


Lipid peroxidation in Gram-negative bacteremia modulates the risk for septic shock and infections by resistant *Klebsiella pneumoniae*

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Abstract Controversies in outcomes with the parenteral administration of antioxidants as adjuvant therapies led to the measurement of malondialdehyde (MDA), a product of lipid peroxidation, in serum collected from 120 patients with primary Gram-negative bacteremia during the first 24 h from sepsis onset. MDA was measured by the thiobarbiturate assay, followed by high-performance liquid chromatography (HPLC) analysis. After receiver operator characteristic (ROC) curve analysis, patients were divided into those with high levels of MDA and low levels of MDA. The primary endpoint was the association of the level of MDA with septic shock. The level of MDA as an index of neutrophil function and associations with outcome and with infections by carbapenem-resistant *Klebsiella pneumoniae* were the

secondary endpoints. In total, 63 patients had high and 57 had low MDA levels; 27% and 49.1%, respectively, had septic shock ($p = 0.015$). The rate of the concentration of MDA to the total neutrophil count was used as an expression of neutrophil function; this was lower among patients with septic shock. The odds ratio (OR) for death among patients without septic shock and low level of MDA was 4.00; this was 0.48 for patients with septic shock ($p = 0.020$ between the two ORs). The OR for resistance to carbapenems among patients with bacteremia by *K. pneumoniae* and low level of MDA was 7.50 ($p = 0.011$ compared to patients with bacteremia by other pathogens). Low level of circulating MDA is associated with susceptibility to septic shock and infections by carbapenem-resistant *K. pneumoniae*.

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Introduction

Lipid peroxidation and generation of free radicals is a major mechanism leading to organ dysfunction in sepsis. Studies have shown that, in patients with sepsis-associated organ dysfunction, circulating oxidative products are increased and that circulating antioxidants are decreased [1, 2]. This led to the assumption that the administration of antioxidants such as selenium might prevent organ dysfunction and lead to survival benefit. Three randomized clinical trials have been conducted where the efficacy of selenium supplementation was compared to placebo treatment. These trials provided conflicting results [3–5]. A hypothesis that may explain these conflicting results is that the activation of lipid peroxidation varies among patient populations. Support for this hypothesis comes from a study of 50 patients; oxidative products were increased only in the event of hepatic dysfunction and coagulopathy, but not in the event of respiratory and circulatory failure [6]. The need

for study of circulating products of lipid peroxidation in homogeneous cohorts becomes obvious.

The Hellenic Sepsis Study Group (<http://www.sepsis.gr>) has been prospectively collecting biosamples of patients with severe infections since May 2006 from 65 departments across Greece. We selected a homogeneous patient population with bacteremia by Gram-negative pathogens. All these patients also had infection-associated organ dysfunction according to the new Sepsis-3 definition [7, 8]. We explored the relationship between lipid peroxidation and organ dysfunction using measurements of malondialdehyde (MDA), which is an indirect expression of the generation of free radicals synthesized by neutrophils. Free radicals attack polyunsaturated fatty acids embedded into cell membranes and lead to the formation of MDA [9]. As a consequence, circulating MDA is an indirect expression of the capacity of neutrophils for the generation of free radicals and for their involvement in sepsis-associated organ dysfunction. Secondly, we studied the risk for infections by carbapenemase-producing *Klebsiella pneumoniae*, since patients with impaired neutrophil function may be susceptible to infections by these hospital pathogens.

Patients and methods

Patient population

In this prospective collection starting from May 2006, blood was collected within the first 24 h from the onset of signs of systemic inflammatory response syndrome (SIRS) among patients with well-defined infections. Screened patients for eligibility were patients either admitted for suspected infections in the emergency department or developing infections at least 48 h after admission to an intensive care unit (ICU). Patients with at least two signs of SIRS due to an infection were included. The definitions of SIRS, of organ failures, and of infections relied on international criteria [10, 11]. Adult patients were enrolled after written consent provided by themselves or by first-degree relatives for patients unable to consent. The study protocol was approved by the ethics committees of the participating hospitals.

Exclusion criteria were: (a) infection by the human immunodeficiency virus (HIV); (b) neutropenia defined as an absolute neutrophil count below $1000/\text{mm}^3$ attributed to causes other than SIRS; and (c) chronic intake of corticosteroids defined as the daily intake of more than 0.4 mg/kg of equivalent prednisone for at least 15 days. These exclusion criteria were applied because they can influence the neutrophil function.

From this prospective collection, patients enrolled between 2009 and 2013 with primary bacteremia by Gram-negative bacteria and meeting the criteria of sepsis set by the Sepsis-3 definitions were further analyzed. This subanalysis was approved by the Ethics Committee of ATTIKON University

Hospital (approval 209/25-05-2009). Primary bacteremia was considered as the isolation of one Gram-negative pathogen species from the bloodstream of a patient without any central catheter and where thorough clinical and radiological investigation failed to identify any primary site of infection [11]. Patients were retrospectively validated for the Sepsis-3 definition of sepsis as recently described [8].

For every patient, a complete diagnostic work-out was done comprising past and present medical history, physical examination, laboratory exams, urinalysis, blood and urine cultures, and chest X-ray. Further radiology investigation with abdominal ultrasound and chest and abdomen computed tomography was done if considered necessary. Mortality after 28 days was recorded.

Laboratory investigation

Fifteen milliliters of blood was sampled after venipuncture of one forearm vein under aseptic conditions. Five milliliters were collected into pyrogen-free tubes (Vacutainer, Becton Dickinson, Cockeysville, MD). The tubes were immediately centrifuged and serum was kept refrigerated at $-70\text{ }^{\circ}\text{C}$ until assayed. MDA was measured in serum as already described [12]. Briefly, a 0.1 mL aliquot of each sample was mixed with 0.9 mL of trichloroacetic acid 20% (Merck, Darmstadt, Germany) and centrifuged at $12,000 \times g$ and $4\text{ }^{\circ}\text{C}$ for 10 min. The supernatant was removed and incubated with 2 mL of thiobarbituric acid 0.2% (Merck) for 60 min at $90\text{ }^{\circ}\text{C}$. After centrifugation, a volume of 10 μL of the supernatant was injected into a high-performance liquid chromatography system (HPLC, Agilent 1100 Series, Waldbronn, Germany), with the following characteristics of elution: Zorbax Eclipse XDB-C18 ($4.6 \times 150\text{ mm}$, $5\text{ }\mu\text{m}$) column under $37\text{ }^{\circ}\text{C}$; mobile phase consisting of a 50 mM K_3PO_4 (pH: 6.8) buffer and methanol 99% at a 60/40 ratio with a flow rate of 1 mL/min; fluorometric detection with signals of excitation at 515 nm and emission at 535 nm. The retention time of MDA was 3.5 min and it was estimated as mM by a standard curve created with 1,1,3,3-tetramethoxypropane (Merck, Darmstadt, Germany). All determinations were performed in duplicate.

Another 10 mL of blood was inoculated into ready-prepared culture vials (bioMérieux, Marcy l'Etoile, France) and incubated. Identification and susceptibility testing through measurement of the minimum inhibitory concentrations (MICs) of antimicrobials was done by the VITEK 2 automated system. Susceptibility was reported based on the Clinical and Laboratory Standards Institute (CLSI) susceptibility criteria. Resistance of enterobacteria to carbapenems was defined as any MIC above or equal to $4\text{ }\mu\text{g/mL}$, provided that the disk carbapenemase test was positive [13].

Study endpoints

The primary study endpoint was the association of the level of circulating MDA with the development of septic shock. The secondary endpoints were: (a) the role of MDA as an index of neutrophil function and its relationship to the development of septic shock; (b) the association of the level of circulating MDA with 28-day outcome; and (c) the association of the level of circulating MDA with infection by carbapenem-resistant *K. pneumoniae*, which is a major health problem in Greece [14].

Statistical analysis

The distribution of concentrations of MDA was studied by Kolmogorov's and Smirnov's statistics. A receiver operator characteristic (ROC) curve was designed to define a cut-off of MDA that can be the best trade-off of sensitivity and specificity for septic shock. Patients were then divided into two groups; patients with MDA above the cut-off or high level and patients with MDA below the cut-off or low level. The odds ratio (OR) and 95% confidence intervals (CIs) for septic shock at that cut-off were calculated according to Mantel and Haenszel. Comparisons of quantitative baseline characteristics between the two groups were done by Student's *t*-test and of qualitative baseline characteristics by Fisher's exact test. To develop an index of neutrophil function, we took into consideration that MDA is the end product of the effect of free radicals produced by neutrophils [9]. To this end, the rate of MDA to the absolute neutrophil count was considered an

index of neutrophil function. ROC curve analysis was also done to identify the best trade-off of the neutrophil index for septic shock. The ORs and 95% CIs of mortality after 28 days for patients with high and low levels of MDA were calculated according to Mantel and Haenszel. Comparisons between ORs were done by Breslow–Day's and Tarone's tests. Using the same approach, we explored the relative distribution of resistant bacteria among the two groups of patients. Any value of *p* below 0.05 was considered statistically significant.

Results

Selection of patients

The study flow chart is shown in Fig. 1. A total of 218 patients with Gram-negative bacteremia were prospectively selected for inclusion in the study. After retrospective validation for the Sepsis-3 definitions, 120 patients were further selected for measurements of MDA.

Primary study endpoint

The concentration of MDA was skewed from a normal distribution. Using ROC curve analysis, a concentration lower than 2.70 mM provided the best trade-off for sensitivity and specificity for septic shock. In total, 63 patients had MDA more than or equal to 2.70 mM and 57 had MDA less than 2.70 mM. As shown in Table 1, these two groups of patients did not differ in their baseline characteristics. However, 27.0%

Fig. 1 Study flow chart of the selection of sepsis patients for the measurement of malondialdehyde (MDA). *SIRS* systemic inflammatory response syndrome

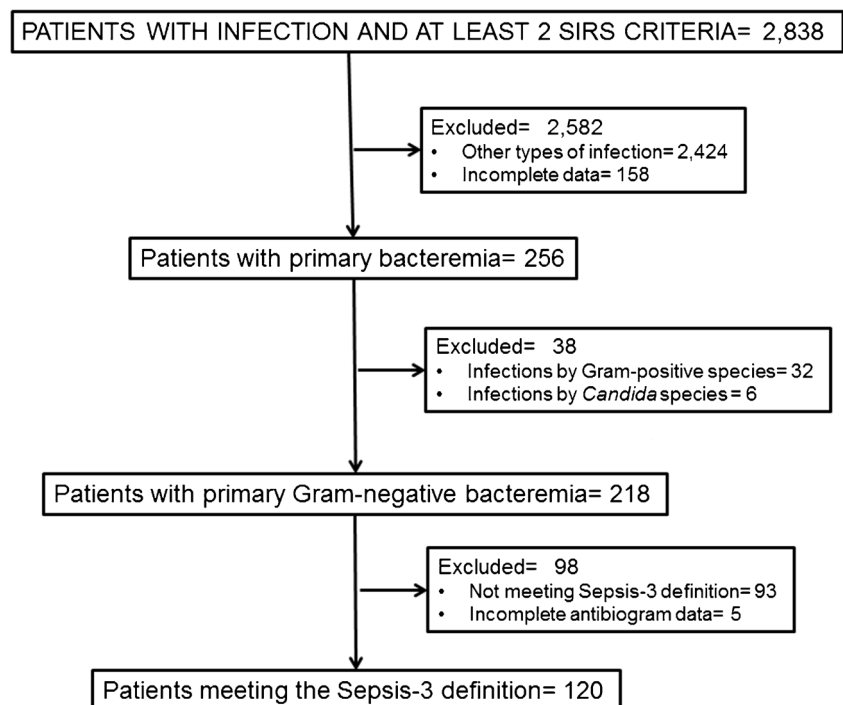


Table 1 Demographics of patients enrolled in the study in relation to the level of circulating malondialdehyde (MDA)

	MDA \geq 2.70 mM (n = 63)	MDA <2.70 mM (n = 57)	p-Value
Male gender (n, %)	35 (55.5)	35 (61.4)	0.706
Age (years, mean \pm SD)	68.9 \pm 16.2	66.5 \pm 16.1	0.438
Total white blood cell count (/mm ³ , mean \pm SD)	14,854.9 \pm 8493.8	18,489.1 \pm 13,879.0	0.087
Total neutrophil cell count (/mm ³ , mean \pm SD)	12,433.6 \pm 7109.3	15,475.4 \pm 11,616.7	0.082
APACHE II score (mean \pm SD)	18.4 \pm 6.6	20.4 \pm 6.0	0.146
SOFA score (mean \pm SD)	6.36 \pm 3.43	7.63 \pm 4.06	0.067
Type of organ failure (n, %)			
ARDS	22 (34.9)	23 (40.4)	0.575
Acute kidney injury	11 (17.5)	11 (19.3)	0.817
Acute coagulopathy	19 (30.2)	20 (35.1)	0.697
Septic shock	17 (27.0)	28 (49.1)	0.015
Isolated pathogen (n, %)			
<i>Escherichia coli</i>	10 (15.9)	11 (19.3)	0.639
<i>Klebsiella pneumoniae</i>	14 (22.2)	12 (21.1)	1.00
<i>Acinetobacter baumannii</i>	7 (11.1)	6 (10.5)	1.00
<i>Pseudomonas aeruginosa</i>	7 (11.1)	6 (10.5)	1.00
Other Gram-negative bacteria	10 (15.9)	9 (12.3)	0.475
Co-existing disorders (n, %)			
Type 2 diabetes mellitus	21 (33.3)	14 (24.6)	0.320
Chronic heart failure	16 (25.4)	18 (31.6)	0.544
COPD	12 (19.0)	15 (26.3)	0.386
Chronic renal disease	5 (7.9)	5 (8.8)	1.00
Death after 28 days (n, %)	16 (25.4)	23 (40.4)	0.060

ARDS acute respiratory distress syndrome, COPD chronic obstructive pulmonary disease

of patients with MDA more than or equal to 2.70 mM had septic shock compared to 49.1% of patients with MDA lower than 2.70 mM. The OR for septic shock with MDA lower than 2.70 mM was 2.61 (95% CI: 1.22–5.59, $p = 0.015$). These findings showed a positive association between low circulating MDA and septic shock, which was the study's primary endpoint (Fig. 2a).

Secondary endpoint

The first secondary study endpoint was the relationship of the index of neutrophil function to septic shock. As described above, the index was generated by dividing circulating MDA by the absolute neutrophil count. The index was greater among patients without septic shock than among patients with septic shock (Fig. 2b, c). An index lower than 0.164 was associated with septic shock at OR 3.20 (95% CI: 1.42–7.23).

The second secondary study endpoint was the association of MDA with 28-day outcome. Presence of septic shock was an independent driver for 28-day mortality. More precisely, 27 out of 45 patients with septic shock died (60%), contrary to 12 out of 75 patients without septic shock (16%, $p < 0.0001$).

However, a trend towards greater mortality was found between patients with MDA lower than 2.70 mM (40.4% vs. 25.4% of patients with MDA greater than or equal to 2.70 mM; Table 1) This trend led us to further explore the association of MDA with outcome in light of the presence of septic shock or not.

As shown in Table 2, circulating MDA lower than 2.70 mM was accompanied by greater mortality among patients without septic shock. When circulating MDA was lower than 2.70 mM, the OR for death among patients without septic shock was 4.00; this was 0.48 among patients with septic shock (p of comparisons of the two ORs = 0.020 by Breslow–Day's test and $p = 0.020$ by Tarone's test).

Regarding the third secondary endpoint, *K. pneumoniae* was isolated as a pathogen from 26 patients; the isolate was carbapenem-resistant in 12 cases (46.2%). From the 94 pathogens isolated from the remaining patients, a carbapenem-resistant pathogen was isolated from only 12 (12.8%, $p < 0.0001$). The analysis shown in Table 3 indicated that the OR for the acquisition of a carbapenem-resistant isolate was associated with the level of circulating MDA. More precisely, the OR for a non-*K. pneumoniae* carbapenem-resistant

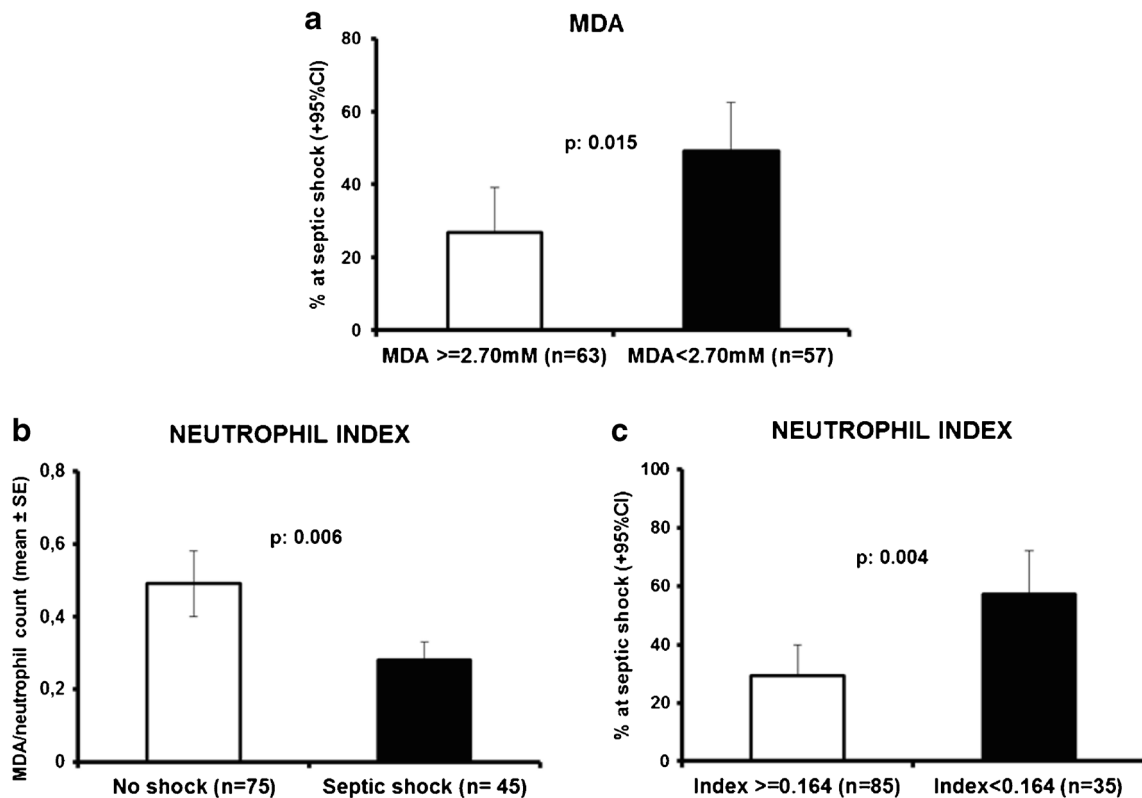


Fig. 2 The association of MDA and septic shock. **a** Frequency of septic shock in relation to circulating MDA. **b** Comparison of neutrophil index between patients with septic shock and patients without septic shock. **c** Frequency of septic shock in relation to the neutrophil index. The ratio of

MDA to the absolute neutrophil count was considered an index of neutrophil function. *p*-Values indicate respective statistical comparisons. *CI* confidence interval

pathogen was 0.50 when the MDA was lower than 0.27 mM; it was increased to 7.50 for a *K. pneumoniae* carbapenem-resistant pathogen (*p* of comparisons of the two ORs = 0.011 by Breslow–Day’s test and *p* = 0.011 by Tarone’s test).

Discussion

The present study shows that the level of circulating MDA is modulating the disease severity and outcome of sepsis. Patients with low MDA are prone to shock. Although the mortality from septic shock is not affected by the level of MDA, patients not in shock render a greater risk for unfavorable outcome when MDA is low. Patients with low MDA

infected by species of *K. pneumoniae* were found to carry a greater risk for the pathogen to be resistant to carbapenems.

Our data suggest that MDA can be considered an indirect index of the potential of neutrophils for the production of oxygen free radicals by the azurophilic granules. Free radicals attack phospholipids of cell membranes and this leads to the production of MDA [9]. This index is decreased in septic shock and it is compatible with the exhaustion of the innate immune defense that takes place when septic shock develops [15]. The functional significance of the index is fully demonstrated among the non-shocked population. In that population, where shock does not exist as a driver for mortality, circulating MDA lower than 2.70 mM indicates the presence of neutrophil exhaustion and this can independently predispose to death.

Table 2 Association of circulating MDA with 28-day outcome in relation to the absence or presence of septic shock

	MDA (<i>n</i>)	Survival (<i>n</i> , %)	Death (<i>n</i> , %)	<i>p</i> -Value	OR for death (95% CI)
Absence of septic shock	≥2.70 mM (46)	42 (91.3)	4 (8.7)	0.033	4.00 (1.08–14.82)
	<2.70 mM (29)	21 (72.4)	8 (27.6)		
Presence of septic shock	≥2.70 mM (17)	5 (29.4)	12 (70.6)	0.208	0.48 (0.13–1.73)
	<2.70 mM (28)	13 (46.4)	15 (53.6)		

CI confidence interval, *n* number of observations, *OR* odds ratio

Table 3 Association of circulating MDA with resistance to carbapenems in relation to the isolation of *Klebsiella pneumoniae* or not as a bloodstream pathogen

	MDA (n)	Survival (n, %)	Death (n, %)	p-Value	OR for death (95% CI)
Non- <i>K. pneumoniae</i> isolate	≥2.70 mM (49)	41 (83.7)	8 (16.3)	0.361	0.50 (0.14–1.79)
	<2.70 mM (45)	41 (91.1)	4 (8.9)		
<i>K. pneumoniae</i> isolate	≥2.70 mM (14)	10 (71.4)	4 (28.6)	0.047	7.50 (1.31–43.03)
	<2.70 mM (12)	3 (25.0)	9 (75.0)		

CI confidence interval, n number of observations, OR odds ratio

As far as the predisposition to carbapenemase-producing *K. pneumoniae* is concerned, there are a couple of explanations. First of all, these patients have a low MDA, which implies an exhaustion of neutrophil function. This may lead to more acquisition of prevalent hospital pathogens. A more attractive explanation comes from the recent findings of our group. We have recently shown that isolates of *K. pneumoniae* which produce carbapenemase suppress T17 cells and subsequent production of interleukin (IL)-17 [16]. IL-17 is a major chemoattractant for neutrophils at the infection site. Low MDA could come from suppressed production of IL-17.

The results of our study may also explain, in part, the controversial findings of trials of antioxidants as adjuvant therapy of sepsis. Most of the available clinical studies have been conducted with the parenteral administration of selenium [3–5]. Our findings would suggest that antioxidants are not needed when MDA is low and neutrophil exhaustion prevails. When they are given at a state of neutrophils overactivation, they may prevent worsening of the patient. In this context, a small recent clinical randomized trial of 29 mechanically ventilated critically ill patients receiving selenium parenterally showed decrease in the development of new episodes of ventilator-associated pneumonia over follow-up compared to placebo-treated patients [17].

In conclusion, our findings suggest that circulating neutrophils present signs of functional exhaustion for the production of oxygen free radicals in Gram-negative sepsis, making patients susceptible to septic shock. This is also associated with infections by carbapenem-resistant *K. pneumoniae*. These findings underline the need for more personalized therapies taking into consideration the level of neutrophil function.

Compliance with ethical standards

Funding The study was funded by the Hellenic Institute for the Study of Sepsis. The study sponsor had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

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

Ethical approval The study protocol was approved by the Ethics Committee of ATTIKON University Hospital (approval 209/25-05-2009). Adult patients were enrolled after written consent provided by themselves or by first-degree relatives for patients unable to consent.

Informed consent Patients were enrolled after written consent provided by themselves or by first-degree relatives for patients unable to consent.

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