Original Paper

Levels of Proinflammatory Cytokines, Oxidative Stress, and Tissue Damage Markers in Patients with Acute Heart Failure with and without Cardiorenal Syndrome Type 1

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Keywords

Acute decompensated heart failure · Acute kidney injury · Cardiorenal syndrome · Cardiorenal cross talk · Inflammation · Cytokines · Tissue damage · Oxidative stress

Abstract

Background: Cardiorenal syndrome type 1 (CRS type 1) is characterized by a rapid worsening of cardiac function leading to acute kidney injury (AKI). Inflammation and oxidative stress seem to play a pivotal role in its pathophysiology. In this in vivo study, we examined the putative role of inflammation and humoral markers in the pathogenesis of the CRS type 1. **Methods:** We enrolled 53 patients with acute heart failure (AHF); 17 of them developed AKI (CRS type 1). The cause of AKI was presumed to be related to cardiac dysfunction after having excluded other causes. We assessed the plasma levels of proinflammatory cytokines (TNF- α , IL-6, IL-18, sICAM, RANTES, GM-CSF), oxidative stress marker (myeloperoxidase, MPO), brain natriuretic peptide (BNP), and neutrophil gelatinase-associated lipocalin (NGAL) in AHF and CRS type 1 patients. **Results:** We observed a significant increase in IL-6, IL-18, and MPO levels in CRS type 1 group compared to AHF ($p < 0.001$). We found higher NGAL at admission in the CRS type 1 group compared to the AHF group ($p = 0.008$) and a positive correlation between NGAL and IL-6 (Spearman's $\rho = 0.45$, $p = 0.003$) and between IL-6 and BNP (Spearman's $\rho = 0.43$, $p = 0.004$). We observed lower hemoglobin levels in CRS type 1 patients compared

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to AHF patients ($p < 0.05$) and inverse correlation between hemoglobin and cytokines (IL-6: Spearman's $\rho = -0.38$, $p = 0.005$; IL-18: Spearman's $\rho = -0.32$, $p = 0.02$). **Conclusion:** Patients affected by CRS type 1 present increased levels of proinflammatory cytokines and oxidative stress markers, increased levels of tissue damage markers, and lower hemoglobin levels. All these factors may be implicated in the pathophysiology of CRS type 1 syndrome.

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Introduction

Heart and kidney functions are firmly interconnected, and communication between these two organs occurs through many pathways, including hemodynamic and nonhemodynamic mechanisms. In particular, cardiac disease may contribute to worsening kidney function and vice versa.

According to the definition proposed by the Consensus Conference on Acute Dialysis Quality Initiative Group, the term cardiorenal syndrome (CRS) has been used to define different clinical conditions in which heart and kidney dysfunctions are overlapping [1]. Five subtypes of CRS have been described [2]. In this work, we focused on CRS type 1, in which an abrupt worsening of cardiac function leads to acute kidney injury (AKI). CRS type 1 occurs in approximately 20–30% of patients with acute heart failure (AHF) and is associated with an increase in mortality and in-hospital length of stay [3, 4].

The pathophysiology of CRS type 1 is complex and poorly understood. Many factors, such as hemodynamic instability, potentially nephrotoxic medications (such as vancomycin, gentamycin, loop diuretics), use of iodinated contrast media for diagnostic procedures, upregulation of the renin-angiotensin-aldosterone system and the sympathetic nervous system, collaborate in this multiorgan dysfunction. Furthermore, the increase in proinflammatory cytokines, the dysregulation of apoptosis, and the increase in oxidative stress may play a central role in the pathogenesis of this syndrome [5].

In previous works, we tried to deepen our knowledge of CRS type 1 pathophysiology using different in vitro experimental models. Monocyte cells treated by CRS type 1 plasma showed upregulation of apoptosis compared with those treated by AHF plasma. Furthermore, we observed an increase in plasma IL-6 and IL-18 in CRS type 1 compared with AHF. Proinflammatory cytokines may induce apoptosis and necrosis through activation of death-signaling receptors and, indirectly, through increase in reactive oxygen substrate production [6, 7].

Similarly, we reported that renal tubular epithelial cells incubated with CRS type 1 plasma increased proinflammatory production, release of tubular damage markers as neutrophil gelatinase-associated lipocalin (NGAL) and apoptosis. Moreover, kidney tubular cells are important components for both the initiation and prolongation of inflammation [8]. In addition, in a recent work, we also observed a significant increase in oxidative stress markers in CRS type 1, such as myeloperoxidase (MPO), nitric oxide, and copper/zinc superoxide dismutase [7].

Alterations in the immune response, apoptosis, cytokine release, oxidative stress, small noncoding RNAs, extracellular vesicles, inflammation, and the change in immune cell function have been postulated as potential mechanisms involved in the pathogenesis of CRS type 1 syndrome [9–12]. We supposed that inflammation and immune dysfunction may greatly contribute to the final nonhemodynamic pathways of organ dysfunction in the heart-kidney cross talk and may be mediators of this pathological communication [13]. The complexity of this syndrome presents a key challenge for diagnostic or treatment approaches.

Fig. 1. The flowchart of patient enrollment.

In this *in vivo* study, we evaluated the putative role of different inflammatory and humoral factors that may be implicated in the development of AKI in AHF patients. In particular, we evaluated plasma levels of proinflammatory cytokines, markers of AKI and oxidative stress, to better understand the pathophysiology of CRS type 1.

Materials and Methods

Subjects

Patients admitted to the Internal Medicine Department of San Bortolo Hospital between September 2011 and February 2012 were screened. A total of 80 patients with AHF were further examined for inclusion in the study. Patients with AKI prior to the episode of AHF or with other potential causes of AKI were excluded from this study. In the enrolled patients, the cause of AKI was presumed to be related to cardiac dysfunction.

Furthermore, patients with estimated glomerular filtration rate (eGFR) <45 mL/min/1.73 m² ($n = 14$) or with a history of kidney transplantation ($n = 1$) were excluded. Septic patients ($n = 2$) and hypotensive patients who required inotropic support prior to the diagnosis of AKI ($n = 3$) were not included in the study. Patients exposed to contrast media in the 72 h preceding AKI ($n = 0$) were excluded from the study. Furthermore, we excluded patients without a baseline level of serum creatinine (sCr; $n = 7$) (Fig. 1). We considered as the baseline value, at least one sCr level of the last 3 months (maximum) before the admission of all the patients enrolled in the study. The baseline value was obtained by Biochemical Database of the Central Laboratory of San Bortolo Hospital or by patient's previous clinical checkup.

Finally, we enrolled 53 AHF patients. Subsequently, we identified 17/53 patients who exhibited AKI at the time of admission (caused by AHF) or developed AKI during the course of hospitalization and were classified as CRS type 1.

Clinical data, blood pressure, sCr, blood urea, hemoglobin, and brain natriuretic peptide (BNP) were evaluated and collected at admission. Echocardiograms were performed within 6 h from the admission into the Internal Medicine ward. AHF was defined by the European Society of Cardiology (ESC) guidelines [14]. AKI was defined by the Acute Kidney Injury Network (AKIN) criteria [15]. Scr was measured with an enzymatic method, IDMS (isotope dilution mass spectrometry) traceable by an automatic analyzer (Dimension Vista, Siemens Healthcare, Tarrytown, NY, USA), and eGFR was calculated with the CKD-EPI equation [16]. CRS type 1 was defined according to the current classification system [17–19]. Hypertension was defined by the European Society of Cardiology (ESC) guidelines for the management of arterial hypertension (normal

range: systolic blood pressure 130–139 mm Hg and diastolic blood pressure 85–89 mm Hg) [20]. Obesity was defined by OMS classification (normal range body mass index <29.9) [21–23]. Diabetes was defined according to American Diabetes Association (ADA) guidelines [24].

All the procedures were in accordance with the Helsinki Declaration. The protocol and consent form were approved by the Ethics Committee of San Bortolo Hospital. All the patients were informed about the experimental protocol and the objectives of the study before providing informed consent and blood samples.

Sample Collection

Peripheral venous blood samples were collected from all patients within 8 h of admission to the Internal Medicine ward and on the third day of in-hospital stay. For AHF patients, we used the blood sample from the admission; we used the blood sample within 24 h of AKI for patients who developed CRS type 1. Blood samples were collected in EDTA tubes and subsequently centrifuged for 10 min at 1,600 rpm. Following centrifugation, plasma was immediately separated from blood cells and stored at -80°C until use. All samples were processed within 4 h after collection. Collection and processing of control samples from healthy volunteers followed the identical protocol.

Determination of NGAL Levels

Quantitative determination of NGAL was performed with an Alere Triage NGAL Panel (Alere, San Diego, CA, USA) on admission and the third day of hospitalization. Plasma NGAL levels were measured by fluorescence-based immunoassay with a Triage point-of-care analyzer (Alere), which allows the rapid quantitative measurement of NGAL concentrations in EDTA plasma. NGAL concentrations are expressed as nanograms per milliliters.

Cytokines and Markers of Tubular Injury Enzyme-Linked Immunosorbent Assay

Quantitative determination of TNF- α , IL-6, IL-18, soluble I form of cell adhesion molecule (sICAM), regulated on activation normal T cell expressed and secreted (RANTES), granulocyte-macrophage colony-stimulating factor (GM-CSF), and MPO in EDTA plasma were performed by Human Instant ELISA kit (eBioscience, San Diego, CA, USA). All determinations were performed according to the manufacturer's protocol and instructions. Optical density was read using a VICTORX4 Multilabel Plate Reader (PerkinElmer Life Sciences, Waltham, MA, USA) at 450 nm. The concentration values for these molecules were calculated from standard curves, according to the manufacturer's protocols. All tests were performed in triplicate.

Statistical Analysis

Statistical analysis was performed using the SPSS 15 software package. Categorical variables were expressed as percentages; continuous variables were expressed as means $\pm$ standard deviation (parametric variables) or median and interquartile range (IQR) (nonparametric variables). The Mann-Whitney U or *t* test was used for comparison of two groups, as appropriate. Correlation coefficients were calculated with the Spearman rank correlation coefficient test. A *p* value of <0.05 was considered statistically significant.

Results

Patient Baseline Characteristics

The mean age of 36 patients with AHF was 76 ± 10 years, and 58% of these patients were male. The median baseline sCr of AHF subjects was 1.0 mg/dL (IQR 0.82–1.15), the median eGFR was 68 mL/min/1.73 m² (IQR 55–81). The median peak of sCr during hospitalization was 1.0 mg/dL (IQR 0.92–1.25). The median length of in-hospital stay was 8 days (IQR 6–10) in the AHF group; 44 and 92% of subjects in the AHF group had diabetes and hypertension, respectively; 3% of AHF patients died during the in-hospital stay.

The mean age of 17 patients with CRS type 1 was 75 ± 9 years, and 59% of these patients were male. The median baseline sCr of CRS type 1 patients was 1.24 mg/dL (IQR 1.04–1.52), the median eGFR was 53 mL/min/1.73 m² (IQR 47–66). The median peak of sCr during hospitalization was 1.5 mg/dL (IQR 1.4–2.1). The median length of in-hospital stay was 8 days (IQR 7–11) in the CRS type 1 group; 71 and 88% of CRS type 1 subjects had diabetes and hyper-

Table 1. Characteristics and clinical parameters of AHF and CRS type 1 patients at admission

	AHF	CRS type 1	<i>p</i>
Age, years	76±10	75±9	0.63
Weight, kg	75 (64–91)	80 (71–90)	0.42
Diabetes	44%	71%	0.22
Hypertension	92%	88%	0.80
Peripheral vascular disease	42%	35%	0.78
Cardiovascular disease	22%	24%	0.49
Obesity	22%	29%	0.49
Dyslipidemia	36%	41%	0.61
Baseline serum creatinine, mg/dL	0.99 (0.82–1.2)	1.0 (0.91–1.2)	0.22
BNP at admission, pg/mL	432 (300–737)	795 (355–1,963)	0.09
Hemoglobin at admission, g/dL	13 (11.9–14.7)	11 (10–12.7)	0.05
Urea at admission, mg/dL	51 (41–68)	85 (58–121)	0.03
Mortality during in-hospital stay	3%	18%	0.05

Data are presented as means ± standard deviation, median (IQR), or as stated.

Table 2. Medication of CRS type 1 and AHF patients

	ADHF	CRS type 1	<i>p</i>
Angiotensin-converting enzyme inhibitor	50%	53%	NS
Angiotensin II receptor blockers	19%	24%	NS
β-Blockers	53%	59%	NS
Calcium channel blockers	19%	18%	NS
Diuretics	97%	100%	NS
Statins	36%	41%	NS
Nonsteroidal anti-inflammatory drugs	3%	6%	NS

tension, respectively; 18% of CRS type 1 patients died during the in-hospital stay. 7/17 patients classified as CRS type 1 developed AKI during hospitalization. Urea ($p = 0.17$), hemoglobin ($p = 0.6$), BNP ($p = 0.4$), and NGAL (at admission $p = 0.58$, on the third day $p = 0.69$) were not different between patients presenting AKI at admission and developing AKI during hospitalization. In contrast, sCr at admission was higher in CRS type 1 patients presenting AKI at admission ($p = 0.03$).

The characteristics of CRS type 1 and AHF patients are described in Table 1. Medications of CRS type 1 and AHF patients are described in Table 2.

Comorbidities and medication treatments were similar in CRS type 1 and AHF patients. In particular, the amount of diuretics administered was similar in these two groups. No patients developed the need for mechanical ventilation and renal replacement therapy. Urea, hemoglobin and sCr levels were significantly different at admission in CRS type 1 and AHF patients. In contrast, BNP levels were not significantly different between groups at admission. NGAL levels were significantly higher at admission in CRS type 1 patients when compared to AHF patients (391 ng/mL; IQR 219–1,301 vs. 197 ng/mL; IQR 145–282, $p = 0.008$). sCr and NGAL was significantly higher in CRS type 1 patients at admission and at the third day of in-hospital stay (Table 3) (Fig. 2). The median variation of sCr was significantly higher in CRS type 1 group (0.35 mg/dL, IQR 0.30–0.51) when compared with AHF patients (0.08 mg/dL, IQR 0.04–0.12) ($p = 0.05$). Mortality was significantly higher in CRS type 1 patients ($p = 0.05$).

Table 3. NGAL and serum creatinine levels of AHF and CRS type 1 patients at admission and on the third day

	AHF	CRS type 1	<i>p</i>
Plasma NGAL at admission, ng/mL	197 (145–282)	391 (219–1,301)	0.02
Plasma NGAL at day 3, ng/mL	203 (111–395)	312 (233–923)	0.03
Serum creatinine at admission, mg/dL	1.0 (0.92–1.25)	1.24 (1.04–1.52)	0.01
eGFR at admission, mL/min/1.73 m ²	68 (55–81)	53 (47–66)	0.03
Serum creatinine at day 3, mg/dL	0.97 (0.86–1.11)	1.17 (1.07–1.42)	0.05
eGFR at day 3, mL/min/1.73 m ²	65 (56–82)	46 (44–64)	0.07

Data are presented as median (IQR).

Fig. 2. Inflammatory, oxidative stress, and NGAL profile in AHF and CRS type 1 patients. Plasma levels of IL-6, IL-18, and MPO were significantly higher in CRS type 1 patients compared with AHF patients ($p < 0.001$). NGAL was significantly higher in CRS type 1 patients at admission and on the third day of in-hospital stay.

Inflammatory and Oxidative Stress Profile

Inflammatory and oxidative marker levels were measured by ELISA in plasma to examine potential immune mediators involved in CRS type 1 pathogenesis.

TNF- α , sICAM, GMCSF, and RANTES levels in plasma were not significantly different in AHF when compared with CRS type 1 patients (Table 4). Furthermore, plasma levels of proinflammatory cytokines were significantly higher in CRS type 1 compared with AHF patients ($p < 0.001$) (Fig. 2). Specifically, the mean values of IL-6 and IL-18 were around 1.5 and 5 times higher in CRS type 1 patients compared with AHF subjects. In a similar way, the mean value of MPO was around 1.5 times higher in the CRS type 1 group than in the AHF group (Fig. 2). Furthermore, we performed a subanalysis in the CRS type 1 group, and inflammatory and oxidative marker levels were similar between patients presenting AKI at admission and developing AKI during hospitalization (IL-6 $p = 0.07$, TNF- α $p = 0.32$, sICAM $p = 0.39$, GMCSF $p = 0.32$, RANTES $p = 0.95$, IL-18 $p = 0.12$, MPO $p = 0.24$).

In addition, we observed a negative significant correlation between Hb levels and IL-6 (Spearman's rho = -0.38 , $p = 0.005$), IL-18 (Spearman's rho = -0.32 , $p = 0.02$), GMCSF (Spear-

Table 4. Inflammatory and oxidative stress profiles in AHF and CRS type 1 patients

	AHF	CRS type 1	<i>p</i>
TNF- α , pg/mL	34 (29.5–39.2)	33.1 (27.4–38.0)	0.386
sICAM, pg/mL	3 (2.3–4.1)	3 (2.8–4.9)	0.360
GM-CSF, pg/mL	13 (12.4–14.7)	14 (13.0–15.8)	0.253
RANTES, pg/mL	8,846 (6,446–10,409)	9,511 (7,845–10,156)	0.322
IL-6, pg/mL	19 (16.2–23)	87 (76.6–98.6)	<0.001
IL-18, pg/mL	71 (46.3–84.5)	107 (87.8–124.4)	<0.001
MPO, pg/mL	423 (360–523)	747 (699–974)	<0.001

Data are presented as median (IQR).

man's $\rho = -0.39$, $p = 0.003$), and MPO (Spearman's $\rho = -0.31$, $p = 0.02$). Furthermore, we observed a positive correlation between IL-6 levels and NGAL (Spearman's $\rho = 0.45$, $p = 0.003$), BNP (Spearman's $\rho = 0.43$, $p = 0.004$), and IL-18 (Spearman's $\rho = 0.52$, $p < 0.01$). In addition, we observed a positive correlation between inflammation and oxidative stress, i.e. MPO (IL-6: Spearman's $\rho = 0.62$, $p < 0.01$; IL-18: Spearman's $\rho = 0.42$, $p = 0.002$).

Discussion

Many recent investigations indicate that immune-mediated, inflammatory, and apoptotic mechanisms may play a role in the pathogenesis of CRS type 1 [6, 19, 25, 26]. In this study, we investigated the role of immune-mediated inflammatory markers in the pathophysiology of CRS type 1.

CRS type 1 patients showed significantly higher plasma levels of proinflammatory cytokines, such as IL-6 and IL-18, compared to AHF patients. Furthermore, we found a positive correlation between inflammation markers and between IL-6 and BNP. Many hypotheses are suggested to explain immune system activation in AHF patients: immune activation by direct antigenic stimulation could happen during viral myocarditis, secondary to cardiac injury because of exposition of new antigen by injured myocardium and cytokine release by cardiac tissue in response to hemodynamic stress [27]. Inflammation associated with hemodynamic imbalance caused by acute cardiac pump dysfunction may induce renal endothelial damage, vasodilatation, and coagulation system impairment, complement activation with consequent vasoconstriction and capillary obstruction and final AKI [5]. Recently, Fanola et al. [28] observed that in patients with acute coronary syndrome, higher levels of IL-6 are associated with higher risk of cardiovascular death and heart failure.

Based on these observations, different therapeutic interventions were proposed to decrease inflammation: from highly specific to broad-spectrum therapies, from anti-TNF- α therapy to intravenous immune globulin therapy to immune-adsorption and immune-modulation therapy. Unfortunately, none of the interventions showed a clinical benefit [29–32].

We also observed higher plasma levels of MPO in CRS type 1 patients. MPO is an enzyme stored in azurophilic granules of neutrophils and macrophages and it is involved in the inflammatory process and oxidative stress. This result confirmed the significant increase of oxidative stress markers in a small group of AHF patients developing AKI observed in a previous work [7]. Furthermore, our findings highlighted a strong link between inflammation and oxidative stress: a positive correlation between MPO and inflammation markers and between IL-6 and -18 and NGAL. In our study, NGAL levels at admission and on the third day were higher in CRS

type 1 patients compared to AHF patients, but there was no difference between patients presenting AKI at admission and those developing AKI during hospitalization. NGAL is a 25-kDa polypeptide; it is expressed by neutrophils and a number of epithelial cells; it was a promising AKI marker, particularly for proximal tubular cell damage [33, 34]. NGAL was well evaluated in pediatric cardiac surgery [35]. Cardiac surgery-associated AKI is classified as a form of CRS type 1. In several studies, NGAL showed a good performance in predicting AKI 2 h after surgery [36, 37]. NGAL could be also considered a marker of inflammation; it correlates with proinflammatory cytokines, such as IL-6 and IL-10, and endothelial adhesion molecule [38]. It is also predictive of severe sepsis and septic shock [39, 40].

In patients with AHF, increased serum NGAL at admission was associated with increased risk of subsequent CRS type 1 [41]. Alvelos et al. [42] found that at a cut-off level of 170 ng/ml, NGAL predicted the development of CRS type 1 within 72 h with a sensitivity of 100% and a specificity of 86.7% with an AUC of 0.93. In addition, we hypothesized that the positive correlation between MPO and NGAL and between IL-6 and IL-18 and NGAL indicate proximal tubular cell damage induced by inflammation and oxidative stress in CRS type 1.

Based on these descriptive results, we may hypothesize an important cross talk between inflammation, oxidative stress, and development of AKI in AHF patients.

In addition, we observed lower hemoglobin levels in CRS type 1 patients and a negative correlation between hemoglobin and IL-6 levels. In this context, proinflammatory cytokine overexpression may be implicated in the pathogenesis of anemia in patients affected by heart failure and, in particular, in patients with CRS type 1. Recent studies reported a decrease in bone marrow responsiveness to erythropoietin throughout induction of apoptosis of red cell precursors and downregulation of erythropoietin receptors [43, 44]. Furthermore, IL-6 may induce overexpression of hepcidin, an acute-phase protein, with consequent iron metabolism impairment [45]. Other authors observed that administration of exogenous erythropoietin might reduce circulating levels of proinflammatory cytokines and soluble apoptosis mediators [46]. Relative hypoxia associated with lower levels of hemoglobin in CRS type 1 patients may induce mitochondrial and intracellular modifications with consequent cell death throughout necrosis and apoptosis [47]. In this context, we hypothesized that lower hemoglobin levels may be a risk factor to developing CRS type 1 in AHF patients; in fact, we did not find a difference between patients presenting AKI at admission and patients developing AKI later on.

This study explores the premise of humoral, inflammatory, and oxidative processes in the development of AKI in AHF patients. Our results can stimulate further exploration of novel pathophysiological mechanisms in CRS type 1 and assign a specific role for these factors in CRS type 1. Although these findings are provocative, the design of the study does not allow us to make conclusions about causality.

In conclusion, hemodynamic and nonhemodynamic factors may be implicated at the onset of CRS type 1 syndrome. These factors may be associated with upregulation of inflammatory pathways with consequent increase in circulating proinflammatory cytokines, like IL-6 and IL-18, and oxidative stress markers such as MPO, resulting in tissue damage. Inflammation may affect endothelial kidney function with the exposition of the proinflammatory and prothrombotic profile, vasoconstriction and capillary obstruction leading to AKI. Furthermore, proinflammatory cytokines may lower bone marrow red cell production by destroying red cell precursors and by reducing erythropoietin receptor expression leading to anemia. Reduction of renal oxygen delivery secondary to hypoperfusion of nephrons and to the low level of hemoglobin may alter aerobic cellular metabolism leading to cellular death. All these factors may be implicated in the pathogenesis of CRS type 1 syndrome. Unfortunately, there exists no specific therapy to interrupt proinflammatory cytokine cascade. Many trials did not find significant improvement in survival using specific immune-modulation therapy.

CRS type 1 syndrome represents a diagnostic and therapeutic challenge. We need to recognize at-risk patients early in order to immediately make the diagnosis or prevent it, allowing individualized approach, for example avoiding unnecessary pharmacological and instrumental diagnostic treatments. In this scenario, biomarker utilization, such as NGAL, may be helpful. In fact, NGAL was a sensitive and specific marker of proximal tubular cell damage with a good diagnostic value for AKI development in AHF patients.

Further studies are needed to better understand the pathogenesis of CRS type 1 syndrome allowing prevention, early diagnosis, and specific therapy.

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Statement of Ethics

This study was approved by the Ethics Committee of San Bortolo Hospital in Vicenza, and the procedures were in accordance with the Helsinki Declaration.

Disclosure Statement

The plasma NGAL assays were donated by Alere Inc. Alere Inc. did not participate in the protocol development, analysis, or interpretation of the results. C. Ronco is a consultant for Alere, and a member of speakers bureau for Abbot Diagnostics. The other authors declare no conflict of interest.

Author Contributions

All persons listed have contributed sufficiently to the project to be included as authors, and all those who are qualified to be authors are listed in the author byline.

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