which explored the effect of vitamin D on exacerbations, only 1.4% of patients with COPD not taking supplements had baseline 25OHD levels above 40 ng/ml (2). Despite differences in latitude, it seems likely that most patients of the study by Kunisaki and coworkers with highest 25OHD levels were also supplemented. When excluding them from the analysis, it is then striking to see that the subgroup of patients with COPD with the most deficient vitamin D levels (<10 ng/ml, n = 8.4%) had the shortest time to first exacerbation and experienced the highest number of exacerbations. Moreover, when pooling together patients with 25OHD levels ranging from 10 to 30 ng/ml, a dose–response relationship may even appear for both outcomes. We cannot test this hypothesis for statistical significance, but the observation that patients with COPD in the United States with very deficient baseline levels (<10 ng/ml) have the highest number of exacerbations is in line with the baseline data of our Belgian randomized controlled trial (2). Interestingly, a *post hoc* analysis of our study indicated potential benefit of supplementation for this particular subgroup.

Overall, it should be stressed that observational studies exploring relationships between 25OHD levels and outcomes should focus on individuals not taking vitamin D supplements. Only then can important bias be prevented and correct hypotheses on the potential of interventions be formulated, which need to be tested in randomized trials.

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Reply

From the Authors:

Heulens and colleagues hypothesize that patients with 25-hydroxyvitamin D (25[OH]D) blood levels greater than 40 ng/ml are likely to be vitamin D supplement users. Although this is a well-reasoned hypothesis, vitamin D supplementation data were not collected in our study (1), so we cannot confirm or refute this hypothesis.

TABLE 1. MEAN FEV_1 PERCENT PREDICTED, STRATIFIED BY BASELINE PLASMA 25(OH)D LEVEL IN THE COPD CLINICAL RESEARCH NETWORK AZITHROMYCIN TRIAL

25(OH)D Level (ng/ml)	n	Mean FEV_1% Predicted (95% Confidence Interval)
0–9.99	82	38.4 (34.9–42.0)
10–19.99	229	37.9 (35.8–39.8)
20–29.99	322	39.9 (38.2–41.6)
30–39.99	233	41.0 (39.0–43.1)
⩾40	107	40.6 (37.6–43.5)

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; 25(OH)D = 25-hydroxyvitamin D.

In regard to exclusion of supplement users from observational studies, we agree that supplement use has been associated with many other confounders such as engagement in other healthy behaviors, better adherence to medications, and higher educational status (2–4). Such associations tend to overestimate the potential benefits of dietary supplements, and we found no such potential benefit, making health user bias unlikely. Conversely, Heulens and colleagues suggest that vitamin D supplement users in our study may have had more severe chronic obstructive pulmonary disease (COPD) and therefore biased our results toward the null hypothesis. Again, without supplement data, we cannot perform a formal analysis to address this issue of potential confounding by indication. However, the 107 patients with 25(OH)D greater than or equal to 40 ng/ml had a mean FEV_1, the most commonly used marker of COPD severity, that was not different from those at lower 25(OH)D levels (Table 1).

Randomized controlled trials (RCTs) remain the gold standard for evaluating the effects of medical interventions, and the null results of Lehouck and colleagues' well-designed RCT in 182 patients with COPD suggest no effect of vitamin D supplementation on COPD exacerbation risk (5). Their *post hoc* subgroup analysis of 30 patients with 25(OH)D levels less than 10 ng/ml and our *post hoc* observational data are consistent in suggesting a potential effect of vitamin D on exacerbation rate in patients with COPD with severe vitamin D deficiency. However, placebo-controlled RCT confirmation of these hypothesis-generating data is not ethical due to well-documented skeletal health benefits of vitamin D supplementation in patients with such profoundly low 25(OH)D levels (6).

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Investigative *In Vitro* Study about Red Blood Cell Concentrate Processing and Storage

To the Editor:

We read with interest the elegant study by Kor and colleagues evidencing no difference in measures of pulmonary function, immunologic features, and coagulation status, in groups of patients transfused with fresh versus random red blood cell component (RBCC) units (1). Previous studies showed that aged RBCCs favor inflammation and acute transfusion reactions (2). However, the investigators did not examine what we believe is an important factor, namely the processing of donated blood. The quality of RBCCs is thought to be influenced by collection, processing, and storage. Although systematic leukoreduction of blood components has successfully limited inflammation associated with leukocyte debris and chemokines, the collection, processing, and storage of RBCCs may lead to membrane and cytoplasmic changes that result in hemolysis and release of proinflammatory products (3). We here report an *in vitro* observation concerning 20 whole-blood (WB) units collected into citrate phosphate dextrose anticoagulant and processed either immediately, that is, within 2 hours of collection (referred to as instant WB [iWB]), or held overnight at room temperature (22 ± 2°C) prior to processing (referred to as RT hold/RTH-WB). Leukofiltered WB was separated into one plasma unit and one packed RBCC containing 100 ml saline, adenine, glucose, and mannitol. Soluble cytokine/chemokine levels (IL-8 [Abcyss, Paris, France] and Gro-α [growth-regulated oncogene-α], ENA-78 [epithelial neutrophil-activating protein 78], MIP-1α [macrophage inflammatory protein-1α], NAP-2 [neutrophil-activating protein-2], and SDF-1 [stromal cell–derived factor] [R&D Systems Europe Ltd., Lille, France]) were measured (by ELISA) in RBCC supernatants, in triplicate. Those cytokines/chemokines increased with storage duration (Day 1 [d1] to d42; Table 1) as described previously for Gro-α (growth-regulated oncogene-α), IL-8, and ENA-78 (epithelial neutrophil-activating protein 78) (4). There was only a significant increase of NAP-2 (neutrophil-activating protein-2) concentration on d1 in WB versus RTH-WB. In contrast, on d42, all cytokine/chemokine concentrations levels in the RTH-WB units were consistently higher than in the RT hold units WB within 2 hours of collection (WB). Further, human neutrophils, freshly isolated and purified according to our current laboratory techniques, and originating from blood given by healthy donors at the Blood Bank, were treated with RBCC supernatants or 1.0 nM *N*-formyl-methionyl-leucyl-phenylalanine (positive control). Flow cytometric analysis of surface expression of leukocyte activation/differentiation antigen was performed with fluorescein isothiocyanate–conjugated monoclonal antibody. We observed that the mean ± standard deviation mean fluorescence intensity of CD11b, an activation marker evaluating the functional activity of neutrophils, was significantly ($P < 0.05$; two-way ANOVA) more elevated on leukocytes exposed to supernatants of RTH-WB compared with iWB, at three separate time points (d1: 77.89 ± 2.98 vs. 54.25 ± 0.4, $P = 0.00047$; d21:82.8 ± 0.64 vs. 62.84 ± 0.8, $P = 0.00038$; d42—the expiry date: 89.47 ± 2.26 vs. 70.69 ± 1.88, $P = 0.00097$). Similar results were obtained by using—in parallel—the HL60 neutrophil cell line as targets (not shown).

TABLE 1. PRODUCTION OF CYTOKINES AND/OR CHEMOKINES IN RBCCs PREPARED OVER TIME

	RTH-WB			WB		
	d1	d21	d42	d1	d21	d42
NAP-2, pg/ml	14267.7	16924.9	29442.4*	23732.9†	19130.7	15420.9†
SD	124.8	599.1	1855.3	1495.6	1205.5	971.8
SDF-1, pg/ml	950	2878.6*	3718.7*	765.8	2320.4*	2997.6*†
SD	265.2	699.3	912.1	213.7	563.7	735.2
MIP-1α, pg/ml	2.1	139.8*	96.3*	1.7	112.7*	77.6*
SD	0.2	55.5	21.5	0.2	44.7	17.4
ENA-78, pg/ml	9.8	62.3*	185.8*	7.9	50.2*	149.8*†
SD	1.4	14.1	25.9	1.1	11.3	20.9
IL-8, pg/ml	17.5	100.5*	211.9*	14.1	81*	170.8*†
SD	9.7	12.4	34.7	7.8	10	28
Gro-α, pg/ml	0	52*	214.1*	0	41.9*	172.5*†
SD	0	10.3	31.9	0	8.3	25.7

Definition of abbreviations: d = day; ENA-78 = epithelial neutrophil-activating protein 78; Gro-α = growth-regulated oncogene-α; NAP = neutrophil-activating protein; RBCC = red blood cell component; RTH-WB = whole blood after overnight room-temperature hold; SD = standard deviation; SDF = stromal cell–derived factor; WB = whole blood within 2 h of collection.

d42 represents the last day of storage for which RBCCs can be used for transfusion. Values shown are deducted from background levels. Data (mean ± SD; n = 5 experiments) are expressed in picograms per milliliter.

* Significant ($P \leq 0.05$; statistical analysis—two-way ANOVA), d1 vs. d21 or d42 (n = 5).

† Significant ($P \leq 0.05$; statistical analysis—two-way ANOVA), WB vs. RTH-WB (n = 5).

Several studies suggest that transfusion-induced dysregulation of neutrophils may in part be responsible for acute transfusion reactions and transfusion-related acute lung injury (5); we propose that RBCC processing, in addition to the age of blood products, must be considered in patient studies as it may contribute to storage lesions. The same may hold true also for platelet components (6).

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