RESEARCH ARTICLE

Predictive ability of circulating osteoprotegerin as a novel biomarker for early detection of acute kidney injury induced by sepsis

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ABSTRACT. ***Background:*** **Though significant progress has been made towards new diagnostic approaches for early detection of acute kidney injury (AKI) induced by different factors, there is still an urgent demand for a more specific and predictive biomarker for each type. The aim of this study is to unravel the potential diagnostic utility of circulating osteoprotegerin (OPG) in septic patients who developed AKI in the ICU, compared to cystatin C (a renal function maker) and KIM-1 (a kidney damage marker).** ***Methods:*** **Eighty patients (male = 43, female = 37) with ages ranging from 42 to 46 years and with sepsis, 40 of whom developed AKI, and 30 healthy controls were enrolled in this prospective study.** ***Results:*** **Results revealed significant progressive elevation of OPG, along with cystatin C and KIM-1, among sepsis, severe sepsis, and sepsis-AKI patients. The progression of OPG levels paralleled the deterioration of kidney and endothelial functions from sepsis to sepsis-AKI, revealed as progressively increased levels of serum E-selectin (15.3%), endothelin-1 (ET-1) (19.6%), and decreased nitric oxide (NO) (29.7%), associated with elevations of TNF-α (25.5%) and TGF-β (18%). Their comparative prognostic validity of sepsis-AKI was assessed using ROC analysis, which revealed that OPG, KIM-1, and cystatin C showed similar AUCs (0.827-0.83) but with different sensitivities, viz., 84%, 88%, and 92%, respectively. Although cystatin showed 82% specificity, OPG showed a higher, similar specificity to KIM-1 of 85%, indicating its potential function as a marker of renal damage such as KIM-1.** ***Conclusion:*** **This study revealed a significant elevation of circulating OPG in septic patients with different levels of severity and those who progressed to AKI. Moreover, OPG showed a significant correlation to KIM-1 and cystatin, as well as conventional renal, inflammatory, and endothelial markers. Having a similar specificity to KIM-1, as evidenced by the ROC analysis, OPG has the potential to serve as a reliable biomarker of kidney damage in cases of sepsis-AKI.**

Key words: osteoprotegerin, KIM-1, cystatin C, sepsis, acute kidney injury

Abbreviations

AKI	acute kidney injury
GFR	glomerular filtration rate
ET-1	endothelin-1
DN	diabetic nephropathy
ECM	extracellular matrix
ICU	intensive care unit
ICAM-1	intercellular adhesion molecule
IFN-γ	interferon gamma
IL	interleukin
OPG	osteoprotegerin
KIM-1	kidney injury molecule 1
NGAL	neutrophil gelatinase-associated lipocalin
NO	nitric oxide
iNOS	inducible NO synthase
eNOS	endothelial NO synthase
ROC	receiver operating curve
RANKL	receptor activator of NF-kappa B ligand
TNF-α	tumor necrosis factor-α
TNF-R	tumor necrosis factor-α receptor
TGF-β	transforming growth factor-β
TNFSF	tumor necrosis factor-α superfamily
TRAIL	TNF-related apoptosis-inducing ligand
TWEAK	TNF-like weak inducer of apoptosis
LPS	lipopolysaccharide
LBP	lipopolysaccharide binding protein
WBC	white blood count
VCAM-1	vascular cell adhesion molecule

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Notwithstanding the increasing ability to support vital organ systems during sepsis, acute kidney injury (AKI) remains a threatening sequel of sepsis in the intensive care unit with high mortality rate in critically ill patients [1]. Current strategies focus on clinical risk identification by early detection of injury, early appropriate antimicrobial therapy to alleviate poor outcomes, and prevention of long-term sequelae of kidney damage among survivors [2].

Significant progress has been made, over the years, towards new diagnostic approaches for early detection of AKI *via* delineating the pathophysiologic mechanisms that predispose to a high incidence of AKI in sepsis. Conventional urinary biomarkers, such fractional excretion of sodium and casts, were found to lack specificity and sensitivity as predictive or diagnostic markers of AKI. Regrettably, serum creatinine is no more considered the only robust sensitive marker for AKI, as it is dependent on several nonrenal factors and is incapable of discriminating the various kinds of AKI (tubular and glomerular damage) [3]. Thus, there is an urgent demand for a biomarker that may ensure earlier sensing of an acute insult than that provided by serum creatinine and timely differentiate between glomerular and tubular injury sites. Regrettably, the routine urinary biomarkers were found to exhibit several limitations, especially in patients with oliguria, in whom the availability of enough samples is a challenge. The hydration status and diuretic usage could also impact the urinary biomarker levels [4].

Although a number of interesting risk markers have been proposed to provide additional prognostic information for AKI, a precise cutoff and biomarker pattern able to recognize AKI patients has not been reported yet. Plasma and urine levels of neutrophil gelatinase-associated lipocalin (NGAL) [5], as well as cystatin C [6, 7], are considered the most promising biomarkers, in addition to kidney injury molecule 1 (KIM-1) and others [8, 9].

Biomarkers have been categorized into two types, namely: those reflecting changes in renal function (*e.g.*, serum creatinine or cystatin C and urine flow rate) and those representing renal damage (*e.g.*, KIM-1, NGAL, interleukin [IL]-18, etc.). The simultaneous utility of both kidney functional and damage markers provides an easy method to classify patients with AKI [10].

Regarding KIM-1, it acts as an adhesion molecule to reduce epithelial cells shedding, and therefore, it is considered an established marker for proximal tubular damage by ischemia. Moreover, KIM-1 functions as a phosphatidylserine receptor that recognizes and binds to its ligand on the apoptotic cells and internalizes them, thereby reducing tubular obstruction. It also functions as a scavenger receptor, mediating the uptake of modified low-density lipoprotein and necrotic cell debris [8, 9]. However, its role as a reliable marker for sepsis-induced AKI is yet to be assessed.

Cystatin C is a protease inhibitor excreted solely by the kidney, and not affected by muscle catabolism. The assessment of serum cystatin C is evidenced to be more precise than creatinine for detecting the glomerular filtration rate, especially in patients with significant muscle wasting or diabetes. Serum and urinary cystatin C concentrations are intimately linked to kidney function with a higher accuracy in estimating amikacin excretion compared to creatinine clearance [6]. Yet, relatively lesser data are available on the interval from detecting elevated cystatin C concentrations to the incident of an AKI.

Osteoprotegerin (OPG) is considered a soluble decoy receptor for the TNF superfamily (TNFSF), cytokine receptor activator of NF-kappa B ligand (RANKL), and TNF-related apoptosis-inducing ligand (TRAIL) as well, with a weaker affinity of the latter for OPG than its transmembrane receptors. Having a wide tissue distribution, that is not restricted to bone or immune tissues [11], OPG plays a key regulatory function in osteoclastogenesis, carcinogenesis, as well as central thermoregulation. It is also reported to be involved in nonapoptotic signaling as well as playing a protective role against TRAIL-induced apoptosis during kidney injury. TRAIL and its potential decoy receptor OPG were found to be the two most upregulated death-related genes in human diabetic nephropathy (DN) that are correlated with parameters of kidney injury [12]. Though recent data support the biological relevance of interaction of TRAIL/OPG in various *in vitro* cell systems, further studies are warranted to unravel the relation between TRAIL, OPG, and AKI that could clarify potential cross-regulatory mechanisms [13].

The aim of this study is to assess the circulating levels of OPG in sepsis-AKI *versus* septic patients to unravel its potential utility for early diagnosis of AKI in the ICU. Therefore, OPG circulating level will be compared to KIM-1 (marker of kidney damage) and cystatin C (reflecting renal function).

PATIENTS AND METHODS

Subjects

Prior to initiation, this study received approval from the Ethical Committee of the Faculty of Medicine at Cairo University and conformed to the ethical guidelines of the 1975 Helsinki Declaration. All participating patients agreed to provide a written informed consent to participate in our study and to publish.

This prospective study included eighty patients (male/female: 43/37), with ages ranging from 42 to 46 years and with sepsis, 40 of whom developed AKI. The included patients were referred from the Nephrology outpatient clinics to the ICU at Kasr ElAini Hospital, Cairo University, Cairo, Egypt, during the period from December 2013 to September 2014.

The diagnosis of sepsis is confirmed by the evidence of infection along with the presence of systemic inflammatory response syndrome (SIRS), based on the definition of the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM), revised at the SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference 2001, and as per the latest Surviving Sepsis Campaign Guidelines [14].

Diagnosis of sepsis was confirmed when two or more of the following criteria were met as a result of systemic infection:

– temperature >38 C or <36 C;
– heart rate >90/min;
– respiratory rate >20/min or pCO_2 <32 mm;
– white blood count (WBC) >12,000/mm^3, <4,000/mm^3, or >10% immature (band) forms.

Table 1
RIFLE and AKIN criteria for diagnosis and classification of AKI, adopted from Palevsky *et al.* (2013).

RIFLE			AKIN	
Class	SCr	Urine output (common to both)	Stage	SCr
Risk	Increased SCr to >1.5 × baseline	Urine output <0.5 mg/kg/h for >6 h	**1**	Increase in SCr to ≥0.3 mg/dL or increase in SCr to ≥150%-200% of baseline
Injury	Increased SCr to >2 × baseline	Urine output <0.5 mg/kg/h for >12 h	**2**	Increase in SCr to >200%-300% of baseline
Failure	Increased SCr to >3 × baseline, or increase of ≥0.5 mg/dL to a value of ≥4 mg/dL	Urine output <0.3 mg/kg/h for >12 h or anuria for >12 h	**3**	Increase in SCr to >300% of baseline or to ≥4 mg/dL, with an acute increase of ≥0.5 mg/dL or on RRT
Loss	Need for RRT for >4 weeks			
End stage	Need for RRT for >3 months			

The specific criteria of AKI diagnosis comply with the Risk Injury Failure Loss of kidney function End-stage renal disease (RIFLE) classification [15] and Acute Kidney Injury Network (AKIN) classification [16], as described in *table 1*. These criteria are also compatible with the KDIGO guidelines, which combines both RIFLE and AKIN guidelines and describes AKI as any of the following: increase in SCr by ≥0.3 mg/dL (≥26.5 nmol/L) within 48 hours; or increase in SCr to ≥1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or urine volume <0.5 mL/kg/h for 6 hours [16].

For all enrolled patients, a detailed record of the patient's history was obtained and documented; physical and laboratory assessments were performed and documented; data are shown in *table 2*.

Patients were grouped according to the extent of organ failure and sepsis severity in the first hours of admission. The criteria set by Surviving Sepsis Campaign Guidelines criteria (14) were used for determination of severity of sepsis and possible concomitant organ failure.

Severe sepsis

Severe sepsis is defined as the presence of sepsis and at least one of the following signs of insufficient organ perfusion or function: hypoxemia [PaO_2 <10 kPa (<75 mmHg)], metabolic acidosis (pH<7.30), oliguria (output <30 mL/h), lactic acidosis (serum lactate level >2 mmol/L), or an acute alteration in mental status without sedation (*i.e.*, a reduction by at least three points from baseline value in the Glasgow Coma score).

Septic shock

Septic shock is defined as the presence of sepsis accompanied by a sustained decrease in systolic blood pressure (<90 mmHg or a drop of 40 mmHg from baseline systolic

Table 2
Laboratory test results in septic patients with and without AKI as well as those in the control group. (n = 40).

	Sepsis (n = 40)	**Sepsis-induced AKI (n = 40)**	**Control (n = 30)**
Age (Year)	42-46	45-51	
Gender (M/F)	21/19	22/18	16/14
SCr (mg/dL)	1.97 ± 0.517^{a}	$2.14 \pm 0.27^{a,b}$	0.92 ± 0.131
Urinary Creatinine/24hrs (g/24 h)	0.788 ± 0.19^{a}	$0.63 \pm 0.068^{a,b}$	1.14 ± 0.08
BUN (mg/dL)	47.36 ± 1.52^{a}	$51.57 \pm 6.06^{a,b}$	28.4 ± 4.14
Albumin (g/dL)	3.41 ± 0.34^{a}	$3.32 \pm 0.24^{a,b}$	3.94 ± 0.15
Uric acid (mg/dL)	8.64 ± 1.87^{a}	$10.88 \pm 1.31^{a,b}$	4.73 ± 0.42
Ca (mg/dL)	4.6 ± 0.3	5.0 ± 0.5	4.7 ± 0.5
Pi (mg/dL)	5.2 ± 0.6	4.5 ± 0.5	3.7 ± 0.7
WBC (10^3/mm^3)	9.56 ± 1.56^{a}	$15.52 \pm 1.52^{a,b}$	6.32 ± 1.52
Culture	G (-ve)	G (-ve)	-
Number of patients treated with the following antibiotics	Amoxicilin/Clavulanate (12) Amikacin (16) Ceftrioxone (12)		

Data are presented as means ± SD. Values are statistically significant at $P < 0.05$. Parametric data were analyzed *via* ANOVA, while nonparametric ones *via* Friedman's test. [a] Significant difference from normal group. [b] Significant difference from Sepsis group; all at P<0.05

blood pressure) despite fluid resuscitation and the need for vasoactive amines to maintain adequate blood pressure.

The development of organ failure was first assessed in the first 24 h of ICU admission. Predictive Acute Physiology and Chronic Health Evaluation II (APACHE II) and Sequential Organ Failure Assessment (SOFA) scores were calculated on the day of ICU admission.

To assess the development of complication and the outcome of the disease, we monitored patients for 28 days from the onset of sepsis.

The following patients were excluded from the study: those aged below 18 years, having performed previous dialysis or kidney transplantation, or having history of kidney disease. In addition, diabetic and pregnant patients and those with hypovolemia responsive to fluids were dismissed as well. To prevent any confounding factors intervening with OPG assessment, patients with overt osteoporosis or bone pathology were not enrolled in the study, which was further confirmed by assessing serum levels of Ca, and Pi in all patients. Patients were also excluded if proven to have the following predisposing factors for AKI: nephrotoxic, radiographic contrast given within three days before the defined increase in serum SCr concentration, decreased renal perfusion or systemic hypoperfusion with overt hypotension (systolic BP less than 80 mmHg), or symptomatic systolic heart failure.

Thirty healthy volunteers served as controls and were selected to match to the patient population by sex (male = 16, female = 14) and age (45-51 years). They were all seemingly healthy on physical examination and laboratory assessment, with normal renal, hepatic, and cardiac function tests.

Septic patients were empirically treated by the antibiotic regimens after drawing blood cultures before starting the study therapy, and were followed up after six weeks of antibiotic therapy along with the normal control group.

Blood sampling and biochemical assays

Venous blood samples (5 mL) were collected, sera separated, and analyzed on the day of hospital admission to determine standard renal function tests; these tests were performed in addition to BUN and SCr, as well as uric acid, serum albumin, and WBC. Separation of sera blood samples was done by centrifugation of unheparinised blood cat 3500 × g for 10 min, and the sera were kept frozen at -70 °C until further analyses.

Serum levels of sE-selectin and tumor necrosis factor-a (TNF-α) were determined using commercial monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) kits (Ray-Bio® Human, RayBiotech Inc.), while endothelin-1 (ET-1) (QuantiGlo) was measured using sandwich ELISA Human Immunoassay (R and D Systems, Minneapolis, Minn., USA). Total serum nitric oxide (NO) level was determined using the kit provided by Assay Designs Inc., USA. Transforming growth factor-β (TGF-β) was determined using a quantitative sandwich immunoassay using the kit provided by ANOGEN, Canada. Cystatin was estimated using the Immuno-Biological Laboratories (IBL)-America human cystatin C ELISA kit, which is based on standard sandwich enzyme-linked immunosorbent assay technology (IBL-Minneapolis, America). KIM-1 was assessed using Human Kidney Injury Molecule (KIM-1) ELISA (Kamiya Biomedical Company, Seattle, WA, USA).

OPG was assessed by the human OPG ELISA kit, (AVISCERA BIOSCIENCE INC, Santa Clara, CA, USA). Some consideration during OPG assessment was taken into account. OPG molecule primarily circulates as a homodimer, but also monomer or complexes with RANKL or TRAIL may be present, which may interfere with the measurements. The specificity of the kit recognizes both natural and recombinant human OPG/Fc chimera. The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference: mouse OPG 17%, human CD40 0%, human sTNF RI 0%, and human sTNF RII 0%.

Results were calculated by refering to standard curves. The intra- and interassay coefficient of variation (CVs) for serum E-selectin and TNF-α is 10 and 12%, respectively, whereas that of ET-1 was 3 and 6.7%, NO was 3.2 and 4.8%, and TGF-β was 5.3 and 8.8%. Limits of detection were as follows: sE-selectin <30 pg/mL, TGF-β1: 9 pg/mL, ET-1: 0.064 pg/mL, NO: 1.35l μmol/L, and TNF-α: 30 pg/mL.

Statistical analysis

Continuous variables are expressed as means ± standard deviation (SD) and as medians and interquartile ranges if nonnormally distributed. Normality was assessed by the Shapiro-Wilk test. Differences between groups were assessed by one-way analysis of variance (ANOVA), and if it was breached we use Welch's correction. Friedman's test was used for non-parametric data. Association between the normally distributed parameters was determined using the Pearson's correlation coefficient. Receiver operating characteristic (ROC) curve analysis was used to assess the predictive ability of the assessed biomarkers; the area under the curve (AUC) and the confidence intervals (CI) were calculated with the Wilcoxon and Mann-Whitney tests. All reported probability values were two-tailed, and *P* value <0.05 was considered statistically significant. Statistical analysis was performed using the SPSS 20.0 statistical software package (SPSS Inc., Chicago, Ill., USA).

RESULTS

Patient's characteristics and laboratory test results of sepsis *versus* sepsis-AKI patients, as well as of those belonging to the comparative normal group, are illustrated in *table 2*. After clinical assessment of sepsis, the laboratory results of the septic AKI patients, compared to healthy subjects, revealed that these patients were having culture-positive sepsis (n = 40, gram-negative bacteria) and significantly increased levels of SCr (2.14-fold), BUN (40%), uric acid (1.8 times), and WBC count (1.5 times); in addition, they had significant decline in the levels of albumin (13.5%) and urinary creatinine (30.8%). The impact of AKI in addition to the presence of sepsis had more profound effect on the assessed levels, compared to sepsis alone. Further increase of urinary markers was observed in sepsis-AKI patients in terms of increased septic levels of SCr (7.9%), BUN (8.2%), uric acid (20.58%), and WBC count (38.4%), added to decreased levels of albumin (2.63%) and urinary creatinine (20%), as compared to septic patients.

Table 3
Serum levels of circulating endothelial dysfunction indicators and inflammatory markers in septic patients with and without AKI as well as those in the control group.

	Sepsis (n = 40)	**Sepsis-induced AKI (n = 40)**	**Control (n = 30)**
Endothelial dysfunction indicators			
E-selectin (ng/mL)	128.9[a] (109.6-167.3)	152.3[a,b] (138.7-171.6)	24.9 (21.6-26.3)
ET-1 (pg/mL)	131.2[a] (114.3-171.3)	163.2[a,b] (149.6-181.4)	94.5 (80-120)
NO (μmol/L)	17.37 ± 6.51[a]	12.2 ± 2.43[a,b]	33.72 ± 3.43
Inflammatory markers			
TNF-α (pg/mL)	43.5 ± 11.5[a]	58.4 ± 11.48[a,b]	12.58 ± 2.2
TGF-β 1(pg/mL)	30.616 ± 8.8[a]	37.34 ± 9.41[a,b]	11.63 ± 1.52
Osteoprotegerin (pg/mL)	276. 3 ± 29.29[a]	384.2 ± 35.2[a,b]	34.9 ± 6.08
KIM-1 (ng/mL)	3.51[a] (2.63-6.27)	4.79[a,b] (7.3-9.4)	0.61 (0.49-0.95)
Cystatin C (ng/mL)	4.61 ± 1.17[a]	8.25 ± 1.51[a,b]	0.941 ± 0.154

Data are presented as means ± SD. Values are statistically significant at $P < 0.05$. E-selectin, ET-1, and KIM-1 are presented as medians (25^{th}-75^{th} percentile range). Parametric data were analyzed *via* ANOVA, while nonparametric ones *via* Friedman's test.
ET-1: endothelin-1; NO: nitric oxide; TNF-α: tumor necrosis factor-α ; TGF-β: transforming growth factor-β.
[a] Significant difference from normal group; at $P < 0.05$.
[b] Significant difference from sepsis group; at $P < 0.05$.

Concerning the Pi and Ca levels in sepsis and sepsis-AKI groups, they were observed to be within normal range as the healthy subjects.

Surrogate markers for endothelial dysfunction in septic patients were assessed by the increased levels of serum E-selectin (5.2-fold) and ET-1 (1.4-fold) and decreased NO (48.5%), all at $P < 0.05$, as compared to control subjects (*table 3*), reflecting impaired vasorelaxation mediated by endothelium. The results also revealed increased serum levels of both inflammatory mediators, TNF-α (3.5 times) and TGF-β (2.6 times), compared with controls ($P < 0.05$).

Sepsis-induced AKI caused further deterioration of the aforementioned parameters. A significant elevation in levels of E-selectin, ET-1, TNF-α, and TGF-β (15.36%, 19.6%, 25.5%, and 18%, respectively) and decreased NO (29.7%) were evident compared with their sepsis values ($P < 0.05$) (*table 3* and *figure 1B,C*).

The progressive elevation of OPG, cystatin C, and KIM-1 in sepsis, severe sepsis, and sepsis-AKI patients is illustrated in *figure 1D-F*. Significant elevation of OPG was observed from the normal level to sepsis recorded as 34.9-276.3 pg/mL and from severe sepsis to sepsis-AKI recorded as 320-384.2 pg/mL (*figure 1D*). In a similar pattern, progressive elevation of cystatin C was observed from the normal level to sepsis recorded as 0.95-4.62 and from severe sepsis to sepsis-AKI (6.82, 8.25 ng/mL), as illustrated in *figure 1E*. Furthermore, the levels of KIM-1 ranged from 0.49 to 0.95 ng/mL in the healthy group, which was increased to 2.63-6.27 ng/mL in the septic group, to 5.2-7.4 ng/mL in the severe sepsis group, and reaching 7.3-9.4 ng/mL in the sepsis-AKI group (*figure 1F*).

The significant positive correlations between OPG and each of KIM-1 (r = 0.904; $P < 0.05$) and cystatin C (r = 0.911; $P < 0.05$) are illustrated in *figure 2*, reflecting its potential usefulness adjunct to KIM-1 and cystatin C as an active indicator of septic AKI. Moreover, the association between each of OPG, KIM-1, cystatin C, and the assessed markers of AKI, inflammatory signals, and surrogate markers of endothelial dysfunction in sepsis and septic AKI are presented in *table 4*. OPG was significantly positively correlated to SCr in septic patients (r = 0.923) as well as sepsis-AKI patients (r = 0.91), both at $P < 0.001$. OPG with urinary Creat/24 h was negatively correlated in septic patients (r = -0.832) and sepsis-AKI patients (r = -0.78), both at $P < 0.001$.

These indicate the involvement of increased OPG added to KIM-1 and cystatin C level in inflammatory cascades and endothelial dysfunction in AKI.

Furthermore, ROC analysis was applied to detect the specificity and sensitivity of OPG, KIM-1, and cystatin C as predictive markers for sepsis-induced AKI (*table 5*, *figure 3*). The curve presents the comparative prognostic validity of OPG, KIM-1, and cystatin C and showed that the three markers revealed an analogous AUC (0.827-0.83), but with different sensitivities and specificities. The cut-off level of OPG was >275.65 pg/mL, which can identify septic AKI patients with good accuracy (84% sensitivity and 85% specificity). The same analysis for KIM-1 demonstrated that a cutoff level of >3.625 ng/mL had also a good accuracy (88% sensitivity and 85% specificity). In the same line, cystatin C >4.17 ng/mL reflected an accuracy of 92% sensitivity and 82% specificity.

DISCUSSION

Although the detailed pathophysiology of sepsis-induced AKI is not yet thoroughly understood, it is widely perceived that it has a multipronged injury pathway distinct from nonseptic AKI. This form of AKI exhibits components of ischemia-reperfusion injury, direct inflammatory injury, as well as stimulation of the fibrinolytic cascades and coagulation, ultimately resulting in apoptosis and endothelial injury [17]. The predictable downward spiral of bacteremia-induced sepsis triggers the release of LPS,

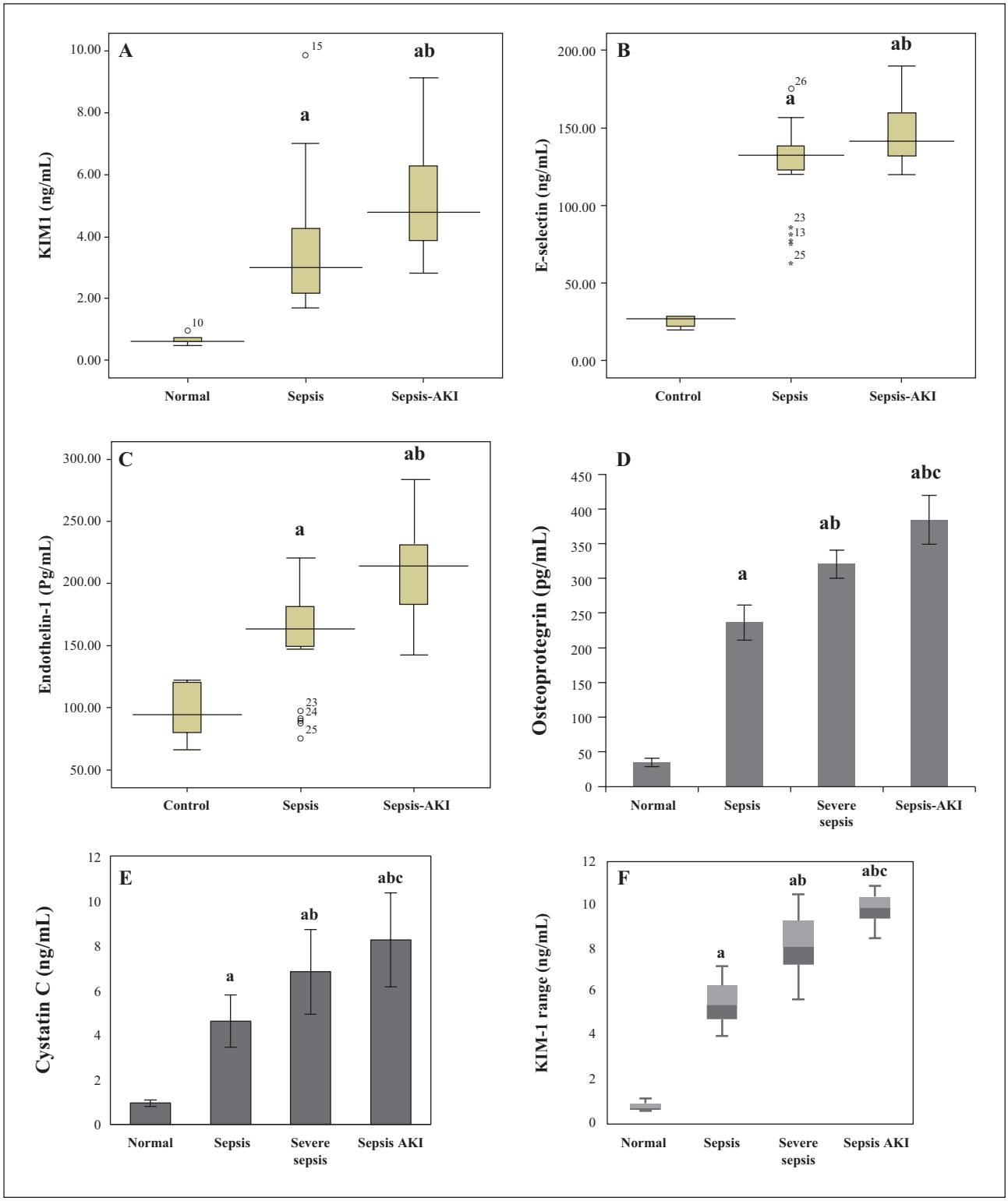

Figure 1

Descriptive presentation of KIM-1 **(A)**, E-selectin **(B)**, and Endothelin-1 **(C)** using Box and Whisker plot**.** Concentrations of OPG levels **(D),** Cystatin C **(E),** and KIM-1 in patients with sepsis, severe sepsis, and sepsis-AKI groups. (a) $P < 0.05$ compared with normal group, (b) compared with septic patients, and (c) compared with severe sepsis patients.

then cytokines, and consequently NO. The proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1, and interferon (IFN)-γ, following LPS exposure, bind to their specific receptors on different cell types and organs [18]. Thereafter, such cytokines intercede with various cellular and molecular processes that may result in impaired immune responses. In the kidney, TNF ligand binds to two receptors: TNF receptor 1 (TNFR1) on glomerular endothelial cells and TNF receptor 2 (TNFR2) on renal tubular epithelial cells [19]. Current strategies focus on early detection of kidney injury and investigation of new diagnostic approaches for early detection of AKI and prevention of the high incidence of AKI due to sepsis.

This study highlights the potential utility of OPG, a new promising kidney biomarkers that could be used for prognostication of AKI complications and is expected to

Table 4
Correlations of serum Osteoprotegerin, KIM-1, and Cystatin C levels in sepsis and sepsis-AKI patients with indicators of kidney function, markers of inflammation, and endothelial dysfunction.

Parameters	Osteoprotegerin		KIM-1		Cystatin C	
	Sepsis	Sepsis-AKI	Sepsis	Sepsis-AKI	Sepsis	Sepsis-AKI
Kidney functions						
SCr	$r = 0.923$ $P = 0.001$	$r = 0.91$ $P = 0.001$	$r = 0.876$; $P = 0.001$	$r = 0.79$ $P = 0.023$	$r = 0.9$; $P = 0.001$	$r = 0.89$ $P = 0.023$
UCreat /24 h	$r = -0.832$ $P = 0.001$	$r = -0.78$ $P = 0.001$	$r = -0.8$ $P = 0.001$	$r = -0.76$ $P = 0.021$	$r = -0.823$; $P = 0.001$	$r = -0.86$ $P = 0.022$
Urea	$r = 0.856$; $P = 0.001$	$r = 0.79$ $P = 0.001$	$r = 0.77$; $P = 0.021$	$r = 0.79$ $P = 0.021$	$r = 0.797$ $P = 0.001$	$r = 0.82$ $P = 0.021$
Albumin	$r = -0.856$ $P = 0.001$	$r = -0.79$ $P = 0.001$	$r = -0.841$; $P = 0.001$	$r = -0.89$ $P = 0.021$	$r = -0.869$; $P = 0.001$	$r = -0.79$ $P = 0.001$
Uric acid	$r = 0.856$; $P = 0.001$	$r = 0.89$ $P = 0.001$	$r = -0.749$; $P = 0.001$	$r = -0.75$ $P = 0.021$	$r = 0.784$; $P = 0.001$	$r = 0.82$ $P = 0.001$
Endothelial dysfunction						
E-selectin	$r = 0.758$; $P = 0.021$	$r = 0.82$ $P = 0.001$	$r = 0.631$; $P = 0.023$	$r = 0.69$ $P = 0.021$	$r = 0.666$; $P = 0.021$	$r = 0.79$ $P = 0.001$
ET-1	$r = 0.67$; $P = 0.021$	$r = 0.71$ $P = 0.001$	$r = \mathbf{0.536}$ $P = 0.033$	$r = 0.61$ $P = 0.021$	$r = \mathbf{0.541}$ $P = 0.033$	$r = 0.62$ $P = 0.021$
NO	$r = -0.806$ $P = 0.001$	$r = -0.69$ $P = 0.021$	$r = -0.736$; $P = 0.021$	$r = -0.82$ $P = 0.001$	$r = -0.771$; $P = 0.021$	$r = -0.82$ $P = 0.001$
TNF-α	$r = 0.934$; $P = 0.001$	$r = -0.89$ $P = 0.001$	$r = 0.89$; $P = 0.001$	$r = 0.91$ $P = 0.001$	$r = 0.91$; $P = 0.001$	$r = 0.95$ $P = 0.001$
TGF-β	$r = 0.855$; $P = 0.001$	$r = 0.82$ $P = 0.001$	$r = 0.817$; $P = 0.001$	$r = 0.92$ $P = 0.001$	$r = 0.827$; $P = 0.001$	$r = 0.84$ $P = 0.001$

Values at $P < 0.05$ are statistically significant, bold values have subtle significance.

synergize the sensitivity and specificity of conventional serum markers. Our results revealed significant progressive elevation of circulating OPG among sepsis, severe sepsis, and sepsis-AKI patients. Moreover, OPG level was found to increase in correlation with the assessed indicators of AKI, renovascular inflammatory markers, and endothelial functions; these were findings that were not tested before. The exact rationalization of this association is not known, but is expected to be linked to TNFSF, RANK and TWEAK-induced inflammatory processes, and related vascular calcification. A recent study of Morena *et al.* in 2015 reported a significant association of decline in renal functions with a significant increase in OPG levels, but in a state of a chronic renal failure [20]. The studies of Benito-Martin *et al.* in 2013 pointed out that the functional TNFSF proapoptotic cytokines including TNF, Fas ligand, TRAIL, and TWEAK are potentially significant for the pathophysiology of kidney tubular cells. According to a human kidney transcriptomics approach, it has been found that the chronic kidney disease (CKD) is triggered by such apoptosis-related genes as TRAIL and its decoy receptor OPG (OPG/OCIF/TNFRSF11B) that are exceedingly expressed in DN [21]. Previous data also provide strong evidence that certain environmental conditions including glomerulonephritis, inflammatory and ischemic conditions in DN, aging, and CKD enhance the sensitivity of renal tubular cells to TRAIL-induced death. Moreover, expression of OPG by tubular cells is reported to guard against TRAIL-induced apoptosis, as verified by the stimulated apoptosis cascade when OPG antibody was used in tubular cells [22]. Enhanced tubulointerstitial TRAIL/OPG may not limit itself to DN; as a matter of fact, it may also rise, although to a lesser extent, in the tubulointerstitium of lupus nephritis, hypertensive nephropathy, and membranous nephropathy, as well as in sepsis-induced AKI [23]. Survival regulation and stress are closely related to the TRAIL pathway *via* transcriptional master regulator NF-κB. OPG intercedes with both TRAIL-induced NF-κB activation and TRAIL-induced loss of tubular cell survival implying that TRAIL and OPG interaction may serve as one of the OPG pathways to modulate tissue injury. The effect of the TRAIL/OPG interplay in vivo seems to rely on the relative levels of local tissue of both molecules in the cell microenvironment, similar to other cytokine and soluble receptor systems [24].

Some studies also support the involvement of OPG in vascular calcification regulation [25], plaque destabilization, and vascular atherosclerosis, since they are linked to the coronary calcification degree [26] and prediction of cardiovascular diseases (CVD) [27]. The former has been related to severity and pervasiveness of cerebrovascular disease, peripheral vascular disease, and coronary artery disease [25].

The association of the synthesis of OPG in osteoblastic lineage cells with increased cytokines, such as interleukin (IL)-1, TNF-α, and transforming growth factor-β (TGF-β) [28], explains the current association of OPG with TNF-α and TGF-β. The OPG-induced vascular pathology may be also linked to endothelial dysfunction indicators, E-selectin and ET-1.

Kim *et al.* in 2012 [29] investigated diagnostic and prognostic usage of decoy receptor 3 (DcR3) *versus* OPG as surrogate markers of sepsis alternative to procalcitonin,

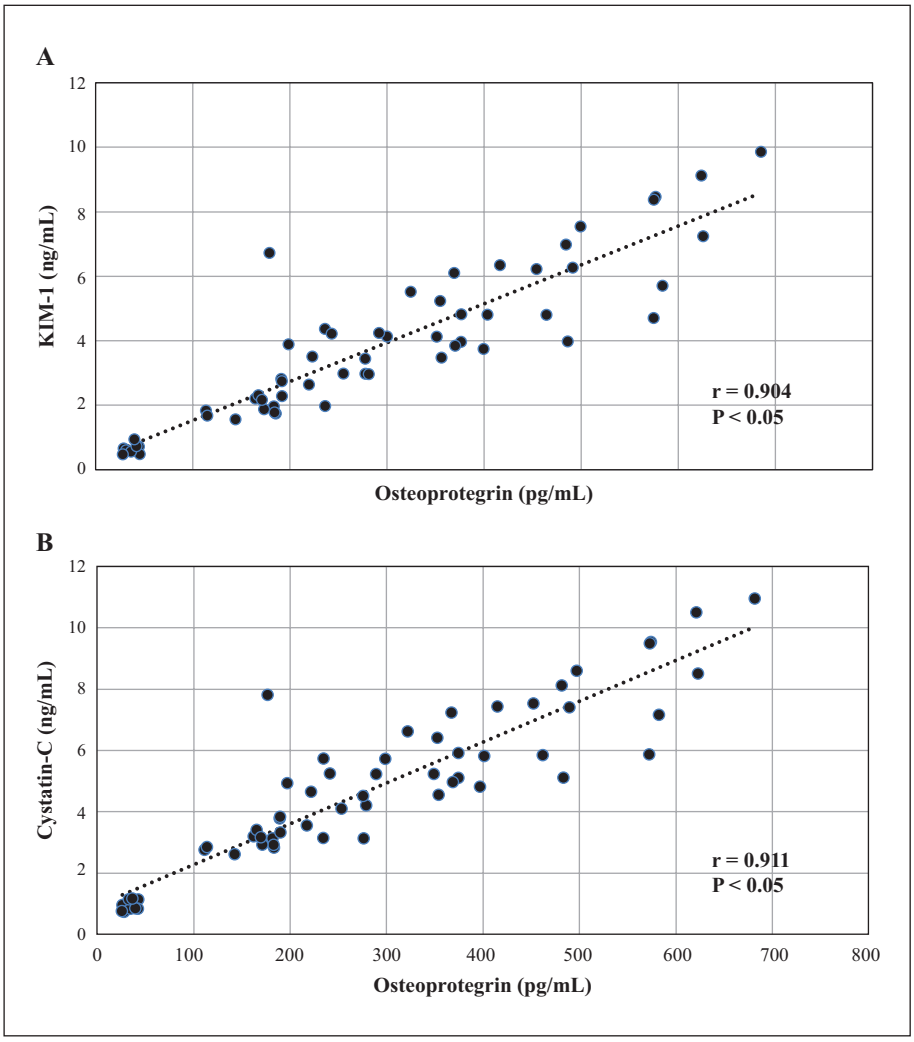

Figure 2
Correlation of serum levels of Osteoprotegerin with KIM-1 **(A)** and Cystatin C **(B)** in sepsis-AKI patients, $P < 0.05$.

which was reported to have no direct impact on prognosis and did not predict survivors from nonsurvivors [30]. Intriguingly, their study revealed a significant elevation of OPG in septic patients compared to those with SIRS; however, the area under the ROC curve of OPG was inferior to DcR3, which better predicted sepsis from SIRS. Moreover, they revealed that the reliability of OPG, as a potential biomarker in sepsis, was less significant compared to decoy receptor 3 (DcR3). OPG showed significant elevation in sepsis arm in both studies, though their comparative cohort group was different from ours. They compared the sepsis arm with SIRS patients and we compared it with sepsis-induced AKI, which may rationalize the discrepancies in the results.

Results of this study presented an increase in KIM-1 and cystatin C levels in septic AKI patients, which were thereafter further elevated in septic AKI patients. KIM-1 is undetectable in the renal tissue under physiological conditions and considered a sensitive biomarker that is specific for renal injury [31], as proposed by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) [32]. It is upregulated in the proximal tubule after ischemia and high urinary levels of KIM-1 of acute tubular necrosis patients have been suggested as an early biomarker of renal proximal tubule injury, as in acute exposure to gentamicin, mercury, and chromium. Though acute drug exposure has been the cause of necrosis in almost half of all proximal tubules, only urinary KIM-1 was increased, whereas serum urea, creatinine, and urinary NAG activity did not increase compared to controls signifying that these were too insensitive to reflect tubular injury [33]. Though the diagnostic utility for urinary KIM-1 in sepsis and/or AKI has been proposed, evaluation of serum levels in clinical settings warrants further studies.

In a cohort study of Haase *et al*. in 2009, cystatin C level was significantly correlated with duration and severity of AKI after adult cardiac surgery and therefore considered an independent predictor of AKI severity. Cystatin C appears rather to be a marker of chronic renal impairment than of

Table 5
The AUC and best cutoff value of osteoprotegerin (OPG), KIM-1, and cystatin C as predictive markers for sepsis-induced AKI, as determined by the receiver operating characteristic curve (ROC curve).

Parameter	Osteoprotegerin	KIM-1	Cystatin C
Best cutoff value	275.65	3.62	4.17
Area under the curve	0.827	0.83	0.82
Sensitivity	0.84	0.88	0.92
Specificity	0.85	0.85	0.82

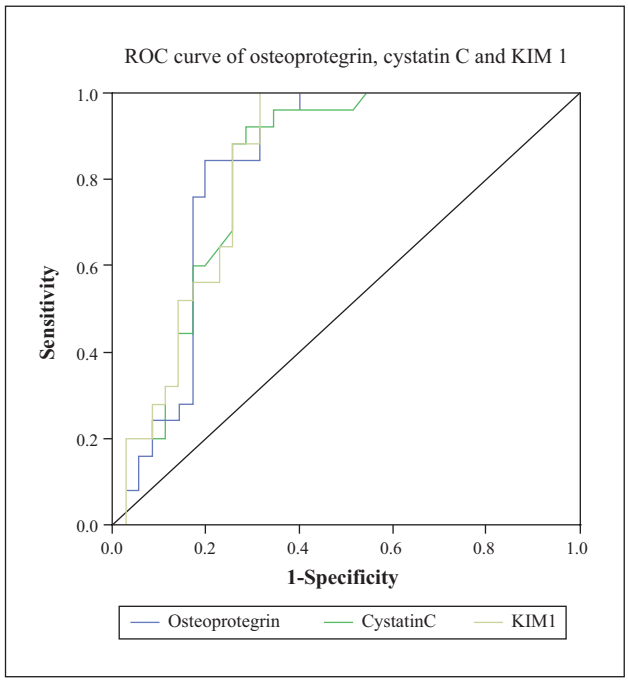

Figure 3
The sensitivity and specificity of Osteoprotegerin (OPG), KIM-1, and Cystatin C as predictive markers for sepsis-induced AKI, as determined by the Receiver Operating Characteristic curve (ROC curve).

acute worsening renal function in this setting [34]. Furthermore, Perianayagam *et al.* in 2009 [35] measured serum cystatin C levels in 200 patients with AKI and found that serum cystatin C had an AUC of 0.65 for the composite endpoint of death. Liangos *et al.* in 2007 [36] and Nejat *et al.* in 2010 [37] revealed that AUC-ROC of KIM-1 for the prediction of renal replacement therapy or death was 0.61 and comparable to those of serum creatinine and urine output. AKI could also be predicted by the elevation of plasma cystatin C concentrations in patients with liver cirrhosis and critically ill patients with and without coexistent sepsis [38, 39]. For patients who developed acute septic kidney injury, the plasma cystatin C levels increased before the classical markers of renal function. Cystatin C also constitutes a severity biomarker that correlates with progression to RIFLE and mortality. This could be used as preventive measure from the possible progression of renal dysfunction [40, 41].

Concerning the association of cystatin C with OPG and RANKL, it has only been limitedly investigated in healthy individuals, where no significant correlation was found. Moreover, Kulcsar-Jakab *et al.* in 2015 [42] reported that in addition to age, which was the stronger predictor, cystatin C was also a significant predictor of OPG, and that the association between cystatin C and OPG was more evident with increased age . Elevated OPG levels have been reported by Fekih *et al.* in 2017 [43] in patients with diabetes 1 (T1D) complications. They noted a significant higher frequency of children with increased cystatin C levels in the group with elevated plasma level of OPG compared with those with normal levels, which may suggest OPG as a potential biomarker of cardiovascular risk in T1D,

In this study, ROC analysis of OPG was compared to KIM-1 and cystatin C for its potential utility as predictive marker for sepsis *versus* sepsis-AKI cases. It showed that the three markers revealed an analogous AUC (0.827-0.83), but with different sensitivities and specificities. While Cystatin C showed the highest sensitivity (92% *versus* 84%, 88% for OPG and KIM-1), OPG and KIM-1 had similar specificity (85%), reflecting its suitability to function as a marker of renal damage.

Although systemic hypotension resulting in renal ischemia is a crucial pathological factor, it is not the sole factor in septic AKI. The change in microvascular function is a hallmark in sepsis, including vasoconstriction, capillary leak syndrome, tissue edema, and leucocyte and platelet adhesion with endothelial dysfunction. Being directly released from the endothelium, ET-1, a vasoactive protein, and NO are cellular endothelial mediators that play a key function in endothelial injury [44, 45]. The formation of NO is a product of assembly of iNOS protein, due to translation of iNOS mRNA. During sepsis, induced hypotension, renal ischemia, and septic shock could be due to the production of large amounts of NO, which is responsible for systemic vasodilatation.

Results of this study verify previous reports on the involvement of endothelial markers in septic AKI patients and further highlight the involvement of OPG, KIM-1, and cystatin C as dynamic players in endothelial dysfunction cascade in septic AKI. The significant elevation in serum levels of E-selectin, ET-1, and TNF-α in our septic AKI patients, compared with controls, demonstrate endothelial dysfunction as reported in previous studies [46]. The current findings presented significant correlations of serum levels of TNF-α with markers of endothelial dysfunction in our septic AKI patients; data reflect the multifaceted crosstalk of inflammation, and endothelial dysfunction in AKI [47, 48]. Higher plasma levels of TNF-α are evidenced in patients with acute/chronic renal failure compared to healthy controls [49, 50]. Interestingly, the experimental induction of renal apoptosis by TNF-α is mediated by binding with TNF receptor 1 on glomerular cells and the TNF receptor 2 sites on renal tubular cells [51].

The current increase of serum TGF-β1 level in AKI patients in our study may reflect elevated renal TGF β1 expression [52], potentially induced by the injury factors including TNF-α, suggesting that TGF-β1 may be a crucial key player in renal repair. The exact mechanistic role of TGF-β in cellular injury and repair remains controversial. Previous data reported that TGF-β1 biosynthesis in human proximal TEC HK-2 cells is significantly stimulated by TNF-α. The renoprotective role of TGF-β1 from renal ischemia-reperfusion injury (IRI) in TGF-β1-deficient mice was addressed in a previous study [50], in which the authors proposed that the upregulation of TGF-β1 protein contributed to the survival mechanism resisting the kidney injury and provided renal protection from apoptosis, causing antiapoptotic Bcl-2 expression [52].

Moreover, AKI-induced endothelial dysfunction is reflected as impaired vasorelaxation, events that are presented in our septic AKI patients as decreased serum levels of NO and increased ET-1. Moreover, a negative correlation of serum levels of NO with ET-1 was revealed in our septic AKI patients, which aligns with previous results of Sadik *et al.* (2012) [53]. According to its concentration and subtype at the site of action, NO has been shown to contribute to several key functions in the kidney [54]. Several forms of cellular NO synthase (NOS) are expressed: inducible NO synthase (iNOS) and

endothelial NOS (eNOS), where iNOS was associated with cytotoxicity in epithelial cells of the renal tubules [55]. The opposite effect is exhibited by endothelium-derived NO (eNOS), as it has potential improving effect on ischemic and toxic renal injury. This is mediated by its vasodilating impact, inhibition of leukocyte adhesion, and platelet aggregation. Moreover, the defective production of endothelial NO may ultimately lead to vascular congestion and destruction of tubular epithelial cells. This phenomenon of "no reflow" was proposed by Goligorsky *et al.* in 2002 [56]. Notably, the ameliorative effect of NO on ischemic AKI, *via* experimental L-arginine supplementation, has been previously evidenced [57]. In this research, the diminished serum NO level in our AKI patients could be because of possible diminished NO generation by injured endothelium, which could be explained by reduction of eNOS expression or loss of neuronal NOS, or both [58].

CONCLUSION

This study demonstrates a significant elevation of circulating OPG in sepsis-AKI patients compared to septic patients with different severities. Moreover, the association between OPG and the assessed renal markers of AKI, inflammatory signals, and surrogate markers of endothelial dysfunction in sepsis and septic AKI indicates the involvement of increased circulating OPG level in inflammatory cascades and endothelial dysfunction in AKI.

Interestingly, our data revealed significant positive correlations between OPG and each of KIM-1 and cystatin C, reflecting its potential diagnostic usefulness in adjunction to KIM-1 (as a marker of kidney damage) and cystatin C (as a marker of renal function) in septic AKI patients. This is further confirmed by the ROC analysis applied to OPG in comparison to KIM-1 and cystatin C to detect the specificity and sensitivity; this analysis showed that the three markers revealed an analogous AUC (0.827-0.83), but with different sensitivities and specificities. The cutoff level of OPG $>$275.65 pg/mL can identify septic AKI patients with good accuracy (84% sensitivity and 85% specificity). Having a similar specificity to KIM-1, as evidenced by the ROC analysis, OPG has the potential to serve as a reliable biomarker of kidney damage in cases of sepsis-AKI.

Limitation of the study

The authors acknowledge the small sample size included in the study due to the difficulty in recruitment of patients in the ICU setting.

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