Estradiol Metabolites as Biomarkers of Endometrial Cancer Prognosis After Surgery

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Abstract

Endometrial cancer (EC) is the most common gynecologic malignancy prevailing after menopause. Defining steroid profiles may help predict the risk of recurrence after hysterectomy, which remains limited due to the lack of reliable markers. Adrenal precursors, androgens, parent estrogens and catechol estrogen metabolites were measured by mass spectrometry (MS) in preoperative serums and those collected one month after hysterectomy from 246 newly diagnosed postmenopausal EC cases. We also examined the associations between steroid hormones and EC status by including 110 healthy postmenopausal women. Steroid concentrations were analyzed in relation to clinicopathological features, recurrence and overall survival (OS). The mean follow-up time was 65.5 months and 26 patients experienced relapse after surgery for a recurrence incidence of 10.6% (6.4% Type I and 29.5% Type II). Recurrence and OS were related to a more aggressive disease but not linked to body mass index. Preoperative levels of estriol (E_3) and estrone-sulfate (E_1-S) were inversely associated with recurrence in a multivariate logistic regression analysis (Hazard ratios (HRs) of 0.31, *P*=0.039 and 3.01, *P*=0.024; respectively). All circulating steroids declined considerably after surgery almost reaching those of healthy women, except 4-methoxy-E_2 (4MeO-E_2) for which postoperative levels increased by 35% and were associated to a 68% decreased risk of recurrence (HR=0.32, *P*=0.015). Women diagnosed with both histological types of EC present significantly higher levels of steroids, in support of their mitogenic effects. The estrogen precursor E_1-S, the anticancer metabolite 4MeO-E_2, and E_3 that exert mixed antagonist and agonist estrogenic activities and immunological effects, are potential independent prognostic factors.

Keywords: Catechol estrogens, Mass spectrometry, Endometrial cancer, Recurrence.

1. Introduction

Endometrial cancer (EC) is the most common gynecologic cancer and the fourth most frequent neoplasm in women in North America, predominantly occurring in postmenopausal women. Furthermore, EC is the only gynecologic cancer with a rising incidence and mortality [1]. Curative surgery, alone or combined with adjuvant radiation therapy, is performed when cancer is limited to the uterus. However, a subset of EC patients experience recurrence, shorter survival and display inadequate response rates to cytotoxic chemotherapy [2]. The prognosis of EC is determined primarily by disease stage, grade and histologic subtype, reinforcing the need to explore novel prognostic markers.

EC is a heterogeneous disease comprising two types based on histology. The most common type, which accounts for nearly 80% of cases, is the endometrioid or Type I adenocarcinoma, associated with unopposed estrogen stimulation and generally has good prognosis. Type II is nonendometrioid that includes serous, clear cell, mixed carcinoma, with higher-grade histology and carries an adverse prognosis. Studies originally described Type I EC as estrogen-dependent whereas Type II was not. However, recent studies indicate that steroid hormones may play a significant etiological role in both types [3]. EC prevails after menopause when ovaries have ceased to secrete potent estrogens. Obesity is a known risk factor of EC [4] and this may be partly related to the fact that adipose tissue represents a major source of estrogen synthesis in postmenopausal women, actively converting adrenal and androgen precursors to estrogens resulting in increased serum bioavailable estradiol (E_2) [5,6]. Previous work by our group and others has revealed that the potent estrogen E_2 primarily derive from conversion of estrone-sulfate (E_1-S) in EC tumors rather than aromatization of androgens by the aromatase (CYP19), which has barely detectable expression levels in EC cells [7-10]. Besides, E_2 and E_1 may be converted into numerous biologically active derivatives with varying mitogenic and genotoxic properties by the action of various cytochrome P450 and catechol-O-methyl transferase enzymes [11]. This metabolism involves the irreversible hydroxylation (OH) at the C-2, C-4, or C-16 positions of the steroid ring and the methylation of C-2 or C-4 hydroxyl group. The latter prevents formation of mutagenic catechol quinones derived from hydroxyl estrogens that form stable and depurinating DNA adducts. In vitro and in vivo studies further support that 2-methoxyestradiol (2-MeOE_2) has strong anticancer activity [12,13]. In addition, these metabolites can be converted to their inactive sulfate and glucuronide conjugates. Recent

studies have assessed the risk of EC in relation to steroid hormones, yet none have explored their association with prognosis [4,14].

In a cohort of 246 postmenopausal women undergoing hysterectomy for a newly diagnosed endometrial cancer, we analyzed the levels of 27 steroids in serums collected the morning of surgery and one month after surgery. Steroid measures included the assessment of endogenous concentrations of adrenal precursors, androgens, potent estrogens and catechol estrogens using sensitive and specific mass spectrometry (MS) validated assays. Our primary goal was to evaluate the association between circulating steroid levels, clinicopathological features and the risk of recurrence after surgery. A group of 110 healthy postmenopausal women was also included to examine the association between steroid hormones and EC status.

2.Materials and Methods

2.1. Study populations

All participants provided a written informed consent for their participation to the study and the use of their specimens. The current study was reviewed and approved by our institutional review boards. Recruitment of healthy postmenopausal women, as well as specimen collection and treatments, have been described elsewhere [15]. Briefly, women were recruited in a mammography clinic in Quebec City (QC, Canada) between July 2003 and March 2004. To be eligible, women had to: 1) be of postmenopausal status, 2) have no history of health problems related to steroid hormone metabolism, 3) have no history of hepatic, thyroid, or adrenal diseases, and 4) have not taken hormone replacement therapy (HRT) during the three months preceding enrolment. Recruitment methods and specimen collection of EC cases have been described [16]. Participants were all recruited at the Hôtel-Dieu de Québec Hospital (Québec City), between 2002 and 2013. All women were of postmenopausal status, undergoing surgery for EC (hysterectomy and bilateral salpingo-oophorectomy) and had not taken HRT in the three weeks prior to surgery. Blood samples were collected the morning of surgery and one month after surgery as part of a follow-up appointment. Samples were immediately processed, separated in aliquots and stored at -80°C until analysis. EC recurrence was ascertained by computerized tomography scan. For both cohorts, demographic and anthropometric data were collected through nurse-administered questionnaires, whereas information regarding drug use (including oral contraceptive and HRT) and obstetric history were collected at the same time. A pathologist assessed the histopathological characteristics of the hysterectomy specimen. Systematic assembling and review of medical records was performed by one of the treating gynecologic oncologist (J.G.).

2.2. Reagents and material

Parent estrogens standards were purchased from USP reference standard (Rockville, MD, USA), while other steroids were purchased from Steraloids (Newport, RI, USA). Deuterated standards were from C/D/N Isotopes (Montréal, QC, Canada), except d3-DHEA, which was synthesized by the Organic Synthesis Service of the CHU de Québec Research Center (Québec, QC, Canada). All chemicals and solvents used in this study were HPLC or reagent grade. Methanol, chlorobutane, dichloromethane, ethyl

acetate and acetone were purchased from VWR (Montréal, QC, Canada). Ascorbic acid, sodium bicarbonate, β-glucuronidase/sulfatase (*Helix Pomentia* Type HP-2) and dansyl chloride were purchased from Sigma (Oakville, ON, Canada).

2.3. Steroids and SHBG quantification

Steroids and SHBG were quantified using validated methods [16,17]. Internal standards (deuterated steroids) were added to samples and quality controls were included in each run. The measured steroids and their limits of quantification were as follows: dehydroepiandrosterone (DHEA; 100 pg/mL); androstenediol (5-diol; 50 pg/mL); testosterone (30 pg/mL); dihydrotestosterone (DHT; 10 pg/mL); androsterone (ADT; 50 pg/mL); estrone (E_1; 5 pg/mL); estradiol (E_2; 1 pg/mL); androstenedione (4-dione; 50 pg/mL); ADT-glucuronide (ADT-G; 1 ng/mL); androstane-3α, 17β-diol 3-glucuronide (3α-diol-3G; 0.25 ng/mL); 3α-diol-17-G (0.25 ng/mL); DHEA-sulfate (DHEA-S; 0.075 mg/mL); estrone-sulfate (E_1-S; 0.075 ng/mL). Briefly, gas-chromatography (GC) coupled to mass spectrometry (MS) were used to quantify levels of DHEA, ADT, 5-diol, 4-dione, testosterone, DHT, E_1 and E_2 using 250 µL of serum, while liquid-chromatography (LC) tandem MS was used for conjugated steroids using 20 µL for sulfates and 100 µL for glucuronides in two independent assays. All metabolite coefficients of variation (CV) were <10% and no samples had undetectable hormone levels.

We also measured 14 catechol estrogens with another MS-based assays, namely i) catechol 2OH: 2-hydroxyestrone (2-OHE_1), 2-hydroxyestradiol (2-OHE_2), ii) catechol 4OH: 4-hydroxyestrone (4-OHE_1), 4-hydroxyestradiol (4-OHE_2), iii) catechol 16OH: estriol (E_3), 16α-hydroxyestrone (16α-OHE_1), 16-ketoestradiol (16-ketoE_2), 16-epiestriol (16-epiE_3), and 17-epiestriol (17-epiE_3), and iv) catechol MeO: 2-methoxyestrone (2-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$), 4-methoxyestrone (4-$MeOE_1$) and 4-methoxyestradiol (4-$MeOE_2$). The quantification method was performed after some adjustments (described below) by stable isotope dilution LC/MS-MS based on method published by Xu *et al* [18] , which detected 13 catechol estrogens in addition to E_1 and E_2 and used 500 µL for a reported lower limit of quantification (LLOQ) of 8 pg/mL. In our study, we used 250 uL of serum for extraction to measure 14 catechol estrogens with a LLOQ of 5 pg/mL (corresponding to 16.56-18.52 pmol/L depending on the estrogen metabolite). LLOQ was defined as the minimum value at which the ratio of signal-to-noise was ≥5:1. Also, values of catechol estrogens observed below LLOQ

(even if detected above the limit of detection) were considered as undetected. To measure total catechol estrogens corresponding to the sum of conjugated plus unconjugated forms, β-glucuronidase/sulfatase was included in sample preparation. Briefly, catechol estrogens were extracted from 250 µL of serum with ethyl acetate:chlorobutane (25:75, v/v) and evaporated to dryness. Derivatization was conducted with dansyl chloride (0.5 mg/mL final in 50% acetone and 50 mM sodium bicarbonate, pH 9.0). Samples were heated for 5 minutes at 60°C, mixed with 15 volumes of water:methanol (80:20, v/v) and loaded on pre-conditioned Strata X 60 mg SPE columns (Phenomenex, Torrance, CA, USA). After being washed with water and water:methanol (10:90, v/v), catechol estrogens were eluted with dichloromethane:methanol (50:50, v/v). Eluates were evaporated to dryness at 45°C under nitrogen gas, reconstituted in 100 µL of acetone:water (75:25, v/v) and injected into a high performance liquid chromatograph (HPLC) Waters (Milford, MA, USA). The chromatographic separation was achieved with an Synergie RP Hydro column containing 2.5 µm packing material, 100 X 3 mm (Phenomenex, Torrance, USA). The mobile phases consisted of water with 0.0375% formic acid (solvent A) and MeOH with 0.0375% formic acid (solvent B). The flow rate was 0.5 ml/min. The analytes were eluted with the following program: 0-8 min, isocratic 22.5% B; 8-18 min, linear gradient 22.5-35% B; 18-23 min, isocratic 35% B; 23-23.1 min, linear gradient 35-95% B; 23.1-28 min, isocratic 95% B; 28.0-28.1 min, linear gradient 95-22.5% B and 28.1-33 min, isocratic 22.5% B. Catechol estrogens were detected with an API5500 QTRAP MS (Concord, ON, Canada) equipped with a turbo ion-spray source set in positive ion mode, and operated in multiple reaction monitoring mode (MRM). Electrospray ionization was performed with an ionization voltage of 5500 V, declustering potential voltage of 180 V, collision energy of 42 V, and a heater probe temperature of 650°C. The catechol estrogens were detected using the following mass transitions: E3, 16-epi-E_3 and 17-epi-E_3: 522.3 to 171.0; 16-keto-E_2 and 16OH-E_1: 520.3 to 171.0; 2MeO-E_2 and 4MeO-E_2: 536.1 to 171.0; 2MeO-E_1, 3MeO-E_1 and 4MeO-E_1: 534.1 to 171.0; 2OH-E_1 and 4OH-E_1: 753.3 to 170.0; 2OH-E_2 and 4OH-E_2: 755.3 to 170.0. Quality controls were prepared in non-adsorbed serum samples to obtain low, medium or high analyte concentrations and were included in each run, along with a seven-point calibration curve prepared by spiking, as well as blanks. All catechol estrogen metabolites coefficients of variation were below 10%.

2.4. Statistical analyses

Statistics were conducted using SAS Statistical Software v.9.2 (SAS Institute, Cary, NC, USA) and SPSS Statistics v.23 (IBM Corporation, Armonk, NY, USA). Age and body mass index (BMI) between cases and controls were compared with Student's *t*-test. For analysis of nominal data, chi-square (χ^2) tests were conducted, and the Fisher's exact test was applied when required. Odds of EC were assessed using binary logistic regressions to calculate odds ratios (ORs) and 95% confidence intervals (95% CIs). Backward stepwise (likelihood ratio) was used for the selection of covariates included in the final model, in which hormone concentrations (categorized upon tertiles or the median when specified) were added. For assessment of risk of recurrence, a similar method was used, with the median level of EC cases as the category threshold. For survival and recurrence analyses, patients were also categorized as low or high risk of poor prognosis, based on histological types (T) and grade (G): TI-G1 or TI-G2 were considered at low risk, whereas TII and TI-G3 were considered at high risk [19]. Differences in steroid concentrations between groups were assessed using the analysis of covariance (ANCOVA) on log-transformed data, and untransformed data are presented to facilitate understanding. Fold changes were calculated upon the median of each group. For pairwise comparison of more than two groups, the Tukey-Kramer *post hoc* test was used. The hormone variation between paired blood samples (preoperative and postoperative samples were available for 187 cases) was analyzed using Wilcoxon signed rank test, whereas variation between groups was compared by ANCOVA on the difference of paired preoperative and postoperative levels (log-transformed). The overall survival (OS) (all-cause mortality) was estimated with the Kaplan-Meier method and tested using log-rank test, while Cox regressions were used for further adjustments using backward stepwise (likelihood ratio) for the selection of covariates. Cancer-specific survival could not be not analyzed. Because of the exploratory nature of the study and the significant interrelations between circulating steroids, two-sided *P*-values were considered statistically significant at $P<0.05$, without adjustment for multiplicity. Covariates adjustments are specified in figure and table legends.

3. Results

3.1. A cohort of 246 postmenopausal EC cases treated by hysterectomy

We studied a cohort of 246 postmenopausal EC cases prospectively recruited at a single center and all treated by hysterectomy performed for curative intent. Most cases (82%) presented with a Type I adenocarcinoma and 18% were histologically characterized by serous, clear cell, mucinous, or mixed carcinoma; combined as Type II (**Table 1**). The mean follow-up time after recruitment was 65.5 months and 26 patients experienced recurrence after surgery for a recurrence incidence of 10.6%. (6.4% Type I and 29.5% Type II). The estimated 5-year recurrence incidence was of nearly 10% (n=24 cases). Of note, the majority of Type I cases who experienced relapse (12 out of the 13) were of low-grade and particularly of grade 2 (67%), whereas for Type II, 82% recurrent cases were of grade 3. EC relapse and OS were related to a more aggressive disease (myoinvasive tumors, presence of metastatic nodes) but not BMI (**Table 2**). Only OS was associated with age (HR=1.08, 95% CI=1.04-1.12; *P*<0.001 for OS).

3.2. Estradiol metabolites are associated with recurrence and survival

In serum samples collected on the morning of surgery and one month later, we initially profiled 13 unconjugated (using gas chromatography-mass spectrometry (GC-MS)) and conjugated (using liquid chromatography-mass spectrometry (LC-MS/MS)) steroids including adrenal precursors (DHEA, DHEA-S, 4-dione, 5-diol), androgens (testosterone, DHT, ADT, ADT-G, 3α-diol-3G, 3α-diol-17G) and parent estrogens (E_1-S, E_1, E_2) (**Fig.1**). Steroid hormones were detected above the LLOQ for all cases. None of these steroids measured prior or after surgery were associated with clinicopathological characteristics, i.e. histological type, grade, stage, lymph-vascular space invasion and metastases. Consistent with the notion that adipose tissue is a major source of estrogens in postmenopausal women; E_2 levels were 3.3-fold higher in obese EC cases (*P*<0.001) compared to those of normal weight **(Fig.2a).** A similar trend was observed for the other parent estrogens E_1-S and E_1. This observation is in line with the significant association between BMI and low-grade (1 and 2) endometrial tumors ($\chi^2_{df=4}$=28.9, *P*<0.001), mainly characterizing Type I adenocarcinomas. In contrast, for both Type I and Type II cases, circulating levels of adrenal precursors and androgens were not significantly associated with obesity, except for levels of

3α-diol glucuronide metabolites derived from the potent androgen dihydrotestosterone (DHT) (**Supplemental Table 3**).

LC-MS/MS–based analysis was also used to profile 14 catechol estrogen derivatives in preoperative and postoperative serums of EC cases. Note that only steroid concentrations accurately measured above the LLOQ of 5 pg/mL were considered as detectable. Detection rates are presented in **Table 3** and were similar in preoperative and postoperative serums. In particular, four metabolites were most abundant and detected in more than 60% of EC cases, namely E_3, 2OH-E_1, 2MeO-E_1 and 4MeO-E_2. As observed for the parent estrogens E_1 and E_2, levels of these catechol estrogens were significantly affected by BMI, except 4MeO-E_2, which remained constant across BMI categories (**Fig.2b**). In relation to clinicopathological characteristics, a significant association was only observed for E_3, which levels were 1.6-fold lower in myoinvasive tumors (≥50%) compared to less myoinvasive tumors (<50%) (21.0 vs 33.2 pg/mL, *P*=0.011; adjusted for age and BMI). Compared to cases with preoperative E_3 levels below median (≤30.5 pg/mL), those with higher levels (>30.5 pg/mL) were less likely to experience recurrence after surgery (Log-rank *P*=0.001) during the 5 years following diagnostic; an association that remained significant in the fully adjusted model for all available follow-up (HR=0.27, 95% CI=0.09-0.80; *P*=0.018; **Fig.3a; Supplemental Table 4**). Preoperative levels of E_3 were also linked to an improved OS, as shown by the Kaplan-Meier curves for OS within 5 years post surgery (Log-rank *P*=0.002; **Fig.3b**) and for all available follow-up (Log-rank *P*=0.021; **Supplemental Table 5**), but was not significant upon further adjustment (HR=1.15, 95% CI=0.45-2.92; *P*=0.766; **Supplemental Table 5**). Furthermore, levels of the abundant estrogen precursor E_1-S were associated with a higher risk of recurrence (HR=2.67, 95% CI=1.02-6.99; *P*=0.045; **Fig.3a**). Of note, circulating levels of E_1-S and E_3 were not significantly correlated (not shown).

One month after surgery, nearly all hormone levels were considerably reduced compared to preoperative levels (**Fig.3d; Table 4**). Hence, postoperative E_1-S and E_3 levels were not associated with recurrence in the fully adjusted model (**Fig.3a; Supplemental Table 4**). In contrast, postoperative serum levels of the anticancer metabolite 4MeO-E_2 were significantly increased compared to preoperative levels, with a mean variation of 35% and a median elevation of 6.3 pg/mL (paired data of 187 EC cases). Moreover, EC cases with higher postoperative levels of 4MeO-E_2 were less likely to experience recurrence upon

adjustment for prognostic factors in multivariate analysis (HR=0.34, 95% CI=0.14-0.86; *P*=0.022; **Fig.3a**). Lastly, postoperative levels of the androgen inactive metabolite 3α-diol-17G were linked to an improved OS (HR=0.26, 95% CI=0.07-0.89; *P*=0.031), whereas preoperative levels were not (**Supplemental Table 5**).

3.3. EC cases have higher levels of circulating steroid hormones compared to healthy women

In a last series of analyses, preoperative levels of steroids in EC cases were compared to those of 110 healthy postmenopausal women (**Supplemental Tables 1-2**) [15]. After adjustment for parity, use of oral contraceptives (OC), use of hormone replacement therapy (HRT), as well as age and BMI, levels of endogenous steroids were significantly associated with increased odds of EC, for both histological types, with ORs ranging from 2.19 to 17.07 (**Fig.4**). In turns, SHBG had a protective effect for Type II (OR=0.18, 95% CI=0.04-0.80; *P*=0.024) but did not reach significance for Type I (OR=0.50, 95% CI=0.24-1.05; *P*=0.067). Obese women (BMI>30 kg/m^2) had increased odds of EC (OR=2.45, 95% CI=1.33-4.48; *P*=0.004) and particularly for Type I adenocarcinomas (OR=2.99, 95% CI=1.60-5.57; *P*<0.001) but not for Type II (OR=0.88, 95% CI=0.36-2.18; *P*=0.783) (**Fig.2c**).

4. Discussion

In this study, we profiled by MS a total of 27 steroid derivatives including adrenal precursors, androgens, parent estrogens and catechol estrogens in circulation of postmenopausal women newly diagnosed with EC undergoing hysterectomy for curative intent. Despite the exploratory nature of our study, to the best of our knowledge, this report provides one of the most comprehensive analysis of circulating steroid hormones by MS in the context of EC, and is the first to report steroid levels prior and after surgery in relation to clinicopathological characteristics, recurrence and survival.

None of the steroids measured were associated with known prognostic factors, except E_3, for which higher levels (>30.5 pg/mL) were linked to non-myoinvasive tumors, lower risk of recurrence and improved OS. This contrasts with higher levels of the most abundant estrogen precursor E_1-S (>0.31 ng/mL), which were associated with an increased risk of relapse, in line with the role of E_1-S as a predominant source of E_2 for endometrial tumor cells, and the proliferative effect of estrogens. Similar to the parent estrogens E_1 and E_2, levels of catechol estrogens varied according to BMI, except for 4MeO-E_2. In fact, this anticancer metabolite was the only steroid increased in circulation of EC cases after surgery with postoperative 4MeO-E_2 levels significantly associated with a lower risk of recurrence. Our observations require further investigations but imply that circulating levels of specific E_2 metabolites, namely E_1-S, E_3 and 4MeO-E_2, may represent independent prognostic markers of cancer recurrence after curative therapy.

Other groups reported similar levels of E_2 derivatives in circulation of endometrial, ovarian and breast cancer cases, as well as for healthy postmenopausal women [14,20-23]. In our study, preoperative levels of E_1-S and E_3 were inversely associated with recurrence, in line with their opposing biological roles and the stimulatory action of E_2 on EC cells. High levels of the most abundant circulating estrogen E_1-S were associated with poor outcome. An increased exposure to this estrogen precursor may favour E_2 synthesis and enhance stimulatory effect on tumor cells. In contrast, cases with higher levels of the E_2 metabolite, E_3, were less likely to experience recurrence. The protective role of E_3 is reinforced by its inverse relationship with myometrium invasion whereas higher E_3 level persisted as an independent marker after adjusting for well-known prognostic factors. E_3 displays modest and mixed antagonist and agonist estrogenic activities in addition to immunological effects [24-26]. Recent studies support that EC cancer,

and particularly the most aggressive forms of the disease, are sufficiently immunogenic to be candidate for immunomodulation [27]. It is thus possible that fluctuations of E_3 in this particular microenvironment might affect angiogenic profile and/or antitumor immunity.

In the analysis of E_2 derivatives in serums collected one month after surgery, we observed that postoperative levels of 4MeO-E_2 predicted risk of relapse independently of prognostic factors. In contrast to quantities of all other steroids, including levels of E_1-S and E_3 that considerably declined after surgery, those of 4MeO-E_2 increased, and EC cases with higher levels were less likely to experience recurrence. This observation could be explained by the fact that an increased methylation activity would reduce the genotoxic effects of 4-hydroxylation pathway catechols such as 4OH-E_2 through a decrease of their levels [11]. In line, less extensive methylation is associated with a higher risk of postmenopausal breast cancer whereas enhanced 2-hydroxylation is associated with a lower risk [28,29]. Furthermore, concentrations of 4MeO-E_2 were not influenced by obesity by opposition to other estrogens, suggesting that its synthesis would not predominantly originate from adipose tissue.

Compared to healthy postmenopausal women, EC cases of both histological types presented significantly higher levels of circulating steroid hormones, with concentrations similar to those reported in previous studies [21,30-33]. The increased exposure to adrenal precursors, androgens and estrogens is thus consistent with an enhanced production of steroids directly or indirectly driven by the tumor, regardless of the histological type. The role of estrogens is well delineated in EC, and most particularly in endometrioid Type I carcinomas recognized as hormonally driven [34]. One month after hysterectomy, circulating steroid levels were considerably reduced, almost reaching those of healthy postmenopausal women, supporting that a significant portion of the steroidogenic activity is driven by the presence of the primary tumor. Besides, local estrogen synthesis in EC tumors has been established to occur predominantly through conversion of E_1-S to E_2 compared to androgen aromatization by the aromatase (CYP19), which expression is barely detectable in EC tumors [7]. Adipose tissue is a primary site of aromatization after menopause, consistent with a greater concentration of potent estrogens and several of their metabolites in obese women, whom had higher odds of Type I endometrioid adenocarcinomas, in line with previous studies [35-39]. In turns, reduced SHBG levels in cancer cases may also contribute to the increased bioavailability of potent steroids [40]. EC cases diagnosed with non-endometrioid type II lesions also

presented with significantly higher levels of adrenal precursors and androgens but not estrogens, suggesting an influence of the tumor on the adrenal secretion and on peripheral conversion of adrenal precursors to androgens with no subsequent impact on aromatization. This is in agreement with recent studies sustaining that androgens may play an etiological role in Type II EC, independent of their conversion to estrogens [41-43]. Furthermore, certain risk factors such as cigarette smoking and alcohol intake might influence cancer risk by altering adrenal precursors and androgen levels [44].

Our study has several strengths including long follow-up period and detailed clinicopathological parameters, and to the best of our knowledge, is the only prospective study of estrogen metabolism in the context of EC outcome. We further used fully validated sensitive and specific bioanalytical methods based on mass spectrometry to yield reliable results and portrait a vast array of steroids including precursors, potent androgens and estrogens, and their biologically active and inactive metabolites. Our study excluded cases that have taken HRT prior to surgery to avoid potential confounding effects on hormonal assays. The analytical assay for catechol estrogens was based on a method published by Xu *et al.* [18] that reported a LLOQ for each estrogen metabolite in serum of 8 pg/mL (~29 pmol/L) using 500 uL of serum for extraction. Here, we used 250 uL with a LLOQ of 5 pg/mL (~18 pmol/L). In our study, only levels of catechol estrogens quantitatively determined with suitable precision and accuracy were reported (i.e. above LLOQ). Likewise, our statistical analyses were limited to metabolites detected in most EC cases in order to avoid biases caused by potentially unreliable steroid concentrations measured above detection limit but below LLOQ, potentially corresponding to semi-quantitative or qualitative data [45]. Finally, we are not aware of other studies reporting changes in circulating steroids after curative surgery for EC cases. Limitations of our study need to be considered and include a limited number of relapse events (10%), which prevented us to apply multiple testing adjustments. Also, because of a small number of events, cancer-specific survival could not be assessed and we were unable to determine the association of hormone levels with outcomes by histologic subtypes that may vary. Furthermore, assessment of hormone levels was performed after disease onset and compared to a limited number of healthy postmenopausal women. A single hormone measurement at two different time points was performed with samples from EC cases collected the morning of the surgery and one month after. In

addition, BMI has not been assessed at follow-up visit one month after surgery, preventing us from correcting for this potential confounding factor.

In conclusion, despite the exploratory nature of our study, it may help establish a non-invasive stratification of patients as high-risk and low-risk categories using preoperative and postoperative measures of circulating steroids, which could be repeated during follow-up. In addition, a better understanding of estrogen metabolism may provide insights into the mechanisms underlying EC progression. Additional larger studies are warranted to elucidate the relationships between estrogen levels, recurrence and survival of EC cases undergoing hysterectomy for curative intent.

Conflicts of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

Study design and supervision: CG

Patient's recruitment: JG, MP, PA

Sample preparation and analytics: PC, VT, LV, CG

Statistical analysis and data analysis: YAD, DS, CG

Writing of the manuscript: YAD, CG

All authors revised and accepted the manuscript.

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Abbreviations: DHEA: dehydroepiandrosterone; DHEA-S: DHEA-sulfate; 5-Diol: androstenediol; 4-Dione: androstenedione; DHT: dihydrotestosterone; ADT: androsterone; ADT-G: ADT-glucuronide; 3α-diol-3G: androstane-3α, 17β-diol 3-glucuronide; 3α-diol-17G: androstane-3α, 17β-diol 17-glucuronide; E_1: estrone; E_1-S: estrone-sulfate; E_2: estradiol; E3: estriol; 2OH-E_1: 2-hydroxyestrone; 2OH-E_2: 2-hydroxyestradiol; 4OH-E_1: 4-hydroxyestrone; 4OH-E_2: 4-hydroxyestradiol; 2MeO-E_1: 2-methoxyestrone; 2MeO-E_2: 2-methoxyestradiol; 4MeO-E_1: 4-methoxyestrone; 4MeO-E_2: 4-methoxyestradiol; 16OH-E1: 16α-hydroxyestrone; BMI: body mass index; LVSI: lymph-vascular space invasion; ANCOVA: analysis of

covariance; 95% CI: 95% confidence interval; CYP: Cytochrome P450; SRD5A: Steroid 5α-reductase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; 3α-HSD: 3α-hydroxysteroid dehydrogenase; SULT: Sulfotransferase; CYP19: aromatase; OC: oral contraceptives; HRT: hormone replacement therapy; LC: liquid chromatography; GC: gas chromatography; MS: mass spectrometry; OR: odds ratio.

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Figure 1. Schematic representation of steroid biotransformation pathways from adrenal precursors to estrogen metabolites. CYP: Cytochrome P450, SRD5A: Steroid 5α-reductase, 17β-HSD: 17β-hydroxysteroid dehydrogenase, 3α-HSD: 3α-hydroxysteroid dehydrogenase, SULT: Sulfotransferase, CYP19: aromatase; UGT: uridine diphospho-glucuronosyltransferase; G: glucuronide; S: sulfate, E: parent estrogens E_1 and E_2. E_3 may also be produced through 16-hydroxylation of E_2.

Figure 2. Estrogen levels in relation to BMI categories in postmenopausal women with endometrial cancer. Median concentrations of parent estrogens **(a)** and catechol estrogens **(b)** are presented by BMI categories for EC cases. For statistics, data were log-transformed and adjusted for age. Detection rates for all tested catechol estrogens in EC cases are available in **Table 3**. **(c)** Odds of endometrial cancer for BMI categories and histological types. Odds ratios (ORs) were calculated using multinomial logistic regression with adjustment for age. 95% CI: 95% confidence interval. * P<0.05, ** P<0.01, *** P<0.001.

Figure 3. The risk of recurrence of EC cases is associated with preoperative and postoperative steroid levels **(a)**. Hazard ratios (HR) were calculated using Cox regression for all available follow-up and comparing hormone categories separated upon median, with adjustment for age, BMI, histological type and myometrial invasion and SHBG levels. Overall survival (OS) within five years after surgery for preoperative **(b)** and postoperative **(c)** circulating E_3 levels. Similar results were obtained when analyses were performed with all available follow-ups. **(d)** Mean variation in levels of circulating steroids after surgery depicted by comparing the difference in serum levels of each woman, collected on the morning of surgery (preoperative) and one month after surgery (postoperative). * P<0.05, ** P<0.01, *** P<0.001. Analyses of recurrence for all hormones are presented in **Supplemental Table 4** and those for OS in **Supplemental Table 5**.

Figure 4. Higher circulating steroid levels are associated with endometrial cancer in postmenopausal women. Odds ratios (OR) were calculated using logistic regression comparing hormone tertiles (Q1 (reference) and Q3) or according to median (5-Diol, 3α-Diol-3G, 3α-Diol-17G), adjusted for age, BMI,

parity, use of oral contraceptives and use of hormone replacement therapy. **Supplemental Table 2** displays steroid levels.

Table 1. Clinicopathological features of endometrial cancer cases treated by radical hysterectomy.

Features	**Endometrial cancer cases (n = 246)**	
	Mean ± SD	
Age (yr)	65.1 ± 8.9	
Weight (kg)	75.0 ± 19.0	
Height (cm)	158.4 ± 6.4	
Follow-up (months)	65.5 ± 36.7	
5-year survival (%)	90.2	
5-year recurrence rate (%)	9.8	
	n	(%)
Body mass index (BMI)[1]		
Normal Weight	70	(28)
Overweight	66	(27)
Obese	108	(44)
Missing	2	(1)
Histological Type[2]		
Type I	202	(82)
Type II	44	(18)
Grade		
1	90	(37)
2	94	(38)
3	61	(25)
Missing	1	(0)
Stage		
1	197	(80)
2	12	(5)
3	28	(11)
4	9	(4)
Myometrial invasion		
< 50 %	187	(76)
≥ 50 %	59	(24)
Lymph-vascular space invasion		
Absence	183	(74)
Presence	58	(24)
Undetermined	5	(2)
Presence of metastatic nodes		
No	220	(89)
Yes	26	(11)
Relapse after surgery[3]		
No	220	(89)
Yes	26	(11)

[1]Categories of BMI according to WHO Guidelines: normal weight: BMI<25 kg/m^2, overweight: BMI between 25 and 30 kg/m^2, and obese: BMI≥30 kg/m^2

[2]Type I only comprises endometrioid carcinomas. Included in Type II are papillary serous carcinoma, mixed carcinoma, clear cells carcinoma, undifferentiated carcinoma, and malignant mixed Müllerian tumor.

[3]Clinicopathological features of endometrial cancer cases in relation to recurrence post-surgery are presented in Table 2.

Table 2. Clinicopathological features of endometrial cancer cases in relation to recurrence after surgery for curative intent and overall survival (all-cause mortality).

	Recurrence after surgery					Overall survival				
	No recurrence		Recurrence			Alive		Deceased		
Characteristics	N=220	(%)	N=26	(%)	Log-rank *P*	N=204	(%)	N=42	(%)	Log-rank *P*
BMI										
<25	60	(27)	10	(38)	0.752	57	(28)	13	(31)	0.932
25 to 30	59	(27)	7	(27)		54	(26)	12	(29)	
>30	99	(45)	9	(35)		91	(45)	17	(40)	
Missing	2	(1)	0	(0)		2	(1)	0	(0)	
Histological type										
Type I	189	(86)	13	(50)	**<0.001**	176	(86)	26	(62)	**<0.001**
Type II	31	(14)	13	(50)		28	(14)	16	(38)	
Grade										
1	87	(40)	3	(12)	**0.015**	81	(40)	9	(21)	**0.016**
2	83	(38)	11	(42)		80	(39)	14	(33)	
3	49	(22)	12	(46)		42	(21)	19	(45)	
Missing	1	(0)	0	(0)		1	(0)	0	(0)	
FIGO stage										
1	184	(84)	13	(50)	**<0.001**	177	(87)	20	(48)	**<0.001**
2	12	(5)	0	(0)		10	(7)	2	(5)	
3	19	(9)	9	(35)		14	(5)	14	(33)	
4	5	(2)	4	(15)		3	(2)	6	(14)	
Invasion of myometrium										
< 50 %	174	(79)	13	(50)	**<0.001**	165	(81)	22	(52)	**<0.001**
≥ 50 %	46	(21)	13	(50)		39	(19)	20	(48)	
Lymph-vascular space invasion (LVSI)										
Absence	170	(77)	13	(50)	**<0.001**	162	(79)	21	(50)	**0.001**
Presence	46	(21)	12	(46)		38	(19)	20	(48)	
Undetermined	4	(2)	1	(4)		4	(2)	1	(2)	
Metastatic nodes										
No	203	(92)	17	(65)	**<0.001**	191	(94)	29	(69)	**<0.001**
Yes	17	(8)	9	(35)		13	(6)	13	(31)	
Poor prognosis[1]										
Low	167	(76)	12	(46)	**0.001**	158	(77)	21	(50)	**0.001**
High	53	(24)	14	(54)		46	(23)	21	(50)	
Overall survival[2]										
Alive	198	(90)	6	(23)	**<0.001**					
Deceased	22	(10)	20	(77)						

[1]Risk of poor prognosis is categorized as low-risk corresponding to type I (TI) with low-grade G1 and G2 whereas TI-G3 and TII are considered as high risk. [2] *P*-value is derived from a chi-square test. Significant differences are indicated in bold.

Table 3. MS-based measures of 14 catechol estrogens in preoperative and postoperative serums of endometrial cancer cases.

	Percent of detection (%) (LLOQ = 5 pg/ml)	
	Pre-hysterectomy (n=233)	Post-hysterectomy (n=198)
Catechols 2OH		
2OH-E_1	78.1	79.3
2OH-E_2	3.0	1.5
Catechols 4OH		
4OH-E_1	3.0	1.5
4OH-E_2	0.4	0.5
Catechols 16OH		
E_3	90.6	88.4
16OH-E_1	48.1	32.3
16-epi-E_3	24.1	13.6
16-keto-E_2	22.7	16.7
17-epi-E_3	5.2	4.6
Catechols MeO		
2MeO-E_1	61.8	53.0
2MeO-E_2	11.2	21.2
3MeO-E_1	7.7	7.6
4MeO-E_1	0.9	0.0
4MeO-E_2	85.8	93.9

Detection is defined as levels of estrogens above the lower limit of quantification of 5 pg/mL (LLOQ). Catechol estrogens above LLOQ in the majority of cases (shaded grey) were the focus of subsequent analyses.

Table 4. Comparison of median (10th and 90th percentile) paired pre- and post-operative serum levels of 187 endometrial cancer cases, and those of healthy postmenopausal women.

Hormones	Paired serum samples from EC cases (n=187)			Serum levels of healthy women (n = 110)	Fold change[1] (Post-operative *vs.* Healthy)
	Pre-operative	Post-operative	Median variation[2] (Post vs Pre)		
Adrenal Precursors					
DHEA-S (mg/mL)	0.64 (0.24-1.42)	0.61 (0.24-1.42)	**-0.05**[a]	0.60 (0.23-1.27)	**1.00**[b]
DHEA (ng/mL)	2.62 (0.98-7.38)	1.84 (0.82-4.97)	**-0.68**[a]	1.91 (0.85-4.24)	**0.97**[b]
5-diol (pg/mL)	336.5 (139.4-700.7)	276.4 (50.0-529.0)	**-94.0**[a]	230.0 (100.0-495.0)	**1.20**[c]
4-dione (ng/mL)	0.63 (0.36-1.36)	0.46 (0.26-0.92)	**-0.20**[a]	0.44 (0.24-0.80)	1.05
Androgens					
Testosterone (ng/mL)	0.24 (0.13-0.54)	0.13 (0.05-0.28)	**-0.11**[a]	0.14 (0.06-0.24)	0.96
DHT (pg/mL)	37.30 (17.78-80.15)	26.18 (5.00-61.63)	**-10.60**[a]	30.00 (10.00-70.00)	0.87
ADT (pg/mL)	132.3 (62.6-347.4)	97.3 (25.0-240.3)	**-34.4**[a]	n/a	---
ADT-G (ng/mL)	20.45 (6.78-44.45)	18.54 (4.98-42.59)	**-1.30**[a]	14.16 (5.45-28.17)	**1.31**[c]
3α-diol-3G (ng/mL)	0.80 (0.27-1.77)	0.70 (0.13-1.78)	**-0.02**[c]	0.57 (0.25-1.18)	**1.23**[b]
3α-diol-17G (ng/mL)	0.58 (0.13-1.65)	0.50 (0.13-1.42)	**-0.14**[a]	0.25 (0.25-1.41)	2.00
Estrogens					
E_1-S (ng/mL)	0.31 (0.04-0.99)	0.20 (0.04-0.59)	**-0.10**[a]	0.17 (0.04-0.49)	1.21
E_1 (pg/mL)	31.56 (12.84-77.11)	20.56 (5.00-49.32)	**-9.05**[a]	18.36 (10.17-35.07)	1.12
E_2 (pg/mL)	6.46 (2.25-19.90)	3.96 (1.00-11.56)	**-2.35**[a]	3.35 (1.00-9.49)	1.18
Catechol estrogens					
E_3 (pg/mL)	30.4 (5.0-114.0)	31.1 (2.5-143.6)	0.00	n/a	n/a
2OH-E_1 (pg/mL)	23.1 (2.5-77.1)	29.6 (2.5-73.1)	0.00	n/a	n/a
2MeO-E_1 (pg/mL)	7.6 (2.5-22.8)	5.7 (2.5-16.0)	**0.00**[a]	n/a	n/a
4MeO-E_2 (pg/mL)	13.6 (2.5-51.5)	21.7 (6.9-82.6)	**6.30**[a]	n/a	n/a
SHBG (nmol/L)	64.2 (30.5-123.1)	65.2 (29.6-113.9)	**-1.60**[c]	83.0 (25.9-135.1)	0.79

[1]For statistical analysis, data were log-transformed and adjusted for age and BMI. Untransformed values are shown.

[2]Variation in hormone levels between pre- and post- operative levels were established using Wilcoxon signed rank test for paired data.

n/a: not available. ADT and catechol estrogens could not be measured in healthy postmenopausal women. Significant differences are indicated in bold.

[a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$.

Figures

Figure 1

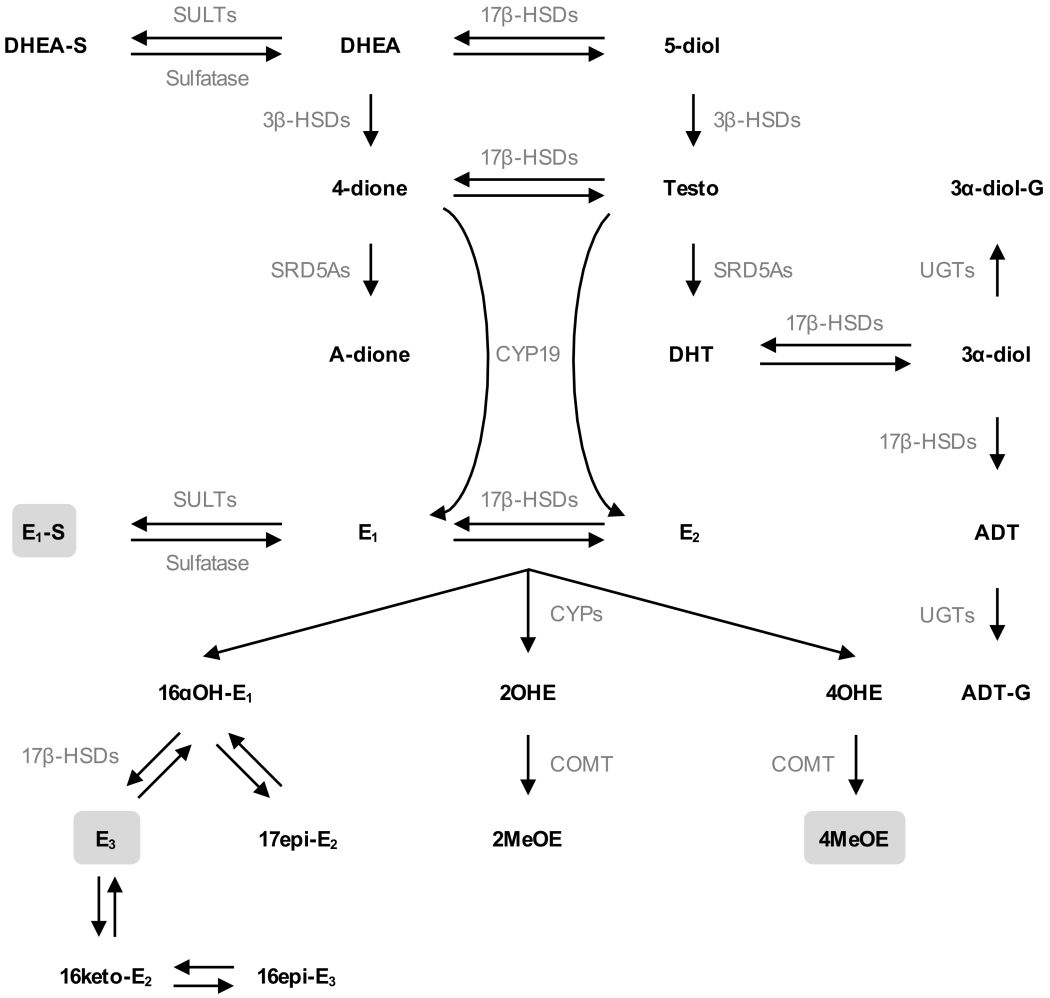


Figure 2

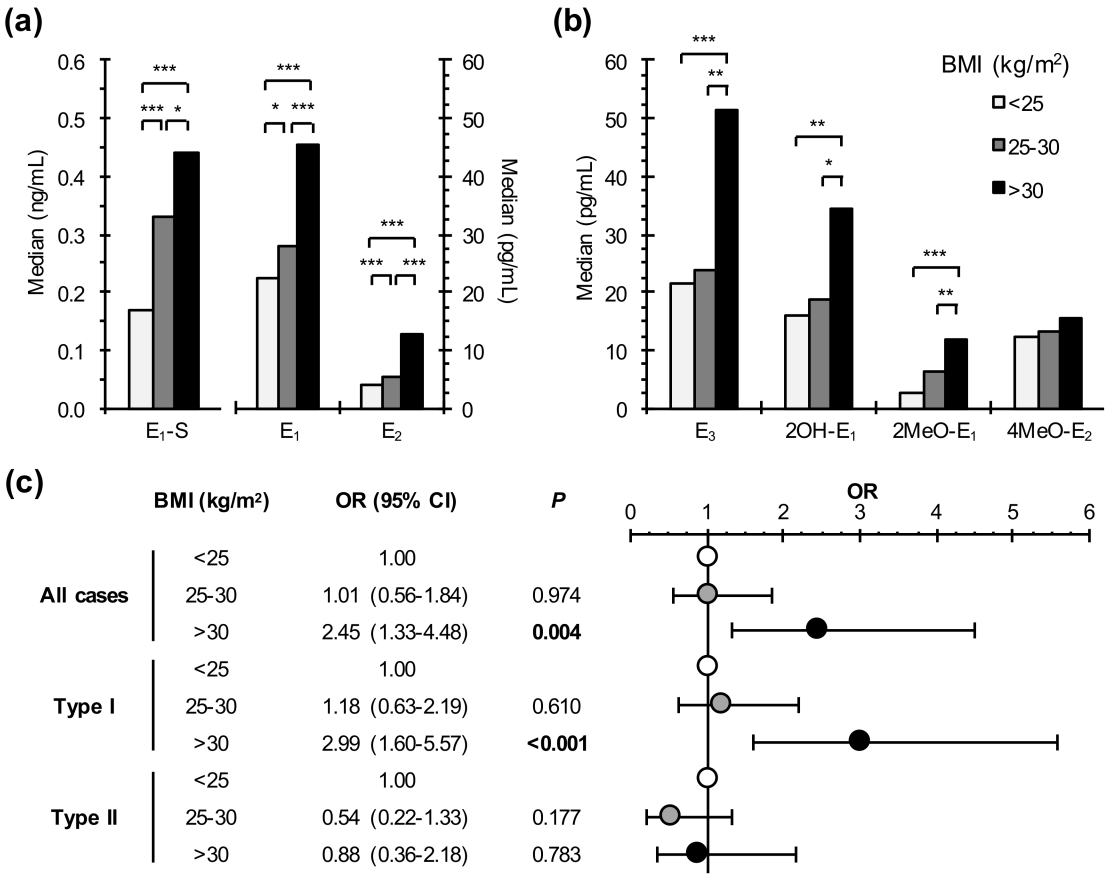

(a)
Median (ng/mL)
Median (pg/mL)
E1-S
E1
E2
(b)
Median (pg/mL)
BMI (kg/m2)
<25
25-30
>30
E3
2OH-E1
2MeO-E1
4MeO-E2
(c)
BMI (kg/m2)
OR (95% CI)
P
OR
All cases
Type I
Type II
<25 1.00
25-30 1.01 (0.56-1.84) 0.974
>30 2.45 (1.33-4.48) 0.004
<25 1.00
25-30 1.18 (0.63-2.19) 0.610
>30 2.99 (1.60-5.57) <0.001
<25 1.00
25-30 0.54 (0.22-1.33) 0.177
>30 0.88 (0.36-2.18) 0.783

Figure 3

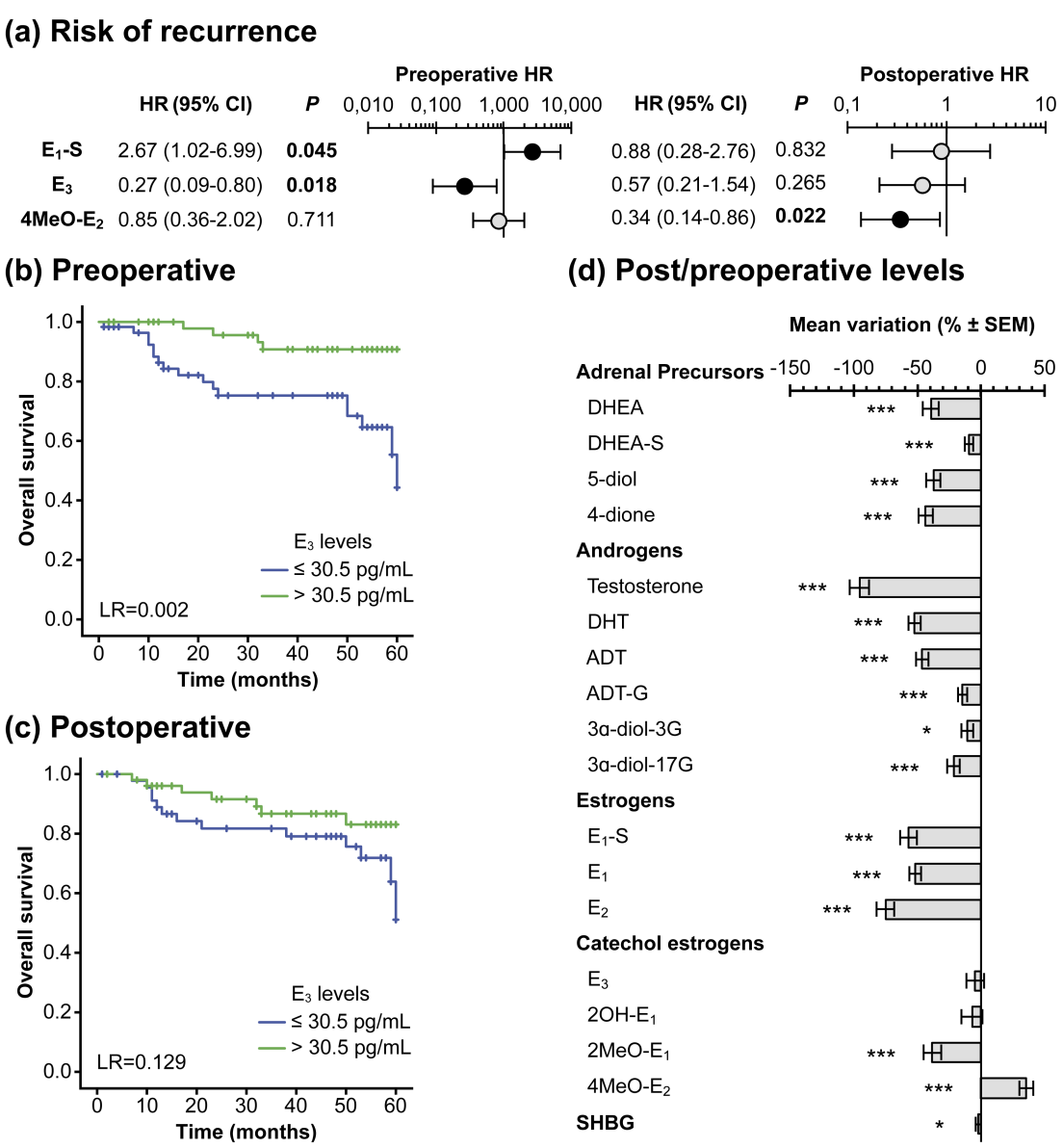
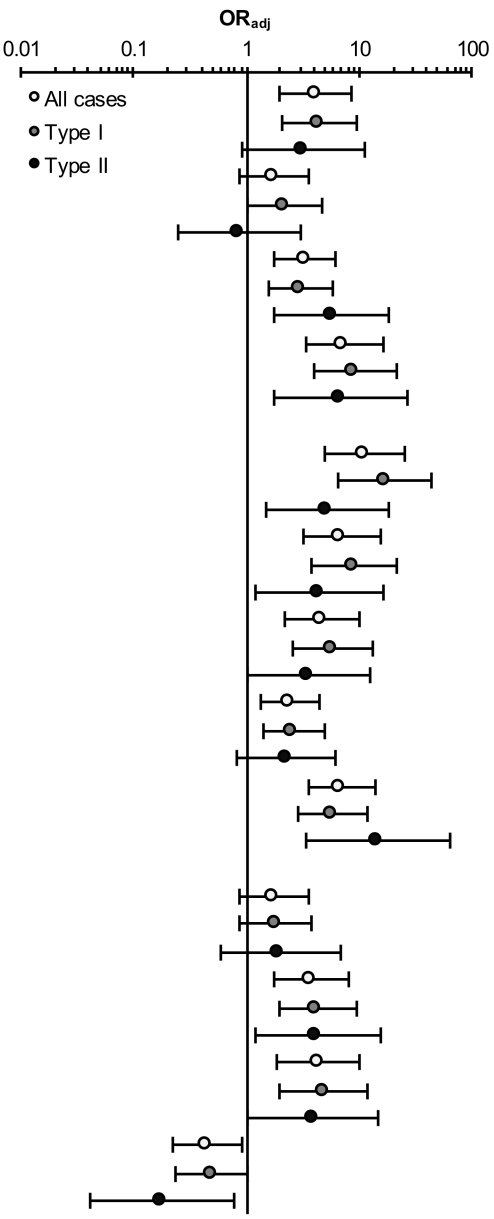

(a) Risk of recurrence
Preoperative HR
HR (95% CI)
P
0,010 0,100 1,000 10,000
E1-S 2.67 (1.02-6.99) 0.045
E3 0.27 (0.09-0.80) 0.018
4MeO-E2 0.85 (0.36-2.02) 0.711
Postoperative HR
HR (95% CI)
P
0,1 1 10
0.88 (0.28-2.76) 0.832
0.57 (0.21-1.54) 0.265
0.34 (0.14-0.86) 0.022
(b) Preoperative
Overall survival
E3 levels
≤ 30.5 pg/mL
> 30.5 pg/mL
LR=0.002
Time (months)
(c) Postoperative
Overall survival
E3 levels
≤ 30.5 pg/mL
> 30.5 pg/mL
LR=0.129
Time (months)
(d) Post/preoperative levels
Mean variation (% ± SEM)
-150 -100 -50 0 50
Adrenal Precursors
DHEA
DHEA-S
5-diol
4-dione
Androgens
Testosterone
DHT
ADT
ADT-G
3α-diol-3G
3α-diol-17G
Estrogens
E1-S
E1
E2
Catechol estrogens
E3
2OH-E1
2MeO-E1
4MeO-E2
SHBG

Figure 4

Hormones	Group	OR_{adj} (95% CI)	*P*
Adrenal Precursors			
DHEA	All Cases	4.13 (1.98-8.65)	**<0.001**
	Type I	4.43 (2.03-9.69)	**<0.001**
	Type II	3.17 (0.90-11.12)	*0.072*
DHEA-S	All Cases	1.76 (0.87-3.56)	0.116
	Type I	2.19 (1.03-4.62)	**0.040**
	Type II	0.86 (0.25-2.99)	0.808
5-Diol	All Cases	3.37 (1.81-6.28)	**<0.001**
	Type I	3.07 (1.60-5.88)	**<0.001**
	Type II	5.77 (1.78-18.63)	**0.003**
4-Dione	All Cases	7.37 (3.38-16.07)	**<0.001**
	Type I	9.08 (3.90-21.12)	**<0.001**
	Type II	6.82 (1.73-26.84)	**0.006**
Androgens			
Testosterone	All Cases	11.29 (4.93-25.85)	**<0.001**
	Type I	17.07 (6.52-44.72)	**<0.001**
	Type II	5.22 (1.50-18.12)	**0.009**
DHT	All Cases	6.91 (3.14-15.21)	**<0.001**
	Type I	9.21 (3.85-22.03)	**<0.001**
	Type II	4.47 (1.19-16.77)	**0.026**
ADT-G	All Cases	4.63 (2.16-9.93)	**<0.001**
	Type I	5.75 (2.52-13.10)	**<0.001**
	Type II	3.61 (1.02-12.82)	**0.047**
3α-Diol-3G	All Cases	2.43 (1.35-4.37)	**0.003**
	Type I	2.63 (1.42-4.88)	**0.002**
	Type II	2.28 (0.82-6.34)	0.113
3α-Diol-17G	All Cases	6.91 (3.48-13.71)	**<0.001**
	Type I	5.84 (2.90-11.75)	**<0.001**
	Type II	14.95 (3.47-64.30)	**<0.001**
Estrogens			
E_1-S	All Cases	1.76 (0.86-3.58)	0.119
	Type I	1.84 (0.87-3.88)	0.110
	Type II	2.00 (0.59-6.72)	0.264
E_1	All Cases	3.75 (1.77-7.98)	**<0.001**
	Type I	4.31 (1.95-9.55)	**0.000**
	Type II	4.30 (1.17-15.78)	**0.028**
E_2	All Cases	4.34 (1.87-10.07)	**<0.001**
	Type I	4.95 (2.02-12.17)	**<0.001**
	Type II	3.90 (1.00-15.15)	**0.049**
SHBG	All Cases	0.45 (0.22-0.94)	**0.032**
	Type I	0.50 (0.24-1.05)	*0.067*
	Type II	0.18 (0.04-0.80)	**0.024**



Supplemental Tables

Supplemental Table 1. Characteristics of endometrial cancer cases and healthy women.

	Postmenopausal women			
	Endometrial cancer (n = 246)		**Healthy** (n = 110)	
	Mean ± SD		Mean ± SD	
Age (year)	65.1 ± 8.9		58.3 ± 5.6**	
Weight (kg)	75.0 ± 19.0		68.8 ± 14.0*	
Height (cm)	158.4 ± 6.4		159.6 ± 5.2	
BMI (kg/m^2)	29.9 ± 7.3		27.0 ± 5.4**	
	n	(%)	n	(%)
Full term pregnancy				
Never	68	(28)	27	(25)
Ever	167	(68)	83	(75)
Missing	11	(5)	0	(0)
OC use				
No	145	(59)	19	(17)
Yes	91	(37)	91	(83)
Missing	10	(4)	0	(0)
HRT				
Never	157	(64)	40	(36)
Ever	80	(33)	70	(64)
Missing	9	(4)	0	(0)

OC: Oral Contraceptive, HRT: Hormone Replacement Therapy. **P< 0.001; *P=0.002

Supplemental Table 2. Median hormone levels (10th and 90th percentile) for healthy postmenopausal women and Type I and Type II EC cases.

Hormones	Healthy women (n = 110)	Type I EC cases (n = 202)	Fold change (TI vs Healthy)	Type II EC cases (n = 43)	Fold change (TII vs Healthy)
Adrenal Precursors					
DHEA-S (µg/mL)	0.60 (0.23-1.27)	0.63 (0.24-1.39)	1.04[b]	0.56 (0.28-1.17)	0.93
DHEA (ng/mL)	1.91 (0.85-4.24)	2.58 (1.02-7.13)	1.35[c]	2.28 (1.24-5.37)	1.20[b]
5-diol (pg/mL)	230.0 (100.0-495.0)	345.1 (144.6-734.9)	1.50[c]	322.6 (134.7-652.4)	1.40[c]
4-dione (ng/mL)	0.44 (0.24-0.80)	0.64 (0.34-1.28)	1.45[c]	0.55 (0.37-1.31)	1.25[c]
Androgens					
Testosterone (ng/mL)	0.14 (0.06-0.24)	0.24 (0.13-0.55)	1.78[c]	0.24 (0.11-0.38)	1.78[c]
DHT (pg/mL)	30.00 (10.00-70.00)	38.32 (17.90-82.18)	1.28[c]	33.19 (17.28-66.19)	1.11
ADT (pg/mL)[2]	n/a	132.3 (62.5-324.4)	n/a	99.4 (65.3-310.5)	n/a
ADT-G (ng/mL)	14.16 (5.45-28.17)	19.60 (7.36-47.90)	1.38[c]	20.50 (5.97-35.40)	1.45[a]
3α-diol-3G (ng/mL)	0.57 (0.25-1.18)	0.71 (0.27-1.68)	1.26[b]	0.83 (0.13-1.79)	1.46
3α-diol-17G (ng/mL)	0.25 (0.25-1.41)	0.60 (0.13-1.65)	2.38	0.45 (0.13-1.13)	1.81
Estrogens					
E_1-S (ng/mL)	0.17 (0.04-0.49)	0.34 (0.08-1.06)	2.03[c]	0.25 (0.04-0.51)	1.51
E_1 (pg/mL)	18.36 (10.17-35.07)	33.97 (15.02-72.78)	1.85[c]	25.89 (14.50-50.34)	1.41
E_2 (pg/mL)	3.35 (1.00-9.49)	7.16 (2.62-19.91)	2.14[c]	4.67 (1.00-9.88)	1.39
Catechol estrogens					
E_3 (pg/mL)	n/a	33.3 (5.7-114.0)	n/a	20.6 (2.5-129.1)	n/a
2OH-E_1 (pg/mL)	n/a	24.5 (2.5-78.3)	n/a	19.4 (2.5-80.6)	n/a
2MeO-E_1 (pg/mL)	n/a	8.1 (2.5-2.0)	n/a	5.9 (2.5-25.5)	n/a
4MeO-E_2 (pg/mL)	n/a	14.0 (2.5-51.7)	n/a	8.4 (2.5-53.2)	n/a
SHBG (nmol/L)	83.0 (25.9-135.1)	63.8 (31.1-123.1)	0.77	74.7 (30.8-155.0)	0.90

Fold change is calculated upon median of each group.

For statistical analysis, data were log-transformed and adjusted for age and BMI. Untransformed values are showed. No significant differences in hormone levels between histological types were detected and thus, fold changes are not shown. n/a: not available. [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$.

Supplemental Table 3. Median hormone levels (10th and 90th percentile) for endometrial cancer cases by BMI categories.

	Endometrial cancer cases			Fold Change		
Hormones	**Normal Weight BMI = 25 kg/m^2** (n=66)	**Overweight BMI = 25-30 kg/m^2** (n=62)	**Obese BMI > 30 kg/m^2** (n=103)	Overweight vs Normal	Obese vs Normal	Obese vs Overweight
Adrenal Precursors						
DHEA-S (µg/mL)	0.74 (0.20-1.41)	0.60 (0.26-1.40)	0.61 (0.27-1.27)	0.81	0.82	1.02
DHEA (ng/mL)	2.65 (1.05-6.77)	2.37 (1.02-5.85)	2.45 (1.12-6.81)	0.89	0.92	1.03
5-diol (pg/mL)	347.0 (118.2-677.2)	328.5 (152.0-754.7)	325.8 (139.3-641.8)	0.95	0.94	0.99
4-dione (ng/mL)	0.57 (0.35-1.37)	0.65 (0.34-1.49)	0.63 (0.39-1.27)	1.14	1.11	0.97
Androgens						
Testosterone (ng/mL)	0.22 (0.10-0.51)	0.25 (0.15-0.59)	0.23 (0.13-0.48)	1.14	1.05	0.92
DHT (pg/mL)	43.98 (18.43-92.92)	38.22 (16.04-71.97)	32.88 (17.90-65.92)	0.87	0.75	0.86
ADT (pg/mL)	121.5 (63.8-348.5)	116.7 (68.4-310.5)	128.9 (57.2-268.9)	0.96	1.06	1.10
ADT-G (ng/mL)	19.55 (7.02-53.44)	18.50 (4.70-42.10)	20.60 (7.90-44.50)	0.95	1.05	1.11
3α-diol-3G (ng/mL)	0.67 (0.13-1.57)	0.56 (0.13-1.48)	0.91 (0.32-2.01)	0.84	1.36[c]	1.63[c]
3α-diol-17G (ng/mL)	0.46 (0.13-1.13)	0.46 (0.13-1.61)	0.71 (0.32-1.65)	1.00	1.54[a]	1.54[c]
Estrogens						
E_1-S (ng/mL)	0.17 (0.04-0.51)	0.33 (0.08-0.92)	0.44 (0.17-1.31)	1.94[a]	2.59[a]	1.33[c]
E_1 (pg/mL)	22.37 (5.00-41.02)	27.80 (14.50-56.43)	45.25 (20.46-83.36)	1.24[c]	2.02[a]	1.63[a]
E_2 (pg/mL)	3.94 (1.00-7.62)	5.56 (3.29-12.22)	12.79 (4.65-22.93)	1.41[a]	3.25[a]	2.30[a]
Catechol estrogens						
E_3 (pg/mL)	21.5 (2.5-84.5)	24.0 (5.2-75.9)	52.2 (10.1-131.0)	1.12	2.43[a]	2.18[b]
2OH-E_1 (pg/mL)	16.2 (2.5-51.9)	18.7 (2.5-72.8)	34.2 (2.5-93.9)	1.15	2.11[b]	1.83[c]
2MeO-E_1 (pg/mL)	2.5 (2.5-12.1)	6.5 (2.5-21.5)	11.9 (2.5-25.5)	2.60	4.76[a]	1.83[b]
4MeO-E_2 (pg/mL)	12.1 (2.5-45.1)	13.2 (5.9-51.9)	15.6 (2.5-52.3)	1.09	1.29	1.18
SHBG (nmol/L)	92.7 (45.0-155.0)	69.5 (37.0-132.1)	46.9 (28.3-105.2)	0.75	0.51[a]	0.68[b]

Fold change is calculated upon median of each group.

For statistical analysis, data were log-transformed and adjusted for age. Untransformed values are displayed. BMI: Body mass index. [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$.

Supplemental Table 4. Risk of EC recurrence estimated in relation to steroid levels.

	Preoperative serum levels					Postoperative serum levels				
Hormones	**LR *P***	**HR_{adj}**	***P***	**HR_{Fadj}**	***P***	**LR *P***	**HR_{adj}**	***P***	**HR_{Fadj}**	***P***
Adrenal precursors										
DHEA-S	0.838	1.00 (0.44-2.28)	0.997	1.01 (0.45-2.30)	0.976	0.706	1.19 (0.46-3.05)	0.720	1.29 (0.51-3.28)	0.594
DHEA	0.527	1.54 (0.66-3.59)	0.321	1.42 (0.60-3.33)	0.425	0.502	0.72 (0.27-1.96)	0.526	0.82 (0.30-2.25)	0.700
5-diol	0.830	1.17 (0.51-2.67)	0.715	1.07 (0.46-2.54)	0.869	0.544	1.05 (0.39-2.80)	0.920	1.06 (0.38-2.94)	0.915
4-dione	0.888	1.10 (0.49-2.49)	0.821	1.22 (0.53-2.77)	0.639	0.117	0.46 (0.13-1.61)	0.223	0.53 (0.15-1.83)	0.316
Androgens										
Testosterone	0.492	0.54 (0.23-1.30)	0.169	0.41 (0.16-1.05)	*0.063*	0.383	0.82 (0.18-3.69)	0.796	0.83 (0.18-3.74)	0.805
DHT	0.637	0.87 (0.38-1.99)	0.733	0.72 (0.31-1.71)	0.458	0.143	0.68 (0.18-2.60)	0.573	0.68 (0.18-2.60)	0.572
ADT	0.537	1.22 (0.74-2.02)	0.427	1.27 (0.77-2.09)	0.355	0.297	1.03 (0.61-1.73)	0.918	1.00 (0.59-1.71)	0.992
ADT-G	0.932	1.00 (0.43-2.30)	0.996	1.08 (0.46-2.49)	0.863	0.482	0.98 (0.37-2.55)	0.959	1.04 (0.40-2.67)	0.938
3α-diol-3G	0.816	1.14 (0.51-2.56)	0.756	1.08 (0.47-2.46)	0.861	*0.055*	0.46 (0.17-1.26)	0.129	0.50 (0.18-1.38)	0.184
3α-diol-17G	0.822	1.58 (0.67-3.75)	0.295	1.59 (0.68-3.74)	0.284	0.775	1.99 (0.68-5.80)	0.206	2.09 (0.73-60)	0.169
Estrogens										
E_1-S	0.260	2.37 (0.97-5.78)	*0.058*	2.67 (1.02-6.99)	**0.045**	0.286	1.00 (0.33-3.05)	0.994	0.88 (0.28-2.76)	0.832
E_1	0.163	0.91 (0.37-2.27)	0.847	0.81 (0.32-2.09)	0.669	0.856	1.44 (0.48-4.35)	0.517	1.24 (0.40-3.88)	0.708
E_2	0.308	1.29 (0.46-3.64)	0.635	1.26 (0.45-3.52)	0.654	0.241	0.98 (0.30-3.25)	0.974	0.81 (0.24-2.76)	0.735
Catechol estrogens										
E_3	**0.001**	0.29 (0.10-0.86)	**0.026**	0.27 (0.09-0.80)	**0.018**	**0.044**	0.63 (0.23-1.69)	0.357	0.57 (0.21-1.54)	0.265
2OH-E_1	0.798	1.05 (0.45-2.44)	0.912	0.98 (0.41-2.36)	0.971	0.625	1.69 (0.66-4.28)	0.272	1.62 (0.64-4.13)	0.312
2MeO-E_1	0.873	1.33 (0.57-3.13)	0.512	1.19 (0.49-2.87)	0.704	**0.040**	0.47 (0.13-1.67)	0.243	0.43 (0.12-1.55)	0.195
4MeO-E_2	0.490	0.78 (0.33-1.84)	0.574	0.85 (0.36-2.02)	0.711	**0.008**	0.32 (0.13-0.80)	**0.015**	0.34 (0.14-0.86)	**0.022**
SHBG	*0.086*	1.56 (0.64-3.83)	0.330	---		0.276	1.50 (0.55-4.08)	0.423	---	

LR *P*: Log-rank *P* from Kaplan-Meier analysis for all available diagnostic; HR_{adj}: Hazard ratio and 95% confidence interval (95% CI), calculated with Cox regression for all available follow-up and adjusted for age, BMI, histological type and myometrial invasion. HR_{Fadj}: Cox regression was calculated as above, and was further adjusted for SHBG levels. Results that are significant in either adjusted models are shaded in grey. When hazard ratio and 95% CI were calculated with a 5-year follow-up after surgery, results were similar.

Supplemental Table 5. Overall survival (OS) of EC cases in relation to steroid levels.

	Preoperative serum levels					Postoperative serum levels				
Hormones	**LR *P***	**HR_{adj}**	***P***	**HR_{Fadj}**	***P***	**LR *P***	**HR_{adj}**	***P***	**HR_{Fadj}**	***P***
Adrenal precursors										
DHEA-S	0.793	1.64 (0.73-3.68)	0.233	1.77 (0.79-3.94)	0.165	0.773	1.07 (0.42-2.75)	0.886	1.08 (0.41-2.82)	0.876
DHEA	0.400	0.84 (0.37-1.93)	0.688	0.77 (0.34-1.74)	0.530	0.272	0.56 (0.18-1.76)	0.323	0.61 (0.19-1.98)	0.408
5-diol	0.898	0.76 (0.36-1.59)	0.463	0.72 (0.33-1.58)	0.407	0.298	0.76 (0.27-2.10)	0.595	0.69 (0.24-2.00)	0.497
4-dione	0.578	0.89 (0.41-1.95)	0.772	0.77 (0.36-1.66)	0.508	0.735	1.14 (0.36-3.62)	0.821	1.06 (0.32-3.54)	0.929
Androgens										
Testosterone	0.394	0.72 (0.35-1.52)	0.393	0.72 (0.34-1.52)	0.388	0.642	0.65 (0.17-2.46)	0.523	0.47 (0.11-1.93)	0.293
DHT	0.759	0.97 (0.44-2.14)	0.946	0.95 (0.43-2.07)	0.893	0.118	0.54 (0.15-1.92)	0.339	0.43 (0.11-1.61)	0.209
ADT	0.532	1.47 (0.69-3.11)	0.316	1.08 (0.68-1.72)	0.746	0.695	0.72 (0.39-1.30)	0.273	0.73 (0.40-1.34)	0.311
ADT-G	0.744	1.30 (0.59-2.86)	0.513	1.36 (0.62-2.99)	0.447	0.302	0.66 (0.23-1.86)	0.429	0.71 (0.25-1.99)	0.511
3α-diol-3G	0.325	1.53 (0.73-3.19)	0.259	1.56 (0.75-3.24)	0.231	0.019	0.39 (0.14-1.11)	*0.079*	0.37 (0.13-1.06)	*0.065*
3α-diol-17G	0.583	1.03 (0.43-2.45)	0.949	1.09 (0.46-2.56)	0.845	0.147	0.25 (0.08-0.84)	**0.025**	0.26 (0.07-0.89)	**0.031**
Estrogens										
E_1-S	0.516	1.04 (0.45-2.38)	0.929	1.01 (0.43-2.36)	0.980	0.601	1.33 (0.48-3.66)	0.587	1.39 (0.51-3.78)	0.514
E_1	*0.098*	1.34 (0.56-3.24)	0.512	1.27 (0.53-3.03)	0.586	0.621	1.15 (0.38-3.49)	0.803	1.02 (0.33-3.21)	0.968
E_2	0.345	1.55 (0.59-4.05)	0.371	1.49 (0.57-3.91)	0.414	0.637	1.91 (0.53-6.91)	0.321	1.60 (0.43-5.93)	0.483
Catechol estrogens										
E_3	**0.021**	1.12 (0.43-2.91)	0.821	1.15 (0.45-2.92)	0.766	0.402	0.92 (0.36-2.34)	0.865	0.74 (0.27-2.03)	0.561
2OH-E_1	0.500	1.80 (0.82-3.96)	0.144	1.82 (0.84-3.93)	0.130	0.416	0.83 (0.32-2.17)	0.711	0.98 (0.36-2.66)	0.971
2MeO-E_1	0.303	1.52 (0.67-3.45)	0.321	1.53 (0.66-3.57)	0.326	0.445	0.65 (0.23-1.87)	0.428	0.51 (0.18-1.46)	0.207
4MeO-E_2	0.334	1.15 (0.51-2.57)	0.737	1.30 (0.58-2.92)	0.530	0.028	0.76 (0.28-2.10)	0.599	0.84 (0.30-2.33)	0.738
SHBG	0.331	0.85 (0.51-1.42)	0.540	---		0.144	1.05 (0.37-2.96)	0.932	---	

All-cause mortality. LR *P*: Log-rank *P* from Kaplan-Meier analysis for all available follow-up; HR_{adj}: Hazard ratio and 95% confidence interval (95% CI), calculated with Cox regression for all available follow-up and adjusted for age, BMI, low/high risk categories, metastases, lymph-vascular space invasion (LVSI) and recurrence; Low/High risk: TI-G1/G2 are categorized as low risk, while TI-G3 and TII are high risk. HR_{Fadj}: Cox regression was calculated as above, and was further adjusted for SHBG levels. Results that are significant in either adjusted models are shaded in grey. When hazard ratio and 95% CI were calculated with a 5-year follow-up after surgery, results were similar.