Original

Measuring ascites and urinary presepsin levels may be useful for diagnosing local infection: a retrospective cross-sectional study

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ABSTRACT

Presepsin (P-SEP) is a well-known biomarker for diagnosing sepsis. Although recent reports have demonstrated the usefulness of measuring P-SEP in the blood, there is insufficient evidence in other samples. This study aimed to explore the clinical usefulness of measuring P-SEP in pleural fluid, ascites, and urine. We used a fully automated analyzer (STACIA) to measure P-SEP levels and compared P-SEP levels with other clinical data. Receiver operating characteristic curve analysis, which was used to separate cases of positive culture test using ascites P-SEP level, showed that the area under the curve was 0.832. The sensitivity and specificity were 94.1% and 85.7%, respectively, when 1,842 pg/mL of ascites P-SEP was used as the cutoff value. In urine, the P-SEP/creatinine ratio of patients who showed pyuria was significantly higher than that of the control group. The results suggest the possible usefulness of P-SEP in ascites and urine, for evaluating local infection.

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Key Words
presepsin, ascites, urine, biomarker, abdominal infection

I. Introduction

Presepsin (P-SEP) is a 13-kDa soluble N-terminal fragment of CD14, which is a receptor for lipopolysaccharide and lipopolysaccharide-binding protein complex [1)2)]. Two main mechanisms have been proposed for P-SEP induction: (1) Monocytes directly produce P-SEP after phagocytosing bacteria. (2) Monocytes phagocytose neutrophils forming NETs, and CD14 on the surface of the monocyte cell membrane is degraded intracellularly, resulting in P-SEP secretion [3)4)].

In clinical practice, measuring serum P-SEP levels is useful for diagnosing sepsis and its severity [5)–10)]. Considering the aforementioned mechanisms, P-SEP is useful for detecting bacterial infection. Thus, theoretically, the measurement of P-SEP may be useful for diagnosing not only systemic bacterial infections such as sepsis but also local bacterial infections.

In fact, recent reports have demonstrated the usefulness of measuring P-SEP not only in the blood but also in other samples. High levels of P-SEP are observed in the pleural fluid of patients with empyema and parapneumonic effusion [11)]. P-SEP levels in synovial fluid are useful for the differential diagnosis of native joint septic arthritis and crystal arthritis with a sensitivity and specificity of 100% [12)13)]. P-SEP in cerebrospinal fluid is also useful for

diagnosing bacterial meningitis, and its diagnostic ability is superior to that of leukocytes or proteins in cerebrospinal fluid[14].

However, the modulations of P-SEP in urine by bacterial urinary tract infection remain unknown. Moreover, there is still insufficient evidence regarding the modulation of P-SEP in pleural fluid and ascites, especially in the aspects of bacterial infection. Therefore, in order to explore the usefulness of P-SEP in samples other than the serum, the present study aimed to validate the measurement of ascites, pleural fluid, and urinary P-SEP levels and to evaluate their diagnostic ability for local bacterial infection.

II. Materials and methods

Samples

For ascites and pleural fluid, 46 ascites and 45 pleural fluid samples were obtained from residual specimens from routine clinical laboratory testing collected by body cavity puncture. In ascites fluid, samples with a serum–ascites albumin gradient (SAAG) of less than 1.1 were determined to be exudate. In pleural fluid, Light's criteria were used to determine whether the fluid was transudate or exudate. Briefly, the samples were determined as exudates if at least one of the following exists: (1) The ratio of pleural fluid protein to serum protein is <0.5. (2) The ratio of pleural fluid lactose dehydrogenase (LDH) to serum LDH is <0.6. (3) The pleural fluid LDH is <2/3 of the upper reference limit. The serum clinical laboratory data on the same day were used to determine whether the sample was transudate or exudate. If culture tests were performed during the same hospital stay, the results were obtained from the medical records (Supplemental **Table 1**). Once a positive culture test result was obtained, it was regarded as a culture-positive group.

For urine, subjects who were clinically diagnosed with specific kidney diseases, including those diagnosed with renal biopsy, were enrolled. A total of 138 residual urine specimens were collected after routine clinical laboratory testing from 6 subjects with diabetes mellitus, 10 subjects with renal sclerosis, 4 subjects with chronic glomerulonephritis, 17 subjects with IgA nephropathy, 13 subjects with vasculitis syndrome, 6 subjects with systemic lupus erythematosus (SLE), 2 subjects with minimal change disease, 7 subjects with membranous nephropathy, 4 subjects with focal segmental glomerulosclerosis, 1 subject with membranoproliferative glomerulonephritis, and 11 subjects with other renal diseases. A total of 32 subjects with >10 white blood cells (WBCs)/μL in their urine as quantified by flow cytometry were included in the pyuria group. Meanwhile, 25 subjects with no evidence of proteinuria and pyuria as verified by dipstick test and flow cytometry, respectively, were included in the control group. All laboratory data, except P-SEP, were extracted from medical records.

Collection of samples

Residual ascites fluid, pleural fluid, or urine samples were collected after routine clinical laboratory testing and stored at −80 ℃ until analysis. Ascites and pleural fluids were collected from a collection tube with EDTA-2K powder, whereas urine was collected from a plain collection tube. Then, samples were centrifuged to separate the supernatant and material components (at 2,300 g for 5 min for ascites and pleural fluids and at 1,700 g for 5 min for urine before collection). For the method validation experiments, pooled samples were prepared by mixing residual samples after routine testing of pleural fluid, ascites, and urine submitted to the clinical laboratory from September 2018 to April 2021 in. any proportion.

Measurement of P-SEP

P-SEP was measured using the fully automated clinical analyzer STACIA and STACIA CLEIA Presepsin (PHC Corporation, Tokyo, Japan), which was based on chemiluminescent enzyme immunoassay (CLEIA). Briefly, a sandwich complex was formed between anti-P-SEP antibody-coated paramagnetic latex beads and alkaline phosphatase (ALP)-labeled anti-P-SEP antibody, if P-SEP was present in the sample. Next, nonmagnetic materials were washed out with a wash buffer under a magnetic field. Then, disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3′,2(5-chlorotricyclo[3.3.1.1^{3,7}]decan])-4-yl]-1-phenyl phosphate was added and hydrolyzed by ALP in the complex, emitting light. Finally, the results were determined as pg/mL using a calibration curve generated by five-point calibration. Because the measurement range in the manufacturer's instrument reaches up to 20,000 pg/mL, samples exceeding 20,000 pg/mL were diluted and measured using the manufacturer's diluent. Both devices have been approved by the Pharmaceuticals and Medical Devices Agency in Japan.

Method validation

The precision of the assay for 10 replicates was investigated using two pooled samples composed of pleural fluid and ascites for validation of the pleural fluid and ascites measurement. For validation of urine measurement, a pooled urine sample for 20 replicates was measured. According to the manufacturer's instructions, the coefficient

of variation is less than 10% when the control samples are measured. Linearity was investigated using two types of pooled samples composed of pleural fluid and ascites or urine samples from one patient. Briefly, each sample was diluted up to 10 times with the manufacturer's diluent. In the urine experiment, one sample was diluted up to five times.

Spike and recovery tests were performed using pleural fluid, ascites, urine, and the manufacturer's standard. The standard was diluted with the manufacturer's diluent at approximately 500 pg/mL before being used in the experiments. Two pleural fluid, ascites, and urine samples were mixed with the standard in the proportions shown in Table 2. The recovery rate was calculated using the following method: (1) The P-SEP levels of mixed samples were measured. (2) Expected values were calculated by the proportion of mixture of the sample and standard. (3) The recovery rate was determined by the ratio of the measured value to the expected value when the expected value was 100%.

Statistical analysis

The Mann–Whitney U test was used for comparisons between two groups. Spearman's rank correlation coefficient was calculated to evaluate the correlations between urinary P-SEP levels and other clinical laboratory data. Statistical analyses were performed using IBM SPSS Statistics 27 (NY, USA). Steel's multiple comparison test, which was used for comparisons between the control and other groups, and receiver operating characteristic curve analysis were performed using JMP Pro 16 (Cary, USA). Statistical significance was considered at $p<0.05$.

Ethical consideration

The present study was conducted in accordance with the ethical guidelines established in the Declaration of Helsinki. Informed consent was obtained in the form of an opt-out form on the website for participants: patients were informed about the study on the website (http://labtky.umin.jp/clinical_research/3333_161.pdf), and those who were unwilling to be enrolled in the study were excluded. If the participants were minors, their parents or guardians could waive participation in this study on their behalf. The study design was approved by the University of Tokyo Medical Research Center Ethics Committee, which waived the need for written informed consent because only archived specimens were used and data for this retrospective study were retrieved from medical records (3683 and 3333-161).

III. Results

Method validation

First, we verified the repeatability with 10 replicates for pleural fluid and ascites and 20 replicates for urine using pooled samples. The precision of the assay was 3.1%–3.5% for P-SEP measurement in pleural fluid and ascites and 4.8% for P-SEP measurement in urine (**Table 1**).

Linearity was validated using linear regression analysis and both pooled pleural fluid and ascites and urine from a patient. The linearity curves of P-SEP in the pleural fluid, ascites, and urine samples are shown in **Figure 1**. The curves exhibited good linearity up to 19,482 pg/mL for P-SEP in pleural fluid and ascites and 967 pg/mL for P-SEP in urine.

To explore the accuracy of P-SEP measurement in pleural fluid, ascites, and urine, spike and recovery tests were performed using two or three types of samples. The recovery rates ranged from 94.8% to 104.8% for pleural fluid, from 93.0% to 103.1% for ascites, and from 95.5% to 101.6% for urine (**Table 2**).

Levels of P-SEP in pleural fluid and ascites

To investigate the association of P-SEP with Light's criteria or SAAG classification of transudate/exudate, we measured P-SEP in 43 pleural fluid samples for which Light's criteria could be evaluated and 42 ascites fluid samples for which SAAG could be calculated. P-SEP levels were compared between the transudate and exudate groups. As shown in Figures 2A and B, the median and range of P-SEP in the transudate pleural fluid, exudate pleural fluid, transudate ascites, and exudate ascites groups were 741.0 (322–12,341), 1,084.0 (296–11,604),

Table 1 Repeatability of P-SEP measurement in pleural fluid, ascites, and urine

	Number of replications	Mean (pg/mL)	SD (pg/mL)	CV (%)
Pleural fluid and ascites 1	10	517.8	17.87	3.5
Pleural fluid and ascites 2	10	3,150.6	97.57	3.1
Urine	20	167.9	8.02	4.8

P-SEP was measured in pooled pleural fluid and ascites and urine with 10 or 20 replicates. SD, standard deviation; CV, coefficient of variation

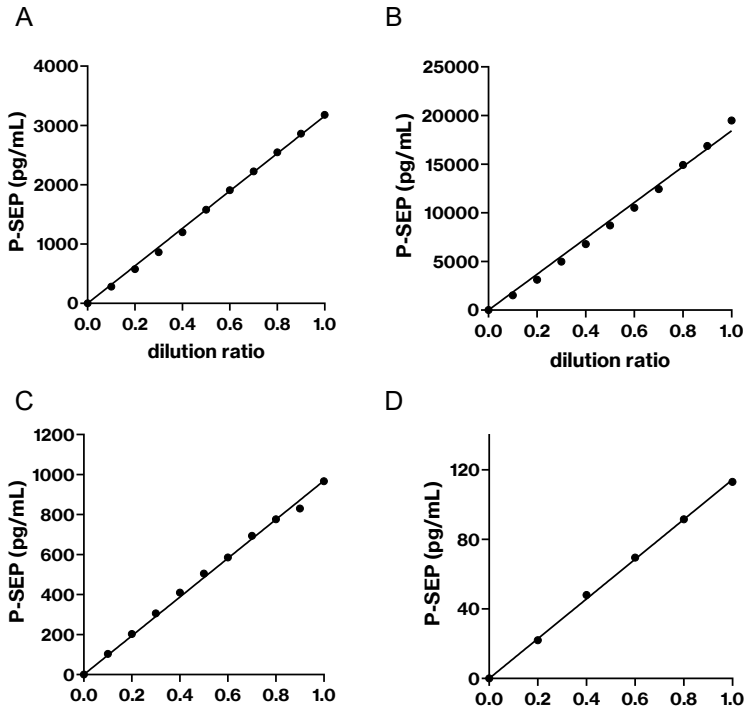

Figure 1 Linearity analysis of pleural fluid and ascites and urinary P-SEP.
The dilution linearities of pleural fluid and ascites (A, B) and urine (C, D) were investigated. A sample was diluted 5 to 10 times with the manufacturer's diluent. Then, each sample was analyzed with two replicates.

Table 2 Recovery rates of spike and recovery tests in pleural fluid, ascites, and urine

	Sample conc. (pg/mL)	Standard conc. (pg/mL)	Recovery rate (%)						
Mixture ratio (Sample:Standard)			1:19	2:18	5:15	10:10	15:5	18:2	19:1
Pleural fluid 1	522	594	101.4	102.6	106.3	99.9	94.2	100.5	98.9
Pleural fluid 2	2,075	537	101.3	101.5	103.2	100.6	97.0	96.7	94.8
Ascites 1	373	515	103.1	99.5	97.2	96.2	98.6	98.1	94.7
Ascites 2	2,648	579	93.0	96.2	101.0	102.5	102.4	102.3	101.6
Urine 1	254	544	98.4	96.2	98.5	97.5	99.2	97.7	101.4
Urine 2	1,049	484	95.5	98.9	97.7	100.5	95.8	101.6	99.5

Pleural fluid, ascites, and urine samples were mixed with standard solution in several proportions and measured. The recovery rate was calculated by the ratio of the measured P-SEP level to the expected P-SEP level when the expected P-SEP level in each mixture ratio was defined as 100%. All values represent the average of three replicates.

1,076.5 (434–21,664), and 1,537.0 (583–5,304) pg/mL, respectively. All samples exceeded 50 pg/mL, which is the lower limit of the measurement range in the manufacturer's instructions. In addition, there was no difference in P-SEP levels between transudate and exudate in either pleural fluid or ascites. However, when the pleural fluid and ascites P-SEP levels were compared between the groups of cases in which the culture test became positive or negative, there was no significant difference in P-SEP levels in pleural fluid between the two groups, whereas the P-SEP levels in ascites fluid were significantly higher in the culture-positive group (**Figures 2C** and **D**).

Usefulness of P-SEP measurement for detecting culture-positive ascites

To further understand the significance of P-SEP in ascites, we performed receiver operating characteristic curve analysis to separate cases of positive culture test using the P-SEP level or WBC in ascites, which are used to diagnose acute peritonitis. The WBC levels in the culture-positive group were significantly higher than those in the culture-negative group (Supplemental **Figure 1**).

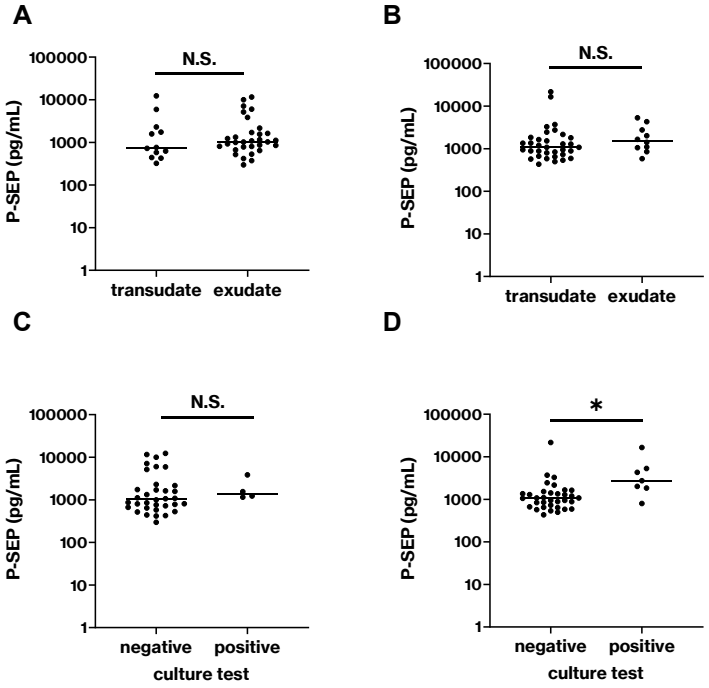

Figure 2 Comparisons of P-SEP levels in pleural fluid and ascites.
P-SEP levels in pleural fluid and ascites samples were measured. (A) Comparison of P-SEP level in pleural fluid samples between transudate (n=12) and exudate (n=31) divided by Light's criteria. (B) Comparison of P-SEP level in ascites between transudate (n=34) and exudate (n=10) divided by SAAG classification. (C) Comparison of P-SEP level in pleural fluid between culture test-negative (n=34) and positive cases (n=4). (D) Comparison of P-SEP level in ascites between culture test-negative (n=34) and positive cases (n=7). *, $p<0.05$; N.S., not significantly different

The area under the curve for ascites P-SEP was 0.832 (95% confidence interval, 0.649–1.000), and that for ascites WBC was 0.910 (95% confidence interval, 0.773–1.000). Based on Youden's index, the optimal cutoff values for ascites P-SEP and ascites WBC were identified as 1,842 pg/mL and 3.3 $\times 10^9$ counts/L, respectively (**Figure 3A**, **Table 3,** Supplemental **Figure 1** and Supplemental **Table 2**).

Next, we evaluated whether a combination of cutoff values for ascites P-SEP and WBC would improve the diagnostic ability to be culture-positive. The specificity improved from 94.1% (when only ascites WBC was measured) to 100% (when ascites WBC and P-SEP were measured) when the cutoff values for culture-positive cases were set as 1,842 pg/mL for P-SEP and 3.3 $\times 10^9$ counts/L for ascites WBCs (**Figure 3B**, **Table 3B**). On the other hand, the sensitivity for culture-positive cases remained as high as 85.7%.

Usefulness of measuring urinary P-SEP for detecting bacterial urinary tract infection

We compared the P-SEP/creatinine ratios in the urine of patients with pyuria, nephritis, or other kidney diseases with those of controls to investigate the possible usefulness of urinary P-SEP. **Figure 4A** shows that the urinary P-SEP/creatinine ratio in the pyuria group was significantly higher than that in the control group. Next, we investigated the correlations between the urinary P-SEP/creatinine ratio and estimated glomerular filtration rate (eGFR) or urinary WBC. **Figures 4B** and **C** show a significant negative correlation between the urinary P-SEP/creatinine ratio and eGFR and a significant weakly positive correlation between the urinary P-SEP/creatinine ratio and WBC count. These negative correlations were also observed when P-SEP was not correlated with urinary creatinine (**Figures 4D** and **E**).

IV. Discussion

We validated a method for quantifying P-SEP in pleural fluid, ascites, and urine using the fully automated clinical analyzer STACIA. First, the repeatability of pleural fluid, ascites, and urine was not inferior to that of the manufacturer's instrument for serum P-SEP, which was less than 10% (**Table 1**). These results suggested that this measurement system provided relatively stable data even if P-SEP in pleural fluid, ascites, and urine was measured. The linearity was good (up to 19,482 pg/mL for pleural fluid and ascites and 967 pg/mL for urine)

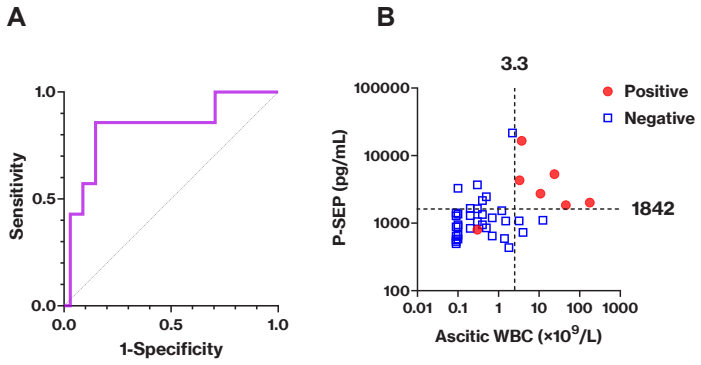

Figure 3 Diagnostic ability of ascites P-SEP for culture-positive case.
(A) Receiver operating characteristic curve analysis was performed to separate culture test-positive cases (n=7) from culture test-negative cases using the P-SEP level in ascites and to show the area under the curve. (B) Two-dimensional plot with ascites P-SEP levels on the vertical axis and ascites WBC levels on the horizontal axis. Blue squares indicate negative cases for culture test, whereas red circles indicate positive cases for culture test. In the WBC counting tests, data under the mechanical detection limit (0.1 cells/μL) were regarded as 0.1 cells/μL.

Table 3 Diagnostic ability of ascites P-SEP for culture-positive case

(A)

Number of cases	Culture-positive case	7
	Culture-negative case	34
AUC		0.832
95% CI		0.649 –1.000
Cutoff value		1,842 pg/mL
p value		<0.001

(B)

	Sensitivity	Specificity
P-SEP only	85.7%	85.3%
WBC only	85.7%	94.1%
Combination of P-SEP and WBC	85.7%	100%

Receiver operating characteristic curve analysis was performed. (A) The results of receiver operating characteristic curve analysis for culture-positive case using ascites P-SEP level are shown. The cutoff value was determined based on Youden's index. (B) The sensitivity and specificity to diagnose culture-positive case were calculated if ascites P-SEP alone, ascites WBC alone, and both were used for diagnosis. In WBC counting tests, data under the mechanical detection limit (0.1 cells/μL) were regarded as 0.1 cells/μL.

(**Figure 1**). The linearity for urine above 967 pg/mL was unclear because a sufficient volume of urine containing high concentration of P-SEP was collected. Several urine samples contained more than 967 pg/mL of P-SEP; however, we measured these samples without dilution because the manufacturer's instrument defined the measurable range as 50–20,000 pg/mL. The recovery rate for measuring P-SEP in pleural fluid and ascites ranged from 93.0% to 106.3% (**Table 2**). The recovery rate of pleural fluid 2 slightly decreased with increasing proportion of sample. However, a proportional systematic error was not observed in pleural fluid 1. For urine, a proportional systemic error was not observed, regardless of how the ratio of sample to standard changed. These results suggest that no specific matrix effects for the measurement of P-SEP exist in pleural fluid, ascites, and urine. Based on these results, the application of the method for measuring serum and plasma P-SEP, which has already been approved by the Pharmaceuticals and Medical Devices Agency in Japan, to pleural fluid, ascites, and urine provides a performance equivalent to that of serum.

Pleural fluid and ascites P-SEP levels may be higher than serum P-SEP levels. The serum P-SEP level from 128 healthy subjects was reported to be 190 pg/mL [6]. This difference can be explained by the fact that pleural fluid and ascites reflect the local production of P-SEP. Consistent with the present study, Watanabe et al. reported that pleural fluid P-SEP levels were significantly higher than those in plasma, whereas the level of C-reactive protein in pleural fluid was significantly lower than that in plasma [11].

With regard to clinical usefulness, ascites P-SEP of the culture test-positive group was significantly higher than that of the negative group, whereas no significant difference in ascites P-SEP levels was observed between the transudate and exudate groups (**Figures 2B** and **D**).

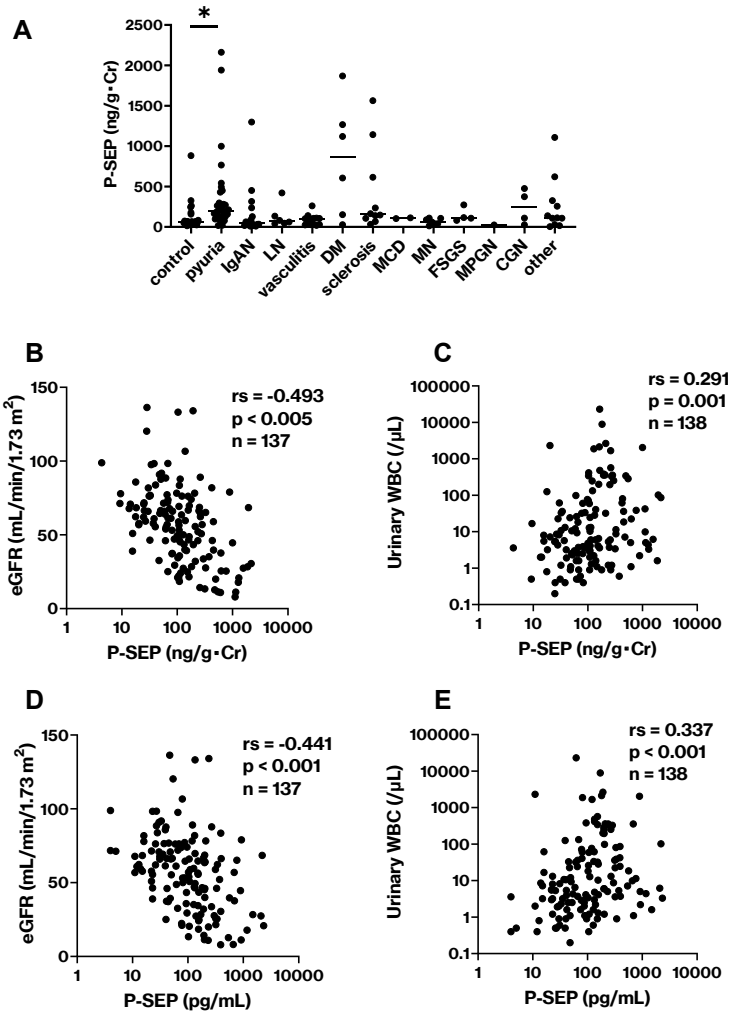

Figure 4 Urinary P-SEP levels in patients.
(A) The urinary P-SEP/creatinine ratio was compared among various patients with renal dysfunction. (B–D) The urinary P-SEP levels and urinary P-SEP/creatinine ratio were compared in relation to eGFR and urinary WBC. IgAN, IgA nephropathy; LN, lupus nephritis; DM diabetes mellitus; MCD, minimal change disease; MN, membranous nephropathy; FSGS, focal segmental glomerulosclerosis; MPGN, membranoproliferative glomerulonephritis; CGN, chronic glomerulonephritis; rs, Spearman's rank correlation coefficient. *, $p<0.05$.

According to this result, we speculate that WBCs migrating into the peritoneal cavity produce P-SEP to exclude bacteria. In fact, a positive correlation between ascites WBCs and P-SEP was observed ($r=0.409$, $p<0.01$). The reason that exudative ascites, which is classically considered as an accumulation with inflammation including bacterial infection, was not significantly higher may be because of the method used to calculate SAAG or the fact that ascites does not emerge in healthy individuals. In this study, three cases of positive culture test were classified into the transudate group because their SAAG values were 1.1. These cases might have been classified as the transudate group due to measurement error because the smallest unit of albumin measurement was 0.1. In addition, SAAG has a 96.7% positive diagnostic rate for the presence of portal hypertension [15]. Therefore, when SAAG is used alone, its performance may be inadequate with respect to its ability to discriminate peritonitis. In this study, pleural fluid P-SEP level of the culture test-positive group was not significantly higher than that of negative group. Two possible explanations can be considered. One is the small number of pleuritis cases (n=5), and the other is the inclusion of several empyema cases in the culture-negative group.

The area under the curve of ascites P-SEP to separate positive or negative results of culture test was relatively high but inferior to that of ascites WBCs. Thus, we attempted to combine these values to discriminate positive or negative culture tests. As a result, the specificity was improved and reached 100%. Ascites WBC and P-SEP tests can be completed quickly and may be useful for diagnosing abdominal bacterial infection prior to culture

testing.

A widely known inflammatory marker is procalcitonin. Viallon et al. reported that the AUC of 0.96 when diagnosing spontaneous bacterial peritonitis (SBP) using ascites procalcitonin [16]. It is higher than that of ascites P-SEP in our study (AUC 0.832). The sensitivity of diagnosing SBP using procalcitonin was better than that of ascites WBC and P-SEP test. However, there is a difference in that the previous study regarded patients with ascites WBC more than 2.5×10^9 count/L with diagnosis of SBP as the positive group, whereas in this study, patients with positive ascites culture test were regarded as the positive group. Elsadek et al. reported that the PEC index, which was calculated using procalcitonin (PCT), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) as follows: PEC index = PCT × (ESR + CRP), efficiently diagnosed SBP in cirrhotic patients [17]. Actually, the PEC index had high sensitivity and specificity for SBP diagnosis (98.33% and 96.67%, respectively, AUC 0.977). The PEC index may have superior sensitivity and specificity compared to our presepsin-based method in the diagnosis of SBP even though the definition of abdominal infection and the disease background of the patient population differ from our study. On the other hand, the ascites WBC and P-SEP test in our study has three advantages in routine clinical practice in the laboratory: 1. only one tube containing EDTA-2K required, 2. no need for complex calculation, 3. measurement completed within one hour.

Whether there is a correlation between ascites P-SEP level and severity of disease is an interesting question. In this study, the patient with the highest ascites P-SEP level of 21,664 pg/mL in culture-positive ascites was not severe and was discharged from the hospital 36 days after ascites collection without being admitted to the intensive care unit. On the other hand, the patient with a P-SEP level of 4,312 pg/mL had severe peritonitis and died 45 days after ascites collection. In this study, the results did not suggest a correlation between P-SEP level and severity.

Regarding the urinary levels of P-SEP, the median urinary P-SEP level of healthy subjects was 48.5 pg/mL, which is considerably lower than that of the pleural fluid, ascites, and serum. We hypothesized that the lower urinary P-SEP level resulted from the urine production process, which was increasingly produced regardless of the pathological condition. As shown in Figures 4B and D, the negative correlation between eGFR and urinary P-SEP may indicate that decreased tubular reabsorption capacity rather than decreased glomerular filtration rate may contribute to the excretion of P-SEP into urine. To our knowledge, this study is the first to clarify the relationship between urinary P-SEP and renal function, although some reports have shown that elevated blood P-SEP levels were observed in patients with kidney dysfunction [18)19)]. Additionally, we found a positive correlation between urinary WBC count and P-SEP (**Figures 4C** and **E**). These results suggest that WBCs migrating to urine are considerably involved in the production of P-SEP. However, the origin of urinary P-SEP remains unclear because most WBCs migrating to urine are neutrophils, contradicting reports that monocytes/macrophages are strongly involved in the production of P-SEP [3].

Regarding the clinical usefulness of measuring urinary P-SEP, the urinary P-SEP levels in the pyuria group were significantly higher than those in the control group (**Figure 4A**). Moreover, urinary P-SEP levels were positively correlated with urinary WBC counts (**Figures 4C** and **E**). These results suggest that WBCs migrating to the urinary tract may produce P-SEP, and measuring urinary P-SEP might be useful to analyze urinary inflammation. However, because urinary P-SEP was elevated in patients with renal dysfunction, we searched for the eGFR in patients whose urinary P-SEP exceeded 500 ng/g･Cr. Of the numbers of urine samples with P-SEP above 500 ng/g･Cr, eGFR below 30 mL/min/1.73 m^2 were 0 of 1 in the control group, 2 of 5 in the pyuria group, 1 of 1 in the IgA nephropathy group, 4 of 4 in the diabetes mellitus group, 2 of 3 in the sclerosis group, and 2 of 2 in other groups. Except for the control and pyuria groups, elevated urinary P-SEP levels might not reflect infection but renal dysfunction. Therefore, further study is required because measurement of urinary P-SEP may be valuable in the diagnosis of urinary tract infections and evaluation of disease status.

This study had several limitations. First, because the study was a retrospective cross-sectional study, future prospective longitudinal studies are needed to confirm the results of the present study. Second, further analysis should consider clinical symptoms because we evaluated the diagnostic ability of ascites P-SEP level not for peritonitis but for culture-positive cases. Finally, it remains unclear why the urinary P-SEP level was increased in cases without pyuria.

In conclusion, we have validated a method of measuring P-SEP in pleural fluid, ascites, and urine instead of the blood, which is broadly used to measure P-SEP. Here, we demonstrated that measuring ascites and urinary P-SEP may be useful to evaluate local bacterial infection. However, further studies are needed to confirm the usefulness of P-SEP as a biomarker of local bacterial infection.

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Authorship contributions

R. Y. participated in the experiments and data analysis and drafted the initial manuscript; Y. M. participated in the experiments, participated in the discussion, and helped in drafting the manuscript; H. S. participated in the experiments; N. Y., M. T., Y. O., and H.I. participated in the discussion and helped in drafting the manuscript; M. K. conceived the study, coordinated the study design, and helped in drafting the manuscript. All the authors have read and approved the final manuscript.

Disclosure of conflicts of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rin Yokoyama reports financial support and equipment were provided by PHC Corporation. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The present study was performed under a joint research project at the University of Tokyo Hospital with PHC Corporation.

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Abbreviations:

ALP, alkaline phosphatase; CGN, chronic glomerulonephritis; CLEIA, chemiluminescent enzyme immunoassay; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy; LDH, lactose dehydrogenase; LN, lupus nephritis; MCD, minimal change disease; MN, membranous nephropathy; MPGN, membranoproliferative glomerulonephritis; P-SEP, presepsin; SAAG, serum–ascites albumin gradient; SBP, spontaneous bacterial peritonitis; WBC, white blood cell

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Original

Comparison of Sysmex UF-5000 bacterial count results and urine culture for urinary tract infection screening

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ABSTRACT

Background: Sysmex UF-5000 urine particle analyzer can simultaneously measure urinary organic constituents and provide bacterial information (BACT-info). Reported here are findings of a retrospective review of BACT-info bacterial flag information provided by Sysmex UF-5000 and urine culture findings, with focus on bacterial count.

Patients and methods: A total of 1011 patients were enrolled, including 465 males and 546 females. Sysmex UF-5000 shows bacterial count as BACT-info, and at the same time estimates bacterial count and classifies the findings into Gram-positive, Gram-negative, Gram-positive/negative, and Unclassified.

Results: The positive predictive value (PPV) of a BACT-info bacterial count ranging from 100-99,999/μL and urine cultures was 40.1% for all Gram-positive samples, and after dividing UF-5000 positive samples into groups A (100-999/μL), B (1000-9999/μL), and C (10,000-99,999/μL) was 34.4%, 46.2%, and 48.4%, respectively ($P<0.05$). For Gram-negative samples, the PPV was 86.0% overall, and 52.1%, 87.8%, and 95.5% ($P<0.001$) for Groups A, B, and C, respectively. In both Gram-positive and -negative samples, the PPV increased significantly with higher bacterial count.

Conclusions: A higher BACT-info bacterial count leads to a more accurate determination of bacteria, suggesting that BACT-info could lead to appropriate selection of antimicrobial agents in the treatment of urinary tract infections.

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Key Words
BACT-info, UF-5000, bacterial count, urinary tract infection, urine culture

I. Introduction

A urinary tract infection is one of the most frequent types of infections encountered and mainly caused by enteric bacteria. The effects range from mild cystitis to severe pyelonephritis, with prompt diagnosis and treatment required, as both community and nosocomial infections are possible. The guidelines in Japan for treatment of infectious diseases state that it is important to first determine the morphology of the causative organism using urinary sediment and flow cytometry examinations before starting treatment for a urinary tract infection [1], though antibacterial agents are often prescribed in clinical practice as empirical treatment for suspected cases.

While Gram staining of a urine sample for bacteriological testing is rapid, a disadvantage is differences among technologists, making it difficult to obtain immediate and accurate results for all specimens in the course of busy daily work. In addition, several days are required for urine culture results to be determined, thus a test able to

quickly reveal causative organisms is required for appropriate timely antimicrobial therapy.

Sysmex UF-5000 (Sysmex Corporation, Kobe, Japan) urine particle analyzer can simultaneously measure urinary plasmablasts and provide bacterial information (BACT-info) based on the principle of flow cytometry, as the dot distribution pattern of the scattergram displayed during quantitative measurement of bacteria can be analyzed by the device to estimate Gram stainability [2]. The present study was conducted to retrospectively examine BACT-info flag results provided by UF-5000 and urine culture findings, with focus on bacterial count. To the best of our knowledge, this is the first reported comparison of urine culture and bacterial count obtained with UF-5000 for urinary tract infection cases.

II. Materials and methods

2.1. Urine samples

A total of 1,011 patients treated at our hospital for a suspected urinary tract infection between September 2022 and June 2024 with a bacterial count of 100-99,999/μL, whose results included BACT-info obtained with UF-5000 and also urine culture findings, were enrolled in this study (**Table 1**). A bacterial count of 100/μL is equivalent to 1×10^5/mL of bacteria in a urine culture. A diagnosis of urinary tract infection cannot be made when the bacterial count is less than 100/μL, while in cases with a count of 100,000/μL or more, urine turbidity is too great for accurate determination by UF-5000.

Table 1 Characteristics of the study population

n	1011	
Age, mean (range)	76.4 (0-101)	
Sex, n (%)	Male	465 (46.0)
	Female	546 (54.0)
Bacterial count (/μL), n (%)	A : 100-999	345 (34.1)
	B : 1000-9999	296 (29.3)
	C : 10000-99999	370 (36.6)

2.2. UF-5000 analysis

Urine specimens are usually measured with UF-5000 within one hour, or four hours at the latest, to ensure accuracy. In addition to bacterial count, a dot distribution pattern of the scattergram is analyzed to determine Gram-positive, Gram-negative, Gram-positive/negative, and Unclassified [3].

2.3. Microbiological analysis

Urine cultures were performed with blood agar and bromothymol blue lactose agar for up to two days. Identification of developing bacteria was performed using a Pos Combo Panel.

2.4. Statistical analysis

Statistical analyses were performed using StatMate, ver. 4.01 (ATMS Co., Ltd., Tokyo, Japan). Comparisons between groups and associations among categorical variables were analyzed using a chi-square test, with a P value <0.05 considered to indicate significance.

2.5. Ethical approval

This study received approval from the ethics committee of Kawanishi City Medical Center (approval number: 24009) and was conducted in compliance with their ethical guidelines for human medical research.

III. RESULTS

Results of 1,011 patients, 465 (46.0%) males and 546 (54.0%) females with an average age of 76.4 (range 0-101) years, were examined. Of the 1,011 urine cultures of cases, cultures were detected in the urine in 797 cases and not in 214 cases. In 797 urine cultures, 921 bacteria species were detected, some of which contained more than one species,346 were Gram-positive and 575 were Gram-negative. The Gram-positive cases showed Enterococcus spp. in 128 (13.9%), Streptococcus spp. in 103 (11.2%), Staphylococcus spp. in 84 (9.1%), Corynebacterium spp. in 27 (2.9%), and others in 4 (0.4%),

Table 2 Microorganisms identified in the urine samples yielding significant bacterial growth on agar plates

Gram-positive (n=346)	n (%)
Enterococcus spp.	128 (13.9)
Streptococcus spp.	103 (11.2)
Staphylococcus spp.	84 (9.1)
Corynebacterium spp.	27 (2.9)
others	4 (0.4)

Gram-negative (n=575)	n (%)
Escherichia coli	353 (38.3)
Klebsiella spp.	95 (10.3)
Pseudomonas aeruginosa	39 (4.2)
Citrobacter spp.	28 (3.0)
Proteus spp.	22 (2.4)
Enterobacter spp.	17 (1.8)
Serratia spp.	3 (0.3)
others	18 (2.0)

while Gram-negative cases showed Escherichia coli in 353 (38.3%), Klebsiella spp. in 95 (10.3%), Pseudomonas aeruginosa in 39 (4.2%), Citrobacter spp. in 28 (3.0%), Proteus spp. in 22 (2.4%), Enterobacter spp. in 17 (1.8%), Serratia spp. in 3 (0.3%), and others in 18 (2.2%) (**Table 2**).

As mentioned above,a bacterial count of 100/μL is equivalent to 1×10^5/mL of bacteria in a urine culture, the amount of bacteria is equivalent to a urinary tract infection. Therefore, the patients were classified into three groups based on bacterial count, as follows: group A, 100-999 (n=345); group B, 1000-9999 (n=296); and group C, 10000-99999 (n=370), with the positive predictive value (PPV) shown by BACT-info flag and urine culture findings compared. The PPV obtained with urine cultures for Gram-positive patients in groups A, B, and C was 34.4%, 46.2%, and 48.4%, respectively, ($P<0.05$), while that with urine cultures in Gram-negative groups A, B, and C was 52.1%, 87.8%, and 95.5%, respectively ($P<0.001$). In BACT-info flag findings, PPV for both Gram-positive and -negative samples showed a significant increase in association with higher bacterial count. On the other hand, the PPV for all Gram-positive and -negative urine cultures was 22.6% overall, while that for Gram-positive groups A, B, and C was 25.0%, 19.4%, and 23.3%, respectively, indicating a low PPV regardless of bacterial count. These results may have been due to the low number of cases, though the results for both Gram-positive and -negative patients were considered to be unreliable (**Table 3A-D**).

Table 3 A-D Discrimination of BACT-Info flags by UF-5000 compared to urine culture results by bacteral count

3A. bacteral count 100 – 99999/μL

100 – 99999/μL		Bacterial species				Total	PPV
		Pos	Neg	Pos/Neg	No growth		
UF-5000 BACT-info	Pos flag	190	91	52	132	465	40.10%
	Neg flag	6	239	12	21	278	86.00%
	Pos/Neg flag	23	102	42	19	186	22.60%
	Unclassified	18	18	4	42	82	
	Total	237	450	110	214	1011	
	CR	80.20%	53.10%	38.20%			

3B. bacteral count 100 – 999/μL (Group A)

100 – 999/μL		Bacterial species				Total	PPV
		Pos	Neg	Pos/Neg	No growth		
UF-5000 BACT-info	Pos flag	76	41	17	87	221	34.40%
	Neg flag	2	25	3	18	48	52.10%
	Pos/Neg flag	1	2	1	0	4	25.00%
	Unclassified	16	11	4	41	72	
	Total	95	79	25	146	345	
	CR	80.00%	31.60%	4.00%			

3C. bacteral count 1000 – 9999/μL (Group B)

1000 – 9999/μL		Bacterial species				Total	PPV
		Pos	Neg	Pos/Neg	No growth		
UF-5000 BACT-info	Pos flag	84	30	26	42	182	46.20%
	Neg flag	1	65	6	2	74	87.80%
	Pos/Neg flag	9	13	7	7	36	19.40%
	Unclassified	2	2	0	0	4	
	Total	96	110	39	51	296	
	CR	87.50%	59.10%	18.00%			

3D. bacteral count 10000 – 99999/μL (Group C)

10000 – 99999/μL		Bacterial species				Total	PPV
		Pos	Neg	Pos/Neg	No growth		
UF-5000 BACT-info	Pos flag	30	20	9	3	62	48.40%
	Neg flag	3	149	3	1	156	95.50%
	Pos/Neg flag	13	87	34	12	146	23.30%
	Unclassified	0	5	0	1	6	
	Total	46	261	46	17	370	
	CR	65.20%	57.10%	74.00%			

CR：Concordance rate
PPV：Positive Predictive Value

As noted above, samples shown as Gram-negative by BACT-info had a PPV of 93.0% with a BACT quantitative count of 1,000/μL or higher considered to be reliable, while Gram-positive samples had a PPV of 48.4% even with a bacterial count of 10,000/μL or higher, indicating a low level of reliability (**Figure**). Finally, when all 1,011 cases were divided into males (n=465) and females (n=546), BACT-info flag and urine culture PPV results showed that 82.5% and 88.8%, respectively, were Gram-negative, not a significant difference (P=0.133), whereas 51.4% and 31.6%, respectively, were Gram-positive, a significant difference between genders (P<0.001) (**Table 4A**, **B**).

IV. Discussion

UF-5000 is an instrument that uses flow cytometry as its principle method to quantitatively determine numbers of erythrocytes, leukocytes, epithelial cells, squamous cells, columns, and bacteria in non-centrifuged urine. The dot distribution of the BACT scattergram displayed when quantitatively determining bacteria is analyzed by an in-instrument algorithm to show Gram-positive and -negative bacteria based on differences in cell wall structure using forward scattered light and lateral fluorescence. With this instrument, it is possible to determine the presence of a mixture of Gram-positive and -negative bacteria in samples, as well as those with only Gram-positive or -negative strains. However, the low PPV limits application of UF-5000 for diagnosis urinary tract infection and determination of treatment [4]. Nevertheless, UF-5000 analyzer is considered to be a reliable diagnostic tool for UTI screening, as the displayed BACT-info flags allow for quick decision making regarding diagnosis by the clinician [5].

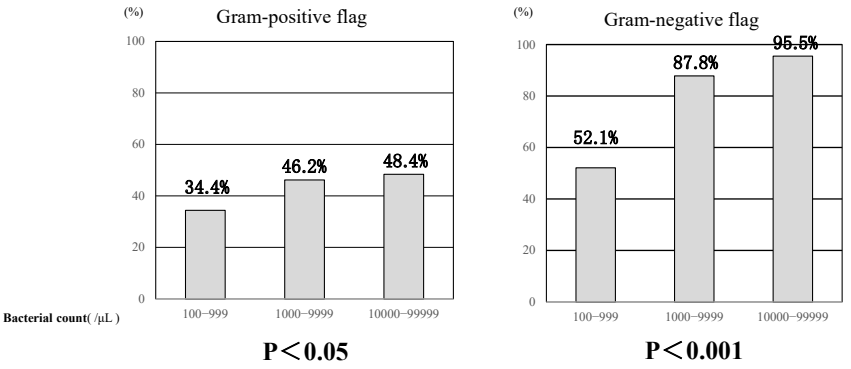

Figure 1 PPV of comparison of BACT-info flag by bacterial count

Table 4 A-B Discrimination of BACT-Info flags by UF-5000 compared to urine culture results by sex

4A. Male (n=465)

100 – 99999/μL		Bacterial species				Total	PPV
		Pos	Neg	Pos/Neg	No growth		
UF-5000 BACT-info	Pos flag	112	32	17	57	218	51.40%
	Neg flag	3	104	7	12	126	82.50%
	Pos/Neg flag	19	26	17	8	70	24.20%
	Unclassified	13	10	2	26	51	
	Total	147	172	43	103	465	
	CR	76.20%	60.50%	39.50%			

4B. Female (n=546)

100 – 99999/μL		Bacterial species				Total	PPV
		Pos	Neg	Pos/Neg	No growth		
UF-5000 BACT-info	Pos flag	78	59	35	75	247	31.60%
	Neg flag	3	135	5	9	152	88.80%
	Pos/Neg flag	4	76	25	11	116	21.60%
	Unclassified	5	8	2	16	31	
	Total	90	278	67	111	546	
	CR	86.70%	48.60%	37.30%			

CR：Concordance rate
PPV：Positive Predictive Value

In a previous study of UF- 5000 results obtained with 94 samples, PPV was 90% in cases determined to be Gram-negative and 54% in those determined as Gram-positive [2], while another study of 179 samples reported PPV of 93% for Gram-negative and 75.0% for Gram-positive cases [6]. The main causative organisms of UTI are Escherichia coli and other Gram-negative organisms, thus a PPV greater than 90% for the Gram-negative flag suggests that UF-5000 device may be helpful for selection of an appropriate antimicrobial agent. UF-5000 sensitivity and specificity were shown to be 80.0% and 88.2%, respectively, for Gram-negative, and 70.0% and 86.5%, respectively, for Gram-positive cases [7]. Additionally, another study reported that UF-5000 showed good sensitivity and specificity for Gram-negative samples as compared to its predecessor the UF-1000i, with improved detection sensitivity for Gram-positive samples as well [8].

It is important to note that the PPV for Gram-positive flagged and urine cultures was lower as compared to that for the Gram-negative cultures. A possible explanation is that Gram-positive strains are less likely to be stained than Gram-negative strains because they are more often found to be clumped, resulting in a grape-like, lenticular, or fenestrate-like morphology. Furthermore, UF-5000 also counts debris in the urine specimen and analysis of the scattergram in cases with debris shows a morphology similar to that of a Gram-positive sample, resulting in possible false-positive findings for Gram-negative specimens. One of the debris components in urine specimens is squamous cells, which in females are more likely to have a vulvar or vaginal origin even in the absence of urinary tract system abnormalities. When a urine specimen is microscopically examined in the laboratory, contamination is considered to be positive when five or more epithelial cells are present in one field of view, while contamination by vaginal secretion, epithelial cells, or indigenous skin bacteria is reported to be found in 25% or more cases [9]. According to the European Urinalysis guidelines, the presence of squamous cells from the vulva or urethra in urine obtained outside of the fertile period is a problem with specimen collection that must be considered [10]. As for UF-5000, the presence of squamous cell contamination in female urine samples was reported to be the reason for the low PPV of Gram-positive and Gram-positive/negative samples noted by the BACT-info flag [11)-14)]. Gilboe et al. examined urine specimens for contamination and noted that most had only small amounts of squamous cells, while a few contained large amounts, though they did not consider squamous cells to be a parameter useful for predicting contamination [15]. In the present study, we also examined PPV in female urine samples that contained 5 or more squamous cells per field of view, but this did not significantly affect the results, and the degree of contamination was difficult to determine based on squamous cells alone.

The present results indicate that the bacterial flag becomes more accurate as the bacterial count in BACT-info increases. In addition, PPV was lower in the Gram-positive than -negative samples, and Gram-positive samples from females had a significantly lower PPV as compared to those from males, suggesting that contamination by debris such as squamous cells may affect the BACT-info flag.

This study has some limitations, including its retrospective nature and analysis of results obtained from a single center aggregate. Nevertheless, it is considered that the findings obtained are valuable for demonstrating the efficacy of the UF-5000 particle analyzer.

In conclusion, it is suggested that a higher bacterial count in BACT-info findings provided by UF-5000 can help with more accurate determination of bacteria present in the patient. It should be kept in mind that the PPV for a Gram-positive flagged sample in BACT-info results can be as low as 48.4%, even when the bacterial count is 10,000/μL or greater, while the PPV is potentially lower in females due to contamination. With a Gram-negative flag, a bacterial count of 1,000/μL or greater is considered to be reliable with a PPV of 90% or more, suggesting that BACT-info could lead to appropriate selection of antimicrobial agents in the treatment of urinary tract infections.

Additional studies are needed to establish the accuracy of these results, including prospective and multicenter studies.

Authors' contributions

Seiji Nagasawa: Data curation, investigation, writing
Shimpei Yoshioka: Data curation
Yohei Kaizuka: Data curation
Kohei Kusakari: Data curation
Yoshikazu Togo: Data curation, review, editing
All of the authors have read and approved the final version of the manuscript.

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Conflicts of interest

There are no conflicts of interest to declare.

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Report

Antiphospholipid antibodies in patients with ovarian cancer: A prospective pilot study

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ABSTRACT

Background; Ovarian cancer is associated with a high incidence of thromboembolism, and it has been suggested that the mechanisms, particularly when apoptosis is induced by chemotherapy, share common underpinnings with antiphospholipid syndrome (APS) -associated thromboembolism. A clinical study was conducted to elucidate the expression of antiphospholipid antibody (aPL) in patients undergoing various treatment steps.

Methods; Fifteen patients with newly diagnosed ovarian cancer, primary peritoneal cancer, or fallopian tube cancer were prospectively and consecutively enrolled to measure lupus anticoagulants, anti-cardiolipin antibodies, and anti-PS/PT antibodies. The observational period for thrombotic events after blood sampling ranged from 5 to 13 months.

Results; Six patients received systemic chemotherapy as neoadjuvant or adjuvant therapy before blood sampling; four patients had thromboembolic diseases, including cerebral infarction; and four patients had clear cell carcinoma. None of the patients showed aPL, regardless of chemotherapy induction. Univariate analysis showed no major clinical characteristics (advanced age, history of thromboembolisms, clear cell carcinoma, large tumor diameter, high body mass index, and advanced FIGO stage) that correlated with thrombosis, while the histological subtype of clear cell carcinoma was associated with elevated plasma D-dimer levels above 8.45 mg/L (P=0.03).

Conclusions; Based on this pilot study with a limited number of patients, ovarian cancer and its treatment may have no direct association with the induction of APS or aPL, regardless of the clinicopathological background or induction of chemotherapy with cytotoxic agents. Further research in this area is warranted.

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Key Words
Ovarian Cancer, Antiphospholipid Syndrome, Antiphospholipid Antibody, Chemotherapy

I. Introduction

Cancer-associated thrombosis is the second leading cause of mortality among cancer patients following disease progression [1]. The risk of thromboembolism in individuals with cancer is intricately linked to tumor characteristics, including primary site, histological grade, or tumor node metastasis stage, as well as to cancer treatments, such as surgery, hospitalization, central venous catheter placement, systemic chemotherapy, radiothera-

py, anti-angiogenesis agents, immunomodulatory drugs, hormonal therapy, erythropoiesis-stimulating agents, and red blood cell or platelet transfusions [1]. The mechanisms underlying cancer-associated thrombosis are multifaceted, involving direct activation of platelets by cancer cells and procoagulant molecules such as tissue factor (TF) and P-selectin derived from tumor cell microvesicles [2]. These pathways illustrate the complex interplay between malignancy and hypercoagulability.

Antiphospholipid syndrome (APS) is a systemic autoimmune condition characterized by arterial, venous, or microvascular thrombosis, recurrent pregnancy loss, and non-thrombotic manifestations in the presence of persistent anti-phospholipid antibodies (aPLs) [3]. The pathogenic role of aPLs is well documented. These autoantibodies induce a prothrombotic state primarily through the activation of vascular endothelial cells and monocytes, resulting in increased TF production [4]. This mechanism, known as the procoagulant cell activation theory, underscores the pivotal role of blood-borne TF in APS-associated hypercoagulability. The representative aPL anti-cardiolipin antibodies are auto-antibodies that target plasma proteins, primarily β2-glycoprotein I (GPI), which binds to anionic phospholipids [5][6]. Phosphatidylserine-dependent anti-prothrombin antibodies (aPS/PTs) exhibit structural characteristics similar to those of antiphospholipid antibodies by binding to prothrombin, which undergoes conformational changes upon interaction with negatively charged phospholipids. These antibodies also possess prothrombotic properties, and are increasingly recognized as novel or alternative antiphospholipid antibodies, potentially substituting lupus anticoagulants (LACs) in certain diagnostic contexts [4][7]. Emerging evidence suggests a potential association between APS and thrombosis in cancer patients, possibly mediated by the overexpression of phosphatidylserine on cell surfaces, including in ovarian cancer [8]-[11]. Although the precise mechanisms remain elusive and may vary by cancer type, this link warrants further exploration.

Ovarian cancer is the most lethal malignancy of the female reproductive system, claiming the lives of over 200,000 women each year [12]. The standard treatment is debulking surgery to achieve no gross residual tumor, followed by adjuvant platinum-based chemotherapy in both primary and recurrent disease [13]. In addition, several clinical trials demonstrated that the anti-angiogenesis agent bevacizumab improved the prognosis of patients with advanced [14] and recurrent ovarian cancer [15], and combination drug therapy with bevacizumab is currently used as a control arm to develop novel treatments in randomized clinical trials [16]. Notably, ovarian cancer has a disproportionately high incidence of thromboembolism relative to other malignancies [17][18] and has been associated with Trousseau syndrome as a cerebral infarction [19]. Chemotherapy has been implicated in the externalization of negatively charged phospholipids, such as phosphatidylserine [20], due to apoptosis in ovarian cancer [21], further increasing the thrombotic risk. The shared pathophysiological underpinnings of APS-associated thrombosis and cancer-associated thrombosis, particularly the formation of fibrin clots, suggest a potential overlap between these conditions [22]. This prompted our hypothesis that aPLs may be more frequently detected in patients with ovarian cancer. Recognizing the critical importance of anticipating thrombotic events and instituting appropriate prophylactic measures, we explored the utility of aPL testing. However, the clinical relevance of aPLs in ovarian cancer remains controversial, as the current data are limited to case reports and small-scale studies. To address this gap, we conducted a prospective study to elucidate the correlation between the expression of aPL and clinical thrombotic events, with a focus on their timing relative to the administration of chemotherapy. Through this investigation, we sought to advance the understanding of thrombotic risk in ovarian cancer and refine strategies for its management.

II. Materials and methods

Ovarian Cancer Patients

From 2016 to 2017, we consecutively invited eligible Japanese patients from Hokkaido University Hospital and its affiliated hospitals to participate in our clinical study. These patients were histologically diagnosed with borderline epithelial ovarian, ovarian, primary peritoneal, or fallopian tube cancer, as confirmed by certified pathologists, irrespective of their clinical characteristics. Body mass index (BMI), history of thromboembolisms before the initial diagnosis of each patient's gynecological tumors, chemotherapy before blood sampling, disease stage, tumor diameter, and disease status were confirmed at registration. All thromboembolic events associated with the diagnosis of each patient's gynecological tumors were confirmed 5 months after the last subjects were registered in November 2017. Thromboembolisms were assessed when they were suspected by ultrasonography or contrast-enhanced computed tomography at the physicians' choice, and we did not perform routine screenings. Certified radiologists checked all the results of imaging tests to diagnose thromboembolism. D-dimer levels were checked in all patients at the same time as blood sam-

pling for this study.

This study was conducted according to the 1964 Declaration of Helsinki. The Institutional Review Board of Hokkaido University approved all experiments on the human genome for this study (Registration ID: 014-0106). Plasma and serum samples were prospectively collected from the patients, and antiphospholipid antibodies were measured as follows. Samples were collected in tubes containing a one-tenth volume of 0.105M sodium citrate and centrifuged immediately at 4℃. Plasma samples were depleted of platelets by filtration and stored at −80℃.

Antiphospholipid antibody (aPL) tests

Clotting tests to determine the activated partial thromboplastin time (aPTT) and dilute Russell's viper venom time (dRVVT) were performed for LAC determination using a semiautomated hemostasis analyzer (STart 4; Diagnostica Stago) according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis 23. IgG and IgM anti-cardiolipin antibodies (aCL) were assayed using a standard enzyme-linked immunosorbent assay (ELISA) [24]. Anti-PS/PT antibodies were detected by an ELISA as previously described [25].

Statistical analysis

Using a univariate analysis, we analyzed any correlations between the clinical parameters and thrombosis or high D-dimer values and set P values of 0.05 as being statistically significant for Fisher's exact test. All statistical analyses were performed using the JMP® Pro software program (ver. 14.0.0; SAS Institute, Cary, NC, USA).

III. Results

Disease characteristics of the study patients

We prospectively enrolled 15 patients with newly diagnosed ovarian, primary peritoneal, or fallopian tube cancer. All approached patients agreed to participate, resulting in complete consecutive enrollment. The clinical characteristics of the patients are summarized in **Table 1**. The median age and BMI at registration were 63 years (range 41 – 82) and 23.4 kg/m^2 (range 16.1 – 34.3), respectively. Blood samples were collected either before or during treatment for the primary disease, except for one patient with recurrent disease. None of the patients had a history of thromboembolism unrelated to their current treatments for gynecological tumors, nor did they have untreated severe hypertension, diabetes, or hyperlipidemia. Two patients in the cohort were smokers; neither had experienced thromboembolism before blood sampling was conducted. None of our patients used pro-coagulant drugs at the time of blood sampling. We histologically diagnosed gynecological tumors using surgical specimens in 14 patients (93.3%) and a pleural effusion cell block in one patient (6.7%). The distribution of histological subtypes among the patients was as follows: serous carcinoma (33.3%), clear cell carcinoma (26.7%), mucinous carcinoma (20.0%), and endometrioid carcinoma (13.3%). Forty percent of patients (n=6) received systemic chemotherapy as neoadjuvant or adjuvant therapy before blood sampling. The median number of chemotherapy cycles was 3 (range, 1–7), and the regimen included tri-weekly paclitaxel and carboplatin (TC), dose-dense TC, tri-weekly TC with bevacizumab, gemcitabine as a single agent, and a combination of irinotecan and nedaplatin. **Figure 1** illustrates each patient's treatment course and the timing of the blood sampling. One patient (no.2 in **Figure 1**) received chemotherapy with bevacizumab before blood sampling. None of the patients had previously undergone radiotherapy. The follow-up period for thrombotic events after study registration ranged from 5 to 13 months. Three patients had preoperative deep venous thrombosis (DVT). In these three patients, blood samples were obtained from two patients before the diagnosis of DVT. In the other patient, we obtained a blood sample after the diagnosis of DVT, and this patient was not taking anti-coagulant drugs because the thrombi were organized. In addition, another patient with systemic thrombosis and cerebral infarction was diagnosed with Trousseau's syndrome and was taking warfarin during blood sampling for this study. The remaining 11 patients either had no thromboembolisms (n=8) or had temporal operation-related DVT (n=3).

Results of blood examinations and aPLs

None of the patients tested positive for syphilis at the time of the diagnosis of the gynecological tumor. The D-dimer level at study registration ranged from 0.5 to 24.96 mg/L, and 10 of 15 patients (66.7%) were positive when we set the cutoff value to the general threshold up to 1.0 mg/L. Regarding aPLs, only one patient was positive for the dRVVT screening test (**Table 2**). However, this patient was negative for all the other screening tests (no.9 in **Figure 1**). Therefore, none of the patients–including one with cerebral infarction of Trousseau's syndrome and six who were registered after chemotherapy–showed the presence of aPLs (**Table 2**). We subse-

Table 1 Disease characteristics and antiphospholipid antibody expression of the study patients (n=15)

Characteristic			
Age - year	Median (range)	63	41 - 82
Race	Asian - no. (%)	15	100.0%
Body mass index (kg/m^2)	Median (range)	23.4	16.1 - 34.3
	≤ 25 - no. (%)	12	80.0%
	> 25 - no. (%)	3	20.0%
History of thromboembolisms			
	Yes - no. (%)	2	13.3%
	No - no. (%)	13	86.7%
Smoker at diagnosis			
	Yes - no. (%)	2	13.3%
	No - no. (%)	13	86.7%
Surgery for diagnosis and/or debulking of present disease			
	Yes - no. (%)	14	93.3%
	No - no. (%)	1	6.7%
Complication of thromboembolism			
	Preoperative DVT - no. (%)	3	20.0%
	Multiple thrombosis with cerebral infarction - no. (%)	1	6.7%
	Postoperative DVT - no. (%)	3	20.0%
	None - no. (%)	8	53.3%
Use of anticoaglants at blood sampling			
	Yes - no. (%)	1	6.7%
	No - no. (%)	14	93.3%
Chemotherapy before blood sampling			
Yes - no. (%)		6	40.0%
	Number of cycles (median, range)	3	1-7
	Interval between last chemotherapy and blood sampling (week, median and rage)	5	0-33
No - no. (%)		9	60.0%
Histologic features			
	High-grade serous carcinoma - no. (%)	5	33.3%
	Clear cell carcinoma - no. (%)	4	26.7%
	Mucinous carcinoma - no. (%)	3	20.0%
	Endometrioid carcinoma - no. (%)	2	13.3%
	Unspecified - no. (%)	1	6.7%
FIGO stage			
	I / II - no. (%)	7	46.7%
	III / IV - no. (%)	8	53.3%
Maximal tumor diameter			
	> 15 cm - no. (%)	4	26.7%
	≤ 15 cm - no. (%)	11	73.3%
Disease status			
	primary - no. (%)	14	93.3%
	recurrent - no. (%)	1	6.7%
D-Dimer			
	< 8.5 mg/L - no. (%)	11	73.3%
	≥ 8.5 mg/L - no. (%)	4	26.7%
Syphilis test			
	positive - no. (%)	0	0.0%
	negative - no. (%)	15	100.0%
Antiphospholipid antibody			
	negative - no. (%)	15	100.0%
	positive - no. (%)	0	0.0%

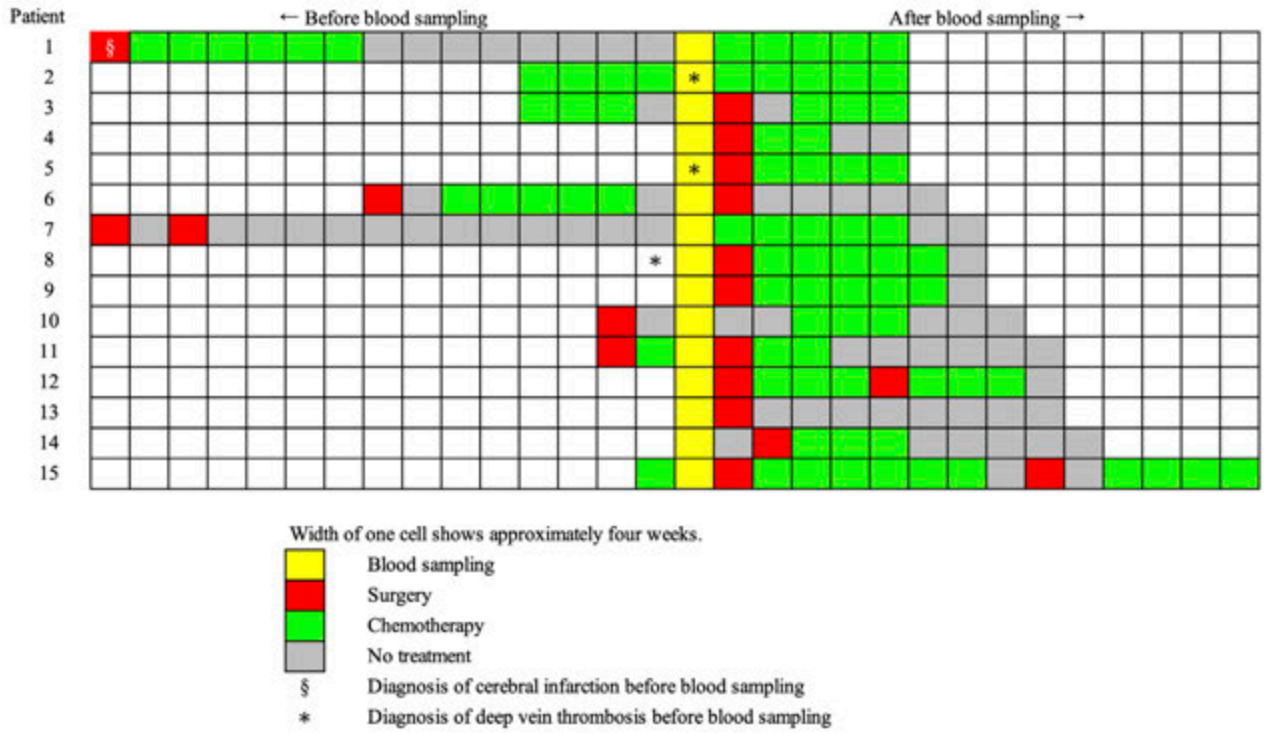

Figure 1 Clinical course and the timing of blood sampling

Table 2 Results of antiphospholipid antibodies (n=15)

	Antiphospholipid antibody (> normal range)					
Patient	PTT-LA	dRVVT >1.3	aCL-IgG >19.2	aCL-IgM >23.4	aPS/PT-IgG >1.2	aPS/PT-IgM >5.2
1	negative	1.16	low	low	low	low
2	negative	1.11	low	3.7	low	low
3	negative	1.02	6.1	low	low	low
4	negative	1.17	low	2.3	low	low
5	negative	1.11	low	low	low	low
6	negative	1.13	3.0	low	low	low
7	negative	1.21	low	low	low	low
8	negative	1.19	low	low	low	low
9	negative	1.43	low	low	low	low
10	negative	1.01	low	low	low	low
11	negative	1.07	11.2	low	low	low
12	negative	1.14	8.1	low	low	low
13	negative	1.15	8.3	10.5	low	low
14	negative	1.13	3.1	low	low	low
15	negative	1.06	low	low	low	low

quently asked 5 of the 15 (33.3%) patients to provide additional blood samples from 1 to 6 months after the first sampling, regardless of the timing of cancer treatment, and no patients had aPLs again (not shown in **Figure 1**). We also measured the expression of aPLs in four patients with mucinous borderline ovarian tumors using the same methods, and none of these patients had aPLs (data not shown).

A univariate analysis for risk factors of elevated D-dimer

Age (> 60 years), history of thromboembolisms, large tumor diameter (> 150 mm), high body mass index (> 25), histological subtypes of clear cell carcinoma, and ad-

vanced FIGO stage (III of IV) were not significantly correlated with thrombosis during the study period. These factors were also not significantly correlated with an elevated plasma D-dimer level at study registration when we set the cutoff value to the general threshold up to 1.0 mg/L. On the other hand, when we set the cutoff value of D-dimer to 8.45 mg/L, which was the mean value of acute phase patients with Trousseau's syndrome in a previous study 26, the histological subtype of clear cell carcinoma was significantly correlated with an elevated plasma D-dimer level ($P = 0.03$, **Table 3**). Although the limited sample size and heterogeneity of clinical backgrounds preclude definitive conclusions regarding the correlation between thrombotic events and prognosis, the 5-year overall survival rate was comparable at 57.1% among patients with and without thrombotic events, except for patient 4 in Figure 1, whose prognosis remains unknown.

IV. Discussion

To our knowledge, this study included the largest cohort of ovarian cancer patients to be investigated in this context. Clinically significant thrombotic events, excluding postoperative DVT, a common complication of abdominal surgery, were observed in 4 of the 15 patients. This finding aligns with existing reports indicating a high incidence of thrombotic events in ovarian cancer. The lack of detectable aPL in ovarian cancer patients in this study may be explained by several factors. First, ovarian cancer cells, whether treatment-naïve or following chemotherapy, might not prominently expose negatively charged phospholipids on their cell surfaces, which are essential for aPL production. Second, even if such phospholipids are expressed on cell surfaces, additional genetic predispositions associated with autoimmune diseases may be necessary to trigger the generation of aPL. Further basic and clinical investigations are required to confirm these hypotheses. Finally, the current finding–that ovarian cancer, despite its strong association with thrombosis, exhibits minimal aPL expression–raises the possibility that aPLs may have relatively limited involvement in cancer-associated thrombosis in other malignancies as well. However, this remains to be validated. This cohort included four patients with clear cell carcinoma, a rare histologic subtype in Western countries. This subtype is reported to carry the highest risk of serious thromboembolism among ovarian carcinomas [19], probably due to an excessive production of tissue factor [27]. Although our study was unable to demonstrate the risk factors for thromboembolism in patients with ovarian cancer due to the small number of subjects, the findings of our univariate analysis did suggest a correlation between clear cell carcinoma and elevated plasma D-dimer levels which strongly correlate with an increased risk of thrombosis, particularly venous thromboembolism, in ovarian cancer patients [28].

Previous studies have shown that treatment with chemotherapy is an independent risk factor for symptomatic and incidental venous thromboembolism in patients with endometrial, cervical, ovarian, tubal, or peritone-

Table 3 Univariate analysis of the factors associated with high D-dimer values

Univariate analysis of the factors associated with thrombosis

	n	Any thrombosis (%)	Odds ratio	95% confidence interval	P value
Age > 60	11	45.5 % (5/11)	0.83	0.08 - 8.24	0.77
History of thromboembolisms	2	50.0 % (1/2)	1.17	0.06 - 22.94	0.73
Large tumor diameter (> 150 mm)	4	25.0 % (1/4)	0.28	0.02 - 3.57	0.95
FIGO stage III / IV	8	62.5 % (5/8)	4.16	0.47 - 36.73	0.21
Body mass index > 25	3	0.0 % (0/3)	0.00	-	1.00
Clear cell carcinoma	4	50.0 % (2/4)	1.20	0.12 - 11.86	0.66

Univariate analysis of the factors associated with high D-dimer values

	n	D-dimer > 8.45 mg/L	Odds ratio	95% confidence interval	P value
Age > 60	11	27.3 % (3/11)	1.12	0.08 - 15.50	0.73
History of thromboembolisms	2	50.0 % (1/2)	3.33	0.16 - 70.90	0.47
Large tumor diameter (> 150 mm)	4	25.0 % (1/4)	0.89	0.06 - 12.3	0.76
FIGO stage III / IV	8	37.5 % (3/8)	3.60	0.28 - 46.36	0.34
Body mass index > 25	3	33.3 % (1/3)	1.50	0.10 - 23.06	0.64
Clear cell carcinoma	4	75.0 % (3/4)	30.00	1.41 - 638.2	0.03

al cancer [29)30)]. In addition, it has been suggested that chemotherapy, which is a mainstay in the treatment of ovarian cancer, may induce aPL via the externalization of negatively charged phospholipids due to apoptosis. Regarding elevated anionic phospholipid expression associated with tissue damage, aPLs are known to transiently appear during acute infections such as COVID-19 [31)]. Therefore, we hypothesized that aPLs could potentially be expressed at various stages in ovarian cancer, where prolonged tumor growth or treatment-related modifications may induce high anionic phospholipid expression levels. However, our study found no significant correlation between aPLs and thromboembolic events in general patients with ovarian cancer, and no aPL induction by chemotherapy with cytotoxic agents. Therefore, with the exception of infrequent situations suspicious for serious catastrophic anti-phospholipid syndrome–characterized by the rapid chronological development of fulminant thrombotic complications leading to multi-organ failure [32)] –clinicians do not need to routinely check aPLs during chemotherapy with cytotoxic agents in patients with ovarian cancer. However, we cannot discuss the influence of bevacizumab because of the small number of patients.

Although aPLs are generally reported to be present in 1–12% of healthy individuals 31, no positive cases were identified among the 15 patients in this cohort. The presence of aPLs has been proposed to be a potential risk factor for malignancy and mortality. A previous study demonstrated a 2.6-fold increase in the risk of cancer-related mortality in patients positive for anti-cardiolipin antibodies [33)]. Additionally, a prospective study of 1,000 APS patients with APS identified malignancies as one of the most frequent causes of death in this population [34)]. A recent large-scale study on obstetric APS (n=517) reported a significant association between aPL positivity and an increased incidence of malignancy after long-term follow-up (hazard ratio, 2.22), although no ovarian cancer cases were observed [35)]. However, the frequency of aPL induction in ovarian cancer remains poorly understood. Few case reports have documented patients with ovarian cancer who incidentally test positive for aPLs [36)-38)]. Fewer reports suggest that APS, characterized by the rapid onset of severe thromboembolisms, may arise as a paraneoplastic syndrome in ovarian cancer [36)39)]. For other malignancies, prior research reported an aPL positivity rate of 5.7% among patients with various solid tumors, with higher rates observed in VTE-positive colon cancer (11.3%), breast cancer (7.9%), head and neck cancer (7.7%), and lung cancer (4.7%) [40)]. However, it found no significant difference in thrombosis-free survival between aPL-positive and aPL-negative patients [41)]. Data specific to ovarian cancer were notably absent from these reports, suggesting that aPL positivity may remain infrequent in patients with ovarian cancer.

The tumor microenvironment of ovarian cancer is suggested to be procoagulant, where tumor cell-induced activated platelets interact with endothelial cells, pericytes, mesenchymal stem cells, cancer-associated fibroblasts, adipocytes, immune cells, and extracellular matrix elements through direct interactions or by releasing various modulatory factors and platelet microparticles [42)]. In this study, we did not evaluate intratumoral thrombosis induced by procoagulant factors other than aPLs and its association with systemic thrombosis, including pre-operative DVT or cerebral infarction, and further study is needed. Our study showed that aPLs are not generally associated with systemic venous and arterial thrombosis. Antiphospholipid antibody detection encompasses various types (including lupus anticoagulant), which require multiple tests for confirmation. Another key marker, aPS/PT, is a frequently observed and characteristic autoantibody of APS; however, its diagnostic utility has only recently been established. This study was associated with the common limitations of antiphospholipid antibody testing. However, based on this pilot study with a limited number of patients and previous studies, ovarian cancer and its treatment may have no direct association with the induction of APS or aPL. Further research in this area is warranted.

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Authorship Contributions

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Fujieda (Methodology; Supervision; Validation; Writing review & editing), Hiroshi Asano (Data curation; Formal analysis; Investigation; Supervision; Validation; Writing - review & editing), Chisa Shimada (Data curation; Investigation; Writing - review & editing), Ayako Nozaki (Investigation; Validation; Writing - review & editing), Daisuke Endo (Investigation; Writing - review & editing), Hidemichi Watari (Supervision; Writing - review & editing).

Disclosure of Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Characterizing low platelet count samples with discrepant results using a reference method and a fully automated comprehensive hematology analyzer, Alinity hq

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Key Words
Hematology analyzer, Platelet count

Text

Dear Editor,

Automated cell counters currently used in many laboratories can rapidly deliver accurate blood count parameters based on multiple principles of cell counting. Among these blood cell parameters, platelet count is an important decision-making factor for platelet transfusion therapy; thus, an accurate platelet count (especially at a low count) is imperative for appropriate and safe transfusion therapy.

The fully-automated comprehensive hematology analyzer Alinity hq (Abbott, Abbott Park, IL; hq) is based on the flow cytometry-based technique. It measures complete blood count and white blood cell differential by analyzing optical signals obtained by the Multi Angle Polarized Scatter Separation (MAPSS™) technology [1)2)]. Additionally, it can enumerate platelets without switching measurement channels and does not require a dedicated staining reagent. We previously reported that measurements of low platelet counts ($<100 \times 10^9$/L) by hq were consistent with those obtained by a flow cytometry-based reference method and the PLT-F and PLT-O methods applied in the XN-series (Sysmex Corporation) [3)]. However, results of a small number of peripheral blood samples with a platelet count $<100 \times 10^9$/L deviated from those of the reference method. Therefore, we further analyzed these samples. This study was approved by the Keio University School of Medicine Ethics Committee (approval number: 20170393).

A total of 68 EDTA-2K-treated peripheral blood samples was obtained from outpatients. Of these, 63 samples were analyzed in a previous study [3)], and the remaining 5, once excluded due to suboptimal quality, were also included in the present analysis. The reference method was developed by the Blood Cell Counting Standardization Subcommittee of the Japanese Society for Laboratory Hematology (JSLH), wherein the absolute platelet count was calculated using a flow cytometry-based method compliant with ICSH/ISLH2001 (indirect method) [4)]. The samples were initially analyzed using the JSLH method, followed by the hq method and blood smear preparation. The correlation coefficient between the JSLH and hq methods indicated a strong correlation, as observed previously [3)]. However, samples from three cases showed a deviation of more than 2 SD in the Bland-Altman analysis

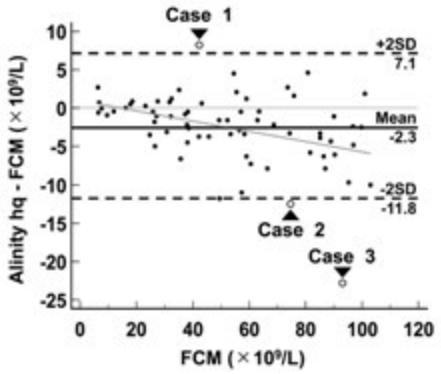

Figure 1 Bland-Altman analysis of 68 peripheral blood samples comparing hq measurements with the reference values obtained by the JSLH method. Three samples indicated by circles with arrowheads exhibited deviations exceeding ± 2 standard deviations.

Table 1 Platelet counts in the three samples whose results were inconsistent when analyzed by the JSLH and hq methods.

Method	JSLH* (× 10^9/L)	Alinity hq (× 10^9/L)
Case 1	42.3	50.6
Case 2	74.6	62.1
Case 3	93.1	70.4

* Reference method developed by the Blood Cell Counting Standardization Subcommittee of the Japanese Society for Laboratory Hematology (JSLH).

between the JSLH and hq methods (**Figure 1**). These samples were further investigated using blood smears and (IAS × ALL [WBC]) scattergrams were generated using hq (**Table 1**).

In Case 1, the platelet count was 42.3 × 10^9/L and 50.6 × 10^9/L by the JSLH method and hq, respectively, with the morphological flag "rst RBC", indicating that RBCs were resistant to hemolytic process in hq, which resulted in a higher value in hq measurement. An abnormal cluster was observed in the area of lower-than-normal fluorescence intensity on the scattergram, and the blood smear showed poikilocytes and Howell-Jolly bodies. In Case 2, the platelet count was 74.6 × 10^9/L and 62.1 × 10^9/L by the JSLH method and hq, respectively, with the morphological flag "Plt Clump?" indicating platelet clumps, which resulted in a lower value in hq measurement. An abnormal cluster was also found on the hq scattergram, although it was smaller than that observed in case 1, and the blood smear showed no platelet aggregation; instead, a small number of giant platelets were recognized. In Case 3, the platelet count was 93.1 × 10^9/L and 70.4 × 10^9/L by the JSLH method and hq, respectively, with the morphological flag "Plt Clump?", again resulting in a lower value in hq measurement. The hq scattergram showed a tiny abnormal cluster; however, no apparent platelet aggregation was observed in the blood smear (**Figure 2**).

The hq results for these three cases showed minor deviations compared with those obtained using the JSLH method (9-23 × 10^9/L), These deviations were asso-

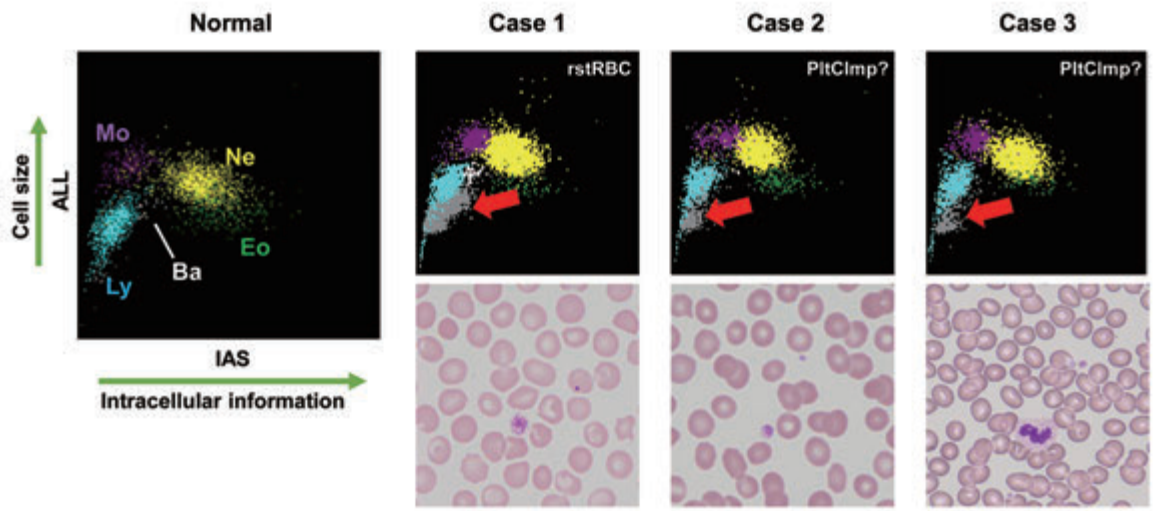

Figure 2 The hq scattergrams and blood smears for three samples with inconsistent results when analyzed using the JSLH and hq methods. Red arrows in the scattergrams indicate abnormal clusters (grey dots). Flags presented in the hq analysis are shown at the top right corner of each scattergram. ALL, axial light loss; Ba, basophils; Eo, eosinophils; IAS, interme angle scatter; Ly, lymphocytes; Mo, monocytes; Ne, neutrophils; PltClmP, platelet clumps; rstRBC, resistant red blood cells.

ciated with morphological flags such as "Plt Clump?" and "rst RBC", as well as abnormal clusters on the hq scattergram. These clusters may indicate the presence of platelet aggregation or hemolysis-resistant red blood cells, which may be underrepresented by microscopic observation. Therefore, special attention should be paid to these abnormal clusters and associated morphological flags, as they may affect the accuracy of platelet counts determined by hq.

In conclusion, although hq delivers highly reliable platelet counts even for samples with very low platelet count, samples with morphological flags in real-world applications should be carefully investigated by observing the scattergrams and blood smears and comprehensively determining whether they are consistent with measured results.

Author contributions

Conceptualization: Arai T and Yokota H
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Visualization: Arai T, Nagai Y and Yokota H
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Writing – review & editing: Nakayama Y, Mitsuhashi T and Matsushita H

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None

Conflicts of interest:

Yoichi Nakayama is an employee of Abbott Japan LLC, whereas Takayuki Mitsuhashi is a medical advisor of Abbott Japan LLC. There are no other conflicts of interest to declare.

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