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SHORT COMMUNICATION

Two Methods for Evaluating the Effect of pre-Analytical Handling Procedures on Interleukin 6, Interleukin 8, and Tumor Necrosis Factor α

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SUMMARY

Background: **Except host and environmental factors influencing individual human cytokine responses, pre-analytical handling procedures and detection methods also affect cytokine levels.**
Methods: **In this study, we used cytometric bead array (CBA) and chemiluminescence (ECL). These two methods were used to test serum and plasma samples from 50 healthy adult volunteers and 50 rheumatoid arthritis (RA) patients' cytokine levels. We evaluated the impact of storage temperature, collection times, and additives in collection tubes on the measurement of IL-6, IL-8, and TNF-α.**
Results: **The finding of this study first indicated that the CBA assay for IL-6 and IL-8 showed excellent agreement with ECL for the same analysis, but the CBA assay and ECL for TNF-α measurement showed less agreement. Furthermore, we used two detection methods to find plasma cytokines showing more stability than serum cytokines, when the samples were stored at 4°C. Additionally, IL-8 concentration was affected by the storage conditions of whole blood from the time of collection until further processing at room temperature. We also found heparin tubes showed higher levels of IL-6 and TNF-α than other tubes at room temperature.**
Conclusions: **In general, the best values for IL-6, IL-8, and TNF-α were found in EDTA samples, stored at 4°C, and centrifuged quickly within 2 hours. The effect of pre-analytical handling procedures and detection methods on cytokine levels we identified would provide a basis for clinical cytokine detection.**
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KEYWORDS

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INTRODUCTION

Cytokines exert a vast array of immune regulatory actions which are critical to human biology and disease [1]. Cytokines and their receptors have not only been associated with normal and pathological status of human immune system, but also become the therapeutic targets in clinic [2]. It is becoming increasingly evident that IL-6 can mediate acute inflammatory disease, such as sepsis and macrophage activation syndrome. IL-6 signaling also contributes to the progression of many autoimmune diseases [3,4]. IL-8, a chemokine in regu-

lating leukocyte infiltration in inflammation, plays an important role in the intersection of cancer plasticity, angiogenesis, and immune suppression [5,6]. TNF-α is a central cytokine in inflammatory reactions, not only directly induces inflammatory gene expression but also indirectly induces cell death [7]. Therefore, detecting the levels of these cytokines, can not only assess the immune status of the body, but also monitor disease progression and response to therapy.

Cytokine concentrations are thought to be affected by pre-analytical sample handling procedures, such as collection tubes, storage temperature, collection times, transport situation, freeze-thawing cycles, and so on [8]. Although there are many studies which report the effect of pre-analytical handling procedures on cytokines, they used only one method to detect the cytokine concentration, and often give confusing results [9,10]. Given the emerging importance of cytokines in human health and multiple influencing reasons due to pre-analytical handling procedures, it is important to use various methods to evaluate the concentration of cytokines.

In this study, samples of cytokine levels were derived from 50 healthy adult volunteers and 50 rheumatoid arthritis (RA) patients. We evaluated the effect of storage temperature, collection times, and additives in collection tubes on detecting IL-6, IL-8, and TNF-α levels in serum and plasma samples by two methods, cytometric bead array (CBA) and chemiluminescence (ECL), in order to provide a basis for clinical cytokine detection in future.

MATERIALS AND METHODS

Sample handling

We used blood samples drawn from 50 healthy adult volunteers and 50 rheumatoid arthritis (RA) patients. Fifty healthy adult volunteers came from Wuhan No. 1 hospital physical examination center. Individuals, who had blood drawn at the center, were asked for their willingness to donate additional blood for research purposes. Patients fulfilling the American College of Rheumatology/EULAR 2010 classification criteria for RA, also came from Wuhan No. 1 hospital. For the study on the stability of cytokines in plasma, whole blood samples were collected into EDTA tubes and heparin tubes. After storage at 4°C or room temperature (RT) for 2 hours, whole blood samples were immediately centrifuged, plasma was separated from serum at 2 hours, 8 hours, and 24 hours, and then stored at -80°C until used. For the study on the stability of cytokines in serum, whole blood samples were collected into clot activator tubes and no additive tubes. After being stored at 4°C or room temperature for 2 hours, whole blood samples were immediately centrifuged, serum was separated at 2 hours, 8 hours, and 24 hours, and then stored at -80°C until used.

Cytometric bead array (CBA) assay

According to manufacturer's instructions, the CBA Human Inflammation Kit was used to detect IL-6 (Weimi, lot: 3060030079), IL-8 (Weimi, lot: 3060030080) and TNF-α (Weimi, lot: 3060030087) by the flow cytometry method. For a single detection, a total volume of 25 μL was required. Samples and standards were incubated for 2.5 hours with capture beads and detection antibody at room temperature in the dark. After being washed and centrifuged, samples and standards were run on a flow cytometer (Beckman Coulter, Navios, Germany). The data were analyzed with BD CBA software (BD Biosciences, USA).

Chemiluminescence (ECL) immunoassay

IL-6, IL-8, and TNF-α were measured with the ECL immunoassay IIMULITE 1000 (SIEMENS, Germany) and on the fully automatic IMMULITE system. For a single determination, a total volume of 150 μL was required.

Statistical analysis

Results were expressed as median ± interquartile ranges, because the cytokine concentrations were skewed. Correlation of the concentration of IL-6, IL-8, and TNF-α between CBA and ECL were compared using non-parametric Spearmen's correlation test. For statistical analysis, GraphPad Prism 7.0 was used (GraphPad software, USA), and two-tailed p-values < 0.05 were considered significant.

RESULTS

Characteristics of study group

The characteristics of the study population, including group number, age, and gender, were shown in Table 1. Subjects were health controls and RA patients. The median concentration of IL-6, IL-8, and TNF-α evaluated via two methods, CBA and ECL. Results were shown in Table 1 after being stored at 4°C for 2 hours.

Correlation of the concentration of IL-6, IL-8, and TNF-α between CBA and ECL methods

An overview of IL-6, IL-8, and TNF-α concentration were performed using two different commercially available immunoassays methods, CBA and ECL. The results of CBA analysis were compared with those of ECL analysis for serum and plasma at different times and temperatures. The concentrations of the majority of samples measured with CBA did concur with the concentrations that were determined with the ECL system (Figure 1). High correlation coefficients (R) between CBA and ECL for IL-6 and IL-8 were 0.8992 and 0.9484, respectively. There was also a significant correlation between the CBA and ECL for TNF-α measurement, but the relationship for TNF-α measurement ($R = 0.5845$) was poorer than the relationship for IL-6 and IL-8 measurements.

Table 1. Characteristics of study group.

	Health controls	RA	p
Number	**50**	**50**	**-**
Age (years)	**41 (33 - 54)**	**42 (32-57)**	**-**
Male/female (n/n)	**29/31**	**24/36**	**-**
IL-6			
CBA (pg/mL)	**1.52 (0.01 - 4.94)**	**3.31 (2.00 - 5.36)**	**< 0.05**
ECL (pg/mL)	**4.01 (2.00 - 6.17)**	**7.46 (2.00 - 28.66)**	**< 0.05**
IL-8			
CBA (pg/mL)	**1.72 (0.10 - 3.52)**	**4.17 (2.93 - 11.73)**	**< 0.05**
ECL (pg/mL)	**4.02 (2.0 - 19.90)**	**10.25 (3.53 - 16.46)**	**< 0.05**
TNF-α			
CBA (pg/mL)	**0.59 (0.04 - 3.14)**	**1.02 (0.09 - 5.05)**	**> 0.05**
ECL (pg/mL)	**3.77 (2.46 - 9.64)**	**9.43 (6.38 - 14.55)**	**< 0.05**

Subjects of health controls and RA patients were group number, age, and gender. Samples were stored at 4°C for 2 hours and measured for IL-6, IL-8, and TNF-α by CBA and ECL methods. Data were shown as median (interquartile). RA, rheumatoid arthritis patients; CBA, cytometric bead array; ECL, chemiluminescence.

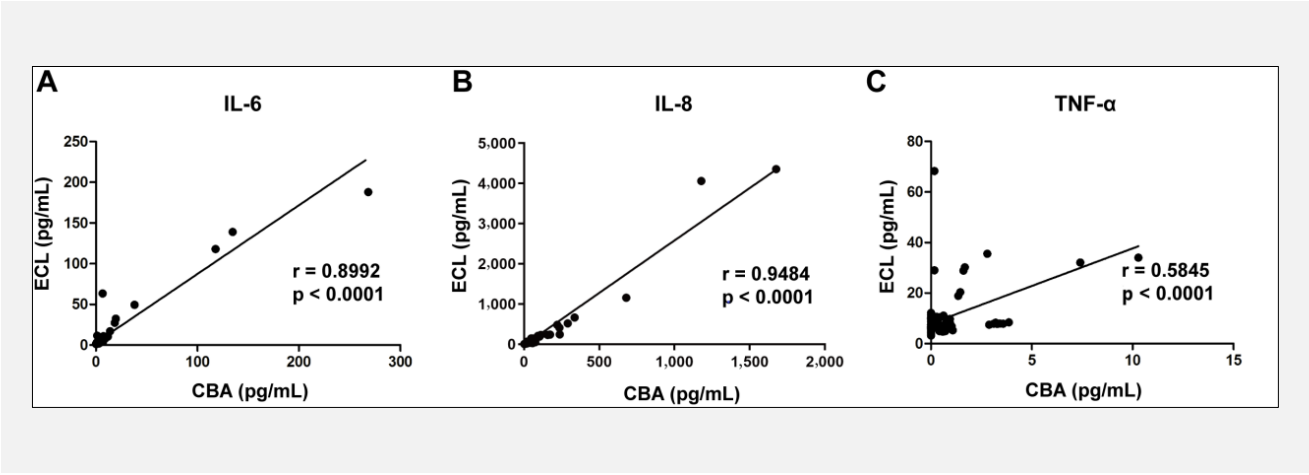

Figure 1. Correlation of the concentration of IL-6, IL-8, and TNF-α between CBA and ECL methods.

The results of IL-6, IL-8, and TNF-α concentration with CBA measurement were compared with those of ECL measurement for serum and plasma samples at different times and temperatures. The correlation coefficients (R) and p-value were shown.

Cytokines' stability in plasma and serum when stored at 4°C

We used the CBA and ECL methods to detect the changes of IL-6, IL-8, and TNF-α. Figure 2 showed the changes of concentrations of IL-6, IL-8, and TNF-α at 4°C in EDTA plasma, clot activator serum, and no additive serum from 2 hours to 24 hours. In general, the concentration of TNF-α was identified to be stable at different times after separation, in both plasma or in serum. Similarly, the concentration of IL-6 and IL-8 were also stable in separated plasma. However, the concentration of IL-6 and IL-8 were significantly declined in serum tubes regardless of the detection methods, especially for 24 hours at 4°C (Figure 2).

Cytokines' stability in plasma and serum when stored at RT

We used two methods, CBA and ECL, to detect the changes of IL-6, IL-8, and TNF-α. Figure 3 showed the changes of concentrations of IL-6, IL-8, and TNF-α at room temperature (RT) in EDTA plasma, heparin plasma, clot activator serum, and no additive serum from 2

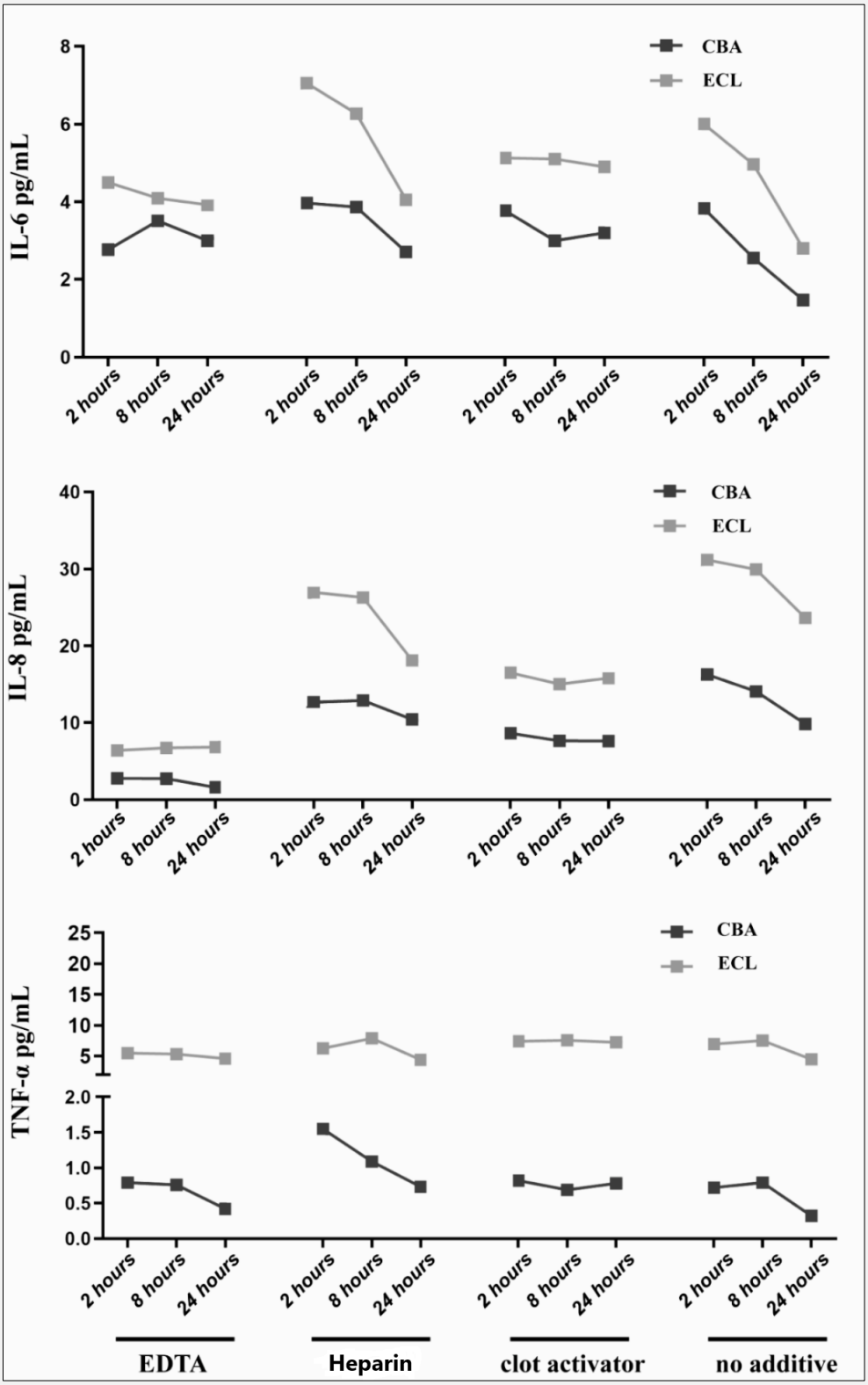

Figure 2. Cytokines' stability in plasma and serum when stored at 4°C.

Plasma (EDTA, heparin) and serum (clot activator, no additive) samples were collected and stored at 4°C for 2 hours, 8 hours, and 24 hours before being processed. Cytokines of IL-6, IL-8, and TNF-α were determined with CBA and ECL methods.

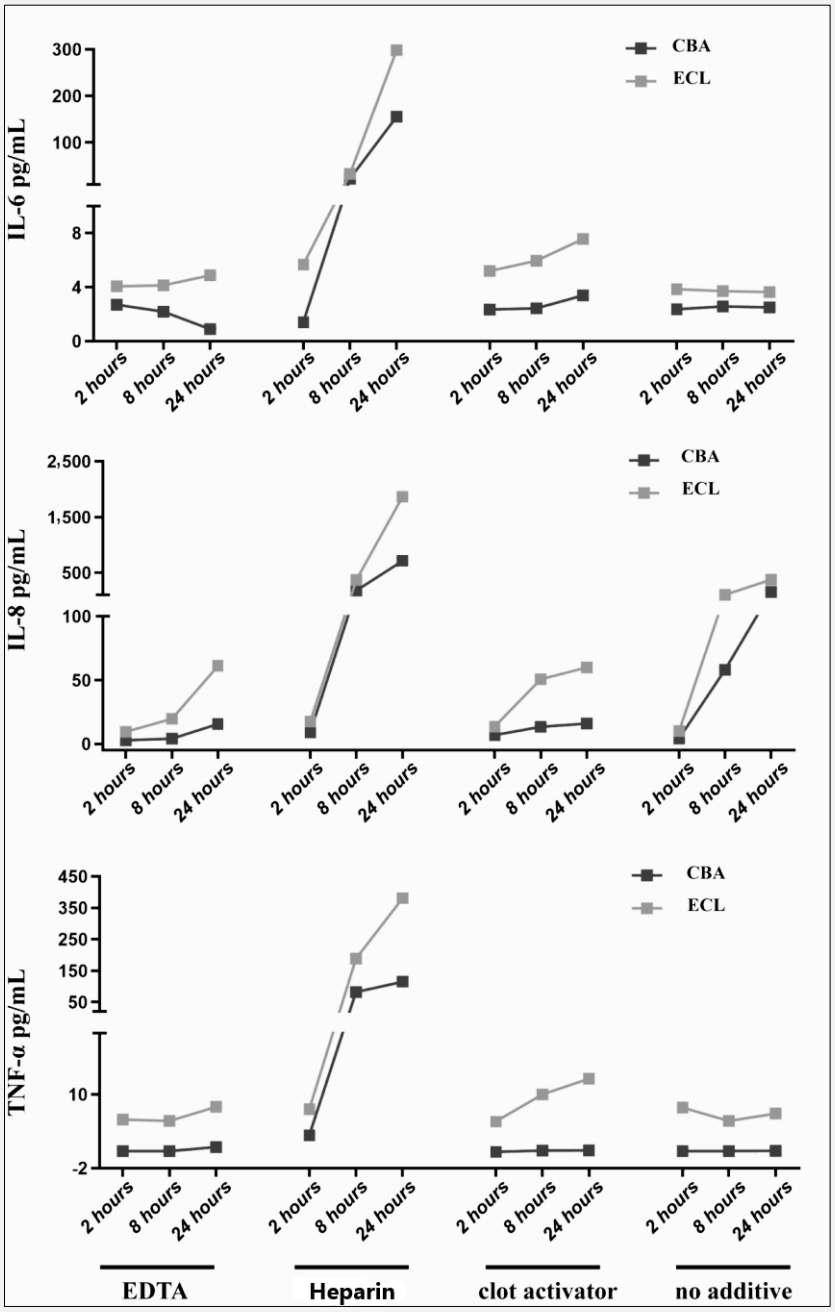

Figure 3. Cytokines' stability in plasma and serum when stored at RT.

Plasma (EDTA, heparin) and serum (clot activator, no additive) samples were collected and stored at RT for 2 hours, 8 hours, and 24 hours before being processed. Cytokines of IL-6, IL-8, and TNF-α were determined with CBA and ECL methods.

hours to 24 hours. We found heparin plasma concentrations of IL-6, IL-8, and TNF-α were all significantly increased after 2 hours at RT, no matter whether they were detected by CBA or ECL methods. We also found both plasma and serum concentration of IL-8 were increased very high degree at 8 hours and 16 hours. The two detection methods showed high consistency (Figure 3).

DISCUSSION

Accurate detection of cytokines is challenging because of their trace amounts (pg/mL) in the body [11]. The most commonly used methods for cytokine quantification are the enzyme linked immunosorbent assays (ELISA) [12]. This method is reliable but time-consuming. ECL immunoassay is easier and quicker to perform than ELISA, with a higher sensitivity, but it also suffers from the problematic "matrix" effect. Cytometric bead array can measure many cytokines simultaneously in a single assay with a small volume requirement. But the pre-processing requires manual operations. In conclusion, different detection methods have their own advantages and disadvantages.

In this study, in order to eliminate the error caused by detection methods, we used two methods to detect the cytokine levels of serum and plasma samples from 50 healthy adults and 50 RA patients. The finding of this study first indicated that the CBA method for IL-6 and IL-8 showed excellent agreement with ECL method for the same analysis. The correlation coefficients were 0.8992 and 0.9484. The CBA and ECL methods for TNF-α measurement showed less agreement, and the correlation coefficient was 0.5845. When the sample was stored at 4°C, the cytokine level of plasma showed less degradation than serum. Additionally, we found IL-8 concentration was affected by the storage conditions of whole blood from the time of collection until further processing at room temperature. We also found heparin tubes showed higher IL-6 and TNF-α level than other tubes at room temperature. In general, the best values for IL-6, IL-8, and TNF-α were found in EDTA samples, stored at 4°C, centrifuged quickly within 2 hours. The degradation of cytokines is a dynamic process. In our results, the concentrations of IL-6 and IL-8 were significantly declined in serum tubes at 4°C regardless of the detection methods, especially at 24 hours. However, plasma cytokines were more stable than serum cytokines at 4°C. Thus, we concluded that plasma was recommended for cytokine analysis. This result expanded on previous reports that plasma could maintain the stability of cytokines. They concluded that during the preparation of serum, some circulation cytokines would be degraded by calcium-dependent proteases [13]. In the coagulant tubes, blood coagulation promotes the formation of thrombin, as a protease, which promotes the degradation of cytokines. However, others indicated that cytokine levels were more stable in serum compared to plasma. A scientist compared samples obtained from patients with SLE and health controls and found protein levels were more stable in serum compared to plasma [14]. Some studies reported that plasma was not suitable for cytokine detection, because of the release of cytokines in vitro during coagulation [8]. Scientists also reported that serum cytokine concentrations were unaffected by storage at room temperature for less than four hours. The storage at -80°C generally results in optimal cytokine stability, and the repeated freezing/thawing of samples stored at -80°C did not significantly affect the cytokine concentration [15]. The reasons for these differences are not clear, but differences in collection and processing protocols or differences between separated and unseparated serum are critical factors for sure.

With the increase in storage temperature, many cytokines were not stable. At RT, we found a pronounced rise in IL-8 in any kind of anticoagulant tube regardless of the detection methods. IL-8 is the best known representative chemotactic cytokine with leukocyte specificity, which can recruit neutrophils into inflammatory sites and protect neutrophil-associated organ damage [16,17]. However, it has been shown that in addition to the circulating IL-8, which is measured in serum or plasma, a high percentage of IL-8 binds to the Duffy antigen on the surface of erythrocytes, and IL-8 bound to red cells is incapable of stimulating neutrophils [18]. Scientist found when freshly drawn blood was stored at room temperature, total IL-8 increased after 3 hours in cell lysis solution [19]. We thought the increase of the storage time resulted in increased red blood cell lysing; thus, IL-8 concentration was significantly increased at room temperature. Therefore, we recommended that the blood samples could not be stored at room temperature for cytokine measurement. As a consequence of our study, we also found heparin plasma concentrations of IL-6 and TNF-α were all significantly increased after 6 hours and 24 hours at RT, no matter if they were detected by the CBA method or the ECL method. However, in EDTA plasma tubes or in serum tubes we did not find the increase. Scientists have confirmed bacterial lipopolysaccharide (endotoxin) contamination is known to cause spontaneous cytokine production by monocytes, and heparin could significantly enhance the stimulatory effect on TNF-α release [20]. These studies give reasons to believe that heparin is impure, contaminated with LPS, which may induce the release of IL-6 and TNF-α. We should be careful in using heparin in detection of TNF-α, and endotoxins stimulating cellular responses through contamination with LPS may lead to systemic mistakes. But there were also some contrary conclusions. Hoffmann investigated different anticoagulants interfered with measurements of plasma TNF-α, found that no biologically active TNF-α was detected in the plasma with heparin anticoagulation, whereas EDTA plasma showed fairly high variability of TNF-α [21]. These different results may be due to the different laboratory conditions.

Our data point to the conclusion that cytokine concentration determination is complicated. Accurate detection of cytokines is challenging because of their dynamic secretion processes *in vitro* and instability in serum. In general, the most appropriate values for all cytokines were found in EDTA samples stored at 4°C. Gong and Kristin also found that the unseparated EDTA plasma remained steady over time [22]. Scientists found the lowest values for all cytokines were found in EDTA samples [8] Additionally, it is recommended to centri-

fuge tubes quickly following collection for accurate cytokine measurement. Consistent with our conclusions, Kristin Skogstrand also reported some cytokines were increased 1,000-fold, compared to serum and plasma isolated and frozen immediately [10]. Efforts to optimize standard operating procedures for serum sample collection, processing, and storage are needed at present.

In practical work, there are potential limitations in this study. First, the sample size was small in each subgroup. Second, we only tested the cytokine levels of IL-6, IL-8, and TNF-α, and found these cytokines were more stable in EDTA tubes than other tubes. How about other cytokines we have not tested, and scientists said that there was no optimal sample preparation that was clearly superior for the measurement of all analyses measured [23]. Thus, prospective study is needed for validation.

Declaration of Interest:

The authors have no conflict of interest to disclose.

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