Letter to the Editor

Gregory J. Eisinger*, Wissam Osman, Evan R. Prather, Mark W. Julian, Mikhail A. Gavrilin, Elliott D. Crouser and Mark D. Wewers

Blood collection in heparin vs. EDTA results in an inflammasome-independent increase in monocyte distribution width at 4 h

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To the Editor,

Sepsis remains a leading cause of morbidity and mortality around the world [1], with early recognition and implementation of appropriate antimicrobial therapy representing the cornerstone of effective treatment [2]. However, prompt diagnosis is often challenging due to nonspecific presentations and delayed availability of confirmatory tests such as cultures, prompting a need for accurate, affordable, and rapidly available biomarkers. A growing body of literature has demonstrated the value of monocyte distribution width (MDW) as a biomarker for sepsis [3]. MDW is an automated measurement of the standard deviation of monocyte cell size performed by certain hematology analyzers as part of a routine complete blood count (CBC) analysis. Its availability within 2 min without additional cost or need for pretest suspicion of sepsis are unique advantages compared to other sepsis biomarkers.

Prior research has consistently shown higher MDW values in blood samples collected in tripotassium (K3) vs. dipotassium (K2) ethylenediaminetetraacetic acid (EDTA) tubes [4–6], necessitating different diagnostic cut points depending on the anticoagulant used. Our recent laboratory research has examined the cellular mechanisms responsible for increased MDW in sepsis, with a focus on the role of inflammasome activation as measured by interleukin-1β (IL-1β) release [7]. Due to the known impact of EDTA on cytokine expression [8, 9], particularly suppression of IL-1β release [10, 11], we conducted some of our experiments using heparin sodium (HS) tubes rather than EDTA. Consequently, this investigation seeks to characterize the impact of HS compared to EDTA on MDW values in human blood.

After obtaining informed consent, whole blood was collected from healthy volunteers in both K2EDTA and HS vacutainer tubes (Becton-Dickinson; Franklin Lakes, NJ). Samples were either left untreated or were treated with bacterial endotoxin (LPS) at a concentration of 1 g/mL. MDW was then measured using the Unicel DxH900 analyzer (Beckman-Coulter; Brea, CA) at baseline (time zero) and after a 4 h incubation in polypropylene culture tubes under humidified, 37 °C, 5 % CO2 conditions. After MDW measurement, plasma was collected by centrifugation and IL-1β levels were measured by sandwich ELISA in order to confirm inflammasome activation.

Statistical analysis was performed using the JMP PRO software package, version 16 (SAS Institute Inc., Cary, NC). Nonparametric distributions were assumed for all variables. Continuous variables are described by median (interquartile range [IQR]) and compared between groups using the Wilcoxon signed-rank test. The human subjects protocol was approved by The Ohio State University’s Biomedical Sciences Institutional Review Board (4/2/2012, study number 2011H0059 – “Immunological characterization of blood of normal healthy individuals”). Procedures were followed in accordance with the ethical standards of the institutional committee on human experimentation and with the Helsinki Declaration of 1975.

Samples were collected from 8 subjects, 75 % of whom were female with a median age of 31 years. Figure 1A shows the results for MDW according to the time point and
anticoagulant used. There was no significant difference in MDW in HS vs. EDTA samples at time zero (median MDW 16.4 in both groups). However, after a 4 h incubation, median MDW was significantly higher in HS tubes (21.3, IQR 2.4) vs. EDTA tubes (16.1, IQR 2.6; difference=5.2, \(p=0.0005\)). Samples treated with LPS had higher median MDWs at 4 h in both groups, reflecting the septic response, with a similar difference seen between the HS and EDTA groups (30, IQR 3.9 vs. 24.6, IQR 5 respectively; difference=5.4, \(p=0.002\)).

Figure 1B shows IL-1\(^\beta\) levels at each time point for HS vs. EDTA samples. As expected, no IL-1\(^\beta\) release was seen in the untreated samples at any time point. At 4 h post-treatment, the LPS-treated samples had significant IL-1\(^\beta\) release in both groups, reflecting inflammasome activation. This was numerically significantly higher in the HS samples (2.78, IQR 3.65 ng/mL) compared to EDTA (1.78, IQR 2.37 ng/mL) but did not reach statistical significance given the small sample size (\(p=0.21\)).

Our results demonstrate that compared to K\(_2\)EDTA, the use of heparinized tubes in blood collection results in higher values for MDW when measured after a 4 h incubation. The etiology of this effect is not known and was not explored in detail by the current study. Since the increased MDW in HS samples not treated with LPS was not associated with IL1\(^\beta\) release, it is unlikely that the effect is mediated by inflammasome activation. Additionally, the difference between HS and EDTA at 4 h was not more exaggerated in the LPS-treated samples, arguing against an accentuated LPS effect as the underlying mechanism. Though not evaluated by our data, it is more likely that the differences relate to the osmotic properties of the different salts or the tendency of HS to result in clumping of cells.

The numerically higher values for IL-1\(^\beta\) release we observed in HS compared to EDTA has been previously described [10] and may relate to EDTA’s inhibition of LPS binding to the cell membrane compared to HS [11]. Given the well-described anti-inflammatory properties of heparin [12], it is unlikely that this response reflects enhanced inflammasome activation.

Because heparin is known to interfere with blood counts due to the clumping of cells (particularly platelets) [13], and the liquid K\(_3\) formulation of EDTA causes hemodilution and osmotic shrinkage of erythrocytes, most experts recommend the use of K\(_2\)EDTA for CBC analysis [14]. Since nearly all clinical laboratories use some form of EDTA for performing CBC analysis, our results would seem to be of primary interest to those engaged in laboratory research on MDW, rather than clinicians. However, our results do also call into question whether the clinical use of heparin for therapeutic or prophylactic anticoagulation may have an impact on MDW, resulting in false positives in sepsis screening. Further research is needed to evaluate this question. It is notable that the median MDW at 4 h in HS samples not treated with LPS (21.3) was well above the most common clinically used diagnostic threshold for K\(_2\)EDTA of 20.

This study has a number of limitations, primarily related to the small sample size, the enrollment of young patients, and the absence of detailed osmotic properties of the different salts.

Figure 1: Monocyte Distribution Width and Interleukin-1 beta response in heparin vs. EDTA. (A) Blood samples collected in heparin sodium tubes had higher values for monocyte distribution width at 4 h than those collected in K\(_2\)EDTA, both with and without treatment with LPS. (B) Despite significantly higher MDW, no IL-1\(^\beta\) release was seen in heparinized samples at 4 h without LPS stimulation. *Statistically significant; NS, not significant; LPS, lipopolysaccharide; K2, dipotassium; EDTA, ethylenediaminetetraacetic acid.
healthy volunteers rather than septic patients, and the limited scope of investigation into the etiology of the observed effects. Additionally, our assessment of MDW at 4 h was chosen for the purposes of optimizing inflammasome activation. In clinical use, the manufacturer recommends that MDW measurement be completed within 2 h of sample collection. Lastly, since we did not assess MDW beyond 4 h, it is not known whether the difference between HS and EDTA remains stable or may continue to widen or eventually result in cell lysis. Further research is needed to confirm and extend our findings.

Blood collection in HS vs. EDTA results in a time-dependent increase in MDW both in the unstimulated blood of healthy volunteers and after inflammasome activation using an in vitro sepsis model. More research is needed to characterize the underlying mechanisms and evaluate the potential clinical importance of this finding, particularly in regard to the diagnostic threshold for MDW in patients receiving heparin.

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**Research ethics:** The research related to human use has complied with all the relevant national regulations, institutional policies, and in accordance with the tenets of the Helsinki Declaration, and has been approved by The Ohio State University’s Biomedical Sciences Institutional Review Board (4/2/2012, study number 2011H0059 – “Immunological characterization of blood of normal healthy individuals”).

**Informed consent:** Informed consent was obtained from all individuals included in this study.

**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

**Competing interests:** Dr. Crouser is the clinical lead for a project jointly funded by the US Department of Defense (BARDA grant) and Beckman-Coulter examining the use of MDW as part of a machine learning algorithm for early sepsis detection in the hospital. He receives additional funding from the NIH and several pharmaceutical companies for his work on sarcoidosis, which is unrelated to the current manuscript. The remaining authors state no conflict of interest.

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