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Exploratory Study of Serum Lactoferrin and Anti-Lactoferrin Antibody Concentrations in Patients with Endometriosis

Kaya Mori-Yamanaka,[1] Fuminori Kimura,[1,2] Akiko Nakamura,[1] Tetsuro Hanada,[1] Jun Kitazawa,[1] Aina Morimune,[1] Shunichiro Tsuji[1] and Takashi Murakami[1]

[1]Department of Obstetrics and Gynecology, Shiga University of Medical Science, Otsu, Shiga, Japan
[2]Department of Obstetrics and Gynecology, Nara Medical University, Kashihara, Nara, Japan

Endometriosis is a disease that is characterized by the ectopic presence of the endometrium or its similar cells. A high prevalence of patients with autoimmune diseases has been reported among patients with endometriosis although the cause of endometriosis remained unknown. Recently, the anti-lactoferrin antibody is reported to be highly detected in autoimmune diseases. This study focused on lactoferrin and anti-lactoferrin antibodies to explore the pathology of endometriosis. Lactoferrin is a substance that regulates inflammation and is produced by neutrophils. Anti-lactoferrin antibody is a type of perinuclear antineutrophil cytoplasmic antibody. The serum lactoferrin and anti-lactoferrin antibody levels were compared among patients with or without endometriosis, revealing significantly higher levels in patients with endometriosis. Additionally, a decreased serum anti-lactoferrin antibody level was observed after surgical endometriosis resection. The receiver operating characteristic curve analysis determined the reference values for the serum lactoferrin and anti-lactoferrin antibody levels. Patients whose serum level exceeded the reference anti-lactoferrin antibody value were significantly higher in more than 40% of cases in the endometriosis group. The rate is comparable to that of autoimmune diseases. This is the first report that anti-lactoferrin antibody is frequently observed in patients with endometriosis, adding a new perspective to the understanding of the pathology of endometriosis although precisely elucidating the mechanism by which lactoferrin and anti-lactoferrin antibody appear in endometriosis in the future is necessary.

Keywords: anti-lactoferrin antibody; autoimmune disease; endometriosis; lactoferrin; neutrophil
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Introduction

Lactoferrin (LTF) is a 78-kDa single-chain glycoprotein that is present in human mucosal secretions and milk (Peen et al. 1996; Pacora et al. 2000) and is produced and stored in neutrophil granules and released during degranulation (Afeltra et al. 1997; Shida et al. 2016). LTF is known to possess anti-inflammatory properties and regulate the spread of inflammation in tissues (Conneely 2001; Polak et al. 2007). It not only inhibits inflammatory substance synthesis, oxygen radical formation, and complement activation (Kuizenga et al. 1987; Polak et al. 2007; Lepanto et al. 2019), but also endogenously inhibits neutrophil extracellular trap (NET) formation (Shida et al. 2016).

NET is an innate immune function of neutrophils that actively release their DNA, a new defense mechanism that physically captures pathogens in a mesh-like structure containing DNA, histones, and intracellular antimicrobial proteins, such as neutrophil elastase, and kills microbes with the antimicrobial proteins (Brinkmann et al. 2004). Recently, NET formation and persistence are reported to be related to autoimmune disease development (Hakkim et al. 2010; Frese and Diamond 2011; Warnatsch et al. 2015).

LTF is an antigen of anti-lactoferrin antibody (aLF), which is a type of perinuclear antineutrophil cytoplasmic antibody (pANCA), in patients with autoimmune connec-

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Correspondence: Akiko Nakamura, M.D., Department of Obstetrics and Gynecology, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan.
e-mail: akikon@belle.shiga-med.ac.jp
Fuminori Kimura, M.D., Ph.D., Department of Obstetrics and Gynecology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan.
e-mail: kimurafu@naramed-u.ac.jp
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tive tissue diseases, such as arthritis and systemic lupus erythematosus (Locht et al. 1999; Manolova 2003). Recently, aLF has been reported to promote NET formation and is associated with disease activity in eosinophilic granulomatosis with polyangiitis, which is an autoimmune disease (Shida et al. 2016).

Thus, LTF may regulate inflammation via NET formation inhibition, but its antibody, aLF, may exacerbate the disease activity by promoting NET formation in autoimmune diseases. In this way, LTF and aLF may have opposing functions concerning the development of autoimmune diseases, which are characterized by chronic inflammation.

Endometriosis is a chronic inflammatory disease that is characterized by the ectopic presence of the endometrium or similar cells. Immunocompetent cells, such as neutrophils and macrophages, are present in endometriosis tissue, which is closely associated with the development of endometriosis (Hogg et al. 2020; Symons et al. 2020; Xu et al. 2020). Endometriosis has not yet been proven as an autoimmune disease, but it is associated with an altered immune system and linked to several autoimmune diseases (Nothnick 2001; Ferrari-Souza et al. 2022). Endometriosis is similar to autoimmune diseases because immune cells are present and chronic inflammation is induced in a specific tissue (Martinez et al. 2007), which is known to activate neutrophils (Borish et al. 1989). Moreover, NET formation is increased in ascites and peripheral blood of patients with endometriosis (Berkes et al. 2014; Munros et al. 2019).

We speculated that LTF secretion and aLF induction may differ in patients with endometriosis. Therefore, the present study performed a preliminary case-control study to compare the circulating serum LTF and aLF levels in patients with endometriosis.

Materials and Methods

Ethics

This study conformed to the Clinical Research Guidelines of the Shiga University of Medical Science and was approved by the research ethics committee. Written informed consent was obtained from all patients to participate in this study (registration number R2020-013).

Patients and sample collection

This study enrolled patients who were admitted to the Department of Obstetrics and Gynecology at Shiga University of Medical Science Hospital for surgery from November 2020 to May 2022. All patients were Japanese females who still exhibited a menstrual cycle. All included patients were diagnosed as patients with (the endometriosis group) or without endometriosis (the non-endometriosis group) based on the surgical and postsurgical histopathology findings. All patients with endometriosis were classified as stages I-IV according to the revised American Society for Reproductive Medicine classification (Canis et al. 1997).

Patients' age, gravidity, parity, smoking status, and surgical indications were documented. Blood samples from patients who underwent surgery at our hospital were used and are surplus in blood tests as preoperative tests. Blood samples were collected after confirming the consent from the patients. The samples for the analysis of serum levels of LTF and aLF were stored in a deep freezer (−80°C) until use. Similarly, surplus serum samples collected 1 month postoperatively were stored for patients who continued to visit our hospital.

Blood sample analysis

The serum levels of LTF and aLF were measured by ELISA kits (Human Lactoferrin SimpleStep ELISA Kit, Product No. ab200015, Abcam, Cambridge, UK; AntiLactoferrin ELISA Kit, Product No. RUO527, ORGENTEC Diagnostika GmbH, Mainz, Germany) following the manufacturer's instructions. Then, the plates were read at a wavelength of 450 nm (reference: 600 nm) using the Microplate reader (TECAN Infinte 200 PRO M Plex; FUJIFILM Wako Pure Chemical Corp., Tokyo, Japan), following the manufacturer's instructions.

Statistical analysis

All statistical analysis, except for power analysis, was performed using Graph Pad Prism version 6.07 (GraphPad Software Inc., La Jolla, CA, USA). Each dataset was analyzed for a normal distribution using the Kolmogorov–Smirnov test. Student's t-test or the nonparametric Mann–Whitney U test was used depending on the distribution pattern. p values of < 0.05 were considered significantly different.

The serum LTF and aLF levels were compared between the endometriosis and non-endometriosis groups, and their differences were compared among the non-endometriosis, early-stages endometriosis (I + II), and advanced-stages endometriosis (III + IV) groups using the analysis of variance or Kruskal–Wallis test.

Moreover, the difference in the serum aLF level between pre- and postoperative periods for endometriosis was analyzed using Wilcoxon's signed rank test. And the difference in the serum LTF level between pre- and postoperative periods for endometriosis was analyzed using paired t-test.

The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to assess the diagnostic values of serum LTF and aLF levels in discriminating between patients with and without endometriosis. Then, the AUC of the ROC curve and the cut-off value were determined by Youden's index. The statistical difference in the rates of patients exceeding the cut-off in the serum LTF or aLF levels was examined by Fisher's exact test between the endometriosis and non-endometriosis groups.

Results

This study included 68 patients. Patients without visible endometriotic foci who underwent treatment of benign

Table 1. Summary of patient characteristics.

	Non-endometriosis	Endometriosis	
	N = 17	N = 51	*p* value
Age, year, median (IQR)	37.00 (35.00-41.00)	36.00 (32.00-42.00)	0.9047
Gravidity, median (IQR)	0.00 (0.00-2.50)	0.00 (0.00-1.00)	0.2036
Parity, median (IQR)	0.00 (0.00-2.00)	0.00 (0.00-1.00)	0.0813
Smokers	0	0	Not applicable
Surgical indications			
Endometriosis	0	27	
Uterine myoma	8	5	
Cesarean scar syndrome	2	10	
Benigh ovarian tumor	4	5	
Uterine adenomyosis	0	3	
CIN3 (Hysterectomy)	1	1	
Asherman syndrome	1	0	
Hydrosalpinx	1	0	
Stage of endometriosis	Not applicable	I 15	
		II 4	
		III 18	
		IV 14	

CIN, cervical intraepithelial neoplasia; IQR, interquartile range.

ovarian tumors and uterine myomas, cesarean scar syndrome, Asherman syndrome, and hydrosalpinx were assigned to the non-endometriosis group. The endometriosis and the non-endometriosis groups consisted of 51 and 17 patients, respectively. Patient characteristics are shown in Table 1. No significant differences were observed between both groups in age and gravidity, although parity was higher in the non-endometriosis group than in the endometriosis group. None from either group were current smokers, but two patients in the endometriosis group had a smoking history. The r-ASRM classification revealed that 15, 4, 18, and 14 patients have stages I, II, III, and IV endometriosis, respectively.

The serum LTF and aLF levels in the endometriosis and non-endometriosis groups were shown in Fig. 1. The serum LTF and aLF levels were significantly higher in the endometriosis group than in the non-endometriosis group ($p = 0.016$ and $p = 0.028$, respectively) (Fig. 1A, B).

No significant difference was found in the serum LTF levels among the early-stage endometriosis (I + II), advanced-stage endometriosis (III + IV), and the non-endometriosis groups. No significant difference was found in the serum LTF levels between the early-stage endometriosis (I + II) and non-endometriosis groups ($p = 0.054$) (Fig. 2A). A significant difference was found in the serum LTF levels between the advanced-stage endometriosis (III + IV) and non-endometriosis groups ($p = 0.024$).

No significant difference was found in the serum aLF level among the early-stage endometriosis (I + II), advanced-stage endometriosis (III + IV), and non-endometriosis groups, as well as between the early-stage endometriosis (I + II) and non-endometriosis groups ($p = 0.208$) (Fig. 2B). The serum aLF level was higher in the advanced-stage endometriosis (III + IV) than in non-endometriosis groups ($p = 0.016$).

Pre- and postoperative blood sampling was conducted on 21 patients. The differences in the serum LTF and aLF levels preoperatively and postoperatively were shown in Fig. 3A, B, respectively. The serum aLF level was significantly lower postoperatively than preoperatively [preoperatively: 36.01 ± 23.65 (mean ± SD) vs. postoperatively: 25.40 ± 20.92; $p < 0.001$], although the LTF levels were not different between them (preoperatively: 0.796 ± 0.279 vs. postoperatively: 0.709 ± 0.254; $p = 0.102$).

The accuracy of endometriosis detection by the serum LTF level was examined using ROC curves. The AUC was 0.694 and the cut-off value with the highest Youden's index was 0.822 (Fig. 4A). The sensitivity and specificity were 0.51 and 0.82, respectively. Positive patients were significantly higher in the endometriosis than the non-endometriosis group ($p = 0.023$) when the patient was defined as positive if the serum LTF level exceeded the cut-off, as shown in Table 2A.

The accuracy of endometriosis detection by the serum aLF level was examined using ROC curves. The AUC was 0.678 and the cut-off value with the highest Youden's index was 24.4 (Fig. 4B). The sensitivity and specificity were 0.43 and 0.94, respectively. Positive patients were significantly higher in the endometriosis than the non-endometriosis group ($p = 0.006$) when the patient was defined as posi-

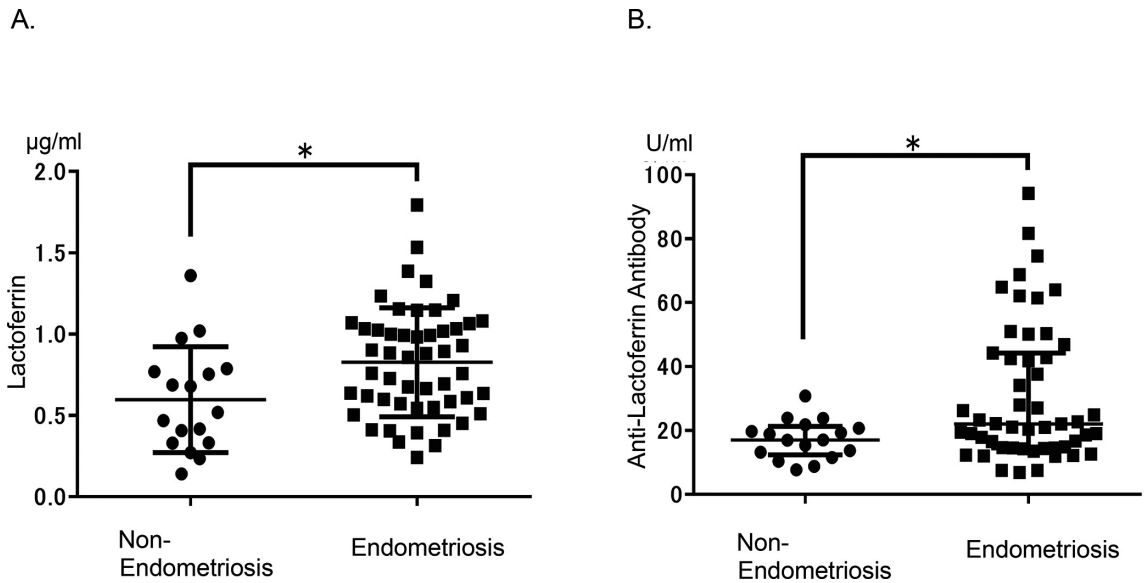

Fig. 1. Serum lactoferrin and anti-lactoferrin antibody levels in patients with or without endometriosis.
A. Serum lactoferrin levels in the endometriosis and non-endometriosis groups. Horizontal bars represent means with standard deviation (SD). Differences between groups were analyzed by the unpaired t-test. *$p < 0.05$. B. The serum levels of anti-lactoferrin antibody in the endometriosis and non-endometriosis groups. Horizontal bars represent medians with interquartile range (IQR). Differences between groups were analyzed by Mann–Whitney U tests. *$p < 0.05$.

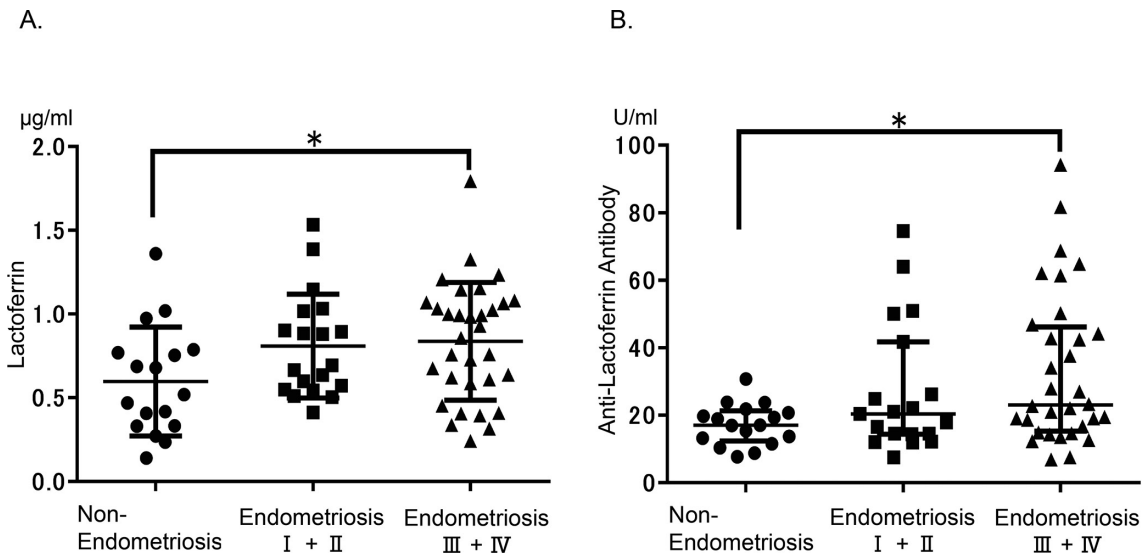

Fig. 2. Serum lactoferrin and anti-lactoferrin antibody levels in patients with early-stage endometriosis, advanced-stage endometriosis, or non-endometriosis.
A. Serum lactoferrin levels in patients with early-stage endometriosis (endometriosis I + II), advanced-stage endometriosis (endometriosis III + IV), or non-endometriosis. Horizontal bars represent means with SD. Comparisons among the three groups were performed using one-way analysis of variance. Differences between the two groups were analyzed by the unpaired t-test. *$p < 0.05$. B. Serum anti-lactoferrin antibody levels in patients with early-stage endometriosis (endometriosis I + II), advanced-stage endometriosis (endometriosis III + IV), or non-endometriosis. Horizontal bars represent medians with IQR. Comparisons among the three groups were performed using the Kruskal–Wallis test. Differences between the two groups were analyzed by Mann–Whitney U tests. *$p < 0.05$.

tive if the serum aLF level exceeded the cut-off, as shown in Table 2B.

Discussion

The present study measured the serum LTF and aLF levels in patients diagnosed with or without endometriosis based on surgical findings and pathology results and revealed that both serum LTF and aLF levels were higher in patients with endometriosis. These values did not differ between early-stage endometriosis and non-endometriosis and were significantly different between advanced-stage endometriosis and non-endometriosis. Additionally, a sig-

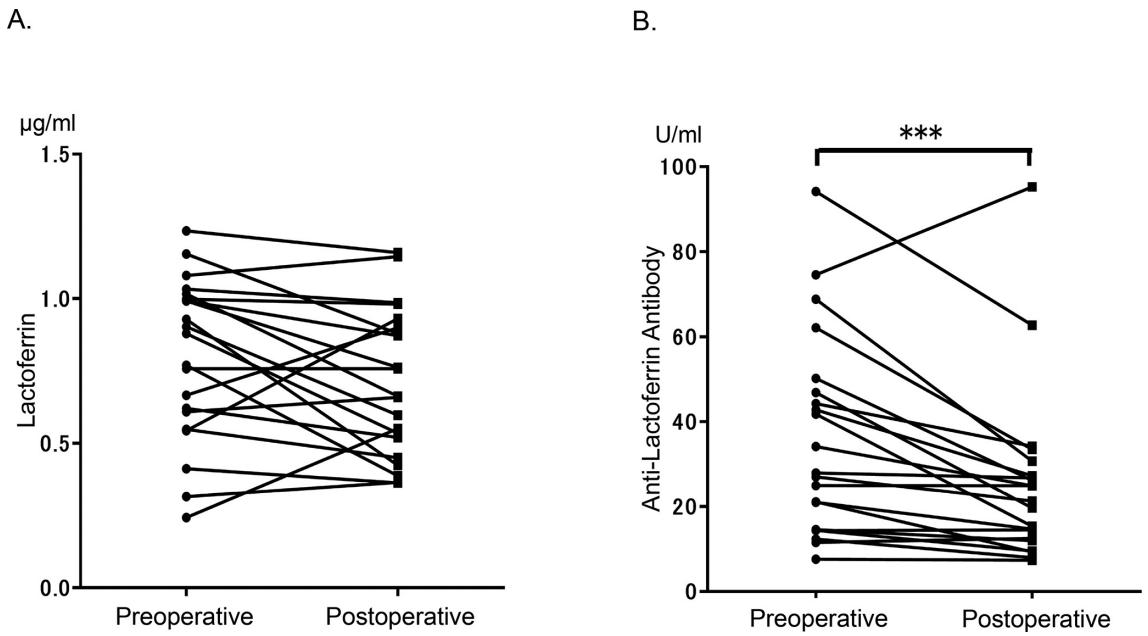

Fig. 3. Serum lactoferrin and anti-lactoferrin antibody levels in patients with endometriosis preoperatively and postoperatively.
The same patient was connected by a line. A. Serum lactoferrin levels in patients with endometriosis preoperatively and postoperatively. Differences between groups were analyzed by the paired t-test. B. Serum anti-lactoferrin antibody levels in patients with endometriosis preoperatively and postoperatively. Differences between groups were analyzed by the Wilcoxon matched-pairs signed rank test. ***$p < 0.001$.

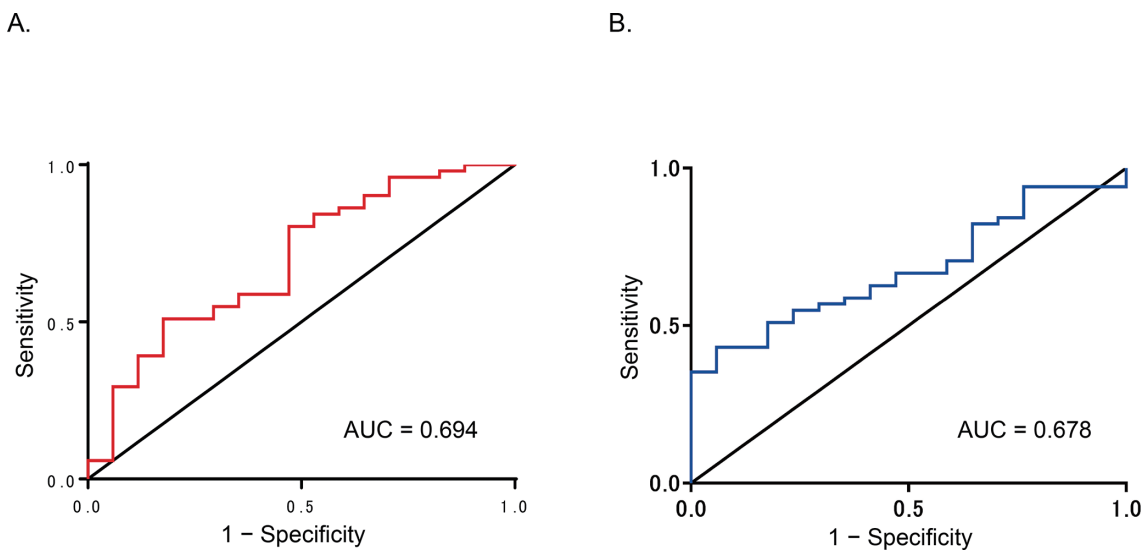

Fig. 4. Receiver operating characteristic curve for the serum lactoferrin and anti-lactoferrin antibody levels.
A. Receiver operating characteristic curve for the serum lactoferrin level. B. Receiver operating characteristic curve for the serum anti-lactoferrin antibody level.

nificantly decreased serum aLF level was observed postoperatively than preoperatively in endometriosis, suggesting that serum LTF and aLF levels are affected by the development and/or volume of endometriosis tissue. The presence of aLF may affect the development of endometriosis because aLF may induce inflammation and exacerbate inflammatory diseases (Shida et al. 2016). However, the present study revealed that surgical reduction of endometriosis tissue volume in the same patient with endometriosis led to a decreased serum aLF level, suggesting that aLF is induced by the presence of endometriosis.

LTF is produced and released by neutrophils, and its serum concentration is related to the total amount of neutrophils (Birgens 1985). Neutrophils are present in the lesions of patients with endometriosis (Milewski et al. 2011; Abramiuk et al. 2022) and a microenvironment in the lesions influences neutrophil induction (Symons et al. 2018). Additionally, systemic circulating neutrophils from

Table 2. Patients who equal or exceed the reference value for lactoferrin (LTF) and anti-lactoferrin antibody (aLF) in the endometriosis and non-endometriosis groups.

A. LTF

	Non-endometriosis	Endometriosis	*p* value
Patients with equal to or greater than the reference value	3	26	0.023
Patients with lower than the reference value	14	25	

The cut off of LTF concentration in serum was 0.822 μg/ml.

B. aLF

	Non-endometriosis	Endometriosis	*p* value
Patients with equal to or greater than the reference value	1	22	0.006
Patients with lower than the reference value	16	29	

The cut off of aLF concentration in serum was 24.4 U/ml.
Fisher's exact test was performed.

patients with endometriosis exhibit distinct transcriptomic differences compared to neutrophils from healthy controls (Symons et al. 2020). These findings suggest that increased neutrophils and their functional changes may lead to an increase of serum LTF level in patients with endometriosis.

LTF levels in ascites fluid were reported to be not different between advanced-stage endometriosis and non-endometriosis (Polak et al. 2007). Neutrophils are present in similar concentrations in the peritoneal fluid of females with and without endometriosis. Furthermore, a study reported no polymorphonuclear granulocytes in the ascites of patients with endometriosis and healthy subjects (Wang et al. 1997). The difference in the results between the present study and the previous one studying the LTF levels in ascites may be due to the difference in the immune environment between endometriosis tissue, which affects the systemic environment, and ascites.

Conversely, LTF levels did not differ pre- or postoperatively although the serum aLF level was decreased postoperatively. The cause of this result cannot be firmly concluded from the present study. However, an increased postoperative serum LTF level was observed in seven cases, of whom the serum aLF levels were all decreased. Endometriosis is a systemic inflammatory disease, and neutrophils may remain active postoperatively since not all endometriosis could be surgically removed. In addition, a decreased postoperative aLF level may have affected blood LTF levels.

ROC curves were drawn to establish reference values to investigate the potential of LTF and aLF as diagnostic markers. The reference values of the serum LTF and aLF levels were 0.822 μg/ml and 24.4 U/ml, respectively. The reference values of the present study were considered appropriate because the mean ranges of serum LTF and the reference value for serum aLF in other previous studies were 0.2-0.4 μg/ml and 20 U/mL, respectively (Venge et al. 1984; Barthe et al. 1989). However, the AUC in the ROC curve was low and the sensitivity was low even using the criteria derived from the Youden index. Specificity was acceptable when using the criteria derived from Youden's index, but the present study targeted 51 cases of endometriosis and 17 cases of non-endometriosis. Thus, the number of patients is three times more in the endometriosis group than in the control. The prevalence of endometriosis is generally considered as 6.0%-18% (Parasar et al. 2017; Moradi et al. 2021; Rowlands et al. 2021). Therefore, the number of people exceeding the reference values of LTF and aLF in the general population would be expected to markedly increase, thereby possibly decreasing the specificity. Thus, using the serum LTF and aLF levels as markers to detect endometriosis was considered difficult.

The percentages of patients higher than the reference values regarding the serum LTF and aLF levels were calculated for the endometriosis and non-endometriosis groups, respectively. Patients exceeding the reference LTF value were 51% (26/51) and 18% (3/17) for the endometriosis and non-endometriosis groups, respectively. Patients exceeding the reference aLF value were 43% (22/51) and 6% (1/17) for the endometriosis and non-endometriosis groups, respectively. The percentage of patients exceeding the reference aLF value was surprisingly high and comparable to the rates seen in autoimmune diseases (Oudkerk Pool et al. 1993; Muratori et al. 2001; Gajic-Veljic et al. 2022).

To investigate the significance of LTF and aLF, we examined CA125, a marker of endometriosis in the same patients. Similar to previous reports (Huhtinen et al. 2009; Karimi-Zarchi et al. 2016), the serum CA125 level was significantly increased in the presence of endometriosis, and the difference was more significant at the advanced-stage endometriosis in the present study (Supplementary Fig. S1A, B). This significance was stronger than those of the serum levels of LTF and aLF. In cases with ovarian endometrioma, CA125 correlated with the surface area and volume (Supplementary Fig. S2A, B), but not with LTF or aLF (data not shown). The surface area and volume of ovarian

endometrioma are considered to be proportional to the amount of endometriotic tissue. Therefore, CA125 appears to correlate with the amount of endometriosis, whereas LTF and aLF seems to not simply reflect the amount of endometriosis.

Endometriosis is known to spontaneously subside, as the lesion of endometriosis can be assessed as red, black, or white lesions depending on the state of endometriosis (Nisolle et al. 1993; Brosens 1994; Donnez et al. 1998). aLF has been reported to be associated with disease activity in autoimmune diseases by the promotion of NET formation. Given the fact, we speculate that the serum aLF level might be elevated during the development of endometriosis from the early stage to the advanced stage, and aLF might appear when endometriosis is actively developing. Therefore, it would be valuable to investigate the exact mechanism by which aLF is induced in patients with endometriosis. This may lead to a better understanding of endometriosis development in the future.

This study included patients with surgical cases, confirmed the presence or absence of endometriosis, and excluded clinical endometriosis diagnosed by ultrasound findings or other means. This is the first study that shows that serum LTF and aLF are elevated in patients with endometriosis. The strength of the present study is the inclusion of the control group which consisted of patients with gynecological diseases who had undergone surgery. Thus, the controls were not disease-free without endometriosis, which is the limitation of the present study.

In conclusion, we found that serum LTF and aLF concentrations were higher in patients with endometriosis. A surprisingly large percentage of patients with endometriosis exceeded the reference LTF and aLF values. This is the first report of an association of endometriosis with the serum LTF and aLF levels. The mechanism of the elevated levels in patients with endometriosis and its effect on endometriosis should be investigated in the future.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
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