Evaluation of sterol-o-acyl transferase 1 and cholesterol ester levels in plasma, peritoneal fluid and tumor tissue of patients with endometrial cancer: A pilot study

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Abstract. Endometrial cancer (EC) is the most prevalent gynecological malignancy. Abnormal accumulation of sterol-O-acyl transferase 1 (SOAT1) and SOAT1-mediated cholesterol ester (CE) contributes to cancer progression in various malignancies, including ovarian cancer. Therefore, it was hypothesized that similar molecular changes may occur in EC. The present study aimed to evaluate the diagnostic and/or prognostic potential of SOAT1 and CE in EC by: i) Determining SOAT1 and CE levels in plasma, peritoneal fluid and endometrial tissue from patients with EC and control subjects; ii) performing receiver operating characteristic curve analysis to determine diagnostic performance; iii) comparing SOAT1 and CE expression to that of the tumor proliferation marker Ki67; and iv) assessing the association between SOAT1 expression and survival. Enzyme-linked immunosorbent assay was used to determine the levels of SOAT1 protein in tissue, plasma and peritoneal fluid. The mRNA and protein expression levels of SOAT1 and Ki67 in tissues were detected by reverse transcription-quantitative polymerase chain reaction and immunohistochemistry, respectively. CE levels were determined colorimetrically in plasma and peritoneal fluid. SOAT1-associated survival data from the cBioPortal cancer genomics database were used to assess prognostic relevance. The results revealed that SOAT1 and CE levels were significantly elevated in tumor tissue and peritoneal fluid samples collected from the EC group. By contrast, the plasma levels of SOAT1 and CE in the EC and control groups were similar. Significant positive associations between CE and SOAT1, SOAT1/CE and Ki67, and SOAT1/CE and poor overall survival in patients with EC suggested that SOAT1/CE may be associated with malignancy, aggressiveness and poor prognosis. In conclusion, SOAT1 and CE may serve as potential biomarkers for prognosis and target-specific treatment of EC.

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Abbreviations: SOAT1, sterol-O-acyl transferase 1; ACAT-1, acyl-CoA cholesterol acyl transferase; CE, cholesterol ester; FC, free cholesterol; TC, total cholesterol

Key words: endometrial cancer, SOAT1, CE, Ki67

Introduction

For endometrial cancer (EC), poor prognostic factors driving tumor recurrence are directly related to mortality (1). Tumor grade, histological subtype, degree of myometrial invasion, cervical involvement, tumor size, lymph-vascular space invasion (LVSI), and lymph node status are the most important clinicopathological prognostic markers in patients diagnosed with EC (2). However, these markers have been proven to be of limited utility, particularly in the case of recurrent or high-grade EC (2,3). It is therefore crucial to identify prognostic biomarkers for metastatic and/or recurrent EC to estimate disease risk and treatment options. High-grade and metastatic/recurrent EC patients have few therapeutic options due to the ineffectiveness of traditional platinum and taxane chemotherapy regimens and the limited impact of newer agents such as Lenvatinib, Pembrolizumab and Bevacizumab (4,5). New therapeutic approaches are necessary to address this critical unmet need. A deep understanding of the molecular alterations associated with EC offers the opportunity of adding targeted therapies to the current treatment arsenal.

Excess lipids and cholesterol are converted to triglycerides and cholesteryl esters (CE) in cancer cells (6). Intra-tumoral CE has been shown to increase tumor cell proliferation, invasiveness, and survival (7-10); thus, inhibiting CE synthesis may be a useful anti-cancer therapeutic strategy (10). Because sterol O-acyltransferase (SOAT1), also known as acyl-CoA

cholesterol acyltransferase1 (ACAT1) is involved in maintaining appropriate levels of CE within cells by converting excess cholesterol to CE, this enzyme became the target of research in tumor cells. SOAT1 expression and CE levels were reported to be abnormal in a variety of cancers, including ovarian cancer, leukemia, glioma, prostate cancer, pancreatic cancer, breast cancer, and colon cancer (11-16). However, information on the role of SOAT1/CE accumulation in EC is limited. We have recently reported increased SOAT1 and CE levels in ovarian cancer cell lines, tumor tissue, and peritoneal fluid compared to the non-malignant group, confirming SOAT1-mediated CE accumulation as a cancer-specific event (15). The expression of these mediators correlated with malignancy and tumor aggressiveness in ovarian cancer (16,17). These findings prompted us to investigate SOAT1/CE as potential prognostic or therapeutic targets in advanced EC.

The Cancer Genome Atlas (TCGA) has reported SOAT1 expression (mRNA and protein) levels in normal and tumor tissue from EC patients; however, to date, there are no data documenting SOAT1 levels in peritoneal fluid or plasma of EC patients. Consequently, we examined the levels of SOAT1 and CE in tumor tissue, peritoneal fluid, and plasma from subjects diagnosed with EC and subjects with normal endometrium (controls) to ascertain the relationship between SOAT1/CE levels and a variety of factors, including malignancy, tumor aggressiveness (ki67 expression), and survival. Additionally, possible correlations of SOAT1/CE levels between peritoneal fluid, plasma and tumor tissue were assessed to evaluate their diagnostic potential. A strong, positive, linear correlation between increasing BMI and disease incidence, as well as a strong, negative, linear correlation between increasing BMI and oncological outcomes have been previously reported (18). Moreover, comorbidities such as obesity, hyperlipidemia, diabetes, hypertension and hyperthyroidism are known to alter cholesterol metabolism; therefore, BMI and other comorbidities were adjusted in logistic regressions to analyze their potential influence on the association between SOAT1/CE levels and malignancy.

Materials and methods

Study design. This was an observational, cross-sectional pilot study involving patients scheduled for an oophorectomy, bilateral salpingo-oophorectomy (BSO), hysterectomy, hysterectomy/BSO, staging and/or debulking via laparotomy or laparoscopy for surgical management of biopsy confirmed EC, between 2016 and 2021, at the Division of Gynecological Oncology, Department of Obstetrics & Gynecology, Southern Illinois University School of Medicine, Springfield, IL. Patients scheduled to undergo the aforementioned procedures for the management of other gynecologic diagnoses (e.g., pelvic prolapse), were enrolled within the divisions of General Gynecology and Urogynecology. Exclusion criteria included a previous malignancy, chemotherapy or radiation therapy prior to surgery. All sample collections were performed on the day of surgery. After surgery, subjects were grouped into three study cohorts based on their diagnosis: i) Subjects with a confirmed diagnosis of EC ('EC' group; N=32); and ii) subjects with normal endometrium ('control' group; N=16). Relevant clinical information was collected from SIU electronic health records, including: age, menopausal status, cancer diagnosis, FIGO stage/grade (confirmed by independent pathologists), and presence of comorbidities such as obesity, dyslipidemia, diabetes, hypertension and hypothyroidism.

For analysis, data from subjects diagnosed with stage I and stage II were pooled together into the 'early stage EC' group (N=18) whereas data from subjects diagnosed with stage III and stage IV patients were pooled into the 'advanced stage EC' group N=14). The clinical and pathological characteristics of the study population are summarized in Table I.

Ethic statement, standard protocol approvals, registrations and patient consents. This study was approved by the local Institutional Review Board (Springfield Committee for Research Involving Human Subjects) under protocol 16-493. Eligible patients (age ≥30 years) were invited to participate during their preoperative evaluation and diagnostic workup. If patients expressed interest in participation, written informed consent was obtained at this preoperative visit.

Peripheral blood, peritoneal fluid and tumor tissue sample collection. Peripheral blood was collected into sodium heparin tubes just prior to surgery. Peritoneal fluid was collected during the surgical procedure as previously described (19). To summarize, collection involves aspiration of ascites, infusion of saline and re-aspiration of the fluid. Plasma and peritoneal fluid samples were centrifuged at 1,500 r/min for 10 min and stored at -80˚C before being tested. Fragments (≥1 cm^3) of fresh tissue without necrotic areas were collected from the endometrium of subjects immediately after the hysterectomy was completed. A macro-dissection of the tissue samples was performed to remove fatty tissue and exclusively collect tumor or normal endometrial tissue. The tissue specimens were flash frozen in liquid nitrogen and stored at -80˚C until analyzed.

SOAT1 protein quantification by enzyme-linked immunosorbent assay (ELISA). SOAT1 protein concentrations in plasma, peritoneal fluid, and tissue lysates were determined using the SOAT1 ELISA Kit (Human) from Mybiosource according to the manufacturer's protocol. Quantification of SOAT1 in plasma and peritoneal fluid was done in 50 μl aliquots of sample. Tissue lysates for ELISA were prepared as described previously (16,17). Protein concentrations were quantified using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad). Absorbance was measured at 450 nm using a Synergy H1MFD (Hybrid multimode) microplate reader (BioTek). Concentration of SOAT1 (pg/ml) was calculated by interpolation from a standard curve.

Quantitative analysis of CE, free cholesterol (FC) and total cholesterol (TC) from plasma and peritoneal fluid. We used the Total Cholesterol and Cholesteryl Ester Colorimetric Assay Kit from Biovision to quantify TC, FC, and CE from plasma and peritoneal fluid, as previously described (15). Following the manufacturer's instructions, concentrations were determined in 50 μl aliquots of sample. The absorbance at 570 nm was measured using a Synergy H1MFD (Hybrid multimode) microplate reader (BioTek). Interpolation from a standard curve was used to calculate TC concentrations (mg/dl).

Table I. Clinical and pathological characteristics of samples.

Parameter	Control	EC
Sample size, n[a] (%)	16 (33)	32 (67)
Age, median years (min-max)	61.5 (52-81)	63 (49-83)
BMI, median kg/m^2 (min-max)	26.7 (20.9-35.3)	35.9 (19.8-52.0)
Premenopausal, n (%)	2 (13)	1 (3)
Postmenopausal, n (%)	14 (88)	31 (97)
Obesity, n (%)	4 (25)	17 (53)
Diabetes, n (%)	3 (19)	7 (22)
Hypertension, n (%)	3 (19)	17 (53)
Hypothyroidism, n (%)	3 (19)	8 (25)
FIGO stage, n (% of EC)		
Stage I		16 (50)
Stage II		2 (6)
Stage III		12 (38)
Stage IV		2 (6)
FIGO grade, n (% of EC)		
Grade 1		15 (47)
Grade 2		7 (22)
Grade 3		4 (13)
No information		6 (19)
Histotype, n (% of EC)		
Endometrioid		27 (85)
Serous		3 (9)
Other		2 (6)

BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; EC, endometrial cancer. aIndicates total number of patients eligible for the study.

Immunohistochemistry (IHC). As described previously, immunohistochemical analysis for SOAT1 was performed on paraffin-embedded endometrial tissue sections generated by Springfield Memorial Hospital Laboratory as surgical pathology standard of care samples. We also purchased endometrial cancer disease spectrum tissue microarray slides from US Biomax (T091a and T094) for SOAT1 and Ki67 staining. IHC was performed as per standard protocol previously described (16,17). Briefly, the slides were deparaffinized, rehydrated and heated to unmask the antigenic sites. The slides were further incubated with appropriate primary antibodies (SOAT1 1:500 dilution, Ki67 1:500 dilution) and their respective secondary antibodies (Abcam), followed by detection with 3.3'-diaminobenzidine (DAB) substrate (Abcam) and counterstain with hematoxylin. Negative controls were performed without the addition of any primary antibody to rule out any nonspecific staining of the secondary antibodies. Images were captured using an inverted microscope (Olympus H4-100, CCD camera) with a 20X objective. Five images were recorded in each core, and 1 μm wide z-stacks were obtained. ImageJ software (NIH) was used to analyze the images. For pathological investigation, one slide per sample was stained with hematoxylin and eosin. We have included appropriate IgG isotype negative controls to establish the background staining during IHC method optimization studies. We used recombinant Anti-Ki67 (ab92742) and anti-SOAT 1/SOAT1 (ab39327) primary antibodies from Abcam.

RNA extraction and cDNA synthesis. Total RNA from EC tumors and normal endometrial tissues were isolated using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After assessing the yield and quality by spectrophotometry, the RNA was stored at -80˚C until use. A total of 1 μg RNA from each sample was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad).

Gene expression analyses by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). SOAT1 and Ki67 mRNA levels were determined by real-time RT-qPCR using their respective specific primers purchased from Integrated DNA Technologies, Inc. RPl4 and β-actin were used as housekeeping genes. Transcript analysis was done as described previously using PowerUP SYBR-Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Applied Biosystems 7500 Real Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for RT-qPCR analysis. The thermal expression levels were measured in triplicate. The threshold cycle (Ct) values were normalized to RPl4 and β-actin and relative mRNA expression was determined using the ΔΔCt method (20).

Statistical and bioinformatics analysis. The clinical/pathological variables and comorbidities were described using descriptive statistics. Categorical data are presented as frequencies (percentages) and continuous data as medians (interquartile ranges). Continuous variables were compared between the control group and EC group using the Mann-Whitney non-parametric U test. When more than two groups were compared (i.e., control, early stage EC and advanced stage EC), statistical significance was determined using Kruskal-Wallis test with Dunn's post hoc correction. $P<0.05$ was considered significant. Analysis of correlation between variables was assessed using Spearman's rank correlation coefficient test. To assess the diagnostic potential of biomarkers, ROC curve analysis was used to determine the area under the curve (AUC), sensitivity, specificity and optimal cut-off values of individual biomarkers. Logistic regressions were performed to understand the influence of confounding variables (BMI and comorbidities) on the SOAT1/CE content in malignancy (EC). Model 1 is an unadjusted model whereas model 2 adjusts predictor variables with different cofounder variables individually. Differences were considered statistically significant when adjusted $P<0.05$. Bioinformatics analysis for SOAT1 expression and its prognostic significance was retrieved from UALCAN and cBioPortal platforms. Kaplan Meier curves were generated to understand the relationship between SOAT1 gene and overall survival. Log rank test was used to compare the survival curves of samples with high gene expression and low gene expression. All statistical analyses were performed using GraphPad Prism 7.04 and SPSS statistical software (SPSS Inc.).

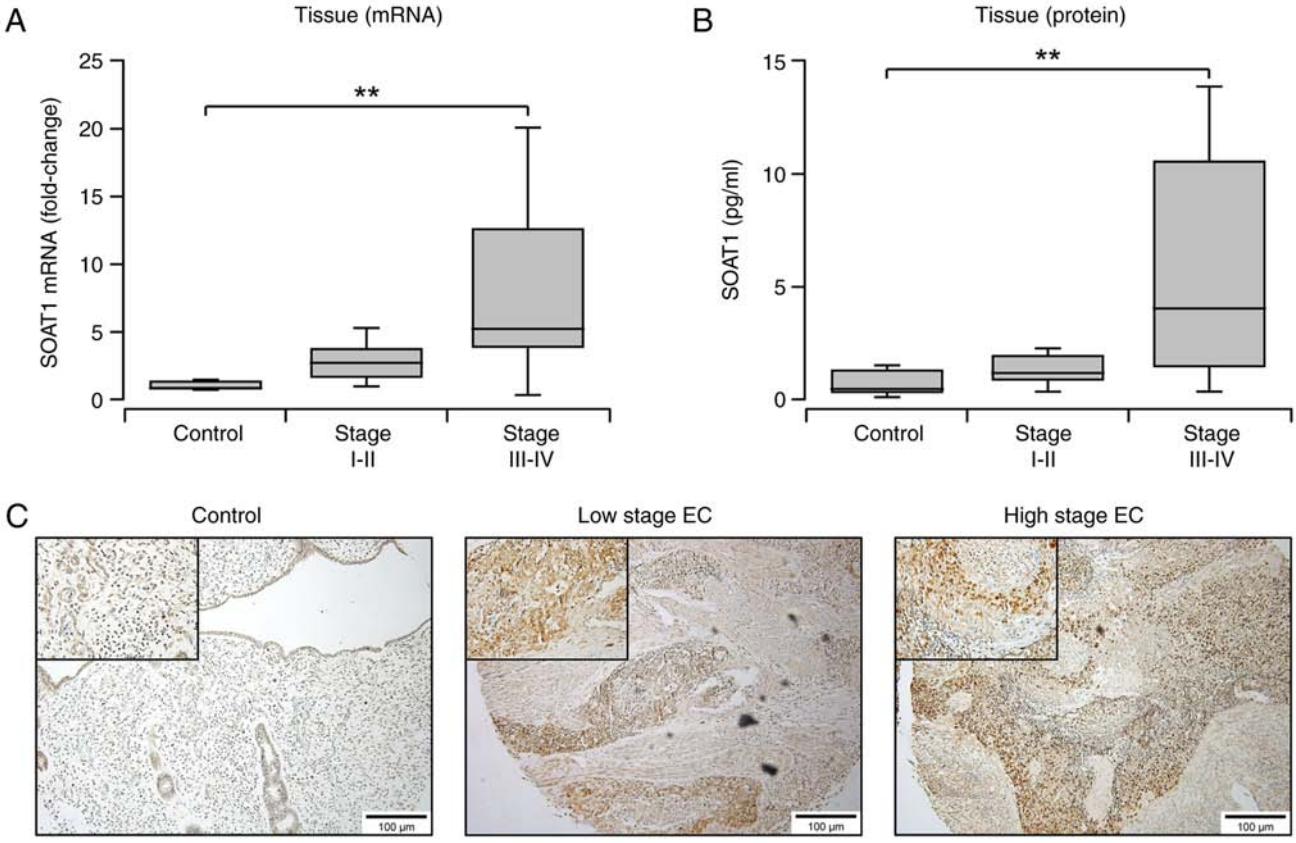

Figure 1. SOAT1 mRNA and protein levels in tissue samples. Samples were collected from control subjects and endometrial cancer (EC) patients diagnosed with early (Stage I-II) or advanced (Stage III-IV) disease. Box plots show medians (interquartile ranges) and whiskers (the minimum and maximum values). Asterisks indicate statistically significant difference compared to control, **P<0.01. (A) SOAT1 mRNA transcript levels assessed in endometrial tissue via RT-qPCR (Control, n=11; early stage EC, n=9; advanced stage EC, n=8; triplicate experiments). (B) SOAT1 protein expression levels in tissue assessed via ELISA (control, n=15; early stage EC, n=9; advanced stage EC, n=8; triplicate experiments). (C) SOAT1 expression shown by DAB staining (brown) in human normal endometrium, low stage and high stage EC tumor samples. Representative images were taken with an inverted microscope (Olympus H4-100, CCD camera) and a 10X objective. n, number of samples. Insets show images obtained with a 40X objective. EC, endometrial cancer; SOAT1, sterol-o-acyl transferase 1.

Results

SOAT1 mRNA and protein expression increased in EC tumor tissues. EC tumor tissue contained significantly higher levels of SOAT1 mRNA than control endometrial tissue (P=0.0002; Mann-Whitney U test; data not shown). When dividing the EC group into early EC (stage I-II) and advanced EC (stage III-IV), only the advanced EC group had significantly higher SOAT1 mRNA levels than the control (P=0.0010; Kruskal-Wallis test with Dunn's post hoc correction; Fig. 1A). No significant difference was observed between early and advanced EC groups. Similar to mRNA, SOAT1 protein levels were also higher in malignant EC tissue compared to normal endometrial tissue (P=0.0006; Mann Whitney non-parametric U test; data not shown). When dividing the EC group by disease stage, SOAT1 protein levels were significantly higher only in the advanced stage EC group compared to the control group (P=0.0013; Kruskal-Wallis test with Dunn's post hoc correction; Fig. 1B). No significant difference was observed between early and advanced EC groups. IHC analysis further confirmed increased SOAT1 expression in tumor tissue collected from EC subjects (evidenced by increased DAB staining) compared to normal tissues (Fig. 1C). There was a positive correlation between SOAT1 protein and mRNA levels in tissue samples (Table SI; Spearman correlation analysis, r=0.721, P<0.0001).

SOAT1 expression evaluation in UALCAN and cBioportal cancer databases. We used the UALCAN platform (http://ualcan.path.uab.edu) to validate SOAT1 expression at both mRNA and protein levels in endometrial cancer and matched normal tissues. Statistical analysis in the UALCAN database revealed that SOAT1 expression was significantly higher in the primary EC group compared with the normal group (P<0.0001) at both mRNA and protein levels (Fig. S1). According to the cBioPortal cancer genomics database (https://www.cbioportal.org/), genetic alteration of SOAT1 gene was reported in 6% of EC patients (83 of 1638 patients), most of which are copy number amplifications.

SOAT1 elevation in peritoneal fluid of EC patients. Peritoneal fluid collected from subjects diagnosed with EC had significantly higher levels of SOAT1 protein compared to those in peritoneal fluid collected from control subjects (P=0.0082; Mann-Whitney non-parametric U test; data not shown). When dividing the EC group by stage, statistical significance was only observed in the advanced stage group (P=0.0015; Kruskal-Wallis test with Dunn's post hoc correction; Fig. 2A).

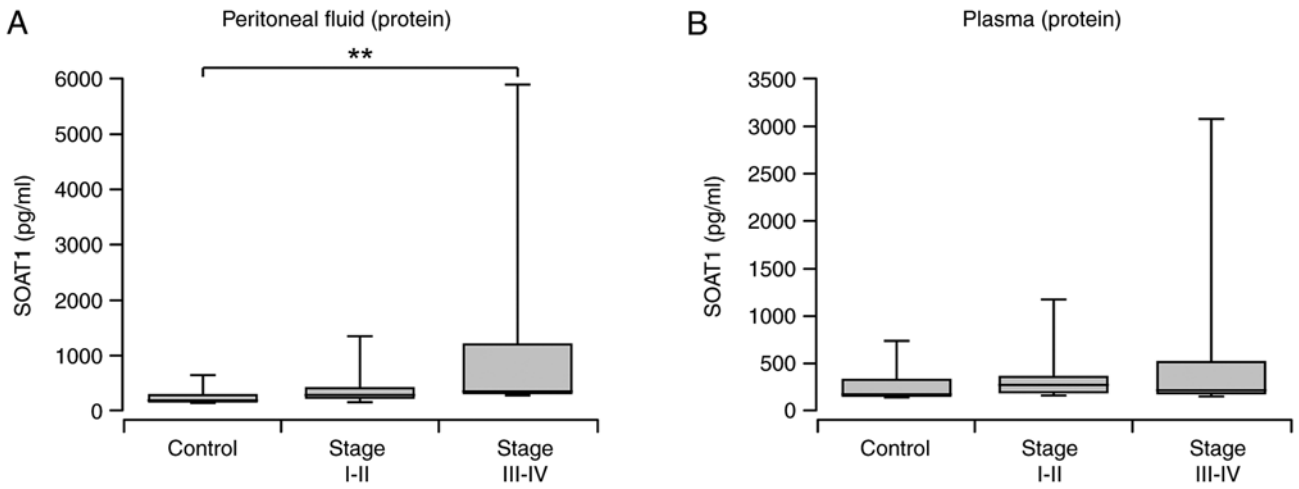

Figure 2. SOAT1 protein levels in peritoneal fluid and plasma samples. Samples were collected from control subjects and endometrial cancer (EC) patients diagnosed with early (Stage I-II) or advanced (Stage III-IV) disease. Box plots show medians (interquartile ranges) and whiskers (the minimum and maximum values Asterisks indicate statistically significant difference compared to control, **P<0.01. (A) SOAT1 protein levels (ELISA; triplicate experiments) in peritoneal fluid collected from control (n=12), early stage EC (n=13) and advanced stage EC subjects (n=12). (B) SOAT1 protein levels (ELISA, triplicate experiments) in plasma collected from control (n=12), early stage EC (n=17) and advanced stage EC subjects (n=14). EC, endometrial cancer; SOAT1, sterol-o-acyl transferase 1.

No significant difference was observed between early and advanced EC groups. As shown in Table SI, peritoneal fluid SOAT1 protein levels positively correlated with tissue SOAT1 mRNA (Spearman r=0.506, P=0.008) and tissue SOAT1 protein (Spearman r=0.388, P=0.049).

Plasma SOAT1 concentration in EC and control group. Plasma SOAT1 levels did not differ between the EC and control groups (P>0.05; Mann-Whitney non-parametric U test; data not shown). Early or advanced stages did not differ significantly from the control group or between stages (P>0.05; Kruskal-Wallis test with Dunn's post hoc correction; Fig. 2B). The plasma SOAT1 protein and tissue SOAT1 mRNA transcript levels did not correlate (Table SI; Spearman r=0.342, P=0.081).

Correlation between SOAT1 protein concentrations in tissue, peritoneal fluid, and plasma. To determine if peritoneal fluid and plasma SOAT1 levels can predict tumor SOAT1 status, we assessed the correlation between peritoneal fluid, plasma and tissue SOAT1 protein levels. As shown in Fig. S2A, peritoneal fluid and tissue SOAT1 levels correlated positively (Spearman r=0.388, P=0.049) but plasma SOAT1 did not correlate with tissue (Fig. S2B, Spearman r=0.324, P>0.05) or peritoneal fluid SOAT1 levels (Fig. S2C, Spearman r=0.231, P>0.05).

CE, TC, and FC levels in peritoneal fluid and plasma. TC, FC, and CE levels did not differ significantly in the peritoneal fluid of women with EC compared to the control group (P>0.05; Mann-Whitney non-parametric U test; data not shown). However, comparing the control group to the early and advanced stage EC groups separately, significantly higher levels of TC and CE were observed in advanced stage EC group than control group (P=0.0473 and P=0.0293, respectively; Kruskal-Wallis test with Dunn's post hoc correction; Fig. 3A-C). Interestingly, significant difference in CE levels were observed between early and advanced EC groups (P=0.0033; Fig. 3C). There was a strong positive correlation between TC, FC, and CE levels in peritoneal fluid (P<0.0001). FC levels did not differ significantly between malignant and control groups (Fig. 3B). Plasma levels of TC, FC, and CE did not differ between the EC and control groups (P>0.05; Mann-Whitney non-parametric U test; data not shown). No significant differences were observed when compared between control, early and advanced stage groups (P>0.05; Kruskal-Wallis test with Dunn's post hoc correction; Fig. 3D-F).

Correlation between SOAT1 and CE levels and in endometrial tissue, plasma and peritoneal fluid. Table SI shows a significant correlation between CE and SOAT1 protein levels in peritoneal fluid (spearman r=0.453, P=0.005). Interestingly, a strong correlation was also observed between peritoneal fluid CE levels and tissue SOAT1 protein (Spearman r=0.467, P=0.016). This may also imply that tissue SOAT1 regulates CE secretion into the peritoneal fluid, and that CE levels in peritoneal fluid reflect tissue SOAT1 levels and tumor aggressiveness. In contrast, plasma CE did not show any correlation with plasma, tissue or peritoneal fluid SOAT1 protein.

Correlation between Ki67 expression and SOAT1 (protein, mRNA), CE, TC and FC levels. Tumor tissue collected from subjects diagnosed with EC has higher levels of Ki67 mRNA transcripts than endometrial tissue collected from control subjects (P=0.0012; Mann-Whitney non-parametric U test; data not shown). When multiple comparisons were done between the control, early EC (stage I-II) and advanced EC groups (stage III-IV), Ki67 mRNA levels were significantly higher only in advanced EC group (P=0.0001, Kruskal-Wallis test with Dunn's post hoc correction, Fig. 4A). Interestingly, significant difference in Ki67 mRNA levels were observed between early and advanced EC groups (P=0.0152; Fig. 4A). Spearman correlation analysis (Table II) indicated a significant positive correlation between tissue Ki67 and SOAT1 mRNA transcripts (r=0.581, P=0.0015). Additionally, Ki67 mRNA

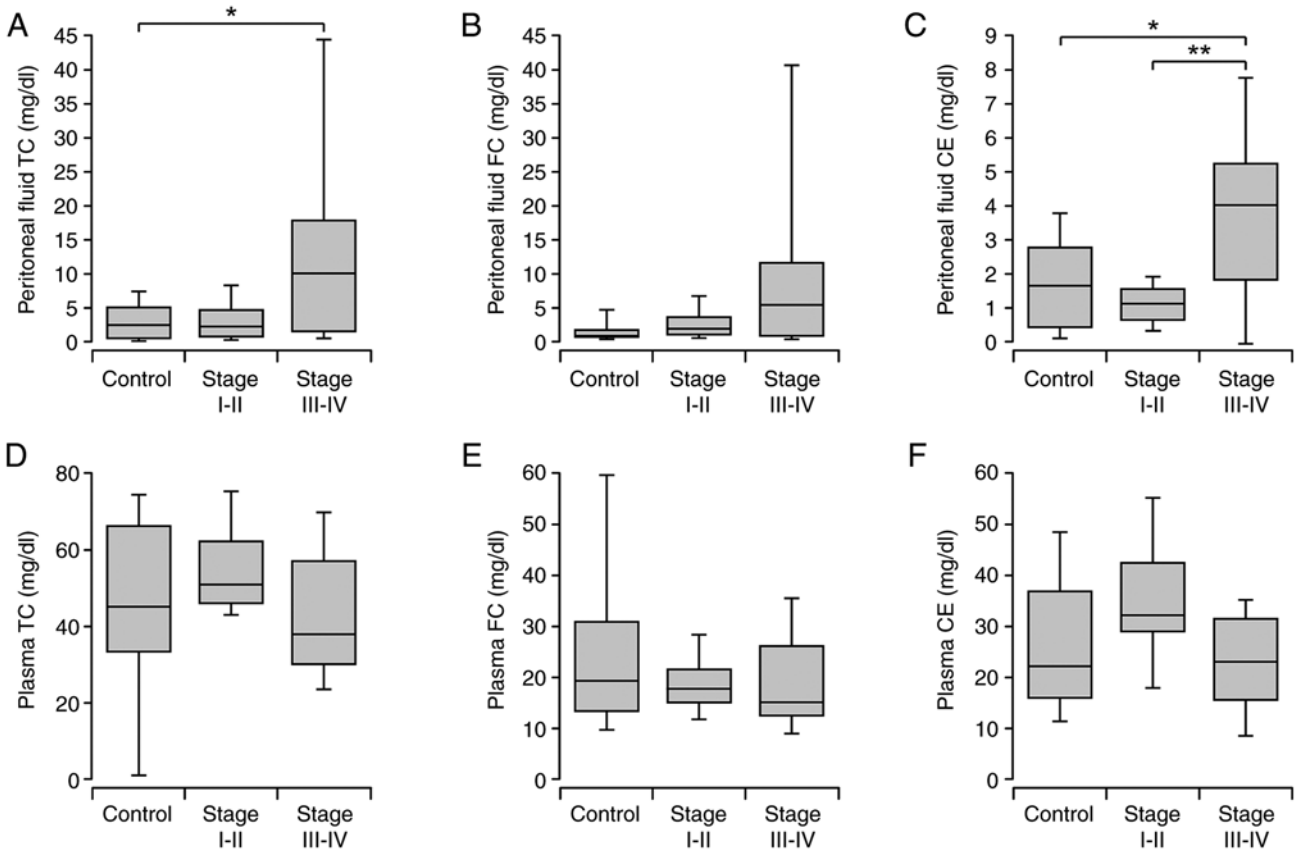

Figure 3. Total cholesterol (TC), free cholesterol (FC) and cholesteryl ester (CE) levels in biological samples. Samples were collected from control subjects (n=12) and endometrial cancer (EC) patients diagnosed with early (Stage I-II; n=14) or advanced (Stage III-IV; n=14) disease. Lipids from peritoneal fluid and plasma were quantified using Total Cholesterol and Cholesteryl Ester Colorimetric Assay Kit. Box plots show medians (interquartile ranges) and whiskers (the minimum and maximum values). Asterisks indicate statistically significant difference between the indicated groups, *P<0.05; **P<0.01. Experiments were performed in triplicate. (A) TC in peritoneal fluid from control, early stage and advanced stage EC patients; (B) FC in peritoneal fluid from control, early stage and advanced stage EC patients; (C) CE in peritoneal fluid from control, early stage and advanced stage EC patients; (D) TC in plasma from control, early stage and advanced stage EC patients; (E) FC in plasma from control, early stage and advanced stage EC patients; (F) CE in plasma from control, early stage and advanced stage EC patients. EC, endometrial cancer; SOAT1, sterol-o-acyl transferase 1; TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester.

correlated significantly with SOAT1 protein from tissue (r=0.506, P=0.007) and peritoneal fluid (r=0.614, P=0.0011). A positive association was also seen between Ki67mRNA and peritoneal fluid CE (r=0.628, P=0.0008) and TC levels (r=0.562, P=0.0035). Moreover, high SOAT1 immunostaining was associated to high Ki67 expression in tissue sections (Fig. 4B). EC tumors of advanced stage and high grade had the highest levels of SOAT1 immunostaining. There was no significant correlation between Ki67 and any of the plasma analytes.

Assessment of SOAT1 and CE as diagnostic markers for EC. The diagnostic potential of SOAT1 and CE in plasma, peritoneal fluid, and tissue was evaluated using ROC curves to determine optimal cut-off levels for SOAT1 and CE in EC diagnosis. As shown in Table SII, assessing SOAT1 levels in peritoneal fluid and EC tissue has better diagnostic power as compared to plasma levels. Tissue SOAT1 mRNA and protein has the highest area under the curve (AUC) of ROC (0.834 and 0.893 respectively) followed peritoneal fluid SOAT1 (0.767). Other peritoneal fluid or plasma analytes have not shown significant diagnostic potential and thus may not be ideal for diagnostic assessments. Tissue SOAT1 protein had a sensitivity of 59% and a specificity of 100% at a cut off concentration of >1.56 pg/mg protein. Tissue SOAT1 mRNA had a sensitivity of 82% and a specificity of 100% at a cut off concentration of >1.67-fold change. Peritoneal fluid SOAT1 level had a sensitivity of 80% and a specificity of 67% at a cut off concentration of >234 pg/ml.

Prognostic evaluation of SOAT1 expression in endometrial cancer (Bioinformatics analysis). Survival data could not be analyzed in our study population due to the short time span of the study and the small sample size. However, we used cBioPortal database to evaluate the significance of SOAT1 expression as a prognostic biomarker in EC. According to cBioportal, comparison of groups based on their median SOAT1 gene expression, revealed that subjects with low SOAT1 expression survived significantly longer than those with high SOAT1 expression (Log Rank test P<0.0001; Fig. S3). This suggests that SOAT1 expression can be a potential prognostic marker for EC. Further bioinformatics analyses are needed to confirm the utility of SOAT1 expression and to establish prognostic algorithms.

Assessment of the potential influence of comorbidities on the association of SOAT1/CE with EC. BMI differed significantly between the non-malignant and EC groups (P=0.003, data not

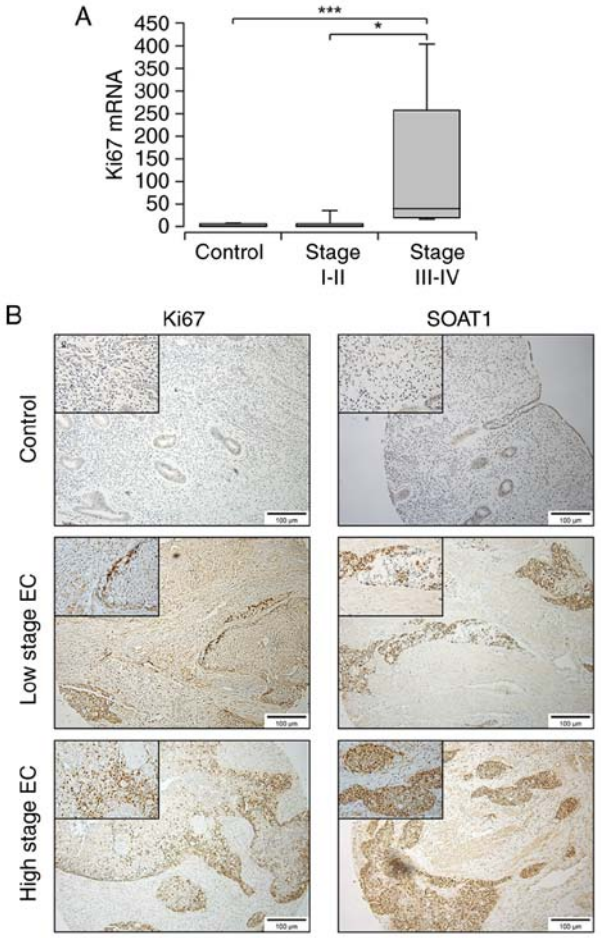

Figure 4. Ki67 expression in tissue samples. (A) Ki67 mRNA transcript levels assessed via RT-qPCR in normal endometrium (Control, n=11), early stage EC (Stage I-II, n=8) and late stage EC (Stage III-IV; n=8). Box plots show medians (interquartile ranges) and whiskers (the minimum and maximum values). Asterisks indicate statistically significant difference between the indicated groups, *P<0.05; ***P<0.001. Experiments were performed in triplicate. (B) Ki67 protein immunostaining in tissue. Ki67 expression shown by DAB staining (brown) in human normal endometrium, and low stage (I-II) EC and high stage (III-IV) EC tumor samples. Representative images were taken with an inverted microscope (Olympus H4-100, CCD camera) and a 10X objective. Insets show images obtained with a 40X objective. EC, endometrial cancer; SOAT1, sterol-o-acyl transferase 1.

shown). This result is consistent with the previously established correlation between BMI and increased EC risk (14). Spearman correlation analysis showed a significant correlation between BMI and EC grade and stage (spearman r=0.362, P=0.025, and r=0.298, P=0.049, respectively). Moreover. BMI positively correlated with tissue SOAT1 levels (spearman r=0.387, P=0.042). Comorbidities such as obesity, hyperlipidemia, diabetes, hypertension, and hyperthyroidism are known to alter cholesterol metabolism, therefore we analyzed the impact of BMI and other comorbidities on SOAT1 and CE levels. When these co-variables were adjusted in logistic regressions (Model 2), the association of SOAT1 or CE levels with EC remained statistically significant (P<0.05; Table SIII).

Discussion

Despite advances in the development of new therapeutic strategies, the prognosis for advanced EC patients remains dismal. Indeed, histologic subtype and FIGO staging alone may not effectively predict prognosis due to the molecular heterogeneity of EC (21). There is an urgent need to identify sensitive and specific molecular markers for prognosis to achieve personalized treatment and improve clinical outcomes. In this study, the prognostic relevance of SOAT1 in EC was investigated. SOAT1 expression in tumor tissue, peritoneal fluid and plasma were comprehensively analyzed in patients diagnosed with EC and normal healthy subjects. SOAT1 and CE levels, were significantly elevated in tumor tissue and peritoneal fluid samples collected from the EC group. Significant positive associations between CE and SOAT1, SOAT1/CE and Ki67, and SOAT1/CE and poor overall survival in EC patients suggest that SOAT1/CE may be associated with malignancy, aggressiveness, and poor prognosis, thus may serve as potential biomarker(s) for prognosis and target-specific treatment in EC.

Chemo-resistance and metastatic spread continue to be key challenges for EC patients, and various chemotherapy resistance mechanisms have been proposed. Many malignancies have abnormal cholesterol metabolism, which contributes to tumor aggressiveness and resistance to treatment (6-9,11-14). As a result, cholesterol metabolism has been identified as a potential cancer therapeutic target. Numerous studies have established SOAT1/CE as a new prognostic marker in various cancers (11-14), but few have examined its role in endometrial cancer. Previously, we demonstrated the prognostic significance of CE and SOAT1 in ovarian cancer (15,17). Ovarian and endometrial malignancies have similar epidemiological and genetic characteristics (22). Ovulatory cycles are linked to both endometrial and ovarian cancer risk due to accumulation of p53 or PTEN mutations associated with reproductive tissue turnover (23). Due to these common factors, it is intriguing to investigate SOAT1/CE levels in endometrial cancer as well. In this study, we systematically analyzed SOAT1 expression and CE levels in peritoneal fluid, plasma and tumor tissue in EC patients and compared them to normal controls. We also correlated the expression of these mediators with a marker of tumor aggressiveness. Ki67.

SOAT1/CE levels in plasma were not significantly different between normal and EC cohorts, implying that SOAT1 cannot be used as a non-invasive biomarker for diagnosis or prognosis. In tumor tissue, significant SOAT1/CE levels were observed in both early and advanced stage EC group compared to normal group. Our result is in accordance with TCGA data and other similar studies which reported 3-7 fold higher SOAT1 levels in endometrial cancer tissue as compared to normal secretory endometrium (24). SOAT1 and CE-rich tumors were associated with higher aggressive potential and poor survival in many cancers (16). cBioportal reported poor overall survival with higher SOAT1 expression. Consistent with these reports, we observed a significant positive correlation between SOAT1 (protein and mRNA), CE levels and Ki67, an established tumor proliferation marker known to predict disease outcome in many human malignancies. Many studies demonstrated a positive relation between high proliferation rates and poor survival or increased recurrence (25).

SOAT1 expression in peritoneal fluid, on the other hand, was low in the normal and early stage groups but significantly higher in advanced stage EC. The peritoneal fluid (tumor

Table II. Spearman's rank coefficient analyses of Ki67 correlation with SOAT1, TC, FC and CE levels.

Ki67 (mRNA)	Spearman r	P-value
vs. Grade	0.660	0.0010[a]
vs. Tissue SOAT1 protein	0.506	0.0070[a]
vs. Tissue SOAT1 mRNA	0.581	0.0015[a]
vs. Peritoneal Fluid SOAT1 protein	0.614	0.0011[a]
vs. Plasma SOAT1 protein	0.469	0.0157[b]
vs. Peritoneal Fluid TC	0.562	0.0035[a]
vs. Peritoneal Fluid FC	0.439	0.0280[b]
vs. Peritoneal Fluid CE	0.628	0.0008[c]
vs. Plasma TC	-0.383	0.0533[d]
vs. Plasma FC	-0.287	0.1554[d]
vs. Plasma CE	-0.314	0.1180[d]

SOAT1, sterol-O-acyl transferase; CE, cholesterol ester; TC, total cholesterol; FC, free cholesterol; NS, non-significant. [a]P<0.01; [b]P<0.05; [c]P<0.001; [d]NS.

microenvironment) contains oncogenic cellular and acellular mediators that promote tumor invasiveness and treatment resistance (26,27). In ascites, cholesterol levels are greatly elevated and may be utilized as a marker for malignant ascites (28). Indeed, our research revealed SOAT1 and CE as additional key components of the EC tumor microenvironment that vary based on stage/grade and may contribute to cancer aggressiveness and treatment resistance.

While cholesterol is required for cell proliferation, excessive cellular cholesterol is toxic (29). SOAT1 mediated cholesterol esterification has been hypothesized to keep signaling pathways active and protect cells from FC toxicity, while also evading feedback inhibition and maintaining the high metabolic activity required for disease progression (7-10). Corroboration for this idea was found in the form of a significant positive connection between SOAT1 and CE levels (12-17). As a result, inhibiting CE generation may suppress tumor proliferation and disease progression. Indeed, suppression of SOAT1 by pharmacologic (avasimibe) or genetic (SOAT1 shRNA) agents decreased cancer cell proliferation, migration, and invasion *in vitro* and *in vivo*, as observed in colon, pancreas, prostate, and EOC models (12,14,15,30). The mechanism(s) underlying the relation between CE accumulation and cancer aggressiveness has not been precisely established. Inhibiting CE generation was linked to inactivation of SREBP1 leading to downregulation of SREBP1 regulated processes such as caveolin-1/MAPK activation, reduced LDLr expression and reduced LDLr mediated uptake of essential fatty acids, such as arachidonic acid, a proliferation factor in many cancers (13,14,31).

According to Yue *et al* 2014, CE accumulation is a consequence of the loss of the tumor suppressor PTEN (phosphatase and tensin homolog), and of the subsequent activation of the PI3K/AKT pathway (13). Other possible signaling mechanisms include the downregulation of Wnt/β-catenin, pAkt and ERK1/2 pathways and inhibition of TLR4 (Toll-like receptor 4), all of which play significant roles in cancer cell proliferation, metastatic cancer recurrence, and chemotherapy resistance (32,33). For all of the gynecologic malignancies, Wnt signaling is being evaluated as a possible therapeutic target (34-38), therefore this pathway can be targeted via inhibition of CE and SOAT1.

Chemoresistance and metastatic dissemination remain major hurdles for EC patients. Abnormal accumulation of SOAT1/CE may lead to resistance to drugs such as tamoxifen, gemcitabine, imatinib and cisplatin as shown in various *in vitro* and *in vivo* cancer models (15,30,39,40). Therefore, inhibition of CE accumulation may enhance the sensitivity of cancer cells to drugs. We previously shown that SOAT1-inhibited SKOV-3 and IGROV-1 cell lines were more sensitive to cisplatin than their respective controls (15). Although we do not have evidence supporting a specific mechanism(s), others have reported that SOAT1 inhibition and depletion of CE lead to inhibition of PI3K/Akt, caveolin and MAPK pathways contributing to increased sensitivity to drugs (13,40). Further studies are needed to fully elucidate the mechanisms linking cholesterol metabolism and cancer drug resistance in EC.

We concluded that SOAT1/CE levels are associated with malignancy and tumor aggressiveness, and thus can be considered druggable targets that reflect molecular modifications during endometrial cancer, especially in advanced stages. Tissue and peritoneal fluid SOAT1/CE levels, in addition to predictable clinical-pathological characteristics, could be utilized to categorize patients into groups with varying risk of recurrence for better treatment guidance. Additional research and a larger sample cohort are needed to validate SOAT1/CE as therapeutic targets in EC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LB and ABF provided resources and acquired funding for the investigation. VNA and LB supervised the study, and participated in its conceptualization and design. LB, PDS, EMS, KG and TW collected clinical samples, administered sample allocation, developed and submitted the clinical research protocol, and reviewed and reporting any unanticipated problems and

protocol deviations to the Institutional Review Board. ABF participated in the processing and storage of clinical samples. VNA, ML and ZP conducted all the experiments. VNA, ML and PDS participated in the formal analysis of data. VNA and ML confirm the authenticity of all the raw data. VNA wrote the original draft. VNA and PDS designed the figures and tables. All authors reviewed, edited, read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the local Institutional Review Board (Springfield Committee for Research Involving Human Subjects) under protocol 16-493. Written informed consent was obtained from all participants for participation in the study and use of their biological samples and clinical data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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