Impact of High-Dose Intravenous Vitamin C on Cell-Free DNA and Syndecan-1 in Patients with Sepsis-Associated ARDS

Markos G. Kashiouris (mkashiouris@vcu.edu)

Research

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Abstract

Background:

We set out to examine the effects of high dosage intravenous vitamin C (HDIVC) infusion on plasma cell-free DNA and Syndecan-1, two mortality biomarkers that represent neutrophil extracellular trap (NET) formation and degradation of the endothelial glycocalyx.

Methods: Post-hoc analysis of plasma cell-free DNA and syndecan-1 in patients enrolled in the randomized placebo-controlled trial: Vitamin C Infusion for Treatment in Sepsis-Induced Acute Lung Injury, the CITRIS-ALI trial. Setting: Seven intensive care units in hospitals located in five different states in the U.S. Patients: Septic adults with ARDS between September 2014 to November 2017, final follow-up January 2018.

Results: In 167 study patients, baseline plasma cfDNA levels in HDIVC (84 patients) and placebo (83 patients) were 2.18 ng/μL (SD 4.20 ng/μL) and 2.65 ng/μL (SD 3.87 ng/μL), respectively, p=0.45. At 48-hours, the cfDNA reduction was 1.02 ng/μL greater in HDIVC, compared to placebo, p=0.05. Mean baseline plasma syndecan-1 levels in HDIVC and placebo were 9.49 ng/mL (SD 5.57 ng/mL) and 10.83 ng/mL (SD 5.95 ng/mL) respectively, p=0.14. At 48 hours, patients in the placebo arm exhibited a 1.53 ng/mL (95% CI, 0.96 to 2.11) increase in syndecan-1 vs. 0.75 ng/mL (95% CI, 0.21 to 1.29), in HDIVC patients, p=0.05. The 48-hour plasma syndecan-1 levels in patients treated with HDIVC exhibited a linear association with improved oxygenation (PaO2/FiO2, beta= -18.9, p=0.004).

Conclusions:

HDIVC infusion significantly attenuated plasma cell-free DNA and syndecan-1, biomarkers known to be elevated in sepsis-induced ARDS. These results support the conclusion that high dosage intravenous vitamin C infusion reduces sepsis-induced vascular injury.

Trial Registration: ClinicalTrials.gov identifier: NCT02106975

Introduction

Acute respiratory distress syndrome (ARDS) is an inflammatory lung disease with a mortality rate of 35–46%. [1–3]

Sepsis can trigger vascular injury that leads to ARDS through systemic and local inflammation, damaging lung barrier function by both alveolar epithelial cell and lung capillary endothelial cell injury. Loss of barrier integrity leads to interstitial and alveolar flooding, surfactant damage, and collapsed lung units. The cumulative result is severe lung function impairment characterized by diminished compliance, increased shunt, and severe hypoxemia.

Clinical and laboratory evidence suggest that High-Dose Intravenous Vitamin C (HDIVC) may have a role in the ARDS treatment. [4–7] A recently completed double-blind, randomized placebo-controlled trial revealed that HDIVC significantly reduced 28-day mortality and organ failure in sepsis-associated ARDS. [8, 9] We hypothesized that important mechanisms of action of HDIVC are the regulation of Neutrophil Extracellular Trap (NET) formation and preservation of the endothelial glycocalyx. [10–12]

A key biomarker of glycocalyceal integrity is the proteoglycan syndecan-1, an important structural component of the glycocalyx which lines luminal endothelial vascular surfaces, including alveolar capillaries. [13] Endotoxemia and bacteremia that lead to sepsis disrupt the glycocalyx, one of the earliest and most significant injury sites. [14] Early phase injury leads to degradation of the glycocalyx barrier and shedding of syndecan-1 into the circulation. [15] Syndecan-1, a biomarker of the degree of glycocalyx damage, is associated with ARDS development. [10, 16] Loss of glycocalyx integrity results in movement of cells, protein, and fluid from the vascular space into lung perivascular interstitial and alveolar spaces, a hallmark of ARDS. Therapies that target protection or restoration of the glycocalyx may benefit septic patients and theoretically reduce ARDS-associated mortality. [17]

NETs, a recent discovery in innate immunity, are composed of granules and nuclear content extruded from neutrophils which kill bacteria extracellularly. [18] A key plasma biomarker of NET formation is cell-free DNA (cfDNA), which is elevated in sepsis and ARDS.[19–21] Prior studies have investigated the prognostic utility of cfDNA and syndecan-1 at the onset of sepsis and ARDS. [15, 22, 23] To date, no study has investigated an intervention in septic individuals that directly reduces NET formation and glycocalyx degradation. Patients receiving HDIVC in the CITRIS-ALI trial exhibited improved mortality and organ failure. [8, 9] Given these results, we hypothesized that HDIVC would reduce plasma cfDNA and syndecan-1 when compared to placebo patients, receiving only standard of care for sepsis and ARDS. We further hypothesized that cfDNA and syndecan-1 are associated with objective clinical oxygenation indices in patients with ARDS.

Materials And Methods

Population and Setting

A post-hoc quantitative analysis of cell free DNA and syndecan-1 was performed on plasma specimens obtained from patients enrolled in the CITRIS-ALI trial, a recently completed multi-center, double-blind, randomized, placebo-controlled trial. [8] Virginia Commonwealth University's Institutional Review Board approved the post-hoc study (HM20014768). At enrollment, all patients or legally authorized representatives provided informed consent for trial entry and for future use of patient plasma. The Food and Drug Administration provided oversight for CITRIS-ALI performance with IND: 113856. Patient enrollment occurred at Virginia Commonwealth University, Richmond, VA, Medical College of Wisconsin and St. Luke's Medical Center, Milwaukee, WI, Cleveland Clinic and Fairview Medical Center, Cleveland, OH, The University of Kentucky, Lexington, KY, and Emory University, Atlanta, GA from September 2014 through January 2018. In the CITRIS-ALI trial, patients with new-onset sepsis-associated ARDS were

randomized to receive 30-minute intravenous infusions of ascorbic acid (n = 84) (McGuff Pharmaceuticals, Santa Ana, CA, USA) at 50 mg/kg admixed in 50 ml dextrose 5% in water every 6 hours, for 96 hours vs. placebo (n = 83). All patients received the current standard of care for sepsis-associated ARDS.

Blood collection

Whole blood was drawn into sterile Vacutainer tubes (BD 367863, Lavender Top, K_2EDTA). Plasma was separated by centrifugation (1000g, 10 min, 4°C), aliquoted and frozen at -80°C for batch analysis.

Plasma Cell-free DNA Quantification

Plasma cfDNA levels were quantified using the Invitrogen Quant-iT PicoGreen dsDNA assay kit, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Fluorescence intensity was measured on a SpectraMax Gemini XPS microplate reader with excitation at 490 nm and emission at 525 nm, with 515 nm emission cutoff filter (Molecular Devices, San Jose, CA).

Plasma Syndecan-1 Quantification

Plasma syndecan-1 levels were analyzed using a human magnetic bead Luminex assay system (LXSAHM), according to the manufacturer's instructions (R&D Systems, Minneapolis MN) and quantified using a Luminex LX200 instrument with xPONENT 3.1 software (Luminex Corporation, Austin, TX). Biomarker concentrations were calculated from standard curves of Median Fluorescence Intensity (MFI) by generating a five-parameter logistic (5-PL) curve-fit and multiplying by the dilution factor. Specimens outside the standard range were further diluted and assays repeated.

Statistical Analysis

Analyses were conducted using Stata Statistical Software (Rel.16.1, TX StataCorp LP). Multiple linear regressions were applied to assess biomarker differences at 48 hours, adjusting for baseline biomarker levels, comparing them among the two randomized groups (HDIVC and Placebo). Post-estimation plots represent the findings graphically. Regression residuals for normalcy were then assessed. In instances where residuals were not normally distributed the non-parametric Wilcoxon test was applied. [24] Multiple logistic regression was applied to evaluate the adjusted effect of biomarkers on mortality. We evaluated the models with the area under the receiver operator characteristic curve, and the Hosmer-Lemeshow goodness-of-fit test. [25]

Results

Study Participants

Patients with sepsis-induced ARDS were enrolled at the time of ARDS onset (n = 167). Patients were randomized to receive HDIVC (n = 84) or placebo (dextrose 5% in water, n = 83). Baseline plasma samples were analyzed for cfDNA (n = 167) and syndecan-1 (n = 166). At 48 hours, 82 (97.6%) HDIVC and 72

(86.8%) of the placebo patients survived and remained in the ICU (P = 0.009). Plasma specimens from the survivors were analyzed. A summary of study patients and specimens can be found in Table 1. The study population is described extensively elsewhere. [8]

Table 1
Patient and Specimens Characteristics from the randomized controlled trial.

	Baseline (N = 167)		48-Hours (N = 154)	
	HDIVC	**Placebo**	**HDIVC**	**Placebo**
Survived, and in the ICU, % (N)	50·3% (84)	49·7% (83)	53·2% (82)	46·8% (72)
Age, years (mean, SD)	52·7 (17·5)	56·8 (15·7)	52·3 (17·3)	55·8 (15·7)
Gender (% men)	53·6% (45)	54·2% (45)	53·7% (44)	54·2% (39)
Number of patients with ABG, % (N)	48% (80)	49·1% (82)	40·3% (62)	39·6% (65)
P_aO_2/F_iO_2 ratio, mean (SD)	189·3 (182·8)	214·5 (95·9)	233·2 (115·4)	246 (105·1)
Patients with cfDNA available plasma, % (N)	100% (84)	100% (83)	52·3% (81)	45·5% (70)
Patients with syndecan-1 available plasma, % (N)	49·7% (83)	49·7% (83)	52·3% (80)	45·8% (70)

Abbreviations: ABG: Arterial blood gas, cfDNA: cell-free deoxyribonucleic acid, HDIVC: High-Dose, Intravenous Vitamin C. P_aO^2/F_iO^2: Arterial partial oxygen pressure divided by the fraction of inspired oxygen, Number, SD: Standard Deviation.

Effects of Intravenous Vitamin C on Plasma cfDNA

Mean baseline (day 0), cfDNA levels in HDIVC treatment and placebo arms were 2.18 ng/μL (SD 4.20 ng/μL) and 2.65 ng/μL (SD 3.87 ng/μL), respectively. There was no statistical difference (p = 0.46). After 48 hours, mean cfDNA levels in HDIVC and placebo arms were 1.78 ng/μL (SD 1.73 ng/μL) and 2.80 ng/μL (SD 5.0 ng/μL), respectively. The mean change (delta, Δ) following 48 hours of HDIVC treatment was minus 0.45 ng/μL (95% CI, -1.16 to 0.25), indicating a decrease in cfDNA levels. The mean change following 48 hours in placebo patients increased by 0.57 ng/μL (95% CI, -0.19 to 1.33 ng/μl). Adjusting for the different baseline cfDNA levels, patients receiving HDIVC treatment exhibited a mean 48-hour decrease of 1.02 ng/μL (p = 0.05) compared to placebo (Fig. 1).

Effects of Intravenous Vitamin C on Plasma Syndecan-1

Mean baseline (day 0) syndecan-1 levels in HDIVC and placebo arms were 9.49 ng/mL (SD 5.57 ng/mL) and 10.83 ng/mL (SD 5.95 ng/mL) respectively with no statistical difference (p = 0.14). At 48 hours,

syndecan-1 levels in HDIVC patients increased to 10.43 ng/mL (SD 6.05 ng/mL) while corresponding levels in placebo patients increased to 11.22 ng/mL (SD 5.31 ng/mL). The change in patients randomized to placebo at 48 hours (+ 1.53 ng/ml, 95% CI, 0.96 to 2.11) was twice that of patients randomized to HDIVC (0.75 ng/ml, 95% CI, 0.21 to 1.29), p = 0.05 (Fig. 2). Increased syndecan-1 levels at 48 hours correlated significantly with increased plasma cfDNA levels (0.39, 95% CI, 0.17 to 0.61, p = 0.001).

Effect of cfDNA and Syndecan-1 on Changes in Oxygenation

Forty-eight-hour Δsyndecan-1 plasma level elevations were significantly correlated with worsened oxygenation. Increases in syndecan-1 levels by one ng/mL corresponded with diminished P_aO_2/F_iO_2 ratios by -8.85 (95% CI, -17.50 to -0.19; p = 0.045). HDIVC treatment had a larger (-18.9 vs. -4.4) and significant (p = 0.004 vs. 0.48) impact on $\Delta P_aO_2/FiO_2$ ratio compared with placebo (Fig. 3). The 48-hour ΔcfDNA exhibited no significant effects on P_aO_2/F_iO_2 ratios (-2.72 [95% CI, -9.66 to 4.20], p = 0.44).

Association of cfDNA and Syndecan-1 with Mortality

Baseline plasma syndecan-1 levels and both 48-hour ΔcfDNA and Δsyndecan-1 levels predicted 28-day all-cause hospital mortality. Table 2 outlines the Odds Ratio (OR) of death for every incremental unit increase of the corresponding biomarker.

Table 2
Effect of cfDNA and syndecan-1 on 28-day all-cause hospital mortality. Adjusted (multiple) logistic regression table outlining Odds Ratio (OR) of death for each incremental unit increase in baseline and 48-hour change (delta, Δ) of the plasma cfDNA and syndecan-1 levels.

Predictor	Odds Ratio	Standard Error	z	PValue	95% Confidence Intervals	
Baseline syndecan-1 each unit increase, ng/ml	1.3	0.1	4.9	< 0.001	1.2	1.4
48-hour Δsyndecan-1 each unit increase, ng/ml	1.3	0.1	3.2	0.001	1.1	1.6
Baseline cfDNA each unit increase, ng/μL	1.1	0.2	0.5	0.650	0.8	1.5
48-hour ΔcfDNA each unit increase, ng/μL	1.8	0.5	2.1	0.035	1.0	3.0
Constant	0.0	0.0	-5.7	< 0.001	0.0	0.1
Abbreviations: cfDNA, cell-free deoxyribonucleic acid; Δ, delta, change from baseline.						
The model's area under the receiver-operating characteristics curve was 0.82. The Hosmer-Lemeshow goodness-of-fit p = 0.56, indicating a good model fit.						

Discussion

The present study reports that a 96-hour HDIVC infusion in patients with sepsis-associated ARDS attenuated increases in 48-hour cfDNA and syndecan-1 plasma levels. Attenuated syndecan-1 levels correlated with improved lung function, as gaged by improved 48-hour P_aO_2/F_iO_2 ratios (Fig. 3). HDIVC's impact on syndecan-1 and cfDNA levels independently predicted lower 28-day all-cause mortality. Elevated cfDNA and syndecan-1 levels in the plasma of septic patients with ARDS provides fresh insight into the extent of systemic inflammation and the molecular mechanisms that produce vascular injury, leading to ARDS onset.

Neutrophil extracellular traps are highly linked to endothelial damage and organ failure, crucial events in sepsis. [19] NET formation is a neutrophil effector mechanism whereby neutrophils extrude a web of chromatin fibers complexed to granule-derived antimicrobial peptides and enzymes. This process occurs following neutrophil activation and is implicated in producing endothelial damage. [26] Hirose et al. identified NETs in peripheral blood smears of critically ill patients. [27] In septic patients, circulating cfDNA levels correlated with the degree of lung injury, as higher concentrations were found in patients who developed moderate or severe ARDS than septic patients without ARDS. [28] LeFrancais et al found that attenuating NET formation in an acute lung injury mouse model led to increased survival. [21]

Activated endothelial cells induce neutrophil NET formation and are themselves susceptible to NETosis-mediated cell death, [29] thus, promoting a self-perpetuating damage that ultimately leads to hypercoagulable states. [30] The association of syndecan-1 with endothelial damage and neutrophilic inflammation has focused attention on a biomarker indicative of vascular injury. [15, 17, 31] Plasma syndecan-1 levels in septic patients are increased at baseline and may remain elevated for up to 72 hours. [32] Further, in these septic patients, elevations of syndecan-1 are associated with heightened risks of developing respiratory failure and increased mortality. [33] Both cfDNA and syndecan-1 levels are reported for mortality predictions in septic patients and are associated with adverse clinical outcomes (e.g., development of multiple organ failure, ARDS). [14, 34] Correlations between the two biomarkers to clinical outcomes pertain to their roles as surrogates for NET formation and glycocalyx integrity. Plasma syndecan-1 elevations are a robust marker of glycocalyx degradation and development of ARDS. [31] To our knowledge, this is the first human randomized placebo-controlled study of sepsis-associated ARDS to examine an interventional therapy's effect on these biomarkers.

CITRIS-ALI is the first study to show that a 96-hour infusion of HDIVC decreased human plasma cfDNA and attenuated the rise in syndecan-1 levels at 48-hours (Figs. 1 and 2). [8] A re-analysis of the CITRIS-ALI data, accounting for the missing SOFA scores due to the large survival differences among the two arms, (i.e., Survivorship Bias), disclosed improved overall organ-function (modified SOFA Scores) in ARDS patient who received HDIVC infusion.[9] Murine models of polymicrobial sepsis using high dose vitamin C have demonstrated attenuation of lung NET formation, and circulating cfDNA.[5] Other studies reveal that high dose vitamin C reduced multiple organ failure, neutrophilic capillaritis and increased extravascular lung water in septic mice. [6] Thus, decreased circulating cfDNA may represent a surrogate marker of high dose vitamin C's ability to reduce neutrophil cell death, thereby reducing NET formation and the ensuing inflammatory vascular injury. The present study found significant correlations between decreased plasma syndecan-1 and improved P_aO_2/F_iO_2 ratios (Fig. 3) and 28-day all-cause hospital mortality (Table 1). Taken together, these findings suggest that a 96-hour infusion HDIVC may improve ARDS recovery by protecting or restoring glycocalyx integrity.

Limitations

The study has several limitations. First, the specific origin of circulating cfDNA was not determined. One study suggests that surges in cfDNA in sepsis results from cellular necrosis. [35] However, other studies show that cfDNA in septic patients is host derived and the cfDNA base pair length is consistent with neutrophil NET formation and not cellular necrosis. [36] Second, syndecan-1 was measured, but not other glycocalyceal structures (i.e., endocan, heparan sulfate, hyaluronan). Multiple studies show that syndecan-1 levels in sepsis correlate strongly with other markers of glycocalyx degradation. [14, 15] Third, missing data points at 48-hours due to early deaths in placebo patients (13.3%) vs. HDIVC patients (2.4%) may have biased the ability to detect an even greater difference in biomarker levels.

Conclusion

HDIVC treatment reduced 48-hour cfDNA and syndecan-1 plasma levels in patients with sepsis-associated ARDS. The dynamic changes of these biomarkers were strongly associated with lung oxygenation and 28-day all-cause mortality. These results suggest that HDIVC reduces the severity of illness by decreasing neutrophil activation and glycocalyx degradation. Syndecan-1 and cfDNA signal pathophysiological processes that lead to vascular injury in sepsis-associated ARDS. Future studies will clarify the role of these biomarkers in directing the care of patients with sepsis-associated ARDS.

Declarations

Acknowledgments

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Figures

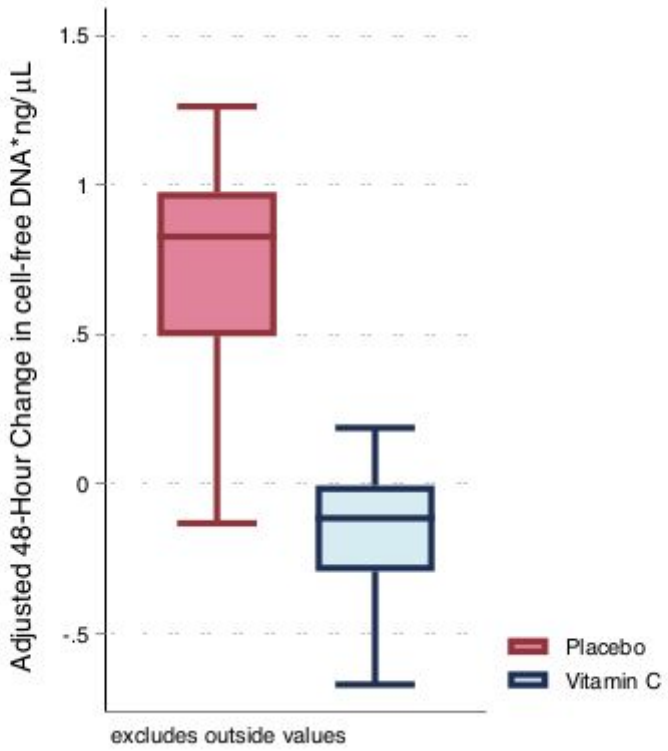

Figure 1

Forty-eight hour increase in cell-free DNA (cfDNA) in the two groups, Placebo and HDIVC. Median values, the top and bottom of the boxes show the interquartile range (IQR), whiskers indicate 95% CI. The extreme outliers outside the 95% confidence intervals were omitted. Abbreviations: cfDNA, cell-free deoxyribonucleic acid; CI, confidence intervals; IQR, interquartile range; HDIVC, High-Dose Intravenous Vitamin C.

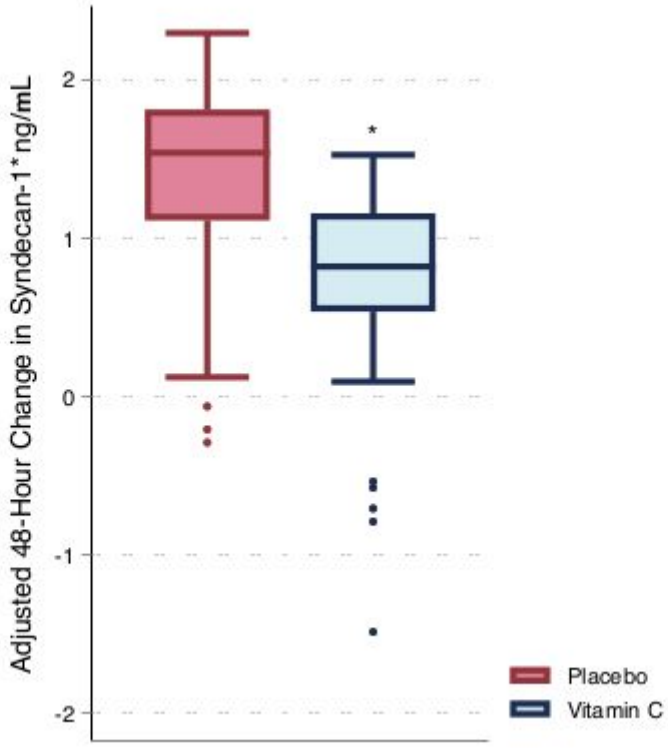

Figure 2

Forty-eight hour increase in syndecan-1 in the two groups, Placebo and HDIVC. Median values, the top and bottom of the boxes show the interquartile range (IQR), whiskers show 95% CI. The extreme outliers outside the 95% confidence intervals have been omitted. Abbreviations: CI, confidence intervals; IQR, interquartile range; HDIVC, High-Dose Intravenous Vitamin C; P/F, PaO2/FiO2 ratio.

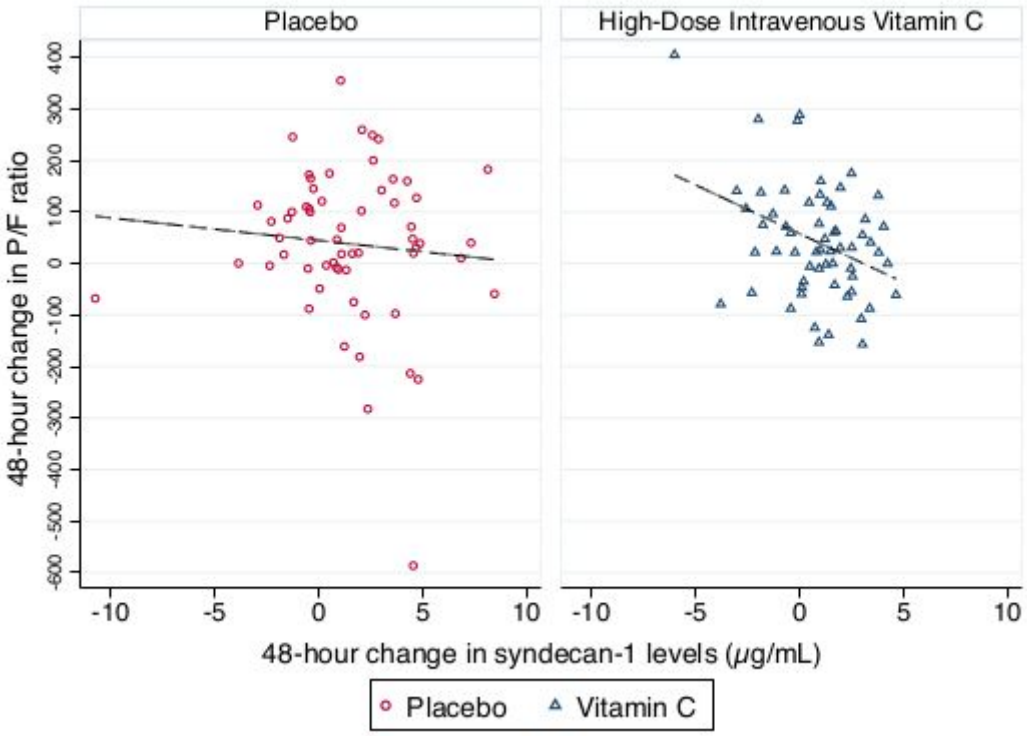

Figure 3

Scatterplots of the 48-hour change in the PaO2/FiO2 ratio and the change in the plasma syndecan-1 during the same time-period. The figure illustrates the change of the PaO2/FiO2 ratio, a marker of oxygenation which corresponds to the change in the plasma biomarker syndecan-1 among the two groups, Placebo and HDIVC. The dotted, linear fitted line corresponds to the regression line for each group: Placebo and HDIVC, respectively. The patients of the placebo group are represented with hollow circles, and the patients of the HDIVC group are represented with crosses. Abbreviations: FiO2, fraction of inspired oxygen; HDIVC, High-Dose Intravenous Vitamin C; PaO2, arterial oxygen pressure.