Enhanced expression of TACE contributes to elevated levels of sVCAM-1 in endometriosis

Running title: TACE and sVCAM-1 in endometriosis

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Abstract

Study Question: Are increased sVCAM-1 and sICAM-1 levels associated with Tumor necrosis factor-alpha- converting enzyme (TACE) activity in endometriosis?

Summary Answer: Here we provide the first functional evidence that induced TACE activity in human endometriotic epithelial cells is at least in part responsible for the enhanced release of sVCAM-1 from these cells.

What is known already: We and others have shown that serum soluble (s) VCAM-1 levels are significantly higher in women with endometriosis, compared to disease free controls. Experimental evidence exists suggesting a role of sICAM-1 and sVCAM-1 in the pathogenesis of endometriosis. TACE was identified as the protease responsible for phorbol 12-myristate 13-acetate (PMA)–induced VCAM-1 release in murine endothelial cells. Additionally, it has recently been shown that TACE is upregulated in the endometrial luminal epithelium of the mid-secretory phase in infertile women.

Study Design, Size, Duration: This study was conducted at the Tertiary Endometriosis Referral Center of the Medical University of Vienna. Samples from a total number of 97 women were collected between July 2013 and September 2014.

Participants/Materials, Setting, Methods: After complete surgical exploration of the abdominopelvic cavity, 49 women with histologically proven endometriosis and 48 endometriosis-free control women were enrolled. 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 ectopic samples). Among the 49 women with endometriosis, 36 matched samples of endometriotic lesions and corresponding eutopic endometrium were collected. In order to detect sVCAM-1 and TACE protein by ELISA, peritoneal fluid (PF) samples were collected from 44 cases and 32 controls during surgery. Expression of *TACE* mRNA was analyzed by qRT-PCR in 111

endometrium tissue samples (28 eutopic control samples, 33 eutopic samples from women with endometriosis, 50 ectopic samples from lesions) and 37 healthy peritoneum samples. Immunohistochemistry was performed in 123 tissue samples (39 eutopic control samples, 42 eutopic samples from women with endometriosis, 42 ectopic samples from lesions) and the relation between tissue TACE protein levels and sVCAM-1 secretion was examined. Phorbol 12-myristate 13-acetate (PMA)–induced sVCAM-1 release, and *TACE*-, and *VCAM-1*-transcripts or proteins were measured in an immortalized endometriotic epithelial cell line (11Z) pre-incubated either with TACE inhibitors or following *TACE* siRNA knockdown.

Main Results and the Role of Chance: Here we demonstrate that TACE protein is overexpressed in epithelium of tissue samples of both eutopic endometrium and ectopic lesions of women with endometriosis compared to disease free controls ($p<0.001$ both) and that the overexpression of the protein in the lesions is due to activation of *TACE* gene transcription ($p<0.001$). Moreover, epithelial TACE protein was significantly higher in ectopic samples than in corresponding eutopic tissue of women with the disease ($p<0.001$). High endometrial tissue TACE protein expression correlated with higher serum sVCAM-1 levels ($p<0.05$), but not with sICAM-1 levels. Inhibition of TACE either by TACE inhibitors or by *TACE* siRNA knockdown resulted in decreased PMA-induced shedding of sVCAM-1 *in vitro* ($p<0.005$ or $p<0.01$, respectively) but the TACE inhibitors did not affect transcription of *TACE* or *VCAM-1*. Additionally, we observed an upregulation of TACE in proliferative endometrial epithelium of infertile ($p<0.005$), compared to fertile women. TACE was increased in infertile women with endometriosis ($p=0.051$) but not in infertile women without endometriosis.

Limitations, Reasons for Caution: Albeit well characterized, our control population included women with other gynecologic diseases, which may have impacted the

levels of sVCAM-1 and tissue TACE expression levels e.g. benign ovarian cysts or uterine fibroids. Thus, the results of our analysis have to be interpreted carefully and in the context of the current experimental settings.

Wider Implications of the Findings: The dysregulation of TACE substrate shedding represents a promising yet relatively unexplored area of endometriosis progression and could serve as a basis for the development of new treatments of the disease.

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Key Words: endometriosis, infertility, ADAM17, TACE, VCAM1, ICAM1

Introduction

Endometriosis is a benign gynecological disease characterized by the presence of endometrial tissue outside the uterine cavity (Giudice and Kao, 2004) and is estimated to affect up to 15% of women in their reproductive age. (de Ziegler *et al.* , 2010). One of the more widely accepted hypotheses is that endometriosis originates from endometrial cells adhering to peritoneal surfaces following retrograde menstruation (Sampson, 1927). However, given that retrograde menstruation occurs in the majority of women, it remains unclear why only some women develop the disease (Halme *et al.* , 1984, Liu and Hitchcock, 1986, O *et al.* , 2017).

It seems that the mechanisms underlying the spreading, attachment and implantation of endometrium within the pelvis, as well as the formation and survival of ectopic endometriosis lesions are influenced by specific cell adhesion molecules (CAMs). In addition to their well-described function in extracellular matrix remodeling, CAMs are

involved in the regulation of inflammatory and immune responses, cell survival, detachment and migration (Jung *et al.* , 2012, Ohene-Abuakwa and Pignatelli, 2000).

Our group, like others, has reported an altered expression of several CAMs, including vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in eutopic and ectopic endometrium and in unaffected peritoneum of women with endometriosis (Kuessel *et al.* , 2017, Kyama *et al.* , 2008, Schutt *et al.* , 2015, Vigano *et al.* , 1998, Wu *et al.* , 2004). Furthermore, it was shown that serum soluble (s) VCAM-1 (Barrier and Sharpe-Timms, 2002, Daniel *et al.* , 2000, Kuessel *et al.*, 2017) and sICAM-1 (Daniel *et al.*, 2000) levels are higher in women with endometriosis, compared to endometriosis free controls. Additionally, endometrial stroma cells of women with endometriosis secrete more sICAM-1, which can disturb adhesion between immune cells and their targets (Somigliana *et al.* , 1996, Vigano *et al.* , 2001). In light of these findings we hypothesize that altered expression of VCAM-1 and ICAM-1 in the endometrium and peritoneum, as well as the soluble forms of these molecules, may play a key role in the pathogenesis of endometriosis.

ICAM-1 and VCAM-1 are transmembrane glycoproteins of the immunoglobulin superfamily, which are ligands for integrins expressed on leukocytes. Proteolytic cleavage of membrane-bound ICAM-1 and VCAM-1 gives rise to soluble circulating forms of those proteins (Garton *et al.* , 2003, Garton *et al.* , 2006, Hundhausen *et al.* , 2007, Mullooly *et al.* , 2016, Smalley and Ley, 2005). In general, members of the Zn^{2+} -dependent protease superfamily, including the matrix metalloproteinases (MMPs), membrane-tethered MMPs, and „A Disintegrin And Metalloproteinases" (ADAMs) have been shown to be responsible for the cleavage of cellular VCAM-1 and ICAM-1 (Garton *et al.*, 2003, Herren, 2002, Mullooly *et al.*, 2016).

Tumor necrosis factor-alpha- converting enzyme (TACE), also known as ADAM17, was identified as the protease responsible for phorbol 12-myristate 13-acetate (PMA)–induced VCAM-1 release in murine endothelial cells (Garton *et al.*, 2003). TACE exists in a full-length precursor and a mature form lacking the prodomain (Schlondorff *et al.* , 2000). TACE can act as sheddase for a broad spectrum of substrates, including a variety of cytokines, growth factors, receptors and adhesion molecules, controlling many processes that are known to be prerequisites for endometriosis, such as immune responses (Lisi *et al.* , 2014, Walcheck *et al.* , 2006) , tissue regeneration (Chalaris *et al.* , 2010, Scheller *et al.* , 2011) , cell migration (Maretzky *et al.* , 2011, Miller *et al.* , 2013, Rosso *et al.* , 2007, Xiao *et al.* , 2012), proliferation (Hu *et al.* , 2018, Lin *et al.* , 2012, Maetzel *et al.* , 2009, Zheng *et al.* , 2012), neovascularization (Dreymueller *et al.* , 2012, Swendeman *et al.* , 2008, Weskamp *et al.* , 2010) and controlling cancer development (Murphy, 2008, Scheller *et al.*, 2011). Therefore, it may serve as a novel therapeutic target in a variety of human diseases (Moss and Minond, 2017)**,** including endometriosis. However, little is still known about the expression levels of TACE and the TACE-mediated mechanism of membrane shedding and release of VCAM-1, ICAM-1, and TNF-alpha in endometriosis.

In this study we have assessed the relationship between the levels of sVCAM1, sICAM1 and TACE expression in serum, peritoneal fluid and tissue samples of women with and without endometriosis and approached the mechanism of TACE - mediated shedding in endometriotic epithelial cells *in vitro.*

Materials and Methods

Study population

For this study, tissue, blood and peritoneal fluid samples were collected in accordance to the protocols of the Endometriosis Marker Austria (EMMA) study, a prospective cohort study conducted at the Tertiary Endometriosis Referral Center of the Medical University of Vienna, which has been previously described in detail by our group (Kuessel *et al.*, 2017). In brief, premenopausal women 18–50 years of age 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 had acute inflammation, known or suspected infectious disease, chronic autoimmune disease or malignancy were excluded from the study.

To experimentally check our hypothesis that increased sVCAM-1 and sICAM-1 levels are associated with TACE activity in endometriosis we have used the same cohort described in our previous study (Kuessel *et al.*, 2017) where the solible levels of VCAM-1 and ICAM-1 were estimated. Therefore, the detailed baseline characteristics of the women in this study and the number of analyzed tissue and serum samples have already been summarized (Kuessel *et al.*, 2017). Briefly, from a total number of n=97 participating women, 49 (50.5%) had endometriosis and 48 (49.5%) were classified as controls. 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). 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%). 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%). Among the 49 cases with endometriosis, we obtained matched samples of endometriotic and eutopic endometrium in 36 cases. For our subgroup analysis concerning infertility, endometrium samples from women with regular menstrual cycles were analyzed. We compared women with known normal fertility (who had at least one successful pregnancy) (n=12) to women with primary unexplained infertility for more than 1 year duration and nulliparity (n=8). In our infertile patients, male factor was an exclusion criterion. Of the 8 women with preoperative unexplained infertility, in 2 cases (one in the control group, one in the endometriosis group) unilateral tubal reconstruction was performed intraoperatively, in 5 women endometriosis was diagnosed.

Ethical approval

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

Sample collection procedure

During the surgical intervention biopsies of the ectopic endometriosis lesion, the eutopic endometrium and unaffected peritoneum were collected for analyses. 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 extirpation and were stored at −80 °C to minimize enzymatic degradation until final analysis. Blood samples were obtained prior to surgery using 9ml Z Serum Separator Clot Activator tubes (VACUETTE® #455010; Greiner bio-one; Kremsmuenster, Austria). Peritoneal fluid samples were collected after lavage of the peritoneal cavity with 10ml of sterile 0.9% $NaCl_2$. All samples were collected in accordance to Endometriosis Phenome and Biobanking Harmonization Project guidelines (Fassbender et al., 2014).

RNA extraction

Frozen tissue samples were homogenized using a Precellys 24 homogenizer (PEQLAB, Erlangen, Germany). 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 and cell line 11Z using TRI reagent (Sigma-Aldrich, MO, USA). RNA concentration and purity were determined by measuring optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 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 template for synthesizing firststrand cDNA using the SuperScript First-Strand Synthesis Kit (Invitrogen) and procedure included random hexamer primers. The reactions were performed in accordance to the manufacturer's instructions. 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 (qRTPCR) using gene-specific primers and fluorescent probes (TaqMan Gene Expression Assay). The qRT-PCR experiments were performed in accordance with standard protocols from life technologies (Thermo Fisher Scientific, MA, USA). The following genes (with the indicated TaqMan probe numbers) were analyzed: *ADAM17* (Hs01041915_m1), *VCAM-1* (Hs01003372_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 *TACE (ADAM17)* and *VCAM-1* 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 $2^{-\Delta Ct}$. The *in vitro* experiments were performed in three biological replicates and all qRT-PCR were analyzed in technical duplicates.

ELISA from serum and peritoneal fluid samples

Serum samples were separated by centrifugation for 10min at 3000 g at 4°C within 1hour (h) of blood collection and were stored in aliquots at −80°C for further analysis. The serum concentrations of soluble VCAM-1 (sVCAM-1) and human TACE/ADAM17 were measured using ELISA. Specifically, the serum samples were diluted 1:50 or 1:100 and analyzed using eBioscience Platinum ELISA kits for sVCAM-1 (Thermo Fisher Scientific, MA, USA), or respectively, the DuoSet humanTACE/ADAM17 (R and D Systems, MN, USA) for TACE. Serum concentrations are presented in pg/ml.

Peritoneal fluid samples were centrifuged at 3000 g at 4°C for 10 min within an hour after sample collection. The clear fluids were aliquoted and stored at -80°C for subsequent quantification of sVCAM-1 and TACE/ADAM17. The peritoneal fluid samples were diluted 1:3 and analyzed using eBioscience Platinum ELISA kits for sVCAM-1 (Thermo Fisher Scientific, MA, USA). Dilution of 1:2 was performed for TACE analysis using the DuoSet HumanTACE/ADAM17 (R and D Systems, MN, USA). All experimental procedures were performed in accordance to the manufacturer's protocols. Internal controls for assay validation were included in each plate by spiking in a known amount of sVCAM-1 or TACE to a single peritoneal fluid sample which was assayed in parallel to the unspiked sample. For statistical evaluation of the experimental data all ELISA results below the detection limit were considered as 0 pg/ml (Gonzalez-Foruria *et al.* , 2015). In order to avoid biases owing to peritoneal fluid concentration or dilution at the moment of obtaining the sample, the peritoneal fluid concentration was measured in all of the samples using the spectroscopic Bradford protein assay method (Gonzalez-Foruria *et al.*, 2015). The

ratio between the sVCAM-1 or TACE result (pg/ml) and the protein concentration (mg/ml) was calcultated for each peritoneal sample individually (values are eventually expressed in pg/mg of protein). The laboratory that performed the ELISA measurements was blinded with respect to the presence or absence of endometriosis.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on formalin fixed, paraffin-embedded tissues. Three-micrometer thick sections were cut and placed on glass slides. Heat antigen retrieval was performed in 10mM Sodium Citrate Buffer pH6. Unspecific background staining was blocked by incubating in H_2O_2 and with Ultra V Block (Thermo Scientific, Ultra Vision LP Kit, TL-060-HL, MA, USA) according to the protocol. Rabbit polyclonal Anti-ADAM17 antibody (#ab39162, Abcam, Cambridge, UK) was applied at a dilution of 1:3000 with Antibody Diluent with Background Reducing Components (Dako, S3022, Glostrup, Denmark). Ultra Vision LP Kit was used for detection (Thermo Scientific, Ultra Vision LP Kit, TL-060-HL, MA, USA). Finally, all slides were incubated with DAB-Substrate (Dako, K346811, Glostrup, Denmark) and counterstained in Hematoxylin before they were dehydrated and mounted.

Scoring and Immunohistochemical Analysis

Endometriotic lesions, consisting of well-defined glandular epithelial and stromal cells, were identified in hematoxylin-eosin stained sections by a pathologist. For each sample a semiquantitative subjective scoring system was applied in order to evaluate the localization, quantity and intensity of immunoreactivity of TACE. In each sample,

the staining for glandular epithelial cells and stromal cells was scored separately. The intensity of the staining was scored using a four-point scoring scale (0, negative staining; 1, weak staining; 2 moderate staining, 3, strong staining). The percentage of positively stained cells was again scored by a four-point scoring scale (0, negative staining; 1, 1-35% positive cells; 2, 36-70% positive cells; 3, >67% positive cells). The two scores were combined by multiplication to derive a final IHC score (0-9) (Proestling *et al.* , 2016). Evaluations were performed blinded by two investigators. Positive (ColonCa and Placenta) and isotypic negative control IgG were run concurrently.

Cell line and reagents

The well characterized endometriotic epithelial cell line 11Z was established and kindly supplied by Professor Starzinski-Powitz (Banu *et al.* , 2008, Zeitvogel *et al.* , 2001). The cell line was cultured in Dulbecco´s modified Eagle´s medium (DMEM) containing antibiotics and 10% v/v fetal calf serum (FCS) and maintained in a 37°C CO_2 humified incubator. Cells were tested and found to be negative for mycoplasm infection. All cell culture reagents were purchased from ThermoScientific (MA, USA) or Sarstedt (Nümbrecht, Germany). The selective TACE-inhibitor, PF-5480090 (WAY-18022 or TMI-002), was provided by Pfizer while the broad-spectrum inhibitor TAPI-1 was purchased from Roche (Rotkreuz, Switzerland). Phorbol-12-myristate-13-acetate (PMA) was obtained from Sigma-Aldrich (MO, USA).

sVCAM-1 shedding after TACE inhibition

Five hundred thousand 11Z cells were grown in 6-well tissue culture plates for 24 hours, starved in DMEM containing 3% v/v FCS for 3 hours, pre-incubated with

inhibitors or with 0.06% v/v DMSO solvent control for 1 hour and stimulated with 1 µM PMA for 3 hours. Medium was removed, centrifuged at 1200 g at 4 °C for 5 minutes, aliquoted and stored at -80 °C. Concentration of sVCAM-1 in culture medium was determined undiluted by Luminex Performance Assay according to the manufacturer's protocols (R and D Systems, MN, USA). For cell number determination, the cells were washed, trypsinized, resuspended in DMEM containing FCS, and centrifuged at 300 g for 5 minutes. The cell pellet was resuspended in Dulbecco´s Phosphate Buffered Saline (DPBS; Gibco, MA, USA) and cells were counted using trypanblue. The results were expressed as pg soluble VCAM-1 per 10^5 cells. The experiment was performed in biological triplicates with technical duplicates.

sVCAM-1 shedding after *TACE* siRNA transfection

One hundred and fifty thousand 11Z cells were grown in 6-well tissue culture plates for 24 hours, and then transfected using Lipofectamine RNAiMAX reagent (Invitrogen, Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Commercially available siRNAs for *ADAM17/TACE* (Ambion ID s13719), *GAPDH* as positive control, or for non-silencing control sequence (Ambion, Thermo Fisher Scientific, MA, USA) were used. The effects that RNA interference had on expression of *TACE* or *GAPDH* mRNA and protein were determined by reverse transcription (RT)-PCR and Western immunoblotting. Transfection was done by growing 11Z cells in the presence of 10nM siRNA 48 hour. Then, cells were starved in DMEM containing 3% v/v FCS for 3 hours and stimulated with 1 µM phorbol 12-myristate 13-acetate (PMA) for 3 hours. The sVCAM-1 concentration in medium and cell numbers were analyzed as described above. The results were expressed as pg

soluble VCAM-1 per 10^5 cells. The experiment was performed in biological triplicates with technical duplicates.

Protein isolation and immunoblotting

The 11Z cells were harvested, counted and lysed in ice-cold Frackelton buffer [10 mM Tris–HCl, pH 7.05; 50 mM NaCl; 30 mM sodium pyrophosphate; 50 mM NaF; 1% v/v Triton X-100; 100 µM Na_3VO_4; 1 tablet of protease inhibitor cocktail (Roche); 1 mM phenylmethylsulphonyl fluoride]. Insoluble material was removed by centrifuging the samples at 20,000 g, 20 min, at 4 °C. The protein concentration for total cell lysates were determined using the spectroscopic Bradford protein assay method (Bradford, 1976). Twenty micrograms of normalized samples were immunoblotted as previously described (Rubiolo *et al.* , 2006) and probed with the following primary antibodies: alpha-tubulin (1:10000; #T5168; Sigma-Aldrich, MO, USA), beta-actin (1:10000; #A5441; Sigma-Aldrich, MO, USA), ADAM17 (1:2500; #ab39162, Abcam, Cambridge, UK) or GAPDH (1:1000; #2118-14C10; Cell Signaling, MA, USA) prior to incubation with peroxidase-conjugated secondary antibodies (Pierce Chemical Co., TX, USA). Bound antibodies were detected by enhanced chemiluminescence plus western blotting detection system (Amersham Pharmacia Biotech, Inc., NJ, USA) and exposed to X-ray films (GE Health Care, UK). Western blots were scanned by hp Scanjet and quantified by ImageJ free web program (Schneider *et al.* , 2012) with normalisation against alpha-tubulin or beta-actin.

Statistical analyses

All statistical tests were performed using SPSS version 17.0 for patient data and Prism (GraphPad Software, La Jolly, CA, USA) for cell culture experiments . Data are expressed either as mean ± SD, 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 by the non-parametric Mann–Whitney U-test. Mann–Whitney U-test and Kruskal– Wallis test were used for further comparisons between subgroups. For paired statistics, the Wilcoxon signed-ranks test was used. The results were adjusted for multiple testing using Bonferroni-Holm correction. For correlation analysis between TACE protein and serum VCAM-1 levels we used Spearman correlation. For evaluation of cell culture experiments, unpaired two-tailed t-tests were used. Cell culture experiments were considered 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.

Results

Increased TACE expression in epithelium of eutopic endometrium and in endometriotic tissue of women with endometriosis

To assess the relationship between the levels of sVCAM1 and sICAM1 in either peritoneal fluid or serum and TACE expression in tissue, we first analyzed the levels of TACE protein in tissue samples of women with and without endometriosis using standard immunohistochemistry (IHC) staining. Our data showed that TACE is expressed in both epithelial and stromal cell compartments (Fig. 1A-F). The

subsequent evaluation of IHC stains (Fig. 1G and H) showed the following: In the ectopic endometriosis lesions, the levels of epithelial TACE were 3.0-fold (adjusted p <0.0001, Mann-Whitney U Test) and of stromal TACE, 2.0-fold (adjusted p=0.0017, Mann-Whitney U Test) higher, compared to eutopic endometrium of women without endometriosis. In contrast to stromal TACE levels, the protein level of TACE in epithelial cells was additionally altered in eutopic endometrium of women with endometriosis. Epithelial TACE protein levels were significantly higher in eutopic samples of women with endometriosis compared to samples of women without endometriosis (2.0-fold median increase; adjusted p=0.0008, Mann-Whitney U Test) and respectively, lower (1.5-fold median decrease; adjusted p=0.0009), when compared to the levels observed in the epithelium of endometriotic lesions (Fig. 1G). The changes of the epithelial TACE levels however, were not due to changes in luminal TACE protein expression (Fig. 1I and Supplementary Fig. 1A). The results of the pairwise analysis of women with endometriosis confirmed the higher expression of epithelial (1.7-fold median increase; p<0.0001, Wilcoxon signed-ranks test) (Supplementary Fig. 1B), but not of stromal (Supplementary Fig. 1C) TACE protein in ectopic versus corresponding eutopic endometrium. Overall, these data strongly suggest that the changes of epithelial TACE protein are associated with the pathogenesis of the disease.

The differential expression of TACE in women with endometriosis is associated with transcriptional activation of *TACE* gene.

Since the levels of TACE protein were higher in endometriotic tissue compared to the eutopic endometrium of women without endometriosis, we further tested the hypothesis that the alterations of tissue TACE expression are due to the

transcriptional activation of the gene. As shown in Fig. 2A, *TACE* mRNA expression was significantly increased in ectopic endometrium compared to eutopic endometrium of both, endometriosis (+1.65 median fold change; adjusted p=0.0006) and control patients (+1.72 median fold change; adjusted p=0.0001; Mann-Whitney U-Test), respectively. This observation was confirmed by our pairwise analysis in the endometriosis sample group, showing that the expression of ectopic *TACE* mRNA is 2.15-fold higher (p<0.0001, Wilcoxon signed-ranks test) compared to the corresponding eutopic endometrium (Supplementary Fig. 1D). Moreover, although not significant, the median *TACE* expression tended to be higher in normal peritoneum of women with endometriosis compared to peritoneum of women without endometriosis (+1.39 median fold change; not significant; Fig. 2A).

TACE mRNA and protein levels do not correlate with lesion entity, menstrual cycle phase and disease severity.

Further, we examined whether TACE mRNA and/or protein levels are correlating with: (i) specific type of endometriosis, (ii) the severity of the disease (i.e. mild versus severe, as classified by rAFS score), (iii) the phase of the menstrual cycle, or (iv) with additional general patient characteristics.

The results of our subgroup analyses revealed that there are no significant differences in TACE expression between any of the subgroups (Supplementary Table I).

The overexpression of tissue TACE is strongly associated with high serum sVCAM-1 levels.

As we recently reported, the levels of circulating serum sVCAM-1 and sICAM-1 differ between women with and without endometriosis (Kuessel *et al.*, 2017). Thus, we further asked whether these differences are associated with changes in TACE levels in tissue, serum and/or peritoneal fluid (PF). To answer this question, we first analyzed the concentration of TACE in serum and peritoneal fluid in women with and without the disease. The data from our ELISA analyses showed that both the serum and PF TACE concentrations do not significantly differ between cases and controls (p=0.268 and 0.518, respectively; Mann-Whitney U-tests; Supplementary Table II). Further, we performed a subgroup analysis, as described in the previous section, and showed that there are no significant differences in TACE biofluid concentration between any of the tested subgroups except for the clinical parameter BMI (Supplementary Table II). The results from this analysis revealed that that the concentration of TACE in peritonial fluid of women with and without endometriosis is significantly higher in obese (BMI>30, median=20.55 pg/mg in women with endometriosis and median=13.79 pg/mg in controls) compared to women with BMI <30 (median=0.00 pg/mg in women with endometriosis and median=0.00 pg/mg in controls, Kruskal-Wallis test; Supplementary Table II).

When looking at the levels of the sVCAM-1 in peritoneal fluid we found no significant difference between women with and without endometriosis (median=4.28 pg/mg vs. median=4.13 pg/mg, p=0.850; Mann-Whitney-U test; Supplementary Table II). However, when we compared the levels of TACE expression in tissue samples to the corresponding levels of sVCAM-1 and sICAM-1 in the serum, we found that the high epithelial TACE protein expression is associated with significantly higher sVCAM-1 but not with sICAM-1 serum levels (p=0.011; median serum sVCAM-1 concentration

in tissues with high TACE protein= 571.3 pg/ml versus median serum sVCAM-1 concentration in tissues with low TACE protein= 402.5pg/ml ; Mann-Whitney U-test; Fig. 2B; Spearman Corr.Coeff.=0.450, p=0.004). In summary, we showed that the overexpression of tissue TACE is associated with higher serum sVCAM-1 levels suggesting an involvement of TACE in the mechanism of VCAM-1 membrane shedding.

TACE is upregulated in the endometrial epithelium of the proliferative phase in infertile women and in infertile women with endometriosis

It has recently been shown that high levels of TACE protein in luminal epithelium can be associated with infertility Thus, we further asked whether this finding is associated with endometriosis. For this subgroup analysis we have defined a cohort (fertiles n=12; infertiles n=8) as described in materials and methods section. When looking at the epithelial TACE protein expression in fertile women and in women with primary infertility, we found a significant increase of TACE protein expression in infertile compared to fertile women (p=0.048, median=3.0 versus median=6.0 ; Mann-Whitney U-test; Fig. 3A). We observed this infertility-associated upregulation of TACE protein only in the proliferative phase (p=0.0014, median= 3.0 versus median=9.0 ; Mann-Whitney U-test; Fig. 3A), but not in the secretory phase of the menstrual cycle irrespective of endometriosis. Further, at borderline significance, we found that the TACE protein is increased in infertile women with endometriosis, but not in infertile women without endometriosis (p=0.0506; median=4.0 versus median=6.0 ; Mann-Whitney U-test; Fig. 3B).

The shedding of VCAM-1 in 11Z epithelial endometriotic cell line is mediated by TACE.

To experimentally approach the mechanism of TACE mediated VCAM-1 shedding in endometriosis epithelia, we used the 11Z stable cell line. Most of the TACE mediated shedding events are known to occur at a low basal rate (Doedens *et al.* , 2003). Thus, we used Phorbol-12-myristate-13-acetate (PMA) stimulation to enhance the rate of the putatively TACE mediated VCAM-1 cleavage. As shown in Figures 4A and 4B, the stimulation of 11Z cells with PMA caused significant activation of *TACE* and *VCAM-1* transcription. The levels of *VCAM-1* mRNA were 19.45-fold and the levels of *TACE* mRNA were 2.26-fold higher in PMA treated compared to solvent treated cells (p=0.0002 and p=0.008, respectively, two-tailed t-test). Additionally, we observed a significant 2.11-fold up-regulation of the sVCAM-1 levels in the medium of PMA treated cells compared to the solvent controls (p=0.0128, two-tailed t-test, Fig. 4C). As expected, the transcriptonal effects of PMA were not abolished by the treatment of 11Z cells with different concentrations of either the non-specific TACE inhibitor TAPI-1 or the specific TACE inhibitor WAY-18022 (Fig. 4A and B). In contrast, both inhibitors alone or in combination were able to significantly suppress the shedding of sVCAM-1 into the medium of the 11Z cells (Fig. 4C). The pretreatment of the cells with 10 µM or 20 µM of the specific TACE inhibitor WAY-18022 before PMA stimulation significantly reduced the sVCAM-1 levels in the medium compared to PMA treated cells (mean fold decrease=-2.78 or -2.18; p=0.004 or 0.03, respectively, two-tailed t-tests, Fig. 4C). Similarly, the PMA induced sVCAM-1 shedding was significantly reduced by the pretreatment with 20 µM TAPI-1 or a combination of 10 µM TAPI-1 and 10 µM WAY-18022 (mean fold decrease=-2.02 or -2.90; p=0.009 or 0.008, respectively, two-tailed t-tests, Fig. 4C). Cell viability was affected neither by PMA stimulation nor by TACE inhibition (Fig. 4D). Based on this

observation, PMA induced TACE mediated shedding of VCAM-1 in 11Z cells occurs at least in part via an increase in enzymatically active TACE protein.

Reduced shedding of sVCAM-1 following TACE siRNA transfection

To confirm the role of TACE in the regulation of sVCAM-1 shedding in endometriosis, we performed knockdown experiments in the endometriotic epithelial cell line 11Z using a specific *TACE* siRNA. After affirming the efficiency of the TACE knockdown (Supplementary. Fig. 2), we performed Western blot analysis and ELISA assays to evaluate the effect of the knockdown on cellular sVCAM-1 release. We showed that the down-regulation of cellular TACE protein levels to 6.0% ($p<0.0001$; two-tailed t-test) was associated with significant reduction of released sVCAM-1 protein (Fig. 5A-C). The viability of the cells was not altered either by siRNA transfection or by PMA stimulation (Fig. 5D). PMA stimulation significantly increased the sVCAM-1 levels in the medium compared to the solvent-control in control siRNA transfected cells (mean fold increase=1.77; $p=0.030$, two-tailed t-test, Fig. 5C). The PMA induced sVCAM-1 shedding was significantly reduced in cells with downregulated TACE protein compared to controls (mean fold decrease=-1.67; $p=0.007$, two-tailed t-tests, Fig. 5C). These observations strongly suggest PMA induced TACE mediated shedding of VCAM-1 in epithelial endometriotic 11Z cells.

Discussion

In this study we found that TACE is overexpressed in the epithelial cell compartment of tissue samples of women with compared to women without endometriosis and that the overexpression of the protein in the lesions is at least in

part due to activation of *TACE* gene transcription. Althought increased levels of TACE protein in glandular and luminal epithelia at ectopic sites of women with endometriosis were previously reported, this enhanced protein expression could not be associated with changes in the levels of the *TACE* transcript (Gottschalk et al., 2000). These cellular changes in the levels of TACE expression were not associated with lesion entity, menstrual cycle phase or disease severity. Thus, there may be biological selection for stronger activation of intracellular TACE regulated processes, supporting the formation and promoting the survival of the ectopic lesions, such as enhanced endometrial cell migration, adhesion and invasion, increased inflammatory and impaired immune responses (Giudice and Kao, 2004, Hull *et al.* , 2008, Koninckx *et al.* , 1998). In agreement with this hypothesis, Miller et al. (Miller *et al.*, 2013) have demonstrated that TACE integrates numerous signaling pathways to direct cell migration in an endometriotic epithelial cell line. In addition, *in vitro* experimental evidence has associated the hyperactivation of TACE/Notch signaling with endometriosis related fibrotic processes that take place in ectopic lesions of patients with deep infiltrating endometriosis (Gonzalez-Foruria *et al.* , 2017).

TACE mediated ectodomain shedding is a regulated proteolytic process that directs the cleavage of cell surface proteins, typically at a juxta-membrane site, resulting in the release of a soluble extracellular domain fragment (Murphy, 2008). The functional implications of ectodomain shedding are diverse, because it can promote formation of soluble agonists and antagonists, as well as regulate the density of receptors and adhesion molecules. TACE is known to process several different substrates, many of which are mediators of inflammation and immune responses, such as VCAM-1 and ICAM-1 (Herren, 2002, Mullooly *et al.*, 2016). Since we have recently reported that the expression of both genes is higher in endometriotic lesions than in eutopic endometrium, and that the *VCAM-1* expression

is also higher in eutopic endometrium of women with endometriosis compared to controls (Kuessel *et al.*, 2017), we questioned whether this protein is a direct substrate for TACE enzyme in endometriosis. By using an *in vitro* experimental system of endometriotic epithelial cells, we provide an important functional proof that TACE is responsible for the enhanced release of sVCAM-1 from endometriotic epithelial cells. These data together with our observation that increased levels of tissue TACE are associated with significantly elevated sVCAM-1 serum levels draws attention to the role of this enzyme in systemic inflammatory processes in endometriosis. Although previous findings from our group (Kuessel *et al.*, 2017) and others (Barrier and Sharpe-Timms, 2002, Daniel *et al.*, 2000, Kuessel *et al.*, 2017) showed that serum sVCAM-1 levels are elevated in women with endometriosis, it should be mentioned that by quantifying these levels it is not feasible to distinguish between sVCAM-1 originating from endothelial, peritoneal mesothelial or endometriosis cells, or VCAM-1 released by angiogenic lymphatic vessels. Here, we did not find significant disease related changes in the levels of *TACE* transcript in healthy peritoneum of women with versus without the disease. Thus, we can exclude the putative involvement of the enzyme in the regulation of mesothelial sVCAM-1 release.

Elevated circulating adhesion molecules may also compete with the membrane bound forms and limit the adhesive interaction of leukocytes and endothelium (Fiore *et al.* , 2002, Meyer *et al.* , 1995, Mullooly *et al.*, 2016, Somigliana *et al.*, 1996, Vigano *et al.*, 2001, Zeller *et al.* , 1994). In addition, cleavage of VCAM-1 by TACE may play a role in regulating the adhesive function of VCAM-1 by rapidly decreasing its levels at the cell surface. In support of this model, it has been shown that L-selectin, another TACE substrate, is rapidly shed from the surface of leukocytes during emigration into sites of inflammation (Faveeuw *et al.* , 2001).

Based on these findings we speculate that increased TACE expression in endometriotic lesions and endometrium of women with endometriosis may facilitate the escape of endometrial tissue from the T cell-mediated immune response, thereby preventing effective immune clearance of the endometriosis implant.

It has been shown that the levels of TACE may reflect the progression of several human disorders (Bertram *et al.* , 2015, Bostanci *et al.* , 2008, Brynskov *et al.* , 2002, Liu *et al.* , 2013), including endometriosis (Gonzalez-Foruria *et al.*, 2015) . For example, elevated TACE activity was found in peritoneal fluid (PF) of women with deep infiltrating endometriosis (DIE) where it significantly correlated with advanced oxidation protein products secreted into PF(Gonzalez-Foruria *et al.*, 2017). The data from our ELISA analysis revealed that both serum and PF TACE levels do not significantly differ between cases and controls. In the cited study the difference in PF TACE activity was tested between controls and DIE cases. In contrast, in our study we have evaluated the levels of expression/secretion of the TACE enzyme. Thus, the discrepancy of our data with the study mentioned above might reflect differences in research approaches and could be linked to the limited number of DIE cases in our study cohort (<5%).

Information about the control of TACE expression levels and/or activity in normal endometrial tissue is limited. Jowicz at al. (Jowicz *et al.* , 2013) have shown that TACE expression levels are highest throughout the menstrual to follicular phases of the cycle. This suggests a role of the protein in tissue remodeling and repair events that occur during and after menstruation and further, the putative implication of the protein in the regulation of endometrial receptivity. This notion is supported by the recent study of Van Sinderen at al. (Van Sinderen *et al.*, 2017), showing that the levels of TACE in luminal epithel during mid-secretory phase of the menstrual cycle are significantly higher in women suffering from primary infertility, compared to fertile

controls. Increased levels of TACE were also found in women with intrauterine adhesions (Liu *et al.*, 2013), a pathologic condition associated with infertility (Valle and Sciarra, 1988). Our current findings delivered an experimental proof for the putative role of the enzyme in endometriosis-associated infertility. We were able to show that the TACE is increased in infertile women with endometriosis, but not in infertile women without endometriosis. It is important to note that the results from this subgroup analysis were obtained from a very small cohort of samples. Thus, the results should be treated with caution and need to be evaluated in a separate larger study to be able to make any meaningful conclusions regarding the role of the enzyme in endometriosis-associated infertility. Moreover we observed this infertility-associated upregulation of TACE only in the proliferative phase of the menstrual cycle. Although endometrial glands and their secretions are essential for embryo implantation and survival, the proliferative phase, when glands are formed, is a critical period which lays the foundation for the subsequent, receptive secretory phase. Therefore, we believe that increased levels of epithelial TACE in proliferative phase of infertile woman with endometriosis might be associated with deregulation of the glandular functions and thereby have an impact on endometrial receptivity.

Albeit well characterized, our control population included women with other gynecological diseases, which may have impacted the levels of tissue and or biofluid TACE e.g. benign ovarian cysts or uterine fibroids. Thus, the results of our analyses have to be interpreted carefully and in the context of the current experimental settings.

In conclusion, we provide the first functional proof that induced TACE activity in human endometriotic epithelial cells is at least in part responsible for the enhanced release of sVCAM-1 from these cells. This putative mechanism of endometriosis-associated deregulation of the levels of circulating sVCAM-1, represents a promising

yet relatively unexplored area of the pathogenesis of endometriosis and could serve as a basis for the development of new alternative treatment approaches for the disease. In addition, we have accumulated experimental evidence supporting the hypothesis that TACE may be an important factor responsible for impaired endometriosis-associated fertility as well as for hindered immune surveillance at the site of endometriotic lesions.

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Authors´roles

KP, IY, HH and LK contributed to the conception and design of the study. HH, LK and RW assisted with sample collection. KP, SG, CH, and LS collected data and performed experiments. KP developed the statistical analyses. KP and IY, wrote the manuscript. HH, MM, LK, RW and CS revised the manuscript for important intellectual content. All authors approved the final manuscript.

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Conflict of interest

The authors have no competing interests to declare.

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	Median *TACE* mRNA expression (number of samples)				Median epithelial TACE protein expression (number of samples)				Median stromal TACE protein expression (number of samples)			
	Eutopic Control	**Eutopic EM**	**Ectopic Lesion**	**Adj. p-value**	**Eutopic Control**	**Eutopic EM**	**Ectopic Lesion**	**Adj. p-value**	**Eutopic Control**	**Eutopic EM**	**Ectopic Lesion**	**Adj. p-value**
	0.0451 (28)	0.0471 (33)	0.0777 (50)	$9.0*10^{-5}$	2.0 (39)	4.0 (42)	6.0 (42)	$7.44*10^{-9}$	2.0 (38)	3.5 (42)	4.0 (41)	*0.020*
Lesion entity												
Ovarian	n.a.	n.a.	0.0798 (38)	0.456	n.a.	n.a.	6.0 (13)	0.992	n.a.	n.a.	4.0 (12)	0.170
Peritoneal	n.a.	n.a.	0.0673 (5)		n.a.	n.a.	6.0 (9)		n.a.	n.a.	4.0 (9)	
DIE/others	n.a.	n.a.	0.0534 (6)		n.a.	n.a.	4.0 (11)		n.a.	n.a.	3.0 (11)	
Disease stage												
rAFS Score I or II	n.a.	0.0463 (13)	0.0825 (6)	0.737^{+}	n.a.	4.0 (19)	6.0 (12)	0.753^{+}	n.a.	4.0 (19)	4.0 (12)	0.498^{+}
rAFS Score III or IV	n.a.	0.0477 (20)	0.0777 (44)		n.a.	4.0 (23)	6.0 (26)		n.a.	3.0 (23)	4.0 (25)	
Menstrual cycle phase												
Proliferative	0.0316 (8)	0.0463 (15)	0.0705 (29)	0.981^{+}	2.0 (19)	3.5 (20)	4.0 (18)	0.278^{+}	3.5 (18)	2.0 (20)	4.0 (18)	0.451^{+}
Secretory	0.0449 (19)	0.0490 (18)	0.100 (21)		2.0 (20)	4.0 (22)	6.0 (24)		2.0 (20)	4.0 (22)	4.0 (23)	
BMI classification												
Underweight <18.5	0.0334 (3)	0.0406 (6)	0.0550 (12)	0.893	9.0 (1)	4.5 (2)	5.0 (4)	0.428	(0)	3.0 (2)	4.0 (4)	0.809
normal weight 18.6-24.9	0.0374 (12)	0.0433 (18)	0.0817 (27)		2.5 (6)	4.0 (13)	6.0 (7)		2.0 (6)	3.0 (13)	4.0 (7)	
Overweight 25-29.9	0.0630 (7)	0.04631 (3)	0.0710 (5)		2.5 (4)	6.0 (1)	(0)		4.0 (4)	4.0 (1)	(0)	
Obesity >30	0.0570 (6)	0.0553 (6)	0.0953 (6)		3.0 (2)	5.0 (4)	3.0 (2)		2.0 (2)	4.0 (4)	4.0 (1)	
Cigarette smoking												
No	0.0390 (15)	0.0524 (27)	0.0788 (42)	0.255^{+}	2.5 (6)	5.0 (18)	6.0 (13)	0.105^{+}	2.0 (5)	3.5 (18)	4.0 (12)	0.294^{+}
Yes	0.0452 (13)	0.0383 (6)	0.0701 (8)		3.0 (7)	4.5 (2)	(0)		2.0 (7)	3.5 (2)	(0)	

Supplementary Table I. TACE expression levels in indicated subgroups of eutopic endometrium of women with and without endometriosis (controls) and ectopic lesions.

TACE mRNA values are presented as the median *TACE* mRNA expression levels normalized to *ACTB* and *GAPDH* as described in Kuessel et al 2017; Epithelial and Stromal TACE protein values are presented as the median TACE protein score evaluated as described in Proestling et al. 2016 and the number of patients (n); All p-values were calculated using Mann-Whitney U-test^{+} or Kruskal-Wallis test. p-values were Bonferroni-Holm adjusted. DIE, deep infiltrating endometriosis; n.a., not applicable; rAFS, revised American Fertility Society; BMI classification using WHO guidelines 2000; EM, endometriosis patients

	Median Serum TACE concentration (pg/ml)			Median Peritoneal Fluid TACE concentration (pg/mg)			Median Peritoneal Fluid sVCAM-1 concentration (ng/mg)		
	Endometriosis (number of samples)	Controls (number of samples)	Adj. p-value	Endometriosis (number of samples)	Controls (number of samples)	Adj. p-value	Endometriosis (number of samples)	Controls (number of samples)	Adj. p-value
	10681,3 (43)	17644,4 (28)	0,268*	0,00 (23)	0,75 (21)	0,518*	4,28 (44)	4,13 (32)	0,850*
Lesion entity									
Peritoneal	7768,9 (7)	n.a.	0,405	1,11 (6)	n.a.	0,650	3,96 (7)	n.a.	0,784
Ovarian	24324,9 (5)	n.a.		4,70 (2)	n.a.		2,84 (5)	n.a.	
Peritoneal and Ovarian	7022,8 (9)	n.a.		1,41 (6)	n.a.		4,00 (9)	n.a.	
Ovarian and DIE	8897,9 (6)	n.a.		0,00 (6)	n.a.		2,98 (6)	n.a.	
Peritoneal and Ovarian and DIE	5069,7 (2)	n.a.		0,00 (2)	n.a.		4,41 (2)	n.a.	
Disease stage									
rAFS Score I or II	15999,1 (15)	n.a.	0,513*	2,21 (9)	n.a.	0,444*	4,30 (15)	n.a.	0,235*
rAFS Score III or IV	9574,4 (28)	n.a.		0,00 (14)	n.a.		4,27 (29)	n.a.	
Menstrual cycle phase									
Proliferative	8581,1 (20)	13758,6 (8)	0,223*	0,00 (9)	0,75 (9)	0,335*	5,08 (21)	4,07 (11)	0,157*
Secretory	14159,3 (23)	18843,2 (19)		4,04 (14)	5,82 (12)		4,03 (23)	4,23 (20)	
BMI classification									
Underweight <18.5	161,1 (8)	17964,9 (3)	0,371	0,00 (4)	0,00 (2)	*0,018*	4,42 (8)	4,02 (3)	0,748
normal weight 18.6-24.9	15379,2 (23)	17344,2 (12)		0,00 (13)	0,00 (7)		4,40 (24)	4,39 (14)	
Overweight 25-29.9	10044,7 (6)	13999,7 (7)		0,00 (3)	0,38 (6)		4,23 (6)	3,73 (9)	
Obesity >30	16709,2 (6)	15474,0 (6)		20,55 (3)	13,79 (6)		3,69 (6)	4,12 (6)	
Cigarette smoking									
No	14079,6 (35)	18843,2 (15)	0,557*	0,00 (18)	1,18 (13)	0,857*	4,36 (36)	4,00 (18)	0,381*
Yes	8954,1 (8)	13111,6 (13)		0,00 (5)	0,00 (8)		4,14 (8)	4,47 (14)	

Supplementary Table II . Concentration of TACE in serum and peritoneal fluid and sVCAM-1 concentration in serum in indicated subgroups of eutopic endometrium of women with and without endometriosis (controls) and ectopic lesions.

Serum values of TACE are presented as the median TACE concentration in pg/ml; Median peritoneal fluid values of TACE and sVCAM-1 concentrations are presented in pg/mg total protein and ng/mg total protein, respectively, and the number of patients (n); All p-values were calculated using Mann-Whitney U-test* or Kruskal-Wallis test. p-values were Bonferroni-Holm adjusted. DIE, deep infiltrating endometriosis; n.a., not applicable; rAFS, revised American Fertility Society; BMI classification using WHO guidelines 2000; EM, endometriosis patients

Figure 1. Immunohistochemical analyses of TACE expression levels in eutopic and ectopic endometrium of women with and without endometriosis are shown. Anti-TACE antibody was applied at a dilution of 1:2000 and yielded weak, moderate, or strong staining in eutopic (**A, B, C**) or ectopic tissue (**D, E, F**) of women with and without endometriosis, respectively. The intensity (0-3) and the percentage (0-3) of the stained cells were combined to derive a final IHC score (0-9). For statistical evaluation of the cellular TACE intensity, epithelial (**G**), stromal (**H**) and luminal (**I**) immunostainings were analyzed separately at magnification = 200x in a cohort of n=39 control patients (EM controls) and n=42 women with endometriosis. The latest included tissue samples from unpaired (n=6) and paired (n=36) eutopic (EM patient) and ectopic (ectopic EM) tissue specimens, respectively. All results are expressed as box and whisker plots ranging from minimum to maximum, including the median and box boundaries at the 25th and 75th percentiles. The significant differences between the groups are indicated by the p-value on the top of the graph. p-values <0.05 are considered as significant. EM, endometrium; TACE, TNF-alpha converting enzyme.

Figure 2.
(**A**) qRT-PCR analyses of *TACE* mRNA expression levels in endometrium and peritoneum samples obtained from women with (eutopic, EM patients, and ectopic, ectopic EM) and without endometriosis (controls) are given. The analyzed cohort contained 28 EM controls, 33 EM patients, 50 ectopic EM, 17 peritoneum samples of controls and 20 peritoneum samples of endometriosis patients. The boxplots in A are showing changes in relative gene expression levels for *TACE* gene in analyzed tissue after their normalization to *GAPDH* and *ACTB* housekeeping genes. (**B**) Serum sVCAM-1 and sICAM-1 concentrations (pg/ml) in women with endometrial tissue samples showing low and high epithelial TACE protein expression are plotted. The cutoff for low (n=24) and high (n=14) TACE protein expression was set at IHC score of ≥6.
All results in this figure are expressed as box and whisker plots ranging from minimum to maximum, including the median and box boundaries at the 25th and 75th percentiles. Significance was determined using the Mann–Whitney *U*-test with Post Hoc Bonferroni-Holm correction. The significant differences between the groups are indicated by the p-value on the top of the graph. p-values <0.05 are considered as significant. TACE, TNF-alpha converting enzyme; EM, endometrium

Figure 3. The expression levels of TACE protein in endometrial tissue samples of fertile women and women with primary infertility with and without endometriosis are shown. Two group comparisons were performed. (**A**) The analysis of the differences between fertile and infertile women independent from their disease status is given in all women or in women in proliferative or secretory menstrual cycle phase. (**B**) Disease-dependent changes of TACE protein levels are plotted. The cohort of analyzed samples in (**A**) included specimens obtained from n=12 fertile and n=8 infertile women, of which 9 fertile and 3 infertile women were in proliferative, 3 fertile and 5 infertile were in secretory menstrual cycle phase. The subgroup analysis in (**B**) included 5 fertile and 3 infertile women of the

control group and 7 fertile and 5 infertile women with endometriosis. All results in the figures are expressed as box and Whisker plots, ranging from minimum to maximum value of TACE protein, including the median and box boundaries at the 25th and 75th percentiles. Significance was determined using the Mann–Whitney *U*-test. The p-values from these analyses are given on the top of each graph. p-values <0.05 are considered as significant. TACE, TNF-alpha converting enzyme

Figure 4. The effects of specific (WAY-18022) and broad spectrum (TAPI-1) TACE inhibitors on *TACE* (**A**) and *VCAM-1*(**B**) transcription in 11Z endometriotic epithelial cell line are given. The mRNA expression levels of both transcripts were measured using quantitative real-time PCR, performed 1 h after initial TACE inhibition with indicated concentrations of inhibitors followed by 3 h of stimulation with either PMA (1 µM) or 0.06% DMSO solvent control. The bar-graphs are showing the changes in relative gene expression levels for the transcripts after their normalization to *GAPDH* and *ACTB* housekeeping controls. (**C**). Effect of TACE inhibition on the levels of extracellular sVCAM-1 release from 11Z endometriotic epithelial cells. The *in vitro* shedding of sVCAM-1 into the culture medium of 11Z cells was measured 1 h after initial inhibition of intracellular TACE with indicated concentration of inhibitors, followed by 3 h of stimulation with either PMA (1 µM) or 0.06% DMSO solvent control, using Luminex Performance technology. The results are presented as averaged pg of sVCAM-1 per 10E5 cells of biological and technical replicates ±SD. Statistics was performed using t-test (GraphPad). Statistically significant differences between the treated groups and respective PMA-stimulation only are indicated with stars on the top of each bar. *, p<0.05; **p<0.005
(**D**) Effect of WAY-18022 and TAPI-1 TACE-inhibitors on 11Z cell viability. Viable cells were counted, after the treatment conditions described in C and after staining with Trypan blue. Data are plotted as a mean of the total number of cells of technical and biological replicates ± SD. Statistics was performed using two-way ANOVA (GraphPad). p-values <0.05 are considered as significant. TACE, TNF-alpha converting enzyme; PMA, Phorbol-12-myristate-13-acetate

Figure 5. (**A**) Western blot analyses of the levels of TACE protein in 11Z cells, transiently transfected with 10 nM of either control or *TACE*-siRNA and treated with either 1 µM PMA or solvent control (0.01% DMSO) for 3 h is given. Beta-actin was used as protein loading control. The duration of siRNA transfection was 48 h. (**B**) The histogram shows the densitometric analysis of mean TACE protein ±SD of biological triplicates. Each band was normalized to the corresponding loading control and relative to those for untreated control siRNA, set to 100%. (**C**) Effect of *TACE* siRNA knockdown on cellular sVCAM-1 release under the culturing treatment conditions plotted below the x-axis of the bar-graph is shown. Shedding of sVCAM-1 into the culture medium of epithelial endometriotic cell line 11Z was measured 3 h after stimulation with PMA (1 µM) or 0.01% DMSO solvent control. Inhibition of PMA induced sVCAM-1 shedding was measured after 48 h siRNA incubation (10 nM). The results from Luminex sVCAM-1 assay are presented as averaged pg per 10E5 cells of biological and technical replicates ±SD, compared to PMA treated control siRNA.
(**D**) Effect of *TACE* siRNA knockdown on 11Z cell viability. Viable cells were counted, after the treatment conditions described in A and after staining with Trypan blue. Data are plotted as a mean of the total number of cells of technical and biological replicates ± SD.

Statistics was performed using t-test (GraphPad). The p-values from these analyses are given on the top of each graph; ***p<0.0001. p-values <0.05 are considered as significant. TACE, TNF-alpha converting enzyme; PMA, Phorbol-12-myristate-13-acetate

Supplementary Figure 1. Immunohistochemical analyses of luminal (**A**), epithelial (**B**) and stromal (**C**), TACE protein levels in 36 paired cases of eutopic and corresponding ectopic endometriotic tissues are expressed on the graphs. The intensity (0-3) and the percentage (0-3) of the stained cells were combined to derive a final IHC score (0-9). (**D**) The levels of *TACE* transcript in 35 paired samples of eutopic and corresponding ectopic tissues are shown. For six eutopic samples more than one corresponding ectopic lesion was analyzed. The boxplots in D are showing changes in relative gene expression levels for *TACE* gene in analyzed tissue after their normalization to *GAPDH* and *ACTB* housekeeping genes. Paired statistics were performed using Wilcoxon signed ranks Test. The significant differences between the groups are indicated by the p-value on the top of the graph. p-values <0.05 are considered as significant. TACE, TNF-alpha converting enzyme; EM, endometrium

Supplementary Figure 2. Levels of *TACE* and *GAPDH* mRNA and protein in 11Z cells, transiently transfected with 5 nM of either control, *TACE*- or *GAPDH*-siRNA for 48h, 72, and 96 h are plotted. The mRNA levels of *TACE* (**A**) and *GAPDH* (**B**) in 11Z cells, transiently transfected with 5 nM of either control-, *TACE*- or *GAPDH* siRNA for 48h, 72, and 96 h is given. The bar-graphs are showing the changes in relative gene expression levels for the transcripts after their normalization to *ACTB* housekeeping control and relative to their respective control siRNA, set to 100%. The graphs show the levels of TACE (**C**) and GAPDH (**D**) protein plotted as optical density of each protein to the Tubulin loading control and relative the respective loading controls, set to 100%. One independent experiment and technical replicates for qRT-PCR are shown. (**E**) Western blot analyses of pro TACE and GAPDH protein in 11Z cells, transiently transfected as described in (B) are given. Tubulin was used as protein loading control. TACE, TNF-alpha converting enzyme; PMA, Phorbol-12-myristate-13-acetate