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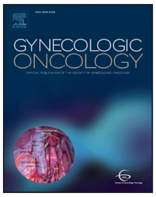

Blood steroids are associated with prognosis and fat distribution in endometrial cancer

Ingvild L. Tangen [a,b], Kristine E. Fasmer [c,d], Gonda F. Konings [e], Arthur Jochems [e,f], Bert Delvoux [e], Sofia Xanthoulea [e], Tomasz Stokowy [g], Elin Strand [a,b], Hege F. Berg [a,b], Seppo Auriola [h], Jone Trovik [a,b], Merja R. Häkkinen [h], Ingfrid S. Haldorsen [c,d], ENITEC [i], Camilla Krakstad [a,b,⁎,1], Andrea Romano [e,1]

[a] *Department of Gynaecology and Obstetrics, Haukeland University Hospital, Bergen, Norway*
[b] *Centre for Cancer Biomarkers CCBIO, Department of Clinical Science, University of Bergen, Bergen, Norway*
[c] *Mohn Medical Imaging and Visualization Centre, Department of Radiology, Haukeland University Hospital, Bergen, Norway*
[d] *Section for Radiology, Department of Clinical Medicine, University of Bergen, Bergen, Norway*
[e] *Department of Gynaecology and Obstetrics, GROW – School for Oncology & Developmental Biology, Maastricht University, Maastricht, the Netherlands*
[f] *The D-Lab: Decision Support for Precision Medicine, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Centre, Universiteitssingel 40, 6229, ER, Maastricht, The Netherlands*
[g] *Department of Clinical Science, University of Bergen, Bergen, Norway*
[h] *School of Pharmacy, University of Eastern Finland, Kuopio, Finland*
[i] *ENITEC: European Network for Individualised Treatment of Endometrial Cancer (within the European Society of Gynaecological Oncology*

HIGHLIGHTS

- Plasma levels for DHEA, DHEAS, P4, 21OH-P4 and E1S significantly increased in patients with long compared to short survival.
- Increased E2 plasma levels are associated with a high visceral fat percentage.
- Increased expression of genes involved in estrogen related signaling in tumors from patients with high E2 plasma levels

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ABSTRACT

Background. Despite being a hormone dependent cancer, there is limited knowledge regarding the relation between level of steroids in blood and prognosis for endometrial cancer (EC) patients.

Methods. In this study we investigated plasma levels of 19 steroids using liquid-chromatography tandem mass-spectrometry in 38 postmenopausal EC patients, 19 with long, and 19 with short survival. We explored if estradiol levels were associated with specific abdominal fat distribution patterns and if transcriptional alterations related to estradiol levels could be observed in tumor samples.

Results. The plasma steroid levels for DHEA, DHEAS, progesterone, 21 OH progesterone and E1S were significantly increased (all $p < 0.05$) in patients with long survival compared to short. Estradiol levels were significantly positively correlated with visceral fat percentage ($p = 0.035$), and an increased expression of genes involved in estrogen related signaling was observed in tumors from patients with high estradiol levels in plasma.

Conclusion. Several of the identified plasma steroids represent promising biomarkers in EC patients. The association between increased estradiol levels and a high percentage of visceral fat indicates that visceral fat is a larger contributor to estradiol production compared to subcutaneous fat in this population.

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1. Introduction

The endometrium is sensitive to hormonal stimulation, and endometrial cancer (EC) is influenced by steroid hormones [1]. Many of the established risk factors for developing EC are linked to hormonal stimulation including exogenous steroids, menstrual and reproductive factors and obesity [2,3]. Consistent with epidemiological data supporting that reproductive and hormonal factors are involved in the etiology of EC,

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⁎ Corresponding author at: Centre for Cancer Biomarkers, Department of Clinical Science, University of Bergen, Norway, Department of Gynaecology and Obstetrics, Haukeland University Hospital, Jonas Lies vei 72, 5020 Bergen, Norway.
URL's: camilla.krakstad@uib.no (C. Krakstad).

[1] Shared authorship.

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studies have shown that increased blood levels of steroids including estrogens, androgens and adrenal precursors are associated with increased risk of EC [4–6]. For instance, obesity is an important EC risk factor as the adipose tissue represents a major source of circulating estrogens in postmenopausal women who comprise the majority of EC patients. It is estimated that almost 40% of EC cases can be linked to overweight and obesity [7,8]. Factors like the obesity epidemic and the higher life expectancy contribute explaining the observed increasing incidence of EC [9]. Adding to the effects of increased estrogen levels in the blood, active steroid hormones and estrogens are generated locally from blood precursors and in turn influence the final exposure of EC tissues to hormone stimulation and cancer risk [10–12].

Although EC is a common cancer type with increasing incidence, improvements in treatment options have been slow. Clinical applicable and robust biomarkers for better prognostication and risk stratification are needed to better guide post-surgical adjuvant treatment and patient care. Several studies have investigated the potential prognostic value of steroid receptors and key factors in the local steroid metabolism in EC [13–18], and estrogen and progesterone receptors in primary tumor are at present the best validated prognostic biomarkers, although not widely used in the routine clinical setting. However, the potential of circulating hormone levels as biomarkers in EC is less thoroughly studied, despite the fact that recent investigations indicated that several steroids are altered in EC patients and may represent novel biomarkers predictive of clinical characteristics including the risk of relapse and overall survival [19].

In this study we investigated if the plasma concentration of a broad range of steroids including estrogens, progestogens, androgens, glucocorticoids and adrenal precursors was associated with outcome in EC. Since adipose tissue is an important source of steroids, circulating steroid levels were compared to fat distribution in patients. Finally, transcription profiling was performed to explore whether differences in plasma steroid concentrations were reflected in transcriptional alterations in the tumor, and to chart the local steroid metabolism in EC tumors.

2. Materials and methods

2.1. Patient series

A population based patient series was prospectively collected from 2001 to 2015 including patients diagnosed with EC in Hordaland County (Norway). Clinical data were collected as previously described [20] and patients were surgically staged according to the International Federation of Gynecology and Obstetrics (FIGO) 2009 criteria. From this patient series a subgroup ($n = 19$, blood samples and mRNA) comprising postmenopausal EC patients with stage 1 (grade 1–3) and stage 2 (grade 2) disease were selected based on short survival time. All these patients recurred within 27 months (average 10 months, standard deviation (SD) 6.8) after primary surgery, and died within 35 months of primary surgery (average 18 months, SD 9.9). These patients were matched with comparable patients ($n = 19$ for blood samples and $n = 14$ for mRNA; having the same type, stage and grade, and similar parity, age and BMI) who had no signs of recurrence after a mean (SD) follow-up of 66 (11) months. All patients were postmenopausal.

The study has been approved according to Norwegian legislation by the Western Regional Committee for medical and health Research Ethics (REK 2009/2315). All included patients had given written informed consent.

2.2. Steroid metabolite analysis by LC-MS/MS

EDTA-blood was obtained from 38 patients with EC before primary surgery. The blood samples were centrifuged at 1600*g* for 15 min and the plasma was stored at −80 °C. The following steroids were measured using liquid-chromatography tandem mass-spectrometry (LC-MS/MS), employing three different protocols. Androsterone (AN), dihydrotestosterone (DHT), dehydroepiandrosterone-sulphate (DHEAS), cortisol and cortisone were measured with commercially available and ISA certified AbsoluteIDQ® Stero17 kit assay (Biocrates Life Sciences AG, Innsbruck, AU) according to the manufacturer's instruction [21]. Samples were prepared through a solid phase extraction in a 96 well plate-format for precleaning and pre-concentration of the target steroid hormones. LC-MS/MS was then performed in multiple reaction monitoring mode (MRM) using a SCIEX API 4000 QTrap, and steroids were quantified by 7-point external calibration curves and 13 isotope-labeled internal standards. These analyses were performed at the Biocrates laboratory.

Estrone (E1), 17β-estradiol (E2), progesterone (P4), 17OH-progesterone (17OH-P4), 21OH-progesterone (21OH-P4), pregnenolone (P5), 17OH-pregnenolone (17OH-P5), androstenedione (A4), dehydroepiandrosterone (DHEA), aldosterone (ALDO), 11-deoxycortisol and corticosterone (CORT) were measured with Agilent 1290 UHPLC - Agilent 6495 QQQ as described earlier [22]. In short, the samples were spiked with isotope-labeled steroids as internal standards, and extracted with toluene prior to LC-MS/MS analysis. Underivatized steroids were separated using a Kinetex biphenyl (100 × 2.1 mm, 1.7u), (Phenomenex) with methanol gradient and 0.2 mM NH_4F as eluent additive.

Estrone-sulphate (E1S) was determined using 300 μl of plasma samples that were pre-processed by adding 650 μl of acetonitrile and 20 ng of the internal standard (d4-E1S). E1S was measured by Thermo Scientific TSQ Vantage system (Thermo Scientific, Breda, The Netherlands) equipped with a HESI-2 ion source in the negative ion mode (ion spray voltage was 3500 V, vaporizer temperature 380 °C, capillary temperature 320 °C and S-lens RF 50) in a targeted SIM (selective ion monitoring) mode. E1S at *m/z* 349.1 and d4-E1S at m/z 353.1. The reversed phase column Acclaim 120 (C18, 3 μm, 2.1 × 150 mm; Thermo Scientific, Breda, The Netherlands) was used at a flow rate of 200 μl/min and a linear gradient from 20% B to 80% B in 10 min. The mobile phase was water with 0.05% ammoniumhydroxide (solvent A) and acetonitrile with 0.05% ammoniumhydroxide (solvent B). At the retention time of six minutes the analytes of interest eluted. 5 μl of standards or samples were injected. The isotope ratio was calculated from the area A349 (E1S) and A353 (d4-E1S). The correction factor f1 was determined for the pure E1S (A353/A349) and f2 for d4-E1S (A349/A353). The calculation of the mass ratios resulted in the following formula: $(A349 - f1 * A353) / (A353 - f2 * A349)$.

2.3. Image analysis on CT scans

Diagnostic abdominal contrast-enhanced Computer Tomography (CT) scans ($n = 20$) were evaluated for assessment of abdominal fat volume. Using the software iNtuition (TeraRecon Inc.; San Mateo, CA, USA), cross-sectional images were analyzed consecutively from the upper right diaphragm to L5/S1-level, using a semi-automated method for volumetric quantification of abdominal fat [23]. This method is based on segmentation of pixels with values for Hounsfield units (HU) corresponding to adipose tissue (−195 to −45 HU) [23]. The correct segmentation between visceral and subcutaneous fat compartments was adjusted by the operator if necessary. Both the visceral abdominal fat volume (VAV; cm^3) and the subcutaneous abdominal fat volumes (SAV; cm^3) were estimated, and the sum of these was the total abdominal fat volume (TAV; cm^3). The percentage of visceral fat was calculated ([VAV / TAV] × 100; VAV%).

2.4. Gene expression analysis

For both microarray and RNA sequencing RNA was extracted from fresh frozen tissue from primary EC tumors using the RNeasy Mini Kit (Qiagen). Gene expression was analyzed using microarrays for 256 samples as previously described. The samples were hybridized to Agilent Whole Human Genome Microarrays 44 k (Cat. No G4112F), scanned and normalized as previously described [24,25]. cDNA libraries were prepared and sequencing performed by Illumina HighSeq 4000 (paired end, 75 bp). Raw RNA-Seq reads were aligned to human genome hg19 using hisat 2.0.5 with Gencode v26 transcriptome reference. Aligned files were processed using Samtools. Furthermore, reads aligned in the coding regions of the genome were counted using Feature Counts. Finally, read counts were normalized using DESeq2, then normalized expression values were subjected to differential analysis (mean based fold change) and statistical testing using the Students *t*-test in the R/Bioconductor programming environment. Gene set enrichment analysis (GSEA) (http://www.broadinstitute.org/gsea) [26] was performed using the Molecular Signatures Database (MSigDB, version 5.1) dataset C2 (Curated gene sets) (www.broadinstitute.org/gsea/msigdb).

2.5. Statistical analysis

Statistical analysis were performed using the software package SPSS 24.0 (SPSS Inc., Chicago, IL), and R-studio. Probability of <0.05 was defined as statistical significant. For categorical variables, the Pearsonχ^2 or Fisher's exact test was used to evaluate associations between groups, and for continuous variables the Mann-Whitney *U* test was used. Correlations were assessed by Spearman's rank correlation ($\rho =$ rho). Univariate analyses were done using the Kaplan-Meier (product-limit) method. Entry date was the date of primary surgery, and time to death due to EC was the endpoint (disease specific survival). Survival between groups was compared using the log-rank test (mantel-cox). All analyses were done blinded for patient characteristics.

3. Results

3.1. Plasma concentrations of progesterone (P4) and progesterone metabolites are increased in patients with long survival compared with patients with short survival

The plasma concentration of nineteen steroids including estrogens (E2, E1, E1S), progestogens (P4, 17OH-P4, 21OH-P4, P5, 17OH-P5), androgens (T, DHT, A4, AN), glucocorticoids (cortisol, cortisone, ALDO, 11-deoxycortisol, CORT) and adrenal precursors (DHEA, DHEAS) was analyzed in 38 postmenopausal patients with EC. Of these patients, 19 had short survival, and died due to the cancer within three years after they were diagnosed, while 19 were long time survivors. The two groups were matched for FIGO stage, histologic type and grade, age, BMI and parity (Table 1). Although patients were not matched for myometrial infiltration or hormone receptor status, there was no statistical difference in these parameters between the two groups, however a tendency towards more patients with deep myometrial infiltration (>50%) and loss of ER or AR was observed for the group with short survival. Differences in circulating steroid levels between groups, analyzed by Mann-Whitney *U* test, revealed that five compounds had a significantly different concentration in the patient group with short survival compared with the patient group with long survival (Table 2). The concentrations of P4, of the adrenal precursors DHEAS and DHEA as well as the corticosteroid precursor 21OH-P4 were significantly higher in the patient group with long survival compared with the group with short survival (Fig. 1). With regard to circulating estrogens, no significant difference in the concentration of unconjugated compound was seen, but E1S was increased in the group with long survival compared with the group with short survival (Fig. 1C and D). When analyses were restricted to patients with endometrioid only or non-endometrioid only EC, high levels of circulating AN, DHEA, A4 and E1 were associated with long survival, however the number of subjects included in these sub-analyses were very small (Supplementary Table S1). Steroid levels in blood were associated with other patient characteristics; DHEA and DHEAS levels inversely correlated with age (Supplementary Fig. S1), and 17OH-P5, A4, DHEA and DHEAS were negatively correlated with BMI (data not shown).

3.2. Increased E2 plasma levels are associated with a high percentage of visceral fat

The majority of circulating estrogens in postmenopausal women is produced from androgens via the enzyme CYP19A1 in the adipose tissue, which also influences the levels of other steroids like androgens and glucocorticoids. Since visceral/abdominal fat depots have different metabolic activities, and BMI alone disregards such information, we explored the relationship between steroid levels in plasma and fat distribution. Of the 38 patients with data on steroid plasma concentration, 20 patients had available preoperative CT scans. For these patients CT-assessed obesity variables including subcutaneous abdominal fat volume (SAV), visceral abdominal fat volume (VAV), total abdominal fat volume (TAV) and VAV% ([VAV/TAV]*100) were measured. There was a significant (moderate) positive correlation between visceral fat percentage and E2-levels, both within the whole patient population ($\rho = 0.47$, $p = 0.035$, Table 3), and within the non-endometrioid subgroup ($\rho = 0.59$, $p = 0.043$, Table 3), also the visceral fat volume tended to the same within the whole patient population ($\rho = 0.42$, $p = 0.068$, Table 3). Neither BMI, TAV, SAV nor other steroids (including E1 and androgens) were found to be significantly correlated to the E2-levels.

3.3. High E2 concentration in plasma is associated with increased expression of genes involved in estrogen related signaling in tumor

We performed whole exome RNA sequencing on tumor specimens from 38 patients of whom 33 overlapped with patients with available steroid profile. A number of genes had significantly different expression between patients with long and short survival (Supplementary Table S2). Expression of several of the top ranked genes was also validated to have prognostic impact in a larger cohort including 256 EC patients [25], and could potentially be useful prognostic biomarkers in EC (Supplementary Table S2). Also genes involved in local steroid metabolism were found to be associated with patient prognosis in the whole patient population, and depending on histologic subtype, these were however not among the top ranked genes (Supplementary Table S3).

We then explored whether circulating steroid levels were associated with specific RNA expression patterns indicating hormone signaling activation. To this end, genes with differential expression between patients with the highest plasma level (upper quartile) of a specific steroid and patients with lower levels (three lower quartiles) were identified. When investigating genes overexpressed in the patient group with high E2 levels in a Gene Set Enrichment Analysis (GSEA), gene sets associated with estrogen related signaling were found to be significantly enriched in patients with high estradiol levels compared to low (Table 4, Supplementary Table S4). The levels of other steroids were not associated with specific gene signatures.

3.4. Local metabolism of steroid hormones

Steroids present in the blood, most of which have little activity, can be locally converted into active steroids through a complex enzymatic network, as illustrated in Fig. 2A. Cluster analysis of circulating steroids revealed that in patients with long survival, precursors or intermediates in the steroid metabolic pathway (like the progestogens P4, P5 and hydroxylated forms and adrenal steroids DHEA and DHEAS) had higher levels than in patients with short survival (Fig. 2B). To examine to what extent EC tissues possess the enzymatic machinery to metabolize these blood steroids, we extracted the expression levels of all genes involved in the local steroid metabolism from the RNA seq data (Supplementary Fig. S2). STAR and CYP11A1 were detectable, although at very low level, suggesting that cholesterol may be converted to P5. Enzymes involved in the subsequent conversion of P5 to androgens

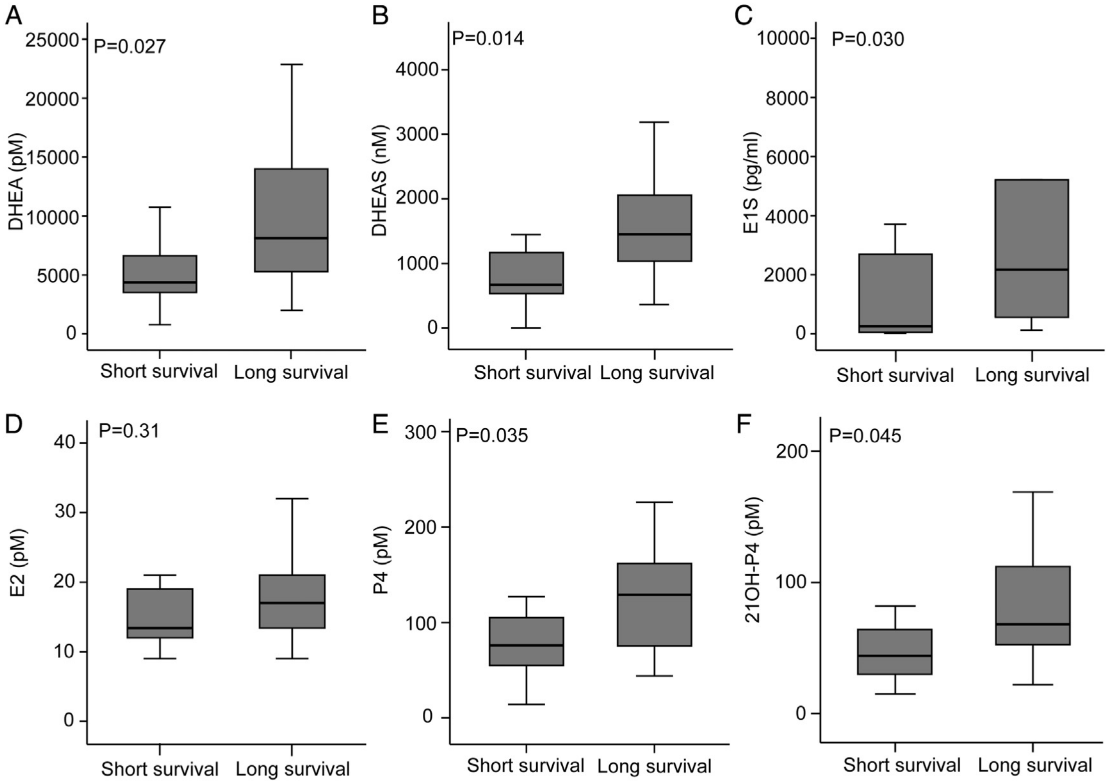

Fig. 1. Increased level of steroids in EC patients with long survival. The levels of DHEA (A), DHEAS (B), E1S (C), progesterone (E) and 21 OH progesterone (F) were increased in patients with long survival compared to short (all *p*-values < 0.05). For estradiol no significant difference in plasma level was observed between patients with long and short survival (D).

(i.e., CYP17A1, HSD3B2) were also detectable, at low levels, indicating that the substrates for these enzymes (P5, 17OH-P5 and DHEA), all highly abundant in the blood (Fig. 2A, left panel), can be converted into A4 in the tumor tissue. Further, the enzymatic machinery to metabolize A4 into other androgens (T, DHT and AN) as well as estrogens is present at relatively high levels (HSD17Bs, AKR1Cs and SRD5As). P4 and 17OH-P4 could serve as substrates for local corticosteroid generation, but the enzymes responsible for this pathway (i.e., CYP21A1 and CYP11Bs) are those with the lowest expression levels (undetectable for CYP11Bs); hence, local generation of corticosteroids in EC from progestogens seems negligible. Nevertheless, cortisone/cortisol balance (both present at high circulating levels, in the nano molar range) can be regulated by the highly expressed HSD11Bs. No expression was detected for CY11B1 and 2, HSD17B8 and HSD3B1. Of interest, SRD5As and AKR1Cs, which are widely involved in the metabolism of both androgens and progestogens with AKR1Cs also having ample substrate specificity and catalytic activity, were highly expressed in EC. AKR1Cs show border line correlation with patient survival ($p = 0.05$; Supplementary Fig. S3).

4. Discussion

Although patients diagnosed with low grade and early stage EC have a favorable prognosis, in some of these patients the disease recur and prognosis is then poor. In this study we used a large prospectively collected cohort to select patients who, although their prognosis was favorable at the time of primary cancer diagnosis, died of their disease within a short period, and matched these women with patients with similar characteristics, but long survival. We aimed at identifying differences between these two patient groups in blood (circulating) steroid profile, tumor gene expression and local steroid metabolism. A cluster analysis revealed that patients with long survival generally had higher steroid levels in plasma compared to patients with short survival, and for DHEA, DHEAS, progesterone (P4), 21OH-P4 and estrone-sulphate (E1S) the difference was statistically significant. Both endometrioid and non-endometrioid ECs were included in this analysis and although endometrioid EC is considered more closely linked to hormonal stimulation than non-endometrioid, our data suggests that non-endometrioid EC is not completely hormone independent, as the increased steroid levels in relation with survival was independent of histologic subtype. This is in line with the contention that different histological subtypes may to a certain extent have similar pathogenesis [27,28]. Some differences between histologic subtypes were observed, and although the sample size is small we observed that also within the non-endometrioid EC an increased level of several steroids was associated with long survival.

Obesity is a risk factor for developing several cancer types, including EC and the specific fat distribution pattern has also been found to be associated with cancer development. Individuals with a high percentage of visceral fat have increased risk of developing breast-, colorectal- and esophageal cancer compared with individuals with less visceral fat [29]. We have previously found that a high percentage of visceral fat is an independent predictor of reduced disease specific survival in patients with EC [24]. After menopause, circulating estrogens are primarily produced in the adipose tissue [30], and it has been shown that an increased BMI is associated with increased circulating E2 concentration in healthy postmenopausal women [31]. The relation between fat distribution and E2 levels is however less studied. In a study by Mongraw-Chaffin et al. a significant positive association between visceral fat and E2 levels and between E2 levels and amount of subcutaneous fat was observed in postmenopausal women [32]. In this study we explored a potential correlation between fat distribution pattern and E2 levels in postmenopausal EC patients. Patients with a high VAV% had significantly increased plasma E2 levels compared to patients with a low VAV%. This could indicate that in postmenopausal women with EC the visceral fat is a larger contributor to E2 production compared to the subcutaneous fat.

Table 1
Clinical-pathological characteristics for included patients with long and short survival.

Variable	Long *n* (%) *n* = 19	Short *n* (%) *n* = 19	*P*-value[a]
Age			0.5
<66	8 (62)	5 (38)	
≥66	11 (44)	14 (56)	
Parity			1.0
0	1 (50)	1 (50)	
≥1	18 (50)	18 (50)	
FIGO-09 stage[c]			0.05
IA	13 (68)	6 (32)	
IB	4 (27)	11 (73)	
II	2 (50)	2 (50)	
Histologic type			1.0
Endometrioid	7 (50)	7 (50)	
Non-endometrioid	12 (50)	12 (50)	
Histologic grade			1.0
Grade 1/2	5 (50)	5 (50)	
Grade 3	14 (50)	14 (50)	
Lymph node status			–
Negative	15 (54)	13 (46)	
Positive	0	0	
Myometrial infiltration			0.1
<50%	13 (65)	7 (35)	
≥50%	6 (33)	12 (67)	
Ploidy			0.7
Diploid	8 (62)	5 (39)	
Aneuploid	6 (46)	7 (54)	
PR			0.5
Positive	8 (57)	6 (43)	
Negative	9 (43)	12 (57)	
ERα			0.2
Positive	9 (64)	5 (36)	
Negative	8 (38)	13 (62)	
AR			0.3
Positive	9 (60)	6 (40)	
Negative	6 (37)	10 (63)	
BMI			0.6[b]
Mean (SD)	24.9 (3.8)	25.5 (2.7)	

[a] Chi-square test.
[b] *t*-Test.
[c] Only FIGO-stage I and II included.

Table 2
Median steroid level (25th percentile and 75 percentile) for endometrial cancer patients with long (*n* = 19) and short (*n* = 19) survival.

Metabolite	Long survival group Median (25th and 75th percentile)	Short survival group Median (25th and 75th percentile)	*p*-Value[*]
E2 (pM)	17 (13.4, 21)	13.4 (11.8, 19.5)	0.31
E1 (pM)	73 (56, 108)	64 (44.5–91)	0.32
E1S (pg/ml)	2174 (489.5, 5213)	249 (48–3704)	0.030
P4 (pM)	129 (65, 167)	76 (53, 107)	0.035
17OH-P4 (pM)	542 (347, 779)	366 (215.5, 544.5)	0.093
21OH-P4 (pM)	68 (52, 112)	44 (29.5, 71)	0.045
P5 (pM)	1094 (533.5, 2048.3)	818 (439.5, 1453)	0.34
17OH-P5 (pM)	1170 (696, 2231)	681 (446, 1068.5)	0.057
T (pM)	759 (307, 1054)	570 (369, 664.5)	0.21
AN (nM)	0.60 (0.53, 0.73)	0.57 (0.44, 0.69)	0.23
DHT (nM)	0.15 (0.012, 0.44)	0.19 (0.00, 0.26)	0.71
A4 (nM)	1.3 (1.1, 2.3)	1.0 (0.8, 1.7)	0.071
DHEA (nM)	8.1 (4.9, 14.5)	4.4 (3.3, 6.7)	0.027
DHEAS (nM)	1452 (991, 2311)	669 (510, 1264)	0.014
Cortisol (nM)	336 (253, 465)	338 (261, 392)	0.94
Cortisone (nM)	47 (44, 55)	48 (43, 53)	0.85
ALDO (pM)	121 (77, 201)	144 (130, 196)	0.38
11-deoxycortisol (pM)	597 (290, 970)	323 (279, 523)	0.12
CORT (nM)	6.4 (3.7, 12.3)	5.4 (3.0, 9.5)	0.36

[*] Mann-Whitney *U* test.

Table 3
Correlations (Spearman rho) between BMI, CT-assessed obesity variables and plasma E2 values.

	All patients		Endometrioid		Non-endometrioid	
	Spearman rho, ρ	*P*-value[*]	Spearman rho, ρ	*P*-value[*]	Spearman rho, ρ	*P*-value[*]
BMI	0.29	0.1	0.57	0.069	0.11	0.61
VAV	0.42	0.068	0.36	0.39	0.35	0.27
SAV	0.005	0.98	0.21	0.61	−0.071	0.83
TAV	0.24	0.31	0.33	0.42	0.15	0.64
VAV %	0.47	0.035	0.33	0.42	0.59	0.043

VAV: Visceral abdominal fat volume, SAV: Subcutaneous abdominal fat volume, TAV: Total abdominal fat volume, VAV%: Visceral fat percentage.
[*] Spearman's rho.

In line with the known protective and anti-estrogenic effect of P4 in EC, this hormone was among the compounds with increased levels in the long survival group. P4 is known to have a tumor-suppressor effect in EC through inhibition of proliferation and induction of differentiation, and progesterone therapy is also used in treatment of patients with EC [1,33]. Other compounds with increased circulating levels in patients with favorable prognosis included precursors or intermediates in the steroid biosynthetic pathway. Together with the observation that most enzymes involved in the local steroid metabolism are expressed in EC (especially the HSD17Bs, ACR1Cs and SRD5As), this suggests that these compounds are further metabolized locally to produce various androgenic and estrogenic compounds with various activities. Since this local metabolism of steroids affects the tissue steroid levels without affecting the blood concentration, it is not surprising that the circulating levels of active estrogens and androgens did not differ between groups. Previous studies showed that unbalanced intracrine metabolism and enzyme levels are associated with EC [18,34–37].

The progestogen 21OH-P4 (also known as deoxycorticosterone), elevated in patients with long survival, is converted into CORT [15] and ALDO in the adrenal gland, but the absence of the enzymes involved in these conversions (CYP11B1 and CYP11B2) indicates that this route in corticosteroid metabolism is absent in EC. Similarly, intracrine formation of 11DOC and cortisol from 17OH-P4 seems negligible in EC due to the absence of the responsible enzymes (CYP21A1 and CYP11B1). However, HSD11B1 and HSD11B2, controlling the balance between active cortisol and inactive cortisone, are present in EC as shown earlier [38]. The role of these steroids in EC is poorly studied in previous investigations.

In this study we investigated whether a difference in gene expression could be observed between patients with long and short survival in a cohort with otherwise similar characteristics. Several genes were differentially expressed between the two groups, and for many, such differences were also validated in a larger cohort. Some of these genes have earlier been linked to cancer and should be further explored as potential biomarkers in endometrial cancer [39–41]. As E2 drives proliferation in endometrial cancer we also explored whether high estradiol levels were linked to a specific gene expression profile in tumor. We observed an enrichment of gene sets associated with estrogen related signaling in tumors from patients with high plasma levels of E2 compared to patients with low levels, indicating that high E2 concentration in blood contribute to increased transcription of ER regulated genes in the tumor.

This study was designed to investigate whether differences in blood (circulating) steroids, tumor gene expression and local steroid metabolism could be observed between two similar patients groups with different survival times. We identified 19 patients in our large cohort that fulfilled the inclusion criteria for the short survival group, and these were matched with 19 patients with long survival. This highly selected, small population is not representative for the whole population of EC patients. Also, the patients in this study were matched for specific criteria (FIGO stage, histologic type and grade, age, BMI and parity) but not for depth of myometrial infiltration or ERα and AR status. Although these parameters did not differ significantly between groups,

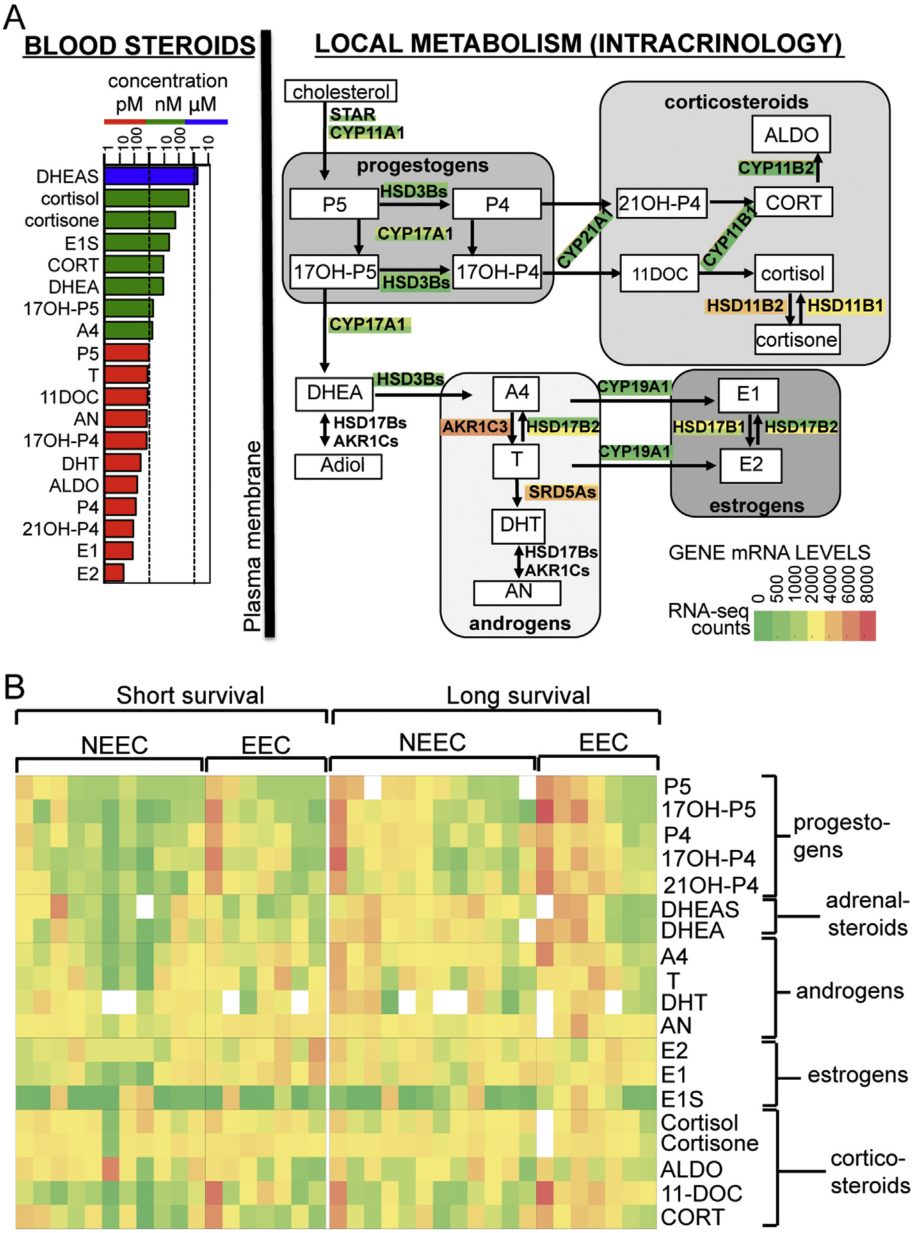

Fig. 2. Systemic steroids and local metabolism in EC. A. The levels of all measured steroids in the blood are shown in the left panel (blood steroids). Pico molar rages are indicated in red, nano molar ranges in green and micro molar ranges are indicated in blue. Mean concentration values of all 38 subjects are shown. At the right, the potential steroid metabolism and conversion are indicated. The mean expression level of each gene is indicated by the color (based on the heat map and levels in Supplementary Fig. S2). The sulfatase pathway consisting of STS and SULTs is not shown. *Adiol = androstenediol and androstanediols were not measured in this study. B. Heat map of the levels of the various analyzed steroids in patients with long compared to short survival.

patients with short survival tended to have deeper myometrial infiltration, and loss of ERα or AR. Both the small population, and the patient selection supports that our findings should be validated in a large population based series to further investigate the role of blood steroids in endometrial cancer patients.

Table 4
GSEA (curated gene sets) of genes upregulated in patients with high level of estradiol.

Rank[a]	Gene set name (selected gene sets)	Genes in overlap	*P*-value	FDR q-value
5	GOZGIT_ESR1_TARGETS_DN	19	2.48e-9	2.35e-6
9	RIGGINS_TAMOXIFEN_RESISTANCE_DN	10	6.03e-8	3.17e-5
17	YANG_BREAST_CANCER_ESR1_UP	5	5.4e-7	1.44e-4
20	DOANE_BREAST_CANCER_ESR1_UP	7	7.24e-7	1.72e-4

[a] Complete list is given in Supplementary Table 3.

Despite being a hormone dependent cancer, there is limited knowledge regarding the relation between level of steroids in blood and prognosis for endometrial cancer (EC) patients. In this study we have identified steroids that might be promising biomarkers in blood samples from patients with EC, and we also observe an interesting association between E2 levels in blood and fat distribution. The sample size in this study is however limited and our findings should be validated in a larger population based patient cohort.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2018.10.024.

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Conflict of interest statement

There are no conflicts of interest to disclose.

Author contribution

ILT, CK, AR designed and planned study.

ILT, HFB, JT gathered clinical samples and collected patient data.

SA, MH performed steroid analysis.

KEF, ISH performed fat distribution analysis.

TS performed RNA sequencing.

ILT, KEF, AJ, TS, ES, CK, AR performed data analyses.

ILT, CK, AR interpreted data, prepared figures and tables and wrote the manuscript.

ILT, KEF, GFK, AJ, BD, SX, SA, MH, TS, ES, HFB, JT, ISH, CK, AR reviewed and approved the manuscript.

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