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SHORT COMMUNICATION

Influence on sCD40L Value According to ELISA Assay Kits

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SUMMARY

Background: **Recent studies show sCD40L as a potential biomarker for thrombotic risk and cancer progression. Accurate measurement of sCD40L level is critical for research and clinical applications. However, variations in pre-analytic conditions, sample types and discrepancies in reference range across ELISA kits pose challenges to standardization in biobanking and research reproducibility.**
Methods: **sCD40L levels were measured using two ELISA kits. Bayesian statistical methods defined reference ranges, and paired *t*-tests and Pearson's correlation assessed differences and correlation between kits.**
Results: **The reference range for sCD40L using the R&D kit was 1,095.48 - 6,603.00 pg/mL, and for the Invitrogen kit, 1,620.00 - 10,405.00 pg/mL. There was a statistically significant difference between kits (p = 0.0019), and a strong correlation (r = 0.88) in serum samples.**
Conclusions: **sCD40L reference values differ by ELISA kit, underscoring the need for institution-specific reference ranges. Serum, not plasma, is preferable for sCD40L measurement. Establishing standardized reference ranges will improve the reliability of sCD40L as a biomarker in research fields.**
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KEYWORDS

sCD40L, reference range, sample types, comparison test, meta-analysis

INTRODUCTION

Recent studies have generated clinical interest in the use of sCD40L levels as a marker of thrombotic risk, inflammatory and autoimmune diseases [1,2]. Also, the soluble form of CD40L (sCD40L) is receiving increased attention as a potential biomarker in cancer diagnosis and progression [3]. Measuring serum sCD40L levels in research involves several methods, with enzyme-linked immunosorbent assay (ELISA) being one of the most commonly used. The ELISA method enables the precise measurement of sCD40L in serum samples, offering an accurate method to evaluate its concentration [1]. Also, soluble CD40L values can be

Table 1. Summary of measurement values according to sCD40L ELISA kits.

Serum (pg/mL)	R&D kit	Invitrogen kit	R&D kit - Invitrogen kit	p-value
n	11	11		
Mean (SD)	3,279.09 (1,736.32)	4,906.36 (2,502.35)	-1,627.27 (1,288.06)	0.0019
95% CI	2,112.62, 4,445.57	3,225.26, 6,587.47		
Median	3,576.38	4,875.00		
Min	1,095.48	1,620.00		
Max	6,603.00	10,405.00		
Range of Reference	1,095.48 - 6,603.00	1,620.00 - 10,405.00		

*** p-value was calculated by paired *t*-test.**
CI Confidence Interval.
Reference was calculated by Bayesian's method.

used as a quality control marker for pre-analytic conditions of human resources transported and collected from other institutions in the biobanking field [4-12]. Therefore, biobanks need to control such pre-analytic variations and must be able to perform appropriate quality control tests on their samples. However, the reference ranges, detection limits, and units of measurement suggested by each ELISA kit are different. There are also controversies depending on the sample type (plasma and serum) applied [2]. There have been few comparative studies of two reagent kits (ELISA) in serum and plasma so far. Therefore, the authors compared two kits that are frequently cited in papers to the sCD40L results from healthy volunteer's serum and plasma. Also we conducted to compare the reference range presented by the manufacturer with the cutoff values for various diseases applied in the other studies.

MATERIALS AND METHODS

After giving informed consent, blood was collected from a total of 11 volunteers. The blood was divided into 2 groups (plasma and serum). This study was approved by the Clinical Study Ethics Committee of HANARO Medical Foundation in 2024 (HNR2024-02). Each sample group was centrifuged within 2 hours after whole blood collection. Separated plasma and serum samples were stored frozen at -80℃ until analysis. sCD40L was measured using Human sCD40L ELISA kits (Invitrogen, Catalog Number BMS293, Thermo Fisher Scientific Inc.) and Human CD40 Ligand Immunoassay (Quantikine, Catalog Number SCDL40, R&D Systems Inc.). All samples were measured in duplicate. The concentrations of sCD40L in plasma and serum were summarized using descriptive statistics, including the number of subjects observed, mean, standard deviation, median, minimum, and maximum values. By applying Bayesian's method, the reference range was based on the 2.5th - 97.5th percentile, excluding sample values that are ± 3 SD from the mean values. The concentration differences between kits were analyzed using a paired *t*-test. All confidence intervals were computed at the 95% level. Scatter plot between 2 ELISA kits in samples was evaluated using Pearson's correlation. The prediction ellipses were presented as 70%, 80%, and 90%. The prediction ellipses represent the probability that the mean of the data will be included within the ellipses and were presented to visually express the correlation between three variables. 90%, 80%, and 70% prediction ellipses were used to visualize the relationship between the two variables more clearly. We used SAS® 9.4 for analysis.

RESULTS

We established a normal reference range by conducting statistical analysis by Bayesian's method as follows: The reference range was determined based on the 2.5th percentile to the 97.5th percentile among all the result values. There were no sample values out of ± 3 SD in our results. The reference range of serum sCD40L using the R&D ELISA kit was 1,095.48 pg/mL to 6,603.00 pg/mL (Mean; 3,279 pg/mL), and the reference range of serum sCD40L using the Invitrogen kit was 1,620.00 pg/mL to 10,405.00 pg/mL (Mean; 4,906 pg/mL) (Table 1). These showed rather different values with reporting mean of serum sCD40L levels from healthy volunteers, 5,461 pg/mL suggested by R&D systems, and by Invitrogen kit of 2.13 ng/mL. The difference in measurement values between Invitrogen and R&D Kit was -1,627.27, showing a statistically significant difference ($p = 0.0019$). In addition, the correlation between the two kits was higher in serum than plasma (correlation coefficient: 0.8766 vs. 0.6436), so the correlation between the two kits appears to be high (Figure 1, 2). Green, red, and blue lines represent prediction ellipses for 70%, 80%, and 90% in scatter plot between the 2 ELISA kits.

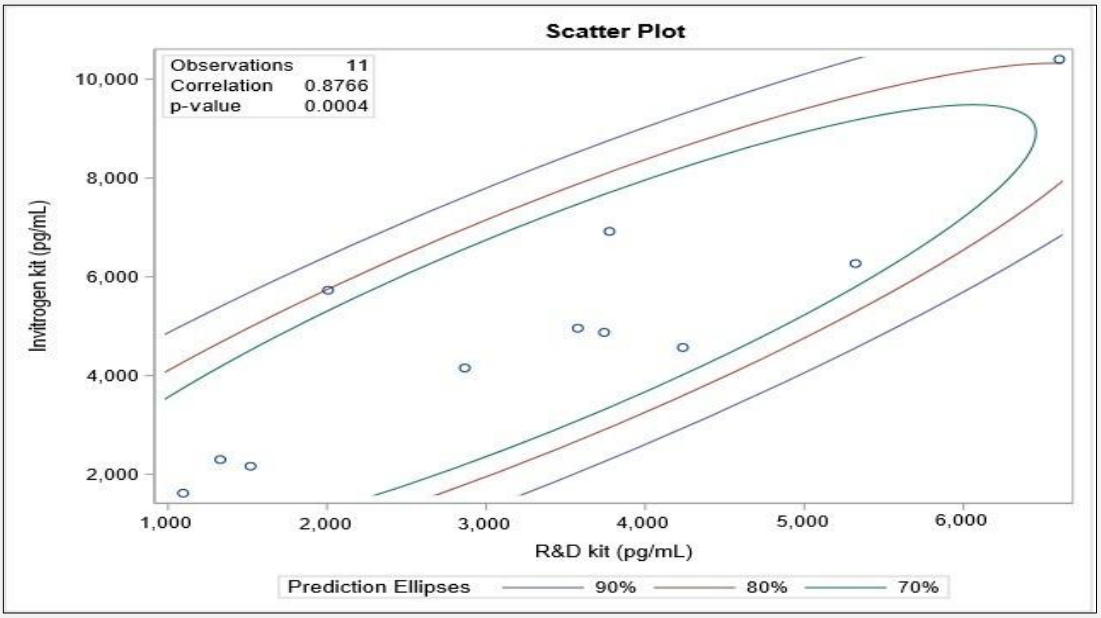

Figure 1. Scatter plot between 2 ELISA kits in serum sample (Pearson's correlation).

Green - Prediction ellipse 70%, Red - Prediction ellipse 80%, Blue - Prediction ellipse 90%.

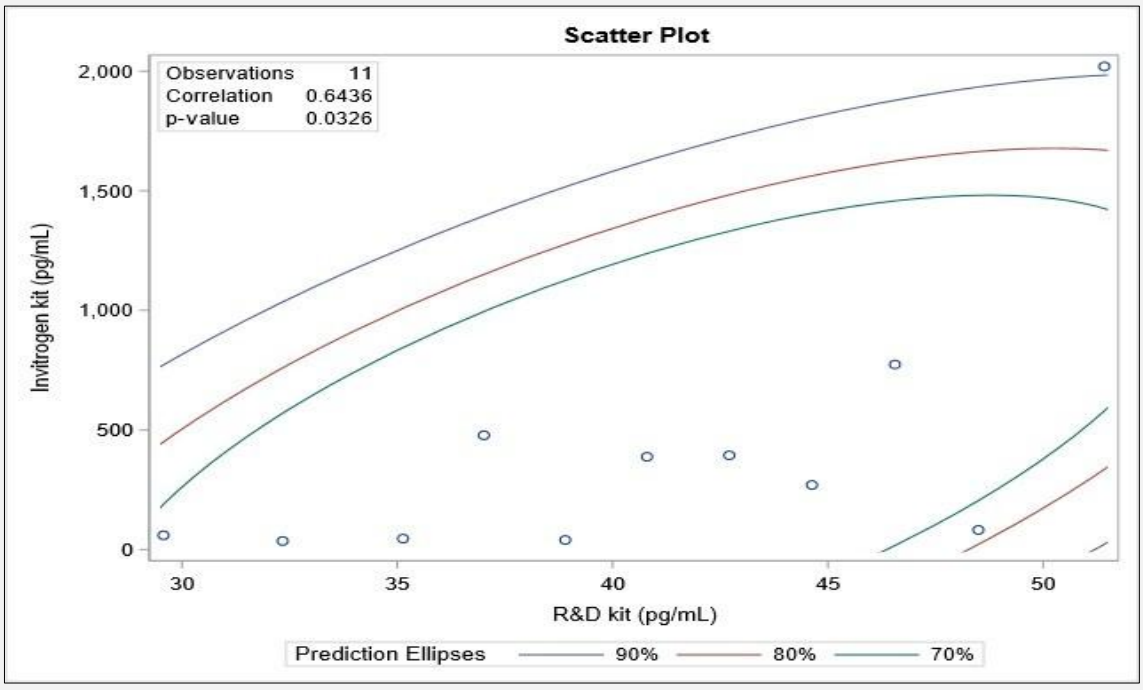

Figure 2. Scatter plot between 2 ELISA kits in plasma sample.

DISCUSSION

The reason soluble CD40 ligand (sCD40L) levels are higher in serum compared to plasma is primarily due to the release of sCD40L from activated platelets during the clotting process. When blood is allowed to clot, as in the preparation of serum, platelets are activated and release sCD40L. In contrast, plasma is prepared by adding anticoagulants to prevent clotting, which minimizes platelet activation and the subsequent release of sCD40L. Therefore, the process of serum preparation inherently results in higher sCD40L levels due to platelet activation and release during clot formation. For that reason, plasma is not an appropriate sample for sCD40L measurement. Bereczki et al. concluded that plasma samples were not appropriate for the study of the association between inflammatory markers and early onset atherosclerosis [13]. According to Varo et al. [14], comparison of serum and plasma (platelet-free) samples from 20 healthy volunteers demonstrated significantly ($p = 0.001$) elevated sCD40L levels in serum samples, yielding 0.65 - 12.93 ng/mL (mean, 5.45 ± 4.55 ng/mL) compared with citrated, EDTA or heparinized plasma (1.03 ± 1.07, 1.43 ± 1.03 or 1.80 ± 1.25 ng/mL, respectively). sCD40L levels in plasma collected in EDTA, citrate or heparin did not differ significantly. Furthermore, serum sCD40L concentrations did not correlate with sCD40L levels in plasma collected using any of the anticoagulants tested. In our study, the correlation between the two kits was higher in serum than plasma (correlation coefficient: 0.8766 vs. 0.6436). In contrast, to get a reference range of sCD40L in healthy individuals, Pedersen et al. assessed the concentration of sCD40L in 100 healthy donors. They found that 61 (61%) of the samples had levels below the detection limit, and thus the medium level is the detection level for 10-fold diluted samples, 0.81 ng/mL, with interquartile range of 4.33 ng/mL and range of 0.81 - 5.14 ng/mL. A linear correlation ($R^2 = 0.9952$) of sCD40L levels in EDTA plasma and matched serum from 23 healthy donors was seen [15]. On the other hand, Weber et al. reported that plasma, not serum, is appropriate for measuring sCD40L [16]. Besides, the sCD40L levels in healthy controls and disease conditions suggested by several studies and ELISA kit manufacturers. The serum sCD40L level in hepatocellular carcinoma patients had a significantly higher AUC value of 0.930 with 90% sensitivity and 86.7% specificity at a cutoff of 7,305.5 pg/mL (Quantikine ELISA kit, R&D systems) [17]. The serum level of sCD40L (3.57 ± 1.63 ng/mL) in the gastric cancer group was significantly ($p < 0.01$) higher than that of healthy group (1.94 ± 0.86 ng/mL) using Bender Medsystems kit [18]. Considering our results and meta-analysis, we concluded that serum, not plasma, is preferable for sCD40L measurement.

A limitation of our study is the small sample size. To complement for such research limitation, we additionally reviewed, compared, and analyzed the reference range of other studies through meta-analysis. For your information, the limitation used with commercially available ELISA kits is the different detection ranges and reference ranges presented by each manufacturer. The different detection ranges and reference ranges of sCD40L presented by each manufacturer are as follows. The mean of serum sCD40L levels from healthy volunteers is 5,461 pg/mL suggested by R&D systems, 2.13 ng/mL by Invitrogen. Due to the different detection ranges and reference ranges presented by each manufacturer, each institution must set its own reference range. For that reason, the Korea Disease Control and Prevention Agency (KDCA) suggested serum 7 - 17 ng/mL as a reference range, recommending to use them as a quality control marker for pre-analytic conditions of human resources transported and collected from other institutions in the biobanking field. Lengelle et al. used the R&D system kit and reported that sCD40L value decreases to below that reference range when exposed to room temperature for a long time before and after centrifugation [5]. Earlier, our study using Human sCD40L ELISA kits (Thermo Fisher Scientific Inc.) suggested that sCD40L values can be used as an indicator indirectly showing that separation of whole blood was delayed and after centrifugation left unattended (data not shown). Taken together, our results suggested here will enhance the usability of sCD40L assay by establishing a normal reference range. After setting their own reference range by applying their correlation coefficient factor, or regression analysis comparing commercially available ELISA kits, it will be comparable to the cutoff values of various diseases in other studies for clinical application. In this regard, additional, multicenter studies with statistically significant sample sizes should be done to derive standardized reference ranges through comparative testing using commercialized kits.

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Declaration of Interest:
The authors declare no conflicts of interest, financial or otherwise.

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