


# Increase of circulating endocan over sepsis follow-up is associated with progression into organ dysfunction

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**Abstract** How circulating inflammatory mediators change upon sepsis progression has not been studied. We studied the follow-up changes of circulating vasoactive peptides and cytokines until the improvement or the worsening of a patient and progression into specific organ dysfunctions. In a prospective study, concentrations of tumor necrosis factor-alpha (TNF $\alpha$ ), interleukin (IL)-6, IL-8, IL-10, interferon-gamma (IFN $\gamma$ ), endocan and angiopoietin-2 (Ang-2) were measured in serum by an enzyme immunoassay in 175 patients at baseline; this was repeated within 24 h upon progression into new organ dysfunction ( $n = 141$ ) or improvement ( $n = 34$ ). Endocan and Ang-2 were the only parameters that were significantly increased among patients who worsened. Any increase of endocan was associated with worsening with odds ratio 16.65 ( $p < 0.0001$ ). This increase was independently associated with progression into acute respiratory distress syndrome (ARDS) as shown after logistic regression analysis (odds ratio 2.91,  $p: 0.002$ ). Changes of circulating cytokines

do not mediate worsening of the critically ill patients. Instead endocan and Ang2 are increased and this may be interpreted as a key-playing role in the pathogenesis of ARDS and septic shock. Any increase of endocan is a surrogate of worsening of the clinical course.

## Introduction

Despite progress made in early recognition and management, sepsis remains the leading cause of death accounting for more than 200,000 deaths every year in the United States [1]. Sepsis is nowadays defined as a life-threatening organ dysfunction induced from the dysregulated response of the host to an infection [2]. This definition is coming from our better understanding of the complex pathophysiology of sepsis acquired in recent years. We have managed to understand that when sepsis develops not all patients react in a similar way; in some hosts a

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pro-inflammatory response prevails whereas in others an anti-inflammatory response emerges [3]. However, this distinction is not clear-cut in all patients. Everyday practice teaches that unfavourable outcome is due to the deterioration of the initial host response to progressive organ dysfunction. It is highly probable that the mechanisms underlying this deterioration are individualized from patient to patient. These mechanisms are poorly studied and their knowledge is mandatory for future immunotherapy studies.

Deterioration of the host from an initial septic state to multiple organ dysfunctions most probably results from the dynamic interaction of circulating cytokines and peptides secreted from endothelial cells. Vascular endothelium is stimulated by cytokines for the production of angiopoietin-2 (Ang-2) and endocan. Ang-2 increases vascular permeability and mediates progression into shock [4] whereas endocan inhibits leukocyte migration in the lungs and kidneys [5]. Our aims were to monitor the changes of circulating levels of pro-inflammatory and anti-inflammatory cytokines and of vasoactive peptides of critically ill patients at well-defined time-points of the clinical course and to understand how these changes mediate progression to organ dysfunction in an individualized way.

## Patients and methods

### Study design

This is a prospective study that was conducted in ten Intensive Care Units (ICUs) participating in the Hellenic Sepsis Study Group from January 2013 until December 2014. The study protocol was approved by the Ethics Committees of the participating hospitals. A patient was enrolled after written informed consent provided by his first-degree relatives.

Inclusion criteria were: (a) written informed consent, (b) age equal to or above 18 years, (c) both genders, (d) at least two signs of the systemic inflammatory response syndrome (SIRS) as defined elsewhere [6], (e) one of the following infections: acute pyelonephritis, community-acquired pneumonia, acute intraabdominal infection, primary bacteremia or ventilator-associated pneumonia. The diagnosis of infections was done by international definitions [7], (f) first blood sampling within the first 24 h from the presentation of the first two signs of SIRS, and (g) repeat blood sampling at a follow-up time point. The follow-up time point could be 72 h from the first blood sampling or within the first 24 h from the onset of any new organ dysfunction provided that this was at least 72 h apart from the first blood sampling. This 72-h time difference between the two time-points of sampling was selected in order to increase the likelihood that the changes described over-time of the studied mediators were associated with the new organ dysfunction and not with the existing baseline inflammatory process.

Exclusion criteria were: (a) infection by the human immunodeficiency virus –1, (b) neutropenia defined as an absolute neutrophil count less than  $1000/\text{mm}^3$  unless due to SIRS per se, and (c) intake of corticosteroids at a daily dose of more than 0.4 mg/kg of equivalent prednisone for at least 15 consecutive days.

Five ml of blood was sampled after venipuncture of one forearm vein under sterile conditions. Blood was poured into pyrogen-free tubes (Vacutainer, Becton Dickinson, Cockeysville, MD) and centrifuged at room temperature. The supernatant serum was immediately aliquoted and aliquots were shipped in dry-ice on the same day into the central lab at the Laboratory of Immunology of Infectious Diseases as the 4th Department of Internal Medicine of ATTIKON University Hospital. Aliquots were stored there at  $-80\text{ }^\circ\text{C}$  until measurement.

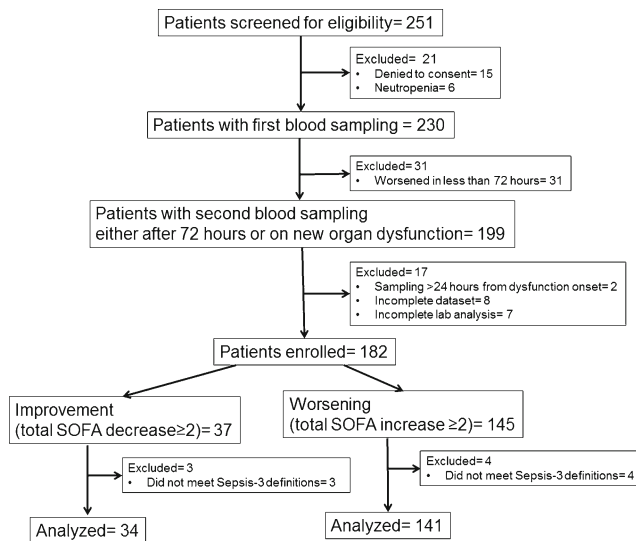
Patients were then followed-up daily for 28 days. Patients who progressed into new organ dysfunctions less than 72 h from the first blood sampling were excluded from the study. A second blood sample was collected from the remaining patients 72 h after the first blood sampling. Patients were then followed daily for 28 days and sequential organ failure assessment (SOFA) score was measured daily. If the patient was presenting with a new organ dysfunction, blood sampling was done within 24 h and the blood sample drawn at 72 h was discarded. If not, the blood sample originally collected at 72 h was processed for analysis to be used as comparator.

Organ dysfunctions were defined as follows: (a) acute respiratory distress syndrome (ARDS) as diffuse shadows in chest X-ray and ratio of partial oxygen tension to fraction of inspired oxygen below 200 [6], (b) acute kidney injury (AKI) as less than 0.5 mg/kg/min of urine output for at least two consecutive hours provided that the negative fluid balance was restored [6], (c) disseminated intravascular coagulation (DIC) as absolute platelet count below  $100,000/\text{mm}^3$  accompanied by an increase of the concentrations of D-dimers and decrease of circulating fibrinogen [8], and (d) septic shock as decrease of systolic arterial pressure below 90 mmHg not responding to fluid resuscitation and requiring the administration of vasopressors [6].

For all patients retrospective evaluation was done using the recently published Sepsis-3 definitions [2]. Those who did not meet the new definitions at study enrolment were excluded from analysis.

### Laboratory measurements

Concentrations of tumor necrosis factor-alpha ( $\text{TNF}\alpha$ ), interleukin (IL)-6, IL-8, IL-10, interferon-gamma ( $\text{IFN}\gamma$ ), angiopoietin-2 (Ang-2) and of endocan were measured by an enzyme immunoassay. Reagents for  $\text{TNF}\alpha$ , IL-6, IL-8, IL-10 and  $\text{IFN}\gamma$  were purchased from eBiosciences (San



**Fig. 1** Study flow chart. *SOFA* sequential organ failure assessment

Diego, USA); those for Ang-2 by R&D (Minneapolis, USA); those for endocan were a kind donation by

Lunginnov s.a.s (Campus de l’Institut Pasteur de Lille, 59,000 Lille, France). The lower limits of detection were 4 pg/ml for TNF $\alpha$ , IL-6, IL-8, IL-10 and IFN $\gamma$ ; 40 pg/ml for Ang-2; and 0.31 ng/ml for endocan.

**Study endpoints**

The primary study endpoint was the correlation between the follow-up changes of each measured parameter from baseline until the improvement or the worsening of a patient. A patient was considered worsened if his total SOFA score was increased on the follow-up time point by at least two points from the baseline SOFA score on study enrolment. A patient was considered improved if his total SOFA score was decreased on the follow-up time point by at least two points from the baseline SOFA score on study enrolment. The secondary endpoint was the comparative changes of the measured parameters for specific organ dysfunctions.

**Table 1** Demographic characteristics of the 175 patients enrolled

Variable	Worsened (n = 141)	Improved (n = 34)	p-value
Male/female (n, %)	86 (61.0)/55 (39.0)	25 (73.5)/9 (26.5)	0.234
Age (years, mean $\pm$ SD)	65.9 $\pm$ 18.1	70.8 $\pm$ 19.6	0.177
APACHE II score (mean $\pm$ SD)	19.0 $\pm$ 8.2	18.0 $\pm$ 6.6	0.505
Baseline SOFA score (mean $\pm$ SD)	5.18 $\pm$ 3.13	6.56 $\pm$ 2.72	0.020
Follow-up SOFA score (mean $\pm$ SD)	9.56 $\pm$ 3.76	2.76 $\pm$ 2.11	3.1 $\times$ 10 <sup>-19</sup>
White blood cells (/mm <sup>3</sup> , mean $\pm$ SD)	13,749.8 $\pm$ 8007.1	15,476.4 $\pm$ 8350.5	0.274
Type of infection (n, %)			
Acute pyelonephritis	18 (13.0)	7 (20.6)	0.242
Community-acquired pneumonia	29 (20.6)	5 (14.7)	0.438
Intrabdominal	19 (13.5)	7 (20.6)	0.303
Primary bacteremia	31 (22.0)	6 (17.6)	0.578
Ventilator-associated pneumonia	33 (23.4)	8 (23.5)	1.000
Hospital-acquired pneumonia	11 (7.8)	1 (2.9)	0.314
Isolated pathogen (n, %)			
<i>Klebsiella pneumoniae</i>	23 (16.3)	5 (14.7)	0.818
<i>Acinetobacter baumannii</i>	13 (9.2)	3 (8.8)	0.942
<i>Pseudomonas aeruginosa</i>	13 (9.2)	5 (14.7)	0.344
<i>Escherichia coli</i>	8 (5.7)	7 (20.5)	0.005
Other	16 (11.3)	2 (5.9)	0.346
Co-morbidities (n, %)			
Type 2 diabetes mellitus	28 (19.9)	12 (35.3)	0.069
Chronic heart failure	19 (13.5)	8 (23.5)	0.184
Chronic obstructive pulmonary disease	27 (19.1)	6 (17.6)	1.000
Chronic renal disease	14 (9.9)	4 (11.8)	0.755
Solid tumor malignancy	14 (9.9)	4 (11.8)	0.755
Multiple injuries	21 (14.9)	4 (11.8)	0.789
28-day mortality (n, %)	65 (46.1)	0 (0)	1.6 $\times$ 10 <sup>-8</sup>

APACHE acute physiology and chronic health evaluation, SOFA sequential organ failure assessment

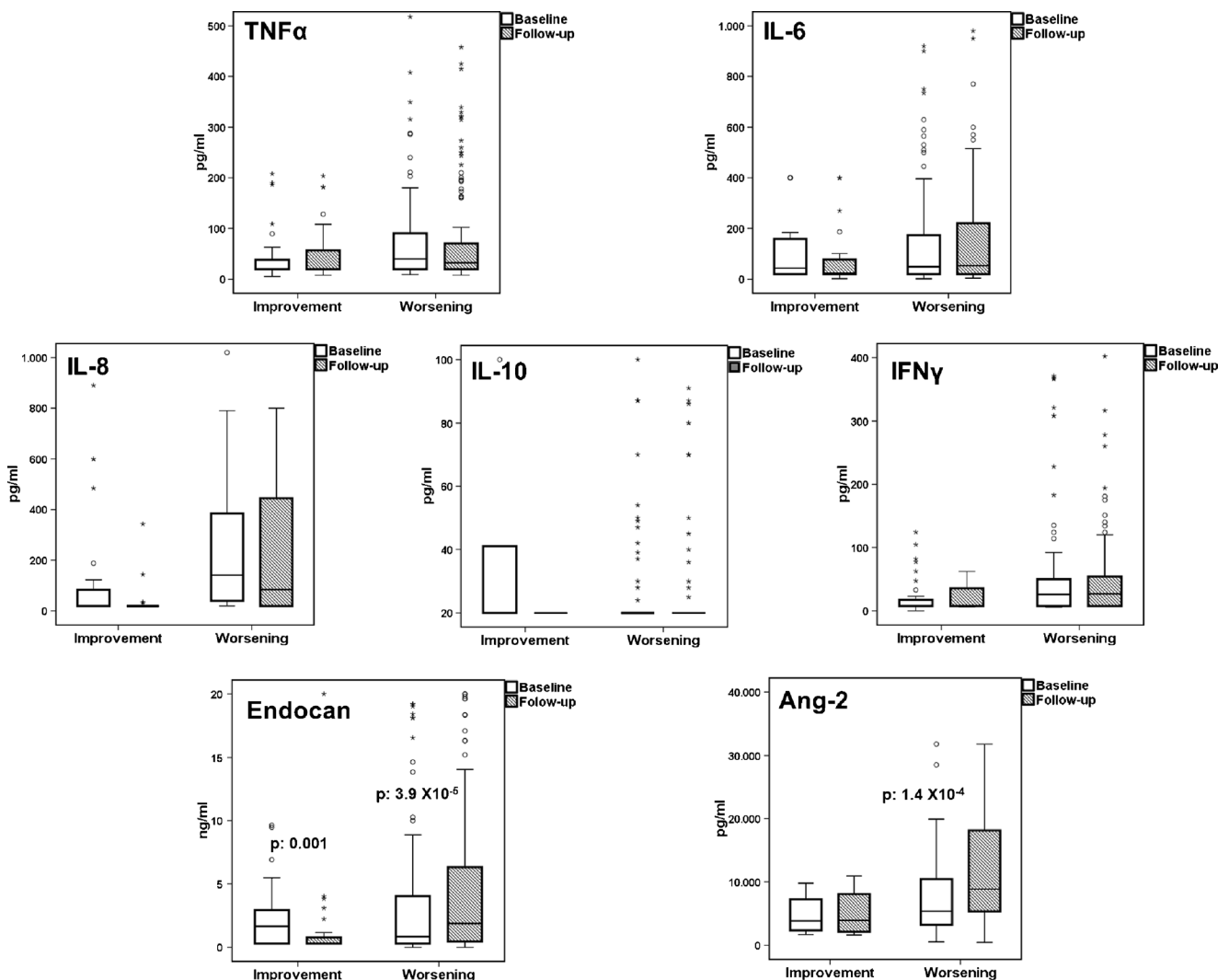
## Statistical analysis

Results of measured parameters were expressed as median and 95% confidence intervals. For the primary endpoint, pair-wise comparisons were done between the two time points of sampling separately for patients worsening and for patients improving by the Wilcoxon test. The percent of change of parameters from baseline was analyzed by receiver operating characteristic curve (ROC) analysis. Using co-ordinate points of the curve, the cut-off with specificity greater than 90% for worsening was chosen. The sensitivity, specificity, positive and negative predictive values of that cut-off were calculated. The odds ratio (OR) and 95% confidence interval (CI) for worsening at the depicted cut-off point were calculated by Mantel and Haenzel's statistics. For the secondary endpoint, pair-wise

comparisons between baseline and follow-up measurements were done within the subgroups of patients developing specific new organ dysfunctions. The association of changes of circulating peptides with the development of a specific organ failure was further analyzed by logistic regression analysis; the presence of at least one comorbidity and baseline severity entered the equation as co-variates. Any value of  $p$  below 0.05 after correction for multiple comparisons was considered significant.

## Results

The study flow-chart is shown in Fig. 1. A total of 251 patients were screened. The average number of beds per participating ICU was ten. Taking into consideration that the median patient



**Fig. 2** Comparative concentrations of endocan, angiotensin-2 (Ang-2) and cytokines on initial presentation of sepsis (baseline) and on follow-up. Patients are divided into those with worsening of their clinical course with a new organ dysfunction ( $n = 141$ ) and into those with improving

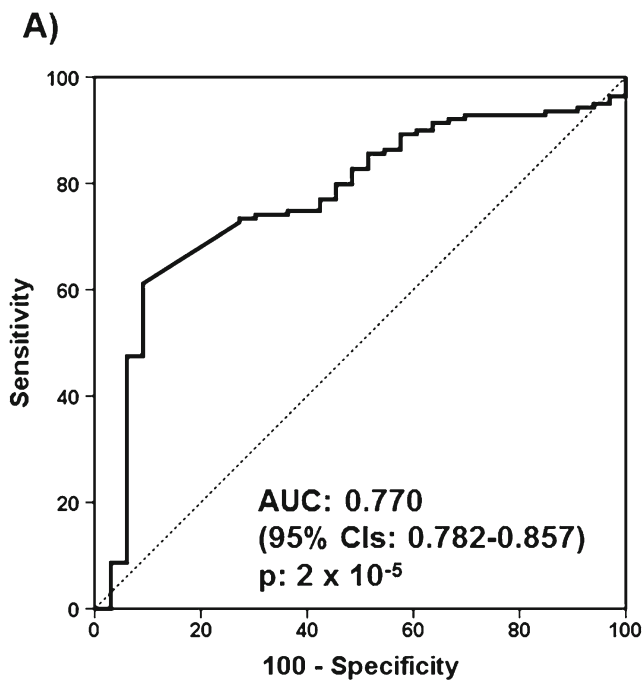
clinical course ( $n = 34$ ). Only the  $p$  values of comparisons that provided statistical significance are provided. *IFN $\gamma$*  interferon-gamma, *IL* interleukin, *TNF $\alpha$*  tumor necrosis factor-alpha

turnaround time per bed was 25 days, the study screening rate is fully explained. A total of 175 patients were finally analyzed; 141 patients progressed into a new organ dysfunction; and 34 patients were improved. Their baseline characteristics are shown in Table 1. In the same table, it is shown that the SOFA score was significantly increased in the subgroup of patients who worsened; it was significantly decreased in the subgroup of patients who improved.

Pair-wise comparisons showed that the only parameters that were significantly increased upon worsening of the patients were endocan and Ang-2 (Fig. 2). Endocan significantly decreased among patients who improved. ROC curves of the percent changes of endocan and Ang-2 from the baseline were designed to explore the use of endocan and of Ang-2 as

surrogate markers of worsening. Only the ROC curve of the change of endocan provided a significant area under the curve (Fig. 3a). Using the coordinate points of ROC curve, it was found that any increase of endocan from baseline could be associated with sepsis worsening with 91.2% sensitivity and 96.7% positive predictive value (Fig. 3b). Any baseline increase of endocan was associated with OR 16.65 (95% CIs: 4.85–57.11,  $p: 8 \times 10^{-6}$ ) for worsening.

When pair-wise comparisons between baseline and follow-up measurements were done within the subgroups of patients developing new organ dysfunctions, it was found that the only parameters significantly changing were endocan and Ang-2. More precisely, both were increased on development of ARDS and septic shock; Ang-2 was also increased on development of AKI (Fig. 4). No changes were found upon development of DIC (data not shown). Logistic regression analysis showed that any increase of endocan from baseline was independently associated with the progression into ARDS. A similar association was shown for the progression into septic shock with a trend towards statistical significance (Table 2).



### Discussion

The present study measured the concentrations of a range of circulating protein molecules in the sera of patients at ICU admission and upon progression into a new organ dysfunction. Results showed that only vasoactive and endothelial-derived molecules like endocan and Ang-2 are increased upon deterioration of the patient into a more severe stage as this is defined by any increase of the total SOFA score by at least two points. Increase of endocan and Ang-2 were mainly found on progression into ARDS and septic shock whereas Ang-2 was also increased among patients who progressed into AKI. Any increase of endocan was associated with increased likelihood for the deterioration of a patient.

In a recently published single-center prospective study, 14 patients with SIRS, nine patients with sepsis, 35 patients with severe sepsis and 92 patients with septic shock were enrolled. Serum levels of endocan were measured on days 1, 3 and 8. Results showed that concentrations of endocan were greater among patients at septic shock, and that day 1 levels of endocan could prognosticate both 30-day and six-month outcome [9]. Although at first glance our study appears similar in design, it presents with some clearly distinctive characteristics in the design: (a) serial sampling at clinically indicative time points of progression into a new organ dysfunction or improvement, (b) analysis based on the new Sepsis-3 definitions, and (c) correlation of changes of baseline endocan with progression into specific organ dysfunctions.

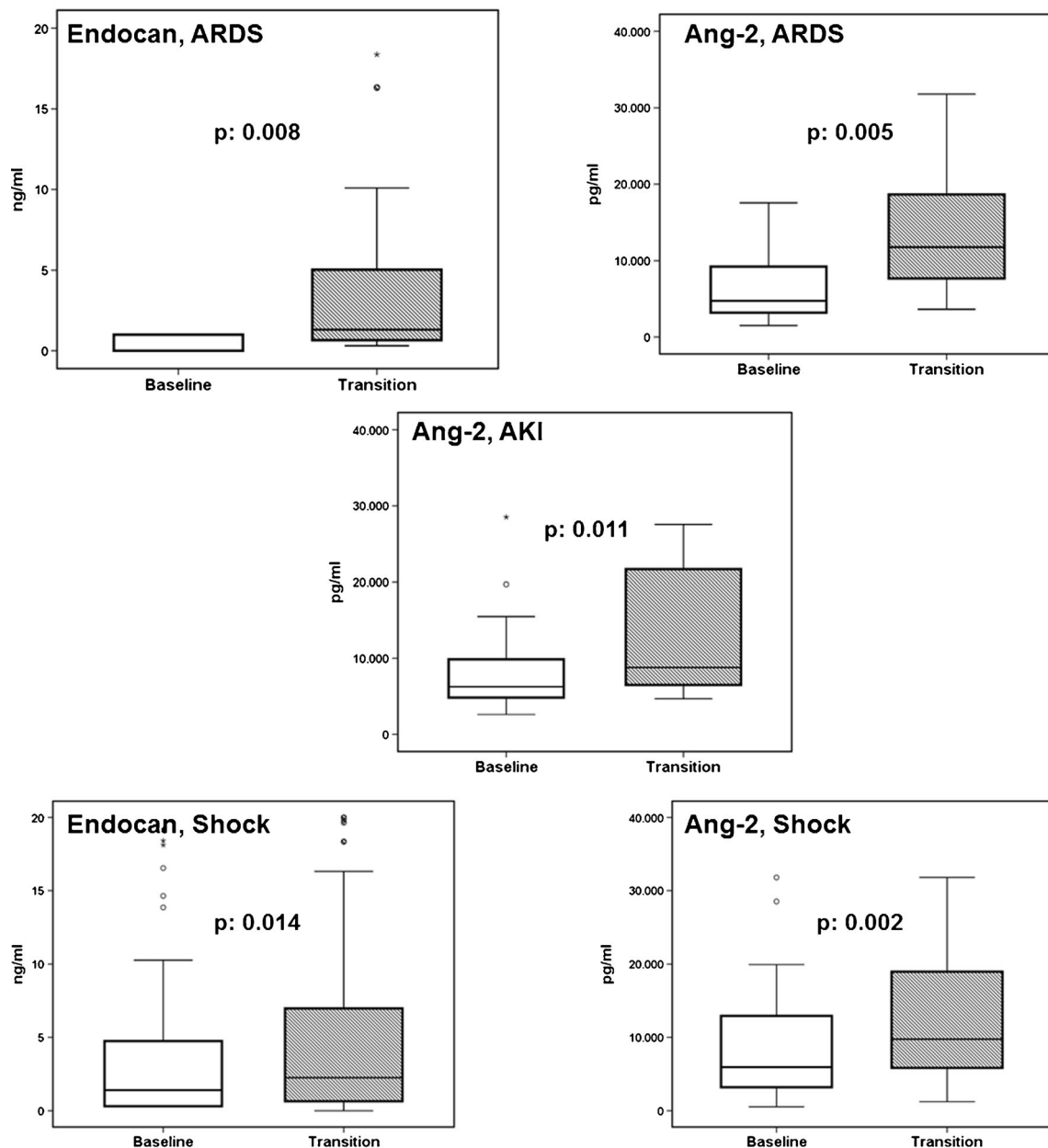
Endocan is a proteoglycan located on endothelial cells of the lungs and kidneys. At the event of a systemic

**B)**

	Worsening	Improvement	Total
<b>Increase</b>	87	3	90
	Sens: 61.7%		
	PPV: 96.7%		
<b>Decrease</b>	54	31	85
	Spec: 91.2%		
	NPV: 36.5%		
<b>Total</b>	141	34	175

**Fig. 3** Change of endocan from baseline as prognostic of the clinical course of sepsis. **a** ROC curve of the % change of baseline endocan for worsening of the clinical course. **b** Distribution of patients into worsening and improvement of their clinical course in relation to an absolute increase or decrease of baseline endocan on follow-up. *AUC* area under curve, *CIs*: confidence intervals, *NPV* negative predictive value, *PPV* positive predictive value, *Sens* sensitivity, *Spec* specificity





**Fig. 4** Concentrations of endocan and of angiopoietin-2 (Ang-2) at sepsis baseline and on progression to the indicated organ dysfunction. *P* values of the indicated comparisons are provided. *ARDS* acute respiratory distress syndrome, *AKI* acute kidney injury

inflammation, the molecule is cleaved through the activity of cathepsin G of neutrophils and a 14 kDa polypeptide is generated at greater concentrations in patients with sepsis than in healthy volunteers [10] and also in patients with severe sepsis and septic shock than in patients with SIRS [9, 11]. Former studies have shown that this glycoprotein may be involved in the pathophysiology of an infection without its contribution been clearly defined. In a prospective study of 78 patients, serum endocan greater than 1.70 ng/ml was associated with the presence of bacteremia [12]. Our overall findings are in general agreement with those of recent studies enrolling a lower number of patients. These studies have explored the

probable associations of admission endocan levels with the development of organ failures within two to five days. In a study of 48 patients with multiple injuries, 24 developed acute lung injury (ALI) and 24 did not. Admission levels of endocan in the ICU were lower among patients who developed ALI compared to those who did not [13]. A similar finding was described in 20 critically ill patients; admission serum endocan was lower among those who developed respiratory failure on day 3 [14]. Their findings may at first appear contradictory to ours. However follow-up measurements were missing so it could be hypothesized that serum endocan might increase on the day of respiratory failure.

**Table 2** Logistic regression analysis of the association of changes of endocan with the development of acute respiratory distress syndrome (ARDS) and septic shock

Measure	Odds ratio	95% CIs	p-value
<b>Progression into ARDS</b>			
Any increase of endocan	2.91	1.47–5.76	0.002
Presence of at least one comorbidity <sup>a</sup>	0.85	0.44–1.65	0.632
Baseline SOFA score	1.08	0.96–1.19	0.182
<b>Progression into septic shock</b>			
Any increase of endocan	2.01	0.93–4.36	0.077
Presence of at least one comorbidity <sup>a</sup>	1.85	0.85–4.01	0.121
Baseline SOFA score	1.05	0.93–1.18	0.444

CI confidence interval, SOFA sequential organ failure assessment

<sup>a</sup>Type 2 diabetes mellitus, chronic heart failure, chronic obstructive pulmonary disease, chronic renal disease, solid tumor malignancy, multiple injuries

Two more studies have linked admission endocan levels with the prognosis of the clinical course of sepsis. The first study on 60 critically ill patients showed that endocan was greater among patients who developed organ failures and MODS within the first 48 h post admission [15]. The second study on 42 patients with ARDS showed that admission endocan levels were greater in non-survivors than in survivors [16].

Our study is not using the traditional design that compares the concentrations of circulating peptides between patients with infection and patients with sepsis. Instead, all enrolled patients had sepsis at baseline and the changes of circulating peptides between those improving and those worsening were compared. Our findings suggest that changes of circulating cytokines do not mediate progression of the clinical course of sepsis. Probably this is explained by the fact that upon deterioration of sepsis the patient is already at the state of immunosuppression where further cytokine production does not take place [17, 18]. Instead endocan and Ang2 are increased and this may also be interpreted as a key-playing role in the pathogenesis of ARDS and septic shock. From the practical aspect, it becomes evident that any increase of endocan is a surrogate of prediction for the overall deterioration of the patient.

Our findings clearly show that endocan and angiopoietin-2 are mediators increased upon deterioration of the clinical course of sepsis. Any baseline increase of endocan is a specific surrogate marker to indicate worsening of the patient. Increases of endocan are independently associated with progression into adult respiratory distress syndrome. The findings require validation by an independent cohort where changes of endocan are correlated with respective changes of the SOFA score.

**Compliance with ethical standards** The study protocol was approved by the Ethics Committees of the participating hospitals.

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**Conflict of interest** None of the authors has any conflict of interest related to this submission.

**Ethical approval** The study protocol was approved by the Ethics Committees of Korinthos General Hospital, Korgialeneion Benakeion General Hospital, ATTIKON University Hospital, "G.Gennimatas" Thessaloniki General Hospital, Sotiria General Hospital, "Aghios Pavlos" Thessaloniki General Hospital, and Tzaneion General Hospital.

**Informed consent** A patient was enrolled after written informed consent provided by his first-degree relatives.

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