013; revised manuscript received October 22, 2013, accepted November 12, 2013.

Blood sampling and measurements. Blood was collected into Becton Dickinson (Franklin Lakes, New Jersey) 3.2% citrate Vacuette tubes after having discarded the first 2 ml to 4 ml of blood to avoid spontaneous platelet activation from a peripheral vein in the same fashion before and after transfusion. Blood samples were processed for platelet function testing within 2 h after drawing. Platelet reactivity was tested at baseline on blood drawn just before RBC transfusion, and a second blood draw was performed 12 to 36 h after the end of RBC transfusion. Plasma samples were obtained for each patient before and after transfusion by centrifugation of blood samples at 3,000 *g* for 20 min, and aliquots of 0.6 ml of plasma were stored at −80°C until assay.

Light transmission aggregometry. Platelet-rich plasma was obtained by centrifugation of citrated whole blood at 100*g* for 10 min at room temperature. Platelet-poor plasma was obtained by further centrifugation at 4,500*g* for 15 min. In vitro platelet aggregation in platelet-rich plasma was measured at 37°C using light transmission aggregometry (LTA) (model 490-4D; Chrono-Log Corporation, Kordia, the Netherlands) and was induced by 4 different agonists: 1) adenosine diphosphate (ADP) 20 μmol/l (Sigma-Aldrich, Saint Quentin Fallavier, France); 2) arachidonic acid (AA) 1.25 mmol/l (Sigma-Aldrich); 3) collagen 2 μg/ml (Helena Biosciences, Tyde and Wear, United Kingdom); and 4) thrombin receptor–activated peptide 20 μmol/l. Maximal platelet aggregation (MPA) and residual platelet aggregation (RPA) measured 6 min after the induction of aggregation were recorded for all agonists. Pre-specified criteria used to define nonevaluable samples were lack of sufficient signal, hemolysis, and platelet-rich plasma platelet count <150,000/ml and an unstable baseline signal.

Flow cytometry. Flow cytometry was performed <2 h after venipuncture for P-selectin measurement. The phosphorylation of vasodilator-stimulated phosphoprotein was measured within 24 to 48 h using a Beckman Coulter FC500 cytometer (Beckman Coulter, Villepinte, France).

To determine platelet P-selectin expression, blood samples previously activated by a dose of 10 or 20 μmol/l of ADP were mixed with saturated concentrations of anti-CD62p-PE (Beckman Coulter) monoclonal antibody and anti-CD41a–fluorescein isothiocyanate monoclonal antibody (Beckman Coulter). After staining with antibodies, samples were incubated for 30 min in the dark and diluted with 1 ml of Isoflow Sheath fluid (Beckman Coulter). Samples were immediately processed for flow cytometric analysis. To determine platelet CD62P expression, individual platelets were identified by size (forward and scatter) and anti-CD41a–fluorescein isothiocyanate immunofluorescence using a logarithmic scaled dot plot. P-selectin expression on the surface of platelets was defined as positive for anti-CD62P-PE. Variation in activation corresponded to the percent of gated platelets after activation by agonist minus the percent of gated platelets at rest. The level of P-selectin expression at rest was subtracted from the raw value of P-selectin expression after activation by ADP to compare the variation in delta of P-selection expression.

Vasodilator-stimulated phosphoprotein platelet reactivity index. Vasodilator-stimulated phosphoprotein (VASP) phosphorylation was measured using platelet VASP kits (Diagnostica Stago, Asnières, France) according to the manufacturer's instructions. Briefly, blood samples were incubated in vitro with ADP and/or prostaglandin E_1 (PGE_1) before fixation. The platelet population was identified on its forward-scatter and side-scatter distributions, and 5,000 platelet events were gated and analyzed for mean fluorescence intensity (MFI). The MFI corresponding to each experimental condition (PGE_1 and ADP + PGE_1) was determined to establish a ratio directly correlated with the VASP phosphorylation state. The VASP platelet reactivity index (PRI) was calculated from the MFI of each condition according to the formula: VASP PRI = $[(MFI_{PGE1} - MFI_{PGE_1+ADP})/MFI_{PGE1}] \times 100$. All measurements were performed in our research laboratory on thrombosis (the Institut National de la Santé et de la Recherche Médicale unit UMRS 937) according to previously published methods (14–17). The technicians were blinded to patients' identities and characteristics.

Biomarkers. Soluble CD40 ligand levels were measured using a human soluble CD40 ligand enzyme-linked immunosorbent bioassay (Biovendor, Karasek, Czech Republic). Tumor necrosis factor–alpha and interleukin-6 levels were measured using Quantikine sandwich enzyme immunoassays (AssayPro, St. Charles, Missouri) according to the manufacturer's instructions. C-reactive protein levels were quantified using commercially available enzyme-linked immunosorbent assay kits (Zymutest; HypenBioMed, Neuville-sur-Oise, France). Plasminogen activator inhibitor-1, D-dimer, and von Willebrand factor antigen concentrations were measured using Asserachrom kits (Diagnostica Stago). Tissue factor pathway inhibitor was measured using sandwich enzyme immunoassays (USCNK, Wuhan, China). All measurements were assessed in duplicate.

Study endpoints. The main endpoint was the relative change in MPA in response to ADP, expressed as the percent of change between baseline and post-transfusion measurement ($[MPA_{post\text{-}transfusion} - MPA_{baseline}]/MPA_{baseline}$). Other endpoints were the relative changes of platelet aggregation with other agonists or other platelet function tests and changes in biomarkers before and after transfusion ([post-transfusion value − baseline value]/baseline value).

Clinical follow-up. Patients were followed for 30 days through outpatient consults. Physicians noted the occurrence of major cardiovascular and cerebrovascular events, including death, stroke, recurrent myocardial infarction, urgent revascularization, and definite and probable stent thrombosis (per the Academic Research Consortium definition), as well as major and minor bleeding events according to the Bleeding Academic Research Consortium (BARC) definition (18).

Statistical analyses. On the basis of our previous study (19), at least 60 transfused patients were needed to yield 80% power with a risk for alpha error of 0.05 to demonstrate a difference of 10% relative change in MPA before and after transfusion.

Means and standard deviations are used to report the results. Data were analyzed using paired Student *t* tests. A confirmatory analysis using the Wilcoxon rank test was also performed to assess statistical significance in case of a possible non-Gaussian distribution. Correlations between RBC storage duration and platelet aggregation data were tested using the Pearson test. Statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). All tests were 2 sided, with a statistical threshold for significance of 0.05.

Results

Study population and RBC transfusion. Sixty-one patients were included in the TRANSFUSION-2 study. Baseline characteristics are presented in Table 1. Thirty-three patients had suspected ACS treated with both $P2Y_{12}$ inhibitors and aspirin, and the remaining 28 patients were hospitalized for other cardiac diseases treated with aspirin only or without any antiplatelet agent. Our study population was a high-risk population: one-fifth had diabetes, and 40.9% had renal insufficiency. Two-thirds of the patients were treated with aspirin and proton pump inhibitors. The 33 patients with ACS treated with dual-antiplatelet therapy received clopidogrel 75 mg (n = 28), and 5 patients received prasugrel 10 mg. The mean baseline hemoglobin level before transfusion was 7.8 ± 1.1 g/dl, and the distribution of ABO blood types was 55.7% (n = 34) in group O, 29.5% (n = 18) in group A, 11.4% (n = 7) in group B, and 3.3% (n = 2) in group AB. The vast majority of the patients received 2 packs of RBCs (80%), leading to a mean and significant rise of 2.3 g/l in hemoglobin. The repartition of ABO blood type of the transfused packs matched perfectly the patients' blood types, according to the transfusion rules. Patients included in this study did not receive any other blood products (plasma or platelet concentrates) during the study period, and there was no substantial modification of the transfused patients' medications between the 2 sampling periods (<24 h).

Effect of RBC transfusion on platelet aggregation measured by LTA. Results of platelet aggregation measured by LTA before and after transfusion in response to ADP are presented in Figure 1. Compared with baseline values, transfusion resulted in an 11.6% relative increase in MPA and a 10.8% increase in RPA after platelet activation by ADP (p = 0.004 and p = 0.005, respectively). The results of LTA in response to other agonists are shown in Table 2. Platelet reactivity was also increased by 11.8% for MPA and 12.7% for RPA with thrombin receptor–activated peptide activation (p = 0.04 and p = 0.02, respectively), but no significant difference was found after activation by a nonspecific agonist (collagen) or by AA, exploring the Cox-1 inhibition pathway.

Table 1 Demographics (n = 61)

Demographics and risk factors	
Age (yrs)	68.5 ± 16.1
Men	38 (62.3)
Active smokers	21 (34.4)
Diabetes	12 (19.7)
Hypertension	16 (26.2)
Dyslipidemia	30 (49.2)
BMI (kg/m^2)	24.6 ± 4.6
Renal insufficiency (creatinine clearance <60 ml/min)	25 (40.9)
Medical history	
CAD	35 (57.4)
Angioplasty	27 (44.3)
Clinical presentation	
STEMI or NSTEMI	24 (39.3)
Unstable angina	9 (14.8)
Cardiac insufficiency	11 (18.0)
Others	17 (27.9)
Reason for RBC transfusion	
Bleeding and anemia	6 (8.2)
Anemia without external bleeding	55 (91.8)
Length of stay in hospital (days)	12 (6–20)
Biology on admission	
Troponin I (μg/ml)	5.8 ± 4.35
Negative troponin	37 (60.6)
Baseline hemoglobin (g/dl)	7.8 ± 1.1
CRP (mg/l)	36 (13.5–75.9)
Fibrinogen (g/l)	5.1 ± 1.8
Platelet count ($\times 10^3/mm^3$)	265 ± 146
Proteins (g/l)	60.1 ± 12.9
Creatinine clearance (ml/min)	56 ± 6.15
Creatinine (μmol/l)	155 ± 123
Antithrombotic treatment	
ASA	45 (73.7)
Maintenance dose (mg/day)	81.9 ± 15.6
Clopidogrel 75 mg	28 (45.9)
Prasugrel 10 mg	5 (8.2)
Loading dose within 24 h	9 (14.7)
GP IIb/IIIa inhibitors	0 (0)
Anticoagulant agents	37 (60.6)
RBC transfusion	
Number of RBC packs	2 (1–6)
RBC storage duration (days)	18.8 ± 12.2
Group O	34 (55.7)
Group A	18 (29.5)
Group B	7 (11.4)
Group AB	2 (3.3)
Post-transfusion hemoglobin (g/dl)	10.1 ± 0.12
Pre-transfusion WBC count ($\times 10^9$/l)	10.04 ± 3.78
Post-transfusion WBC count ($\times 10^9$/l)	9.65 ± 3.50

Values are mean ± SD, n (%), or median (range). Data on clinical presentation represent culprit events within the same hospitalization.

ASA = acetylsalicylic acid; BMI = body mass index; CAD = coronary artery disease; CRP = C-reactive protein; GP = glycoprotein; NSTEMI = non–ST-segment elevation myocardial infarction; RBC = red blood cell; STEMI = ST-segment elevation myocardial infarction; WBC = white blood cell.

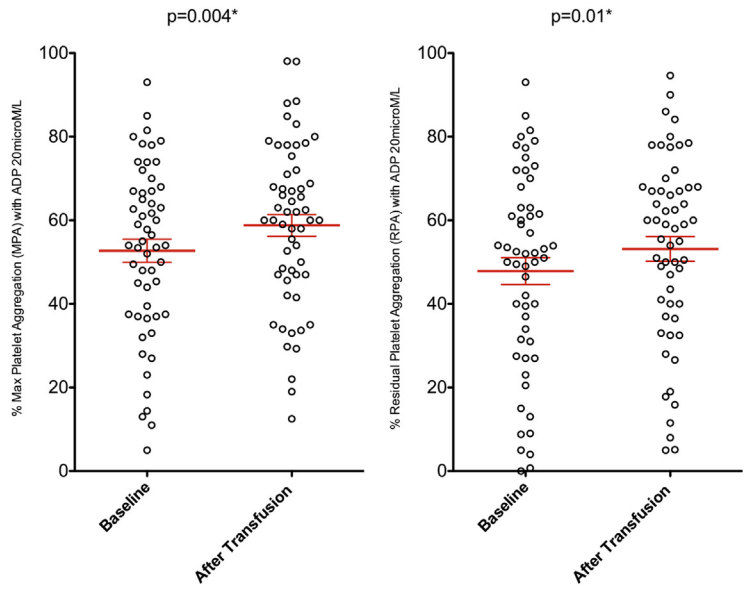

Figure 1 Platelet Reactivity Before and After RBC Transfusion Measured by MPA in Response to ADP 20 μmol/l and by RPA in the Entire Study Population

ADP = adenosine diphosphate; MPA = maximal platelet aggregation; RBC = red blood cell; RPA = residual platelet aggregation. *p < 0.05 for comparison with baseline.

Effect of RBC transfusion on platelet activation measured by flow cytometry. Results for VASP PRI and changes in P-selectin expression are reported in Figure 2. The effect of transfusion on platelet aggregation was confirmed by a relative increase of 20.7% (p = 0.002) in the VASP PRI, a specific marker of the $P2Y_{12}$ receptor and ADP pathway. Conversely, there was a relative increase of 8.3% in P-selectin expression, a marker of platelet activation, after transfusion of RBCs when 10 μmol/l ADP was used as an agonist (p = 0.07). This difference was blunted by the use of 20 μmol/l ADP (2% increase; p = 0.11).

Effect of RBC storage duration and blood type on platelet activation. The median length of conservation of the RBC packs before administration was 18.8 days (interquartile range: 11 to 26 days; maximum 36 days, minimum 3 days). We found no correlation between RBC storage duration and the change in platelet aggregation or platelet activation with LTA (r = 0.07 for ADP, r = 0.03 for collagen, r = 0.17 for AA, r = 0.03 for thrombin receptor–activated peptide). Similar results were obtained for P-selectin expression (r = 0.03 for both ADP conditions). However, we found a significant correlation between the increase in VASP PRI after transfusion and the duration of conservation (r = 0.34, p = 0.009).

There was no clear impact of ABO blood type on platelet reactivity (MPA with ADP 20 μmol/l measured by LTA), as no major difference was found for blood type (+5.89% for group O [n = 34], +6.05% for group A [n = 18], −1.25% for group B [n = 7], and +9.7% for group AB [n = 2]; p = 0.77 for trend).

Effect of RBC transfusion on inflammatory and thrombotic biomarkers. Results of measurements of inflammatory and thrombotic biomarkers and changes before and after transfusion are reported in Table 3. We did not find any significant changes between the pre-transfusion and post-transfusion measurements.

Subgroup analysis. In an exploratory subgroup analysis, we evaluated the results in patients treated with $P2Y_{12}$ inhibitors compared with those not receiving $P2Y_{12}$ inhibitors. As

Table 2 Platelet Reactivity Before and After RBC Transfusion in the Study Population (n = 61) Measured With LTA, Expressed by MPA and RPA

	Baseline		After Transfusion		
Variable	Mean	SD	Mean	SD	p Value
ADP 20 μmol/l					
MPA (%)	52.7	20.5	58.8	19.5	0.004*
RPA (%)	47.9	23.9	53.1	22.2	0.005*
AA 1.25 mmol/l					
MPA (%)	13.4	24.2	17.0	25.0	0.46
RPA (%)	12.9	24.1	15.6	24.3	0.63
Collagen 2 μg/ml					
MPA (%)	21.5	21.4	21.02	20.4	0.55
RPA (%)	20.6	21.5	20.4	20.0	0.86
TRAP					
MPA (%)	49.3	23.4	55.1	24.5	0.04*
RPA (%)	44.7	25.4.9	50.4	27.2	0.02*

*p < 0.05 for comparison with baseline.

AA = arachidonic acid; ADP = adenosine diphosphate; LTA = light transmission aggregometry; MPA = maximal platelet aggregation; RBC = red blood cell; RPA = residual platelet aggregation; SD = standard deviation; TRAP = thrombin receptor–activated peptide.

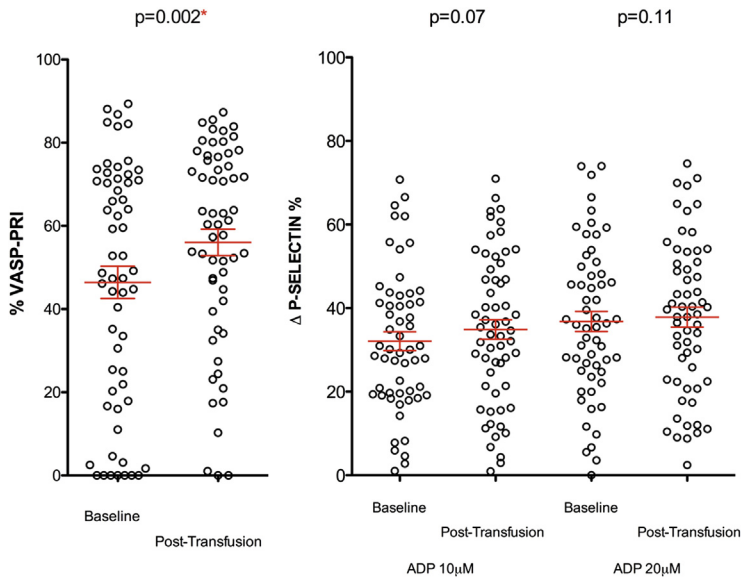

Figure 2 **Variation in Platelet Aggregation and Activation Measured by Flow Cytometry With the VASP PRI and With P-Selectin Expression With 10 μmol/l ADP and 20 μmol/l ADP Measured at Baseline and After Transfusion in the Entire Study Population**

ADP = adenosine diphosphate; PRI = platelet reactivity index; VASP = vasodilator-stimulated phosphoprotein. *p < 0.05 for comparison with baseline.

expected, coronary patients treated with $P2Y_{12}$ inhibitors had lower baseline values of platelet reactivity whatever the test used (data not shown). Platelet reactivity after RBC transfusion was predominantly increased in coronary patients treated with $P2Y_{12}$ inhibitors when measured by LTA (relative increases of 20.4%, p = 0.002, and 15.6%, p = 0.03, for MPA and RPA, respectively) compared with noncoronary patients not receiving $P2Y_{12}$ inhibitors (relative increases of 4.7%, p = 0.20, and 0.7%, p = 0.40, for MPA and RPA, respectively).

Short-term outcomes. Clinical follow-up was obtained in all patients at 1-month follow-up. The mean length of stay in the hospital was 12 days for transfused patients. Three patients, all from the coronary group, died during the hospitalization period, 1 from a fatal myocardial infarction, 1 from gastrointestinal bleeding, and 1 from septic shock. The rate of major cardiovascular and cerebrovascular events was 11.5% (n = 7). The rate of additional bleeding (per the BARC definition), not counting the initial RBC transfusion, was 9.8% (n = 6), including 2 BARC 3 events and 4 BARC 1 or 2 events.

Discussion

The evidence on the impact of RBC transfusion in patients is limited, and although several hypotheses have been described regarding the pathophysiological changes induced

Table 3 **Results of Biomarkers Measured at Baseline and After Transfusion in the Entire Study Population**

	Baseline		After Transfusion		
Biomarker	Mean	SD	Mean	SD	p Value
Inflammation					
TNF-α (pg/ml)	34.6	29.4	39.4	36.2	0.31
CRP (μg/ml)	39.6	44.3	41.7	48.1	0.15
IL-6 (pg/ml)	13.8	22.5	12.8	19.7	0.72
PAI-1 (ng/ml)	13.8	22.5	12.8	19.7	0.61
Thrombosis					
D-dimer (ng/ml)	2,942	2,989	2,947	2,922	0.31
TFPI (ng/ml)	91.3	44.0	98.4	36.1	0.35
sCD40L (ng/ml)	1.59	2.1	1.65	2.5	0.90
vWF:Ag (%)	155.4	98.4	165.5	116	0.44

*p < 0.05 for comparison with baseline.
CRP = C-reactive protein; IL-6 = interleukin-6; PAI-1 = plasminogen activator inhibitor–1; sCD40L = soluble CD40 ligand; SD = standard deviation; TFPI = tissue factor pathway inhibitor; TNF-α = tumor necrosis factor–alpha; vWF:Ag = von Willebrand factor antigen.

after RBC administration (20), we still do not fully understand the mechanisms that could explain such deleterious effects. In the TRANSFUSION-2 study, we explored the consequences of RBC transfusion on platelet reactivity and inflammatory response in anemic patients with or without coronary artery disease and treated accordingly with or without antiplatelet agents.

The results of this study can be summarized as follows. First, we demonstrated that platelet reactivity measured using 3 different platelet function tests increases in anemic coronary and noncoronary patients after RBC transfusion. Second, platelet reactivity increases with platelet function tests exploring the $P2Y_{12}$ receptor pathway (activated by ADP) but not with tests exploring the AA pathway or with nonspecific agonists such as collagen. Third, we found no significant variations in levels of inflammatory or thrombotic biomarkers after RBC transfusion, suggesting a limited systemic effect of this therapy. Finally, we found no relationship between storage duration or blood type and the increase in platelet aggregation after RBC transfusion, with the exception of the increase in the VASP PRI, which seems to be correlated with the duration of conservation of RBC packs.

The present findings obtained in clinical situations are novel and confirm our previous work performed in vitro with blood from healthy volunteers. In the TRANSFUSION-1 study (19), we previously demonstrated a 13.9% relative increase in MPA after RBC transfusion from healthy volunteers, a magnitude of change similar to that reported in the present TRANSFUSION-2 study (11.8%). This finding is supported by our flow cytometric measurements showing a simultaneous increase of VASP phosphorylation, reflecting the degree of $P2Y_{12}$ receptor inhibition and a similar trend for P-selectin expression (not significant), a global marker of platelet activation (21). More important, we were able to show that this increase of platelet reactivity was predominantly found with platelet function tests using agonists of the ADP-$P2Y_{12}$ receptor pathway, as demonstrated by the results of LTA using ADP as an agonist and the major increase in VASP PRI, suggesting again an implication of this pathway in the platelet activation effect of RBC transfusion.

According to our findings, one hypothesis that can be offered is that RBC transfusion might directly affect platelet reactivity through activation of the $P2Y_{12}$ platelet receptor or within the ADP pathway by agonists or mediators contained in RBC packs. The liberal quantities of ADP contained in RBCs have been shown to play a role in platelet adhesion (22) and might represent a potential stimulus for platelet activation and aggregation, a hypothesis already supported by our previous in vitro experiments. The strong correlation found in the present study between increased VASP PRI and the duration of RBC storage reinforce the hypothesis of an activation of this pathway through ADP and a possible effect on outcomes, as suggested in a previous study in critically ill patients (23), although this finding deserves to be explored in further studies.

Another possible explanation of our results is that the increase in platelet reactivity found in the $P2Y_{12}$ pathway is attributable mainly to the group of coronary patients as a consequence of their physicians' decisions to stop $P2Y_{12}$ inhibitors. In recent studies (24,25), platelet recovery was estimated to be as high as 10% within the first 24 h of clopidogrel discontinuation, a magnitude that is consistent with our present finding. However, this hypothesis is unlikely to explain our finding, as only half of the population was treated with $P2Y_{12}$ inhibitors, and the VASP results show increased platelet reactivity in both coronary patients treated with $P2Y_{12}$ inhibitors and noncoronary patients not treated with thienopyridine. Even if these arguments are not supportive of the interruption hypothesis as the unique mechanism for the increase in platelet reactivity found in our study, this hypothesis cannot be excluded.

Study limitations. The lack of a control group should be acknowledged as a theoretical limitation of our study. Several controls, such as anemic or nonanemic patients or healthy volunteers receiving RBC transfusion or another vascular solution, and treated with different regimens of antiplatelet agents, would have been necessary, but such situations are difficult to conceive in clinical research and our previous in vitro model of RBC transfusion in nonanemic healthy volunteers support our findings. Another limitation is whether this effect of RBC transfusion could have been differentiated from that of treatment interruption, for which another study design would have been needed. However, we believe that our study raises platelet recovery as an issue in the context of RBC transfusion in stable patients and that its effect only adds to the increase in platelet reactivity after RBC transfusion. The subgroup analysis of the ACS group was meant only to be exploratory and must be interpreted with caution, as this study was not powered to conduct for such an analysis. Finally, the magnitude of the increase in platelet reactivity found before and after RBC transfusion in our studies, both in vitro and in vivo, can be seen as modest but of similar magnitude to that found in pharmacodynamic studies between carriers and noncarriers of the loss-of-function allele cytochrome P450 2C19*2 (24,25), an effect well correlated with an increase in ischemic events (26,27).

We acknowledge that the increase in platelet reactivity observed in our study is of moderate magnitude, but we believe that it may be a plausible explanation for the detrimental effect of RBC transfusion found in clinical studies (4,6,10,12). In patients with ACS, an increase in platelet reactivity due to the transfusion itself, or potentialized by the cessation of antiplatelet agents, seem to lead to a clinical situation of high risk for ischemic events, especially when added to the detrimental physiological impact of blood loss itself. A recent meta-analysis supports the detrimental effect of blood transfusion in high-risk situations such as myocardial infarction, but there is a lack of analysis of potential confounders such as treatment cessation (13,28). The potential role of the $P2Y_{12}$ pathway highlighted in our study suggests that interruption of clopidogrel, prasugrel, or

ticagrelor in particular may exacerbate the detrimental effects of transfusion, while aspirin interruption may be less of a concern considering the data from our study on the AA pathway.

Conclusions

RBC transfusion exposes patients to an increase in platelet reactivity, an effect that is predominantly observed in the ADP-$P2Y_{12}$ receptor pathway. The link with an increase in ischemic events in transfused patients due to an increase in platelet reactivity remains to be demonstrated, but we suggest that the risks and benefits of transfusion should be weighed on an individual basis in patients with ACS until a randomized controlled trial of conservative versus liberal treatment of anemic patients with ACS clarifies this issue.

Acknowledgments
The authors thank Ghalia Anzaha for her technical assistance and Professor Jean Chastre from the critical care department at Pitié-Salpêtrière Hospital for assistance in the recruitment of patients.

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Key Words: ACS ■ clopidogrel ■ platelet response ■ transfusion.