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Soluble VCAM-1/soluble ICAM-1 ratio is a promising biomarker for diagnosing endometriosis

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STUDY QUESTION: Do cell adhesion molecules play a role in endometriosis, and can they be used as a biomarker for diagnosing endometriosis?

SUMMARY ANSWER: Altered expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in the endometrium and peritoneum may play a key role in endometriosis and the soluble VCAM-1/soluble ICAM-1 ratio is a promising biomarker.

WHAT IS KNOWN ALREADY: Cell adhesion molecules are cell surface proteins that mediate cellular adherence, inflammatory and immune responses, and cancer-related biological processes. Altered expression of VCAM-1 and ICAM-1 in women with endometriosis has been investigated previously; however, gene expression levels in tissues and protein levels in the serum have not been investigated in the same patients.

STUDY DESIGN SIZE, DURATION: We performed a prospective, longitudinal study (the Endometriosis Marker Austria) in patients who underwent a laparoscopy for benign gynecological pathology in a university-based tertiary referral center for endometriosis. From a total of 138 women who were included in the study from July 2013 through September 2014, 97 had not received hormonal treatment for at least 3 months prior to recruitment and were included in the analysis; 49 (50.5%) of these women had endometriosis, and the 48 (49.5%) who did not have endometriosis served as a control group.

PARTICIPANTS/MATERIALS SETTING METHODS: During laparoscopy, tissue samples were obtained from ectopic and eutopic endometrium, and from normal pelvic peritoneum. In addition, serum samples were collected immediately before and 6–10 weeks after surgery. The mRNA levels of VCAM-1, ICAM-1 and epithelial cell adhesion molecule (EpCAM) were measured using quantitative real-time PCR, and serum protein levels of soluble VCAM-1 (sVCAM-1), ICAM-1 (sICAM-1) and EpCAM (sEpCAM) were measured using ELISA and correlated with endometriosis status.

MAIN RESULTS AND THE ROLE OF CHANCE: The mRNA levels of both VCAM-1 and ICAM-1 were higher in ectopic endometriotic lesions than in eutopic endometrium ($P < 0.001$). Moreover, the mRNA levels of both VCAM-1 and ICAM-1 were higher in normal peritoneum samples obtained from women with endometriosis compared to those from controls ($P = 0.038$ and $P = 0.009$). The mRNA levels of VCAM-1 were also higher in the eutopic endometrium samples obtained from women with endometriosis compared to controls ($P = 0.018$). With respect to serum protein levels, compared to controls, the women with endometriosis had lower serum levels of sICAM-1 ($P = 0.042$) and higher levels of sVCAM-1 ($P < 0.001$). Our analysis revealed that the serum levels of sVCAM-1 were not affected by lesion entity, menstrual cycle phase or disease severity. An receiver operating characteristics curve, calculated to determine whether preoperative serum sVCAM-1 concentration can be used to predict endometriosis, found an AUC of 0.868 with 80% specificity and 84% sensitivity at a cutoff value of 370 pg/ml. This predictive performance can be further improved by calculation of the sVCAM-1/sICAM-1 ratio, leading to an AUC of 0.929 with 86.7% specificity and 90.3% sensitivity at a cutoff ratio value of 1.55.

LARGE SCALE DATA: Not applicable.

LIMITATIONS REASONS FOR CAUTION: The relatively small sample size in the expression analyses is a possible limitation of this study.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings could contribute to an improved understanding of the pathogenesis of endometriosis and the role of cell adhesion molecules. In addition, the results may lead to the development of new, non-invasive tools for diagnosing endometriosis. The ability to diagnose patients by measuring serum sVCAM-1 levels or the sVCAM-1/sICAM-1 ratio would have considerable clinical value.

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Key words: endometriosis / biomarker / cell adhesion molecules / VCAM-1 / ICAM-1

Introduction

Endometriosis, which is characterized by the growth of endometrial stroma and glands outside of the uterine cavity, is estimated to occur in 6–10% of women of reproductive age (Giudice, 2010; Falcone and Lebovic, 2011). Although the clinical presentation of endometriosis varies widely, patients typically present with chronic pelvic pain, dysmenorrhea, dyspareunia and infertility (Bulun, 2009).

Non-surgical approaches for diagnosing endometriosis, such as transvaginal sonography, pelvic magnetic resonance imaging and blood tests, could not replace surgery as the current gold standard for diagnosis (Mol *et al.*, 1998; Brosens *et al.*, 2004). Thus, in the majority of cases, laparoscopy is needed in order to establish a definitive diagnosis (May *et al.*, 2010). As a result, the mean interval between the onset of symptoms and obtaining a definitive diagnosis is ~10 years (Hadfield *et al.*, 1996). Given that endometriosis has such severe clinical consequences, significantly reducing quality of life, the development of a non-surgical method (e.g. a serum biomarker) for detecting endometriosis as early as possible would greatly reduce the burden associated with endometriosis and reduce the associated socio-economic costs.

A major obstacle in the development of new strategies for diagnosing endometriosis is our limited understanding of the etiology and pathogenesis of this condition. One of the oldest and most widely accepted hypotheses is that endometriosis originates from endometrial cells that implant on peritoneal surfaces following retrograde menstruation (Sampson, 1927). However, it remains unclear why only some women develop endometriosis, given that retrograde menstruation occurs in the majority of women (Halme *et al.*, 1984; Liu and Hitchcock, 1986; O *et al.*, 2017).

Cell adhesion molecules are cell surface proteins that mediate adherence between cells and the extracellular matrix. In addition to their well-described role in inflammatory and immune responses, cell adhesion molecules are also involved in cancer-related processes, including cell survival, detachment and migration; therefore, these proteins appear to play a key role in tumourigenesis, tumor progression and metastasis (Springer, 1990; Ohene-Abuakwa and Pignatelli, 2000; Jung *et al.*, 2012). In order to better understand the pathophysiology of endometriosis and facilitate the search for a non-invasive candidate biomarker for diagnosing endometriosis, it is essential to gain insight into: (i) the mechanisms involved in the adhesion and invasion of endometrial cells and/or tissue in the peritoneal cavity and (ii) the differences between women with endometriosis and women without endometriosis.

In the present study, we measured the expression levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and epithelial cell adhesion molecule (EpCAM) in the endometrium and pelvic peritoneum of women with endometriosis and women without endometriosis (controls). In addition, we examined whether the soluble forms of these cell adhesion molecules can be measured in the serum as a non-invasive biomarker for diagnosing endometriosis.

Materials and Methods

Study design and study population

Tissue and blood samples were collected in accordance with the protocols of the Endometriosis Marker Austria (EMMA) study, a prospective cohort study conducted at the Endometriosis Referral Center of the Medical University of Vienna. Premenopausal women 18–50 years of age who were undergoing a laparoscopic procedure due to suspected endometriosis, infertility, chronic pelvic pain, benign adnexal masses or uterine leiomyoma were invited to participate in the EMMA study. Women who were pregnant at the time or breastfeeding up to 6 months prior to the beginning of the study and women who had acute inflammation, known or suspected infectious disease, chronic autoimmune disease or malignancy were excluded from the study.

In addition to a biopsy of the ectopic endometriosis lesion, biopsies were also obtained from the eutopic endometrium and unaffected peritoneum during the laparoscopy. Samples of eutopic endometrium were obtained by curettage, and peritoneal biopsies were obtained using sharp dissection of the peritoneum overlying the right or left paravesical space. In patients with endometriosis, the peritoneum was biopsied carefully several centimeters away from an endometriosis implant. The tissue samples were snap-frozen in liquid nitrogen immediately following surgical extraction and were stored at −80°C to minimize enzymatic degradation until final analysis. Blood samples were obtained at the time of the laparoscopy (Visit 1) and at a follow-up appointment 6–10 weeks after laparoscopy (Visit 2) using 9 ml Z Serum Separator Clot Activator tubes (VACUETTE® #455010; Greiner bio-one; Kremsmuenster, Austria). All samples were collected in accordance with the guidelines established by the Endometriosis Phenome and Biobanking Harmonization Project (Fassbender *et al.*, 2014). Demographic data were also collected prior to surgery. In addition, the participants were asked to complete a detailed questionnaire prior to surgery and at their post-operative follow-up visit.

The presence of endometriosis was determined by visual inspection during surgery and was confirmed histologically; the control group of women had no macroscopic or histological evidence of endometriosis at the time of the laparoscopy. Endometriosis was classified in accordance

with the revised American Fertility Society Score (rAFS:1996, 1997). Menstrual cycle phase was determined by histological examination of endometrial samples performed by an experienced pathologist and confirmed by noting the date of the last menstrual period. In four women, the cycle phase could not be clearly determined because the interphasic endometrium showed characteristics of both cycle phases.

From a total of 138 women who participated in the EMMA study from July 2013 through September 2014, 97 women had not taken hormonal treatments for at least 3 months prior to recruitment and were included in our analysis; 49 (50.5%) of these women had endometriosis and 48 (49.5%) did not have endometriosis. The endometriosis group included 18 (36.7%) women with mild disease (rAFS Stage I or II) and 31 (63.3%) women with severe endometriosis (rAFS Stage III or IV). All women in the endometriosis group were scheduled due to suspicion of endometriosis and underwent operative laparoscopy with complete removal of all visible endometriosis. In 4 (8%) women with endometriosis, concomitant uterine fibroids were diagnosed. The control group consisted of women undergoing laparoscopy for uterine fibroids (n = 14, 29%), benign ovarian cysts (n = 9, 19%), fallopian tube disorders (n = 9, 19%), or diagnostic laparoscopy due to unexplained infertility (n = 6, 13%) or chronic pelvic pain (n = 10, 21%). None of the women in the control group had macroscopic or histologic evidence of endometriosis. The baseline characteristics of the women in our study are summarized in Table I, and the number of analyzed tissue and serum samples of sufficient volume and quality are shown in Table II. Each participating woman contributed only one sample of eutopic endometrium and normal peritoneum, and some of the women with endometriosis contributed samples of diverse types of endometriotic lesions. In total, 52 lesions were classified into the following three subgroups: peritoneal lesions (n = 10, 19%), ovarian endometriomas (n = 38, 73%) and deep infiltrating endometriosis (DIE) (n = 4, 8%).

Table I Baseline characteristics of the women with endometriosis and the women without endometriosis (controls).

	n	Endometriosis	Controls	*P*-value
n	97	49	48	
Age (years)	97	32.5 ± 5.9	34.0 ± 5.8	0.227 [a]
BMI	96	22.5 ± 4.8	24.2 ± 5.4	0.121 [a]
Gravidity				
0	97	34 (64.2%)	19 (35.8%)	0.004 [b]
≥1		15 (34.1%)	29 (65.9%)	
Parity				
0	97	37 (56.1%)	29 (43.9%)	0.131 [b]
≥1		12 (38.7%)	19 (61.3%)	
Cigarette smoking				
No	94	38 (61.3%)	24 (38.7%)	0.017 [b]
Yes		11 (34.4%)	21 (65.6%)	
Cycle phase				
Proliferative	93	23 (56.1%)	18 (43.9%)	0.532 [b]
Secretory		25 (48.1%)	27 (51.9%)	

[a]Welch's two-sample *t*-test
[b]Fisher's exact test.
Age and BMI are presented as the mean ± SD; the other variables as presented as the number (%).

Table II Overview of the number of analyzed tissue and serum samples obtained from women with endometriosis and women without endometriosis (controls).

	Total	Endometriosis	Controls
Tissue samples			
Ectopic endometrium	52	52	n.a.
Eutopic endometrium	82	35	47
Normal peritoneum	36	19	17
Serum samples			
Before surgery (Visit 1)	61	31	30
Follow-up (Visit 2)	53	28	25

n.a., Not applicable. Some patients contributed samples of diverse types of endometriotic lesions.

RNA extraction

Frozen tissue samples were homogenized using a Precellys 24 homogenizer (PEQLAB). Subsequently, total RNA was isolated from eutopic and ectopic endometrium using the Agilent Absolutely RNA miRNA kit in accordance with the manufacturer's instructions (DNase I treatment included), and total RNA was isolated from unaffected peritoneum samples using TRI reagent (Sigma). RNA concentration and purity were determined by measuring optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). We defined the quality of the RNA samples to be sufficient when the ratios of OD260/280 and OD260/230 were ~2.

Reverse transcription (cDNA synthesis)

The isolated RNA samples were used as the template for synthesizing first-strand cDNA using the SuperScript First-Strand Synthesis Kit (Invitrogen) in accordance with the manufacturer's instructions, using the random hexamer primers supplied in the kit. The cDNA samples were then used to measure gene expression as described below.

Quantitative real-time PCR

Each sample of cDNA was analyzed by quantitative real-time PCR (qRT-PCR) using gene-specific primers and fluorescent probes (TaqMan Gene Expression Assay). The qRT-PCR experiments were performed in accordance with standard protocols from Applied Biosystems. The following genes (with the indicated TaqMan probe numbers) were analyzed: ICAM-1 (Hs00164932_m1), VCAM-1 (Hs01003372_m1), EpCAM (Hs00901885_m1), and the control genes ACTB (β-actin; Hs99999903_m1) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase; Hs99999905_m1). PCR cycles were performed on an ABI 7500 Fast System (Applied Biosystems) and consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The mRNA levels of ICAM-1, VCAM-1 and EpCAM were normalized to the levels of ACTB and GAPDH in each sample by subtracting the mean Ct (threshold cycle) values of ACTB and GAPDH from the Ct value of the target genes, as described previously (Proestling *et al.*, 2015). This calculation produces a delta-Ct value (ΔCt). Relative RNA expression levels were derived from the ΔCt values as $2^{-\Delta Ct}$.

ELISA

Serum samples were separated by centrifugation with 3000 r.p.m. at 4°C for 10 min within 1 h of blood collection and were stored in aliquots at −80°C until analysis in order to avoid repeated freeze-thaw cycles. The serum concentrations of soluble VCAM-1 (sVCAM-1) and soluble ICAM-1 (sICAM-1) were measured using ELISA. Specifically, the serum samples were diluted 1:50 and analyzed using eBioscience Platinum ELISA kits for sVCAM-1 and sICAM-1 (catalog numbers BMS2001 and BMS232, respectively) in accordance with the manufacturer's protocol. For the measurement of EpCAM, serum samples were diluted 1:5 and analyzed using human TROP1 (EpCAM) ELISA Kit (ab155442, Abcam, Cambridge, GB). The results below detection limit were considered as 0 pg/ml for the statistical analysis. Serum concentrations are presented in pg/ml. The laboratory that performed the ELISA measurements was blinded with respect to the presence or absence of endometriosis.

Ethical approval

Both verbal and written informed consent were obtained from each participant prior to inclusion in the study. Ethics approval was provided by the institutional ethics committee of the Medical University of Vienna (EK 545/2010).

Statistical analysis

All statistical tests were performed using SPSS version 17.0. Data are expressed either as mean ± SD, absolute or relative frequency, or box and whisker plots ranging from minimum to maximum, including the median and box boundaries at the 25th and 75th percentiles. Characteristics between the endometriosis and control groups were analyzed using Welch's *t*-test or Fisher's exact test; the non-parametric Mann–Whitney *U*-test and Kruskal–Wallis test were used for further comparison between groups. For paired statistics, the Wilcoxon signed-ranks test was used. We considered the subgroup analyses to be exploratory; therefore, as recommended by Bender and Lange, we did not adjust for multiple testing (Bender and Lange, 2001). Differences with a *P*-value < 0.05 were considered statistically significant. Finally, receiver operating characteristics (ROC) analysis was used to examine the value of using sICAM-1 or sVCAM-1 levels or the sVCAM/sICAM ratio to diagnose endometriosis. To determine sensitivity and specificity, we calculated the area under the ROC curve (AUC) and the 95% CI.

Results

Increased VCAM-1 expression in ectopic endometrium, eutopic endometrium and peritoneum of women with endometriosis

First, we measured the mRNA levels of VCAM-1 in tissue samples obtained from women with endometriosis and women without endometriosis (controls). VCAM-1 expression was significantly higher in both ectopic (+28.03 median fold change) and eutopic (+0.50 median fold change) endometrium samples from women with endometriosis compared to eutopic endometrium samples from controls (Fig. 1A). In the women with endometriosis, VCAM-1 expression was significantly higher in ectopic endometrium than in eutopic endometrium (+18.37 median fold change; Fig. 1A); similarly, a pairwise analysis of 21 women with endometriosis revealed significantly higher VCAM-1 expression in ectopic endometrium than in the corresponding eutopic endometrium samples obtained from the same woman (+23.80 median fold change; $P < 0.001$; Supplementary data Fig. S1A). Lastly, our analysis revealed significantly higher expression levels of VCAM-1 in samples of normal peritoneum obtained from women with endometriosis compared to samples from controls (+1.02 median fold change; Fig. 1A).

Increased ICAM-1 expression in ectopic endometrium and peritoneum of women with endometriosis

Our analysis also revealed that ICAM-1 expression was significantly higher in ectopic endometrium than in eutopic endometrium in both the endometriosis (+7.23 median fold change) and the control group (+7.81 median fold change; Fig. 1B).

A pairwise analysis of the women with endometriosis revealed significantly higher expression of ICAM-1 in ectopic endometrium compared to the corresponding eutopic endometrium (+7.25 median fold change; $P < 0.001$; Supplementary data Fig. S1B). Moreover, ICAM-1 expression was significantly higher in samples of normal peritoneum from the women with endometriosis compared to samples from controls (+1.75 median fold change; Fig. 1B).

Increased EpCAM expression in eutopic endometrium of women with endometriosis and decreased EpCAM expression in ectopic endometrium of women with endometriosis

Next, we measured EpCAM expression and found higher expression in eutopic endometrium samples from the women with endometriosis compared to controls (+0.17 median fold change; Fig. 1C). We found no difference between the two groups with respect to EpCAM expression in samples of normal peritoneum (Fig. 1C). Interestingly, EpCAM expression was significantly lower in ectopic endometrium samples compared to both groups of eutopic endometrium samples (−0.96 median fold change both; Fig. 1C). Similarly, a pairwise analysis of the women with endometriosis revealed significantly lower expression of EpCAM in ectopic endometrium compared to the corresponding eutopic endometrium samples (−0.96 median fold change Supplementary data Fig. S1C).

Next, we measured the sEpCAM levels in serum obtained at Visit 1. We found no significant differences between sEpCAM concentrations in women with (median = 176.8 pg/ml; range = 22056.2, 0.0–22056.2) and without (median = 0.0 pg/ml; range = 43197.6, 0.0–43197.6) endometriosis ($P = 0.240$). Given that, and given the wide ranges of measured serum EpCAM concentrations in both groups, we excluded EpCAM from our subsequent analysis.

Increased serum sVCAM-1 levels in women with endometriosis

Next, we measured the serum sVCAM-1 and sICAM-1 levels in both groups. Blood samples were collected directly before laparoscopy (Visit 1, V1) and again 6–10 weeks after laparoscopy (Visit 2, V2). At V1, sVCAM-1 levels were significantly higher in the women with endometriosis (median = 473.4 pg/ml, range = 873.0) compared to controls (median = 323 pg/ml, range = 300.8), and this difference remained in V2 (median 530.2 vs 458.0 pg/ml; Fig. 2A). To further investigate the perioperative dynamics of sVCAM-1 levels, we performed pairwise analyses and found increased serum VCAM-1 levels at V2 compared to the corresponding V1 samples in both groups (data not shown; $P = 0.014$ and $P < 0.001$ for the endometriosis and control groups, respectively, Wilcoxon signed-ranks test).

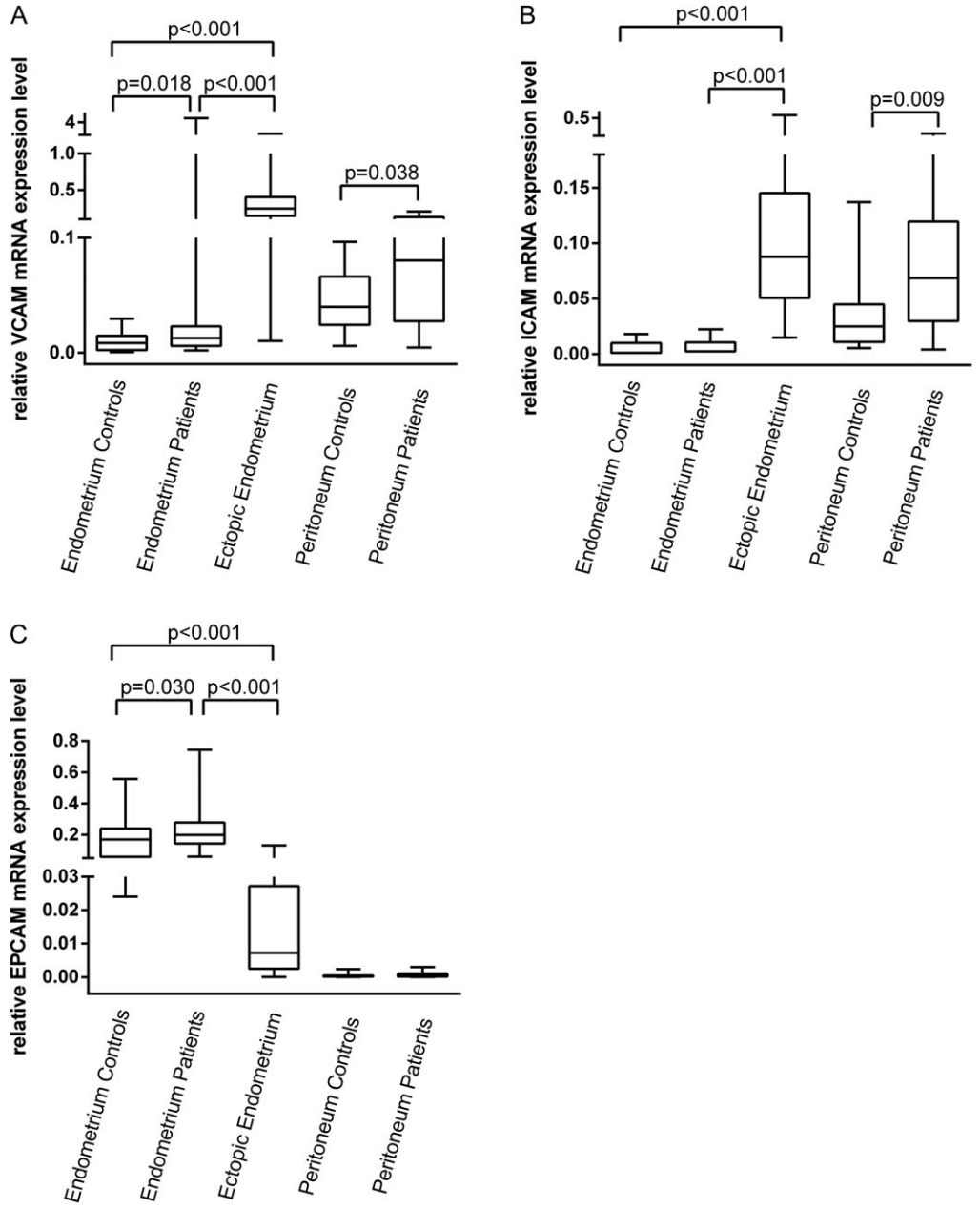

Figure 1 Relative mRNA levels measured in endometrium and peritoneum samples obtained from women with endometriosis and women without endometriosis (controls). Quantitative real-time PCR was performed to measure the mRNA levels of VCAM (**A**), ICAM (**B**) and EpCAM (**C**) in the indicated tissue samples. Expression levels were normalized to β-actin and GAPDH mRNA. Significance was determined using the Mann–Whitney *U*-test. qRT-PCR, Quantitative real-time PCR; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; EpCAM, epithelial cell adhesion molecule.

Decreased preoperative and increased postoperative serum sICAM-1 levels in women with endometriosis

Next, we measured sICAM-1 levels in both groups. Our analysis revealed that before laparoscopy (V1), the serum sICAM-1 levels were lower in the women with endometriosis (median = 240.2 pg/ml, range = 281.6) compared to controls (median = 290.2 pg/ml, range = 310.4; Fig. 2B). In contrast, 6–10 weeks after surgery (V2), the serum sICAM-1 levels were higher in the women with endometriosis (median = 241.4 pg/ml, range = 186.2) compared to controls (199.5 pg/ml, range = 211.4; Fig. 2B). A pairwise analysis of the women with endometriosis revealed no significant difference in serum ICAM-1 levels between the two time points (i.e. V1 vs V2; data not shown); in contrast, a pairwise analysis of the controls revealed a

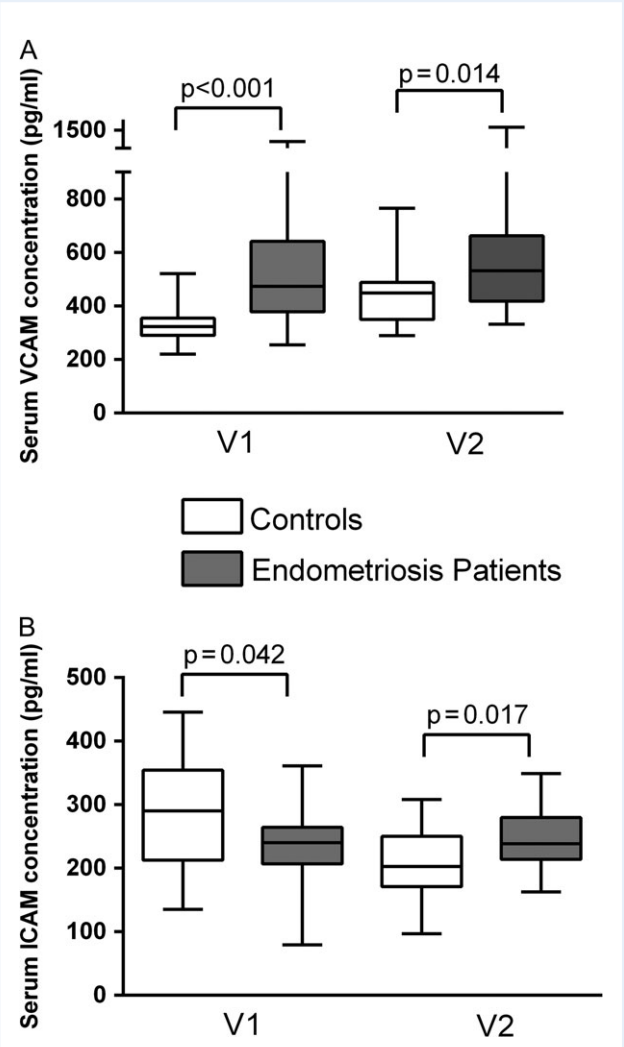

Figure 2 Serum sVCAM-1 and sICAM-1 levels in women with endometriosis and women without endometriosis (controls). The serum concentrations (in pg/ml) of sVCAM-1 (**A**) and sICAM-1 (**B**) were measured using ELISA before (Visit 1; V1) and 6–10 weeks after laparoscopy (Visit 2; V2). Significance was determined using the Mann–Whitney *U*-test.

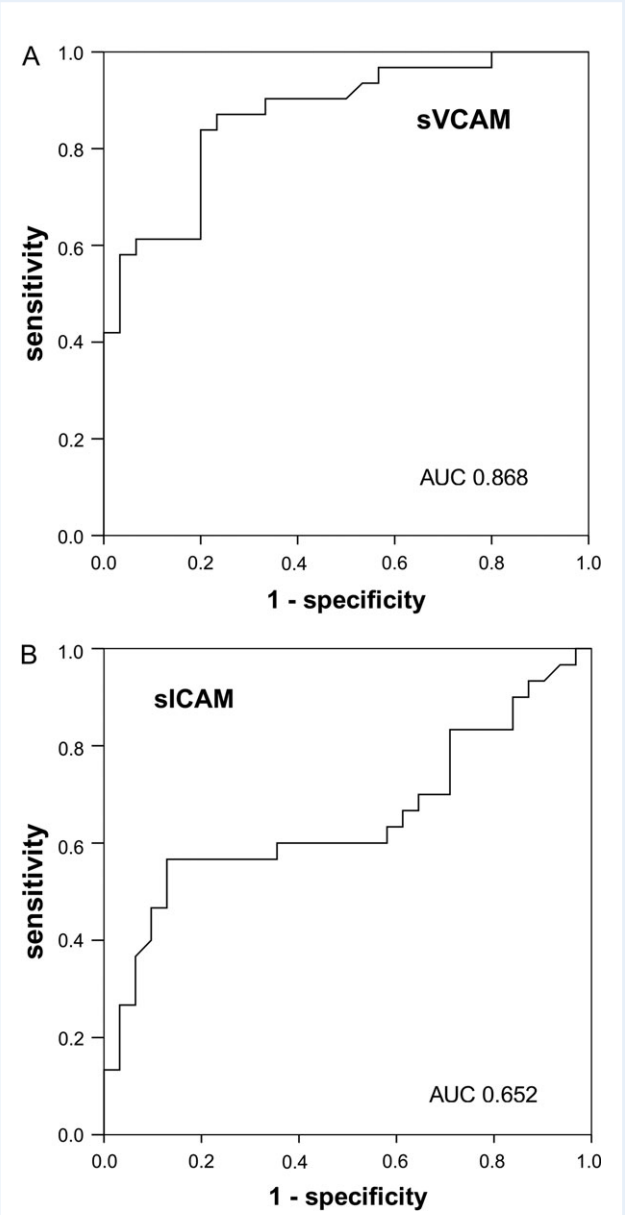

Figure 3 ROC curves showing the sensitivity and specificity of using serum (**A**) sVCAM-1 and (**B**) sICAM-1 concentration to diagnose endometriosis. ROC; receiver operating characteristics.

significant decrease in sICAM-1 levels between the two time points (data not shown; $P < 0.001$, Wilcoxon signed-ranks test).

Serum sVCAM-1 levels can distinguish between women with endometriosis and women without endometriosis

Next, to investigate further the feasibility of using serum sICAM-1 levels and serum sVCAM-1 levels to diagnose endometriosis, we performed ROC AUC analyses on the data collected at Visit 1 (Fig. 3). For sICAM-1, the ROC analyses revealed an AUC of 0.652 (CI: 0.508–0.795), and the cutoff value with the highest sum of specificity and sensitivity was 246.5 pg/ml, providing 65% specificity and 60% sensitivity. For sVCAM-1, our analysis revealed an AUC of 0.868 (CI: 0.779–0.957), and the cutoff value with the highest sum of specificity and sensitivity was 370.1 pg/ml, providing 80% specificity and 84% sensitivity.

Serum sVCAM-1 levels are not correlated with lesion entity, menstrual cycle phase or disease severity

As the ROC analysis indicates that VCAM-1 is a promising biomarker for diagnosing endometriosis, we examined whether serum sVCAM-1 levels were correlated with (i) the types and combination of entities of endometriosis, (ii) the severity of endometriosis (i.e. mild vs severe, as classified by rAFS score) and/or (iii) the phase of the menstrual cycle

in both groups of women; these results are summarized in Table III. Our subgroup analysis revealed no significant differences between any of these subgroups. As the prevalence of cigarette smoking differed among women with and without endometriosis (Table I), we also performed a subgroup analysis comparing smoking with non-smoking women. We did not find any differences in sVCAM concentration between smoking and non-smoking women with ($P = 0.211$) or without endometriosis ($P = 0.722$; Mann–Whitney U-Test).

Table III Serum sVCAM-1 concentration at Visit 1 in the indicated subgroups of women with endometriosis and women without endometriosis (controls).

	Endometriosis	Controls	*P*-value
Lesion entity			
Peritoneal	408.1 (7)	n.a.	0.750
Ovarian	543.7 (5)	n.a.	
Peritoneal and ovarian	467.9 (9)	n.a.	
Peritoneal and DIE	813.5 (1)	n.a.	
Ovarian and DIE	509.8 (6)	n.a.	
Peritoneal and ovarian and DIE	461.7 (2)	n.a.	
Disease stage			
rAFS Score I or II (mild)	461.5 (11)	n.a.	0.650
rAFS Score III or IV (severe)	496.3 (20)	n.a.	
Menstrual cycle phase			
Proliferative	511.2 (12)	327.0 (11)	0.899
Secretory	460.5 (18)	311.6 (18)	

Values are presented as the median VCAM-1 concentration in pg/ml and the number of patients (n). All P-values were calculated using the Mann–Whitney U-test or Kruskal–Wallis test. DIE, deep infiltrating endometriosis; n.a., not applicable; rAFS, revised American Fertility Society.

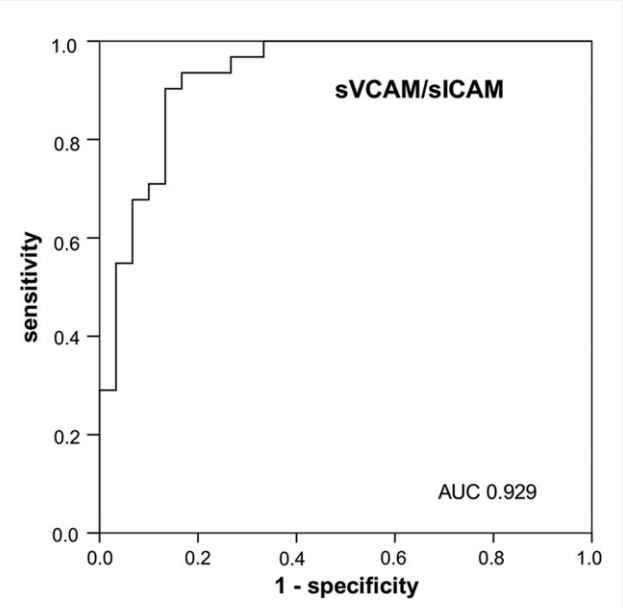

Figure 4 ROC curve showing the sensitivity and specificity of using the serum sVCAM-1/sICAM-1 ratio to diagnose endometriosis.

Calculation of the serum sVCAM-1/sICAM-1 ratio improves the predictive performance

Finally, to evaluate the usefulness of combining sVCAM-1 and sICAM-1 for diagnosing endometriosis, the diagnostic performance of a ratio of these markers was determined using ROC analysis. The ratios were calculated for each sample at Visit 1 from the respective sVCAM-1 and sICAM-1 values. For the sVCAM-1/sICAM-1 ratio, our analysis revealed an AUC of 0.929 (CI: 0.864–0.994), and the cutoff ratio with the highest sum of specificity and sensitivity was 1.55, providing 86.7% specificity and 90.3% sensitivity (Fig. 4).

Discussion

Here, we hypothesized that changes in the expression of cell adhesion molecules play a role in the pathophysiology of endometriosis. To test this hypothesis, we measured expression of the cell adhesion molecules VCAM-1, ICAM-1 and EpCAM in ectopic endometrium, eutopic endometrium and normal pelvic peritoneum obtained from women with endometriosis and women without endometriosis. Moreover, we examined whether serum sVCAM-1 and/or sICAM-1 levels can be used as a biomarker for diagnosing endometriosis.

We found that the expression of both VCAM-1 and ICAM-1 was higher in endometriotic lesions than in eutopic endometrium, and that the VCAM-1 expression was also higher in eutopic endometrium obtained from women with endometriosis compared to controls. Moreover, we found that compared to controls, women with endometriosis had higher VCAM-1 and ICAM-1 expression in normal peritoneum. In addition, we found that the women with endometriosis had lower serum levels of sICAM-1 and higher serum levels of sVCAM-1 compared to controls. Finally, we found that the serum sVCAM-1 and sICAM-1 concentrations can be used to distinguish between women with endometriosis and women without endometriosis.

VCAM-1 is a member of the integrin adhesion protein family, and its expression is up-regulated in activated endothelium, where it facilitates the trafficking of leukocytes through the endothelium into inflamed, infected tissues (Pribila *et al.*, 2004). Interestingly, VCAM-1 also appears to play a role in tumor escape from immune attack; VCAM-1 expressed in tumor cells may promote T cell migration away from the tumor, resulting in decreased numbers of T cells within the tumor (Lin *et al.*, 2007). Specifically, binding of VCAM-1 appears to drive the disassembly of focal adhesions, drives integrin-dependent cell migration via activation of focal adhesion kinases, and stimulates T cell migration mediated by lymphocyte function-associated antigen 1 (Liu *et al.*, 1999; Rose *et al.*, 2003; Lin *et al.*, 2007). Similar mechanisms may also play a role in endometriosis.

In the present study, we could demonstrate that VCAM-1 is overexpressed in ectopic endometriotic lesions, which is a novel finding. Moreover, we provide new evidence that women with endometriosis have significantly increased VCAM-1 expression in eutopic endometrium and normal peritoneum. This finding is consistent with previous reports of altered expression patterns of peritoneal and endometrial VCAM-1. For example, using immunohistochemistry, Schutt *et al.* (2015) reported preferential VCAM-1 staining in both affected and

unaffected peritoneal mesothelial cells in women with endometriosis. Moreover, Kyama *et al.* (2008) analyzed VCAM-1 expression in the endometrium and peritoneum of women both with and without endometriosis in various phases of the menstrual cycle and found increased VCAM-1 expression during the luteal phase. The expression of VCAM-1 in the endometrium was less clear, as the authors found no difference between women with endometriosis and women without endometriosis. This apparent discrepancy between their study and our results may be due to the relatively small sample sizes in both studies.

Based on our findings, we speculate that increased VCAM-1 expression in the peritoneum and endometrium of women with endometriosis contributes to endometrial–peritoneal adhesion and facilitates the escape of endometrial tissue from the T cell-mediated immune response. Furthermore, high expression of VCAM-1 in ectopic endometrium appears to facilitate the maintenance and progression of endometriotic lesions. Motivated by this finding, we compared serum sVCAM-1 levels between women with endometriosis and women without endometriosis. Our analysis revealed that sVCAM-1 levels are significantly higher in women with endometriosis. This finding is consistent with previous studies (Daniel *et al.*, 2000; Barrier and Sharpe-Timms, 2002), although the data obtained by Barrier and Sharpe-Timms did not reach statistical significance. In addition, our ROC analysis revealed that measuring sVCAM-1 in the circulation is a promising tool for diagnosing endometriosis. The feasibility of using sVCAM-1 as a biomarker for endometriosis is strengthened by our subgroup analysis, which revealed that the increased sVCAM-1 levels in women with endometriosis were not dependent upon the lesion entity, disease severity, phase of the menstrual cycle or cigarette smoking.

However, even though expression patterns and serum levels were different between women with and without endometriosis, our study, like others, does not clarify whether the observed differences are a cause for the development of endometriosis or a consequence of prevalent endometriosis. We speculate that alterations in the eutopic endometrium, as well as in the pelvic microenvironment precede manifest endometriosis and contribute to the development and pathogenesis of endometriosis, which may also explain the high recurrence rates of endometriosis after surgery. Overexpression of VCAM-1 in non-endometriotic tissues, such as the eutopic endometrium or peritoneum, may further contribute to elevated serum levels and may contribute to the postoperative persistence of elevated serum sVCAM-1 levels in women with recently resected endometriosis compared to controls.

However, the clinical and/or biological significance of the observed increase in sVCAM-1 levels 6–10 weeks after surgery is currently unknown. We speculate that this increase may be the result of shedding sVCAM-1 induced by peritoneal microinflammation and remodeling triggered by surgery, and that levels would decline to either reach preoperative levels, or potentially normalize after a longer follow-up interval.

Future studies comprising samples of women using hormonal medication, samples of eutopic endometrium after operation, and longer follow-up intervals, should (i) shed light on the peri- and postoperative dynamics of serum sVCAM-1 levels, (ii) address the question of whether sVCAM-1 may serve as a diagnostic marker for prevalent endometriosis, a 'predisposition' for the development or recurrence of endometriosis, or both and (iii) aim to investigate if sVCAM-1 is a specific marker for endometriosis or reflects concomitant pelvic inflammation.

ICAM-1 is a member of the integrin adhesion protein family and is expressed on the surface of many cell types, including endometrial cells, endothelial cells and leukocytes. ICAM-1 affects inflammatory and immune responses and has been implicated in the migration of both tumor cells and healthy cells (Vigano *et al.*, 1994, 2000; Somigliana *et al.*, 1996). Previous studies led to the hypothesis that ICAM-1 may also play a role in endometrial–peritoneal adhesion and in the survival of endometrial cells via protection from immune response-triggered cellular cytotoxicity (Somigliana *et al.*, 1996; Vigano *et al.*, 1998; Somigliana *et al.*, 2002; Wu *et al.*, 2004). Moreover, ICAM-1 mediates lymphocyte–endothelial cell adhesion and is believed to facilitate the migration of lymphocytes along endothelial cells to the site of inflammation (Hayflick *et al.*, 1998). Here, we found increased peritoneal expression of ICAM-1 in women with endometriosis, which is consistent with previous studies (Wu *et al.*, 2004; Kyama *et al.*, 2006). Moreover, we found increased expression of ICAM-1 in ectopic endometriotic lesions, which is consistent with a previous report of ICAM-1 expression in cultures from endometriosis biopsies compared to cultured eutopic endometrium (Vigano *et al.*, 1998); the authors of this study postulated that ectopic endometrial cells lack the mechanisms needed to regulate the release of soluble ICAM-1, leading to increased release of sICAM-1. The soluble form of ICAM-1 (i.e. sICAM-1) is believed to act as an antagonist of ICAM-1 (and its related cellular immune functions after release from the cell surface) by blocking lymphocyte function-associated antigen 1, thereby reducing recognition and the susceptibility to killing by immune cells (Vigano *et al.*, 2000). Although the precise mechanisms underlying the release and function of sICAM-1 are currently unknown, we believe that peritoneal shedding of sICAM-1, due to increased expression of ICAM-1 in the peritoneum, may help the endometrium escape from cellular cytotoxicity after endometrial–peritoneal adhesion.

Based on the above findings, measuring changes in the level of serum sICAM-1 has been proposed for diagnosing endometriosis, although the results obtained to date are inconsistent (Wu *et al.*, 1998; Daniel *et al.*, 2000; Matalliotakis *et al.*, 2001; Barrier and Sharpe-Timms, 2002; Somigliana *et al.*, 2002; Vodolazkaia *et al.*, 2012). Consistent with the largest of these studies, the 2012 study by Vodolazkaia *et al.*, we also found decreased levels of serum sICAM-1 in women with endometriosis. However, the ROC analysis investigating the feasibility of using sICAM-1 for diagnosing endometriosis revealed only a moderate predictive power, which leads to the assumption that serum sICAM-1 is not a promising stand-alone biomarker for predicting endometriotic lesions. Nevertheless, measuring sICAM-1 could be of value for diagnosing endometriosis in combination with measuring sVCAM-1, thereby increasing its predictive power. The sVCAM-1/sICAM-1 ratio seems to perform better as a diagnostic marker for endometriosis than the individual marker values alone and should be investigated in detail in future studies.

EpCAM plays a role in signal transduction, cell migration, cell proliferation and cell differentiation, as well as important functional roles in the onset, development, maintenance, repair and function of the epithelium (Trzpis *et al.*, 2007). In women with endometriosis, EpCAM is expressed in endometriotic lesions, but not in surrounding unaffected tissue (van den Berg *et al.*, 2013). Here, although we found that measuring serum EpCAM levels seems to be of limited value for diagnosing endometriosis, we provide the first report of increased EpCAM expression in eutopic endometrium in women with endometriosis. Moreover, EpCAM expression was higher in eutopic endometrium compared to ectopic endometrium. As excised endometriotic lesions can also contain surrounding peritoneal tissue, which may lead to a lower proportion of epithelial cells in ectopic endometrium samples

compared to eutopic endometrium, this finding might be explained by the capacity of EpCAM as endometrial epithelial cell marker. This issue could be addressed by future studies aiming to examine epithelial and stromal cells separately by applying cell separation technologies.

Currently, laparoscopy is the gold standard for diagnosing endometriosis; however, this procedure is invasive, carries surgery-related risks and contributes to an average delay in diagnosis of 10 years (Hadfield *et al.*, 1996). Therefore, the field has an urgent need for an efficient, sensitive, non-invasive tool for diagnosing endometriosis and researchers have searched for a suitable biomarker for several decades. However, biomarker discovery in this field has focused primarily on investigating a multitude of different candidate markers via single and/or multiplex immunoassay technologies in retrospective studies, unfortunately often leading to inconsistent results.

A possible limitation of our study is the relatively small sample sizes of the subgroups. Therefore, caution should be exercised when interpreting these results. On the other hand, a strength of our study is the prospective nature of the EMMA study, which was designed specifically to study endometriosis and included follow-up visits 6–10 weeks after surgery. This robust design enabled us to investigate the perioperative dynamics of candidate biomarkers and resulted in a well-characterized study cohort that enabled us to investigate several lesion entities in well-characterized patients, thereby facilitating the analysis of mRNA levels in various tissues and hypothesis-driven ELISA measurements in these patients, and relevant subgroup analyses.

Nevertheless, the findings of the present study should not be overestimated, and need to be validated and calibrated in other independent samples of patients. Future studies will help to determine whether serum sVCAM-1 and/or the serum sVCAM-1/sICAM-1 ratio can be used to diagnose endometriosis non-invasively, thereby potentially avoiding the currently prevalent diagnostic delay and surgical interventions.

In conclusion, we provide new evidence of altered expression of cell adhesion molecules in eutopic endometrium and normal peritoneum in women with endometriosis. Moreover, we report that both VCAM-1 and ICAM-1 are overexpressed in ectopic endometriotic lesions. Finally, our results provide new evidence that serum sVCAM-1 is a promising biomarker, potentially providing a simple, non-invasive tool for diagnosing endometriosis. The diagnostic accuracy of this tool can further be increased by calculating the serum sVCAM-1/sICAM-1 ratio.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

L.K., R.W., B.S. and H.H. designed the study. R.W., L.K., G.Y. and P.P. recruited the subjects and collected the data. L.K., R.W., K.P. and H.H. analyzed and interpreted the endpoints and defined the study groups. K.P., S.B., B.S. and I.Y. performed the experiments. K.P. and S.B. conducted the statistical analyses and created the figures. All authors provided discussion of the findings and helped write the manuscript. All authors read and approved the final draft.

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Conflict of interest

The authors declare no competing interests.

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