DAILY VARIATION IN ENDOTOXIN LEVELS IS ASSOCIATED WITH INCREASED ORGAN FAILURE IN CRITICALLY ILL PATIENTS

David J. Klein,* Anastasia Derzko,† Debra Foster,† Andrew J.E. Seely,‡ Fabrice Brunet,* Alexander D. Romaschin,* and John C. Marshall*

*St. Michael's Hospital, University of Toronto; †Spectral Diagnostics, Toronto; and ‡Ottawa Hospital, University of Ottawa, Ottawa, Canada

ABSTRACT—High blood levels of endotoxin on admission to the intensive care unit are predictive of adverse outcomes, including organ failure and death. However, the significance of changes in endotoxin levels over time has not been evaluated. We examined whether dynamic daily changes in endotoxin levels resulted in the development of greater organ dysfunction over time in critically ill patients. The study was a retrospective analysis of data from the longitudinal phase of a prospective observational multicenter cohort study of endotoxin levels in patients admitted to the intensive care unit. We analyzed 345 patients. Daily variation in endotoxin levels was assessed by calculating the number of inflections in the curve generated by plotting endotoxin levels against time. The degree of organ dysfunction over time was analyzed using a calculation of the total area under the curve generated by plotting the Multi Organ Dysfunction Score against time. From 1,301 endotoxin activity assay results, patients with dynamic daily variation in endotoxin levels as measured by a greater number of inflections had a greater degree of total organ dysfunction as measured by Multi Organ Dysfunction Score against time ($P < 0.05$). The arithmetic mean standard deviation of endotoxin activity assay results increased stepwise in the zero, one, and two inflection groups supporting the association between inflections and variability. Endotoxin activity assay variability was found to be independent of infection status ($P = 0.52$). Daily dynamic variation in endotoxin levels is a marker of increased severity of illness as measured by burden of total organ dysfunction over time. Further studies are warranted to assess the role of daily variation in endotoxin levels in the pathogenesis and potential therapy of organ failure in the critically ill.

KEYWORDS—Endotoxin, variability, organ dysfunction, critical care, sepsis

INTRODUCTION

Endotoxin (LPS), lipopolysaccharide from the cell wall of gram-negative bacteria, plays a complex role in health and disease. Gram quantities of endotoxin are a major component of the commensal microbial flora of the gut, and endotoxin has been shown to play an important role in gut development and in the development of innate immunity (1). Since its original description by Pfeiffer and Koch in 1892, endotoxin has also been recognized as a key trigger of sepsis and the inflammatory cascade (2).

We have previously shown that endotoxin levels are low in healthy volunteers (3). However, in sepsis, levels of endotoxin can be substantially elevated—to as much as 1,000 times basal levels (4). We and others have also shown that these levels are elevated most in gram-negative sepsis, but are also elevated in gram-positive infection and in cases of suspected sepsis where no immediate microbiologic cause is identified (4, 5). In these cases, it has been hypothesized that endotoxemia is the direct result of changes in permeability of the gastrointestinal tract and subsequent translocation of LPS (6).

Multiple organ dysfunction is the most common cause of sepsis-associated mortality (7). Infusion of endotoxin in animals and in healthy human hosts activates a signaling cascade analogous to sepsis, which, at high levels, results in organ dysfunction (8). However, the degree of organ dysfunction does not show a direct dose-response relationship with the amount of LPS infused (9). Genetic background and sex have been shown to influence *in vivo* human responses to endotoxin challenge (10). In clinical testing, levels of endotoxin on admission to intensive care unit (ICU) have been shown to be predictive of the development of organ dysfunction in patients with sepsis (5). It is also hypothesized that translocation of endotoxin from the gut may play a role in the ongoing propagation of organ dysfunction over time in critically ill patients (11).

Endotoxemia is common in critically ill patients (5), and levels of endotoxin have been reported to vary over time (12). It is increasingly recognized that homeostasis is characterized by both physiologic stability and measurable variability, and that analysis of patterns of dynamic changes in a variety of parameters over time can provide important insights into states of illness (13). Therefore, we sought to characterize the significance of daily variability in levels of circulating endotoxin in a cohort of critically ill patients and to evaluate the relationship of this variability to clinical course in the ICU.

METHODS

Study design

The Multicenter Endotoxin In Critical Illness (MEDIC) study was a prospective observational cohort study of critically ill patients admitted to 1 of 10 ICUs in Canada, the United States, Belgium, and the UK. Patients were enrolled from January to September of 2000. Detailed inclusion and exclusion criteria for the study as well as definitions of infection and other outcome definitions are available
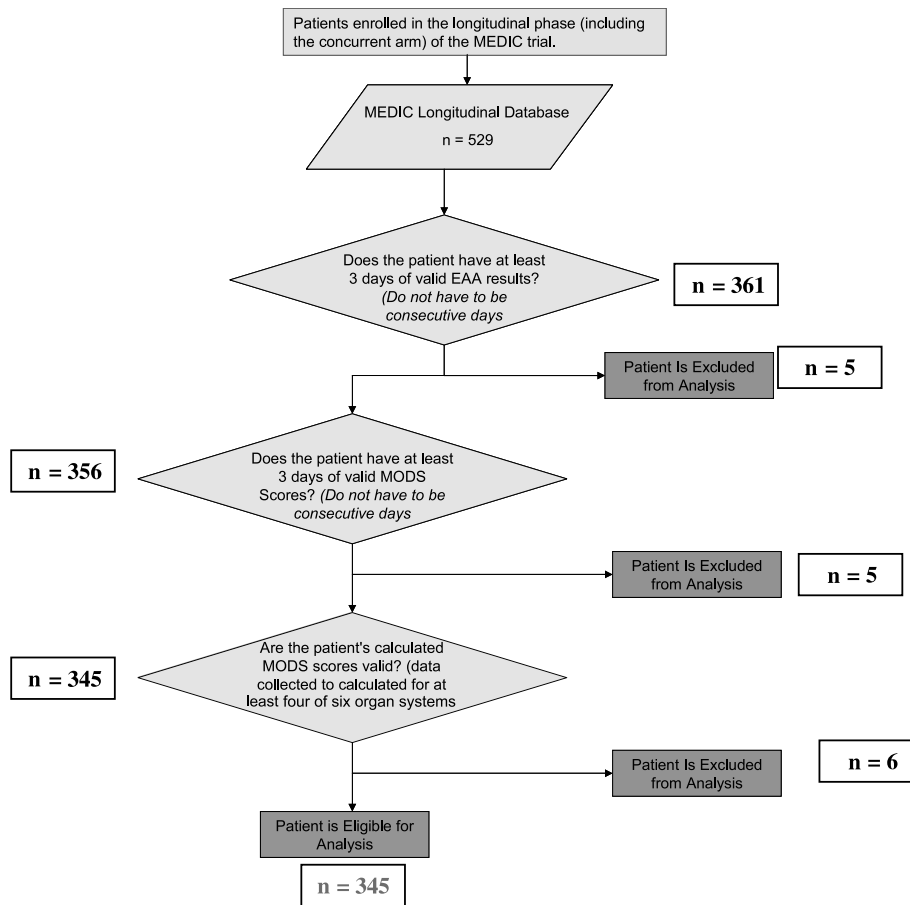
FIG. 1. **Flow diagram showing eligibility determination for analysis for the study cohort.**

elsewhere. In MEDIC, a cohort of 857 patients was studied on admission to ICU. Endotoxin activity (EA) levels were stratified as either low (<0.40 EA units), intermediate (0.40−0.59 EA units), and high (≥0.60 EA units). Endotoxin activity levels were found to be elevated in 57.2% of patients on the day of ICU admission. Rates of severe sepsis were 4.9%, 9.2%, and 13.2% and ICU mortality rates were 10.9%, 13.2%, and 16.8% for patients with low, intermediate, and high EA levels, respectively, in this cohort (5).

In our study, we examined a subset of the MEDIC database of 529 patients that were followed daily after admission and, thus, had multiple endotoxin activity assays (EAAs) performed daily over time. We analyzed patients who had a minimum of three recorded and evaluable EAA values during the first 4 ICU days beginning on ICU admission (Fig. 1). Baseline cultures were collected in all patients along with daily physiologic data for the first 4 days after admission.

Definition of inflections

We defined daily EAA variation by using the number of inflection points in the EAA curve when plotted against time. An EAA inflection was defined qualitatively as an inflection (point when the second derivative changes sign) in the curve generated by plotting EAA values over time using the assumption of a normal biologic polynomial distribution; a convex curve becomes concave, or vice versa. An inflection required a minimum change in the value in EAA (positive or negative) of at least 0.10 EA units to accommodate the intrinsic coefficient of variation of the assay of 0.06 units and to reflect at least a 5-fold difference from the variability seen in healthy controls (0.02 EA units). The sample collection time for each EAA sample was obtained from the MEDIC database, as was the time of admission to the ICU. Using the time of the initial sample collection as a baseline, the number of hours for each of the subsequent samples was calculated. Patients were grouped based on having either zero, one, or two inflections. These three groups accounted for all possible patterns of EAA plotted versus time (Fig. 2).

Outcome variables

To define the degree of organ dysfunction over time, we measured "MODS Burden," the area under the curve generated by plotting the Multiple Organ

FIG. 2. **Samples of potential observed pattern types of longitudinal EAAs and inflection groupings when daily EAAs are plotted against time.**

Dysfunction Score (MODS) against time during the first 4 days of ICU stay (14). Using the trapezoid rule, the area under the MODS versus TIME (in hours) curve (AUC) can be calculated for the 4-day (72-h) period. The cumulative AUC is a measure of total MODS Burden (expressed in hours) in the patient over the 4-day period. Multiple Organ Dysfunction Score Burden (AUC) = A1 + A2 + A3, where $Ax = 0.5 \times ([day\ 2\ (h)] - [day\ 1\ (h)]) \times$ (MODS (day 1) + MODS (day 2) (Fig. 3). We assumed a given level of organ dysfunction to be present over the interval between daily measurements. Baseline severity of illness was quantified using the acute physiology and chronic health evaluation (APACHE) II score, and baseline organ dysfunction using the MODS and the Sequential Organ Failure Assessment scores (14–16). Secondary outcome variables included length of stay, ICU mortality, 28-day mortality, and hospital mortality. In addition, we calculated ICU-free days at a 60-day interval where ICU-free days = 60 − length of stay for survivors. Intensive care unit deaths were calculated as 0 ICU-free days and readmissions were assumed to be 0 free days for the purpose of this analysis. Infection status was adjudicated by a clinical evaluation committee, blinded to EAA level, on the basis of clinical and culture data.

Chemiluminescent assay for endotoxin

Endotoxin activity (EA) in whole blood was measured using the chemiluminescent EAA as recommended by the manufacturer (Spectral Diagnostics, Toronto, Canada). Briefly, samples of 40 μL of whole blood and appropriate controls were incubated in duplicate with saturating concentrations of an antilipid A immunoglobulin M antibody then stimulated with opsonized zymosan. The resulting respiratory burst activity was detected as light release from the lumiphor, luminol, using a chemiluminometer (E.G. & G. Berthold Autolumat LB953, Wildbad, Germany). The LPS/anti-LPS complex primes the patient's neutrophils for an augmented response to stimulation with zymosan; by measuring basal (no antibody) and maximally stimulated (4600 pg/mL LPS) responses in the same blood sample, the EA of the test specimen is calculated by integrating chemiluminescence over time. Levels are expressed as EA units and represent the mean of duplicate determinations from the same sample (17).

Statistical analysis

Quantitative results were compared using one-way ANOVA analysis. Categorical data were compared using the Fisher exact test. Survival rate was analyzed by generating Kaplan-Meier survival curves, and curves were compared using the log-rank test. Infected and noninfected groups were compared using Pearson chi-square for categorical variables and the one-way ANOVA for continuous variables. Statistical significance was assumed for values of $P \leq 0.05$.

RESULTS

Subjects

We studied 345 patients who had samples on at least 3 of the first 4 ICU days. A total of 1,301 EAA test results were evaluated for an average of 3.8 tests per patient (median of four tests per patient; range, three to four tests). The baseline characteristics of the patients are presented in Table 1.

Area Under the MODS Curve
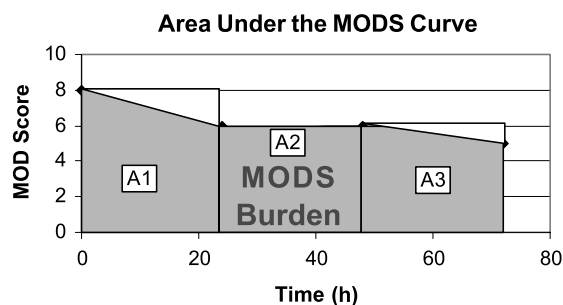
MODS Burden (AUC) = A1 + A2 + A3

where Ax = 0.5 x ([day 2 (h)] - [day 1 (h)]) x (MODS(day 1) + MODS(day 2))

Fig. 3. **A diagrammatic representation of how to calculate of MODS Burden.** The total MODS Burden is expressed in hours as the shaded AUC.

EAA inflections

In a separate study of 15 healthy volunteers, EAA inflections were zero in all patients studied (4). In this study, there were 44 patients with zero inflections, 220 patients with one inflection, and 81 patients with two inflections. In this data set, 23% (79 subjects) had only three evaluable EAA measurements over the 4 days studied. Of those, 72% showed one inflection and 28% showed no inflections in their plotted data. Acute physiology and chronic health evaluation II scores tended to be higher, with increasing numbers of inflection points, and differed significantly between the zero- and two-inflection groups ($P = 0.04$).

Inflections and EAA standard deviation

As an additional measure of dynamic variability, we calculated the average standard deviation of EAA results in each of the groups. In the zero-inflection group, the arithmetic mean of the standard deviation of EAA results was 0.1061 (confidence interval [CI] = 0.0795–0.1327); in the one-inflection group, the mean of the standard deviation was 0.1634 (CI = 0.1516–0.1752); and in the two-inflection group, the mean of the standard deviation of EAA results was 0.1809 (CI = 0.1610–0.2008) over the 4 days of measurement.

EAA inflections and infection/sepsis

Clinical criteria for severe sepsis were met by comparable numbers of patients in each group: 6 of 44 patients in the zero-inflection group (13.6%), 44 of 220 patients in the one-inflection group (20%), and 19 of 81 patients in the two-inflection group (23.5%). Similarly, rates of infection were similar between the groups: 18 of 44 (40.9%) in the zero-inflection group, 107 of 220 (48.6%) in the one-inflection group, and 35 of 81 (43%) in the two-inflection group. The rates of confirmed gram-negative and confirmed gram-positive infection were 12 of 44 (27.3%) and 9 of 44 (20.5%) in the zero-inflection group, 46 of 220 (20.9%) and 63 of 220 (28.6%) in the one-inflection group, and 21 of 81 (25.9%) and 17 of 81 (21.0%) in the two-inflection group. Results were not statistically different between these groups ($P = 0.52$). Although the numbers were too small for statistical comparison, the site of infection and responsible organisms were similar in all groups.

EAA variability and organ dysfunction

Multiple Organ Dysfunction Score Burden increased significantly with increasing variability as measured by number of inflections. Multiple Organ Dysfunction Score Burden hours were 252 ± 214 in the zero-inflection group, 399 ± 207 in the one-inflection group, and 457 ± 224 in the two-inflection group (three-way ANOVA; $P = 0.012$) (Fig. 4). Endotoxin activity assay variability (two inflections) was predictive of MODS burden. (one-way ANOVA; $P < 0.05$) The number of EAA inflections was more predictive of organ dysfunction than the absolute EAA value calculated as the total endotoxin activity load (area under the EAA versus time curve by trapezoidal rule) over the 4-day period by univariate regression analysis ($P < 0.05$ and $P = 0.54$, respectively).

TABLE 1. **Baseline characteristics of the cohort***

Variable	All patients	0 Inflection	1 Inflection	2 Inflections
n	345	44	220	81
Age (mean ± SD; P = 0.13)	58.3 ± 18.2	56.5 ± 17.1	57.3 ± 18.8	61.8 ± 17.0
APACHE II (mean ± SD; P = 0.04)	22.2 ± 9.16	19.8 ± 9.1	21.9 ± 9.0	24.4 ± 9.2
Temperature (fever) no. (%) >38.3°C or <35.6°C (P = 0.77)	201 (58.3%)	25 (56.8%)	126 (57.3%)	50 (61.7%)
WBC no. (%) <4 or >12 (P = 0.82)	203 (58.8%)	26 (59.1%)	127 (57.7%)	50 (61.7%)
MAP no. (%) <80 (P = 0.34)	239 (69.3%)	28 (63.6%)	150 (68.2%)	61 (75.3%)
Pao_2/Fio_2 ratio no. (%) <280 (P = 0.69)	268 (77.7%)	32 (72.7%)	173 (78.6%)	63 (77.8%)
Heart rate no. (%) >90 (P = 0.59)	189 (54.8%)	21 (47.7%)	122 (55.5%)	46 (56.7%)
Respiratory rate no. (%) >20 (P = 0.69)	189 (54.8%)	25 (56.8%)	123 (55.9%)	41 (50.6%)
MODS on day 1 in ICU (mean ± SD) (P = 0.01)	6.1 ± 3.2	5.3 ± 3.7	6.0 ± 3.0	7.0 ± 4.1
SOFA on admission (mean ± SD)	7.7 ± 4.0	6.6 ± 4.4	7.5 ± 3.8	8.6 ± 4.1

*Comparisons of categorical variables performed using a three-sample chi-square test, and comparisons of continuous variables were done using a one-way ANOVA.
SOFA indicates Sequential Organ Failure Assessment.

ICU-free days

Considering an interval of 60 days post-ICU admission, there was a significant difference (one-way ANOVA; P = 0.013) with regard to the mean number of ICU-free days. Patients with zero inflections showed a mean of 39.7 ICU-free days; one inflection, 29.2 ICU-free days; and two inflections, 34.1 ICU-free days.

Other outcomes

Median length of ICU stay was 11 days in all patients and 7 days in the zero-inflection group, 11 days in the one-inflection group, and 13 days in the two-inflection group (P = 0.27 for the differences between the groups). Median hospital length of stay was 32 days in the total cohort, 24 days in the zero-inflection group, 32 days in the one-inflection group, and 33 days in the two-inflection group (P = 0.37 for the differences between the groups). Intensive care unit, hospital, or 28-day mortality rate did not differ statistically between the groups. The ICU, hospital and 28-day mortality rates for each of the inflection groups were as follows: zero inflections (18.2%, 20.5%, and 22.7%, respectively), one inflection (25.9%, 37.7%, and 31.4%, respectively), and two inflections (23.5%, 30.9%, and 22.2%, respectively).

DISCUSSION

Critical illness is typically described by static variables such as admission APACHE score, the admission value of a specific cytokine or biomarker, or other baseline physiologic measures. However, it is increasingly recognized that patterns of change over time in illness are an important indicator of deranged homeostasis and disease severity (13). This pilot study represents the first evaluation of endotoxin variability and its association with organ failure and mortality and generates novel hypotheses regarding the clinical potential of longitudinal endotoxin evaluation in critically ill patients. In this study, we show that levels of endotoxin, a key trigger of the inflammatory cascade in sepsis, fluctuate significantly on a day-to-day basis in critical illness. Moreover, we show that the pattern and degree of these fluctuations seem to have prognostic significance with respect to degree of organ dysfunction and other adverse outcomes. In our patient cohort, ongoing organ dysfunction was associated with increased inflections in endotoxin levels and increased standard deviation in endotoxin levels measured over the first 4 days of admission. Although those with two inflections had more organ dysfunction, patients with a single inflection had fewer ICU-free days. It is apparent that useful prognostic information is contained in understanding the patterns of variability in endotoxin levels in the critically ill. Understanding these patterns of variability may also provide useful learning for understanding the changes that occur in other biomarkers over time and may provide further insights into the pathogenesis of organ dysfunction in critical illness.

Endotoxin levels in the normal host are tightly regulated through a highly conserved series of mechanisms for endotoxin binding, signaling, and clearance (18). Transient endotoxemia has been found in many "healthy" host states, including in marathon runners, Olympic athletes, and smokers
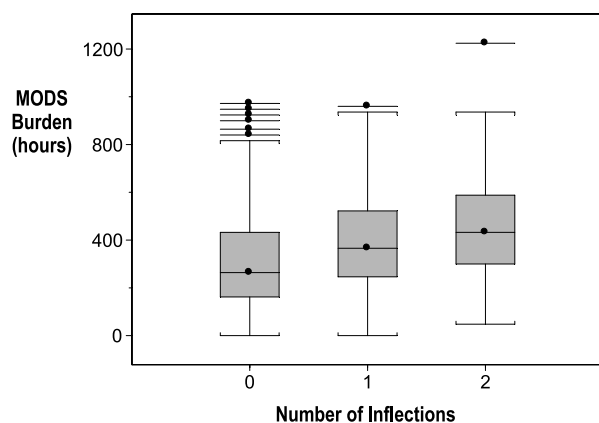
Fig. 4. **Box plot of showing the total MODS burden in patients with increasing daily variability in endotoxin levels.** Multiple Organ Dysfunction Score Burden increases significantly with increasing inflections.

(19, 20). However, in disease, the mechanisms that regulate endotoxin may vary as the inflammatory response becomes uncoupled from the inciting injury (21). The breakdown of these regulatory mechanisms along with ongoing translocation of gut-derived endotoxin may contribute to the fluctuating levels observed in our study. Other potential contributors to fluctuating levels of endotoxin can also include an uncontrolled or recurrent source of gram-negative sepsis.

Traditional models of the inflammatory response have focused on a "single-hit" model, whereby a given insult, such as acute bacterial infection, trauma, or cardiopulmonary bypass, leads to a downstream inflammatory response and subsequent organ failure. However, these models have been difficult to recreate empirically. The concept of linking recurrent exposure to endotoxin to the overwhelming, uncontrolled systemic inflammatory response associated with multiple organ failure in critical illness is attractive. To date, however, studies that have attempted to causally link translocation of LPS to ongoing organ dysfunction have had mixed results (22). In one intriguing supportive model, Pape et al. (23) required multiple boluses of endotoxin every 12 h over a 5-day period after a traumatic surgical insult to induce multiple organ failure in a sheep model of organ failure after trauma. Muenzer et al. (24) also recently published an intriguing "two-hit" animal model of sepsis requiring both cecal ligation and puncture as well as subsequent instillation 72 h later of live bacteria in the lung as a "second hit" to drive the inflammatory response.

If ongoing fluctuations in endotoxin levels play a role not only as a marker of ongoing injury but also as a mediator, it may be possible to measure and modulate this process. A broader understanding of the kinetics of changing levels of endotoxin over time in critical illness may improve therapeutic timing and targeting of specific antiendotoxin therapies (25). Current therapeutic strategies such as selective gut decontamination and early goal-directed therapy may well in part be of benefit because of their role in decreasing gut endotoxin load and in preventing LPS translocation through maintaining gut perfusion and epithelial barrier integrity (26, 27). Further studies examining variability in endotoxin levels over time to monitor and to guide therapy for patients with high endotoxin variability are warranted.

Illness has been characterized by decomplexification, with a change in the complexity and overall variation in different parameters evaluated (28). For example, analyses of heart rate variability in patients with severe sepsis and organ failure have repeatedly shown diminished variability. Rassias et al. (13) demonstrated increased regularity in heart rate, neutrophil function, and plasma cortisol levels in response to endotoxin infusion in healthy volunteers. Reduced breathing variability on the ventilator has been shown to be a negative predictor of extubation success (29). In other physiologic parameters, which include rhythms such as blood pressure, or laboratory tests such as neutrophil count or cortisol level, there is additional clinical information hidden within their patterns of variation and the changes that exist in states of illness (13).

However, interpreting patterns of variability in the setting of critical illness must be done with caution. Little is known about the circadian biology of endotoxin levels or other inflammatory mediators. Although early studies to date have shown some value in measuring changes in certain biomarkers such as protein C and procalcitonin, interpreting more complex changes over time is challenging (30, 31). True variability analysis requires the integration of hundreds of data points over time and the use of a panel of indices reflecting variability based on advanced algorithms requiring computation (32). Fulfilling Nyquest's theorem (which requires the sampling frequency to be twice the highest intrinsic frequency of the underlying signal) would require many more measurements than were feasible in this study. In fact, endotoxin variability may be just one example of dysregulated homeostasis in a tightly controlled physiologic system that contributes to the inflammatory response in critical illness. However, the underlying signal of endotoxin variability is clearly much more complex than the daily sampling included in our study. Nonetheless, we believe daily sampling provides a glimpse of the true variability and carries clinically valuable information.

In summary, we present a novel hypothesis relating increased daily variability in levels of endotoxin to worsening organ dysfunction in critical illness. These fluctuations may reflect ongoing tissue injury and failure of the host to adequately contain and target the inflammatory response. They may also reflect other secondary insults in a critically ill patient. Further studies are needed to assess the potential role in monitoring endotoxin inflections as a marker of adverse outcome and potentially as a therapeutic target and to better understand variability in biomarkers over time in the pathogenesis of critical illness.

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