—Original—

Oxidative Stress and Antioxidant Capacity in Patients with Endometrioma

Go Ichikawa[1,2], Yasuyuki Negishi[1,3], Ryo Tsuchiya[1], Lilika Higuchi[1], Tatsunori Shiraishi[1], Mariko Ikeda[1], Hanako Kaseki[1,2], Rimpei Morita[3] and Shunji Suzuki[1]

[1]Department of Obstetrics and Gynecology, Nippon Medical School, Tokyo, Japan
[2]Department of Obstetrics and Gynecology, Nippon Medical School Chiba Hokusoh Hospital, Chiba, Japan
[3]Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan

Background: Endometriosis has several clinical features, including dysmenorrhea, infertility, and endometrioma (EMO). Although oxidative stress status is closely related to endometriosis, it is unclear how the balance between oxidative stress capacity and antioxidant capacity correlates with treatment of or factors that worsen endometriosis. In this study, we used peritoneal fluid from patients with EMO to investigate the role of oxidative stress capacity and antioxidant capacity.

Materials and Methods: Participants with EMO (n = 30) and without EMO (uterine myoma, n = 13) were enrolled. All peritoneal fluid samples were collected at the beginning of surgery. We evaluated oxidative stress capacity and antioxidant capacity in peritoneal fluid samples by using the diacron-reactive oxygen metabolites (d-ROM) and biological antioxidant potential (BAP) tests, respectively. The d-ROM and BAP values and the d-ROM/BAP ratio were measured, and their correlations with the CA 125 level, revised American Society for Reproductive Medicine (r-ASRM) score, and tumor size were analyzed.

Results: The d-ROM/BAP ratio was significantly higher in patients with EMO than in those without EMO. In addition, the d-ROM/BAP ratio was positively correlated with CA125 level and r-ASRM scores in patients with EMO.

Conclusions: Oxidative stress is correlated with factors that worsen EMO. The d-ROM/BAP test may be useful for assessing disease status in patients with EMO. (J Nippon Med Sch 2024; 91: 146–154)

Key words: endometriosis, endometrioma, oxidative stress

Introduction

Endometriosis is one of the most important gynecological diseases and affects approximately 10% of women[1]. It impairs the quality of life of women of reproductive age and manifests with a variety of clinical features, including dysmenorrhea, infertility, adenomyosis, and endometrioma (EMO). The pathogenesis of endometriosis is related to chronic inflammation in the pelvis[2,3], and the onset of endometriosis has been explained by various theories. The most widely accepted is Sampson's theory, which posits that endometrial debris during retrograde menstruation is engrafted in the ectopic region of the pelvis, Douglas' fossa, peritoneum, myometrium, and ovary[4]. However, it remains unclear why endometrial debris are not eliminated and become viable in the pelvis. Several studies have shown that the immunosuppressive milieu in the pelvic region of patients with endometriosis does not allow endometrial debris to be eliminated[5,6].

Recently, the concept of oxidative stress in diseases such as atherosclerosis, diabetes mellitus, Alzheimer disease, cancer, and endometriosis has gained attention[7]. Oxidative stress is important in chronic inflammation, apoptosis, and immune abnormalities in patients with endometriosis[8–11]. Oxidative stress status is determined by examining the balance between oxidative stress capacity and antioxidant capacity. Oxidative stress capacity is

Correspondence to Yasuyuki Negishi, MD, PhD, Department of Microbiology and Immunology, Nippon Medical School, 1–1–5 Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan
E-mail: negi@nms.ac.jp
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characterized by production of reactive oxygen species (ROS), such as superoxides, hydroxyl radicals, and hydrogen peroxide (H_2O_2)[12]. In peritoneal fluid of patients with endometriosis, iron-induced oxidative stress is important in the pathogenesis of endometriosis. The Fenton reaction—the interaction between Fe^{2+} and H_2O_2—produces ROS via the catalytic form of iron, activating neutrophils and macrophages that lead to chronic inflammation in the pelvis of patients with endometriosis[13]. Therefore, oxidative stress plays a significant role in inducing chronic inflammation and allowing the survival of debris in these patients.

Inflammatory processes are essential for endometriosis. Research has recently focused on endogenous and exogenous agents, called alarmins, as triggers of inflammation in various diseases, cardiovascular disease[14,15], diabetic kidney disease[16], and cancer[17]. In particular, endogenous agents referred to as damage-associated molecular patterns (DAMPs), including the alarmins interleukin (IL)-1 α, IL-33, heat shock protein, and high-mobility group box 1 (HMGB1), have attracted attention as factors causing sterile inflammation. We previously reported upregulation of cytoplasmic HMGB1 in antigen-presenting cells and nonimmune cells obtained from patients with EMO, as compared with those without EMO[18]. HMGB1 is a representative alarmin that is induced by excessive inflammation during reproduction[19–24]. We also found that HMGB1 receptors, including toll-like receptor 4 and receptor of advanced glycation end-products, on antigen-presenting cells were decreased in peritoneal fluid from patients with endometriosis (manuscript submitted for publication). In addition, several studies have suggested that IL-33, which is also a representative alarmin, is an aggravating factor for excessive inflammation in endometriosis[25–27]. Therefore, the kinetics of alarmins might be important in endometriosis in an inflammatory milieu.

There are few studies of the balance between oxidative stress capacity and antioxidant capacity in endometriosis. In this study, we used the diacron-reactive oxygen metabolites (d-ROM) and biological antioxidant potential (BAP) tests. The d-ROM test reflects oxidative stress capacity, the BAP test reflects antioxidant capacity, and the d-ROM/BAP ratio reflects total oxidative stress status. Using these tests, we examined peritoneal fluid from patients with EMO to investigate the role of oxidative stress capacity and antioxidant capacity. Specifically, we examined whether oxidative stress status correlated with CA 125 level, revised American Society for Reproductive Medicine (r-ASRM) score, and tumor diameter, which are typical endometriosis aggravating factors, as well as with alarmins.

Materials and Methods

Samples

Patients with EMO (n = 30) were enrolled in this study. Those with uterine myomas (n = 13) were included in the non-EMO group, which did not include EMO or other endometriosis findings. All peritoneal fluid samples were collected at the beginning of surgery. The obtained peritoneal fluid was centrifuged immediately (3,000 rpm, 4°C, 15 min) and stored at −80°C until assayed. For the EMO group, serum CA125 level (U/mL) was measured preoperatively, and r-ASRM classification score (1997) and tumor size (largest dimension of tumor [mm]) were evaluated postoperatively. Before surgery, 16 and 4 patients with EMO received dienogest and relugolix preoperatively, respectively, and 10 were untreated. In the EMO without pretreatment group, at the time of sample collection (i.e., during surgery), 6 cases were in the proliferative phase and 4 cases were in the secretory phase of menstruation. In the non-EMO group, 12 patients received relugolix preoperatively, whereas 1 patient was untreated. There were no statistically significant differences in age, gravity, parity, body mass index, or history of smoking or drinking in the EMO with pretreatment group, EMO without pretreatment group, and non-EMO group. The clinical characteristics of the present patients are shown in **Table 1**.

All participants provided written informed consent. The Ethics Committee of Nippon Medical School Hospital approved the collection and use of biological materials for research purposes (approval number: 18-HE-165). All experiments were conducted in accordance with relevant guidelines (18-HE-165).

d-ROM and BAP Tests

We evaluated oxidative stress capacity (d-ROM test) and antioxidant capacity (BAP test) in peritoneal fluid by using the Free Radical Elective Evaluator (FREE carpe diem: Diacron International s.r.l., Italy) and d-ROM/BAP kit (Diacron International s.r.l.), respectively, according to the manufacturers' instructions. The d-ROM and BAP tests reflect oxidative stress capacity and antioxidant capacity, respectively. The d-ROM test measures the amount of hydroperoxides, which are byproducts of ROS, and free radicals in CARR units (U. CARR); 1 U. CARR corresponds to 0.08 mg/dL H_2O_2. The BAP test evaluates the reductive reaction of Fe^{2+} to Fe^{3+} and quanti-

Table 1 Clinical characteristics of the patients

	EMO (n = 30)		Non-EMO (n = 13)	*p* value
	EMO with pretreatment Dienogest (n = 16) Relugolix (n = 4)	EMO without pretreatment (n = 10)	Uterine myoma Relugolix (n = 12) No pretreatment (n = 1)	
Age (years) [a]	40.00 (35.00–46.50)	39.00 (35.00–46.50)	35.00 (33.50–39.00)	0.459[b]
Gravity[a]	0.00 (0.00–1.75)	0.0 (0.00–1.00)	0.00 (0.00–0.50)	0.508[b]
Parity[a]	0.00 (0.00–1.00)	0.00 (0.00–1.00)	1.00 (0.00–0.50)	0.636[b]
BMI[a]	23.01 (20.58–26.25)	22.86 (21.22–24.40)	23.42 (20.13–24.41)	0.716 [b]
History of smoking (cases)	n = 3	n = 1 (Unknown 1 case)	n =1 (Unknown 1 case)	0.939[c]
History of drinking (cases) [d]	n = 4 (Unknown 2 cases)	n = 2 (Unknown 1 case)	n = 2 (Unknown 2 cases)	0.989[c]
Phase of the menstrual cycle at surgery	—	Proliferative phase (n = 6) Secretory phase (n = 4)	Proliferative phase (no pre-treat. Group) (n = 1)	—
CA125 (U/mL) [a]	39.60 (20.20–102.5)	61.25 (42.25–97.10)	—	0.397[e]
r-ASRM score[a]	69.00 (36.00–101.5)	82.50 (39.00–105.3)	—	0.837[e]
Greatest tumor dimension of ovary (mm) [a]	5.70 (4.00–7.75)	6.00 (4.40–10.15)	—	0.721[e]

[a]Data are presented as median (interquartile).
[b]Data compared by one-way analysis of variance followed by Tukey posttests.
[c]Data compared by the chi-square test.
[d]Data exclude opportunity drinking.
[e]Data compared between the EMO with pretreatment and without pretreatment groups using Mann–Whitney *U* test.
BMI, body mass index; EMO, endometrioma; r-ASRM, revised American Society for Reproductive Medicine.

fies the amount of reduced iron (μM) in the sample. We obtained the d-ROM and BAP values and calculated the d-ROM/BAP ratio in each sample.

The d-ROM and BAP testing methods are simple and do not require special skills; results are available in approximately 5 minutes per sample.

Alarmin Detection

We analyzed high-mobility group box 1 (HMGB1, n = 20) and IL-33 levels (n = 15) in the peritoneal fluid of patients with EMO. HMGB1 levels were measured with an enzyme-linked immunosorbent assay kit (Shino-test, Kanagawa, Japan). IL-33 levels were measured by electrochemiluminescence using U-PLEX Biomarker Group 1 (human) assays (K15067L-1, Meso Scale Discovery, Rockville, MD, USA). The detection limit is 0.59 pg/mL for IL-33.

Statistical Analysis

We analyzed the statistical data using Prism (GraphPad Software, San Diego, CA, USA). The Mann-Whitney *U* test was used to identify statistical differences between 2 groups. One-way analysis of variance followed by the Tukey posttest was used to identify statistical differences among 3 groups. The chi-square test was used to evaluate categorical data. Linear regression analysis was used to examine correlations between 2 variables. Significance was set at $p < 0.05$.

Results

Oxidative Stress Is Enhanced by EMO

First, we investigated differences in levels of oxidative stress in peritoneal fluid between patients with and without EMO (uterine myomas). Although there was no significant difference in BAP values, d-ROM values significantly differed between the EMO and non-EMO groups (**Fig. 1A**, left and middle panel). The d-ROM/BAP ratio (reflecting total oxidative stress) was also significantly higher in patients with EMO than in those without EMO (**Fig. 1A**, right panel). Next, we evaluated oxidative and antioxidant conditions in patients with EMO. There was no significant difference in d-ROM, BAP, or d-ROM/BAP ratio between patients with EMO who did and did not receive preoperative treatment (**Fig. 1B**). In the EMO without pretreatment group, d-ROM, BAP, and d-ROM/BAP ratio were not associated with the time when peritoneal fluid was sampled (i.e., proliferative or secretory phases). These results indicate that oxidative stress in peritoneal fluid is greater in patients with EMO than in those without EMO. However, oxidative stress was not associated with treatment or menstrual period.

Oxidative Stress Was Correlated with CA125 Level and r-ASRM Score in Patients with EMO

We then attempted to identify factors in peritoneal fluid that were associated with oxidative stress in patients with EMO. We focused on serum CA125, r-ASRM score, and tumor size, as these were aggravating factors for EMO. Interestingly, we observed a slight increase in d-ROM and a small decrease in BAP (**Fig. 2A**, left and middle panels). Additionally, d-ROM/BAP ratio was positively correlated with CA125 level (**Fig. 2A**, right panel) and r-ASRM score (**Fig. 2B**) but not with tumor size (**Fig. 2C**). These results indicate that aggressive EMO, as reflected by serum CA125 and r-ASRM scores, may be associated with oxidative stress in patients with EMO.

Oxidative Stress Was Correlated with HMGB1 in Patients with EMO

We investigated the relationship of oxidative stress in EMO with HMGB1 and IL-33. Although IL-33 was not correlated with any oxidative variable, d-ROM and d-ROM/BAP ratio were significantly correlated with HMGB1 (**Fig. 3A and B**). These results suggest that oxidative stress in peritoneal fluid is associated with an abnormal inflammatory state.

Discussion

We found that the d-ROM/BAP ratio in peritoneal fluid was higher in patients with EMO than in those without EMO. Additionally, d-ROM/BAP ratio, but not d-ROM or BAP, were positively correlated with CA125 and r-ASRM score in patients with EMO. However, among patients with EMO, there was no difference in d-ROM, BAP, or d-ROM/BAP ratio between those who did and did not receive preoperative treatment. These results indicate that both oxidative stress and antioxidant capacities should be measured to fully evaluate oxidative stress in patients with endometriosis.

Oxidative stress status is associated with disturbance of the balance between oxidative stress and antioxidant capacity[12,28–31]. Iron-induced oxidative stress disrupts this balance in endometriosis. In patients with endometriosis, retrograde menstruation causes accumulation of iron in peritoneal fluid, macrophages, and endometrial lesions. Thus, iron-induced oxidative stress is upregulated in the pelvic cavity, leading to production of ROS, superoxides, hydroxyl radicals, and H_2O_2[13,32]. Oxidative stress then induces angiogenesis, allowing the survival of retrograde endometrial tissues[33–35] that affect epigenetic modulation, histone methylation and acetylation, and DNA methylation[36]. Furthermore, oxidative stress induces excessive activation of macrophages, leading to chronic inflammation[37]. These mechanisms may be involved in the abnormalities caused by oxidative stress in endometriosis.

Endometriosis is generally considered to be triggered by chronic severe inflammation[2,3]. However, recent studies indicate that suppression of the immune response, i.e., reduced cytotoxicity of natural killer cells[38–40], alteration of regulatory T cell (Treg)/Th17 balance[41,42], and M2 polarization of macrophages[27,43], impairs elimination of endometrial debris, leading to the survival and growth of ectopic endometrial tissues. These 2 hypotheses are contradictory, and the mechanism by which inflammation is enhanced or suppressed in the pelvis in endometriosis remains unclear. Therefore, we propose that excess inflammation occurs in endometriosis. In this study, HMGB 1 level in peritoneal fluid was correlated with aggravating factors of endometriosis, namely, CA125 level and r-ASRM score. ROS have a significant impact on the immune system, as they reportedly suppress T cell signaling, activation, and proliferation[44]. These findings suggest that, while excessive inflammation is induced by alarmins such as HMGB1, increased oxidative stress prevents the normal immune response from eliminating endometrial debris, further aggravating endometriosis. Additional studies of oxidative stress, chronic inflammation, and alarmins are expected.

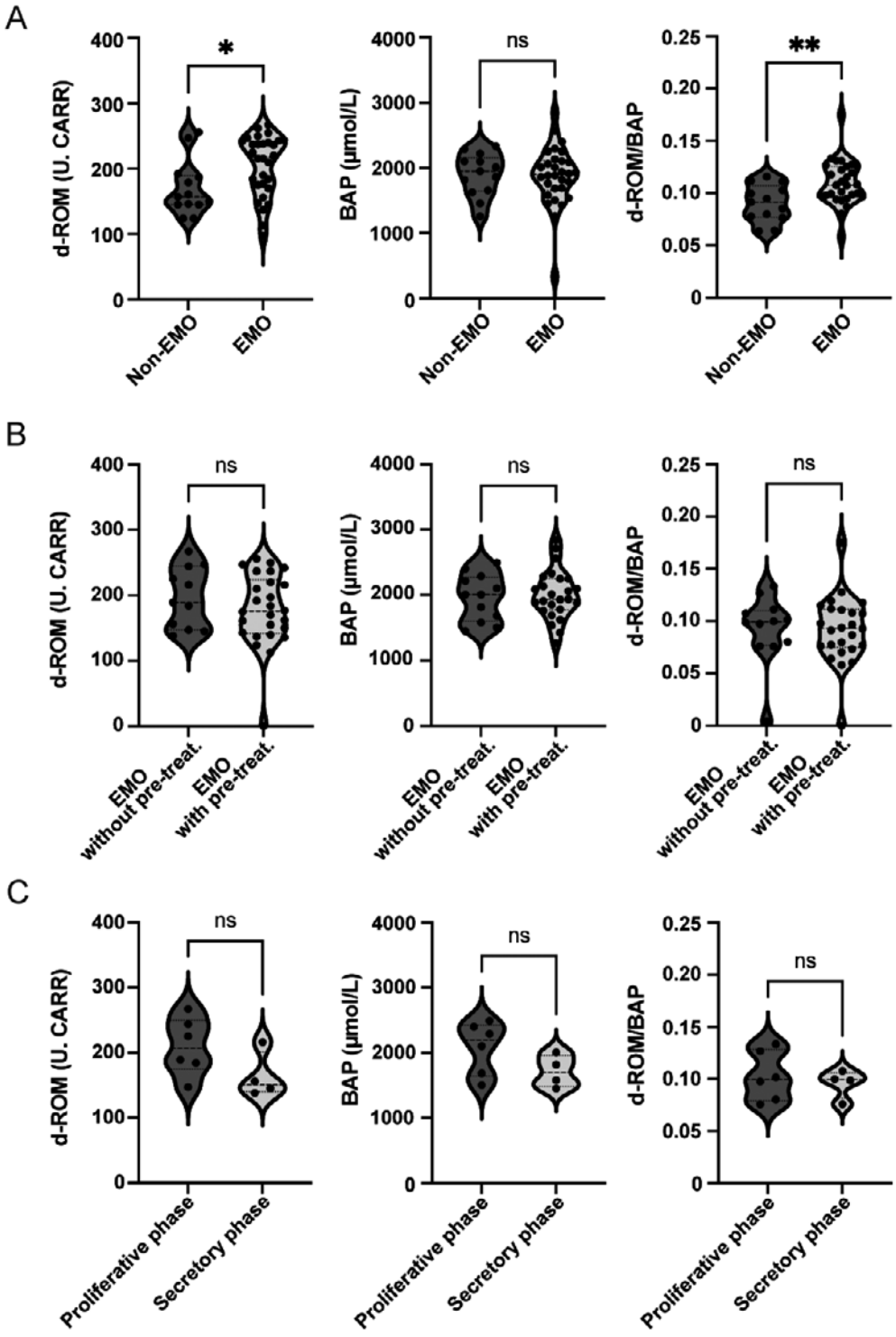

Fig. 1 Oxidative stress status in peritoneal fluid. Oxidative stress status was enhanced in EMO but was independent of preoperative treatment and menstrual period. (A) Comparison of d-ROM, BAP, and d-ROM/BAP ratio in the peritoneal fluid of patients with and without EMO. Data are from individual women: n = 30 for EMO and n = 13 for uterine myoma. *$p < 0.05$; Mann–Whitney U test. (B) Comparison of d-ROM, BAP, and d-ROM/BAP ratio in the peritoneal fluid of patients with EMO who did and did not receive treatment. Data are from individual women: n = 20 with preoperative treatment and n = 10 without preoperative treatment. ns, not significant. (C) Comparison of d-ROM, BAP, and d-ROM/BAP ratio in the peritoneal fluid of patients with EMO who did not receive treatment. Data are from individual women: n = 6 in the proliferative phase and n = 4 in the secretory phase. EMO, endometrioma; d-ROM, diacron-reactive oxygen metabolites; BAP, biological antioxidant potential.

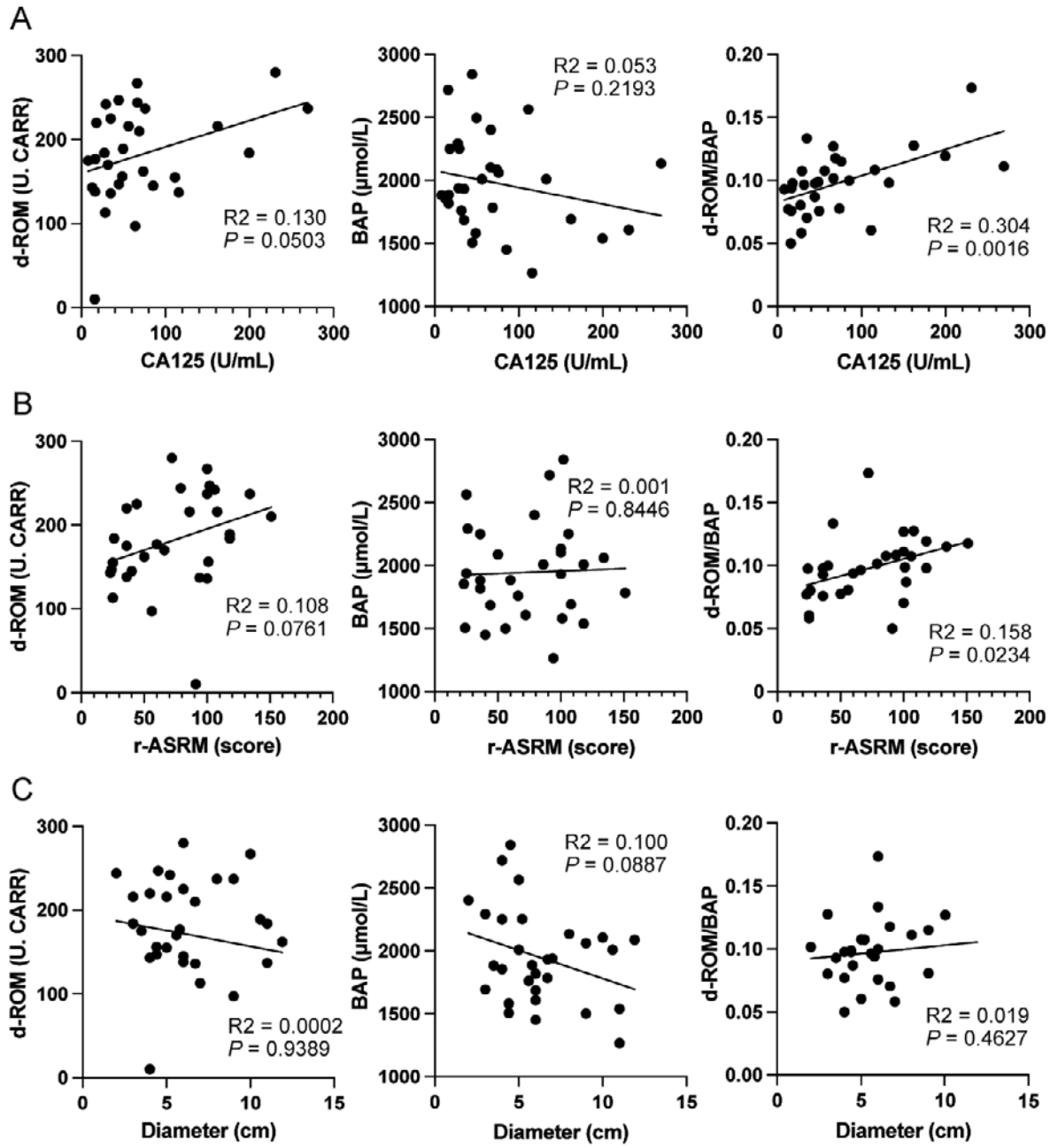

Fig. 2 Correlation of oxidative stress status with aggravating factors of EMO. Correlations of d-ROM, BAP, and d-ROM/BAP ratio with (A) CA125, (B) r-ASRM score, and (C) tumor size in peritoneal fluid of patients with EMO. Linear regression analysis was performed to examine the correlations, and statistical significance was defined as $p < 0.05$. BAP, biological antioxidant potential; d-ROM, diacron-reactive oxygen metabolites; EMO, endometrioma; r-ASRM, revised American Society for Reproductive Medicine.

Limitations of this study include the absence of data on oxidative stress in serum. Since this study revealed that oxidative stress correlates with endometriosis and its aggravating factors, we plan to determine whether oxidative stress in serum of patients also correlates with aggravating factors. The findings will allow us to predict the status of oxidative stress in the pelvis of patients without the need for surgery. Another limitation is that we used patients with uterine myoma as the control because it is a benign, noninflammatory proliferative disease[45]. In another study of endometriosis, patients with uterine myoma and ovarian cysts were used as the control[46,47]. Although normal ovaries should be used as controls, it is ethically impossible to obtain normal ovaries. Hence, in the future, we must examine peritoneal fluid from patients with benign ovarian cysts, especially those with normal CA125 levels and without inflammatory signs.

In summary, we evaluated oxidative stress capacity

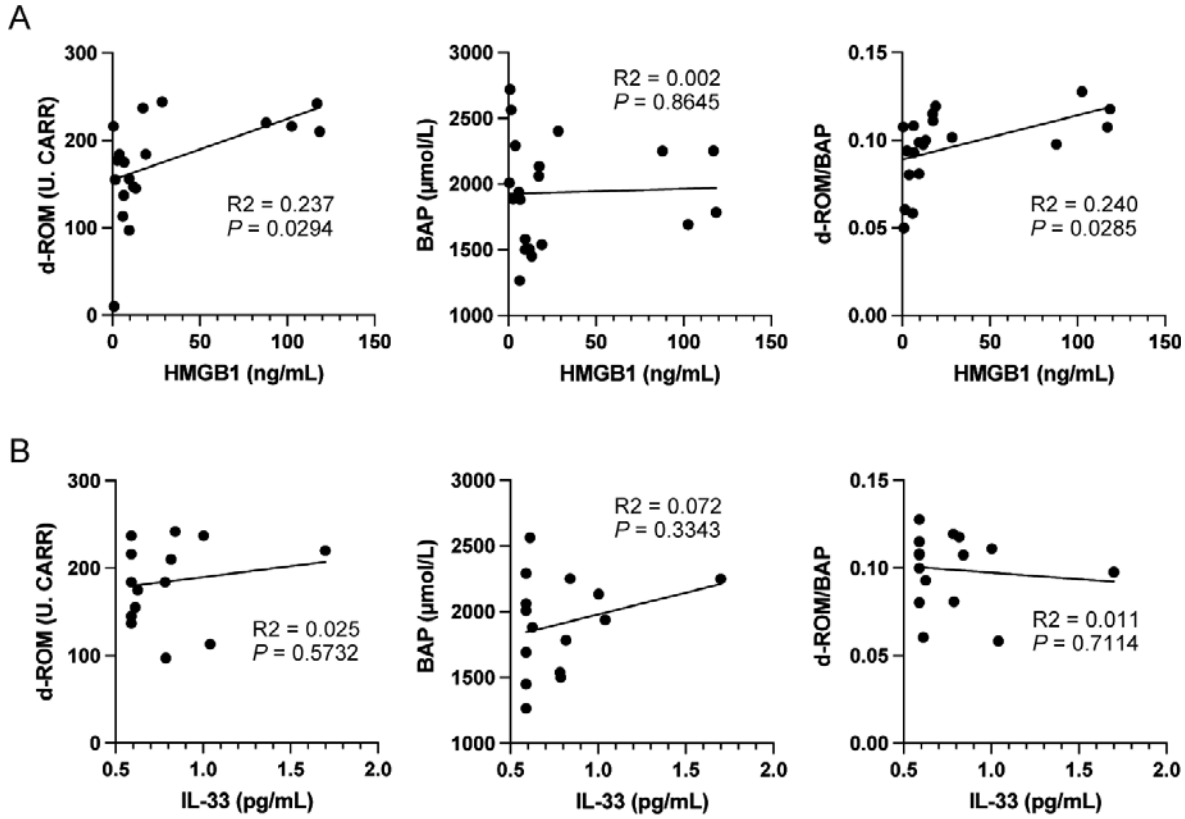

Fig. 3 Correlation of oxidative stress status with alarmins. Correlations of d-ROM, BAP, and d-ROM/BAP ratio with (A) HMGB1 levels (n = 20) and (B) IL-33 levels (n = 15) in peritoneal fluid of patients with EMO. Linear regression analysis was performed to examine the correlations, and statistical significance was defined as $p < 0.05$. BAP, biological antioxidant potential; d-ROM, diacron-reactive oxygen metabolites; EMO, endometrioma; HMGB1, high-mobility group box 1.

and antioxidant capacity, d-ROM, BAP, and d-ROM/BAP ratio in peritoneal fluid from patients with and without EMO. d-ROM/BAP ratio was positively correlated with CA125 level and r-ASRM score, as well as with HMGB1 level in the peritoneal fluid of patients with EMO. Therefore, d-ROM/BAP testing may be useful for determining disease status in patients with endometriosis. Indeed, the methods for d-ROM and BAP testing were simple and easy to apply clinically. Detailed analyses of correlations among pelvic inflammation, oxidative stress, immune response, alarmins, and other factors, including patient symptoms, are expected.

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Conflict of Interest: None declared.

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