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Steroids 139 (2018) 45–52

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

# The sulfatase pathway as estrogen supply in endometrial cancer

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ARTICLE INFO

*Keywords:*
Steroid sulfatase
Estrogen sulfotransferase
Endometrial cancer
Intracrinology
Estrogen metabolism

ABSTRACT

*Objective:* Contradictory results are reported about the level of steroid sulfatase (STS), estrogen sulfotransferase (SULT1E1; together, the sulfatase pathway) and aromatase (CYP19A1) in endometrial cancer (EC). The aim of this study was to explore the levels of these enzymes in a well-characterized cohort of EC patients and post-menopausal controls.

*Materials and Methods:* Endometrial tissues from 31 EC patients (21 grade 1 and 10 grade 2–3) and 19 post-menopausal controls were collected. Levels of mRNA (RT-qPCR) and protein (immunohistochemistry) were determined. STS enzyme activity was measured by HPLC, whereas SULT1E1 enzyme activity was determined using a novel method based on liquid chromatography-mass spectrometry (LC-MS/MS).

*Results:* No significant differences in STS, SULT1E1 mRNA or protein levels and STS:SULT1E1 ratio were found. STS enzyme activity and STS:SULT1E1 activity ratio were significantly decreased in ECs compared with controls. CYP19A1 mRNA levels were lower in ECs than in controls.

*Conclusion:* A novel highly sensitive and accurate protocol to assess SULT1E1 activity is presented. STS enzyme activity and the STS:SULT1E1 activity ratio seem to be lower in ECs than in controls. STS is an important route for estrogen supply in endometrial cells.

## 1. Introduction

Endometrial cancer (EC) is the most common gynaecological malignancy in the western world with 25.7 new cases per 100.000 women diagnosed per year in the period of 2010–2014 [1]. Two types of EC are distinguished: type 1 EC is characterised by an endometrioid histology (well differentiated), it is considered estrogen dependent and is related to risk factors indicative of estrogen exposure such as nulliparity, early menarche, late menopause, high body mass index (BMI) and tamoxifen use. Type II EC has a non-endometrioid histology, is considered less related to estrogen exposure, it is more often diagnosed at an advanced stage and is associated with poorer prognosis [2–10].

Although estrogen-dependent type 1 EC represents more than 80% of the cases, this disease is generally diagnosed in postmenopausal women when the ovaries have already ceased hormone production. However, estrogens can still be generated *in situ* in EC cells using systemic blood precursors such as estrone-sulfate (E1S). E1S is an inactive estrogen present in the blood and due to its high water solubility and long half-life is considered to be a reservoir for the local estrogen synthesis [11,12]. E1S is activated to free estrone (E1) by steroid sulfatase (STS) that catalyses the hydrolysis of sulfate-ester-bonds from a wide range of substrates including E1S [13–15]. The enzyme estrogen sulfotransferase (SULT1E1) catalyses the opposite reaction, i.e. conjugates and inactivates E1 with a sulfate moiety. The combined action of STS and SULT1E1 represents the sulfatase pathway, which ultimately controls the intracellular availability of E1 [13,16]. E1 is a weak estrogen, but it the can be further converted intracellularly to the most potent 17β-estradiol (E2). E1 and E2 interconversion is controlled by the enzymes 17β-hydroxysteroid dehydrogenase 1 (HSD17B1, converting E1–E2) and HSD17B2 (deactivating E2 back to E1) [14,15,17].

Estrogens can be supplied locally also via the aromatase enzyme (CYP19A1), which converts circulating androgens (namely androstenedione and testosterone) into E1 and E2, respectively [15].

Although the role of STS and SULT1E1 was studied in previous investigations at the mRNA and protein levels, few studies determined the activity of these enzymes [18–21] and one study only [19] assessed in the same cohort mRNA, protein and enzyme activity (all literature on the sulfatase pathway was recently reviewed [14,15,22]). Additionally,

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https://doi.org/10.1016/j.steroids.2018.09.002
Received 8 April 2018; Received in revised form 10 August 2018; Accepted 6 September 2018
Available online 12 September 2018
0039-128X/ 

none compared EC specimens with postmenopausal controls, which is the most suitable control tissue.

The purpose of this study was to investigate the sulfatase pathway (STS and SULT1E1) assessing mRNA, protein and enzyme activity in EC specimens and compare the levels of these enzyme in ECs with those seen in postmenopausal control endometrium, the most appropriate control tissue for EC. mRNA levels were assessed by reverse transcription quantitative PCR (RT-qPCR), protein levels by immunohistochemistry and enzyme activities were measured by highly sensitive and non-radioactive methods based on high-performance-liquid-chromatography (HPLC, for STS activity) and a new protocol based on liquid-chromatography tandem mass-spectrometry (LC-MS/MS, for SULT1E1 activity). CYP19A1 mRNA level was also determined by RT-qPCR.

## 2. Materials and methods

### 2.1. Ethical statement

Procedures were conducted in accordance with ethical standards, national, international guidelines according to the Declaration of Helsinki and the study protocol was approved by the local ethics authority (METC 14-4-003).

### 2.2. Chemicals

Estrone 3-sulfate (E1S), 17β-estradiol (E2), estrone (E1), butyl-4-hydroxybenzoate and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands) and were of analytical grade. Deuterium labelled sodium estrone-2,4,16,16-D4 sulfate ($d_4$-E1S) was purchased from Ritmeester B.V. (Nieuwegein, The Netherlands). HPLC grade methanol, acetonitrile, chloroform and liquid chromatography tandem mass spectrometry (LC-MS) grade acetonitrile, water and ammonium hydroxide solution were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

### 2.3. Human specimens

Endometrial carcinoma (EC) tissue was collected from 31 patients undergoing hysterectomy at Maastricht University Medical Centre between 2008 and 2017 (21 EC grade 1, 10 EC grade 2 and 3). Only ECs with endometrioid histology were included (e.g., serous, clear cell and sarcomas/carcinosarcomas were excluded). Normal endometrial control tissue was obtained from 19 postmenopausal women undergoing hysterectomy for endometrial non-malignant indications (cervical lesions; myomas; prolapses). Disease stage was classified according to the International Federation of Gynecologic Oncology (FIGO) [23]. None of the patients had used hormonal medication within the last six months prior to surgery. Fourteen EC patients and nine controls of the present study cohort were common to the patient population studied in a previous investigation [17], and 16 patients were common to the study population of an additional previous investigation [24].

One part of the biopsies was immediately frozen in liquid nitrogen for further processing and one part was fixed in formalin and embedded in paraffin (FFPE) for histology and immunohistochemistry. Frozen biopsies were cut in 15 µm thick slices using a cryotome and lysates from 10 to 20 slices were used for mRNA isolation and enzyme activity of both STS and SULT1E1 measurement. In order to ensure the presence of endometrial material in these specimens, tissue sections at the beginning and at the end of each specimen, and at approximately every 150 µm of thickness, were used for histological staining (see below), and a gynaeco-pathologist (LK) confirmed the presence and the grade of EC or the presence of normal endometrium (healthy controls) in all materials used.

All protocols were approved by the Local Ethical Committee in our Medical Centre as mentioned earlier.

### 2.4. RNA isolation, cDNA synthesis and Real-Time PCR

Protocols were described earlier [17,24–26]. In short, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, US), assessed spectrophotometrically for quantity and purity (260/280 nm and 260/230 nm ratios) and cDNA was synthesised with the iScript cDNA synthesis Kit (BioRad, Hercules, CA, US). Quantitative PCR was performed using the SYBR-Green (BioRad, Hercules, CA, US) and the BioRad MyIQ apparatus (Hercules, CA, US) using the following primers: house-keeping gene β-actin: F 5′-GCC AAC CGC GAG AAG ATG AC, R 5′-GAT GGG CAC AGT GTG GGT GAC; house-keeping Cyclophilin A: F 5′-CCG TGT TCT TCG ACA TTG CCG T, R 5′-AAT CCT TTC TCT CCA GTG CTC AGA; house-keeping Ribosomal protein S18: F 5′- TGC GAG TAC TCA ACA CCA ACA, R 5′- GCA TAT CTT CGG CCC ACA; CYP19A1: F 5′-ATG CGA GTC TGG ATC TCT GG, R 5′-GCC TTT CTC ATG CAT ACC; SULT1E1: F 5′-GGA AAC AGC CAC ATC CTT TG, R 5′-TTG CCA CCT GAA CTT CTT CC; for STS, probe Hs00996679 m1 from Applied Biosystems (CA, US) was used. Gene expression levels were computed using the delta ct system and three house-keeping genes (β-actin, Cyclophilin A and Ribosomal protein S18) were used as reference.

The levels of expression of ER-α (ESR1), ER-β (ESR2), HSD17B1 and HSD17B2 were determined with primers described earlier [17,24,26] and using the same three house-keeping genes as reference (β-actin, Cyclophilin A and Ribosomal protein S18). The levels of these enzymes (ERS1, ERS2, HSD17B1 and HSD17B2) were determined in all specimens used in the present study and that were used also to determine STS, SULT1E1 and CYP19A1 mRNA levels and enzyme activities. However, the mRNA (and activity) levels of HSD17B1 and HSD17B2 from 14 patients and 9 controls, as well as the HSD17B1 mRNA and activity levels from 16 patients included in the present study were already analysed using independent tissue specimens in earlier studies [1,24] and showed good correlation with the results obtained with the new samples used in the present investigation (data not shown). As quality control, all qPCR products were separated on agarose gels.

### 2.5. Histology and immunohistochemistry

Frozen sections were stained with haematoxylin & eosin (Sigma-Aldrich, Zwijndrecht, The Netherlands) and used for histology evaluation. Immunohistochemistry for SULT1E1 and STS on FFPE tissue was performed as described earlier [27]. In short, after heat-induced epitope retrieval in citrate buffer, anti-SULT1E1 (1:100 Sigma-Aldrich, St. Louis, USA) or anti-STS (1:100 Sigma-Aldrich, St. Louis, USA) were used. Chemate Envision and 3,3-diaminobenzidine (DAB) solution (Dako, Glostrup, Denmark) were used to visualise antibody binding. Stainings were scored by two independent observers (KMCC & BD) blindly from each other results, and staining indexes were computed using the Allred method with modifications, i.e., staining proportion (0–5) was multiplied by intensity scores (0–3), resulting in scores ranging between 0 and 15 [26,28].

### 2.6. Steroid sulfatase activity assay

The activity of the STS was determined using a previously published HPLC-based method [29,30]. In short, tissue homogenate was prepared by disrupting 10–20 cryosections (15 µm thick) for 20 s with mini-beadbeater (Biospec Products, Bartlesville, OK, USA) at maximum speed and in presence of glass beads (< 106 µm diameter, Sigma-Aldrich, St. Louis, USA). The total homogenate volume was 200 µl of lysis buffer, containing 100 mM KCl, 10 mM $KH_2PO_4$, 10 mM $Na_2HPO_4$, 1 mM EDTA, (pH 7.5). After centrifugation the cleared supernatant was used for enzymatic assay and protein determination (BC Assay Protein Quantitation Kit, Uptima-Interchim, Montlucon, France). Approximately 25 µg of protein were added to a 500 µl reaction mixture

**Table 1**
Patients' characteristics.

| | Grade 1 EC (n = 21) | | Grade 2–3 EC (n = 10) | | Postmenopausal controls (n = 19) | |
|---|---|---|---|---|---|---|
| | | *Range* | | *Range* | | *Range* |
| Mean age (y) | 73 | 47–91 | 72 | 46–92 | 65 | 55–87 |
| BMI * kg/m$^2$ | 31.3 | 20.1–55.1 | 26.4 | 19.6–32.9 | 27.8 | 17.0–47.9 |
| **FIGO** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| 1A | 12 | 57.1 | 6 | 60.0 | n.a. | – |
| 1B | 5 | 23.8 | 3 | 30.0 | n.a. | – |
| 3A | 1 | 4.8 | 0 | 0 | n.a. | – |
| 3B | 1 | 4.8 | 0 | 0 | n.a. | – |
| 3C | 2 | 9.6 | 1 | 10.0 | n.a. | – |
| Unknown | 0 | – | 0 | – | n.a. | – |
| **ER-α IHC** ^ | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| IHC positive | 9 | 42.9 | 1 | 10.0 | n.a. | – |
| IHC negative | 0 | 0 | 1 | 10.0 | n.a. | – |
| unknown | 12 | 57.1 | 8 | 80.0 | | |
| **ER-α mRNA** ^^ | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| mRNA low | 3 | 14.3 | 7 | 70.0 | 5 | 26.3 |
| mRNA medium | 8 | 38.1 | 2 | 20.0 | 7 | 36.8 |
| mRNA high | 10 | 47.6 | 1 | 10.0 | 7 | 36.8 |
| **ER-β mRNA** ^^ | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| mRNA low | 5 | 23.8 | 4 | 40.0 | 8 | 42.1 |
| mRNA medium | 4 | 19.0 | 5 | 50.0 | 8 | 42.1 |
| mRNA high | 12 | 57.1 | 1 | 10.0 | 3 | 15.8 |
| **HSD17B1 mRNA** ^^ | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| mRNA low | 6 | 28.6 | 3 | 30.0 | 8 | 42.1 |
| mRNA medium | 9 | 42.9 | 3 | 30.0 | 4 | 21.0 |
| mRNA high | 6 | 28.6 | 4 | 40.0 | 7 | 36.9 |
| **HSD17B2 mRNA** ^^ | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| mRNA low | 5 | 23.8 | 1 | 10.0 | 12 | 63.1 |
| mRNA medium | 7 | 33.3 | 5 | 50.0 | 5 | 26.3 |
| mRNA high | 9 | 42.9 | 4 | 40.0 | 2 | 10.5[#] |
| **Myometrium invasion** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| No | 3 | 14.3 | 3 | 30.0 | n.a. | – |
| < 50% | 8 | 38.1 | 4 | 40.0 | n.a. | – |
| > 50% | 7 | 33.3 | 3 | 30.0 | n.a. | – |
| unknown | 2 | 9.6 | 0 | 0 | n.a. | – |
| **LVSI** ** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| negative | 3 | 14.3 | 3 | 30.0 | n.a. | – |
| positive | 2 | 9.6 | 2 | 20.0 | n.a. | – |
| unknown | 16 | 76.2 | 5 | 50.0 | n.a. | – |
| **Recurrence** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| Yes | 1 | 4.8 | 2 | 20.0 | n.a. | – |
| No | 19 | 90.5 | 6 | 60.0 | n.a. | – |
| unknown | 1 | 4.8 | 2 | 20.0 | – | – |
| **Dead of disease** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| Yes | 0 | 0 | 1 | 10.0 | n.a. | – |
| No | 20 | 95.2 | 7 | 70.0 | n.a. | – |
| unknown | 1 | 4.8 | 2 | 20.0 | | |
| **Hypertension** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| Yes | 12 | 57.1 | 6 | 60.0 | 5 | 26.3 |
| No | 9 | 42.9 | 4 | 40.0 | 14 | 73.7 |
| **DM type 2** *** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| Yes | 7 | 33.3 | 0 | – | 1 | 5.3 |
| No | 14 | 66.7 | 10 | 100 | 18 | 94.7 |
| **Parity** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| 0 | 5 | 23.8 | 2 | 20.0 | 3 | 15.8 |
| 1–2 | 9 | 42.9 | 6 | 60.0 | 11 | 57. 9 |
| ≥3 | 6 | 28.6 | 2 | 20.0 | 5 | 26.3 |
| unknown | 1 | 4.76 | 0 | 0 | 0 | 0 |

n.a.: non-applicable.
ER-α: Low FC from 0.46 to 4.00; Medium FC: from 4.01 to 7.99; High FC: from 8.00 to 84.00.
ER- β: Low FC from 0.02 to 2.49; Medium FC: from 2.50 to 7.99; High FC: from 8.00 to 91.14.
HSD17B1: Low FC from 0.35 to 2.99; Medium FC: from 3.00 to 7.99; High FC: from 8.00 to 145.00.
HSD17B2: Low FC from 0.06 to 1.49; Medium FC: from 1.50 to 7.99; High FC: from 8.00 to 57.08.

* BMI: Body mass index.

** LVSI: Lymphovascular-Space Invasion.

*** DM type 2: Diabetes Mellitus type 2.

^ ER-α status by IHC was performed and described earlier. ER-α expression correlated 100% with progesterone receptor expression (PR) [17].

^^ mRNA expression levels of ER-α (ESR1), ER-β (ESR2), HSD17B1 and HSD17B2 were clustered in tertiles of equal number of subjects (15–17). The range in the fold change (FC, compared to the three references genes) of the four genes were as follows:

[#] the proportions of specimens with high HSD17B2 expression among ECs (n = 13) or controls (n = 2) and the proportions of specimens with combined medium/low mRNA levels of HSD17B2 in ECs (n = 18) and controls (n = 17) differed statistically as computed using Fisher exact test (two sided p-value = 0.02).

containing 4 mM $NADP^+$, 25 mM sucrose and 10 nmol estrone-3-sulfate (E1S; final concentration was 20 μmol/l) in PBS (pH 7.4) and incubated at 37 °C for 2 h. At the end of the reaction, the internal standard (IS) butyl-4-hydroxybenzoate was added (500 pg) and the E1 formed was extracted in the organic phase after adding 2 ml of $H_2O$ and 2.5 ml of chloroform. The chloroform phase was evaporated under nitrogen at 45 °C and estrogens were derivatised with 2-(4-carboxy-phenyl)-5,6-dimethylbenzimidazole (0.05%, w/v) in the presence of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC; Pierce, Rockford, IL, USA) at 50 °C for 20 min. Five μl were injected in the HPLC.

The used HPLC system (Shimadzu LC-10AD; Kyoto, Japan) consisted of a SIL-10ADvp autosampler, FCV-10ALvp gradient mixer, LC10Avp pump with high pressure gradient mixing extension, DGU-14A degasser and a RF-10Axl fluorescence detector (excitation wavelength 336 nm, emission wavelength 440 nm). Labsolutions software (Shimadzu, Kyoto, Japan) was used for instrument control, data acquisition and calculation of peak areas. The derivatised steroids were injected into a LiChroCART 250-4 RP 18 column (Merck, Darmstadt, Germany) and eluted with a gradient of methanol/$H_2O$ at a flow rate of 1 ml/minute. The limit of detection (LoD) for this method was 370 fmol E1/injection at a signal to noise ratio of 5.

### 2.7. Estrogen sulfotransferase activity assay

We developed a novel non-radioactive assay to measure SULT1E1 activity using LC-MS/MS. The reaction was carried out using an assay buffer described earlier [19]. The tissue homogenates were prepared in lysis buffer as described for the STS activity and 70 μl of protein lysate containing approximately 25–100 μg of protein were added to 180 μl of the reaction mixture containing 50 mM $KH_2PO_4$ (pH 7.4), 7 mM $MgCl_2$, 40 μM 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and 7 nmol E1, final concentration was 35 μmol/l. Samples were incubated at 37 °C for 16 h and the reaction was stopped by adding 250 μl of acetonitrile and 5 ng of the internal standard ($d_4$-E1S). After centrifugation for 15 min at 13200 rpm (16000*g*), the cleared supernatant was concentrated approximately five times in an Eppendorf concentrator (type 5301, Eppendorf, Nijmegen, The Netherlands). This unit applies gentle heat (45 °C) and vacuum to evaporate the solvent while centrifugation prevents foaming.

Formed E1S was measured by LC-MS/MS with Thermo Scientific TSQ Vantage system (Thermo Scientific, Breda, The Netherlands) equipped with a HESI-2 ion source in the negative ion mode. The following settings were used: ion spray voltage was 3500 V, vaporizer temperature 380 °C, capillary temperature 320 °C and S-lens RF 50. The compounds were measured in a targeted SIM (selective ion monitoring) mode. E1S at *m/z* 349.1 and $d_4$-E1S at *m/z* 353.1. The separation of E1S was done on an Acclaim 120 (C18, 3 μm, 2.1 × 150 mm) column (Thermo Scientific, Breda, The Netherlands). The mobile phase was water with 0.05% ammonium hydroxide (solvent A) and acetonitrile with 0.05% ammonium hydroxide (solvent B). The linear gradient used was as follows: 20–80% B at 0–10 min. The flow rate was 200 μl/minute and 5 μl of standards or samples were injected. The isotope ratio was calculated from the area $A_{349}$ (E1S) and $A_{353}$ ($d_4$-E1S). The correction factor $f_1$ was determined for the pure E1S ($A_{353}/A_{349}$) and $f_2$ for $d_4$-E1S ($A_{349}/A_{353}$). The calculation of the mass ratios resulted in the following formula: $(A_{349}\text{-}f_1 \times A_{353})/(A_{353}\text{-}f_2 \times A_{349})$. The limit of detection (LoD) for this method was 10 fmol E1S/injection at a signal to noise ratio of 5.

### 2.8. Statistical analysis

The statistical tests were carried out using KaleidaGraph V 4.1.3 (Synergy Software). Mean values between cases (all ECs, grade 1, 2 and 3, were pooled) and controls were compared using the *t*-test. Statistical significance was defined as $P < 0.05$. Two by two table analyses were performed using Fischer exact test (Simple Interactive Statistical Analysis, http://www.quantitativeskills.com). The test used is indicated in the tables concerned.

## 3. Results

### 3.1. Patient features

Clinical and demographic characteristics of the 19 postmenopausal controls and 31 ECs are given in Table 1. Subjects with EC were older than postmenopausal controls, had higher prevalence of hypertension whereas body mass index (BMI) was equally distributed between groups. Parity did not differ between cases and controls. Grade 1 ECs had type-2 diabetes more frequently than high grade ECs and controls. As expected, the mRNA levels of the estrogen receptors (ER-α and ER-β) tended to be statistically non-significantly lower in high grade compared with low grade ECs and controls (the prevalence of samples with high expression of the receptors was lower among high grade than low grade ECs and controls; Table 1). In line with previous works [14], the mRNA levels of HSD17B2 were statistically significantly higher in ECs than controls (the prevalence of samples with high HSD17B2 expression was higher among ECs than controls; Table 1).

### 3.2. mRNA expression levels of STS, SULT1E1 and CYP19A1

In the EC group, we could measure STS and SULT1E1 mRNA expression in all 31 cases and CYP19A1 mRNA in 29 cases (due to technical failure in two samples). In the postmenopausal control group, we were able to measure STS, SULT1E1 and CYP19A1 mRNA expression in 17, 15 and 16 (respectively), out of 19 samples, due to low RNA quality in two samples and technical failure of the PCR reaction in the other three cases (excluded from analyses, see also Table 2 for details). Overall, the mean ct value for STS was 30.0 (standard deviation, SD, ± 2.5), for SULT1E1, it was 31.7 ( ± 1.3) and for CYP19A1 the mean ct value was 34.3 ( ± 1.5). There was no significant difference in either STS or SULT1E1 mRNA expression level between postmenopausal controls and ECs (grade 1, 2 and 3 pooled; Table 2). Since STS and SULT1E1 catalyse opposite reactions, the ratio between the levels of these enzymes (STS:SULT1E1) was computed as an estimate of the net reaction balance. STS:SULT1E1 ratio did not change significantly between ECs and controls (Table 2). In contrast, CYP19A1 (aromatase) mRNA expression levels were lower in ECs compared with controls (Table 2).

There was no correlation between STS, SULT1E1 and CYP19A1 mRNA levels and other patient characteristics, including the levels of the estrogen receptors and of the HSD17Bs.

### 3.3. Protein expression of STS and SULT1E1

Immunohistochemistry for STS and SULT1E1 was performed on all tissue samples used in this study. STS showed strong (membrane associated) immunoreactivity, whereas SULT1E1 protein had cytoplasmic localisation with strong reactivity in glandular epithelial cells (Fig. 1). Two independent observers scored the immune reactivity of samples using a modified Allred system (Table 2). They were blinded to the results of each other and there was an inter-observer correlation of 0.94 for STS and 0.93 for SULT1E1. In postmenopausal controls, grade 1 and grade 2–3 EC, STS staining was seen in 81%, 100% and 94% of the cases respectively, whereas SULT1E1 positivity was detected in 100% of all the samples. The staining index for STS and SULT1E1 did not differ between groups (Table 2).

### 3.4. Novel LC-MS/MS method to measure SULT1E1 activity

To measure the SULT1E1 activity, we developed a new non-radioactive and highly sensitive method based on LC-MS/MS able to quantify the product E1S formed by SULT1E1 after incubation with the

**Table 2**
STS, SULT1E1 and CYP19A1 mRNA levels, protein expression and enzyme activity in ECs and postmenopausal controls.

| | Postmenopausal controls (n = 19) | | Grade 1 EC (n = 21) | | Grade 2–3 EC (n = 10) | | All ECs (31) | | p-value (all ECs-controls)[#] |
|---|---|---|---|---|---|---|---|---|---|
| mRNA level (Samples analysed) | Arbitrary units[*] (n = 17) | | Arbitrary units[*] (n = 21) | | Arbitrary units[*] (n = 10) | | | | |
| | mean | range | mean | range | mean | range | mean | range | |
| STS | 12.6 | 3.7–32. 7 | 22.6 | 3.1–57.7 | 19.2 | 1.2–87.4 | 21.5 | 1.2–87.4 | ns |
| SULT1E1 | 58.9 [&] | 1.0–292.0 | 33.0 | 3.2–160.9 | 61.4 | 1.4–519.1 | 42.1 | 1.4–519.1 | ns |
| CYP19A1 | 76.0 [&] | 1.0–393.4 | 29.7 [&&] | 4.3–85.6 | 30.2 | 3.3–139.1 | 29.9 | 3.3–139.1 | 0.029 |
| Protein expression (Samples analysed) | Allred score [**] (n = 19) | | Allred score [**] (n = 21) | | Allred score [**] (n = 10) | | | | |
| | mean | range | mean | range | mean | range | mean | range | |
| STS | 3.3 | 0–15 | 5.8 | 0–15 | 4.8 | 0–15 | 5.5 | 0–15 | ns |
| SULT1E1 | 5.1 | 0–15 | 6.9 | 0–15 | 8.6 | 0–15 | 7.4 | 0–15 | ns |
| Enzyme activity (Samples analysed) | product/mg protein/hour (n = 17) | | product/mg protein/hour (n = 21) | | product/mg protein/hour (n = 10) | | | | |
| | mean | range | mean | range | mean | range | mean | range | |
| STS | 29. 9 nmol | 8.2–105.8 | 18.2 nmol | 7.6–48.6 | 19.7 nmol | 8.8–40.8 | 18.7 | 7.6–48.6 | 0.025 |
| SULT1E1 | 6.1 pmol | 0.8–27.1 | 20.1 pmol | 0.97–115.2 | 18.4 pmol | 1.1–78.2 | 19.5 | 0.97–115.2 | ns |
| | | | | COMPUTED RATIOS STS:SULT1E1 | | | | | |
| mRNASTS:SULT1E1 | 2.1 | 0.02–8.0 | 2.0 | 0.08–16.9 | 4.7 | 0–29.4 | 2.9 | 0–29.4 | ns |
| proteinSTS:SULT1E1 | 0.8 | 0.2–1.0 | 0.9 | 0.09–2.4 | 0.65 | 0.2–1.5 | 0.7 | 0.09–2.4 | ns |
| Activitysts:SULT1E1 | 8.3 | 0.9–21.5 | 4.2 | 0.15–10.2 | 5.2 | 5.2–21.3 | 4.5 | 0.15–21.3 | 0.022 |

ns: not significant.

[*] mRNA arbitrary units: values indicate the fold change compared to the reference expression level (e.g. the mean of three house keeping genes: Ribosomal protein S18, β-actin and Cyclophilin A).

[**] A modified Allred scoring systems (intensity × proportion; range 0–15) described earlier was used [27].

[&] Two control samples analysed for SULT1E1 and one analysed for CYP19A1 showed aberrant qPCR melting curves and agarose gel bands and were excluded.

[&&] Two grade 1 EC samples analysed for CYP19A1 showed aberrant qPCR melting curves and agarose gel bands and were excluded.

[#] p-values were computed using the *t*-test comparing all ECs and controls.

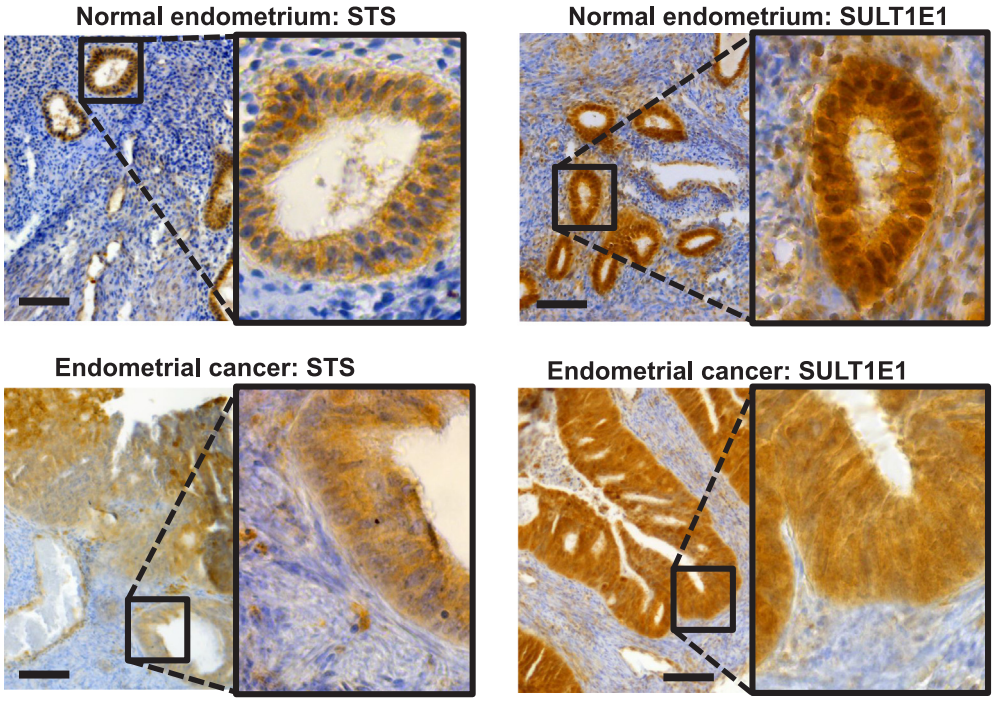


**Fig. 1.** Representative immunohistochemistry images of STS and SULT1E1 in normal postmenopausal endometrium and EC. Scale bar 100 μM.

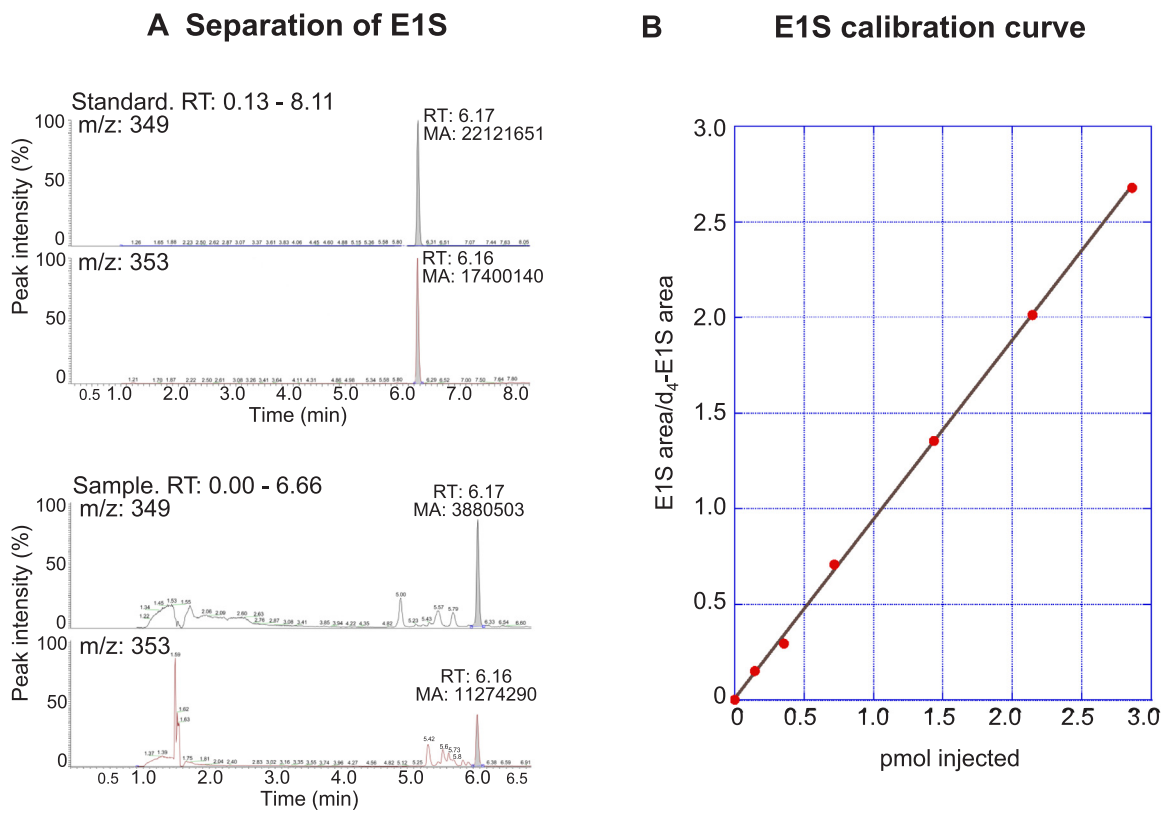


**Fig. 2.** Performance of the SULT1E1 activity measurement by LC-MS/MS A. Separation of E1S and d4-E1S B. E1S calibration curve.

substrates E1 and 3′-phosphoadenosine 5′-phosphosulfate (the sulfate donor in the reaction). A good separation of the E1S and the internal standard $d_4$-E1S from some impurities, on the reversed phase column, was achieved and the analytes of interest eluted around the retention time of six minutes (Fig. 2A). We tested the linearity of the mass ratios (unlabeled:labeled E1S) , that were linear from 0.1 to 2.8 pmol of E1S injected (correlation coefficient: 0.9995; Fig. 2B). The recovery of E1S in the incubation mix after being precipitated with acetonitrile was over 80%.

### 3.5. Enzyme activity of STS and SULT1E1

STS and SULT1E1 activities could be measured in 17 controls and all EC samples. STS enzyme activity ranged between 8 and 106 nmol/mg protein/hour (Table 2). The STS activity was significant lower in ECs (all pooled) compared with postmenopausal controls (Table 2).

SULT1E1 ranged between 0.8 and 115 pmol/mg protein/hour and the mean values did not differ between groups (Table 2).

The enzyme activity ratio STS:SULT1E1 was computed as an estimate of the net reaction balance. STS:SULT1E1 was significantly lower in EC patients compared with postmenopausal controls (Table 2). No further correlations between enzyme activities and other patient clinical features were observed.

We did not observe a clear correlation between mRNA, immunoreactivity and enzyme activity of STS and SULT1E1 for either the EC samples or the postmenopausal controls.

## 4. Discussion

EC, and in particular those lesions with an endometrioid histology, is associated with excessive estrogen exposure. Besides the systemic estrogen levels in the serum, the intracellular milieu, controlled by a complex network of metabolic reactions and referred to as intracrinology [15], is frequently altered in EC towards an increased estrogenicity. Such intracellular estrogen level is controlled, among others, by the enzymes STS, SULT1E1 (sulfatase pathway) and CYP19A1 [14,15]. The present investigation explores the levels of these enzymes in EC cases and postmenopausal controls. No significant differences in the mRNA and protein levels of STS and SULT1E1 between cases and controls were observed. However, STS enzyme activity and the enzyme activity ratio STS:SULT1E1 were lower in EC specimens compared with controls. When the grade was taken into consideration, and controls, grade 1 and high grade (2 and 3) ECs were compared as three groups, the difference tended to be restricted to grade 1 (that had non-significantly lower STS and STS:SULT1E1 levels than postmenopausal controls; p = 0.08 for STS and p = 0.07 for STS:SULT1E1 computed by ANOVA. Data not shown). Although earlier research showed contrasting data regarding the levels of these enzymes, most recent studies are in line with our data and show a decreased level of STS in ECs compared with controls, as recently reviewed [14–16,31].

In the present study, state-of-the-art protocols and technologies were used to measure the enzyme activities of STS and SULT1E1. HPLC was used for STS (previously published [29,30]) and a novel LC-MS/MS method for SULT1E1. The enzyme activity of STS was previously assessed in EC by four studies that used TLC or aqueous/organic phase separation [18–21], as recently reviewed [14]. The STS activity ranged in the magnitude of 1–100 nmol/mg protein/hour, in line with our data and our previously published results in endometriosis specimens [29].

SULT1E1 enzyme activity in EC was assessed by three studies also using TLC or aqueous/organic phase separation (reviewed in [14]) with activities substantially lower than STS, ranging in the pmol/mg protein/hour, in line with our data. Of note, in contrast to our study where EC tissues were compared with postmenopausal endometrium, none of the previously published studies that assessed the activity of STS or SULT1E1 compared the levels between ECs and postmenopausal women. Some studies used tissue from premenopausal women as control, but due to the variation in the expression of some enzymes throughout the menstrual cycle (e.g. SULT1E1), this may create some

biases (as reviewed in [14]).

In contrast to the data of Utsunomiya and co-workers, who found a positive correlation between mRNA, protein and enzyme activity of STS and SULT1E1 [19], these measurements did not correlate in our study, suggesting the presence of post-transcriptional/translational events in the regulation of these genes. There are also important technical differences between the two studies, e.g., mRNA levels were assessed semi-quantitatively, immune reactivity was analysed using a threshold value and activity was measured by organic/aqueous phase separation by Utsunomiya and co-workers [19], whereas, in our study, mRNA levels were assessed quantitatively, immune reactivity was determined as a continuous variable and enzyme activity was measured HPLC or LC-MS/MS.

Our study did not explore those enzymes involved in the transport of sulfated steroids through the plasma membrane, important aid to the sulfatase pathway to influx steroids into the cells. These mechanisms, however, are gaining considerable attention in recent years and should be assessed in future investigations as part of the intracrine networks [16].

In the present study we also explored the mRNA level of CYP19A1, which was decreased in ECs compared with postmenopausal controls. Also in this case, when the grade was taken into consideration, the difference tended to be restricted to grade 1 (where CYP19A1 was non-significantly lower than in postmenopausal controls; $p = 0.09$, computed by ANOVA. Data not shown). CYP19A1 levels were in general very low (high ct. values by RT-qPCR). CYP19A1 immunohistochemistry was not performed because this enzyme is barely detectable, based on previous results from our laboratory [27]. Previous investigations showed contrasting data regarding CYP19A1 mRNA levels in EC. These studies were recently systematically reviewed and most published studies showed a decreased CYP19A1 mRNA in ECs than in controls (although that was not the case for the protein/enzyme activity levels) [14].

The levels of the estrogen receptors, HSD17B1 and HSD17B2 did not show any correlation with the sulfatase or the aromatase pathways.

The important role of estrogen intracrinology in endometrial pathophysiology is corroborated by recent molecular analyses of EC. The Cancer Genome Atlas explored and classified EC according to the molecular signature by integrating various 'omics' datasets (genomics, transcriptomics, epigenomics and proteomics) [32]. Interestingly, genes controlling the local steroid metabolism resulted affected by somatic mutations (SULT1E1 was significantly mutated, with nine mutations in seven out of the 248 cases analysed) or by copy number variations (shown for HSD17B1, HSD17B2, HSD17B3 as well as for the enzymes involved in corticosteroid synthesis HSD11B1 and HSD11B2).

Taken together, our data indicates that the sulfatase pathway is active in human endometrium. STS activity exceeds of some magnitude the activity of SULT1E1 as well as the mRNA level of CYP19A1, suggesting that STS is an important mechanism for intracellular estrogen supply in endometrial pathophysiology and confirming previous data and interpretations [14–16]. CYP19A1 could also contribute to the local estrogen generation in specific subgroups of EC patients with high local CYP19A1 expression, or in obese subjects through the conversion of androgens into estrogens that takes place in adipose tissue – non-local/intracrine effect in this case [14,15].

In a clinical perspective, blocking the estrogen local supply to decrease hormone-dependent tumour growth is an attractive therapeutic approach. STS inhibitors have been tested in both breast cancer and EC. Although in case of EC, the only phase II trial that compared the STS inhibitor Irosustat with standard progestogen treatment (megestrol acetate) in advanced-stage EC was stopped because of no added benefit of Irosustat, novel phase II trial on breast cancer patients show a potential therapeutic value of STS inhibition [14,33,34], and strongly indicate the need to preselect potential responsive patients to obtain therapeutic efficacy [35,36]. Additionally, one should consider that the machinery controlling the local estrogen level is complex and includes reactions other than STS/SULT1E1 (and CYP19A1). E1 supplied by STS can be further activated to the potent E2 by the enzyme HSD17B1, and several non-estrogenic compounds are capable of binding and activate the ERs [14,15,17,27]. Hence, dual/triple drug regimen aimed at blocking multiple steps in this machinery (STS/CYP19A1/HSD17B1), or dual/triple inhibitors able to block simultaneously multiple enzymes carry promises for the future.

## 5. Conclusions

A novel method for a highly sensitive and accurate measurement of SULT1E1 activity level is presented. We confirm that the sulfatase pathway is actively present in the endometrium and in EC. STS seems to be the major route of intracellular estrogen supply. Since the final local estrogen levels in the endometrium are controlled by multiples enzymes and metabolisms, therapeutic approaches should not attempt at blocking one single enzyme but the complete pathways leading to the formation of active estrogens.

## Acknowledgements

The authors would like to thank Dr Freek Bouwman for the assistence in using the LC-MS-MS.

## Conflict of interest

The authors have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Funding information

The study was sponsored by the Dutch Cancer Society ('KWF Kankerbestrijding': www.kwf.nl), contract number UM-2013-5782 granted to A.R.

## Author contribution

All authors contributed to the study either in the design phase, experimental part or both. All authors contributed writing the manuscript and approved the version for submission.

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