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Original Article

Gynaecology

TGFBI as a candidate biomarker for non-invasive diagnosis of early-stage endometriosis

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ABSTRACT

STUDY QUESTION: Can cartilage oligomeric matrix protein (COMP) and transforming growth factor-β-induced protein ig-h3 (TGFBI) alone or in combination with cancer antigen 125 (CA-125) be considered as potential blood biomarkers of endometriosis?

SUMMARY ANSWER: The results of this study indicate that COMP has no diagnostic value. TGFBI has potential as a non-invasive biomarker of the early stages of endometriosis, while TGFBI together with CA-125 has similar diagnostic characteristics as CA-125 alone for all stages of endometriosis.

WHAT IS KNOWN ALREADY: Endometriosis is a common, chronic gynecological disease that significantly affects patient quality of life by causing pain and infertility. The gold standard for diagnosis is visual inspection of pelvic organs by laparoscopy, therefore there is an urgent need for discovery of non-invasive biomarkers for endometriosis to reduce diagnostic delays and allow earlier treatment of patients. The potential biomarkers for endometriosis evaluated in this study (COMP and TGFBI) were previously identified by our proteomic analysis of peritoneal fluid samples.

STUDY DESIGN, SIZE, DURATION: This is a case–control study divided into a discovery (n = 56 patients) and a validation phase (n = 237 patients). All patients were treated between 2008 and 2019 in a tertiary medical center.

PARTICIPANTS/MATERIALS, SETTING, METHOD: Patients were stratified based on the laparoscopic findings. The discovery phase included 32 endometriosis patients (cases) and 24 patients with confirmed absence of endometriosis (controls). The validation phase included 166 endometriosis and 71 control patients. Concentrations of COMP and TGFBI were measured by ELISA in plasma samples, whereas concentration of CA-125 was measured using a clinically validated assay for serum samples. Statistical and receiver operating characteristic (ROC) curve analyses were performed. The classification models were built using the linear support vector machine (SVM) method with the SVM built-in feature ranking method.

MAIN RESULTS AND THE ROLE OF CHANCE: The discovery phase revealed significantly increased concentration of TGFBI, but not COMP, in plasma samples of patients with endometriosis compared to controls. In this smaller cohort, univariate ROC analysis showed fair diagnostic potential of TGFBI, with an AUC value of 0.77, sensitivity of 58%, and specificity of 84%. The classification model built using linear SVM and combining TGFBI and CA-125 showed an AUC value of 0.91, sensitivity of 88% and specificity of 75% in distinguishing patients with endometriosis from controls. The validation phase results revealed similar diagnostic characteristics of the SVM model combining TGFBI and CA-125, with an AUC value of 0.83, sensitivity of 83% and specificity of 67% and CA-125 alone with AUC value of 0.83, sensitivity of 73% and specificity of 80%. TGFBI exhibited good diagnostic potential for early-stage endometriosis (revised American Society for Reproductive Medicine stage I–II), with an AUC value of 0.74, sensitivity of 61% and specificity of 83% compared to CA-125, which had an AUC value of 0.63, sensitivity of 60% and specificity of 67%. An SVM model combining TGFBI and CA-125 showed a high AUC value of 0.94 and sensitivity of 95% for diagnosing moderate-to-severe endometriosis.

LIMITATIONS, REASONS FOR CAUTION: The diagnostic models were built and validated from a single endometriosis center, and thus further validation and technical verification in a multicenter study with a larger cohort is needed. Additional limitation was lack of histological confirmation of disease for some patients in the validation phase.

WIDER IMPLICATIONS OF THE FINDINGS: This study revealed for the first time increased concentration of TGFBI in plasma samples of patients with endometriosis, particularly those with minimal-to-mild endometriosis, compared to controls. This is the first step in considering TGFBI as a potential non-invasive biomarker for the early stages of endometriosis. It also opens a path for new basic research to investigate the importance of TGFBI in the pathophysiology of endometriosis. Further studies are needed to confirm the diagnostic potential of a model based on TGFBI and CA-125 for the non-invasive diagnosis of endometriosis.

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Introduction

Endometriosis is a common gynecological benign disease with a complex pathophysiology that is characterized by endometrial-like tissue outside the uterine cavity (Saunders and Horne, 2021). The disease significantly compromises the quality of life of women and is a major cause of infertility (Zondervan *et al.*, 2020; Saunders and Horne, 2021). Despite major efforts of research groups around the world, endometriosis is still poorly understood. The gold standard for diagnosis is surgical visual inspection of pelvic organs. Current advanced minimally invasive laparoscopy is still an invasive surgical procedure with general anesthesia, endotracheal intubation, and potential perioperative and postoperative complications. Laparoscopy is especially needed to confirm superficial peritoneal endometriosis (PE) that cannot be diagnosed using imaging techniques (Hsu *et al.*, 2010; Nisenblat *et al.*, 2016a). Because of non-specific symptoms and surgery as a standard diagnostic procedure, 6–7 years (on average) can pass before women are diagnosed and properly treated (Nnoaham *et al.*, 2011; Janša *et al.*, 2021b). Therefore, biomarker research was defined as a research priority in 2011 by the World Endometriosis Research Foundation (Rogers *et al.*, 2013, 2017).

In the last decades, numerous molecules identified in biological fluids have been considered as non-invasive biomarkers of endometriosis, including glycoproteins, cytokines, hormones, micro RNAs (miRNAs), long non-coding RNAs (lncRNAs), growth factors, and markers of oxidative stress, apoptosis, cell adhesion, and angiogenesis (Rižner, 2014; Anastasiu *et al.*, 2020; Hudson *et al.*, 2020; Tian *et al.*, 2020; Maier and Maier, 2021; Janša *et al.*, 2021b). Recently, high-throughput technologies (e.g. proteomics, genomics, and metabolomics) have enabled the detection of different proteins, genes, polymorphisms, miRNA molecules, metabolites, and lipids associated with endometriosis (Goulielmos *et al.*, 2020). Among the proposed biomarkers, the most investigated is glycoprotein cancer antigen 125 (CA-125) (Anastasiu *et al.*, 2020). Significantly higher serum CA-125 levels are commonly reported in patients with advanced stages of endometriosis. However, CA-125 measurements alone lack the specificity and sensitivity to detect endometriosis and replace current diagnostic techniques (Mol *et al.*, 1998; Nisenblat *et al.*, 2016b). Nevertheless, several studies showed improved performance of CA-125 when combined with other blood biomarkers (Nisenblat *et al.*, 2016b).

Our research group has identified several individual biomarker candidates in peritoneal fluid and blood and also by using metabolomics and proteomics approaches (Vouk *et al.*, 2012; Kocbek *et al.*, 2015; Vouk *et al.*, 2016; Janša *et al.*, 2021a). Endometriosis is most commonly found in the intraperitoneal space with peritoneal fluid, and thus we hypothesized that investigating peritoneal fluid might help identify blood biomarkers for the non-invasive diagnosis of endometriosis (Rižner, 2015; Janša *et al.*, 2021a). The surface of the peritoneal cavity is large and allows passive dialysis of substances between peritoneal fluid and blood plasma (Koninckx *et al.*, 1998; Bedaiwy and Falcone, 2003; Young *et al.*, 2013; Rižner, 2015). However, peritoneal fluid sampling is more invasive than peripheral blood sampling, and any attempts at identifying clinically useful biomarkers must aim towards being as non-invasive as possible.

We have recently published a prospective case–control study of peritoneal fluid analysis that included a discovery and a validation phase (Janša *et al.*, 2021a). In the discovery phase, we used a proteomics approach with high-content antibody protein microarrays targeting 1360 different proteins with 1830 antibodies (Sciomics GmbH, Heidelberg, Germany). We included 12 women with primary infertility, who were divided into a group of six women with laparoscopically and histologically confirmed endometriosis and a control group of six women with unexplained primary infertility. Peritoneal fluid samples were collected during laparoscopy, and 16 proteins were found to be >1.5-fold upregulated in the endometriosis group compared to the control group. We selected angiotensinogen, transforming growth factor-β-induced protein ig-h3 (TGFBI) and cartilage oligomeric matrix protein (or thrombospondin-5; COMP) for validation. To the best of our knowledge, these proteins have not been previously studied in peritoneal fluid or blood from patients with endometriosis. Thus, we analyzed the concentrations of these proteins in a larger group of patients with endometriosis (n = 32) and controls (n = 24) using commercially available ELISAs. In peritoneal fluid we found significant differences in the concentrations of COMP and TGFBI and small, but non-significant, differences in angiotensinogen. A classification model based on a linear support vector machine (SVM) combining all three proteins revealed very good diagnostic potential with an AUC of >0.83, sensitivity of 81%, and specificity of 100%.

The aim of the current study was to evaluate COMP and TGFBI alone and in combination with CA-125 as potential blood biomarkers of endometriosis. First, we measured the concentrations of COMP, TGFBI, and CA-125 in blood samples of the same cohort of patients as in our peritoneal fluid study (Janša *et al.*, 2021a). Next, we assessed the concentrations of significantly increased proteins (TGFBI and CA-125) in a larger independent validation cohort of patients and built classification models that included concentrations of both proteins.

Materials and methods

Study design and patient selection

The study was designed as a case–control study and was conducted with the approvals of The Medical Ethics Committee of the Republic of Slovenia (No. 0120-049/2016-4 (discovery phase); No. 0120-127/2016-2 and No. 0120-541/2019/7 (validation phase)). Written informed consent was obtained from all participants included in the study.

The study was divided into discovery and validation phases (Fig. 1). The discovery phase comprised the same patient cohort as in our previous study (Janša *et al.*, 2021a): patients with primary infertility (n = 56), who had either endometriosis (cases; n = 32) or unexplained primary infertility (controls; n = 24). The validation phase included a new cohort of 237 patients, which were divided into endometriosis patients or cases (n = 166) and controls (n = 71). All the patients underwent laparoscopy owing to clinical indications (infertility and/or symptoms indicative of endometriosis; Supplementary Data). All the patients included in the discovery phase (Table 1) had a BMI in the normal range, a regular menstrual cycle (21–35 days), and normal results of

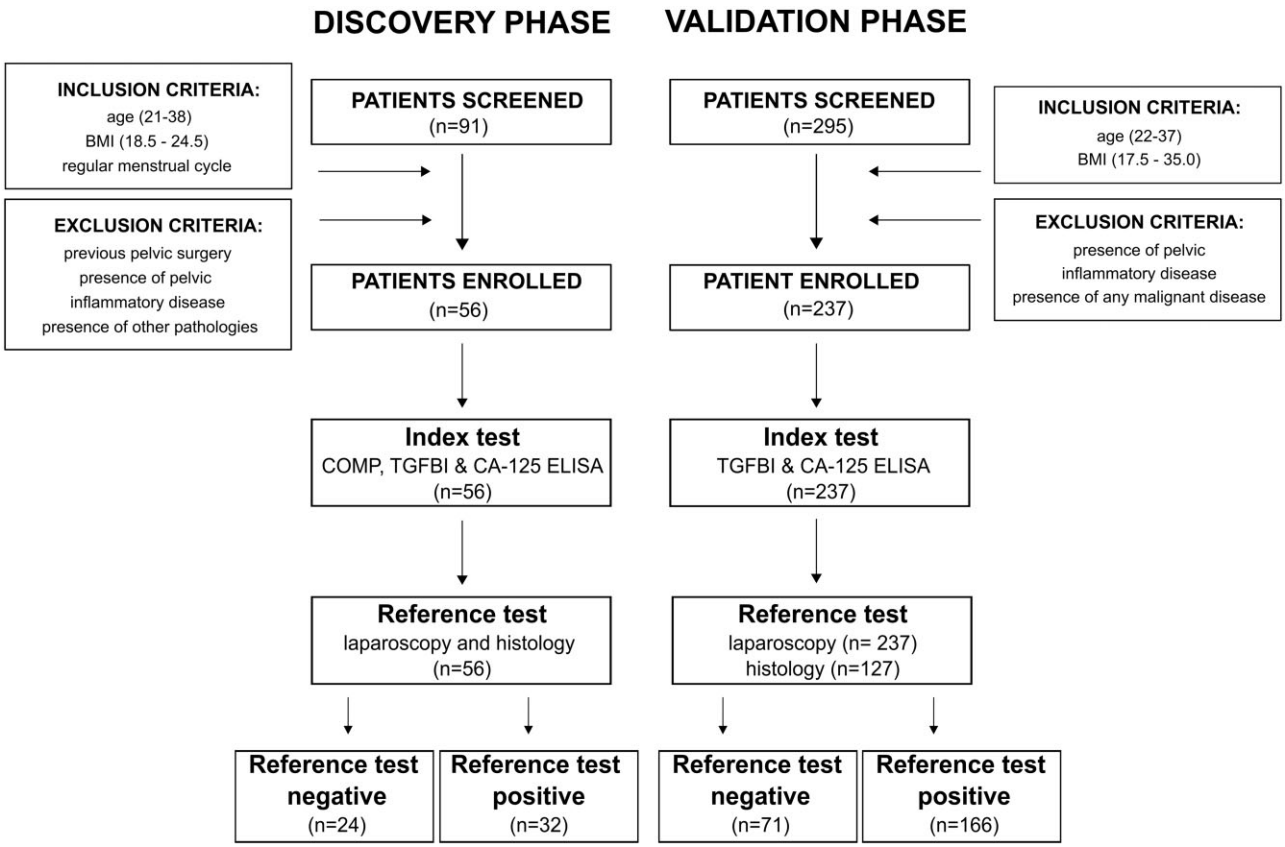

Figure 1. Flowchart of patient recruitment for the discovery and validation phases of a study to identify biomarkers of endometriosis. Cartilage oligomeric matrix protein (COMP); transforming growth factor-β-induced protein ig-h3 (TGFBI), cancer antigen 125 (CA-125). BMI shown in kg/m^2 and age in years.

partner semen analyses. The exclusion criteria for discovery phase were hormonal therapy in the last year, irregular menstrual cycles, autoimmune diseases, malignant or suspected malignant diseases, previous pelvic surgery, previous pelvic inflammatory disease, previous pathologies (controls) other than endometriosis (cases) (as observed by ultrasound examination), leiomyoma uteri, and polycystic ovaries. Patients included in the validation phase (Table 1) had a BMI between 17.5 and 35.0 kg/m^2. The exclusion criteria for validation phase were presence of pelvic inflammatory or malignant disease. Although inclusion/exclusion criteria differed between discovery and validation cohorts, the majority of patients in validation phase had similar characteristics as discovery phase patients. More than 80% of validation phase patients had regular menstrual cycles (89%), normal BMI (83%), and had not undergone previous gynecological surgeries (87%).

Sample and data collection

All the patients who met the inclusion criteria were additionally evaluated. They filled out an extensive questionnaire developed by our research group (Vouk *et al.*, 2012) on their health history, stress levels, medication use, diet, lifestyle habits, and types of pain (dysmenorrhea, dyspareunia, or chronic pain) using a validated visual analogue scale (Wewers and Lowe, 1990). A gynecologist filled out another questionnaire with gynecological and clinical information about each patient, including length and regularity of menstrual cycle, use of peroral contraception and/or hormonal therapy in the past and in the last 3 months before surgery, use of medications 1 week before surgery, type and reason for surgery, type of endometriosis, revised American Society for Reproductive Medicine (rASRM) stage, color of lesions, histological confirmation of endometriosis, menstrual phase determined by ultrasound and additional pathological observations. Stratification was carried out based on the laparoscopic and histological results: the case and control groups included patients with and without endometriosis, respectively.

Blood samples were collected 1 day before surgery according to a strict standard operating procedure (Rizner and Adamski, 2019). Briefly, two tubes of blood (each containing 4 ml of sample) were collected from each patient. To obtain plasma, blood was taken into BD Vacutainer tubes containing EDTA (#368861, Becton Dickinson and Company, NJ, USA). To obtain serum, BD Vacutainer tubes with a gel separator and clot activator (#369032, Becton Dickinson and Company, NJ, USA) were used. Within 1 h after collection, the samples were centrifuged at 2500×*g* (plasma) and 3000×*g* (serum) for 10 min at 4°C. Plasma and serum were aspirated, aliquoted (120 µl), and stored at −80°C until analysis.

ELISAs

Analysis of samples was performed using commercially available ELISA kits according to the manufacturer's instructions. The following ELISA kits were purchased for analysis of plasma samples: TGFBI (MyBioSource, San Diego, CA, USA; Catalogue No. #MBS177286; Lot No. #7481763813 (discovery phase), Lot No. #748171151118 (validation phase)) and COMP (Merck Millipore, Saint Louis, MO, USA; Catalogue No. #RAB1764-1KT; Lot No. #0524I2396). Serum samples were analyzed using a clinically validated electrochemiluminescent immunoassay for CA-125 on an immunoassay analyzer (Cobas e411, Roche Diagnostics GmbH, Manheim, Germany).

Table 1. Clinical characteristics of the patients included in the discovery and validation phase of the study.

		Discovery phase			Validation phase			
Parameter	Detail	Controls, n = 24	Cases, n = 32	P	Controls, n = 71	Cases, n = 166	P	P[a]
Age (years) mean ± SD	/	30.1 ± 4.2	29.1 ± 3.7	0.369	30.3 ± 4.0	30.4 ± 4.2	0.981	0.134
BMI (kg/m^2) mean ± SD	/	23.8 ± 1.7	22.9 ± 3.5	0.371	22.8 ± 3.2	21.9 ± 2.8	0.022	0.374
Reason for surgery, n (%)	Infertility	21 (88)	20 (62)	0.097	46 (65)	55 (33)	<0.001	0.004
	Pain	2 (8)	7 (22)		7 (10)	33 (20)		
	Infertility + pain	0 (0)	0 (0)		1 (1)	28 (17)		
	Cysts	0 (0)	0 (0)		12 (17)	12 (7)		
	Pain + cysts	0 (0)	0 (0)		2 (3)	17 (10)		
	Infertility + cysts	0 (0)	0 (0)		2 (3)	11 (7)		
	Other	1 (4)	5 (16)		1 (1)	10 (6)		
Menstrual phase, n (%)	Follicular phase	11 (46)	20 (63)	0.280	32 (45)	81 (49)	0.283	0.064
	Luteal phase	13 (54)	12 (37)		29 (41)	72 (43)		
	OHC	0 (0)	0 (0)		10 (14)	13 (8)		
Oral contraceptives in the last 3 months, n (%)	No	24 (100)	32 (100)	>0.999	65 (92)	143 (86)	0.286	0.002
	Yes	0 (0)	0 (0)		6 (8)	23 (14)		
Hormonal therapy in the last 3 months, n (%)	No	24 (100)	32 (100)	>0.999	56 (79)	146 (88)	0.068	0.001
	Yes	0 (0)	0 (0)		15 (21)	20 (12)		
Medications in the last week, n (%)	No	21 (87.5)	27 (84)	>0.999	52 (73)	109 (66)	0.289	0.008
	Yes	3 (12.5)	5 (16)		19 (27)	57 (34)		
Smoking status, n (%)	Nonsmoker	18 (75)	25 (78)	0.560	40 (56)	105 (63)	0.248	0.222
	Smoker	4 (17)	3 (9)		18 (25.5)	39 (23.5)		
	Occasional smoker	2 (8)	0 (0)		5 (7)	11 (7)		
	Former smoker	0 (0)	4 (13)		7 (10)	10 (6)		
	Not available	0 (0)	0 (0)		1 (1.5)	1 (0.5)		
Type of endometriosis, n (%)	Peritoneal	/	5 (16)	/	/	48 (29)	/	0.134
	Ovarian	/	9 (28)		/	25 (15)		
	Ovarian plus peritoneal	/	15 (47)		/	51 (31)		
	Deep	/	3 (9)		/	6 (3.5)		
	Peritoneal + deep	/	0 (0)		/	6 (3.5)		
	Ovarian + deep	/	0 (0)		/	11 (7)		
	Peritoneal + ovarian + deep	/	0 (0)		/	19 (11)		
rASRM, n (%)	I	/	8 (25)	/	/	45 (27)	/	0.764
	II	/	0 (0)		/	22 (13.5)		
	III	/	22 (69)		/	61 (37)		
	IV	/	2 (6)		/	37 (22)		
	NA	/	0 (0)		/	1 (0.5)		

n: number; percentages in brackets; rASRM: revised American Society for Reproductive Medicine score; OHC: oral hormonal contraceptives; P: P value of the comparison tests between controls and cases for discovery and validation cohort; P[a]: P value of the comparison tests between discovery and validation cohort.

Statistical analyses

Significant differences in protein concentrations between groups were determined with the following steps. First, the robust outlier removal (ROUT) method with Q set to 1% was used to detect outliers, which were not included in the further analysis. The dataset without outliers was tested for normality using Shaphiro–Wilk tests. For normally and non-normally distributed data, the unpaired Student's t-test and Mann–Whitney test were used, respectively. Statistical analysis was performed using GraphPad Prism 9.3 (GraphPad Software, San Diego, CA, USA). The level of significance was set at $P < 0.05$. The receiver operating characteristic (ROC) curve analysis was performed using the Biomarker Analysis module in the MetaboAnalyst 5.0 software (Pang *et al.*, 2021). In brief, the validation dataset had missing values for CA-125 (n = 13, 5.5%), which were replaced by the column mean value of each group (mean substitution) (de Goeij *et al.*, 2013). For individual proteins, univariate ROC curve analysis was performed, and AUC and 95% CI were calculated (using the 500-bootstrapping method). The optimal cutoff was set using the point closest to the left-top corner (Hoo *et al.*, 2017). For a given cutoff, sensitivity and specificity were calculated. Protein combinations were analyzed using multivariate ROC curve exploratory analysis, and the ROC curves were generated using Monte–Carlo cross validation (Xu and Liang, 2001). The classification models were built using the linear SVM method with the SVM built-in feature ranking method (Li *et al.*, 2002; Chang and Lin, 2008). Numerical characteristics of patients were compared using unpaired Student's *t*-test or Mann–Whitney test, while for comparison of categorical data Fisher's exact test or Chi-square test for trend was used.

Results

Clinical characteristics of patients

Discovery phase

The clinical characteristics of the patients included in the discovery phase (32 cases and 24 controls) are presented in Table 1 and were previously described in detail (Janša *et al.*, 2021a). The mean ages of the patients were 29.1 ± 3.7 years (mean ± SD, cases) and 30.1 ± 4.2 years (mean ± SD, controls). The BMI values were in the normal range: 22.9 ± 3.5 kg/m^2 (mean ±SD, cases) and 23.8 ± 1.7 kg/m^2 (mean ± SD, controls), and the menstrual cycles were regular in the control and case groups, respectively (21–35 days). The main reason for laparoscopy in both case and control groups was infertility and/or pain. In the case and control groups, 63% and 46% of samples, respectively, were collected in the follicular phase of the menstrual cycle. The remaining samples were collected in the luteal phase of the menstrual cycle. There were no significant differences between the case and control groups for any of the characteristics examined. According to

the laparoscopic results, 16% of the patients had PE, 28% ovarian endometriosis (OV), 47% combined ovarian endometriosis with peritoneal lesions (PE+OV), and 9% deep endometriosis (DE) (Supplementary Table S1). Of the cases, 75% had stage III–IV disease, according to the 1996 Revised American Society for Reproductive Medicine Classification of Endometriosis (rASRM, 1997).

Validation phase

The clinical characteristics of the patients included in the validation phase (166 cases and 71 controls) are presented in Table 1. The case and control groups were similar in terms of age, menstrual cycle phase, use of oral contraception or hormonal therapy 3 months before surgery, use of medications 1 week before surgery, and smoking status. The mean ages of the patients in the case and control groups were 30.4 ± 4.2 years (mean ± SD) and 30.3 ± 4.0 (mean ± SD) years, respectively. The BMI was slightly but significantly lower ($P = 0.02$) in the case group (21.9 ± 2.8 kg/m^2, mean ± SD) compared to the control group (22.8 ± 3.2 kg/m^2, mean ± SD). In the control group, infertility was the predominant reason for surgery, while both infertility and pain were mostly present in the endometriosis group. The percentages of patients in the follicular or luteal menstrual cycle phases at the time of sample collection were similar between the control (45% follicular, 41% luteal) and case group (49% follicular, 43% luteal). Laparoscopic examination and histological analysis confirmed that 29% of cases had PE, 15% OV, 31% PE+OV, and 3.5% DE. For the rest of the patients in the case group, DE was diagnosed in combination with peritoneal lesions (3.5%), OV (7%), or both (11%). According to the rASRM classification, the case group included 40% of patients with stage I–II and 60% patients with stage III–IV endometriosis. Statistical comparison of the clinical characteristics of the discovery and validation cohorts revealed no differences between the studied groups in age, BMI, menstrual phase, smoking status, type of endometriosis, and rASRM stage. However, a statistical difference was found in the use of oral contraception 3 months before surgery ($P = 0.002$), the use of hormonal therapy 3 months before surgery ($P = 0.001$), and the use of medication one week before surgery ($P = 0.008$) (Table 1)

TGFBI in combination with CA-125 had similar diagnostic characteristics as CA-125 alone

Guided by our observation of increased TGFBI and COMP concentrations in peritoneal fluid from patients with endometriosis (Janša *et al.*, 2021a), here we analyzed the concentrations of these two proteins in plasma samples from the same cohort of patients (discovery phase). In plasma samples, concentrations of COMP did not differ between cases and controls (Fig. 2A), whereas TGFBI levels were higher in cases compared to controls ($P = 0.0007$; Fig. 2B). In serum samples from the same cohort, concentrations of CA-125 were significantly higher in the cases compared to controls ($P < 0.0001$; Fig. 2C). ROC analysis revealed that COMP has no diagnostic potential, with an AUC value of 0.52, sensitivity of 67%, and specificity of 50% at the 260 ng/ml cutoff point (Fig. 2A). TGFBI showed fair diagnostic potential, with an AUC of 0.77 and 95% CI of 0.61–0.88 (Fig. 2B). With the cutoff point selected nearest to the top left-most corner of the ROC curve (1120 ng/ml), TGFBI showed a sensitivity of 58% and specificity of 84%. CA-125 enabled good separation of cases and controls in the discovery set of patients, with an AUC value of 0.86 (95% CI = 0.74–0.94, sensitivity = 88%, and specificity = 74% at cutoff of 26.4 U/ml) (Fig. 2C). Thus, we created additional classification models based on linear SVM combining different sets of proteins. The created model combining all three proteins reached an AUC of 0.89 with a 95% CI of 0.78–0.98, sensitivity of 88% and specificity of 75%. When TGFBI and CA-125 were combined, the AUC value slightly increased to 0.91 with a 95% CI of 0.79–0.98 (Fig. 2D), whereas the sensitivity (88%) and specificity (75%) remained the same.

Based on the results from the discovery phase, we decided to measure concentrations of TGFBI and CA-125 in blood samples in a larger cohort (validation set of patients). TGFBI and CA-125 concentrations were higher in blood samples of cases compared to controls (each $P < 0.0001$; Fig. 3). The ROC curve analysis showed an AUC of 0.69 with a 95% CI of 0.62–0.76 for TGFBI (Fig. 3A). With the cutoff set to 1500 ng/ml, the sensitivity and specificity of TGFBI were 61% and 74%, respectively. CA-125 showed better separation of cases and controls, with an AUC of 0.83 and a 95% CI of 0.78–0.88. With the cutoff set to 17.7 U/ml, the sensitivity and specificity of CA-125 were 73% and 80%, respectively (Fig. 3B). Compared to CA-125 alone, the ROC curve generated using a linear SVM combining both proteins (CA-125 and TGFBI) showed the same AUC value of 0.83 (95% CI = 0.78–0.88), with an increased sensitivity of 83% and slightly decreased specificity of 67% (Fig. 3C).

The validation cohort included patients with significant differences in BMI between cases and controls. In addition, some of the patients from the validation cohort were taking hormonal therapy in the last 3 months before surgery. Therefore, we performed an additional analysis to assess whether these clinical characteristics had an impact on TGFBI and CA-125 levels in patients' blood samples. The Spearman nonparametric correlation test was performed to check if BMI impacted levels of both TGFBI and CA-125. This analysis revealed a very weak association between the parameters (BMI versus TGFBI and BMI versus CA-125) with Spearman correlation coefficient (r) close to 0 and P values >0.05 (Supplementary Fig. S1). Further, in order to investigate if hormonal therapy affected TGFBI and CA-125 levels, an additional analysis was done after excluding the patients using hormonal therapy in the last 3 months before laparoscopy (Supplementary Table S2). For TGFBI, there was no difference in AUC, sensitivity and specificity values, while for CA-125, slightly higher AUC (0.83 versus 0.76), sensitivity (72.6% versus 68.5%) and specificity (79.5% versus 70.1%) values were obtained before excluding patients with hormonal therapy in the last 3 months before surgery. ROC curve analysis using a linear SVM with combination of both proteins (CA-125 and TGFBI) showed no difference in AUC values (0.83) between these two cohorts (Supplementary Table S2).

TGFBI has potential in diagnosing minimal-to mild-endometriosis

Concentrations of TGFBI were higher ($P < 0.0001$) in patients with minimal-to-mild endometriosis (rASRM I-II) compared to controls, and ROC analysis showed fair separation of cases and controls (AUC = 0.74, 95% CI = 0.68–0.82, sensitivity = 61%, and specificity = 83% at the cutoff of 1510 ng/ml) (Fig. 4A). Although CA-125 concentrations were significantly higher in early-stage endometriosis patients compared to controls ($P = 0.01$), they showed poor diagnostic potential (AUC = 0.63, 95% CI = 0.55–0.73, sensitivity = 60%, and specificity = 67% at the cutoff of 15.2 U/ml) (Fig. 4B). In distinguishing early-stage endometriosis patients from controls, TGFBI performed similarly alone as in combination with CA-125 (SVM model, AUC = 0.74, 95% CI = 0.68–0.86, sensitivity = 68%, specificity = 62%) but with an increased sensitivity of 68% and decreased specificity (Fig. 4C).

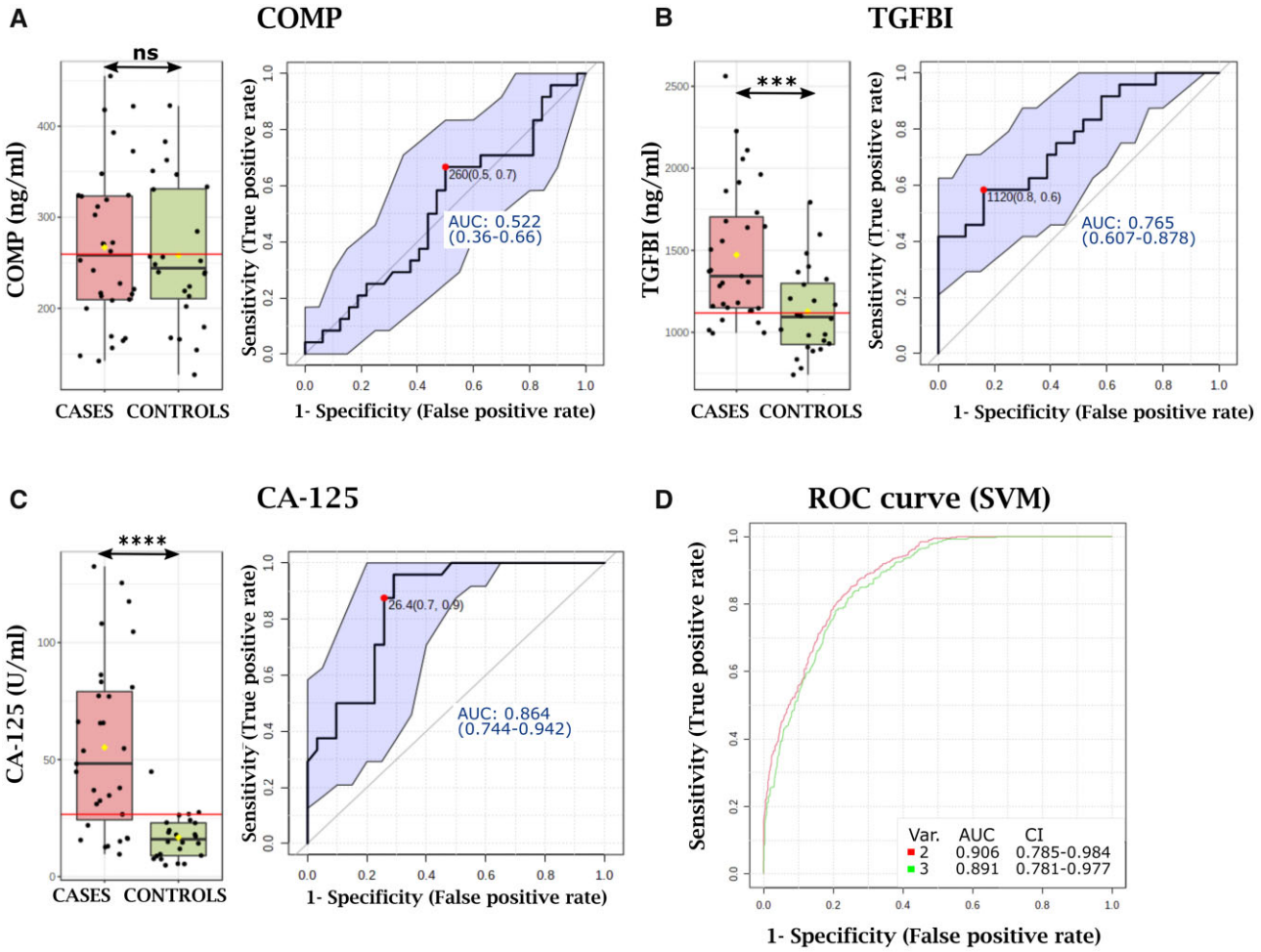

Figure 2. Protein levels of COMP, TGFBI, and CA-125 in patients from the discovery phase of the study. Concentrations and ROC curve analyses of (**A**) COMP, (**B**) TGFBI in plasma, and (**C**) CA-125 in serum samples of patients from the discovery phase of the study (control patients n = 24; patients with endometriosis n = 32). (**D**) The ROC curve for the created model using a linear SVM combining three (COMP, TGFBI, and CA-125) or two (TGFBI and CA-125) proteins. Data are presented as box and whiskers plots with values from lower to upper quartiles (25th to 75th percentile) and median centered. Optimal cutoff values are marked with red dots on the ROC curves and red horizontal lines on the box plots. Statistical differences between groups were determined using the unpaired Student's *t*-test for COMP and Mann–Whitney test for TGFBI and CA-125 (***$P = 0.0007$, ****$P < 0.00001$). ROC: receiver operating characteristic; COMP: cartilage oligomeric matrix protein/thrombospondin-5; TGFBI: transforming growth factor-β-induced protein ig-h3; CA-125: cancer antigen 125; SVM: support vector machine.

Conversely, TGFBI performed more poorly in distinguishing patients with moderate-to-severe endometriosis (rASRM III-IV) from controls (AUC = 0.66, 95% CI = 0.57–0.74, sensitivity = 63%, and specificity = 66%) (Fig. 4D). Compared to TGFBI, CA-125 showed very good diagnostic performance for advanced stages of endometriosis (AUC = 0.93, 95% CI = 0.88–0.96, sensitivity = 86%, and specificity = 83% at the cutoff of 23.8 U/ml) (Fig. 4E). However, its combination with TGFBI slightly improved the diagnostic performance, especially the sensitivity, for advanced stages of endometriosis (AUC = 0.94, 95% CI = 0.9–0.98, sensitivity = 95%, and specificity = 78%), when compared with the ROC curve of CA-125 alone (Fig. 4E and F).

TGFBI performs best in distinguishing PE patients from controls

We also investigated the relation between the concentrations of both proteins (TGFBI and CA-125) and the type of endometriosis. Concentrations of TGFBI were significantly higher in plasma samples of patients with PE ($P < 0.0001$), DE ($P = 0.013$), and DE together with other types of endometriosis (PE+DE ($P = 0.0170$), OV + DE ($P = 0.0003$), PE + OV + DE ($P = 0.016$)), compared to controls (Fig. 5A). Concentrations of CA-125 were significantly higher in patients with OV ($P < 0.0001$) and OV together with other types of endometriosis (PE + OV ($P < 0.0001$), OV + DE ($P < 0.0001$), PE + OV + DE ($P < 0.0001$), compared to controls (Fig. 5B). However, it should be noted that the number of patients with DE, PE + DE, OV + DE, and PE + OV + DE included in the study was relatively low, which can affect results of statistical analysis for the mentioned types of endometriosis.

ROC curve analysis showed that TGFBI performed best in distinguishing PE patients from controls (AUC = 0.76, 95% CI = 0.66–0.85, sensitivity = 58%, specificity = 89%) (Table 2). Although TGFBI showed even higher AUC values in separating controls from patients with DE (AUC = 0.80), PE + DE (AUC = 0.79), and OV + DE (AUC = 0.83), the sample size in these groups was low, and the 95% CI range was wider (Table 2). CA-125 had the highest AUC values for patients with OV (AUC = 0.83) and OV together with other types of endometriosis: PE + OV (AUC = 0.93), OV + DE (AUC = 0.98), and PE + OV + DE (AUC = 0.98). Linear SVM models combining both proteins (TGFBI and CA-125) did not reveal increased performances as compared to individual proteins in predicting any type of the endometriosis (Table 2).

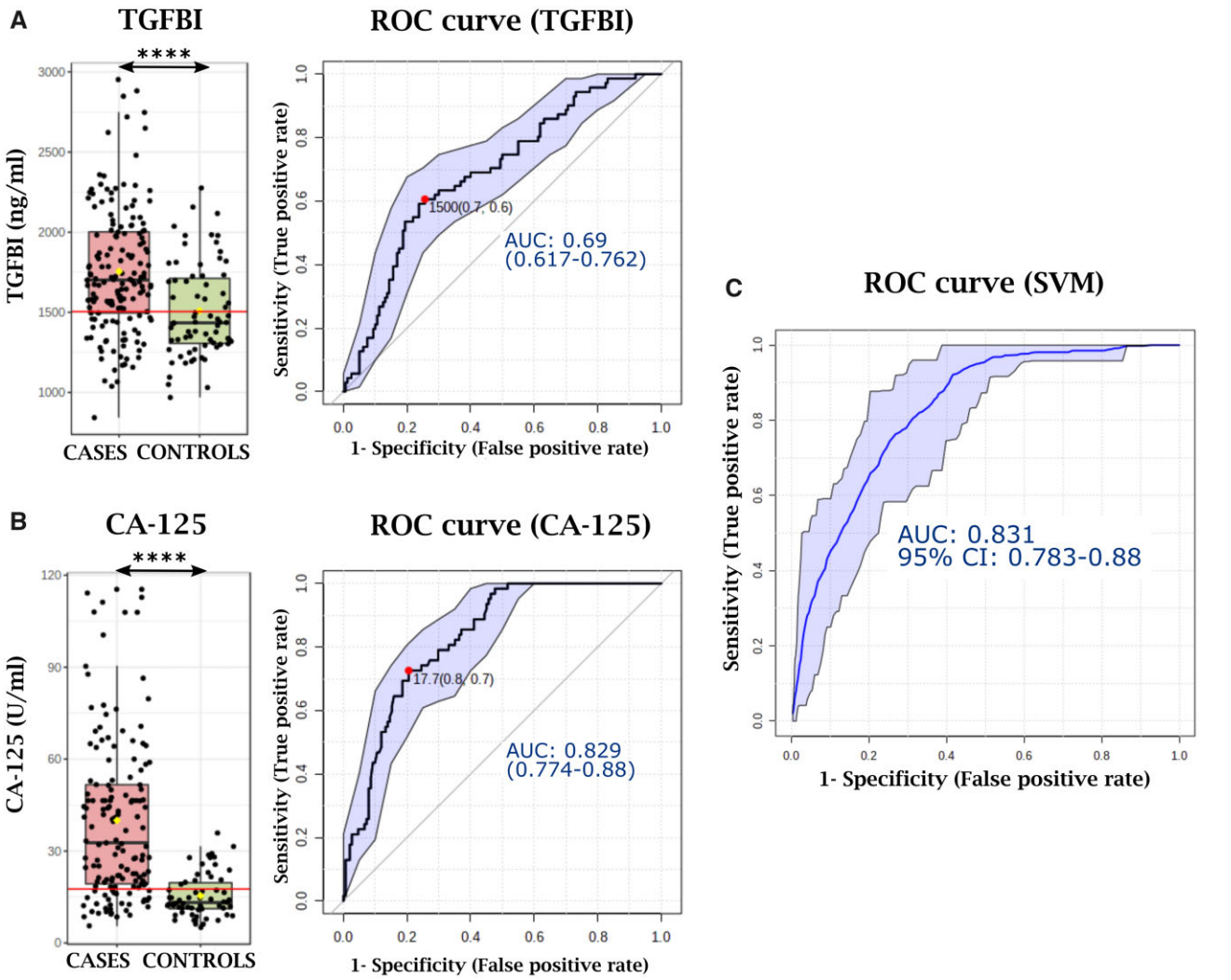

Figure 3. Protein levels of TGFBI and CA-125 in patients from the validation phase cohort. Concentrations and ROC curve analyses of (**A**) TGFBI in plasma and (**B**) CA-125 in serum samples of patients from validation phase cohort (control patients n = 71; patients with endometriosis n = 166). (**C**) The ROC curve for the created model using a linear SVM combining TGFBI and CA-125. Data are presented as box and whiskers plots with values from lower to upper quartiles (25th to 75th percentile) and median centered. Optimal cutoff values are marked with red dots on the ROC curves and red horizontal lines on the box plots. Statistical differences between groups were determined using the Mann–Whitney test (****$P < 0.0001$). ROC: receiver operating characteristic; TGFBI: transforming growth factor-β-induced protein ig-h3; CA-125: cancer antigen 125; SVM: support vector machine.

Diagnostic potential of TGFBI and CA-125 was not impacted by menstrual cycle phase

To investigate the relation between the concentrations of both proteins (TGFBI and CA-125) and the menstrual cycle phase, an additional analysis using patients only in their secretory or proliferative menstrual phase at the time of laparoscopic surgery was performed. The results are presented in Supplementary Fig. S2 and Supplementary Table S3. No significant differences in AUC, sensitivity or specificity values for TGFBI, CA-125 alone or combination of these two proteins was seen between patients in their secretory or proliferative menstrual phase compared to patients in both phases (Supplementary Table S3).

Discussion

In this study, we explored whether COMP and TGFBI have potential as non-invasive blood biomarkers for endometriosis, either alone or in combination with CA-125. The study revealed fair diagnostic potential of TGFBI in detection of minimal-to-mild endometriosis. The SVM model combining TGFBI and CA-125 showed similar diagnostic characteristics as CA-125 for all stages of endometriosis combined. However, the SVM model showed better AUC and sensitivity for early stage endometriosis patients as compared to CA-125. As advanced stages of endometriosis can be diagnosed using imaging techniques, biomarkers for early stage endometriosis are needed in clinical practice, which supports the relevance of this candidate biomarker. In addition, our results demonstrate that TGFBI combined with CA-125 (AUC = 0.94, sensitivity = 95%, and specificity = 78%) can be considered as a triage test to 'rule out' moderate-to-severe endometriosis with high accuracy if there is a negative test result (i.e. a SnOUTtest) and can be used as add-on test, in addition to existing diagnostics. Triage tests (SpIN and SnOUT) were proposed as an initial step in the diagnostic procedure to identify patients who need or do not need further testing (Nisenblat *et al.*, 2016b). To confirm the usefulness of the proposed biomarker, further studies on larger cohorts are needed.

Discovering non-invasive biomarkers is crucial for replacing surgical procedures and reducing diagnostic delay (Tian *et al.*, 2020). To date, most of the discovered potential biomarkers have already been discarded in the preclinical stage, and thus there

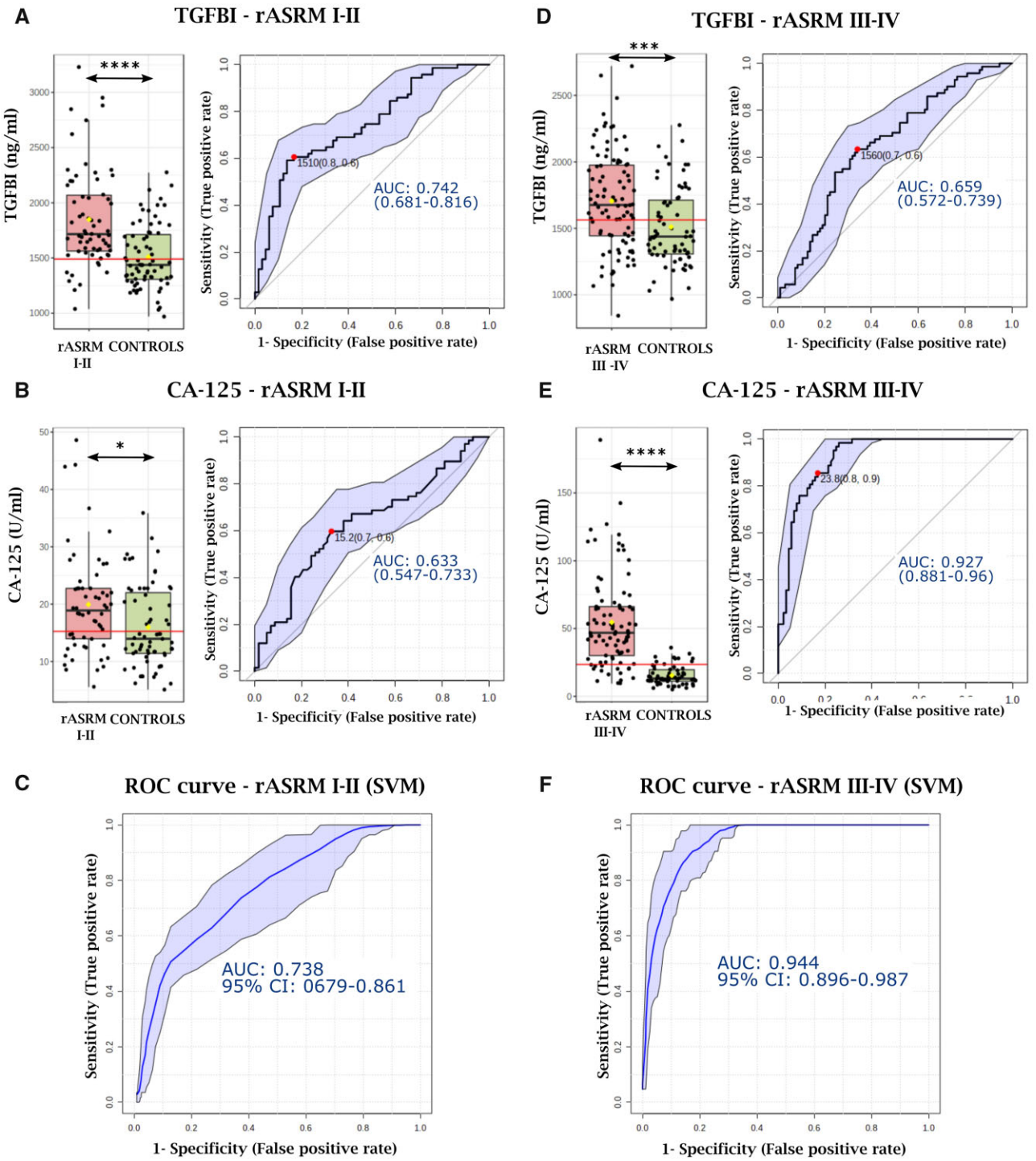

Figure 4. Protein levels of TGFBI and CA-125 in control patients and patients with stage I-II or stage III-IV endometriosis. Concentrations and ROC curve analyses of (**A**, **D**) TGFBI and (**B**, **E**) CA-125 measured in blood samples of controls and patients with (**A**, **B**) rASRM stages I–II or (**D**, **E**) stages III–IV endometriosis. Patients included in this analysis are from the validation phase of the study (control patients n = 71; patients with rASRM stage I–II n = 67; patients with rASRM stage III–IV n = 98). ROC curves for created models combining TGFBI and CA-125 for (**C**) early and (**F**) advanced stages of endometriosis. Data are presented as box and whiskers plots with values from lower to upper quartiles (25th to 75th percentile) and median centered. Optimal cutoff values are marked with red dots on the ROC curves and red horizontal lines on the box plots. Statistical differences between groups were determined using the Mann–Whitney test (*$P = 0.0108$, ***$P = 0.0005$, ****$P < 0.0001$). ROC: receiver operating characteristic; TGFBI: transforming growth factor-β-induced protein ig-h3; CA-125: cancer antigen 125; rASRM; revised American Society for Reproductive Medicine.

are no reliable clinical markers for the diagnosis and prognosis of endometriosis (May *et al.*, 2010; Rižner, 2014; Nisenblat *et al.*, 2016b; Anastasiu *et al.*, 2020; Janša *et al.*, 2021b). Non-invasive tests would be particularly useful for patients with minimal-to-mild endometriosis to avoid unnecessary surgical procedures and enable early disease detection (Fassbender *et al.*, 2013). While

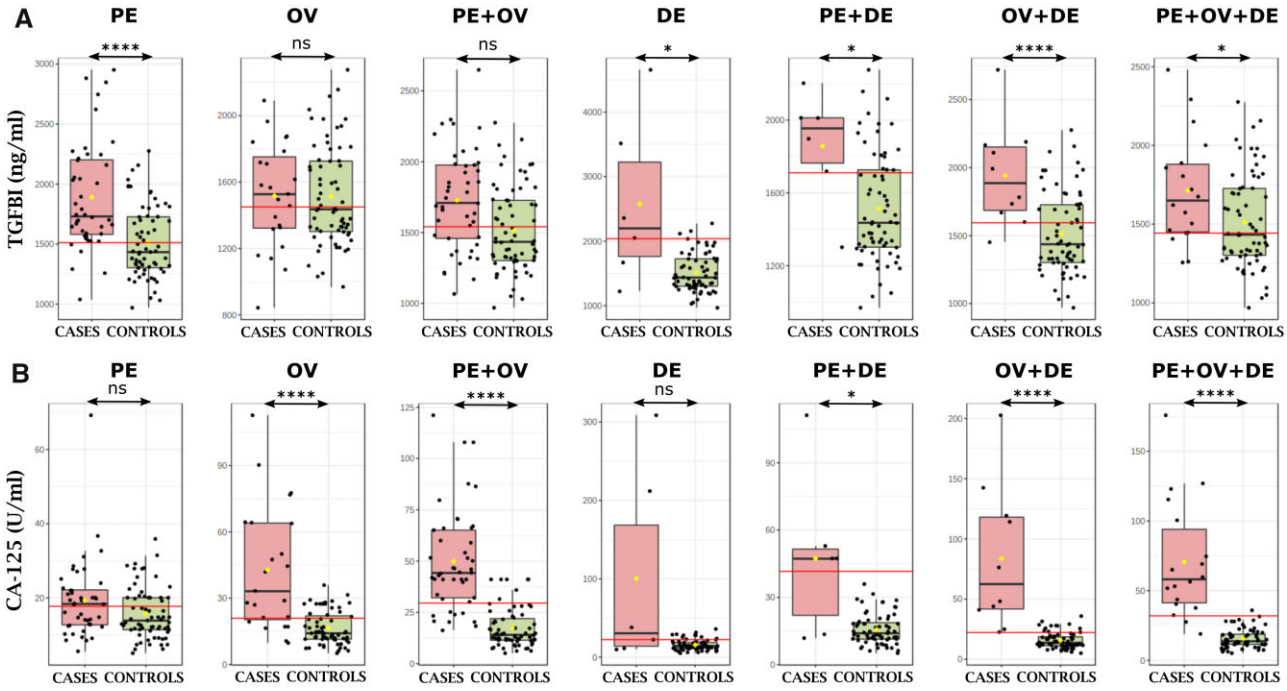

Figure 5. Protein levels of TGFBI and CA-125 in control patients and patients with different types of endometriosis. Concentrations of (**A**) TGFBI and (**B**) CA-125 measured in blood samples of controls and patients with different types of endometriosis. Patients included in this analysis are from the validation phase of the study (control patients n = 71; patients with PE n = 48; patients with OV n = 25; patients with DE n = 6; patients with PE and OE n = 51; patients with PE and DE n = 6; patients with OE and DE n = 11; patients with PE and PE and DE n = 19). Data are presented as box and whiskers plots with values from lower to upper quartiles (25th to 75th percentile) and median centered. Optimal cutoff values are marked with red horizontal lines on the box plots. Statistical differences between groups were determined using the Mann–Whitney test (ns: not significant, $^{*}P < 0.05$, $^{****}P < 0.0001$). PE: peritoneal endometriosis; OV: ovarian endometriosis; DE: deep endometriosis; TGFBI: transforming growth factor-β-induced protein ig-h3; CA-125: cancer antigen 125.

Table 2. Values for the cutoff, AUC, sensitivity, specificity, and 95% CI of TGFBI, CA-125, and SVM models combining TGFBI and CA-125 for different types of endometriosis.

EM TYPE	TGFBI					CA-125					TGFBI and CA-125	
	C/O (ng/ml)	AUC	95% CI	SEN (%)	SP (%)	C/0 (U/ml)	AUC	95% CI	SEN (%)	SP (%)	AUC	95% CI
PE	1510	0.76	0.66–0.85	58	89	17.7	0.61	0.51–0.71	67	55	0.75	0.65–0.86
OV	1450	0.52	0.39–0.67	54	61	20.9	0.83	0.71–0.93	73	74	0.80	0.66–0.94
PE + OV	1540	0.68	0.58–0.78	60	69	29.7	0.93	0.89–0.97	90	80	0.94	0.87–0.98
DE	2040	0.80	0.49–0.99	96	67	22.0	0.72	0.40–0.97	82	67	0.56	0.00–0.99
PE + DE	1710	0.79	0.56–0.96	73	83	41.6	0.81	0.57–1.00	100	67	0.83	0.00–1.00
OV + DE	1600	0.83	0.72–0.93	64	80	22.5	0.98	0.92–1.00	84	100	0.97	0.89–1.00
PE + OV + DE	1440	0.68	0.54–0.80	52	83	31.9	0.98	0.94–1.00	99	89	0.98	0.93–1.00

EM: endometriosis; PE: peritoneal endometriosis; OV: ovarian endometriosis; DE: deep endometriosis; TGFBI: transforming growth factor-β-induced protein ig-h3; CA-125: cancer antigen 125; C/O: cutoff; SEN: sensitivity; SP: specificity.

imaging techniques, such as transvaginal ultrasound and MRI, can be useful in identifying OV and DE, superficial PE can only be confirmed by histological evaluation of tissue collected after laparoscopy (Nisenblat *et al.*, 2016a).

Several biomarkers for early-stage endometriosis have been identified in eutopic endometrial tissue samples of patients with endometriosis. Annexin V was increased in secretory phase endometrium from women with minimal-to-mild endometriosis compared to controls (Kyama *et al.*, 2011). Purine and amino acid metabolites were detected in eutopic endometrium of patients with minimal-to-mild endometriosis (Li *et al.*, 2018). However, they were not validated in the patients' blood samples. Other studies proposed non-invasive biomarkers for early-stage endometriosis. Zachariah *et al.* showed increased levels of circulating cell-free nucleic DNA in the plasma of patients with minimal-to-mild endometriosis compared to controls, with a sensitivity of 70% and specificity of 87%. However, the study had a small number of patients (n = 37) without validation in a larger cohort (Zachariah *et al.*, 2009). Gajbhiye *et al.* suggested a panel of six biomarkers (anti-tropomodulin 3 b-autoAb, anti-TMOD3c-autoAb, anti-TMOD3d-autoAb, anti-tropomyosin 3a-autoAb, anti-TPM3c-autoAb, and anti-TPM3d-autoAb) for the diagnosis of minimal-to-mild endometriosis with a sensitivity of ≥60% and specificity of ≥80% (Gajbhiye *et al.*, 2017). A recently identified potential serum biomarker of endometriosis, galactin-9, showed high diagnostic potential to distinguish patients with endometriosis from controls (AUC = 0.97, sensitivity = 94%, and specificity = 93%); however, in relatively low numbers of patients (n = 77) and healthy controls (n = 30). Another drawback of this study was the lack of validation in a larger independent cohort (Brubel *et al.*, 2017). In recent years, a number of studies have attempted to evaluate the suitability of circulating miRNAs as non-invasive

biomarkers for endometriosis (Maier and Maier, 2021; Monnaka *et al.*, 2021). Some of these studies reported good diagnostic characteristics for certain miRNA molecules (let-7b) (Cho *et al.*, 2015) or miRNA panels (miR-122, miR-145, miR-199a, miR-542-3p) (Wang *et al.*, 2016), (miR-122 and miR-199a) (Maged *et al.*, 2018) for detecting endometriosis. However, these studies have several limitations including small sample size, lack of an independent validation cohort, and conflicting results as the same miRNA was downregulated in blood samples from patients with endometriosis in one study and upregulated in another (Monnaka *et al.*, 2021). Recently, Bendifallah *et al.* presented a saliva-based miRNA signature consisting of 109 miRNAs for diagnosis of endometriosis (Bendifallah *et al.*, 2022). The study included 153 patients with endometriosis and 47 controls, and the miRNA signature was generated using a Random Forest algorithm reaching a sensitivity, specificity, and AUC of 96.7%, 100%, and 98.3%, respectively. The proposed miRNA signature may potentially improve the diagnosis of endometriosis, but its suitability should be confirmed in an independent validation cohort. Compared to the mentioned studies, our study revealed that TGFBI alone exhibits high specificity (83%) and satisfactory sensitivity (61%) for detecting early-stage endometriosis (rASRM stage I–II), while TGFBI combined with CA-125 shows higher sensitivity (68%) but lower specificity (62%). This potential of TGFBI should be further investigated in a larger cohort and multi-center study, and in combination with other biomarkers to assess whether its potential can be improved.

Previous studies reported increased serum CA-125 levels in patients with endometriosis compared to controls, particularly in OV and advanced stages of endometriosis (Hirsch *et al.*, 2016). The low diagnostic potential of CA-125 for early stages of endometriosis is likely the reason why TGFBI alone performed better than in combination with CA-125 when detecting early stages of endometriosis. In addition, several studies have assessed the potential of CA-125 in combination with other blood biomarkers. CA-125 was tested together with CCR1 mRNA and monocyte chemotactic protein-1 for determining the presence or absence of endometriosis and reached a sensitivity of 92% and specificity of 82% (Agic *et al.*, 2008). Furthermore, very good diagnostic values were observed for the following combinations: CA-125 and prolactin (sensitivity = 77% and specificity = 88% for PE patients versus controls) (Bilibio *et al.*, 2014); CA-125, syntaxin-5, and laminin-1 (sensitivity = 95% and specificity = 70% for patients with rASRM stages I-II versus controls) (Ozhan *et al.*, 2014); CA-125, IL-6, IL-8, tumor necrosis factor-α, high-sensitivity C-reactive protein, and cancer antigen CA-19-9 (sensitivity = 91% and specificity = 86% for patients with rASRM stage III–IV versus controls) (Mihalyi *et al.*, 2010); and annexin V, vascular endothelial growth factor CA-125, and sICAM-1/or glycodelin (sensitivity = 81–90% and specificity = 63–81% for patients with rASRM stages I–II or III–IV versus controls in different menstrual cycle phases) (Vodolazkaia *et al.*, 2012). However, most of the mentioned studies did not perform a validation in an independent cohort. The study by Vodolazkaia *et al.* (2012) was the only one that included both discovery and validation sets of patients. In their subsequent study, they conducted additional technical verification and independent validation of proposed biomarker models. Unfortunately, the initially proposed biomarker models were not validated; however, a high correlation was found for CA-125 between the first and second study (Dorien *et al.*, 2019). This suggests that even though CA-125 alone is not sensitive enough to act as an endometriosis marker, it could be used in combination with other potential biomarkers. In our current study, TGFBI alone exhibited good diagnostic values for detecting endometriosis in the validation cohort (AUC = 0.7, sensitivity = 60%, and specificity = 74%). Thus, we built a model combining TGFBI and CA-125, which exhibited improved diagnostic values (AUC = 0.83, sensitivity = 83%, and specificity = 67%), but similar AUC when compared to CA-125 alone (AUC = 0.83, sensitivity =73%, specificity =80%).

Our study confirms that discovery of biomarkers in peritoneal fluid samples may help identify blood biomarkers of endometriosis. Three proteins in peritoneal fluid were selected based on the antibody array analysis, and one of them was increased in the blood samples of patients with endometriosis. The protein TGFBI is located in the extracellular matrix and plays important roles in cell proliferation, differentiation, adhesion, migration, embryonic development, and inflammation (Skonier *et al.*, 1992; Kim *et al.*, 2002; Thapa *et al.*, 2007). The roles of TGFBI have also been established in nephropathy, atherosclerosis, rheumatoid arthritis, corneal disorders, and malignant diseases (Ween *et al.*, 2012). It was demonstrated that TGFBI expressed by peritoneal cells can increase motility and invasion of ovarian cancer cells (Ween *et al.*, 2011). Conversely, TGFBI loss in ovarian cancer cells is protumorigenic, whereas TGFBI overexpression in peritoneal cells aids the metastatic process of ovarian cancer cells on peritoneum. (Ween *et al.*, 2012). In malignant diseases, TGFBI can either suppress or promote tumors, and reports are suggesting that TGFBI can mediate cancer cell invasion and metastasis and enhance cancer cell extravasation (Tang *et al.*, 2009; Ma *et al.*, 2012; Shang *et al.*, 2012; Guo *et al.*, 2018). TGFBI was previously associated with endometriosis in only two studies. Burney *et al.* reported higher *TGFBI* mRNA levels in endometrium from women with moderate to severe endometriosis in their early secretory phase (Burney *et al.*, 2007). Arimoto *et al.* analyzed gene-expression profiles of ovarian endometrial cysts from 23 patients using cDNA microarray consisting of 23 040 genes. They compared expression patterns between endometriotic tissues and corresponding eutopic endometria and found that *TGFBI* was upregulated in endometrial cysts throughout the menstrual cycle (Arimoto *et al.*, 2003). We already detected increased TGFBI levels in peritoneal fluid (Janša *et al.*, 2021a) and are now the first to report higher TGFBI levels in plasma samples of patients with endometriosis. This is also the first study to associate TGFBI with minimal-to-mild endometriosis and to evaluate the combination of TGFBI and CA-125 as blood biomarkers of endometriosis.

The strength of our study is its good design, organized as a continuation of our discovery/validation study using peritoneal fluid and consisting of independent discovery and validation studies using blood samples. Our study included well-characterized cohorts with matched case and control groups in both the discovery and validation phases, and no significant differences in age, menstrual cycle phase, use of hormonal or oral contraception in the last 3 months before surgery, or smoking status. The case and control groups also had similar BMI values in the discovery phase. However, in the validation phase, controls had slightly higher BMI values compared to controls. Additional analysis showed that BMI had no effect on TGFBI and CA-125 levels (Supplementary Fig. S1). Blood samples from both control and case groups were obtained from similar patient populations, both presenting symptoms suggestive of endometriosis (infertility and/or pain). This is also the patient population that would benefit most from biomarker discovery, as such patients are commonly appointed for laparoscopy to confirm or refute the presence of endometriosis after other pathologies have been eliminated. Furthermore, the percentages of patients regarding

the stage of endometriosis (rASRM I-II and rASRM III-IV) and menstrual cycle phase (luteal and follicular) were similar in both subgroups to reduce pre-analytical bias. Additional advantages of our study are the use of previously established protocols for patient recruitment; extensive questionnaires for the collection of patients' personal, clinical, and gynecological data; and blood samples from the same biobank (which minimized potential sample variability). Sample collection, processing, and storage were conducted according to a previously established strict standard operating procedure to ensure analytical reliability and reproducibility (Rizner and Adamski, 2019). Finally, rather than reporting only statistical differences between examined groups, we built a diagnostic model using the linear SVM, and assessed and reported the main results of the diagnostic tests, including the following values: AUC, specificity, sensitivity, and 95% CI at certain cutoff values.

As a limitation of this study one can consider the inclusion of patients on hormonal therapy in the last 3 months before surgery in the validation phase. However, the patients that would benefit most from biomarker discovery are usually treated with hormonal therapy and the percentage of such patients did not differ between controls and cases. Another limitation is lack of histological confirmation of disease for some of the patients included in the validation phase. The current study lacks inclusion of patients from different clinical centers and technical verification of results (execution of experiments by another researcher in a different laboratory setting using ELISA assays from another manufacturer). Finally, the construction of classification models with more than two biomarkers could improve the performance of diagnostic tests. All the stated limitations will be addressed in our future studies. Moreover, future studies should investigate the role of TGFBI in the pathophysiology of endometriosis.

Conclusion

In this study, we investigated the potential of COMP and TGFBI alone or in combination with CA-125 as non-invasive blood biomarkers of endometriosis. To the best of our knowledge, this is the first study to reveal the presence of TGFBI in blood samples of patients with endometriosis and to associate TGFBI with minimal-to-mild and PE. This study consisted of discovery and validation phases, included a selected population of patients, and was conducted following established standard operating procedures. Our validation study revealed TGFBI as a potential non-invasive biomarker for the early stages of endometriosis. Further studies should validate the proposed biomarker in a larger multicenter cohort and investigate the involvement of TGFBI in the pathophysiology of endometriosis.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

V.J.: conception and design of study, sample collection, interpretation of data, writing of the manuscript; M.P.: sample collection, experimental work, data analysis, interpretation of data, writing of the manuscript; H.B.F.: conception and design of study, critical revision and final approval of the manuscript; T.L.R.: conception and design of study, critical revision and final approval of the manuscript, funding.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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