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Elevated peritoneal soluble endoglin and GDF-15 in infertile women with severe endometriosis and pelvic adhesion

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ABSTRACT

Objectives: Chronic inflammation and pelvic adhesion play a critical role in endometriosis-related infertility. Research studies suggest that TGF-β superfamily members, such as soluble endoglin (sEng), growth differentiation factor 15 (GDF-15) and tumor growth factor-beta (TGF-β1) contribute to the regulation of inflammation, angiogenesis and cell adhesion. The objective of this study is to investigate the association between the concentrations of these TGF-β-related members and the clinical parameters of infertile women with endometriosis.
Materials and methods: Sixty-five infertile women who underwent laparoscopy were divided into two groups in this study: those who had endometriosis (n = 33) and control subjects with benign gynecologic disorders (n = 32). The levels of TGF-β- related members in peritoneal fluid and serum were evaluated by the enzyme-linked immunosorbent assay (ELISA). Clinical and hematological parameters were documented and analyzed.
Results: Endometriosis cases had significantly higher levels of sEng, GDF-15 and TGF-β1 in peritoneal fluid ($p < 0.0005$) compared to control subjects, but not in serum. Moreover, serum GDF-15 level was significantly elevated in the late-stage endometriosis compared to the early-stage group. The levels of three TGF-β related molecules in peritoneal fluid showed positive correlations with rASRM score. Blood neutrophil counts have correlation with the peritoneal sEng concentration.
Conclusion: Our novel evidence on the elevated concentration of peritoneal sEng and GDF-15 in endometriosis, specifically in the late-stage, may indicate the essential role of TGF-β-dependent signaling in endometriosis. Serum GDF-15 might serve as a candidate biomarker for endometriosis severity. Further studies are warranted to investigate the role and regulation of these molecules in endometriosis.

1. Introduction

Endometriosis is a chronic inflammatory disorder characterized by the presence of endometrial-like tissue outside the uterine cavity (Zondervan et al., 2020). The main symptoms of endometriosis are dysmenorrhea, infertility, and pelvic pain (Verkauf, 1987). About 30–50% of women with endometriosis experienced infertility (Maceran and Taylor, 2012; American Society for Reproductive Medicine, 2012). Currently, the most frequently used staging system for endometriosis diagnosis based on the revised American Society for Reproductive Medicine (rASRM) (Anon., 1997). Clinicians also use the Endometriosis Fertility Index (EFI) score as a complement of rASRM score to better predict the future outcome of pregnancy (Tomassetti et al., 2013). The index consists of historical factors (age, length of infertility, and previous pregnancy history) and surgical factors (rASRM pelvic adhesion, least function and endometriosis scores).

The pathogenesis of endometriosis has been investigated over the past three decades (Zondervan et al., 2020). However, our understanding of endometriosis-related infertility remains incomplete. Local and systemic inflammatory factors are critically required in the progression of peritoneal endometriosis lesions (Králíčková and Vetvicka, 2015). Enhanced adhesion formation and inflammation in the peritoneal cavity are the major hallmarks of endometriosis (May et al., 2011) The involvement of inflammatory mediators in up-regulation of adhesion factors was reported to be associated with endometriosis severity (Sikora et al., 2017). Some members of transforming growth factor

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(TGF) superfamily were shown to modulate angiogenesis and cellular adhesion (Poniatowski et al., 2015).

The TGF-β superfamily is comprised of over 30 members including the firstly discovered transforming growth factors β 1 (TGF-β1) (Poniatowski et al., 2015; Young et al., 2014). Previous studies have reported that TGF-β1 expression is critically involved in endometriosis lesion development in the murine model and human (Hull et al., 2012; Young et al., 2017) A study by Choi et al. revealed that TGF-β1 enhanced the ectopic endometrial cell adhesion to mesothelial cells through regulation of several integrins (Choi et al., 2017), suggesting the significant role of TGF-β1 in cellular adhesion during endometriosis development. Moreover, TGF-β1 is also known to modulate vascularization in peritoneal endometriosis (Young et al., 2015)

Soluble endoglin (sEng) and growth differentiation factor-15 (GDF-15) are the remote members of TGF-β superfamily (Rossi et al., 2019; Emmerson et al., 2018) Endoglin or CD105 consists of two forms: soluble form (sEng) and membrane-bound endoglin (mEng). mEng, the co-receptor of TGF-β, is widely known as angiogenesis marker due to its abundant expression in the vascular endothelial cells (Dallas et al., 2008). The other cells were also shown to express mEng such as hematopoietic stem cells, bone marrow stromal fibroblasts, activated monocytes, differentiated macrophages, melanocytes, and syncytiotrophoblasts of placenta (Dallas et al., 2008). Beside angiogenesis, mEng also modulates apoptosis and cellular adhesion (Li et al., 1999). On the other hand, the soluble Endoglin or sEng may act as a negative regulator by antagonizing the TGF-β receptor activation leading to angiogenesis impairment, endothelial dysfunction, and increased vascular permeability (Rossi et al., 2019). Recent report by Tan et al. indicated a strong association of endometriosis and cellular dysfunction (Tan et al., 2019). sEng also inhibit cellular adhesion, likely by competing with mEng in integrin-binding (Rossi et al., 2013). The increased expression of mEng was reported in eutopic endometrium of stage III/IV or late-stage endometriosis patients compared to the stage I/II endometriosis and the healthy control (Kim et al., 2001a; Hayrabedyan et al., 2005a), suggesting the role of endoglin in endometriosis pathology. To our knowledge, the expression of sEng has not been reported in endometriosis.

GDF15 or macrophage-inhibitory cytokine-1 (MIC-1) is a stress response molecule and acts via a newly discovered receptor, the glial-derived neurotrophic factor (GDNF) receptor alpha-like or GFRAL (Tsai et al., 2018). The activation of GFRAL receptor by GDF-15 led to the appetite reduction and anti-obesity effect (Tsai et al., 2018; Yang et al., 2017; Hsu et al., 2017; Mullican et al., 2017). Beside this metabolic role of GDF-15, recent accumulating evidence has shown the role of GDF-15 as a pro-angiogenic factor (Wang et al., 2017). The increased levels of sEng and GDF-15 were previously reported in malignancy, metabolic and cardiovascular-related diseases (Rossi et al., 2019; Emmerson et al., 2018; Kopczynska and Makarewicz, 2012, 2012; Adela and Banerjee, 2015). This highlights their additional potential role as biomarker candidate. Nevertheless, the involvement of sEng and GDF-15 in endometriosis-related infertility is largely unknown. In the present study, we provide a novel evidence of sEng and GDF-15 expressions in peritoneal fluid and serum of infertile women with endometriosis. We also evaluated the association between these molecule concentrations and clinical or hematological parameters.

2. Method of the study

2.1. Study design

This study protocol was approved by the Ethical Review Committee Faculty of Medicine, Airlangga University, Surabaya, Indonesia (No. 62/EC/KEPK/FKUA/2020). A total of 65 patients who underwent laparoscopy were consecutively enrolled and signed informed consent. Of them, 33 laparoscopy-confirmed endometriosis and 32 non-endometriosis patients were assigned as case and control groups, respectively. The inclusion criterion for the case group was the clinical diagnosis of endometriosis. The control group consisted of non-endometriosis women with single benign gynecologic disorder related to the fallopian tubes, ovaries, or fibroids. The exclusions criteria were listed as follows: (1) patients with unexplained infertility and presented more than single gynecological disorders and (2) patients with hormonal medications and immunosuppressive drugs three months prior to recruitment. All participants had no known infections, autoimmune diseases, diabetes mellitus, and malignancy before infertility diagnosis. Clinical parameters such as type and duration of infertility, rASRM-EFI score, and dysmenorrheal history including the Visual Analog Scale (VAS) score to assess the menstrual pain were documented (American Society for Reproductive Medicine, 2012; Anon., 1997; Tomassetti et al., 2013; Bourdel et al., 2015; Larroy, 2002).

2.2. Collection of peritoneal fluid and serum

The peritoneal fluid was obtained from the Douglas pouch or perivesical space. In the early step of laparoscopy, peritoneal fluid was collected by aspiration needle through laparoscopic direct vision. Then, the sample was placed in a 15 mL sterile centrifuge tube and immediately centrifuged at 3000 rpm, 4 °C for 10 min. The supernatant was aspirated and placed into 1.5 mL centrifuge tubes. For serum collection, a total of 5 mL blood samples were obtained prior to the laparoscopic examination. The blood samples were collected into BD Vacutainer tubes, and further centrifuged at 3000 rpm, 4 °C for 10 min. Serum sample was isolated in the upper phase and placed into 1.5 mL sterile microtube. All samples from peritoneal fluid and serum were labeled and stored at −80 °C until further analysis.

2.3. Quantification of hematological parameters

Hematological profile was obtained from each patient and consisted of total white blood cells (WBC) count, differential count or percentage of white blood cells, red blood cell count, hemoglobin, hematocrit, and platelet. They were calculated using an automated hematology analyzer (Sysmex KX-21 N, Japan).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The levels of immune parameters in peritoneal fluid and serum of patients were measured by ELISA. The kits of human sEng, GDF-15, and TGF-β1 (R&D Systems, Minnesota, USA) were used according to the manufacturer's instructions. The coefficient variations of intra- and inter-assay were as follows: sEng (2.7–3.2 %; 6.2–6.7%), GDF-15 (2.2–3.1 %; 6.3–7.5%), and TGF-β1 (1.9–2.9 %; 6.4–9.3 %). Plates were developed using tetramethylbenzidine substrate (Thermo Fisher), stopped using 2 N H_2SO_4, and read at a dual-wavelength of 450 nm in a microplate reader (BIO-RAD, USA) to obtain the optical density (OD) and concentration values.

2.5. Statistical analysis

All statistical analysis was conducted using GraphPad Prism version 8.01. The data distribution was assessed by the Shapiro-Wilk normality test. Since all continuous data sets were not normally distributed, the two-tailed Mann-Whitney *U* test was used to determine the differences between the endometriosis group and the control group. The comparison of three or more groups in this study was performed using the Kruskal-Wallis rank test. Correlation analysis was also performed using Spearman's rank test represented as rho (r) between two continuous variables. Rho value indicates the strength of correlations based on Schober et al: 0.0–0.09, negligible; 0.10–0.39, weak; 0.40–0.69, moderate; 0.70–0.89, strong; and 0.9–1, very strong (Larroy, 2002). A p-value below 0.05 was considered statistically significant. The receiver operating characteristic (ROC) curve analysis was performed to

determine the sensitivity, specificity, and cut-off value of serum sEng, GDF-15, and TGF-β1 in endometriosis patients.

3. Result

3.1. Demographic and clinical characteristics

All subjects were ranged in reproductive age from 24 to 41 years with a median age of 30. The primary and secondary infertile women were 83 % and 17 %, respectively. The length of infertility was between 1–16 years with the median of 4 years. No differences in age, type and length of infertility were found between endometriosis and gynecologic control group (Table 1). The presence of dysmenorrhea was equal and no difference between the two groups. However, the pain intensity measured by Visual Analog Scale (VAS) score was significantly higher in endometriosis group compared to the control cases ($p < 0.05$) with median of 5 in endometriosis patients.

Among 33 endometriosis patients, 11 (33 %) and 22 (67 %) patients were respectively classified into early-stage (minimal to mild or stage I/II) and late-stage (moderate to severe or stage III/IV) endometriosis. Control group was comprised of 12 (38 %), 6 (18 %) and 14 (44 %) patients with fallopian-, ovary-, and myoma- associated benign gynecologic pathology, respectively. The median EFI score in this study is 6 and there was no patients with zero EFI score since no patient with age factor above 40. In addition, WBC counts were significantly higher in endometriosis group compared to control (p=0.008). No significant differences in the other hematological parameters were found between endometriosis cases and the control group.

Table 1
Patient's characteristics.

Variables	Gynecologic control (n = 32)	Endometriosis (n = 33)	*p*-value
Age (years)[a]	30 (28–35)	30 (28–33)	0.677[1]
Type of infertility[b]			
Primary	27 (84.38 %)	27 (81.80 %)	>0.999[1]
Secondary	5 (15.62 %)	6 (19.20 %)	
Length of infertility (year)[a]	3 (2–7)	4 (2–6)	0.715[1]
Dysmenorrhea[b]	18 (56.25 %)	17 (51.52 %)	>0.999[1]
Visual Analog Scale (VAS) score[a]	3 (0–5)	5 (2–8)	<0.05[1,*]
Total EFI score[a]	N/A	6 (5–7)	N/A
Hematological Parameters			
WBC count[a,2]	6.75 (5.57–8.11)	8.23 (6.45–9.87)	0.008[*]
Lymphocyte count[c,2]	2.02 (0.60)	2.23 (0.80)	0.292
Lymphocyte %[a]	30.90 (24.28–37.50)	28.00 (22.15–34.85)	0.220
Mixed cells[d] count[c,2]	0.45 (0.17)	0.49 (0.22)	0.494
Mixed cells[d] %[a]	5.50 (4.85–8.20)	6.49 (4.61–8.68)	0.590
Neutrophil count[a,2]	4.20 (3.26–4.91)	4.54 (3.42–6.15)	0.176
Neutrophil %[a]	61.75 (53.28–69.55)	62.75 (57.08–67.70)	0.609
Red blood cell[c,3]	4.47 (0.35)	4.56 (0.43)	0.248
Hemoglobin (g/dL)[c]	12.32 (1.35)	12.57 (1.54)	0.499
Hematocrit %[c]	38.60 (3.70)	39.27 (4.04)	0.493
Platelet[c,2]	290.4 (61.02)	291.9 (61.84)	0.695

[a] median (25 %–75 % percentile).

[b] frequency (percentage) and.

[c] mean (SD).

[d] Mixed cells defines as total population of monocyte, basophil, and eosinophil. Statistical analysis performed with.

[1] two-tailed Mann-Whitney U and.

[2] $10^3/mm^3$ and.

[3] $10^6/mm^3$; N/A not applicable.

[*] p-value <0.05.

3.2. Analysis of sEng, GDF-15, and TGF-β1 in peritoneal fluid and serum

Endometriosis women showed increased peritoneal fluid levels of sEng [24.34, (3.04–164.10) vs. 10.33, (3.02–65.85), p=0.0003], GDF-15 [818, (212–2725) vs. 301, (200–2693), p<0.0001] and TGF-β1 [164 (15–289) vs. 15, (15–235), p<0.0001] when compared to non-endometriosis women (Fig. 1A–C). We further compared the immune parameters according to the endometriosis severity. No differences between early and late-stage endometriosis group in all three molecules. However, if we compared to control, the concentrations of sEng and GDF-15 in the early-stage of endometriosis were significantly higher compared to control, [16.45, (7.98–146,90) vs. 10.33, (3.00–65.85), p=0.0454] and [886, (212–1767) vs. 301, (200–2693), p=0.0012], respectively (Fig. 1D–E). There was a tendency of higher concentration in peritoneal TGF- β1 of early-stage endometriosis compared to control [155, (51–206) vs. 15, (15–235), p=0.0529] (Fig. 1F). Moreover, the late-stage of endometriosis showed significantly higher in all 3 molecules: peritoneal sEng [24.96, (3.04–164.00) vs. 10.33, (3.00–65.85), p=0.0005], GDF-15 [816, (220–2725) vs. 301, (200–2693), p<0.0001], and TGF-β1 [168 (15–289) vs. 15, (15–235), p<0.0001], comparing to control group (Fig. 1D–F).

In blood serum, we could not detect any difference of sEng, GDF-15, and TGF-β1 protein concentrations between endometriosis and gynecologic control patients (all $p > 0.05$) (Fig. 2A–C). Similarly, if we classified the endometriosis cases into early and late-stage, we found no significant differences in serum sEng, GDF-15 and TGF- β1, according to the severity of endometriosis (Fig. 2D–F), except the serum GDF-15 in the early versus late-stage endometriosis (p = 0.0023) (Fig. 2E). Since the serum GDF-15 significantly higher in moderate to severe endometriosis cases, we examined their diagnostic values as a noninvasive biomarker candidate. As shown in Fig. 2G, the area under the curve (AUC) from the receiver operating characteristics (ROC) analysis between the early and late-stage endometriosis were as follows: sEng (AUC = 0.6364, p=0.2076), GDF-15 (AUC = 0.8512, p=0.0012), and TGF-β1 (AUC = 0.6405, p=0.1941). Serum GDF-15 has a sensitivity of 72.73 %, a specificity of 90.91 %, and a cut-off concentration of 251 pg/mL to discriminate between the early and late-stage endometriosis. Moreover, in agreement with the results in Fig. 2D–F, we found that the serum levels of three molecules including GDF-15 (Fig. 2H) as classifiers failed to discriminate the late endometriosis and control group. The p values of sEng, GDF-15 and TGF-β1 were 0.0783, 0.7116 and 0.0698, respectively. Although the trend of p values from serum sEng and TGF-β1 is close to significantly discriminate the two groups, the cut-off calculation in this case is statistically not useful to be determined since the probability of calculated area under the curve (AUC) is by chance. In Fig. 2I, in line with the previous results in Fig. 2A–C, no significant differences in all 3 molecules' concentrations were found between endometriosis and control group in ROC curve analysis.

Next, our analysis was extended to the molecules which showed significantly higher concentrations in serum and peritoneal fluid. We further evaluated the three patient populations within the control group based on the reproductive tract –fallopian, ovary, or myoma related infertility– that significantly different from the severity of endometriosis. As shown in Fig. 3A, peritoneal sEng of late-stage endometriosis had significantly higher levels compared to all three control groups: fallopian, ovary, and myoma related infertility (p=0.0027, p=0.0160 and p=0.0202, respectively). Conversely, the peritoneal sEng of the early-stage endometriosis group (stages I and II) had shown no differences compared to the three control groups. The early-stage peritoneal GDF-15 was evident to have higher concentration compared to the fallopian-related pathology (p=0.0017) and myoma related group

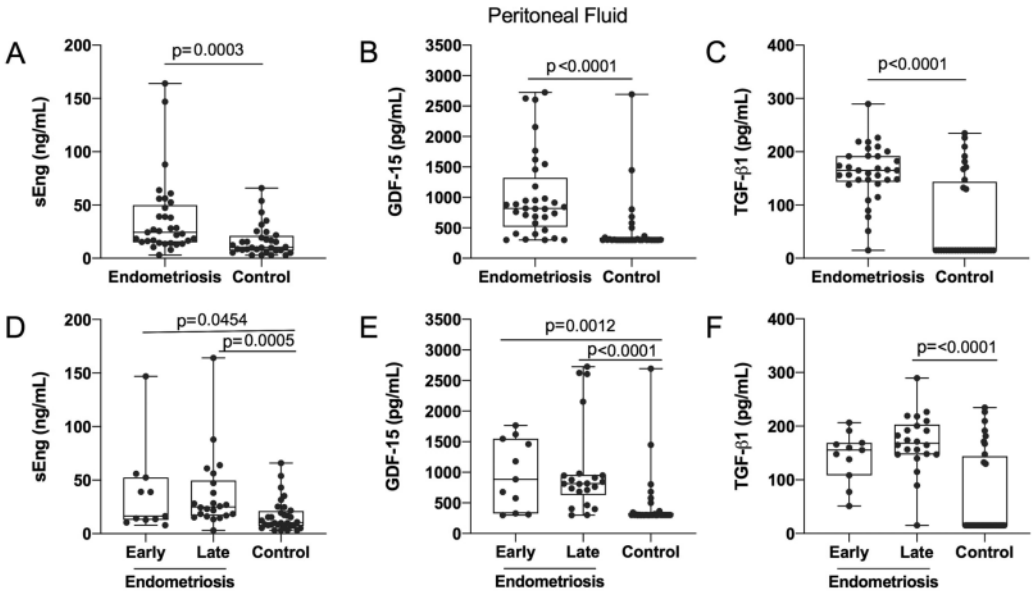

Fig. 1. Peritoneal levels of sEng, GDF-15 and TGF-β1 in endometriosis and gynecologic control patients. Box plot indicates the median and minimum-maximum values with dots representing each patient. The protein concentrations of sEng (ng/mL), GDF-15 (pg/mL), and TGF-β1 (pg/mL) in peritoneal fluid are significantly higher in the endometriosis group compared to the control groups (A—C). There were also significant differences in the severity of endometriosis compared to the control cases (D—F). Significant *p*-values are indicated.

(p=0.0057) (Fig. 3B). Also, the late-stage peritoneal GDF-15 had significantly higher levels compared to the fallopian and myoma related control group (p=0.0001 and p=0.0006, respectively) (Fig. 3B).

In agreement with data in Fig. 1F, the peritoneal TGF-β1 protein of late-stage endometriosis had significantly increased concentrations compared to the fallopian and myoma related control with p=0.0016 and p=0.0109, respectively (Fig. 3C). In contrast, no significant differences were found in the level of peritoneal TGF-β1 in the early-stage endometriosis group compared to the three control populations (Fig. 3C). According to Fig. 3D, we also analyzed further the serum GDF-15 in endometriosis cases and three control groups. Only fallopian-related infertility cases had a significantly lower concentration of serum GDF-15 compared to the late-stage of endometriosis (p=0.0223).

To further analyze the balance of two molecules' concentrations in peritoneal fluid or serum, their ratio was compared between endometriosis and control (Fig. 4). The ratio of TGF-β1/sEng concentrations was significantly different between endometriosis and control in peritoneal fluid and serum (p=0.0023 and p=0.0132, respectively) (Fig. 4A). In peritoneal fluid, the ratio was higher in the endometriosis group, while the serum ratio of TGF-β1/sEng was inversely higher in the control group. Moreover, the ratio of TGF-β1/GDF-15 of endometriosis cases was significantly higher in peritoneal fluid compared to the control group (p=0.0002) (Fig. 4B). Contrary, there were no significant differences in the ratio of serum TGF-β1/GDF-15 and peritoneal or serum GDF-15/sEng (Fig. 4C).

3.3. Correlations between sEng, GDF-15 and TGF-β1 and clinical/laboratory parameters

We also performed the correlation analysis between the three TGF-superfamily members and the laboratory or clinical parameters. As shown in Table 2, there were evident positive correlations between menstrual pain using the VAS score and peritoneal sEng levels (r = 0.358, p=0.003) as well as peritoneal TGF-β1 (r = 0.256, p=0.040). We also found that rASRM pelvic adhesion score had positive correlations with all three molecules in peritoneal fluid: sEng (r = 0.304, p=0.014), GDF-15 (r = 0.238, p=0.047), and TGF-β1 (r = 0.311, p=0.012). Moreover, rASRM total score (endometriosis and adhesion score) had moderate positive correlations with all three molecules in peritoneal fluid (Fig. 5A–C): sEng (r = 0.412, p< 0.001, 95 % CI = 0.180−0.601), GDF-15 (r = 0.464, p< 0.001, 95 % CI = 0.241−0.640), and TGF-β1 (r = 0.530, p< 0.001, 95 % CI = 0.322−0.689). This observation is in line with the Fig. 1A–C. In Fig. 5D–F, the results of correlation analysis between rASRM score and serum levels of sEng, GDF-15 and TGF -β1 have shown non-significant correlations, which is in line with the data in Fig. 2A–0C.

White blood cell (WBC) counts were positively correlated with peritoneal sEng β1 (r = 0.292, p=0.018) and peritoneal TGF-β1 (r = 0.254, p=0.041). The increase concentrations of peritoneal sEng were positively correlated with neutrophil count (r = 0.307, p=0.016) and neutrophil percentage (r = 0.317, p=0.011). There were no differences between serum concentrations of the three TGF-β-related molecules and clinical or laboratory parameters except a positive correlation of serum TGF β1 and mixed cell count (r = 0.317, p=0.018).

4. Discussion

Our novel study demonstrated an elevated concentration of sEng and GDF-15 in the peritoneal fluids of infertile women with endometriosis. Furthermore, these molecules positively correlate with pelvic adhesion in patients. The examination of serum GDF-15 concentration also revealed its capacity to discriminate between early- and late-stage endometriosis, which indicates a potential use as a noninvasive biomarker in endometriosis-related infertility.

The differences between local and systemic inflammatory profiles of TGF-β isoforms in peritoneal endometriosis cases were previously reported (Young et al., 2014, 2017). In agreement with prior studies, we found the increased level of TGF-β1 in peritoneal fluid (Young et al., 2017). The concentration of TGF-β1 tends to be higher in peritoneal compared to the systemic circulation. However, serum TGF-β1 was similar between endometriosis and non-endometriosis patients. This data differed from previous study by Lee et al., which demonstrated

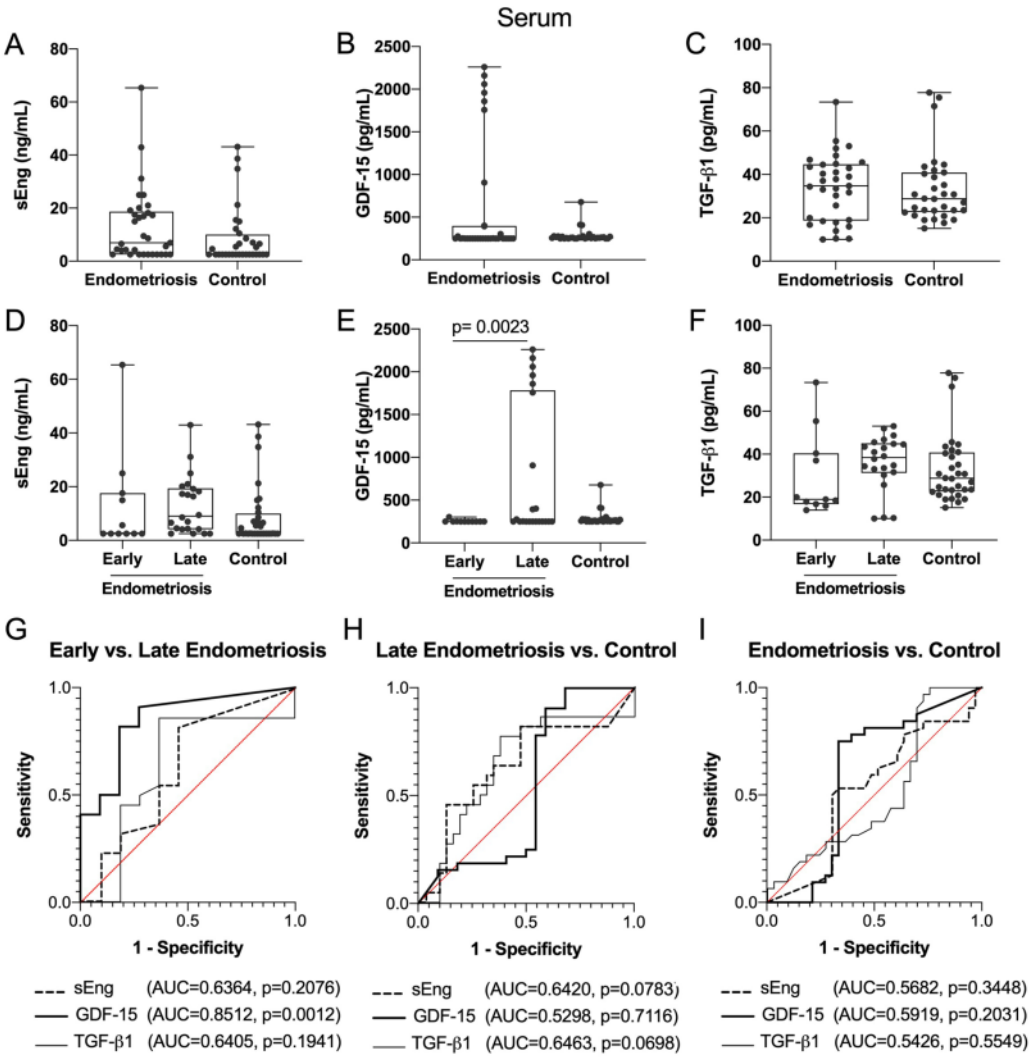

Fig. 2. Serum levels of sEng, GDF-15 and TGF-β1 in endometriosis and gynecologic control patients. The levels of sEng (ng/mL), GDF-15 (pg/mL), and TGF-β1 (pg/mL) in serum are shown according to the endometriosis - control groups (A—C) and the severity of endometriosis and control cases (D—F). There were no significant differences in all three molecules between endometriosis and control group, except the early versus late-stage endometriosis on GDF-15 level ($p = 0.0023$) in Fig. 2E. Box plot indicates the median and minimum-maximum values with dots representing each patient. Receiver operating characteristics (ROC) analysis plots the sensitivity and specificity values of all molecules between early versus late endometriosis (G), late endometriosis versus control (H) and all endometriosis cases versus control group (I). The area under curve (AUC) and p-values are indicated. Only GDF-15 level has shown to significantly differentiate the early and late endometriosis ($p = 0012$).

higher serum TGF-β1 in endometriosis-related cases than the healthy donor (Schober et al., 2018). This discrepancy may be due to the pathological control group in our study that may have a comparable systemic inflammatory profile with endometriosis-related infertility.

During the endometriosis development it is well known that TGF-β isoforms play a critical role in tissue repair and increased adhesion of endometrial tissue in the peritoneal cavity. The presence of the TGF-β1 in this study serves as a comparison molecule to study the other new members of TGF-β superfamily. In line with previous findings, TGF-β1 was increased in the peritoneal fluid of endometriosis cases. The interplay between the activator and negative regulator of TGF-β receptor may be crucial in the development and progression of endometriosis. In this study, we observed some significant differences on the ratio of TGF-β related molecules between endometriosis and control group. Higher TGF-β1/sEng ratio in peritoneal fluid and lower ratio in serum were observed in endometriosis cases. This data may suggest that the relative abundance of TGF-β1 is more locally active in the peritoneum. In addition, membrane-bound mEng was shown to have higher expression in eutopic endometrium tissue of endometriosis patients compared to normal endometrium (Lee et al., 2011; Kim et al., 2001b). Expression of mEng was distributed in microvessels of endometrium, suggesting a possible role of mEng in establishment of angiogenesis. Since sEng is released from the cleavage extracellular domain of mEng (Rossi et al., 2019), it is tempted to speculate that the sEng expressions in the peritoneal fluid also derive from cleavage of ectodomain mEng and act as a negative regulator within ectopic endometrial lesion microenvironment in the peritoneum (Rossi et al., 2019). In addition, endometrial receptivity might be negatively regulated by sEng through the

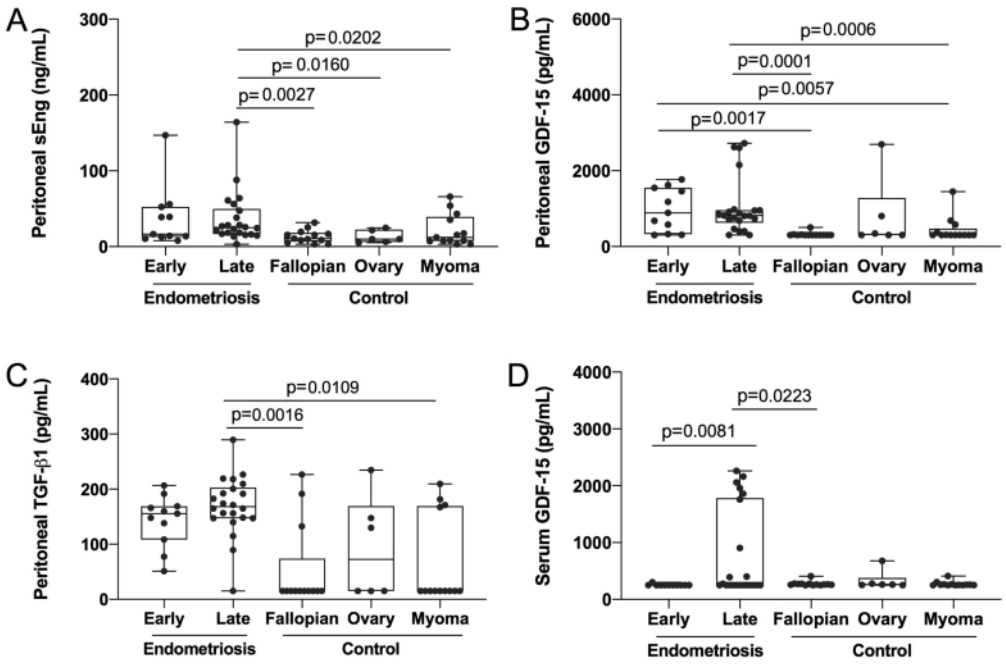

Fig. 3. The concentrations of sEng, GDF-15, and TGF-β1 according to the severity of endometriosis and the type of gynecologic control patients. The peritoneal concentrations of sEng (A), GDF-15 (B), TGF-β1 (C), and serum levels of GDF-15 (D) are indicated. Box plot indicates the median and minimum-maximum values with dots representing each patient. Significant *p*-values are indicated.

anti-angiogenesis and poor trophoblast outgrowth leading to infertility (Hayrabedyan et al., 2005b). The opposing role between membrane bound and soluble form of endoglin on angiogenesis is also reflected by previous evidence on the classical TGF-β1 cytokine. The relative concentrations of TGF-β1 could differentially influence the angiogenic effect on endothelial cells (Chadchan et al., 2016). Low dose TGF-β1 could promote angiogenesis and higher levels inhibit endothelial growth and maturation of blood vessels (Guerrero and McCarty, 2017), suggesting an activation of dose-dependent negative feedback mechanism. For endoglin, the negative feedback mechanism is induced by proteolytic cleavage of mEng when this molecule is in high concentration or abundant on the endometriosis tissue leading to the elevated extracellular production of soluble form sEng (Rossi et al., 2019; Chadchan et al., 2016). sEng may in turn inhibit the proangiogenic role of mEng (Rossi et al., 2019, 2013; Chadchan et al., 2016). Moreover, sEng might contribute to the immunoregulatory state supporting the late-stage development of ectopic endometriosis by lowering the angiogenesis and inhibiting the excessive immune cell infiltrations to clear the ectopic endometrium in the peritoneal microenvironment (Guerrero and McCarty, 2017). In our study, we observe the increasing levels of three TGF superfamily members on severe endometriosis implying the interplay of these molecules in proliferation and differentiation of endothelial cells in peritoneal environment. The further study is warranted to define the exact role and the source of sEng in the pathogenesis of peritoneal endometriosis.

Although the role of soluble mature GDF-15 is largely unknown in endometriosis, accumulating evidence has shown that GDF-15 is generally induced upon stress response-related to tissue injury (Emmerson et al., 2018). Increased serum GDF-15 is positively correlated with prognosis worsening of cardiovascular diseases, chronic kidney disease progression, and poor prognosis of some malignancy cases (Emmerson et al., 2018). In our study, late-stage endometriosis has shown a significant higher level of serum GDF-15 compared to the early-stage endometriosis. Peritoneal GDF-15 was also significantly higher compared to non-endometriosis control, especially in the fallopian and fibroid/myoma-related control pathology, but no difference with ovarian pathology (Fig. 3B). This may suggest that the regulation of this cytokine may be originated from ovarian or peritoneal environment. Indeed, GDF-15 was shown to be produced by ovarian granulosa and follicular cells as well as endometrial cells (Ruiz-Remolina et al., 2017; Monsivais et al., 2017a), supporting our observation on the increased GDF-15 levels in the pathological organs where the cells are highly populated. Hence, by dividing the control group we could gain adeeper insight into the regulation of the studied molecules.

Since the activation of GFRAL receptor by GDF-15 was known to induce anti-obesity effect and, in our study, the GDF-15 level is increased in endometriosis women, we tempted to speculate that there might be metabolic alterations such as lower body mass index (BMI) in endometriosis patients compared to control. Yong et al. has performed a meta-analysis study from 11 reports and suggested that indeed lower endometriosis risk has correlated with increased BMI (Welsh et al., 2003). Of those 11 studies, 3 studies were performed similar to our study subject inclusion with infertile women. Further *in vivo* study may be required to link the GFRAL activation in endometriosis. Beside extracellular expression, GDF-15 is known to accumulate in the cytoplasm and nucleus (Yong and Weiyuan, 2017). A previous study has examined the cellular expression of GDF-15 by immunostaining on endometrium tissues, which revealed significantly lower GDF-15 expression in eutopic endometrium tissue of endometriosis patients compared to healthy donor (Min et al., 2016). Since in a present study, the control is non-endometriotic related infertility cases, the outcome of local expression could differ with that in eutopic endometrium. In addition, the protein expression of eutopic and ectopic endometrium from endometriosis patients could also show a different expression profile due to different local inflammatory milieu (Seo et al., 2010).

An intriguing result from our study is the correlation between peritoneal sEng, GDF-15, TGF-β1, and clinical rASRM score (Fig. 5). Our observation on a positive correlation between peritoneal TGF-β1 and

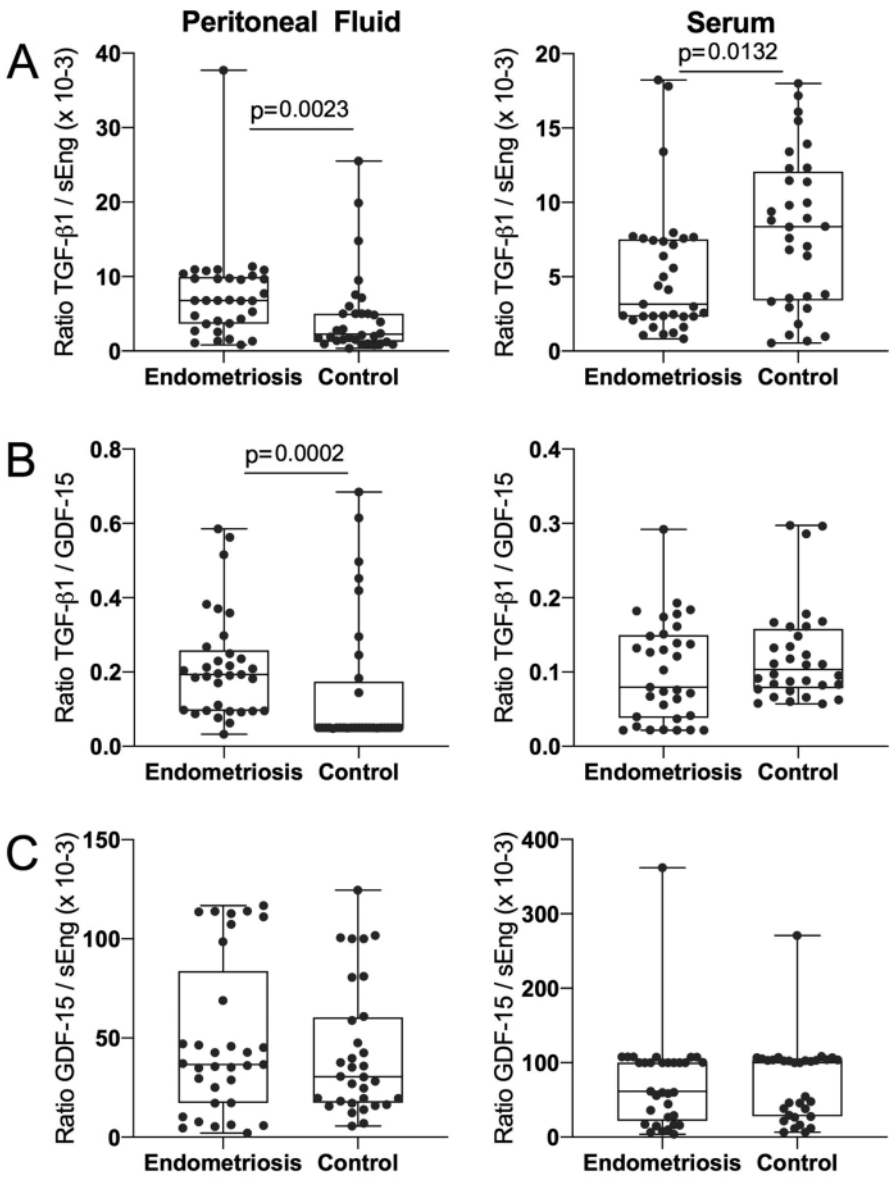

Fig. 4. The ratio of sEng, GDF-15 and TGF-β1 concentration in endometriosis and gynecologic control patients. The ratio of protein concentrations in peritoneal fluid and serum between TGF-β1 and sEng (A), TGF-β1 and GDF-15 (B), and GDF-15 and sEng (C) are shown. Box plot indicates the median and minimum-maximum values with dots representing each patient. Significant *p*-values are indicated where the TGF-β1/GDF-15 ratio in peritoneal fluid and serum, whereas TGF-β1/sEng ratio are significantly different between endometriosis and control group.

rASRM score with adhesion score element coincides well with previous evidence from patients and animal study (Choi et al., 2017; Marianowski et al., 2013; Chegini et al., 2001). Pelvic adhesion is generally considered as the leading cause of pelvic pain and infertility (Jin et al., 2016). A direct effect such as anatomical change of ovary and fallopian tube may lead to infertility. In addition to pelvic adhesion, sEng and TGF-β1 levels in the peritoneal fluid have correlated with dysmenorrheal symptom. Previous work by Tamburro et al. has demonstrated a significant increase of TGF-β1 in the nerve fibers of peritoneal endometriosis compared to non-endometriosis control (Chegini, 2008). In line with our study, they found a significant relationship between TGF-β1 expression and dysmenorrhea. Furthermore, we also found a significant increase of total leukocyte in endometriosis. Although this result was within normal range values, this data resembles the result of a previous study showing higher leukocyte count (WBC) (Tamburro et al., 2003). The possible explanation is because the chronic inflammatory nature of endometriosis (Zondervan et al., 2020). Additionally, the profile of neutrophil has positive correlation with peritoneal sEng, while myelomonocytic cells have a positive correlation with TGF-β1 in serum. TGF-β1 can be produced by a variety of immune cells including myeloid cells in peripheral circulation (Turgut et al., 2019). The mesothelial and myeloid cells in peritoneum from women with endometriosis was reported as the source of TGF-β1 (Young et al., 2014).

A noninvasive biomarker to distinguish the pathological condition in infertile women in various gynecological conditions is one of our main objectives. We chose the pathological control since the access of matched laparoscopic samples and hematological parameters were simultaneously available in the clinic. However, our control group included patients with gynecological pathology, which may also have an alteration in their inflammatory profiles. There were two main

Table 2
Correlation analysis between the levels of TGF-related molecules and clinical or laboratory parameters.

	Peritoneal fluid, r (*p*-value)			*Serum*, r (*p*-value)		
	sEng	GDF-15	TGF-β1	sEng	GDF-15	TGF-β1
Age	−0.100 (0.430)	−0.062 (0.626)	−0.013 (0.918)	−0.036 (0.774)	−0.016 (0.902)	−0.009 (0.943)
Year of infertility	0.187 (0.137)	−0.123 (0.330)	0.001 (0.999)	−0.002 (0.986)	0.067 (0.598)	−0.060 (0.999)
Dysmenorrhea	0.358 (0.003)*	0.106 (0.401)	0.256 (0.040)*	0.209 (0.911)	0.042 (0.737)	0.108 (0.393)
Endometriosis score[a]	0.091 (0.647)	0.258 (0.147)	0.072 (0.689)	0.230 (0.613)	0.035 (0.847)	0.035 (0.489)
Pelvic adhesion score	0.304 (0.014)*	0.238 (0.047)*	0.311 (0.012)*	0.089 (0.480)	0.020 (0.873)	0.138 (0.273)
Least function (LF) score	0.008 (0.948)	−0.141 (0.261)	−0.095 (0.450)	−0.131 (0.297)	0.110 (0.381)	−0.103 (0.415)
Total EFI score[a]	0.131 (0.466)	0.144 (0.425)	0.172 (0.340)	0.003 (0.988)	0.139 (0.442)	0.118 (0.514)
WBC count	0.292 (0.018)*	−0.025 (0.840)	0.254 (0.041)*	0.093 (0.461)	−0.008 (0.948)	0.151 (0.229)
Lymphocyte count	−0.003 (0.984)	0.044 (0.738)	0.085 (0.515)	0.094 (0.471)	0.036 (0.782)	0.087 (0.504)
Lymphocyte %	−0.242 (0.052)	−0.008 (0.950)	−0.088 (0.485)	0.111 (0.377)	0.146 (0.246)	−0.053 (0.676)
Mixed cells count	0.095 (0.488)	0.105 (0.307)	0.250 (0.066)	0.016 (0.270)	0.022 (0.872)	0.317 (0.018)*
Mixed cells %	0.039 (0.771)	0.135 (0.307)	0.214 (0.104)	0.016 (0.902)	0.060 (0.654)	0.184 (0.162)
Neutrophil count	0.307 (0.016)*	−0.102 (0.436)	0.152 (0.242)	0.140 (0.283)	−0.044 (0.737)	0.114 (0.382)
Neutrophil %	0.317 (0.011)*	−0.136 (0.284)	0.085 (0.504)	0.056 (0.660)	0.050 (0.697)	0.110 (0.386)
Red blood cell	0.004 (0.973)	−0.050 (0.693)	0.017 (0.894)	0.092 (0.464)	−0.020 (0.877)	0.025 (0.844)
Hemoglobin	0.114 (0.364)	0.041 (0.749)	0.077 (0.542)	0.062 (0.623)	−0.186 (0.139)	0.046 (0.718)
Hematocrit	0.009 (0.942)	0.074 (0.560)	0.094 (0.460)	0.028 (0.829)	−0.028 (0.825)	0.007 (0.957)
Platelet	−0.059 (0.643)	0.042 (0.741)	0.021 (0.869)	0.031 (0.811)	−0.034 (0.741)	0.236 (0.061)

[a] Within endometriosis cases.

* Significant *p*-value ($p < 0.05$).

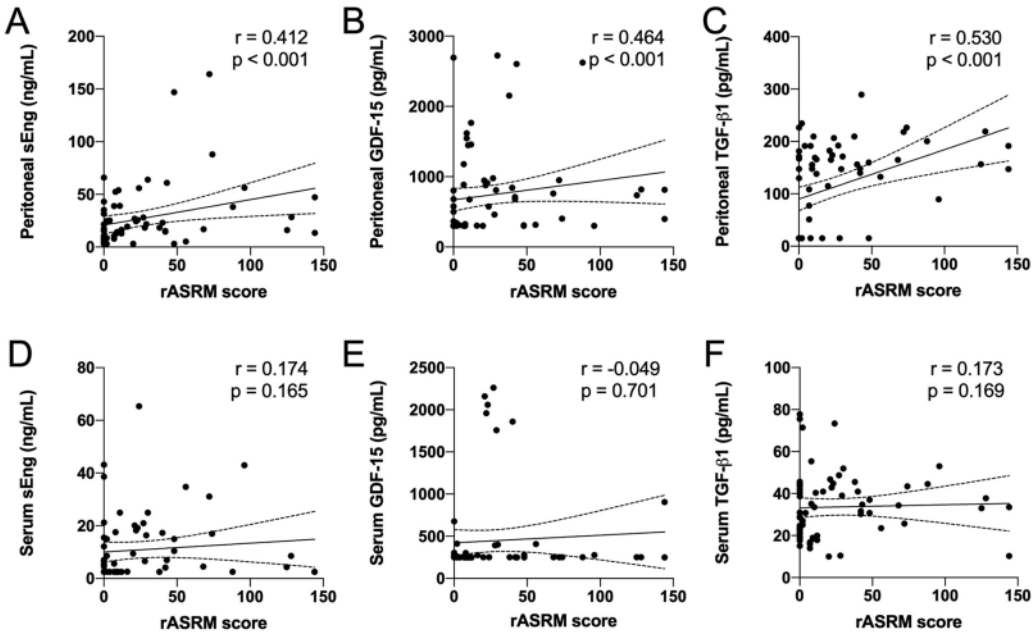

Fig. 5. Correlation of sEng, GDF-15 and TGF-β1 concentrations with rASRM total score. There are significant positive correlations of all three TGF members' concentrations with the rASRM score in peritoneal fluid (A—C), but not in serum (D—F). All rho coefficient r- and *p*-values with 95 % confidence bands are shown in the correlation scatterplot graphs with dots representing each patient.

considerations on the control division into three subcontrol groups. The first reason was the major anatomical localization of pelvic endometrial pathology in the female reproductive tract which primarily consist of fibroid or uterus, fallopian tube and ovaries. The pathology of control cases was non-endometriosis and closely linked to infertility diagnosis. We also realized that there are extrapelvic endometriosis which is considered as rare pathology but not within the scope of our study (Kim et al., 2004). The second reason was the localization of TGF-related members producing cells and their respective receptors. Different cellular compositions in the organ could lead to variable responses towards TGF beta-related signaling which potentially control the proper function of reproductive physiology (Machairiotis et al., 2013). Elucidating the newer members of TGF family expression would lead to our scientific understanding on their role in human reproduction. We observed that some patients with ovary-or myoma- related infertility have high levels of TGF-β-related members. Further study with the healthy control group or fertile endometriosis cases should be considered for more conclusive results.

Beside control inclusion, the other limitation in our study is the use of ELISA as the only technique to assess the soluble molecules. There are indeed various conventional tools to determine the human biomarkers such as western blot, immunohistochemistry and ELISA (Manole et al., 2018). Among those 3 options, ELISA is considered the most suitable option for soluble sample with higher accuracy (sensitivity), shorter processing time and lower cost (Monsivais et al., 2017b). Western blot and immunohistochemistry are mostly suitable for solid samples such as tissues. There are advanced tools as the substitutes for ELISA such as surface plasmon resonance imaging, luminex and bead-based flow cytometry. Further studies are obviously needed to develop the new protocol with these advanced tools to complement the ELISA method and to better assess the newer member of TGF beta family.

In conclusion, our novel data demonstrated the potential new mediators sEng and GDF-15 in endometriosis-associated infertility. The elevated extracellular sEng and GDF-15 expressions in the peritoneal fluid support the notion that TGF-β-related regulation is involved in the development of endometriosis lesion. Serum GDF-15 is a potential biomarker to differentiate the severity of endometriosis. Further studies are warranted to evaluate the role of these molecules in cellular and molecular levels.

Author contribution

B.S. and F.A. developed the concept and design of this study; B.S., N. Y.R., A.S., S.R.D., A.T., M.YA.W., and A.F.M. collected the samples and provided clinical parameters; B.S., F.A., N.Y.R., and A.F.M. wrote the manuscript; F.A., A.F.M, and N.Y.R. performed the experiment and analyzed data; B.S., F.A., A.S., S.R.D., J.Y.A., A.T., and M.Y.A.W. interpreted the data.

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Declaration of Competing Interest

The authors report no declarations of interest.

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6. Elevated peritoneal soluble endoglin and GDF-15 in infertile women with severe endometriosis and pelvic adhesion

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