

# Myeloperoxidase-derived 2-chlorofatty acids contribute to human sepsis mortality via acute respiratory distress syndrome

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**Sepsis-associated acute respiratory distress syndrome (ARDS) is characterized by neutrophilic inflammation and poor survival. Since neutrophil myeloperoxidase (MPO) activity leads to increased plasma 2-chlorofatty acid (2-CIFA) levels, we hypothesized that plasma concentrations of 2-CIFAs would associate with ARDS and mortality in subjects with sepsis. In sequential consenting patients with sepsis, free 2-CIFA levels were significantly associated with ARDS, and with 30-day mortality, for each log increase in free 2-chlorostearic acid. Plasma MPO was not associated with either ARDS or 30-day mortality but was correlated with 2-CIFA levels. Addition of plasma 2-CIFA levels to the APACHE III score improved prediction for ARDS. Plasma 2-CIFA levels correlated with plasma levels of angiotensin-2, E selectin, and soluble thrombomodulin. Endothelial cells treated with 2-CIFA responded with increased adhesion molecule surface expression, increased angiotensin-2 release, and dose-dependent endothelial permeability. Our results suggest that 2-CIFAs derived from neutrophil MPO-catalyzed oxidation contribute to pulmonary endothelial injury and have prognostic utility in sepsis-associated ARDS.**

**Conflict of interest:** S.L. Hazen is named as a coinventor on patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics, is a paid consultant for Procter & Gamble, and has received research funds from Procter & Gamble, Pfizer Inc., Roche Diagnostics, and Takeda. S.L. Hazen reports that he is eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland HeartLab, Esperion, and Frantz Biomarkers LLC. N.J. Meyer and J.D. Christie have received research funds from GlaxoSmithKline for work unrelated to the present manuscript. N.J. Meyer has served on the advisory board for SOBI Inc.

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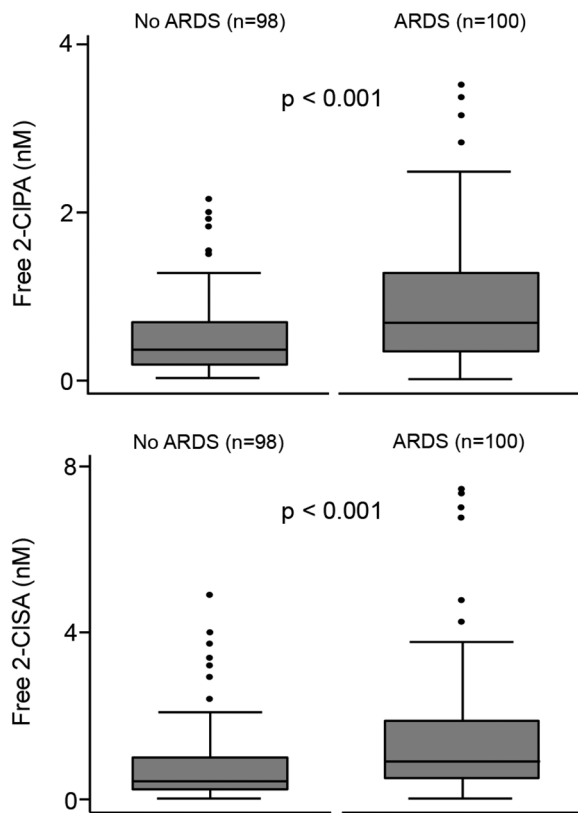
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## Introduction

Sepsis is a major cause of mortality in critically ill patients and is accompanied by development of a dysregulated host immune response to infection (1). Sepsis-induced organ failure is multifactorial and is believed to arise in part due to the interplay among pathogen, blood, and endothelial surface at the microcirculatory level, with the loss of microvascular endothelial barrier integrity and blood-endothelial interface dysfunction (2, 3). Bioactive lipids have received considerable attention as potential mediators of the pathophysiological sequelae of sepsis and inflammation (4–8). Bioactive lipids have also been implicated as potential mediators of the acute respiratory distress syndrome (ARDS), a common sepsis-induced organ failure with high observed mortality rates (9–11). Neutrophils and monocytes are major contributors to the generation of bioactive lipids. We sought to elucidate one potential mechanism of leukocyte-mediated damage during human sepsis by characterizing the plasma 2-chlorofatty acid (2-CIFA) profile and testing the clinical prognostic utility of plasma 2-CIFAs for prediction of ARDS.

Dysregulated balance between the antimicrobial host defense action of leukocytes and leukocyte-mediated tissue injury characterizes sepsis (12–14) and may have a key role in sepsis-associated organ failure (15–19). Autopsy specimens from human sepsis patients reveal neutrophil aggregation and infiltration of the kidney and lung, and the amount of neutrophil infiltration in ARDS patients correlates with lung dysfunction (15–19). Moreover, the gene expression signature of sepsis-associated ARDS is dominated by neutrophil-related genes (20). A major antimicrobial mechanism of neutrophil-associated phagosomes is the generation of reactive oxidant species (13, 14, 21). Myeloperoxidase (MPO), the most abundant granule protein released from neutrophils



**Figure 1. Septic patients with ARDS display higher day 0 plasma concentrations of free 2-CIFAs.** Compared with subjects who never developed ARDS, those who developed ARDS within the first 6 days of sepsis have higher circulating levels of free 2-CIPA and free 2-CISA on the day of ICU admission (compared by the Wilcoxon rank-sum test). All values were included in the analyses; however, outliers higher than the y axis are not shown. Box plots show median and 25th and 75th percentile, and whiskers show minima and maxima for each condition. For the ARDS group, 9 and 11 outliers had values above 4 nM 2-CIPA or above 8 nM 2-CISA, respectively. In the non-ARDS group, there were 2 outliers with values above 8 nM 2-CISA, but none above 4 nM 2-CIPA.

and monocytes, is detectable in human plasma and increased in sepsis compared with sterile inflammation or controls (22). MPO activity amplifies the oxidizing potential of neutrophil-produced hydrogen peroxide ( $H_2O_2$ ) by converting it to hypochlorous acid (HOCl) (23), a potent cytotoxic oxidizing agent. Plasmalogens represent a subclass of diradylglycerophospholipids enriched in vascular and pulmonary endothelial cells. The characteristic vinyl ether bond of plasmalogen phospholipids is highly susceptible to oxidation and anatomically positioned at the surface of the cell, as a major constituent within the plasma membrane. When exposed to HOCl, an oxidant specifically generated by MPO, the plasmalogen oxidation product formed has been shown to be a corresponding free 2-chlorofatty aldehyde (2-CIFALD) (24, 25). 2-CIFALDs were among the first chlorinated lipids found in response to both neutrophil and monocyte activation (24–26). Plasmalogens are abundant phospholipids in the heart, lung, brain, kidney, and smooth muscle tissues as well as neutrophils, monocytes, and endothelial cells (27–34). The vinyl ether-linked aliphatic groups of plasmalogens are predominantly 16 and 18 carbons in length, which are released as 16

and 18 carbon 2-CIFALD molecular species following HOCl targeting (26). Subsequent investigations demonstrated that 2-CIFALDs are oxidized to 2-CIFAs, which can be further metabolized (35–37). Furthermore, HOCl produced by neutrophils has been shown to target plasmalogen in neighboring endothelial cells and extracellular lipoproteins, leading to 2-CIFALD production, and endothelial cells metabolize 2-CIFALD to 2-CIFA, which is released from endothelial cells (25, 26, 37).

2-CIFALDs are short-lived in plasma samples, but our group has detected 2-CIFAs in plasma and demonstrated that plasma 2-CIFA levels are elevated in LPS-treated rats (36) and in Sendai virus-infected mice (35). These rodent studies suggest that chlorinated lipids are a component of the response to bacterial and viral infection. Leukocyte-mediated chlorinated lipid products have been shown to be biologically active species, capable of causing cardiac contractile dysfunction, NF- $\kappa$ B signaling in endothelial cells, endoplasmic reticulum stress, hydrogen peroxide production, and apoptosis (38–40). Accordingly, the present study was designed to investigate the role of 2-CIFAs in human sepsis, with a focus on ARDS given evidence for the contribution of neutrophils to ARDS (20, 41).

## Results

To test the hypothesis that 2-CIFA levels are associated with outcomes in human sepsis, plasma samples were collected upon intensive care unit (ICU) admission (day 0) from 198 sequential subjects enrolled in the *Molecular Epidemiology of Sepsis in the ICU* (MESSI) cohort (see Supplemental Table 1 for MESSI study population characteristics; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.96432DS1>). Informed consent was obtained from patients or their proxies as previously described (42). Plasma was examined for two molecular species of both free (unesterified) 2-CIFA and total (esterified + unesterified) 2-CIFA content, 2-chloropalmitic acid (2-CIPA), and 2-chlorostearic acid (2-CISA) by stable isotope dilution tandem mass spectrometry. These molecular species were chosen because they represent the major aliphatic groups derived from plasmalogens in mammalian tissues (43, 44). Additionally, free and total 2-CIFA were determined to assess short and long half-life forms of 2-CIFA, respectively (36). Admission plasma levels of both free and total 2-CIFAs were higher in subjects who developed ARDS (median 2-CISA 1.12 nM [interquartile range {IQR} 0.48–2.54 nM compared with median 0.43 nM [IQR 0.22–0.98 nM] for non-ARDS,  $P < 0.001$ ) or who died within

**Table 1. Day 0 plasma 2-CIFA concentrations associate with ARDS**

	Adjusted odds ratio for ARDS (95% CI)	P value
Log(free 2-CIPA) nM	1.62 (1.25, 2.09)	<0.001
Log(free 2-CISA) nM	1.82 (1.41, 2.37)	<0.001
Log(total 2-CIPA) nM	1.82 (1.32, 2.52)	<0.001
Log(total 2-CISA) nM	1.78 (1.31, 2.43)	<0.001
Log(MPO) pM ( <i>n</i> = 110) <sup>A</sup>	1.28 (0.89, 1.84)	0.19

The odds for ARDS increase significantly among 198 subjects with sepsis for each log increase in free or total 2-CIPA or 2-CISA, independent of APACHE III score, age, sex, European descent, or pulmonary source of infection. In contrast, increasing MPO did not associate with ARDS in the subset (*n* = 110<sup>A</sup>) with MPO measured. Associations remained unchanged when adjusting for absolute neutrophil count.

30 days compared with sepsis survivors ( $P = 0.0049$ ) (Figure 1 and Supplemental Table 2). For each log increase in free 2-CIFA, the risk for ARDS (Table 1) and mortality (Table 2) significantly increased, and these associations remained robust following adjustment for the Acute Physiology and Chronic Health Evaluation (APACHE) III score, race, age, or sex. The APACHE III score is a validated score to adjust for baseline differences in expected mortality in critical illness (45). It is calculated based on a combination of clinical variables reflecting physiologic derangement, organ injury, age, and chronic health comorbidities that influence survival. Adjustment for absolute neutrophil count had no effect on the association between 2-CIFA concentration and ARDS or mortality. Because ARDS strongly influences sepsis mortality in this population, with odds ratio (OR) 4.7 (3.61–6.25),  $P = 0.0001$ , we used statistical mediation analysis to explain the portion of the 2-CIFA–mortality association that was mediated by increased ARDS risk. For this analysis, ARDS risk was tested as the mediator variable between higher 2-CIFA concentration and mortality risk. As shown in Table 3, the mediation effect of ARDS on mortality was highly significant for both species of 2-CIFA. The development of ARDS accounted for 41%–51% of the mortality risk of higher 2-CIFA levels.

To test whether admission plasma concentrations of 2-CIFA had incremental value for predicting ARDS among septic subjects, we calculated receiver operating characteristic (ROC) curves and assessed the area under the ROC curve (AUROC) for predicting ARDS based on the APACHE III score alone or in combination with plasma 2-CIFA concentrations (Figure 2). The addition of free 2-CISA levels to APACHE III improved the AUROC from 0.66 (95% CI 0.59, 0.74) to 0.76 (95% CI 0.69, 0.82), a significant improvement ( $P = 0.0042$ ). Using the 50th percentile of free 2-CISA as a threshold, the net reclassification index for ARDS was 0.17 compared with APACHE III score alone ( $P = 0.026$ ), and the integrated discrimination improvement (IDI) was 11% ( $P < 0.001$ ). Results were similar for the predictive utility of free 2-CIPA concentrations compared with APACHE score alone. Because 62 of the 100 subjects with ARDS developed ARDS on the same day as ICU admission (and thus the same day as blood draw), we repeated the analyses excluding subjects whose ARDS was present on day 0 and still observed a significant improvement in AUROC ( $P = 0.0030$ ) and in IDI (9.7%,  $P = 0.001$ ). Thus, we concluded that circulating levels of free 2-CIFA have predictive utility for ARDS independent of severity of illness.

Plasma 2-CIFA likely increases during sepsis as a result of systemic activation of neutrophils in response to sepsis. Almost 17% of the MESSI subjects were neutropenic on admission to the ICU secondary to prior

**Table 2. Day 0 plasma 2-CIFA levels associate with sepsis mortality**

	Adjusted odds ratio for death at 30 d (95% CI)	P value
Log(free 2-CIPA) nM	1.34 (1.07, 1.68)	0.011
Log(free 2-CISA) nM	1.45 (1.16, 1.83)	0.001
Log(total 2-CIPA) nM	1.51 (1.13, 2.03)	0.006
Log(total 2-CISA) nM	1.31 (1.00, 1.71)	0.048
Log(MPO) pM ( <i>n</i> = 110) <sup>A</sup>	1.00 (0.68, 1.47)	0.99

Association in 198 septic subjects was tested by logistic regression, adjusting for APACHE III score, age, sex, and race. In the subgroup with MPO tested (*n* = 110<sup>A</sup>), there was no observable association between MPO concentration and mortality. Associations remained unchanged when adjusting for absolute neutrophil count.

**Table 3. Increased ARDS risk explains a significant portion of the association between plasma 2-CIFA concentrations and mortality**

	Adjusted odds ratio for mortality (95% CI)	P value	Proportion mediated by ARDS
<b>Free 2-CIPA</b>			
Total effect	1.06 (1.01, 1.10)	0.02	
Mediation effect	1.03 (1.01, 1.05)	<0.01	50.7%
Direct effect	1.03 (0.98, 1.07)	0.23	
<b>Free 2-CISA</b>			
Total effect	1.08 (1.03, 1.12)	<0.01	
Mediation effect	1.03 (1.01, 1.05)	<0.01	41.6%
Direct effect	1.04 (0.99, 1.09)	0.07	

By mediation analysis, 40%–51% of mortality risk observed for free 2-CIFA concentrations is mediated through ARDS. Each effect shown reflects the average value of 1,000 iterations. The total effect model included the covariates log(2-CIFA), ARDS, ARDS × log(2-CIFA) interaction term, age, APACHE III score, sex, and race. Results are shown separately for each 2-CIFA moiety, 2-CIPA and 2-CISA.

receipt of cytotoxic chemotherapy. Plasma 2-CIFA levels for both 2-CIPA and 2-CISA seem to reflect the activity of circulating neutrophils, since plasma 2-CIFA levels were reduced in septic neutropenic subjects (Table 4). We also tested total and free plasma 2-CIFA concentrations in a small number of healthy volunteers ( $n = 4$ ) and observed significantly lower plasma concentrations in nonsepsis controls compared with sepsis survivors and sepsis nonsurvivors: nonparametric test of trend,  $P = 0.007$  for total 2-CIPA;  $P = 0.003$  for free 2-CIPA;  $P = 0.001$  for free 2-CISA (Supplemental Table 3). In a subgroup analysis of 110 of the subjects with sufficient plasma volume to measure MPO (described in Supplemental Table 4), we observed a strong correlation between plasma MPO levels and free 2-CIFAs and a modest correlation between MPO and total 2-CIFA levels (Spearman rank correlation, total 2-CIPA:  $\rho = 0.385$ ,  $P < 0.001$ ; free 2-CIPA:  $\rho = 0.402$ ,  $P < 0.001$ ; total 2-CISA:  $\rho = 0.497$ ,  $P < 0.001$ ; and free 2-CISA:  $\rho = 0.535$ ,  $P < 0.001$ ). Neutropenic subjects had significantly lower plasma levels of MPO ( $P < 0.001$ ). However, whereas 2-CIFA levels were associated with ARDS and mortality (Tables 1 and 2), MPO levels for these 110 subjects were not significantly associated with ARDS ( $P = 0.19$ , Table 1) or mortality ( $P = 0.99$ , Table 2). Among the small subgroup with neutropenia ( $n = 31$ ), plasma 2-CIFA concentrations did not differ by ARDS status and were very similar to control subjects without sepsis (Supplemental Table 5). Plasma 2-CIFA concentrations did not distinguish Gram-negative bacteria sepsis from non-Gram-negative bacteria sepsis (Supplemental Table 6).

Multiorgan failure associated with sepsis is mediated by microcirculatory collapse, including dysfunction at the blood-endothelial interface where neutrophils interact with endothelial cells leading to injury. We measured plasma concentrations of angiopoietin-2 (Ang-2), vWF, E selectin, soluble thrombomodulin (sTM), and soluble ICAM-1 (sICAM-1) in MESSI subjects to determine the degree of correlation between markers of endothelial activation and plasma 2-CIFA concentrations. Several soluble factors indicating blood-endothelial interface dysfunction, including Ang-2, E selectin, and sTM, were correlated with 2-CIPA or 2-CISA, though the degree of correlation was very weak to weak ( $\rho = 0.16$ – $0.27$ ) (Table 5). In contrast, plasma levels of vWF and sICAM-1 did not correlate with 2-CIFA levels in the MESSI cohort.

To provide better insights into whether the observed association between 2-CIFA and endothelial dysfunction may be mechanistically linked, human lung microvascular endothelial cells (HMVEC-L) were exposed to pathophysiologically relevant levels (10  $\mu\text{M}$ ) of 2-CIPA and indicators of endothelial function were examined. Previous studies have shown local concentrations of 2-CIPA produced by

**Table 4. Total and free plasma 2-CIFA molecular species levels are significantly lower in neutropenic subjects with sepsis**

	Neutropenic ( $n = 33$ )	Nonneutropenic ( $n = 164$ )	P value
Total 2-CIPA nM	0.77 (0.31, 1.67)	1.85 (0.95, 3.06)	<0.001
Free 2-CIPA nM	0.31 (0.16, 0.61)	0.60 (0.28, 1.19)	0.005
Total 2-CISA nM	0.75 (0.34, 2.18)	2.68 (1.26, 4.61)	<0.001
Free 2-CISA nM	0.24 (0.06, 0.70)	0.76 (0.37, 2.91)	<0.001
MPO pM	315 (175, 545)	1519 (871, 2794)	<0.001

**Table 5. Plasma concentrations of 2-CIPA correlate with Ang-2, E selectin, and soluble thrombomodulin**

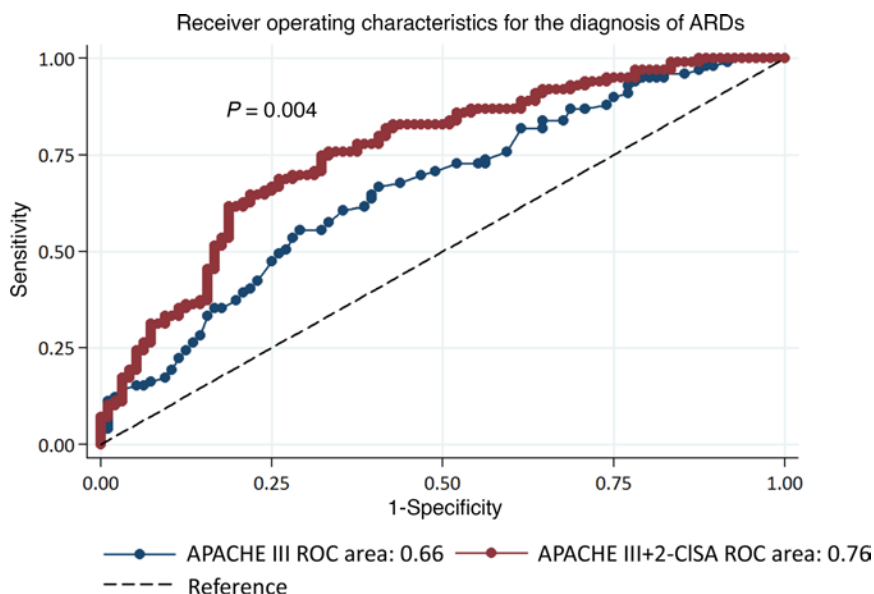
	Ang-2 (pg/ml)	vWF (% control)	E selectin (ng/ml)	sThrombomodulin (ng/ml)	sICAM-1 (ng/ml)
Free 2-CIPA	0.22, $P = 0.0056^A$	0.10, $P = 0.1876$	0.16, $P = 0.0403^A$	0.27, $P = 0.0008^A$	0.08, $P = 0.2974$
Free 2-CISA	0.12, $P = 0.1210$	0.06, $P = 0.4230$	0.24, $P = 0.0020^A$	0.10, $P = 0.2143$	0.10, $P = 0.1968$

Spearman rank correlation ( $\rho$ ) and  $P$  values between plasma endothelial markers and plasma-free 2-CIPA molecular species. <sup>A</sup>Significant correlation. sThrombomodulin, soluble thrombomodulin; sICAM-1, soluble ICAM-1.

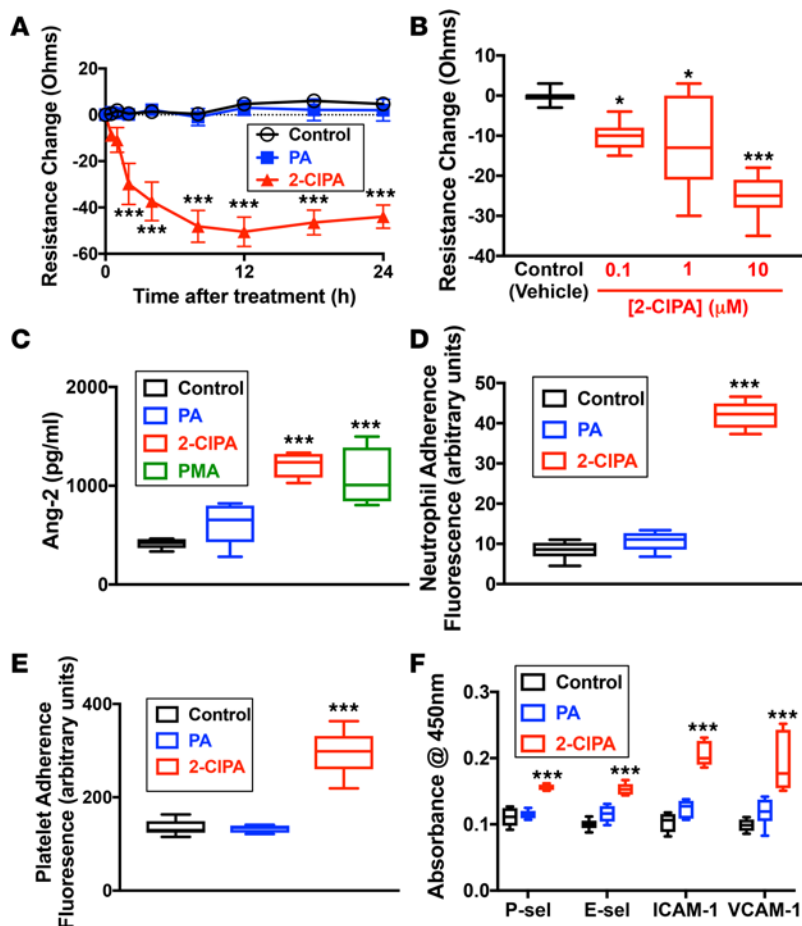
neutrophils and monocytes to be in the 10–30  $\mu\text{M}$  range (25, 35, 40), whereas, in septic plasma, we observed concentrations of 0.5–100 nM. It is likely that systemic levels of 2-CIPA approaching 100 nM represent much higher levels of 2-CIPA produced at sites of inflammation and approach those levels found in activated neutrophils and monocytes. 2-CIPA was shown to elicit loss of endothelial permeability barrier (Figure 3A) but not alterations in cell metabolism, as ascertained by MTT assay (Supplemental Figure 1). In comparison, the nonchlorinated fatty acid precursor, palmitic acid (PA), had no effect on the HMVEC-L permeability barrier. Further studies shown in Figure 3B demonstrate a dose-dependent loss of human pulmonary microvascular resistance in response to 2-CIPA, with significant loss of permeability barrier at concentrations as low as 100 nM (0.1  $\mu\text{M}$ ). Since Ang-2 associated with 2-CIPA in septic subjects and Ang-2 mediates endothelial barrier dysfunction, further investigations revealed the release of Ang-2 from human pulmonary microvascular endothelial cells treated with 10  $\mu\text{M}$  2-CIPA (Figure 3C). In comparison, PA did not cause endothelial Ang-2 release. Moreover, in comparison to the known Weibel-Palade body stimulant, phorbol myristate acetate (46), 2-CIPA was a potent stimulator of Ang-2 release. 2-CIPA, and not PA, also led to increased adhesion of neutrophils (Figure 3D) and platelets (Figure 3E) to HMVEC-L. Exposure to 2-CIPA but not PA also led to enhanced surface expression of P selectin, E selectin, ICAM-1, and VCAM-1 (Figure 3F) in HMVEC-L. Lung endothelium may be particularly at risk to 2-CIPA-mediated injury, as human renal glomerular microvascular endothelial cells manifested a less pronounced fall in endothelial resistance compared with HMVEC-L and did not increase membrane surface expression of P selectin, E selectin, ICAM-1, or VCAM-1 in response to 2-CIPA (Supplemental Figure 2).

## Discussion

The chlorinated lipids, 2-CIFALD and 2-CIPA, are produced as a result of plasmalogen oxidation during neutrophil activation and exposure to the MPO-generated oxidant, HOCl. 2-CIPA has been shown to be present as a relatively stable chlorinated lipid produced as a result of inflammation, including endotoxemia



**Figure 2. Neutrophil-derived plasma 2-CISA levels improve ARDS prediction.** Areas under the receiver operating curve (ROC) for ARDS diagnosis were compared between a logistic regression model predicting ARDS based on APACHE III score alone, which encompasses illness severity and comorbidities contributing to intensive care unit mortality, and a model predicting ARDS based on APACHE III score and log-transformed free plasma 2-CISA concentrations. The improvement in area under the ROC (AUROC) from 0.66 to 0.76 is statistically significant ( $P = 0.0042$  by  $\chi^2$  test).



**Figure 3. 2-CIPA induces Weibel-Palade body mobilization, leukocyte and platelet adherence, and permeability leakiness in human pulmonary microvascular endothelial cells.** In comparison to PA, exogenously added 2-CIPA increases human pulmonary microvascular endothelial cell barrier permeability (A,  $n = 6$  for each data point; mean  $\pm$  SEM) (ANOVA with Tukey's post-hoc test), Ang-2 release (C,  $n = 5$ ) (ANOVA with Tukey's post-hoc test), neutrophil adherence (D,  $n = 10$ ) (ANOVA with Tukey's post-hoc test), platelet adherence (E,  $n = 6$ ) (ANOVA with Tukey's post-hoc test), and surface adhesion molecule expression (F,  $n = 8$ ) (ANOVA with Tukey's post-hoc test). For A and C–F the lipid addition was at  $10 \mu\text{M}$ . (B) Concentration dependence of 2-CIPA ( $0.1$ – $10 \mu\text{M}$  2-CIPA) elicited endothelial permeability barrier disruption ( $n = 6$ ) (ANOVA with Dunnett's post-hoc test). \* $P < 0.05$  and \*\*\* $P < 0.001$  when compared with untreated controls. Box plots show median and 25th and 75th percentile, and whiskers show minima and maxima for each condition.

and Sendai virus exposure (35, 36). Additionally, lung 2-CIFA is elevated in mice exposed to chlorine gas, and intranasal administration of 2-CIFA resulted in increased protein and macrophage levels in bronchoalveolar fluid (47). The reported results demonstrate for the first time to our knowledge that systemic levels of 2-CIFA in sequential septic patients are associated with risk for ARDS and 30-day mortality. Moreover, plasma 2-CIFA concentrations correlate with clinical markers of endothelial dysfunction/injury in humans, and pathophysiologically relevant levels of 2-CIFA promote endothelial damage in microvascular endothelial cells in vitro. Taken together, the present findings indicate strong evidence for plasma 2-CIFA levels as a predictor of sepsis-associated ARDS, likely through effects on pulmonary microvasculature. Two molecular species of 2-CIFA have been assessed, 2-chloropalmitic acid and 2-chlorostearic acid. These species are derived from the saturated aliphatic groups linked to the glycerol backbone of plasmalogens by a vinyl ether bond. The initial chlorinated lipid product from HOCl-targeting plasmalogens is the short-lived 2-CIFALD, which is then oxidized into the free 2-CIFA species. Thus, free 2-CIFA is acutely produced during neutrophil activation, and over time some of the free 2-CIFA is stored in esterified pools, which comprise in part total 2-CIFA levels assessed in these studies. Esterified pools of 2-CIFA may in part reflect long-term storage of 2-CIFA, which may precede sepsis. We observed robust associations between ARDS and both free and total 2-CIFA species (Table 1). Comparing plasma 2-CIPA to 2-CISA, these moieties manifested a similar degree of association with sepsis mortality and ARDS. However, because the molecular species of plasmalogens may vary between target organs, it is possible that 2-CISA and 2-CIPA levels may vary dependent on the target organ infiltrated with neutrophils. This should be considered for future investigations.

Although plasma MPO levels increase in humans with sepsis, indicative of systemic activation of neutrophils (48), MPO has not been shown to predict outcomes in septic patients. Consistent with prior literature (22), we did not observe an association between plasma MPO concentrations and outcomes in the MESSI cohort of sepsis patients. In contrast, plasma concentrations of 2-CIFAs, the stable oxidation products of MPO activity, associated with both ARDS and 30-day mortality, independent of neutrophil count. We posit that MPO is involved in the pathophysiology leading to organ damage, and its catalytic activity leads to the production of sta-

ble metabolites with tighter associations with adverse outcomes. Taken together, these data are consistent with neutrophil activation as a rational mechanism for producing the elevated plasma 2-CIFA levels during sepsis.

The association of plasma 2-CIFA levels with ARDS, as well as with plasma Ang-2 levels, led to further investigations of the direct role of free 2-CIPA on pulmonary microvascular endothelial function. Lung microvascular endothelial cells were exposed to pathophysiologically plausible levels of 2-CIPA, which led to mobilization of Weibel-Palade bodies, resulting in the release of Ang-2 and the surface expression of P selectin and E selectin. In addition to Ang-2 release, 2-CIPA exposure caused permeability barrier dysfunction. Previous studies have shown that plasma Ang-2 levels are increased in human sepsis and have suggested that Ang-2 may contribute to ARDS through its tendency to deactivate Tie-2 receptor function, a key regulator of endothelial permeability (49). In addition to surface expression of P and E selectins, ICAM-1 and VCAM-1 were increased in 2-CIPA-treated endothelial cells. Accompanying the surface expression of adhesion molecules in response to 2-CIPA was an increased adherence of neutrophils and platelets to endothelial cells. Importantly, the changes elicited by 2-CIPA on endothelial permeability, Ang-2 release, surface adhesion molecule expression, and the adherence of neutrophils and platelets were not observed in cells treated with the corresponding nonchlorinated fatty acid, PA. The mechanisms responsible for the changes elicited specifically by 2-CIFA merit further investigation.

As evidenced by the more pronounced barrier dysfunction and the upregulation of markers of endothelial activation in HMVEC-L but not kidney endothelium in response to 2-CIFA, the lung may be particularly vulnerable to MPO-driven injury. The blood and lung fluid cytokine profile of ARDS is dominated by neutrophil chemoattractants IL-8 and G-CSF (50–53), and neutrophils may sequester in the lung microvasculature (54). Although patients with neutropenia do develop ARDS, it may be that neutropenic ARDS is mechanistically distinct from ARDS in subjects with neutrophils (42, 55), just as some animal models of lung injury are neutrophil dependent (56). In our underpowered subgroup of neutropenic subjects, those with ARDS had plasma 2-CIFA concentrations that were not different from non-ARDS subjects and that resembled concentrations observed in healthy controls. Thus, we hypothesize that the mechanism underlying ARDS in neutropenic subjects may be driven by direct epithelial and/or endothelial injury that is not chlorolipid dependent. In large clinical trials of ARDS, a hyperinflammatory subphenotype characterized by high circulating levels of IL-8, high IL-6, and Ang-2 has been described that associates with higher mortality and a differential response to ventilator and fluid management (57, 58). Because 2-CIFA may have additional specificity to identify neutrophil-mediated lung injury, these markers may have utility as potential enrichment factors for trials examining antiinflammatory (59, 60) or antipermeability treatments in ARDS (61).

Our study had several limitations. Our clinical cohort is an ideal design to test association between clinical and biological variation and sepsis-induced organ failure and outcome; however, the observational human portion of our study was not designed to prove causality. Nonetheless, the complementary *in vitro* data demonstrate that 2-CIFAs are sufficient to incite significant lung microvasculature activation and permeability, such as occurs during sepsis-associated ARDS. Our statistical mediation analysis suggests that ARDS is the mechanism by which plasma-free 2-CIFA concentrations associate with mortality, focusing attention on the neutrophil-endothelial interaction as a driver of ARDS. Our population was of modest size and all subjects were enrolled from a single center; thus, replication would improve generalizability. The quantification of 2-CIFA was performed using sophisticated mass spectroscopic techniques, and in order for these markers to be tested as a potential enrichment factor or response indicator, a more widely available and facile assay would be necessary. We observed correlation between free plasma 2-CIFA concentrations and markers of endothelial activation; however, the degree of correlation would be statistically described as very weak ( $\rho < 0.2$ , for 2-CIPA and E selectin) or weak ( $\rho 0.2$ – $0.3$  for Ang-2 and sTM). This degree of correlation is similar to that reported between different vascular markers in a study of comparable size (62) but weaker than the correlation previously reported between Ang-2 and IL-6 (63). Finally, further mechanistic work to understand how 2-CIFA affects endothelial activation, and the degree to which this axis can be manipulated pharmacologically, is needed in order to translate these findings to new ARDS therapies.

In summary, we have used mass spectroscopy to detect markers of neutrophil-mediated injury that may functionally identify the pathologic neutrophil contribution to sepsis-associated ARDS. Circulating levels of 2-chloropalmitic and 2-chlorostearic acid strongly associate with sepsis-associated ARDS and mortality, and these chlorinated lipids incite lung microvascular permeability and upregulation of adhesion molecules that could further promote neutrophil-endothelial interaction. Plasma 2-CIFAs should be investigated as markers of a neutrophil-mediated ARDS endotype that may be amenable to precision therapy.

## Methods

**Patient population.** The MESSI cohort study enrolls adult subjects, with strongly suspected or confirmed infection and new organ dysfunction, who are admitted to the medical ICU at the Hospital of Pennsylvania (42, 64, 65). Informed consent was obtained from patients or their proxies. ARDS was defined in accordance with the Berlin definition, with the added requirement of invasive mechanical ventilation over the first 6 days of ICU admission to be confident that the ARDS was attributable to the first septic episode (66, 67); chest radiographs were interpreted as described previously (42, 68). Acute kidney injury was determined by Acute Kidney Injury Network criteria (69). Neutropenia was defined as an absolute neutrophil count of less than 1,000/microliter on the day of ICU admission as previously described (42). Mortality was defined at 30 days. Plasma was drawn into a 6-ml citrated vacutainer for clinical coagulation testing on arrival to the Emergency Department or upon transfer to the ICU for patients previously on the floor. Samples were spun (3,000 g for 15 minutes) upon receipt, kept at 4°C for 24 hours, and then aliquoted and frozen at -80°C for research purposes. (42). Plasma cytokines and endothelial markers were assayed by ELISA as described previously (42). Healthy controls ( $n = 4$ ) were voluntary donors responding to a solicitation notice at St. Louis University who provided informed consent. Medical history was not obtained. Plasma was collected as described above and frozen at -80°C until analysis.

**Plasma 2-CIFA and MPO analyses.** Twenty-five microliters of plasma were used to determine free and total 2-CIFA levels as previously described (70). In brief, 105 fmol of 2-chloro- $[d_4-7,7,8,8]$ -PA was added during a modified Dole extraction to assess free 2-CIFA. For total 2-CIFA analyses, plasma was first subjected to base-catalyzed hydrolysis prior to Dole extraction. Extracted 2-CIFA was then quantitated using LC/MS with electrospray ionization in the negative ion mode and selected reaction monitoring of individual 2-CIFA molecular species. MPO levels were assessed by using the FDA-cleared in vitro diagnostic assay, CardioMPO (Cleveland HeartLab), on autoanalyzer according to the manufacturer's instructions. For 2-CIFA and MPO analyses, deidentified plasma samples were sent to either DAF (2-CIFA) or SLH (MPO) for analyses, and data for each coded sample were then sent to NJM for data merging into the MESSI data set.

**Lung microvascular endothelial cell studies.** For permeability studies, HMVEC-L were grown to confluence on Transwell polycarbonate filters (6.5-mm diameter, 0.4- $\mu$ m pore size) mounted in a chamber insert. Resistance across cells was monitored daily using an epithelial volt/ohm meter (EVOM, World Precision Instruments). Following consistent resistance for 3 consecutive days, experiments were performed. Resistance was measured for each well prior to the addition of 10  $\mu$ M BSA-conjugated lipids and then at selected time points. To assess Ang-2 release, lung microvascular endothelial cells were plated in 16-mm diameter wells and grown to confluence. Cells were treated with 10  $\mu$ M BSA-conjugated lipids in growth media with 5% FBS for 30 minutes. Supernatant was removed and centrifuged for 2,000 g for 10 minutes to remove cellular debris. Human angiopoietin-2 Quantikine ELISA (R&D Systems) was used to quantify Ang-2 in the supernatant. Fifty microliters were assayed following manufacturer's protocol.

Cell surface expression of adhesion molecules was measured after lipid treatment of HMVEC-L. Cells were plated in 16-mm wells and grown to confluence. Cells were treated with 10  $\mu$ M BSA-conjugated lipids in growth media with 5% FBS for 30 minutes (P selectin), 1 hour (E selectin), or 4 hours (ICAM-1 and VCAM-1). HMVEC-L were fixed with 1% paraformaldehyde overnight. Cells were washed 3 times with PBS and blocked with PBS containing BSA and fish gelatin for 1 hour. Cells were incubated with primary antibodies (1:50) against P selectin (Santa Cruz, catalog sc-8419, monoclonal), E selectin (Santa Cruz, catalog sc-5262, monoclonal), ICAM-1 (Santa Cruz, catalog sc53336, monoclonal), and VCAM-1 (Santa Cruz, catalog 13160, monoclonal) for 1 hour at 37°C. Cells were washed twice with PBS and incubated with HRP-conjugated goat anti-mouse secondary antibody (1:5,000) for 30 minutes at 37°C. Cells were washed 4 times with PBS and incubated with the 3,3',5,5'-tetramethylbenzidine substrate system for 30 minutes in the dark. The color reaction was stopped with 8 N  $H_2SO_4$ , and absorbance was read at 450 nm. Absorbance in the presence of secondary antibody alone was subtracted from each sample absorbance. To assess leukocyte adherence to HMVEC-L, endothelial cells were grown to confluence in 22-mm wells. HMVEC-L were treated with 10  $\mu$ M BSA-conjugated lipids in growth media for 4 hours. Leukocytes were isolated from healthy volunteers (25, 71).  $2 \times 10^6$  leukocytes were subsequently added to lung microvascular endothelial cells and incubated for 20 minutes. Unbound leukocytes were washed away with 3 PBS rinses. The remaining leukocytes were lysed with 0.2% Triton X-100 and transferred to an Eppendorf tube.



Tubes were briefly sonicated to ensure complete lysis of cells. To measure MPO, 400  $\mu$ l cell lysate was transferred to a test tube containing phosphate buffer, Hank's buffer with BSA, 1,9-dimethyl-methylene blue, and 0.5% hydrogen peroxide. Samples were incubated for 15 minutes at room temperature. Sodium azide (1%) was added to stop the reaction. Absorbance was measured at 460 nm. Adherence of platelets to lung microvascular endothelial cells was performed as previously described (72). Briefly, HMVEC-L were grown to confluence in 22-mm wells. Cells were treated with 10  $\mu$ M BSA-conjugated lipids in growth media for 4 hours. Platelets were isolated from healthy volunteers (73). Platelets were labeled with Calcein-AM (2.5  $\mu$ mol/l) for 15 minutes at 37°C in the dark. Fluorescence-labeled platelets were subsequently added to lung microvascular endothelial cells and incubated for 20 minutes at 37°C. Unbound platelets were washed away with 3 PBS rinses. The remaining platelets were lysed in lysis buffer for 10 minutes, and fluorescence was measured with a plate reader (excitation at 492 nm, emission at 535 nm).

*Statistics.* Clinical characteristics were compared between ARDS and non-ARDS subjects using Pearson's  $\chi^2$  test or Fishers exact test for categorical data and 2-tailed Student's *t* or Wilcoxon rank-sum test as appropriate for continuous data. Plasma 2-CIFA concentrations were compared between clinical groups and between experimental conditions using the Wilcoxon rank-sum test or ANOVA. Because the distribution of 2-CIPA and 2-CLSA were right-skewed, we performed log transformation for normality using Stata 12.1. We performed multivariable logistic regression of ARDS using log-transformed 2-CIFA concentrations and APACHE III score, age, sex, European descent, and pulmonary source of infection, given our prior reported associations in this population (42). For multivariable logistic regression of 30-day mortality, we included covariates APACHE III score, age, sex, absolute neutrophil count, and European descent as covariates (42, 45). The lowest absolute neutrophil count on the day of ICU admission was included as a covariate to ensure that associations with 2-CIFA were independent of neutrophil count. Correlation between plasma markers was evaluated with Spearman rank correlation. We employed mediation analysis as a formal approach to explain the mechanism by which an explanatory variable (2-CIFA) influences the outcome (mortality) via a third mediator variable (ARDS) (74). We used logistic regression to estimate the change in ARDS risk for each log change in free 2-CIFA, which we term the mediator effect, and logistic regression of 2-CIFA, ARDS, an interaction term [ARDS  $\times$  log(free 2-CIFA)], APACHE III score, and age to model the total effect of 2-CIFA on mortality, as enacted in R statistical packages. To assess whether plasma 2-CIFA concentrations had predictive utility for ARDS compared with the APACHE III severity of illness score (42, 45), we compared AUROC by  $\chi^2$  test for models predicting ARDS based on APACHE III alone or in combination with log-transformed free 2-CIFA levels (75, 76). Using methodology described by Pencina et al. and enacted in Stata, we calculated the ARDS net reclassification index and the IDI for models that combined APACHE III and plasma 2-CIFA concentration (dichotomized at the median 2-CIFA level) compared to the base model of APACHE III score alone (77, 78). We used the nonparametric test of trend to assess for an association between sepsis severity and 2-CIFA, considering subjects in a stepwise fashion from healthy controls to sepsis survivors to sepsis nonsurvivors. For in vitro experiments, endothelial barrier function was compared among control, PA, and 2-chloropalmitic acid treatment by ANOVA with Tukey's post-hoc test at individual time points. *P* values of less than 0.05 were considered significant.

*Study approval.* Leukocytes and platelets were isolated from healthy volunteers, as approved by Saint Louis University Institutional Review Board protocols 9952 and 12369, respectively. MESSI samples were collected as approved by University of Pennsylvania Institutional Review Board protocol 808542.

### Author contributions

NJM contributed to study conception, experimental design, clinical study data collection, statistical analyses, and wrote the manuscript. JPR contributed to study conception, experimental design, clinical study data collection, statistical analyses, and wrote the manuscript. RF performed statistical analyses and wrote the manuscript. JDC contributed to study conception, experimental design, and wrote the manuscript. SLH contributed to experimental design, plasma MPO studies, and wrote the manuscript. CLH performed endothelial studies and contributed to experimental design. CJA and JDF performed 2-CIFA analyses and contributed to experimental design. JM performed endothelial studies, contributed to study conception, and contributed to experimental design. DAF contributed to study conception, experimental design, 2-CIFA analyses, and wrote the manuscript.

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