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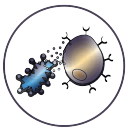

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Plasma Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Combination with Procalcitonin (PCT) and MR-Proadrenomedullin (MR-proADM) in the Diagnosis and Prognosis of Sepsis and Sepsis Associated Acute Kidney Injury

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Abstract

Objective: Early recognition of sepsis is important to prevent progression to severe sepsis and septic shock conditions that may lead to Acute Kidney Injury (AKI). In the present study, the combined measurement of plasma biomarkers NGAL, PCT and MR-proADM in the diagnosis and prognosis of sepsis and sepsis associated acute kidney injury (SA-AKI) was evaluated.

Methods: Twenty patients with sepsis, 18 with localized infections, 17 with SIRS and 24 healthy donors have been consecutively enrolled: NGAL, PCT and MR-proADM were measured at admission (T=0), at 24 hours (T=1) and in the third or fifth day of antibiotic therapy (T=3-5) in septic patients and as a single determination (T=0) in the remaining subjects. PCT and MR-proADM were measured by a time-resolved amplified emission method (Kryptor; Brahms AG, Hennigsdorf, Germany). NGAL was measured by turbidimetric immunoassay (NGAL Test, Bio Porto Diagnostics, Denmark). The creatinine was determined using an enzymatic IDM traced spectrophotometric method (Siemens, Healthcare Diagnostics Inc., Italy). Data were analyzed using the statistical package MedCalc 13.2.2.0.

Results: NGAL values were significantly higher in sepsis than in SIRS and in patients with bacterial localized infections. The discrimination and diagnostic accuracy of NGAL were supported by ROC curve analysis. At the cut-off value of 300 ng/mL, NGAL identified sepsis with high sensitivity and specificity. In sepsis, NGAL was strictly correlated with PCT and MR-proADM. Stratifying subjects of the study population in five different classes on the basis of PCT, NGAL values were correlated with PCT in each class. NGAL, like MR-proADM, was significantly correlated with APACHE II as well as with SOFA scores of disease severity.

Conclusions: Data of the study demonstrated the advantage derived from the combined use of the three markers PCT, MR-proADM and NGAL in septic patients' management and the central role of NGAL in SA-AKI diagnosis and prognosis.

Keywords: NGAL; Sepsis; Multi-marker approach; MR-proADM; Bacterial infection

Introduction

Sepsis is a systemic syndrome characterized by a Systemic Inflammatory Response Syndrome (SIRS) in the presence of a definite or suspected infection. Sepsis is defined "severe" when associated with hypo perfusion or dysfunction of at least one organ system, and can progress to septic shock when severe sepsis is accompanied by persistent hypotension or need for vasopressors [1,2].

SIRS is mediated by innate immune cells, including neutrophils, monocytes and macrophages producing pro-inflammatory cytokines, as Tumor Necrosis Factor (TNF)-α, IL-6, IL1-beta and IL-8 to limit the infection and the consequent tissue damage. In sepsis, the excessive and prolonged production of these cytokines can produce exaggerate inflammatory responses that results more dangerous than the original infection. This is what happen in severe sepsis, where the excessive production of pro-inflammatory cytokines causes tissue injury and lethal multiple organ failure [3,4].

Severe sepsis and septic shock can cause a multiple organ dysfunction syndrome, which induces high morbidity and mortality in critically ill patients [5]. Organ dysfunctions and failures in sepsis patients can be evaluated though the sepsis-related organ failure assessment (SOFA) scores [6].

Sepsis and septic shock are important contributing factors of sepsis associated (SA)-acute kidney injury (AKI) accounting for patients access in the intensive care unit (ICU) and may be an independent risk factor of mortality [7-9]. Sepsis associated AKI usually is accompanied by hyper dynamic circulation [10]. When compared with AKI of non-septic origin, septic AKI is characterized by a distinct pathophysiology and therefore requires a different approach [11]. The pathophysiology, diagnostic procedures, and appropriate therapeutic interventions in sepsis are still highly debatable. Numerous immune modulatory agents showing promise in preclinical studies fail to reduce the overwhelmingly high mortality rate of sepsis. Different markers have been proposed to improve sepsis diagnosis and prognosis.

Procalcitonin (PCT) is a well-established marker for the early diagnosis and staging of sepsis, severe sepsis or septic shock compared to other plasma biomarkers [12]. Recently, another biomarker, the mid-regional proAdrenomedullin (MR-proADM) has been used to differentiate sepsis from non-infectious SIRS with high specificity. The simultaneous evaluation of MR-proADM and PCT in septic patients has been demonstrated to improve the post-test diagnostic probabilities compared to the independent determination of individual markers [13-15].

The diagnosis of AKI is based on plasma creatinine and urine output, as established by the AKI Network (AKIN) criteria [16].

Neutrophil gelatinase-associated lipocalin (NGAL) has been described as a sensitive, specific, and early predictive biomarker for acute kidney injury as described also in a recent meta-analysis where it is reported that 41% of patients with AKI would have been missed using plasma creatinine determination alone [17]. Kim et al. evaluated the diagnostic utility of plasma NGAL to predict SA-AKI in combination with PCT used for sepsis diagnosis and staging support. These authors demonstrated that NGAL was a sensitive marker for AKI in critically ill patients with suspected sepsis and could be useful for the diagnosis and staging of renal failure in sepsis [18]. Recently, the role of NGAL in the identification of SIRS caused by bacterial infection was further evaluated and a specific position of this marker in supporting clinicians to identify bacterial infections identification was suggested [19].

Plasma NGAL levels seem significantly correlated with sepsis severity and AKI progression, but its dosage alone is not sufficient to diagnose sepsis or renal failure with enough specificity. Critically ill patients' evaluation can benefit from a multi-marker approach to confirm sepsis diagnosis, patient prognosis and SA-AKI development.

The aim of the study was to evaluate the combined measurement of plasma NGAL, PCT and MR-proADM in sepsis diagnosis and prognosis and to analyze the role of plasma NGAL as predictive biomarker of worse prognosis in septic patients for its correlation with SA-AKI.

Methods

Patients selection and study design

The study was performed on 55 patients (20 patients with sepsis, 18 patients with localized bacterial infections and 17 patients with not infectious SIRS) consecutively enrolled from the medical and surgical units at the University Hospital Campus Bio-Medico of Rome and 24 healthy donors, between February and July 2015.

Sepsis was defined using the International Guidelines for management of severe sepsis and septic shock: surviving sepsis campaign 2012 based on the presence of a recognized site of infection and evidence of a systemic inflammatory response [20].

SIRS was diagnosed when at least two of the following criteria are evident: (a) body temperature higher than 38°C or lower than 36°C, (b) heart rate higher than 90 beats per minute, (c) respiratory rate higher than 20 breaths per minute or hyperventilation as indicated by an arterial partial pressure of carbon dioxide ($PaCO_2$) lower than 32 mm Hg and (d) a white blood cell count of higher than 12.000 cells/mm3 or lower than 4.000 [20].

Localized bacterial infection was diagnosed when a microorganism was isolated in a culture of biological samples collected from the supposed site of infection and/or in presence of radiological evidence of infectious consolidation or abscess, as reported in the CDC/NHSN Surveillance definitions [21].

Blood samples for blood culture were collected before antibiotic therapy start [20]. Each blood culture comprised three sets (time 0, time 30 and time 60) of one aerobic and one anaerobic broth bottles (Bactec Plus Aerobic/F, Bactec Plus Anaerobic/F, Beckton Dickinson, Franklin Lakes, NJ USA) per patient drawn during 1-h period from cases of clinically suspected bloodstream infection. Blood culture vials were incubated in the Bactec 9240 automated system (Beckton Dickinson, Franklin Lakes, NJ USA). From positive broths, subcultures were prepared and, according to the appearance of colonies on subculture plates, the isolates were identified and the antimicrobial susceptibility test performed by Vitek 2.0 compact instrument (Bio-Merieux, Mercy L'Etoile, France).

Furthermore, depending on the site of infection, further microbiological investigations were carried out: sputum culture, antigen detection of Legionella pneumophila Tipe 1 or Streptococcus peumoniae in the urine, pleural or ascitic fluid culture, urine culture, culture of other collected biological materials (for example abdominal abscesses). Patients and controls characteristics are summarized in Table 1.

The APACHE II score for the 55 patients has been calculated to better define the severity of the infection and the impact of comorbidities on the clinical status of the patients. APACHE II scores were calculated by Medscape, APACHE II scoring system calculator [22]. The SOFA score for the 20 septic patients has been calculated to better define the severity of the sepsis and the renal injury through the renal SOFA sub-score [23,24].

Subjects enrolled in the study population were divided in five different classes on the basis of the PCT values found, as follows [18]:

Class I: PCT<0.05 ng/mL; Class II: PCT=0.05-0.49 ng/mL; Class III: PCT=0.5-1.99 ng/mL; Class IV: PCT2-9.99 ng/mL; Class V: PCT=>10 ng/mL.

Participants provided their written consent to participate in this study. The study was approved by the Ethic Committee of the University Hospital Campus Bio-Medico, and Authors have complied with the World Medical Association Declaration of Helsinki.

Patients	Number	Healthy Controls	Number
Number of patients	55	Number of patients	24
Mean age	74 ± 14	Mean age	65 ± 12
Male	29	Male	9
Female	26	Female	15
Sepsis	20		
Localized infection	18		
SIRS	17		
With comorbidities			
Diabetes	15		
Malignancy	11		
Hypertension	13		
Cardiovascular disease	33		
Gastrointestinal disease	11		
Cerebrovascular disease	08		
Chronic Renal failure	12		

Others	06		
Origin of Sepsis			
Pneumoniae	08		
Intra-abdominal	04		
Urinary tract infection	04		
Other	04		
Site of Localized infection			
Pneumoniae	09		
Intra-abdominal	05		
Urinary tract infection	02		
Other	02		

Table 1: Demographic characteristics of the study population.

Plasma PCT, MR-proADM, NGAL and creatinine measurement

PCT, MR-proADM, NGAL and creatinine were measured at admission (time T=0), at 12-24 hours (time T=1) and in the third or fifth day of antibiotic therapy (time T=3-5) in septic patients, whereas in patients with localized infection or SIRS and in healthy controls as a single determination coinciding with the enrollment time (T=0). PCT and MR-proADM were measured by an automated analyzer using a time-resolved amplified emission method (Kryptor; Brahms AG, Hennigsdorf, Germany), with commercially available assays (Brahms, Germany), as previously described [13,15].

NGAL was measured by the automated new Dimension Vista 1500 (Siemens, Healthcare Diagnostics Inc., Italy), using a particle enhanced turbidimetric immunoassay with the commercially available kit (The NGAL Test, Bio Porto Diagnostics, Denmark). The creatinine was determined using an enzymatic spectrophotometric IDM traced method by the automated analyzer New Dimension Vista 1500 (Siemens, Healthcare Diagnostics Inc., Italy).

Statistical analysis

Normal distribution of PCT, MR-proADM, NGAL and creatinine was analyzed and data subjected to logarithmic transformation to achieve a normal distribution (Kolmogorov-Smirnov test), when necessary.

The Mann-Whitney for independent samples was used to compare at T=0 NGAL, creatinine, PCT and MR-proADM found in the different categories of patients (sepsis, SIRS and patients with localized infection) and in healthy donors. A p-value < 0.05 was considered statistically significant.

The Wilcoxon test for paired samples was used to compare PCT, MR-proADM, NGAL and creatinine values at T=0, T=1 and T=3-5. A p-value < 0.05 was considered statistically significant.

Correlations of the paired NGAL and PCT, MR-proADM and creatinine values in sepsis, in patients with localized infection and with SIRS were assessed by the Spearman rank correlation coefficient (ρ) and p values ≤ 0.05 (two-tailed) were considered significant.

In septic patients the correlation of the paired NGAL and creatinine values was evaluated to ascertain the presence of kidney dysfunction by creatinine measurement and to verify the reported role of NGAL as marker of SA-AKI [18].

In septic patients the correlations between PCT, MR-proADM, NGAL, and APACHE and SOFA scores were evaluated by the Spearman rank correlation coefficient (rho).

Chi-squared for trend has been performed to compare median PCT and NGAL values observed in the five different classes of PCT.

To define the optimal threshold of NGAL and its diagnostic accuracy in sepsis, ROC curve (receiver operating characteristic) and the area under the curve (AUC) were calculated comparing NGAL values in septic patients versus SIRS as well as versus patients with localized bacterial infections [25].

Data were analyzed using the statistical package MedCalc 13.2.2.0 (Med-Calc Software bvba, Belgium).

Results

Patients and controls characteristics

The demographic characteristics of the 55 patients and of the 24 healthy controls enrolled in the study population are summarized in Table 1.

In septic patients, the average APACHE II score was 16.20 (8-29) corresponding to 24% risk of death and the average SOFA score was 4.05 (1-10).

17/20 septic patients developed AKI within three-five days from the onset of symptoms, 3/20 required admission at the Intensive Care Unit (ICU) and 4/20 died for comorbidity complications.

Plasma PCT, MR-proADM, NGAL and creatinine average values comparison (Mann-Whitney for independent samples)

Median, 25th and 75th percentiles of PCT, MR-proADM, NGAL and creatinine in patients with sepsis, localized infection and SIRS and in healthy controls are summarized in Table 2.

PCT, MR-proADM, NGAL and creatinine median values at admission (T=0) were significantly (p<0.01) higher in septic patients than patients with localized infection or SIRS or healthy controls, as reported in Table 3.

Comparison of paired PCT, MR-proADM and NGAL between T0=, T=1 and T=3-5 in septic patients (Wilcoxon test for paired samples)

PCT was significantly reduced between T=0 and T=3-5 (p=0.0003) as well as between T=1 and T=3-5 (p=0.01), whereas it was not between T=0 and T=1. NGAL and MR-proADM were significantly (p<0.05) reduced only between T=1 and T=3-5, because T=0 values were lower than T=1.

T=0									
Study population	**N**	**PCT ng/mL**		**MR-proADM nmol/l**		**NGAL ng/ml**		**Creatinine mg/dl**	
		median	**25th-75th percentiles**	**median**	**25th-75th percentiles**	**median**	**25th-75th percentiles**	**median**	**25th-75th percentiles**
Sepsis	20	2.65	0.17 – 80.20	3.90	2.64 – 6.38	796.30	412.87 – 1091.80	1.92	1.38 – 2.37
LI	18	0.13	0.09 – 0.30	0.97	0.59 – 1.91	250.44	184.00 – 331.00	0.99	0.80 – 1.48
SIRS	17	0.20	0.13 – 0.37	1.00	0.59 – 1.57	204.00	160.73 – 239.48	0.82	0.63 – 1.23
Healthy	24	0.06	0.04 – 0.07	0.50	0.42 – 0.70	98.29	92.83 – 123.97	0.73	0.67 – 0.90
T=1									
Sepsis	20	1.70	0.54 – 20.30	3.94	2.53 – 5.40	763.50	425.42 – 1435.47	1.78	1.26 – 2.30
T=3-5									
Sepsis	20	0.57	0.16 – 4.84	3.09	2.02 – 4.29	519.50	288.17 – 771.08	1.22	1.03 – 1.62

Table 2: Median, 25th and 75th percentiles of plasma PCT, MR-pro ADM, NGAL and creatinine at admission (T=0) in all patients and healthy controls, at T=1 and at T=3-5 in sepsis patients N= number of subjects; LI= Localized infection.

	Sepsis	**LI**	**p**	**Sepsis**	**SIRS**	**p**	**Sepsis**	**Healthy**	**p**
PCT ng/ml T=0	2.65	0.13	<0.0001	2.65	0.20	0.0001	2.65	0.06	<0.0001
MR-proADM nmol/l T=0	3.90	0.97	<0.0001	3.90	1.00	<0.0001	3.90	0.50	<0.0001
NGAL ng/ml T=0	796.30	250.44	0.0003	796.30	204.00	0.0001	796.30	98.29	<0.0001
Creatinine mg/dL T=0	1.92	0.99	0.0002	1.92	0.82	<0.0001	1.92	0.73	<0.0001

Table 3: Median value of plasma PCT, MR-proADM, NGAL and creatinine at admission (T=0): comparison between septic patients vs. patients with localized infection (LI), SIRS and healthy controls (Mann-Whitney test) LI = Localized infection.

Rank-correlation of plasma NGAL with paired PCT, MR-proADM and creatinine values in the patients population

At admission (T=0), in septic patients plasma NGAL values were significantly correlated ($p<0.05$) with PCT, MR-proADM and creatinine values (Figure 1), whereas these correlations were not found in patients with localized infection or SIRS, except for creatinine in SIRS patients ($p<0.05$) (Table 4). At T=1 and T=3-5 NGAL continued to be significantly correlated ($p<0.05$) with PCT and MR-proADM, whereas the correlation with creatinine was present at T=1 but not at T=3-5.

	PCT T=0		**MR-proADM T=0**		**Creatinine T=0**	
	ρ	p	ρ	p	ρ	p
NGAL T=0 sepsis	0.73	0.00003	0.60	0.005	0.57	0.008
NGAL T=0 LI	0.24	n.s.	0.27	n.s.	0.25	n.s.
NGAL T=0 SIRS	0.37	n.s.	0.05	n.s.	0.55	0.023

Table 4: Rank-Correlation (Spearman rank correlation coefficient ρ) between plasma NGAL and PCT, MR-proADM and creatinine at admission (T=0) in sepsis, patients with localized infection (LI) and SIRS.

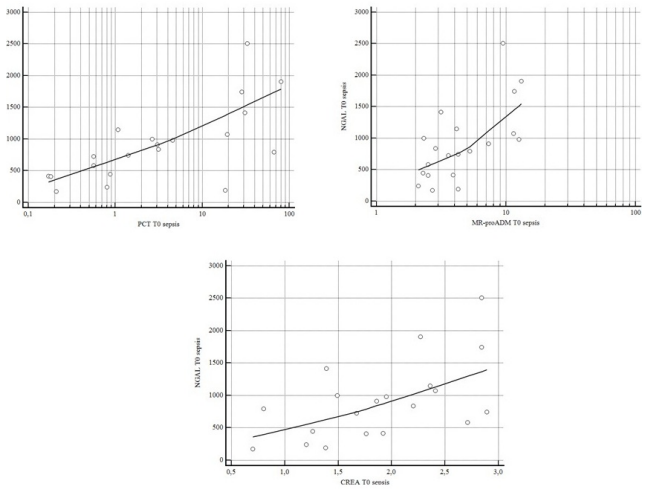

Figure 1: Rank-correlation between NGAL and PCT (logarithmic transformation of data), MR-proADM (logarithmic transformation of data) and Creatinine in septic patients at admission (T=0).

Plasma PCT and NGAL values according to the five PCT classes used to classify subjects enrolled in the study population

Mean and median values of PCT and NGAL found in each PCT class are reported in Table 5. PCT and NGAL values were significantly ($p<0.0001$) correlated in each class as confirmed by the rank-correlation analysis. Sepsis patients are almost distributed in PCT classes III (0.50-1.99 ng/mL), IV (2.0-9.99 ng/mL) and V (>10 ng/mL) corresponding to PCT values above the diagnostic cut-off for sepsis (0.50 ng/mL) than patients with SIRS and localized bacterial infections (Classes I and II) (Table 5 and Figure 2). NGAL values found in septic patients follow the same trend of PCT as shown in Table 5. In class III, IV and V, NGAL median values grow-up from 584 ng/mL to 1419 ng/mL rather than patients with SIRS and localized bacterial infections (Table 5 and Figure 2). Healthy subjects fell into PCT class I and are characterized by low values of PCT and NGAL (Table 5 and Figure 2). PCT and NGAL showed an increased statistically significant trend, according to the five different classes of PCT (Table 5).

Rank-correlation between plasma NGAL, PCT, MR-proADM values and APACHE II and SOFA scores

The analysis of the rank-correlation between NGAL values and APACHE II and SOFA scores showed a significant correlation with APACHE II ($p<0.05$) as well as with SOFA score ($p<0.05$) and within this also with the Renal Sub score ($p<0.05$). MR-proADM was significantly correlated with APACHE and SOFA scores ($p<0.05$) whereas PCT was not.

Plasma NGAL and sepsis diagnostic accuracy: ROC curves and areas under the curves (AUCs) analysis

ROC curves and AUCs analysis was performed comparing NGAL values measured in septic patients and those found in SIRS and localized infections. Based upon this analysis, the optimal decision threshold for plasma NGAL values in the diagnosis of sepsis, allowing distinguishing non-infectious SIRS and localized infections from sepsis, was defined as 300 ng/mL. ROC plots analysis is reported in Figure 3.

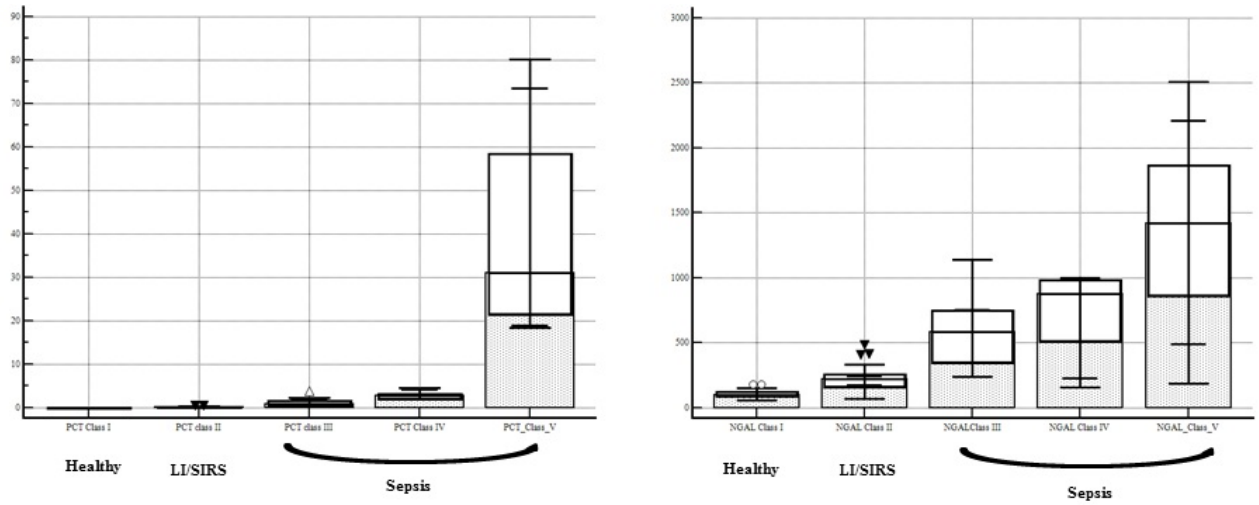

Figure 2: Plasma NGAL values in the five PCT classes and distribution of the study subjects along the different classes of PCT (LI=localized bacterial infections).

	PCT class I (n=24) <0.05 ng/mL		PCT class II (n=33) 0.05-0.49 ng/mL		PCT class III (n=9) 0.50-1.99 ng/mL		PCT class IV (n=6) 2.0-9.99 ng/mL		PCT class V (n=7) >10 ng/mL		^{2} for trend	
	PCT ng/mL	NGAL ng/mL	PCT ng/mL	NGAL ng/mL	PCT ng/mL	NGAL ng/mL	PCT ng/mL	NGAL ng/mL	PCT ng/mL	NGAL ng/mL	PCT	NGAL
Mean	0.02	108.8	0.17	225.7	1.22	594.0	2.92	734.2	39.60	1377.6	7.769 (p<0.001)	4.354 (p<0.05)
CI 95%	0.016-0.024	96.2-121.4	0.13-0.21	192.3-259.2	0.32-2.12	378.1-809.9	1.97-3.87	383.4-1084.9	17.3-61.90	676.8-2088.3		
Median	0.02	98.0	0.14	220.5	0.87	584.0	2.83	876.5	31.00	1419	20.885 (p<0.001)	22.642 (p<0.001)
CI 95%	0.01-0.03	93.7-120.2	0.12-0.18		0.29-2.16	350.5-755.2	2.09-4.27	225.9-996.1	19.90-73.60	490.1-2211.8		

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Table 5: Plasma PCT and NGAL mean, median values, confidence interval (CI) 95% and χ^2 for trend according to the five different classes of PCT.

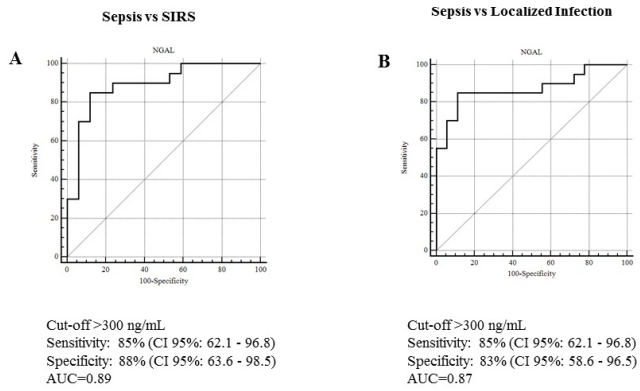

Figure 3: NGAL ROC plots analysis. Comparison between patients with sepsis and patients with SIRS (A) and patients with sepsis and patients with localized infections (B): Areas under the Curve (AUCs) corresponding to the cut-off value of 300 ng/mL.

Discussion

Sepsis is a leading cause of mortality in hospitalized critically ill patients and a prompt diagnosis is needed for a rapid targeted antibiotic therapy start. Early recognition of sepsis is important to prevent progression to severe sepsis and septic shock that can determine multiple organ dysfunction syndromes [5]. Severe sepsis and septic shock are major contributing conditions to AKI, an independent risk factor of mortality especially in critically ill patients [7-9].

NGAL has been described as a sensitive, specific, and early predictive biomarker for AKI [17] and its diagnostic role in combination with PCT to predict SA-AKI has been reported [18,19]. Furthermore, its association with severe sepsis has been recently reported [26].

In this study, the role of plasma NGAL in sepsis diagnosis and prognosis was evaluated in combination with PCT and MR-proADM biomarkers, whose roles in sepsis have been previously described [13-15].

Plasma NGAL values were significantly higher in septic patients than patients with SIRS and patients with bacterial localized infections. These data support the hypothesis that plasma NGAL could distinguish sepsis from other inflammatory conditions as supported by ROC curve analysis (Table 3 and Figure 3). At the cut-off value of 300 ng/mL NGAL distinguish septic patients from SIRS and localized infections with high sensitivity and specificity (Figure 3). Plasma NGAL is increased in septic patients and significantly correlated with PCT and MR-proADM markers that rise in case of sepsis, as previously described [13-15]. This strict correlation could be useful to further confirm the presence of a sepsis and to improve the management of critically ill patients.

Stratifying subjects of the study population in five different classes on the basis of PCT values (Figure 2), plasma NGAL values were significantly correlated with PCT in each class, thus confirming that their proportional increases are in agreement with PCT and sepsis probability, as showed by the chi-squared for trend. Septic patients are distributed in highest PCT classes (III-V) corresponding to PCT values above the cut-off (0.5 ng/mL) currently in use for sepsis diagnosis [15, 18]. Interestingly, in septic patients plasma NGAL values are in a growing range (594 ng/mL-1377 ng/mL) above the cut-off of 300 ng/mL defined in this study by the ROC curve analysis, whereas in patients with SIRS and bacterial localized infections NGAL values are under the cut-off (mean value 225 ng/mL) and positioned in the lower PCT class II (Table 5 and Figure 2).

Being NGAL an early predictive biomarker for AKI [17], its correlation with creatinine has been evaluated to define the presence of acute kidney injury and its role in SA-AKI definition. Plasma NGAL and creatinine were correlated at T=0 and T=1 but not at T=3-5, otherwise PCT and MR-proADM that were correlated at all times. This evidence could suggest that NGAL, besides marker of AKI, as previously described [17], could be determinant in acute kidney injury diagnosis during sepsis (SA-AKI) when measured simultaneously with other specific markers of sepsis.

Plasma NGAL, like MR-proADM already described as marker of sepsis prognosis, was significantly correlated with APACHE II as well as with SOFA scores. These correlations suggest a role of plasma NGAL as marker of disease severity. Furthermore, NGAL demonstrated to be correlated also with the renal sub-score of the SOFA score further confirming its role in SA-AKI diagnosis.

Overall, data from his study, could suggest the advantage derived from the combined use of the three plasmatic markers PCT, MR-proADM and NGAL in septic patients management and the central role of plasma NGAL in SA-AKI. Plasma NGAL values over the cut-off could suggest the need for aggressive therapeutic measure to improve patient outcome by multiple organ failure prevention.

Study Limitation

A limitation of the study could be the limited number of patients included, but given the statistical significance reached we assumed that increasing the population numbers the results will maintain their significance.

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