

Platelet soluble NOX2 as a new biomarker in patients with sepsis

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Research

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Abstract

Background

Substantial evidence supports the view that NOX2 may function as a pivotal regulator in various physiological and pathological processes. However, to date, the interplay between platelet soluble NOX2 levels and sepsis remains unclear. The overall aim of this work is to obtain a better insight into whether or not platelet NOX2 is involved in sepsis.

Methods

The levels of platelet soluble NOX2, from 31 patients diagnosed with Gram-positive sepsis, 36 patients with Gram-negative sepsis and 45 healthy individuals, were measured with sandwich ELISA kits that we developed.

Results

In this work, we showed that platelet NOX2 concentrations were significantly lower in healthy individuals compared to patients with sepsis ($P < 0.01$), and that platelet NOX2 values were significantly correlated with the platelet activation markers, including soluble CD40L and soluble P-selectin. Interestingly, we also found that platelet NOX2 values in patients with Gram-positive sepsis were higher than those in patients with Gram-negative sepsis.

Conclusions

Platelet NOX2 levels maybe an important indicator of the pathogenesis of sepsis-induced platelet activation.

Background

Sepsis is a life-threatening condition caused by an excessive inflammatory immune response to an infection. Remarkably, it has been reported that more than 30 million people worldwide have been suffered from sepsis each year [1]. Currently, it is widely accepted that sepsis can induce the dysregulation of coagulation system, which in turn contributes to the pathogenesis of the sepsis syndrome [2–3]. Over the years, growing evidence indicates that platelets act as crucial regulator in the coagulation system, and that these anucleate cell fragments play an important role in immunity and host defense [4]. Additionally, many micro-organisms including bacteria, viruses and fungi can interact with platelets, which results in the modification of platelet function and contribution to the pathogenesis of bloodstream infection. For example, it has been reported that (i) platelet activation status is a pivotal determinant for platelet lysis and clearance, which leads to thrombocytopenia, in dengue infection [5], (ii)

platelet activation can release platelet factor 4 (PF4), which inhibits HIV-1 infection of adjacent T cells at the stage of virus entry [6], and (iii) *Staphylococcus aureus* and *Streptococcus spp.* can interact with and activate platelets [7]. However, the precise function of platelets in sepsis remains to be established.

At present, accumulating evidence supports the notion that sepsis and platelet activation are close intertwined. This is perhaps best illustrated by the observation that *Streptococcus pyogenes* can induce platelet activation, including integrin activation and alpha and dense-granule release, which may facilitate bacterial infection [2]. Furthermore, a recent study has shown that a sepsis with common Gram-positive pathogens is associated with higher platelet reactivity [8]. Interestingly, Shannon and her colleagues have found that platelet reactivity is higher in early *Streptococcus pyogenes*-induced sepsis compared to late sepsis. This study suggests that platelet activation may be a pivotal biomarker to provide prognostic information during the pathogenesis of invasive *Streptococcus pyogenes* infection [9]. Therefore, examining and analyzing the platelet status may be crucial to identify patients at risk of development to sepsis, which could allow clinicians to perform early preventative treatment.

Reactive oxygen species (ROS) are a group of highly reactive molecules, which act as a double-edged sword in many physiological and pathological processes. Until now, it is widely assumed that an imbalance of ROS generation and degradation can disturb the cellular redox state and damage biomolecules, which in turn initiate and promote a variety of diseases [10]. On the other hand, at physiological low levels, ROS may serve as signaling molecules in various biological pathways [11]. Over the past few decades, a growing body of evidence also suggests that ROS are either directly or indirectly involved in the initiation and progression of platelet activation. Here, it is noteworthy that ROS generated by NADPH oxidase 1 (NOX1) and NOX2 enhance platelet activation via the Syk/PLC γ /calcium signaling pathway [12]. Moreover, a recent study has shown that ROS production may enhance autophagy to promote oxidized low-density lipoprotein (oxLDL)-induced platelet activation via the PI3K/Akt/mTOR pathway [13]. Thus, more future studies are needed to elucidate the role of ROS in platelet activation.

NOX2, encoded by the *CYBB* gene, is a key enzyme associated with generating superoxide anion [14]. It is currently a common belief that this enzyme is mainly present in monocytes, leukocytes, platelets and endothelial cells [15]. Importantly, Violi and his colleagues have been demonstrated that NOX2-derived platelet ROS production is associated with platelet activation *ex vivo* and *in vivo* via NO and isoprostane generation [16]. In addition, the same group also reported that a close interplay between oxidative stress and platelet activation in the intracoronary blood waste and aspirated thrombi of ST-elevation myocardial infarction (STEMI) patients. These data imply that oxidative stress act as an important role in promoting thrombus formation and growth [17]. In this respect, it is essential to mention that HIV infection is involved with increased platelet oxidative stress via enhancing platelet NOX2 activity, which in turn inducing the platelet activation *in vivo* [18]. Unfortunately, little is known about the role of platelet NOX2 in sepsis.

In this study, we aim to investigate the changes of platelet NOX2 levels in patients with sepsis and get a better insight into the interplay between platelet activation and NOX2-mediated ROS.

Methods

Human subjects – From March 2016 to May 2018, 31 patients diagnosed with Gram-positive sepsis, 36 patients with Gram-negative sepsis and 45 healthy individuals were enrolled in the study. Written informed consent was obtained from each subject. Patients with sepsis were diagnosed in accordance with the guidelines on treatment of Chinese severe sepsis/septic shock (2014 version), issued by the Chinese society of Critical Care Medicine. The study was carried out according to the principles of the Declaration of Helsinki and was approved by the medical ethics committee of the Second Affiliated Hospital of Chongqing Medical University. The clinical characteristics of study subjects are demonstrated in Table 1.

Table 1
Clinical characteristics of subjects in this study

	Healthy individuals	Gram-positive sepsis	Gram-negative sepsis
Number of subjects	45	31	36
Age (year, ranger)	22–67	15–88	18–95
(year, mean)	45.1	56.4	51.9
Sex (male:female)	22:23	21:10	24:11
NOX 2 (mean ± SD) pg/ml	781.62 ± 441.53	2993.58 ± 821.14	2293.77 ± 726.63
sCD40L (mean ± SD) ng/ml	3.93 ± 0.96	21.84 ± 6.00	20.30 ± 4.98
sP-Selectin (mean ± SD) ng/ml	14.47 ± 4.04	56.62 ± 14.71	53.16 ± 12.36
WBC (mean ± SD) 10 ⁹ /L	6.19 ± 1.29	10.31 ± 4.82	10.66 ± 3.86
CRP (mean ± SD) mg/L	6.60 ± 5.81	109.39 ± 54.48	113.66 ± 43.88
IL-6 (mean ± SD) pg/ml	1.81 ± 0.51	21.89 ± 9.24	23.68 ± 7.53

Complete blood count (CBC) – A Mindray BC6800 Hematology Analyzer (Mindray, Shenzhen, China) was used to perform complete blood cell counts including white cell differentials.

C - reactive protein (CRP) test – An automatic Immunofluorescence Analyzer Jet-iStar 3000 (Joinstar, Hangzhou, China) was employed to quantitatively detect CRP from EDTA-anticoagulated whole blood specimen.

Platelet preparation – Blood samples were drawn and mixed with 3.8% sodium citrate, then processed in a centrifuge for 15 minutes at 180 g to obtain platelet-rich plasma. As described by Violi and his colleagues, only the top 75% of the platelet-rich plasma was collected to avoid leukocyte contamination [15]. Next, to acquire platelet pellets, platelet-rich plasma was centrifuged for 10 minutes at 300g. In this

process, acid citrate-dextrose (1:7 v/v) was added to avoid platelet activation. Platelet pellets were suspended in HEPES buffer in presence of albumin, pH 7.35 according to Carnevale *et al* [16]. Supernatant was collected by centrifugation for 5 minutes at 300 g and stored at -80°C until analysis.

Analysis of NOX2, sCD40L, sP-selectin and IL-6 – Soluble NOX2-derived peptide was measured by an ELISA method as previously described by Pignatelli and his colleagues [Pignatelli et al., 2010]. Here, it should be mentioned that we modified this protocol to evaluate the serum levels of NOX2 in HBV patients [19]. 100 µL of samples or standard were added into each capture antibody-coated well (Anti-NOX2/gp91phox antibody; Abcam, USA) and incubated 2 hours at room temperature. After aspirating and washing each well 3 times, 100 µL of diluted detection antibody-conjugated HRP (Rabbit anti-human IgG H&G antibody; Abcam, USA) was added and incubated 1 hour at room temperature while gentle shaking. Repeating the aspiration/wash of each well, 100 µL of substrate solution was added and incubated at room temperature for 20 minutes. Next, adding 50 µL of stop solution (1 mol/L H₂SO₄) to each well. The absorbance of the colored solution of NOX2 was measured at 450 nm by using a Multiskan™ FC microplate reader (Thermo Scientific, MA, USA).

Platelet soluble CD40L (sCD40L) and platelet soluble P-selectin (sP-selectin) were analyzed utilizing human sCD40L ELISA kit (Thermo Scientific, MA, USA) and human sP-selectin ELISA kit (Thermo Scientific, MA, USA), according to the manufacturer's guideline. Serum IL-6 was analyzed utilizing human IL-6 ELISA kit (Multisciences, Hangzhou, China), according to the manufacturer's guideline. The absorbance of sCD40L, sP-selectin and IL-6 was also read at 450 nm. All samples were run in triplicate and the mean value was used for statistical analysis.

Statistical analysis – Statistics were performed using SPSS software, version 19.0 for windows (SPSS Inc, IL, USA). The biological and clinical characteristics of human subjects were shown as means ± SD (standard deviation). Two groups were compared with a Mann-Whitney test. Spearman's rank correlation analysis was employed to analyze association between two variables. The significance level was chosen to be $P < 0.01$.

Results

Platelet soluble NOX2 levels in patients and healthy individuals

A number of clinical and experimental studies have revealed that serum NOX2 may act as an important mediator in development of cardiovascular diseases [20]. For example, it has been reported that serum concentrations of NOX2 in hypercholesterolemic patients are significantly higher than those of healthy individuals [15]. In addition, we found that serum NOX2 may serve as a new biomarker for HBV-related disorders [19]. Importantly, several recent studies have reported that there is a good relationship between platelet soluble NOX2 (sNOX2) and platelet activation in various biological processes including STEMI and HIV infection [17–18]. To gain better insight into the crosstalk between platelet sNOX2

concentrations and sepsis, we detected the platelet sNOX2 levels in patients suffered from sepsis. In this study, we showed that (i) the concentrations of platelet sNOX2 in patients with sepsis were significantly higher than those of healthy individuals, and (ii) the platelet sNOX2 values in patients with Gram-negative sepsis were lower those in Gram-positive sepsis patients (Fig. 1).

Platelet activation in patients and healthy individuals

It is currently a common belief that platelet activation is involved in a variety of physiological and pathological pathways. However, to date, the interplay between platelet activation and sepsis remains to be determined. In this series of studies, we explored whether or not sepsis could induce platelet activation. Here, it is informative to note that platelet soluble CD40L and platelet soluble P-selectin are markers of platelet activation [16, 18]. As shown in Fig. 2A, the concentrations of sCD40L in healthy subjects (mean value: 3.93 ng/ml) were significantly lower than those of patients with sepsis (mean value: 21.01 ng/ml, $P < 0.01$). Interestingly, regarding the levels of sCD40L, no significant difference could be observed between patients suffered from Gram-positive sepsis (mean value: 21.84 ng/ml) and those with Gram-negative sepsis (mean value: 20.30 ng/ml, Fig. 2B). Furthermore, we also demonstrated that (i) soluble P-selectin values in patients with sepsis (mean value: 54.76 ng/ml) were considerably higher compared to those with control group (mean value: 14.47 ng/ml, $P < 0.01$, Fig. 2C), and (ii) there was no significant difference between patients with Gram-positive sepsis (mean value: 56.62 ng/ml) and patients with Gram-negative sepsis (mean value: 53.16 ng/ml, Fig. 2D).

Correlation between levels of platelet sNOX2 and platelet activation markers

To further our understanding of the link between platelet sNOX2 and platelet activation in sepsis, we examined whether or not platelet sNOX2 levels are correlated with platelet markers platelet sCD40L and platelet sP-selectin. In our experiments, we found that there is a positive correlation between platelet sNOX2 and platelet sCD40L in patients with sepsis ($r = 0.5982$, $P < 0.0001$, Fig. 3A). Here, it should be pointed out that there is a strong correlation between these two factors in patients with Gram-positive sepsis ($r = 0.6134$, $P < 0.0001$) and patients with Gram-negative sepsis ($r = 0.6526$, $P < 0.0001$, Fig. 3B). In addition, we have demonstrated that the same phenomenon regarding the relationship between platelet sNOX2 and platelet sP-selectin. As shown in Fig. 4B, a significant correlation between platelet sNOX2 and platelet sP-selectin in Gram-positive sepsis patients ($r = 0.5863$, $P < 0.0001$) and Gram-negative sepsis patients ($r = 0.6268$, $P < 0.0001$).

Correlation between levels of platelet sNOX2 and inflammatory markers

So far, mounting evidence has been collected that inflammation plays a crucial role in the initiation and progression of sepsis-related diseases [21]. Additionally, given the fact that inflammatory markers

including CRP and IL-6 can be elevated in patients suffered from sepsis, one may also question whether the observed increases of CRP and IL-6 in patients with sepsis are associated with platelet sNOX2 levels. In this work, we showed that (i) the levels of CRP and IL-6 in patients with sepsis were significantly higher than those of healthy subjects, and (ii) there was no significant difference between patients with Gram-positive sepsis and patients with Gram-negative sepsis in these two inflammatory markers (Fig. 5A, 6A). Importantly, there existed significant correlation between platelet sNOX2 value and inflammatory markers (CRP and IL-6) in patients with sepsis (Fig. 5B, 5C, 6B, 6C).

Platelet soluble NOX2 levels in subgroup of patients with sepsis

Finally, we investigate whether or not the concentration of platelet sNOX2 can distinguish between survivors and non-survivors. These experiments showed that platelet sNOX2 values were markedly elevated in non-survivors compared with survivors (Fig. 7A). Importantly, we found that platelet sNOX2 levels declined during the recovery phase compared to the acute phase of sepsis ($P < 0.01$).

Discussion

NOX2, a major ROS-generating enzyme, plays an important role in cellular redox metabolism. The significance of NOX2 for human health and development is highlighted by the existence of severe metabolic diseases [22]. Furthermore, it has become clear that NOX2 can serve as pivotal players in various physiological and pathological pathways, including chronic granulomatous disease, myocardial infarction and systemic lupus erythematosus (SLE). This postulate about SLE was based on the observations that NOX2 is an essential component of LC3-associated phagocytosis (LAP), and that mice and human lacking NOX2 may contribute to initiate and develop the pathogenesis of SLE [23]. In addition, strong arguments have been put forward that NOX2 activity may affect infection. For example, it has been reported that (i) serum NOX2 levels maybe an important indicator for the pathogenesis of progression of HBV-related disorder [19]; (ii) NOX2 serves as a crucial player in the phosphorylation of κ Ba at Ser32 and of p65 at Ser536 in Human respiratory syncytial virus (RSV) and Sendai virus, which in turn result in the induction of NF- κ B activation [24]; and (iii) the replication of influenza A virus requires the NOX2 activity, which is associated with virus-induced lung inflammation [Vlahos et al., 2011]. Recently, more works have focused on the impact of NOX2-mediated oxidative stress on platelet activation. This is perhaps best illustrated by the observation that HIV infection triggers platelet oxidative stress *in vivo* by upregulating NOX2 activity [18]. However, so far, the biological relevance of platelet sNOX2 levels in sepsis remains to be established. In this study, we have provided novel solid evidence that platelet sNOX2 concentrations are significantly more elevated in patients with sepsis than in healthy individuals. Additionally, we have shown that (i) the levels of platelet sCD40L and platelet sP-selectin, two platelet activation markers, in patient with sepsis are much higher than those in healthy group; and (ii) a significant correlation exists between platelet sNOX2 and these two markers in sepsis patients. Therefore,

our findings support the notion that platelet sNOX2 may be a new candidate biomarker for assessing sepsis-induced platelet activation.

Currently, it is generally believed that Gram-positive sepsis can induce platelet activation. This hypothesis is mainly based on the observations that (i) pathogens isolated from patients with Gram-positive bacteremia can trigger platelet activation from the same infected host *ex vivo* [26]; and (ii) *S. aureus* are able to directly or indirectly bind platelet via releasing toxin or using surface proteins such as protein A and clumping factor A, which result in the platelet activation in *S. aureus* sepsis [27]. Here, it should be mentioned that the crosstalk between Gram-negative sepsis and platelet activation remains unclear and controversial. Numerous experimental data indicated that Gram-negative bacteria such as *E. coli* may activate platelet via FcγRIIa-dependent manner [28]. However, this model is challenged by various research groups, who provide evidence that limited platelet activation occurs in patients with *E. coli* sepsis [8]. Interestingly, in this work, we demonstrated that (i) the levels of platelet activation markers including sCD40L and sP-selectin in patients with sepsis are much higher than those in healthy individuals; and (ii) no difference about platelet activation markers concentrations is observed between patients with Gram-positive sepsis and patients with Gram-negative sepsis. In addition, there is a strong correlation between platelet sNOX2 levels and the platelet activation markers values in patients with sepsis. Here, our data suggest that patients with Gram-positive sepsis display higher platelet activation due to the fact that platelet sNOX2 values are higher in these patients.

Over the years, an increasing number of observations have lent support to the concept that inflammation can function as a crucial regulator in the initiation and development of pathogenesis of sepsis. In our setting, we confirm and extend these findings by showing that the levels of pro-inflammatory markers, including CRP and IL-6, are higher than the corresponding control. Importantly, we found that there is no difference between Gram-positive patients and Gram-negative patients. Hence, our findings imply that inflammation maybe not a determinant factor in sepsis-induced platelet activation, and that NOX2-mediated oxidative stress may serve as an important role in these processes.

Collectively, our evidence, combined with these previous results, strongly indicates that platelet NOX2 may be one of crucial mediators that contributes to the initiation and development of sepsis. These findings open up the way to potential new candidate for assessing clinical severity in sepsis. Importantly, further research with a large number of patients also needs to be done to investigate the molecular mechanisms underlying the role of NOX2 in sepsis-related pathologies.

Conclusions

In this study, we demonstrated that platelet sNOX2 values were higher in patients with sepsis compared to healthy individuals, and that platelet sNOX2 levels were significantly positively correlated with platelet activation markers (soluble CD40L and soluble P-selectin) and inflammatory markers (CRP and IL-6). These results imply that platelet sNOX2 values maybe a new candidate biomarker of the pathogenesis of sepsis. Furthermore, we showed that platelet NOX2 values in patients with Gram-positive sepsis were

higher than those in patients with Gram-negative sepsis. Our findings suggest that platelet NOX2 is a useful tool for assessing clinical severity in sepsis.

Declarations

Ethical Approval and consent to participate

The study was approved by the Ethical Committee of the Second Affiliated Hospital of Chongqing Medical University, (approval no. 39/2015).

Consent for publication

Not applicable

Available of data and materials

Data and materials are available from the corresponding author upon request.

Competing interests

All authors declare no conflict of interest

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Author contributions

BW designed the study. LM, YX, YD, YH, WC produced and analyzed the data. All authors wrote the manuscript.

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Not applicable

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Figures

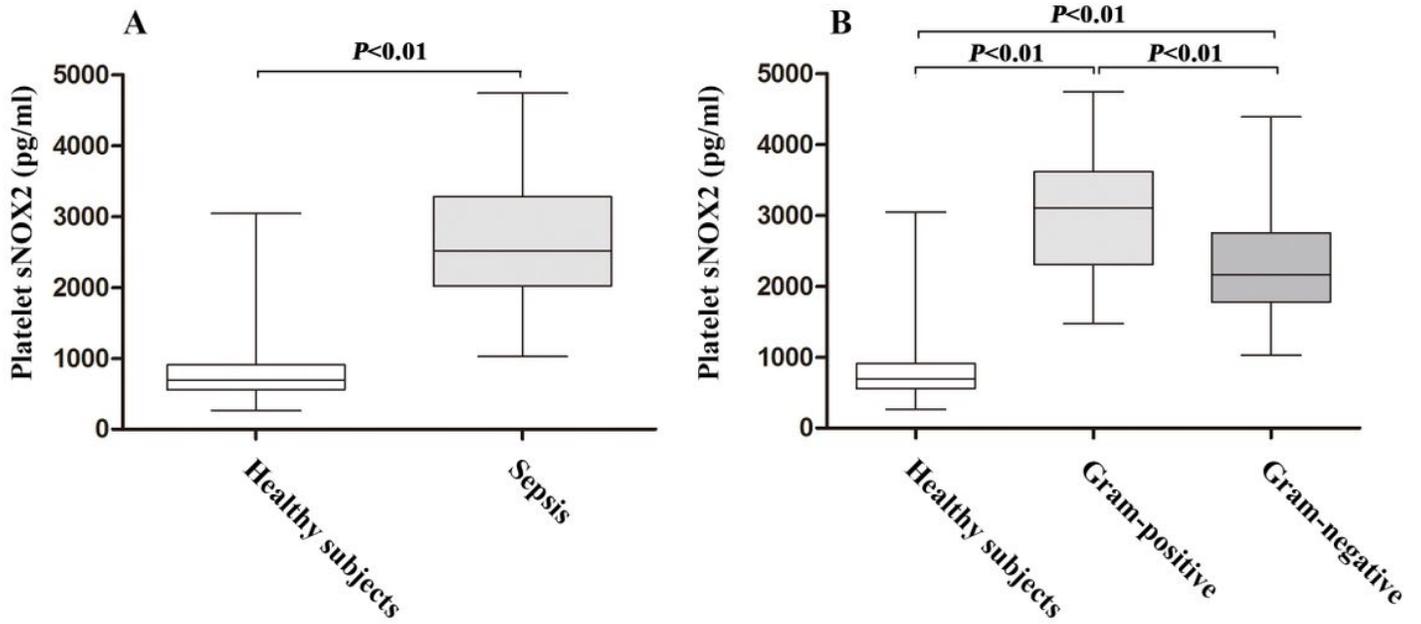


Figure 1

Platelet soluble NOX2 values in subjects. The bottom and top of each box represent the 25th and 75th percentile values, respectively; the horizontal line inside each box represents the median; and the horizontal lines below and above each box represent respectively the mean minus and plus one standard deviation. (A) The box plot shows the levels of platelet soluble NOX2 in healthy individuals and patients with sepsis. (B) The box plot demonstrates the levels of platelet soluble NOX2 in patients with Gram-positive sepsis and patients with Gram-negative sepsis. Statistical significance (P) is indicated.

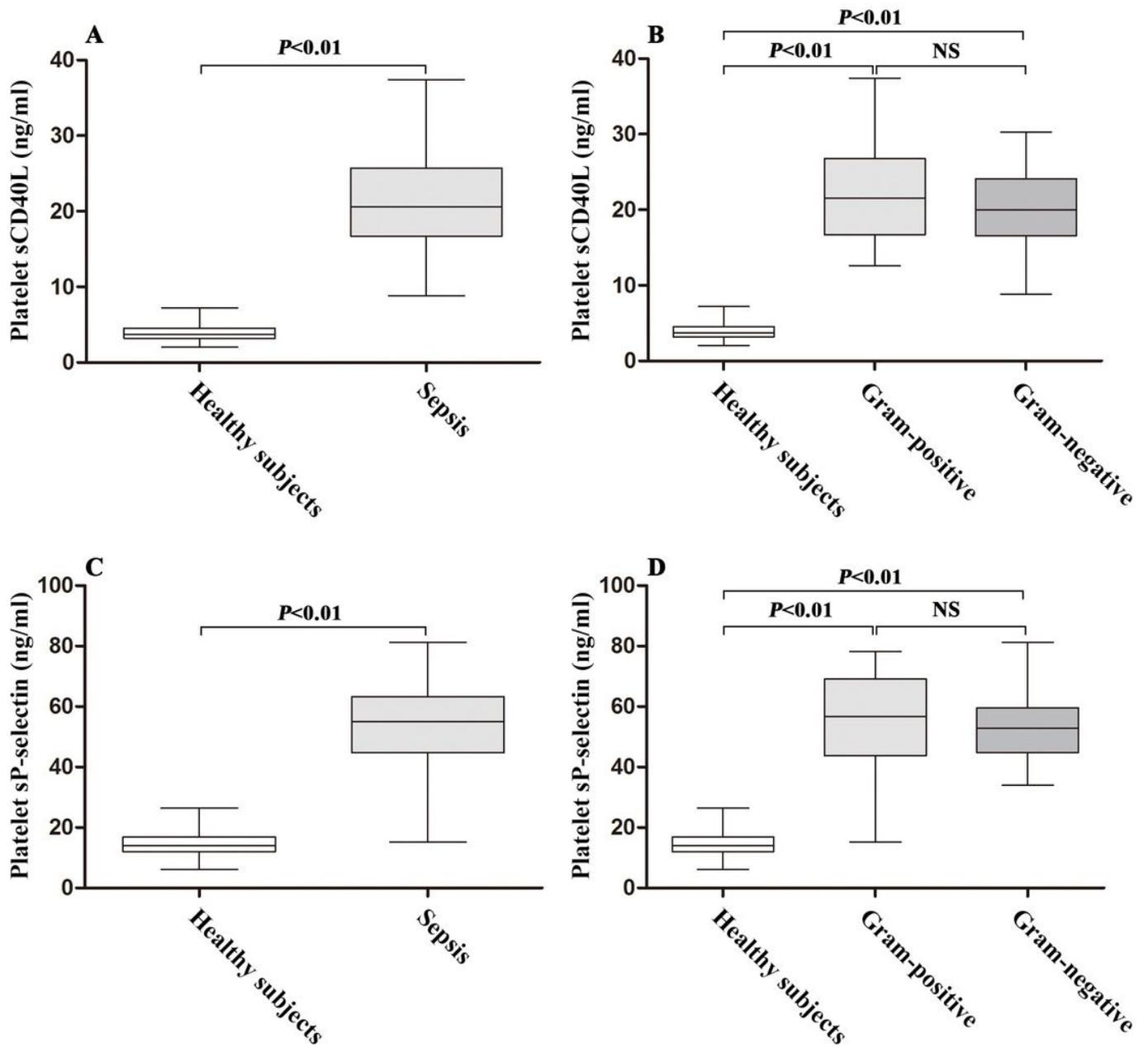


Figure 2

Platelet activation markers values in subjects. The bottom and top of each box represent the 25th and 75th percentile values, respectively; the horizontal line inside each box represents the median; and the horizontal lines below and above each box represent respectively the mean minus and plus one standard deviation. (A) The box plot shows the levels of platelet soluble CD40L in healthy individuals and patients with sepsis. (B) The box plot demonstrates the levels of platelet soluble CD40L in patients with Gram-positive sepsis and patients with Gram-negative sepsis. (C) The box plot shows the levels of platelet soluble P-selectin in healthy individuals and patients with sepsis. (D) The box plot demonstrates the levels of platelet soluble P-selectin in patients with Gram-positive sepsis and patients with Gram-negative sepsis. Statistical significance (P) is indicated.

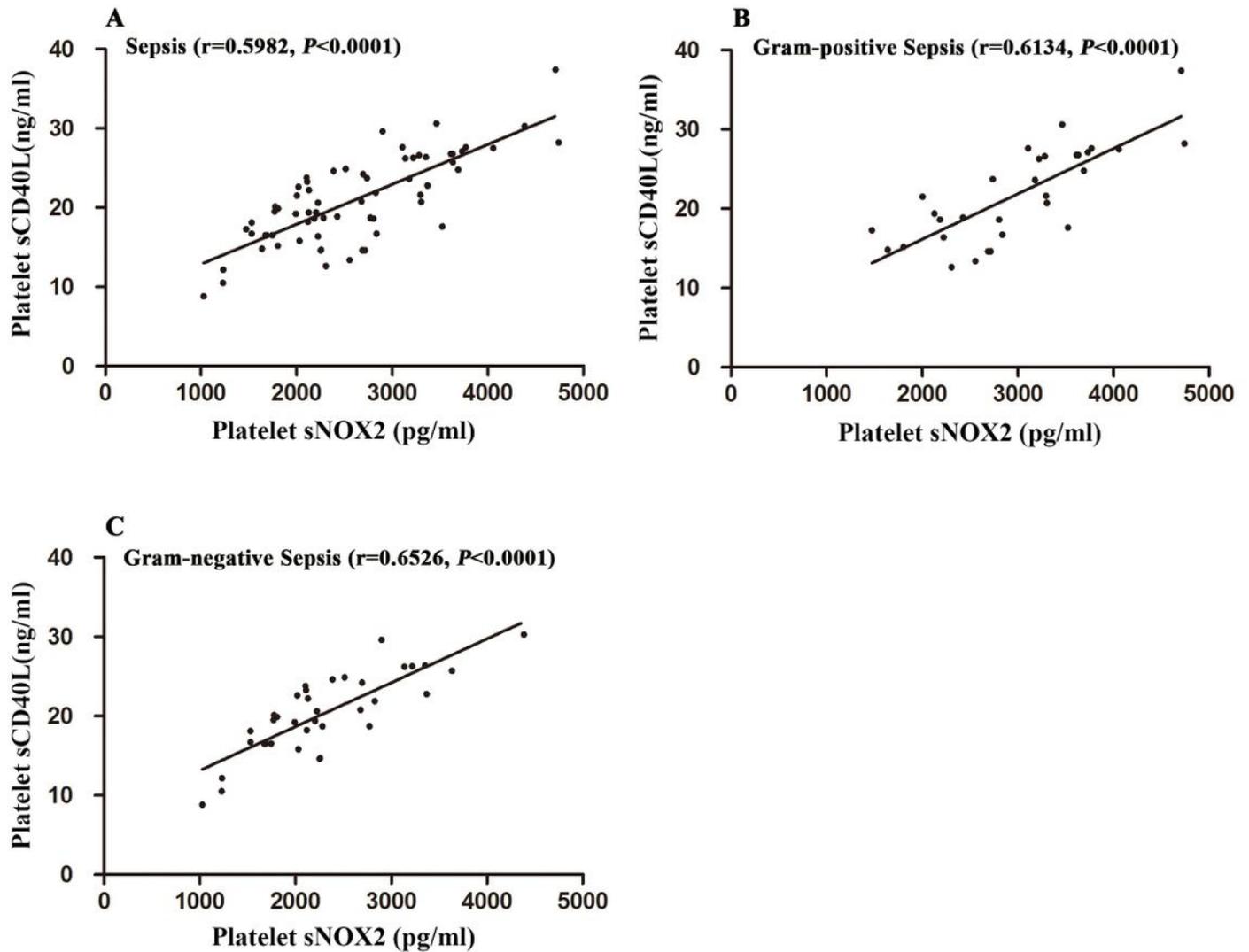


Figure 3

Correlation between platelet soluble NOX2 and platelet sCD40L in subjects. (A) Platelet sNOX2 levels are positively correlated with platelet sCD40L in patients with sepsis. (B) Platelet sNOX2 levels are positively correlated with platelet sCD40L in patients with Gram-positive sepsis. (C) Platelet sNOX2 levels are positively correlated with platelet sCD40L in patients with Gram-negative sepsis.

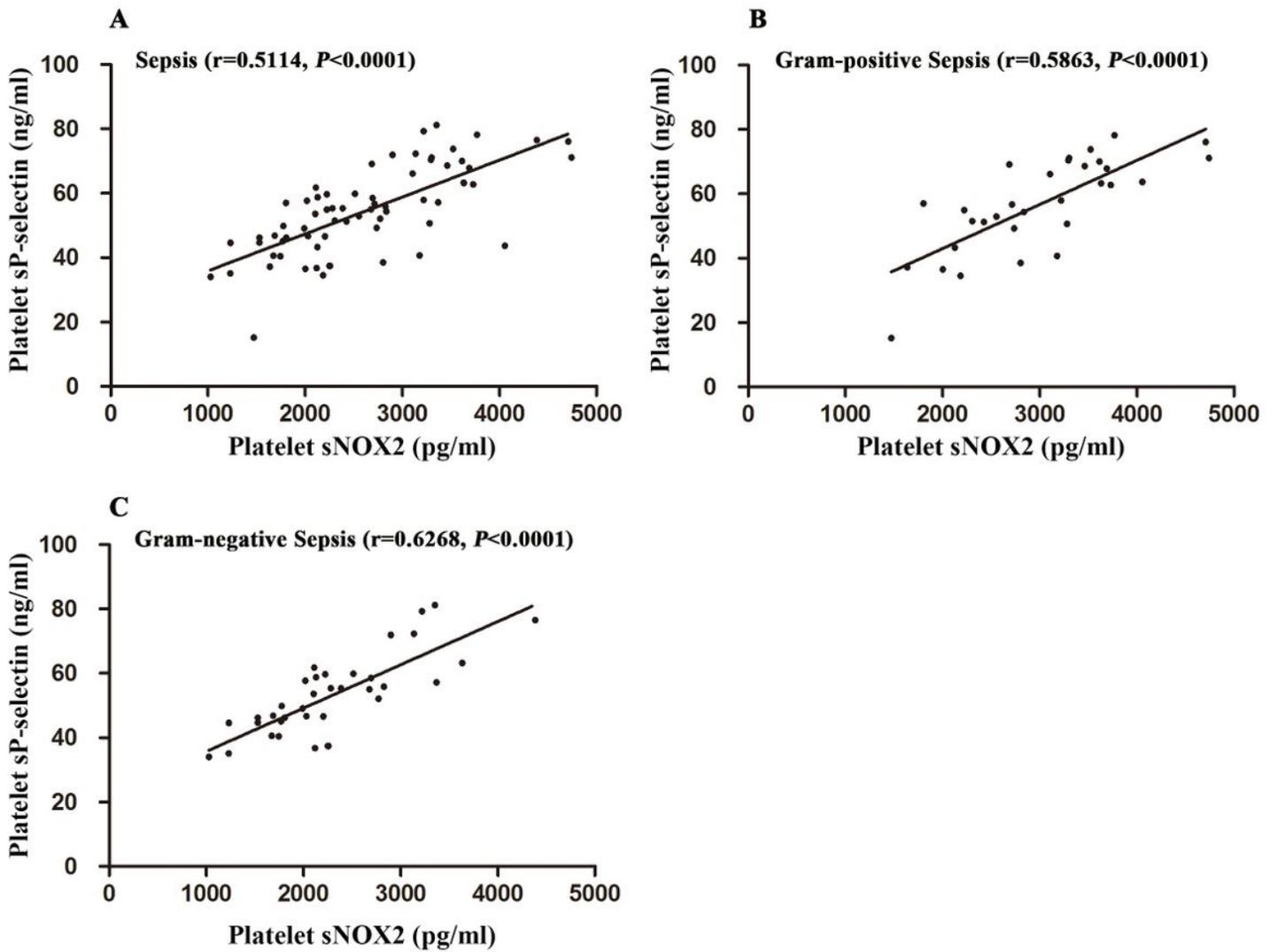


Figure 4

Correlation between platelet soluble NOX2 and platelet sP-selectin in subjects. (A) Platelet sNOX2 levels are positively correlated with platelet sP-selectin in patients with sepsis. (B) Platelet sNOX2 levels are positively correlated with platelet sP-selectin in patients with Gram-positive sepsis. (C) Platelet sNOX2 levels are positively correlated with platelet sP-selectin in patients with Gram-negative sepsis.

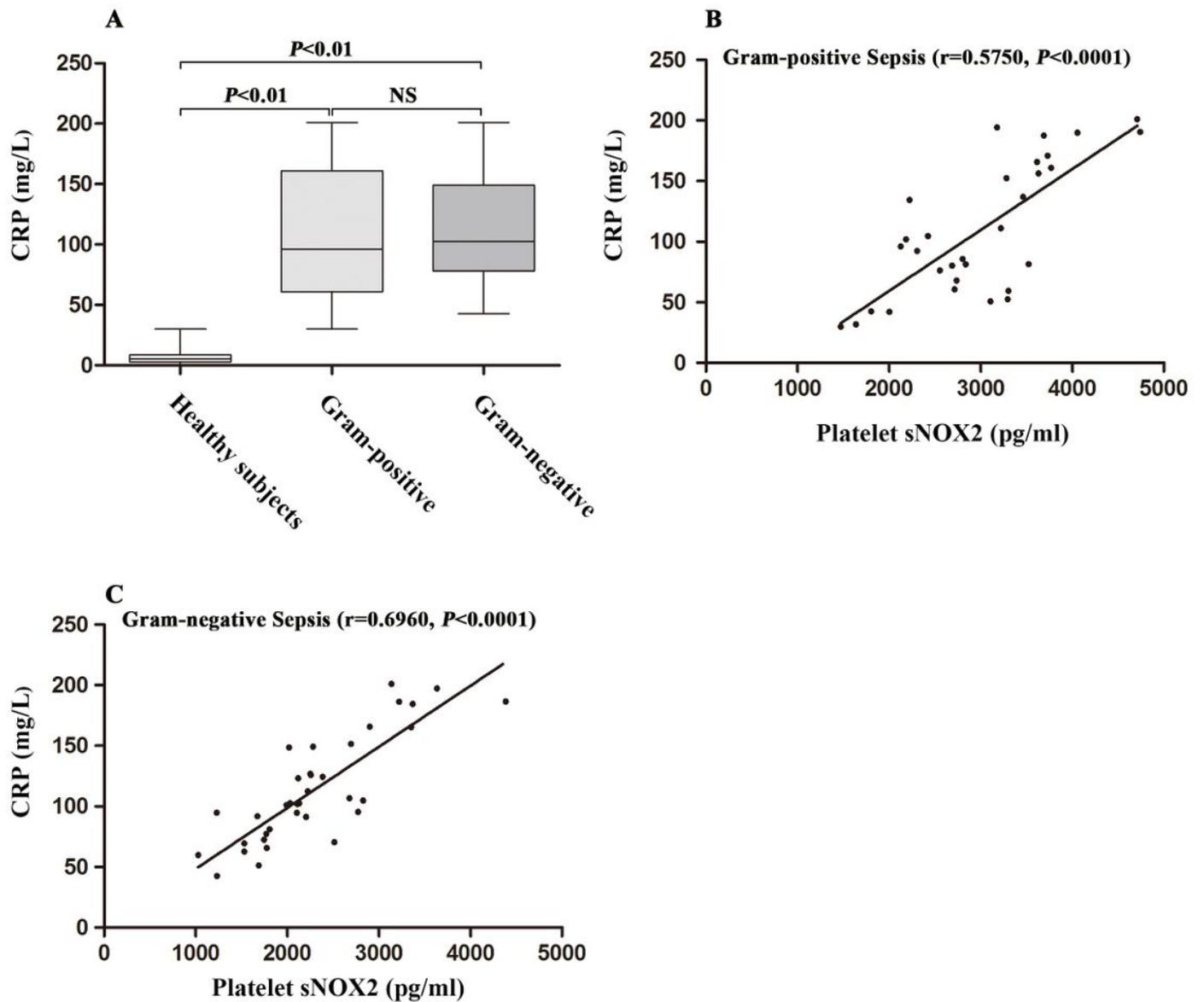


Figure 5

Correlation between platelet soluble NOX2 and CRP in subjects. (A) The bottom and top of each box represent the 25th and 75th percentile values, respectively; the horizontal line inside each box represents the median; and the horizontal lines below and above each box represent respectively the mean minus and plus one standard deviation. The box plot shows the levels of CRP in healthy individuals and patients with sepsis. (B) There is positive correlation between platelet sNOX2 value and CRP in patients with Gram-positive sepsis. (C) There is positive correlation between platelet sNOX2 value and CRP in patients with Gram-negative sepsis. Correlation coefficient (r) and statistical significance (P) are indicated.

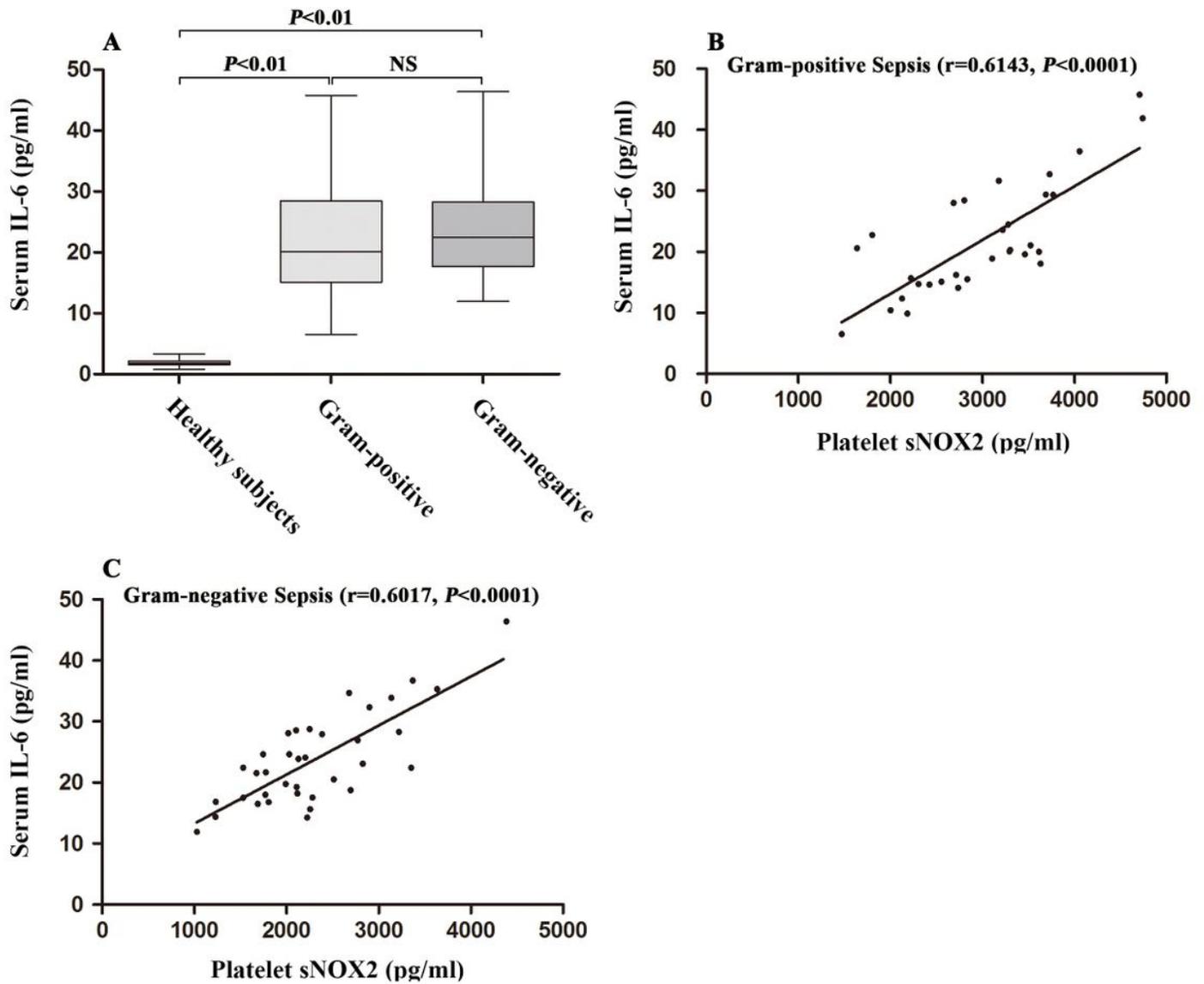


Figure 6

Correlation between platelet soluble NOX2 and serum IL-6 in subjects. (A) The bottom and top of each box represent the 25th and 75th percentile values, respectively; the horizontal line inside each box represents the median; and the horizontal lines below and above each box represent respectively the mean minus and plus one standard deviation. The box plot shows the levels of IL-6 in healthy individuals and patients with sepsis. (B) There is positive correlation between platelet sNOX2 value and IL-6 in patients with Gram-positive sepsis. (C) There is positive correlation between platelet sNOX2 value and IL-6 in patients with Gram-negative sepsis. Correlation coefficient (r) and statistical significance (P) are indicated.

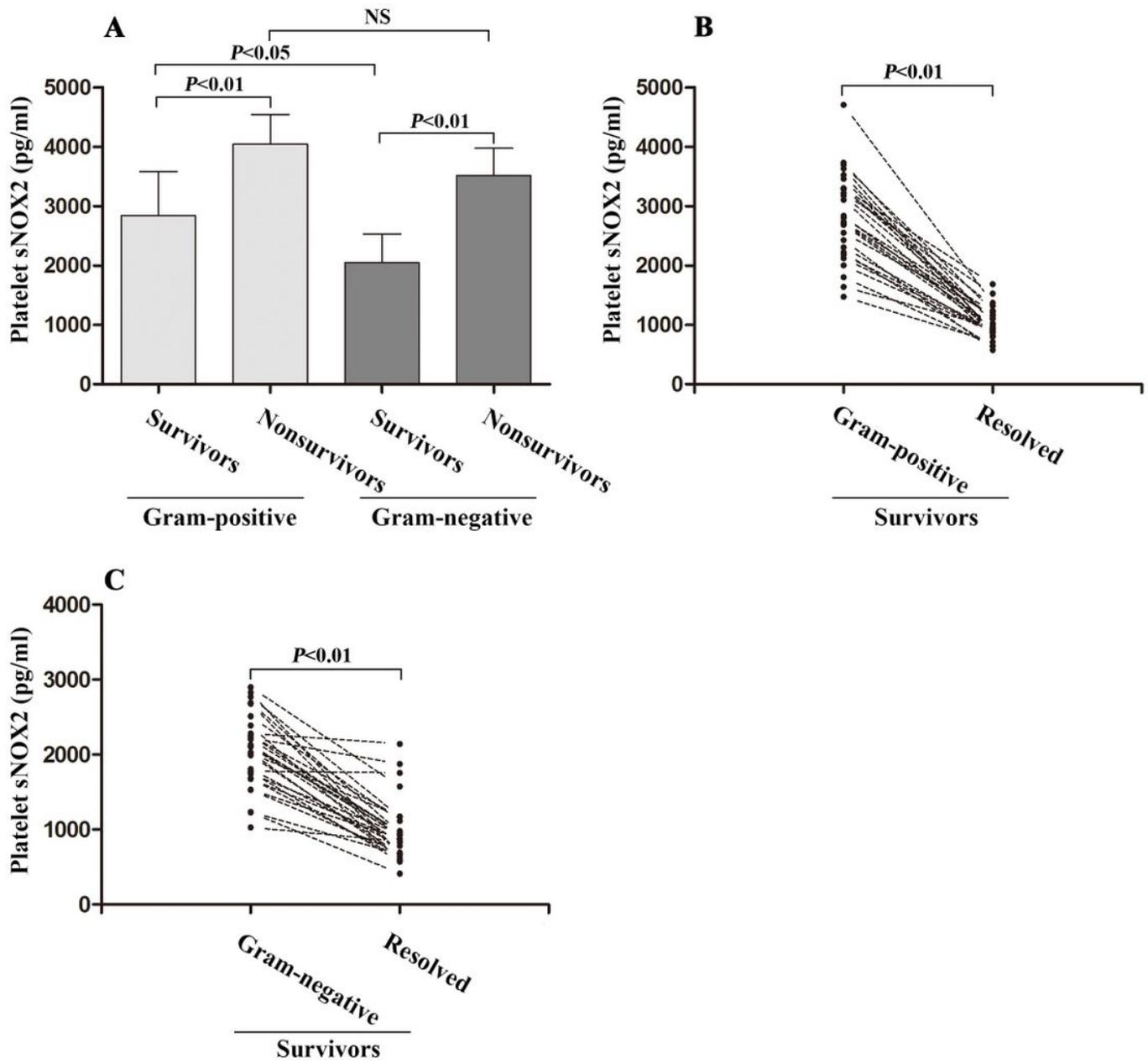


Figure 7

Platelet soluble NOX2 levels in subgroup of patients with sepsis. (A) The column chart shows the concentration of platelet soluble NOX2 in survivors and non-survivors with sepsis. Data presented as mean \pm SD. (B) The scatter plot represents platelet sNOX2 values in severe phase and recovery phase for patients with Gram-positive sepsis. (C) The scatter plot represents platelet sNOX2 values in severe phase and recovery phase for patients with Gram-negative sepsis. Statistical significance (P) is indicated.