Circulating neutrophil-derived microparticles associated with the prognosis of patients with sepsis

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Research

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Abstract

Background

To investigate the relationship between plasma neutrophil-derived micro-particles (NDMPs) and sepsis patients' prognosis.

Methods

Eighty eligible patients were classified as the sepsis and septic shock group according to the international guidelines. Their demographic data, pro-inflammatory mediators (TNF-α, IL-6 and sTREM-1) and sepsis severity assessment index (PCT, APACHE-II scores, MODS scores, mechanical ventilation time, ICU length of stay (LOS) and total hospital length of stay (LOS)) of the post-admission day 1, 3, 5 and 7 were harvested. Their plasma NDMPs were determined with magnetic bead sorting and nanoparticle tracking analyser (NTA). Survival curve against the circulation NDMPs was constructed.

Results

The NDMPs level was higher in the septic shock patients than in the sepsis patients on the post ICU admission day 1, 3 and 5 ($P < 0.05$). The NDMPs levels were significantly increased with a parallel increase of pro-inflammatory mediators and sepsis severity ($P <0.05$) as well as mortality.

Conclusions

Our data suggested that NDMP may be a biomarker of sepsis severity and mortality but how its role on sepsis prognosis warrants further study.

Introduction

Extracellular vesicles (EVs) including membrane-derived exosomes, ectosomes, microvesicles, shedding microvesicles, and microparticles (MPs) have considerable biological functions and can mediate intercellular communications [1, 2]. Neutrophils are the first line immune cells infiltrating into inflamed tissues during acute inflammation and they subsequently produce micro-particles (NDMPs) in response to various inflammatory stimuli. NDMPs (100–1000 nm in diameter) are composed of proteins, lipids and nucleic acids releasing from stilling, activating or injurious neutrophils [3–6]. NDMPs in body fluids can modulate homeostasis and participate in various pathophysiological processes such as host defense suppression [7, 8], pro-inflammatory effect [9, 10] and delayed wound healing [11]. CD15 can serve as a specific cell surface protein marker of NDMPs [12].

Sepsis is characterized by overwhelming acute inflammatory response and impaired immune response [13], resulting in multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF) [14], or even death [15]. Immune cells such as neutrophils, macrophages, dendritic cells (DC), T lymphocytes, regulatory T cells (Tregs), and natural killer T (NKT) cells can initiate or suppress inflammation by

producing pro-inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-6] or inhibitory cytokines (IL-8, IL-10) in septic patients [16–20]. It has been reported that blood biomarkers including soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), procalcitonin (PCT) [21], presepsin (sCD14) [22] can be used to evaluate septic severity and prognosis, but their feasibility is not optimal yet [23]. NDMPs may play a role in the development and progression of sepsis [24], but their predictive value for septic outcome remains unknown. We hypothesize that sepsis can induce NDMPs production, leading to leukocyte inhibition, immunosuppression and poor prognosis. In the current study, the plasma concentration of NDMPs and pro-inflammatory mediators (sTREM-1, TNF-α and IL-6) were measured in sepsis patients, and their correlation with sepsis severity and mortality was analysed.

Materials And Methods

Patients' enrollment

After obtained the written informed consents, 40 sepsis and 40 septic shock patients in Intensive Care Unit (ICU) were recruited together with 10 healthy controls from the Health Surveillance Center in the Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong, China, from January to November 2018. Sepsis was diagnosed and classified using the international guidelines of severe sepsis and septic shock 2016 [18]. Patients were excluded if they had malignant tumor, immunosuppression or poisoning. According to 28-day survival after hospitalization, patients were further divided into the survival and non-survival group. The demographic data including age, gender, primary disease, medical history, laboratory measurements, Acute Physiology and Chronic Health Evaluation (APACHE) score, MODS score, mechanical ventilation time, ICU and total length of hospital stay (LOS), and 28-day survival were recorded.

Isolating of NDMPs by using anti-CD15 conjugated microbeads

The isolation of MVs was performed as previous report [25]. In brief, plasma sample was centrifuged at 300g at 4°C for 15 min and followed by at 2000g at 4°C for 30 min to remove cell debris. The supernatants were centrifuged at 20000g at 4°C for 70 min to pellet microparticles (MPs). The pelleted MPs were incubated with 10μl of Biotin-conjugatedanti-CD15 antibody (Milteny Biotech, Bergisch-Gladbach, Germany) in a 100 μl reaction volume for 2hrs, followed by adding 10 μl of anti-Biotin microbeads (Milteny Biotech) for 15 min. Then the microbeads-labeled MPs from the total MPs suspension was separated by using a DynaMag-2 magnet (Life technology, shanghai, China). After an overnight magnet separation, the fluid was gently removed and the microbeads-bound MPs were resuspended with 100 μl particle-free PBS which was filtered through 20 nm membrane filter (Anotop 25, Whatman, United Kingdom). The multi-sort release reagent (Milteny Biotech) (10 μl) was added to cleave off the microbeads. After 10 min, sample was brought a 250 μl final volume with filtered PBS, and placed on the magnet. On the next day, the MPs in the fluid were collected to be CD15+ MPs. All isolated MPs were enumerated by using the NTA NS300 system (Malvern, United Kingdom).

Nanoparticle tracking analysis

The NanoSight NS300 (Malvern, United Kingdom) was used to detect MPs [26]. The NanoSight polystyrene latex calibration beads, 100 nm and 1000 nm, were used to check the instrument performance. The camera level was maintained at 10 for light scatter mode. Data was analyzed by NTA 3.0 software (Malvern, United Kingdom).

ELISA assay

Plasma levels of sTREM-1 (RayBiotech, Norcross, GA, USA), TNF-α and IL-6 (NeoBioscience, Wuhan, China) were determined by ELISA kits according to the manufacturer's protocol.

Statistical analysis

Measurement data were expressed as means ± standard deviation, dot plot or median and range. Statistical analysis was carried out with GraphPad Prism 5.0 (San Diego, CA, USA) and SPSS22.0 (SPSS, Chicago, IL, USA). A p value of < 0.05 was considered to be statistically significant. Univariate analysis was performed using *t* test, x^2 test, Spearman correlation, and Wilcoxon rank sum test. Cumulative-survival curves were estimated with the Kaplan–Meier method. Receiver operating characteristic (ROC) curve of NDMPs were constructed.

Results

Ninety-four patients were screened, of whom 14 were excluded because of immunosuppressant users (11 cases) and malignant tumor (3 cases). Eighty eligible patients were classified as sepsis (n = 40) and septic shock patients (n = 40), and their baseline characteristics at the ICU admission were listed in the Table 1. Compared to the sepsis patients, the WBC count, APACHE II score and MODS score were significantly higher in the septic shock patients ($p < 0.05$).

Table 1
The baseline characteristics of sepsis and septic shock patients at the ICU admission

Characteristics	Sepsis group (n = 40)	Septic shock group (n = 40)	P value
Age (years)	65 ± 15	69 ± 18	ns
Male/female	25/15	30/10	ns
Temperature (°C)	38.2 ± 1.2	38.9 ± 0.9	ns
WBC counts (× 10^9/L)	13.5 ± 6.2	16.2 ± 7.9	< 0.01
APACHE II score	14.7 ± 0.8	17.8 ± 0.7	0.0085
MODS score	5.8 ± 0.4	7.3 ± 0.3	0.0072
Source of infection (n, %)			
Lung	32 (80%)	34 (85%)	ns
Abdomen	15 (37.5%)	12 (30%)	ns
Blood stream	21(52.5%)	23 (57.5%)	ns
Invasive vessel	31(77.5%)	40(100%)	ns
Skin and soft tissue	9(22.5%)	12(30%)	ns
Other	3(7.5%)	5(12.5%)	ns
Comorbidities			
Current smoker	19(47.5%)	23(57.5%)	ns
Diabetes	12(30%)	17(47.5%)	ns
Alcohol intake	7(17.5%)	11(27.5%)	ns
Hypertension	28(70%)	34(85%)	ns
Coronary disease	14(35%)	19(47.5%)	ns
Chronic heart failure	18(45%)	23(57.5%)	ns
Chronic kidney disease	4(10%)	3(7.5%)	ns
Cirrhosis	2(5%)	5(12.5%)	ns
Chronic obstructive pulmonary	13(32.5%)	17(42.5%)	ns

Nanoparticle tracking analysis of the plasma NDMPs

The NDMPs can be easily seen after immmunostained (Fig. 1a). The plasma NDMPs were detected in the healthy controls, sepsis and septic shock patients (Fig. 1b-d) by Nanoparticle tracking analysis. The plasma concentration of NDMPs in the sepsis and septic shock patients at the ICU admission were significantly higher than those in healthy people (P<0.05) but they were even higher in the septic shock patients (Fig. 1e).

NDMPs, TNF-a, IL-6, sTREM-1, PCT and clinical parameters in sepsis or septic shock patients

The plasma NDMPs concentration in the septic shock patients were significantly higher than those in the sepsis patients during the 1st day to the 5th day of ICU admission (P< 0.05). There was no significant difference in the plasma levels of NDMPs between sepsis and septic shock patients on the 7th day (P> 0.05) (Fig. 2a). Compared with the sepsis group, the pro-inflammatory factors (TNF-a, IL-6, sTREM-1), PCT, APACHE II score and MODS score were significantly higher in the septic shock group from the 1st day to the 7th day of ICU admission (P< 0.05) (Fig. 2b-g). Mechanical ventilation time, ICU LOS and total hospital LOS in the septic shock group were significantly longer than those of the sepsis group (P< 0.05) (Fig. 2h-j).

Relationship between plasma NDMPs concentration and pro-inflammatory cytokines and clinical outcome data

The plasma NDMPs concentration were positively correlated with TNF-α (r=0.828, p<0.01), IL-6 (r=0.955, p<0.001), PCT (r=0.851, p<0.01), sTREM-1 levels (r=0.831, p<0.01), APACHEII scores (r=0.792, p<0.01), MODS scores (r=0.605, p<0.05), mechanical ventilation time (r=0.892, p<0.01), ICU LOS (r=0.309, p<0.05) and total hospital LOS (r=0.933, p<0.001) (Fig. 3a-i). The correlation coefficient of NDMPs and IL-6 was the greatest among those measured variables.

The plasma NDMPs concentration of all patients on the post ICU admission day 1, 3, 5 and 7 were divided into four groups according to ascending quartile analysis (Quartiles cut off points of NDMPs concentration 9.7×10^7/ml, 3.55×10^8/ml, 6.4×10^8/ml). The levels of TNF-a and PCT were increased significantly with the increase of NDMPs concentration (P<0.05). The level of IL-6 showed an increasing trend but did not reach to a statistical significance (P>0.05). With the increase of NDMPs concentration, sepsis severity assessment index (APACHE II score, MODS score and mechanical ventilation time, ICU LOS and total hospital LOS) were also increased significantly (P<0.05) (Fig. 4).

Comparison of various experimental variables between survivals and non-survivals

Various variables including the NDMPs, sTREM-1, TNF-α, IL-6, APACHE II score, MODS score, mechanical ventilation time, ICU LOS, total hospital LOS and 28-days mortality between the survival and non-survival group were analyzed. When compared with the survivals, the NDMPs, sTREM-1, TNF-α, IL-6, APACHE II score, MODS score were significantly higher in the non-survivals from the 1st day to the 7th day of ICU admission (P< 0.05) (Fig. 5a-g). The28-days mortality, mechanical ventilation time, ICU LOS, total hospital

LOS were significantly higher in the non-survivals than those in the survivals (P< 0.05) (Table 2) (Fig. 5h-j).

Table 2
Clinical outcomes in patients with sepsis or septic shock

	Sepsis group (n = 40)	Septic shock group (n = 40)	P value
28-day mortality	11 (27.5%)	15 (37.5%)	0.2123
Mechanical ventilation time (day)	8.3 ± 0.82	17.30 ± 1.63	< 0.001
ICU LOS (day)	12.15 ± 0.81	20.20 ± 1.54	< 0.001
Total hospitalized LOS (day)	20.20 ± 1.54	27.20 ± 1.65	< 0.001
LOS: length of stay			

Predictive value of plasma NDMPs concentration for mortality

Survival curves were derived among 4 quartiles based on the plasma NDMPs concentration from low to high of all patients from the 1st day to the 7th day of the ICU admission ranked by ascending quartile method (the Quartiles cut off points of NDMPs concentration of 9.7×10^7/ml, 3.55×10^8/ml, 6.4×10^8/ml). The ability to predict mortality by the score system was assessed with receiver operating characteristic (ROC) curve analysis. The mortality was increased significantly with the increase of the plasma NDMPs concentration (log-rank test P=0.006). When compared with Quartile 1 as a reference, the hazard ratio at 1.746 (0.655-4.653, p=0.279), 2.66 (1.101-6.167, p=0.029) and 3.723 (1.697-8.165, p=0.002) for Quartile 2, Quartile 3 and Quartile 4, respectively (Fig. 6a). The sensitivity and specificity of NDMPS levels to predict death with operating characteristic (ROC) curve analysis (Fig. 6b) was given the area under curve (AUC) to be 0.77. When the NDMPs concentration set to be 3.55×10^8/ml as the cut-off point, the sensitivity and specificity were 0.882 and 0.630, respectively.

Discussion

This study demonstrated that plasma NDMPs concentration in the septic shock patients were significantly higher than that in the sepsis patients. Furthermore, we also found pro-inflammatory mediators such as IL-6, TNF-α, sTREM-1 and APACHEII scores, MODS scores, Mechanical ventilation time, ICU LOS and total hospital LOS were significantly increased with a parallel increase of NDMPs concentration. We further observed that plasma NDMPs concentration was remarkably higher in non-survivals than that in survivals and the mortality was also increased significantly with the increase of plasma NDMPs concentration.

Sepsis is one of the leading causes of morbidity and mortality world-wide [27–29]. Previous studies have shown that immune cells derive micro-particles, especially those produced by neutrophils, which are

extracellular machinery in communication between cells. NDMPs express extracellular facing proteins that are known to be involved in a variety of important cellular processes and thus can act as bioactive effectors. They have been implicated in a variety of disease processes including host defense [7], inflammatory response [8], and wound healing, lung injury, cancer and sepsis [30, 31]. It was found that NDMPs are phagocytosed by THP-1 cells and they have a divergent effect on the immune response by activating phagocytic cells and deactivating bystander cells during sepsis in patients [31]. NDMPs also cause immune dysfunction in sepsis by blunting the function of neutrophils and macrophages, and increase mortality in a murine model of sepsis [32].

In the present study, we found that a significant increase of plasma NDMPs concentration in patients with sepsis and septic shock when compared with the healthy controls, and as the ongoing sepsis development, at early stage of sepsis, from the day 1 to the day 5 after ICU admission, NDMPs were continuously increased, which suggested that neutrophils were activated by microbes and, in turn, produced abundant NDMPs. This may indicate that NDMPs might be involve in sepsis development and as a biomarker of the pathophysiology of sepsis. Accumulating evidence have suggested that some cytokines (i.e. IL-6, TNF-α), cell surface markers and soluble receptors (i.e. sTREM-1) played important roles in sepsis prognosis [33, 34]. Our data showed that pro-inflammatory mediators such as TNF-α, sTREM-1 and IL-6 were significantly positively correlated with NDMPs, and were increased with a parallel increase of plasma NDMPs concentration, which indicated that NDMPs might promote the release of pro-inflammatory mediators.

In this study, we also found that APACHEII scores, MODS scores, mechanical ventilation time, ICU LOS and total hospital LOS were positively correlated with NDMPs and were increased with the increase of plasma NDMPs concentration, suggesting that NDMPs were associated with the severity of sepsis. In addition, we also found that the plasma NDMPs concentration in non-survivals was significantly higher than that in survivals, and with the increase of plasma NDMPs concentration, the mortality of sepsis patients was significantly increased, which may suggest that NDMPs might be closely related to the prognosis of sepsis. The ROC curve analysis showed that the value of NDMPs to predict mortality of sepsis patient is considerably high.

Diagnosis sepsis and septic shock at early stage is essential, because any delay in the initiation of a proper treatment is associated with poorer prognosis [35]. Therefore, detecting plasma NDMPs immediately after ICU admission might be helpful to understand the severity and prognosis of sepsis and to promote proper treatments. However, we do realize is that all measurements in this study and clinical outcomes reported herein are only associations and are within an observational nature. In addition, a relative small sample size of our study is another limitation. All those make us taking more cautions to interpret our data and to investigate more in this area of research.

Conclusions

In conclusion, we found that NDMPs together with pro-inflammatory mediators were significantly increased in sepsis and further increased in septic shock patients. Those increases were associated with the severity of sepsis. Plasma NDMPs may be considered to be a prognostic biomarker of sepsis severity and mortality but how its role on sepsis prognosis warrants further study.

Declarations

Ethics approval and consent to participate

All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee (2016-093KT) and obtained the written informed consents.

Consent for publication

The authors agree with the consent for publication.

Availability of data and material

Not applicable

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Lie-Hua Deng and Da-Qing Ma conceived and designed research. Hong-Peng Chen and Xiao-Yan Wang performed experiments, analyzed date and drafted manuscript. Xiao-Yan Pan, Wang-Wang Hu and Shu-Ting Cai interpreted results of experiments.

Acknowledgement

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Figures

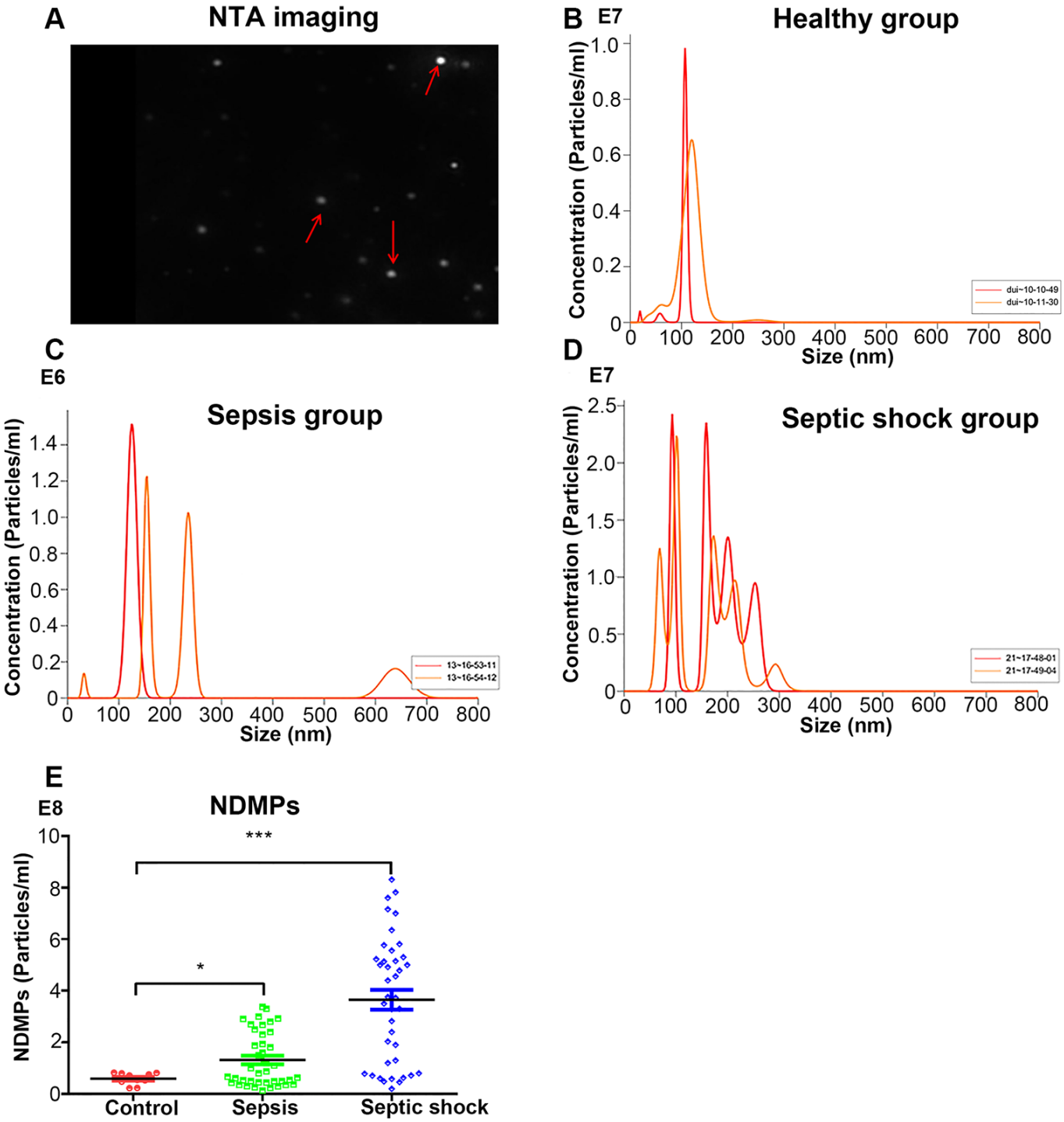

Figure 1

The plasma micro-particles (NDMPs) in healthy controls, sepsis and septic shock patients. (a) Nanoparticle tracking analysis imaging of NDMPs (red arrow). An example measurment trace of a healthy control group (b), sepsis (c) and septic shock patient (d). (e) Dot plot of plasma NDMPs the healthy individuals, sepsis and septic shock patients.*P<0.05 and ***P<0.001.

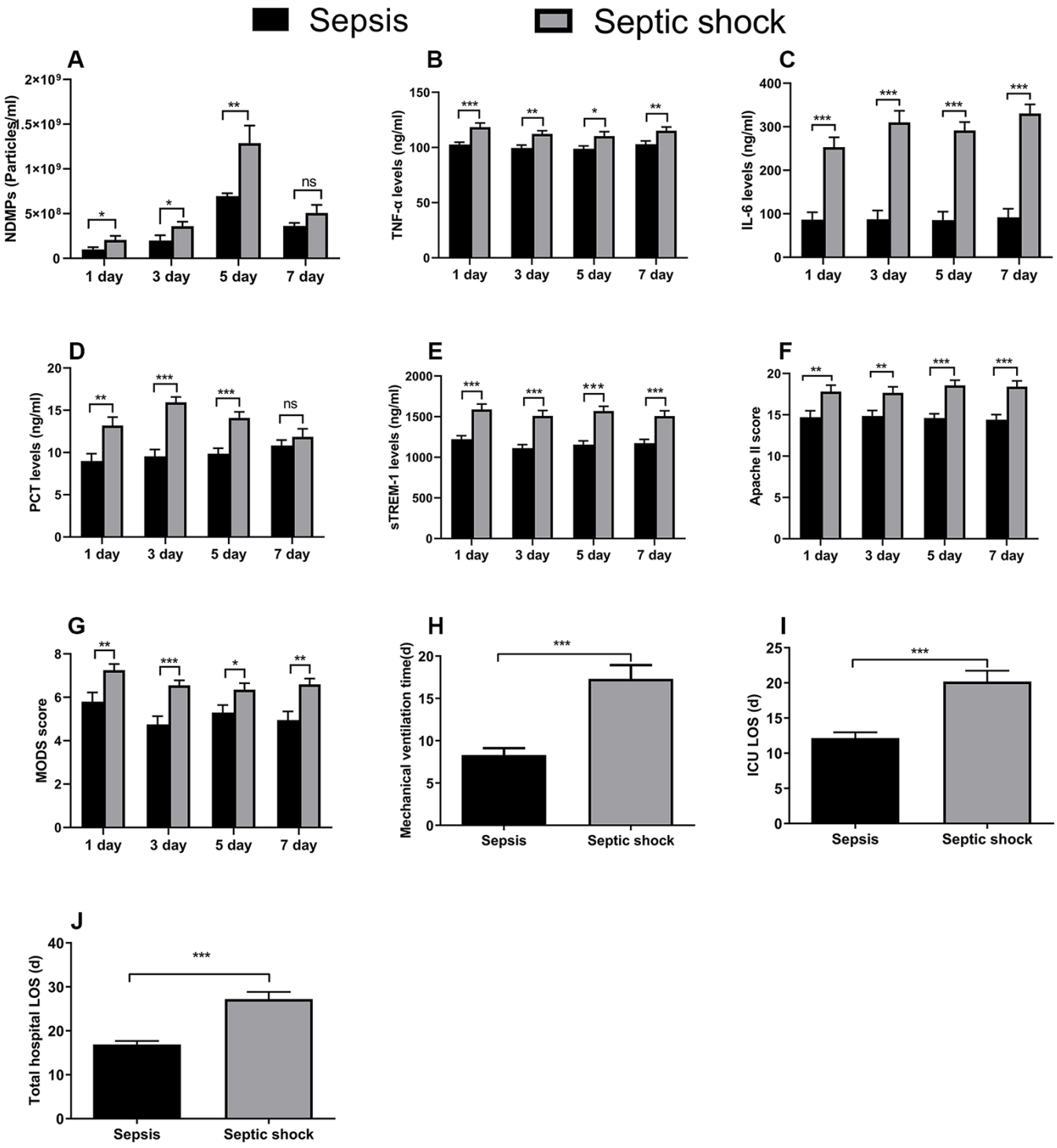

Figure 2

The plasma levels of NDMPs (a), TNF-α (b), IL-6 (c), PCT (d), sTREM-1 (e), Apache II score (f) and MODS score (g) on the days 1 (40 cases in sepsis group versus 40 cases in septic shock group), 3 (38 versus 36 cases), 5 (33 versus 30 cases) and 7 (29 versus 25 cases); mechanical ventilation time (h), ICU LOS (i) and total hospital LOS (j) between sepsis and septic shock groups. Data were mean ± SD and analyzed by t-test. *P<0.05, **P<0.01, ***P<0.001.

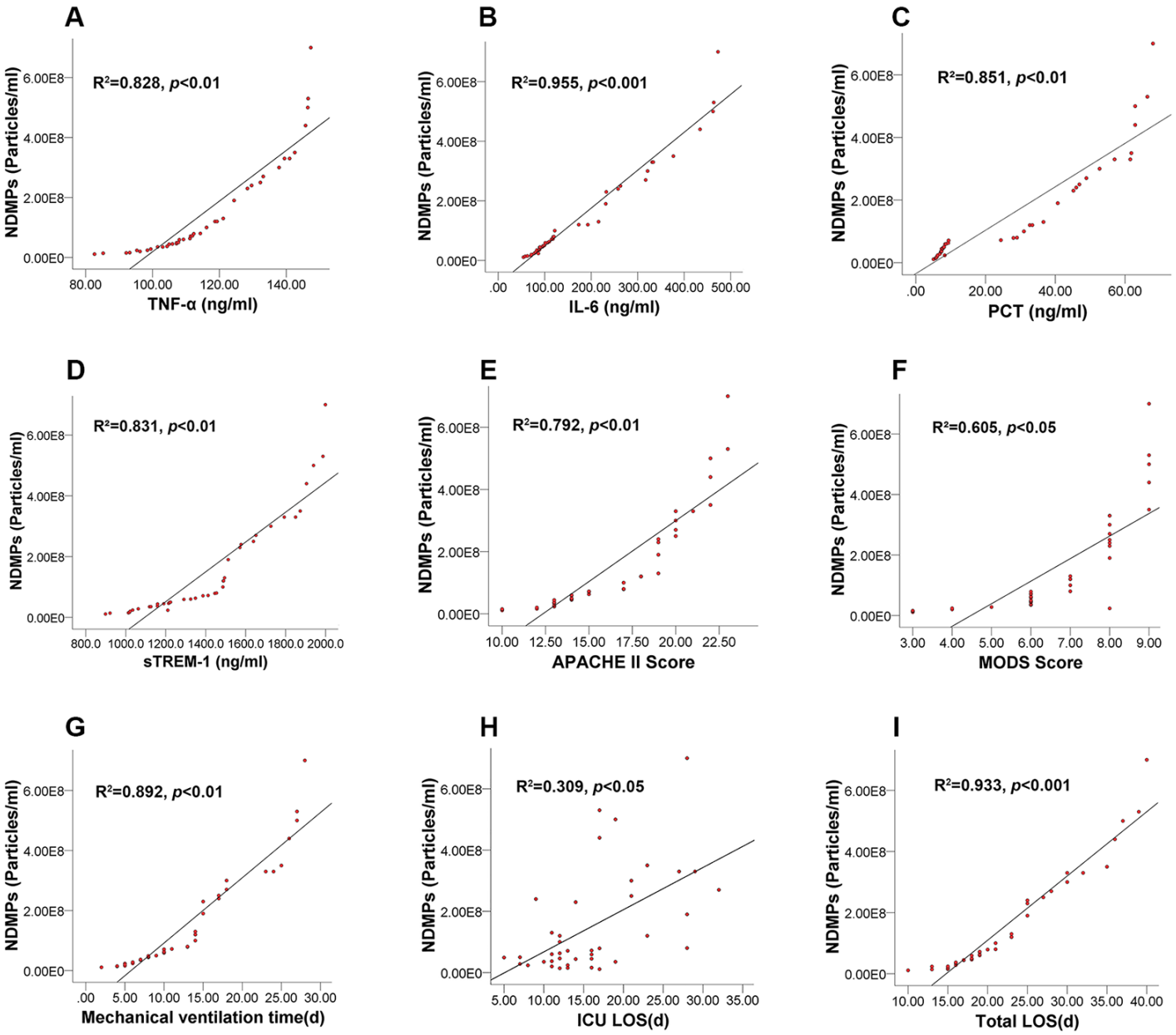

Figure 3

Linear correlation between plasma NDMPs concentration and the levels of TNF-α (a), IL-6 (b), PCT (c) andsTREM-1 (d), Apache II score (e), MODS score (f), mechanical ventilation time (g), and ICU LOS (h), and total hospital LOS (i) in sepsis and septic shock patients.

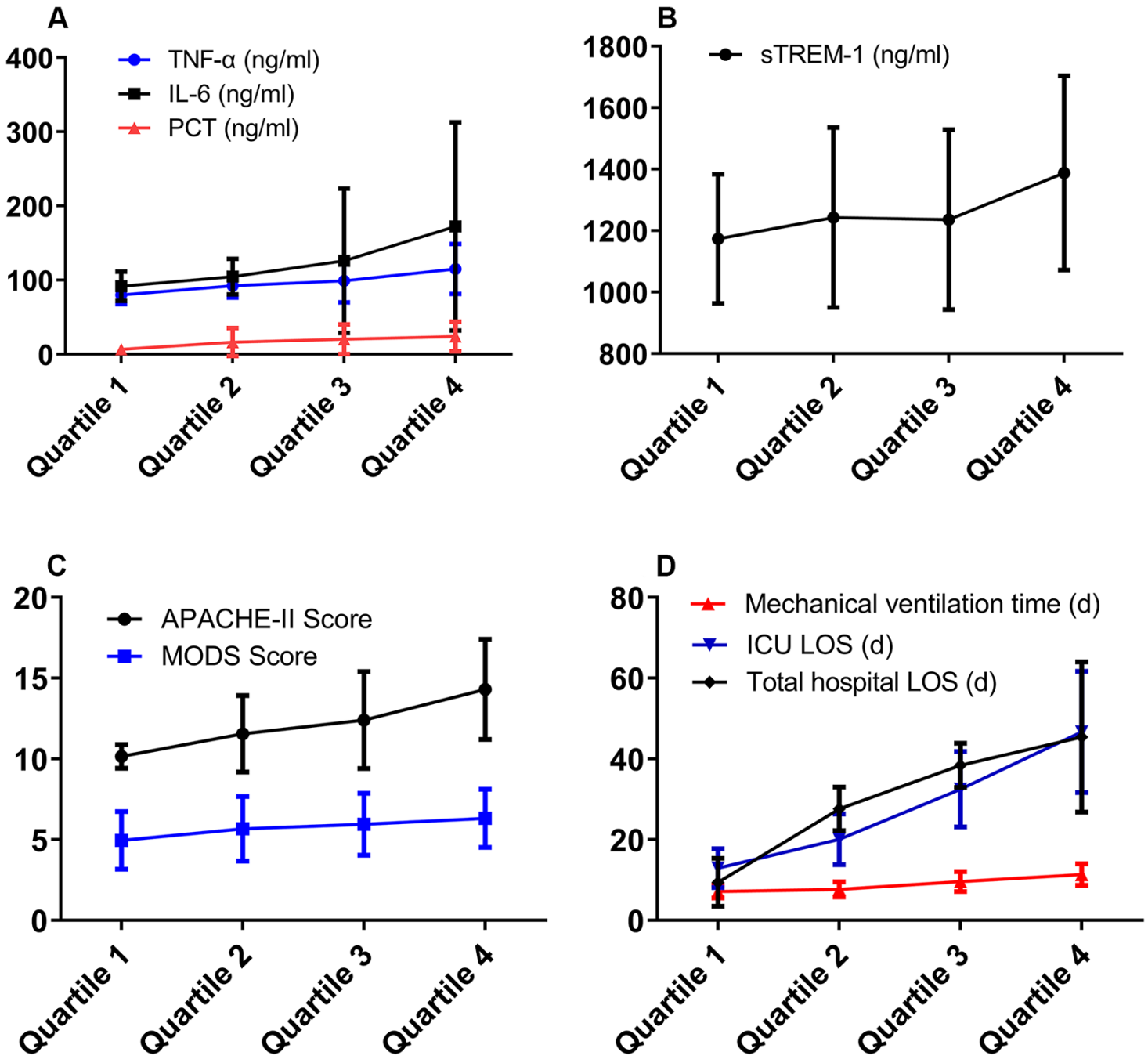

Figure 4

The correlation between plasma NDMPs concentration and mechanical ventilation time, ICU LOS, total hospital LOS, disease severity and inflammatory factors in sepsis and septic shock patients. After ranging the plasma NDMPs concentration of all the patients on days 1,3,5,7 by ascending quartile method (Quartiles cutoff points of NDMPs concentration 9.7×107/ml, 3.55×108/ml, 6.4×108/ml), following the gradual increase of NDMPs concentration, p value for trend- test was (a TNF-α p=0.0045, IL-6 p=0.0537, PCT p=0.0128, b sTREM-1 p=0.0403, c Apache II Score p<0.001, MODS Score p=0.0486, d Mechanical ventilation time p<0.001, ICU LOS p<0.001, Total hospital LOS p<0.001)

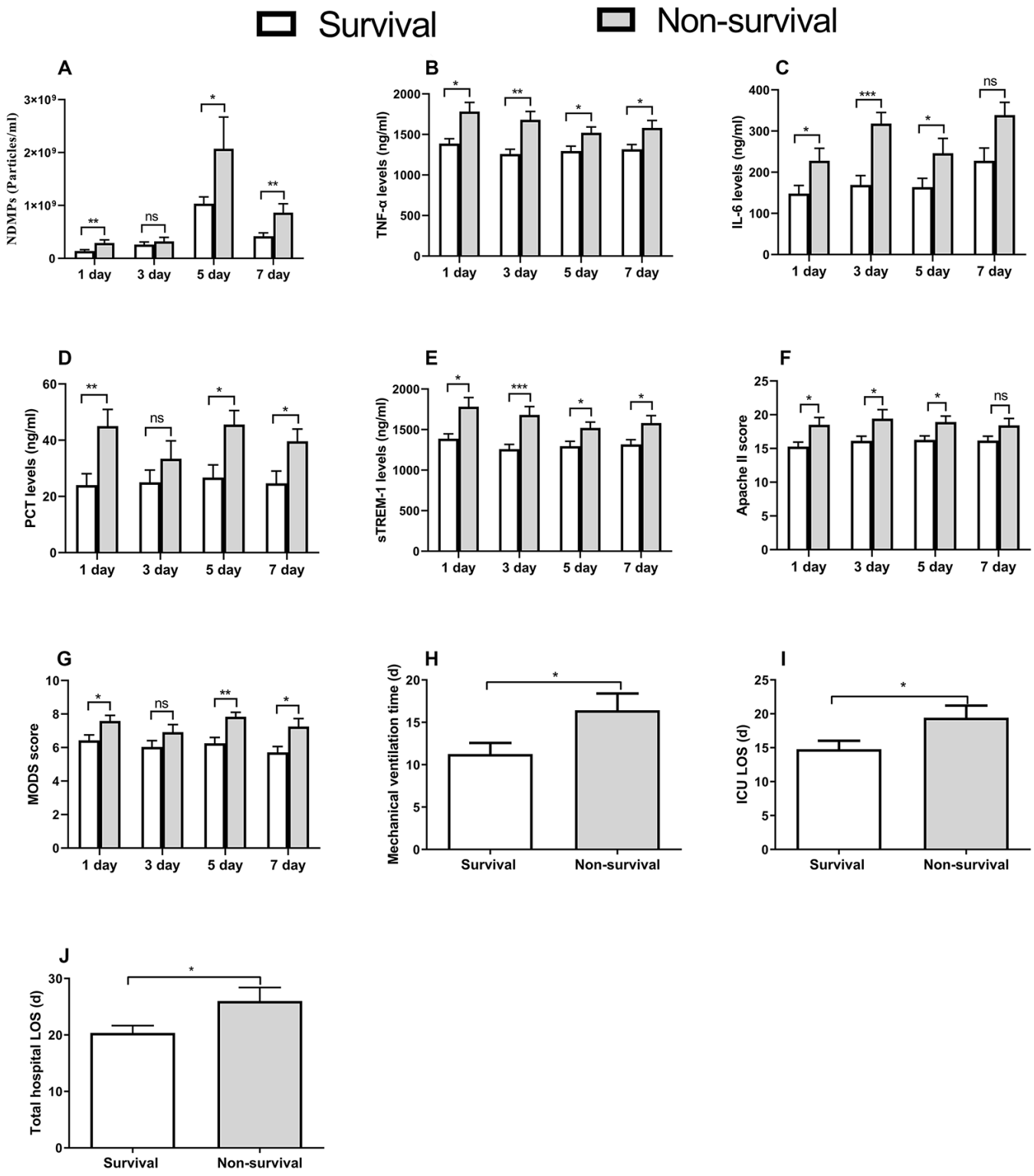

Figure 5

Plasma concentration of NDMPs (a), TNF-α (b), IL-6 (c), PCT (d), sTREM-1 (e), Apache II score (f) and MODS score (g) on days 1 (54cases in survival group versus 26 cases in non-survival group), 3 (52 versus 22 cases), 5 (49 versus 17 cases) and 7 (45 versus 14 cases); and mechanical ventilation time (h), and ICU LOS (i) and total hospital LOS (j) in survival and non-survival groups. Data were mean ± SD and analyzed by t-test. *P<0.05, **P<0.01, ***P<0.001 compared to sepsis group.

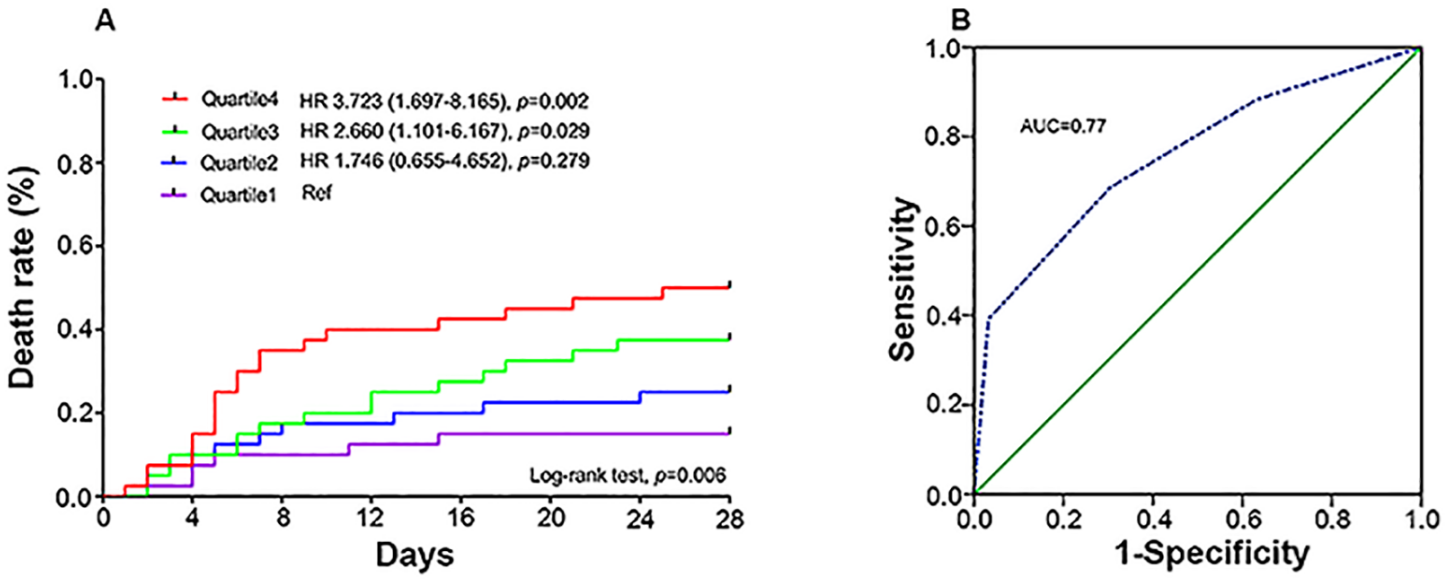

Figure 6

NDMPs vs outcomes. (a) Patient death among 4 quartiles based on the plasma NDMPs concentration from low to high of all the patients on the post admission day 1,3,5 and 7 ranged by ascending quartile method (Quartiles cut off points of NDMPs concentration 9.7×107/ml, 3.55×108/ml, 6.4×108/ml), Log-rank (Mantel-Cox) test **p=0.0061. (b)ROC curve of the sensitivity and specificity of NDMPS levels to predict death in patients with sepsis and septic shock.