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Original contribution

Quantification of Leydig cells and stromal hyperplasia in the postmenopausal ovary of women with endometrial carcinoma☆

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Summary Endometrioid endometrial carcinomas (EECs) are correlated with high serum levels of androgens and estrogen. We hypothesized that Leydig cells and ovarian stromal hyperplasia contribute to postmenopausal ovarian androgen production and are observed more frequently in EEC patients. Ovaries of postmenopausal women with EEC (n = 36) or non-endometrioid endometrial carcinoma (NEEC; n = 19) were examined for the presence of hilar Leydig cells and compared with ovaries resected for benign conditions (n = 22). Leydig cells were counted manually, and a Leydig cell density was calculated per millimeter squared hilar surface. Ovarian stromal hyperplasia was scored as atrophic, moderate hyperplastic, or marked hyperplastic. In all endometrial carcinomas, these findings were correlated with the serum levels of sex steroids and hormone receptor expression in their endometrial carcinomas. In EEC patients, mean number of Leydig cells was 282.8 cells compared with 76.3 cells in NEEC patients and 66.4 cells in controls. Leydig cells, marked stromal hyperplasia, and combined presence were observed more frequently in EEC patients compared with NEEC and controls. Combined presence was associated with higher serum sex steroid levels and increased tumor expression of estrogen and progesterone receptor. A cutoff value for Leydig cell hyperplasia could be proposed at a total of 300 Leydig cells bilaterally, examining a representative cross section of both hili. Concluding, we have quantified hilar Leydig cells and demonstrated that Leydig cells may contribute to the development of EEC by increased androgen production in postmenopausal women. The correlation between sex hormone levels and Leydig cell hyperplasia may support endometrial pathology screening in these women.

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1. Introduction

Well-known risk factors for endometrioid endometrial carcinoma (EEC) are related to prolonged exposure to high estrogen levels due to obesity, early menarche, late menopause, nulliparity, polycystic ovarian syndrome, estrogen-producing tumors, or hormone replacement therapy [1,2]. In postmenopausal women, dehydroepiandrosterone (DHEA) is the main source of both androgens and estrogens. DHEA can be metabolized to testosterone and may be further converted to dihydrotestosterone (DHT), the most potent endogenous androgen receptor (AR) agonist. DHEA can also be converted to androstenedione and subsequently to estrone, or via testosterone to estradiol, mediated by aromatase (CYP19) [3-5]. After menopause, ovarian estrogen production strongly diminishes, but the ovaries continue to produce androgens including DHEA, androstenedione, and testosterone. More specifically, around 20% of circulating DHEA is produced by the postmenopausal ovary and 80% by the adrenal glands [6-8]. Other studies demonstrated that the postmenopausal ovary is responsible for 20% to 40% of the total body amount of androstenedione and testosterone [9-11]. That high postmenopausal serum estrogen levels are related to an increased risk of the development of EEC has been established for a long time. However, also high serum androgen levels are associated with an increased risk for EEC [12-17].

Although several studies have focused on functional aspects of the postmenopausal ovary, only few studies investigated its morphologic aspects. One study found a correlation between the presence of stromal hyperplasia, increased levels of androstenedione and testosterone, and the presence of endometrial carcinoma (EC) [18]. In addition, ovarian Leydig cells are known to produce androgens and are associated with increased serum levels of testosterone and androstenedione [19-21]. Ovarian Leydig cells are morphologically similar to testicular Leydig cells and are usually found in clusters in the ovarian hilus, and rarely in the ovarian stroma [22]. These cuboidal cells, measuring 15 to 25 μm in diameter, have abundant eosinophilic cytoplasm with round, slightly hyperchromatic nuclei with 1 or 2 small nucleoli and sometimes intracytoplasmic inclusion (Reinke crystals) [23]. Increased numbers of Leydig cells are encountered in ovarian Leydig cell hyperplasia (LCH), which is associated with increased serum levels of androgens and virilization. However, systematic criteria for quantification of Leydig cells needed for LCH are lacking. Furthermore, the correlation with EEC has not been properly assessed so far.

Therefore, we aim to quantify the distribution and number of Leydig cells in postmenopausal ovaries of EEC, nonendometrioid endometrial carcinoma (NEEC), and control patients, to propose criteria for LCH. Second, we correlate these findings with serum sex steroid levels and hormone receptor expression in EC.

2. Materials and methods

For this retrospective study, all patients who underwent primary surgical treatment between 1999 and 2009 for EC at the Radboud University Medical Center, with stored preoperative serum, were selected. Patients with a benign uterine condition were identified by the nationwide network and registry of histopathology and cytopathology in the Netherlands (PALGA, http://www.palga.nl) and served as controls. Patients with EC were eligible if they were postmenopausal, had no history of hormonal treatment or contraceptive use within 3 months before diagnosis, and had no ovarian metastases or coexisting ovarian carcinoma. Control patients were eligible if they were postmenopausal, had no history of hormonal treatment or contraceptive use within 3 months before diagnosis, and had inactive endometrium. All patients underwent at least hysterectomy with bilateral salpingo-ophorectomy, and EC patients were staged according to the International Federation of Gynaecology and Obstetrics (FIGO 2009) [24]. Patients were classified into EEC patients, NEEC patients, and control patients with a benign uterine condition. Tumor slides of EEC and NEEC patients were reviewed by a gynecopathologist blinded for other characteristics (J. B.).

Clinicopathological features were extracted from patient records and included patient characteristics, tumor characteristics, imaging results, and treatment and follow-up data.

The study was approval by the Ethics Committee of the Radboud University Medical Center (Nijmegen) and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, http://www.federa.org). Consent has been obtained for biobanking from each patient after full explanation of the purpose and nature of all procedures used.

2.1. Ovarian Leydig cells

All ovaries were retrospectively retrieved from our pathology archives. From each patient, both ovaries were investigated. Our local protocol prescribed that both ovaries were bisectioned in the coronal plane and one-half was paraffin embedded. Sectioning and embedding protocols did not differ between patients with and without EC. Four-micrometer formalin-fixed, paraffin-embedded tissue sections were cut and routinely stained by hematoxylin and eosin.

We manually counted the numbers of Leydig cells in both hili with high-magnification view (×100 and ×200). Despite standardized processing of the ovaries, differences in the total embedded hilar surface were expected. Therefore, we corrected for the total embedded hilar surface area, by calculating a density score. First, the total hilar surface area was calculated. For standardized and reproducible assessment of the hilar surface area, slides were digitalized using a whole-slide scanner (Pannoramic P250 Flash III; 3DHistech, Budapest, Hungary). Images were stored in a proprietary image format (3D Histech), based on jpg compression with a specimen level pixel size of 0.23 μm. Manual delineation of the hilus was performed using ImageJ software (Wayne Resband, National Institute of Mental Health, National Institutes of Health, Bethesda, MD). The following regions for delineation of the ovarian hilus using the ImageJ pen tool were used

(Fig. 1): the junction of the hilus with the medullar stroma, the hilar serosa, and the mesovarian fatty tissue surrounding the hilar vessel structures.

Subsequently, the hilar Leydig cell density was calculated as the number of Leydig cells per mm^2 hilar surface area. The minimum number of Leydig cells needed to define a condition as LCH has never been defined. Based on the mean numbers of Leydig cells and Leydig cell densities in both ovaries, a cutoff value for LCH was proposed.

2.2. Stromal hyperplasia

The degree of stromal hyperplasia was classified into 3 categories according to existing criteria [25]: stromal atrophy (cortex <1 mm), moderate hyperplasia (cortex >1 mm), or marked hyperplasia (cortex >1 mm and apparent cellular cortical stroma present in the medulla). All features were scored by 2 reviewers (J. B., C. R.), blinded for clinical and pathological data.

2.3. Immunohistochemical expression

The most representative paraffin block containing EC tissue was selected for each patient after hematoxylin and eosin staining. Blank 4-µm formalin-fixed, paraffin-embedded sections were cut and mounted on Superfrost slides and were immunohistochemically stained for expression of estrogen receptor (ER), progesterone receptor (PR), and AR. After antigen retrieval with EnVision FLEX High pH Target Retrieval Solution and blocking of endogenous peroxidase with hydrogen peroxide, slides were incubated with either anti-ER (clone EP1, FLEX ready-to-use; Dako, Glostrup, Denmark), anti-PR (clone Pgr-1240, FLEX ready-to-use; Dako), or anti-AR (clone Ab133273, dilution 1:50; Abcam, Cambridge, United Kingdom). Subsequently, they were shortly incubated with EnVision FLEX and visualized with high-pH visualization system according to the manufacturer's instructions for use. The slides were counterstained with hematoxylin, dehydrated, and mounted. All slides were scored by 2 independent reviewers (H. K. V., C. R.) who were blinded for clinical and pathological data. For ER, PR, and AR, slides were considered positive when more than 10% of the tumor cells had a positive nuclear staining, regardless of intensity [26,27].

2.4. Serum sex steroid levels

Preoperative blood samples from EC patients were available and were collected directly before surgery in dry tubes and processed in a standardized matter: centrifuged at 200*g* for 10 minutes and stored at −40°C until assayed. The serum samples were analyzed for DHEAS, testosterone, androstenedione, and estradiol. DHEAS and estradiol (third generation) were measured by the electrochemiluminescence immunoassay on a Modular E170 random access analyzer (Roche, Basel,

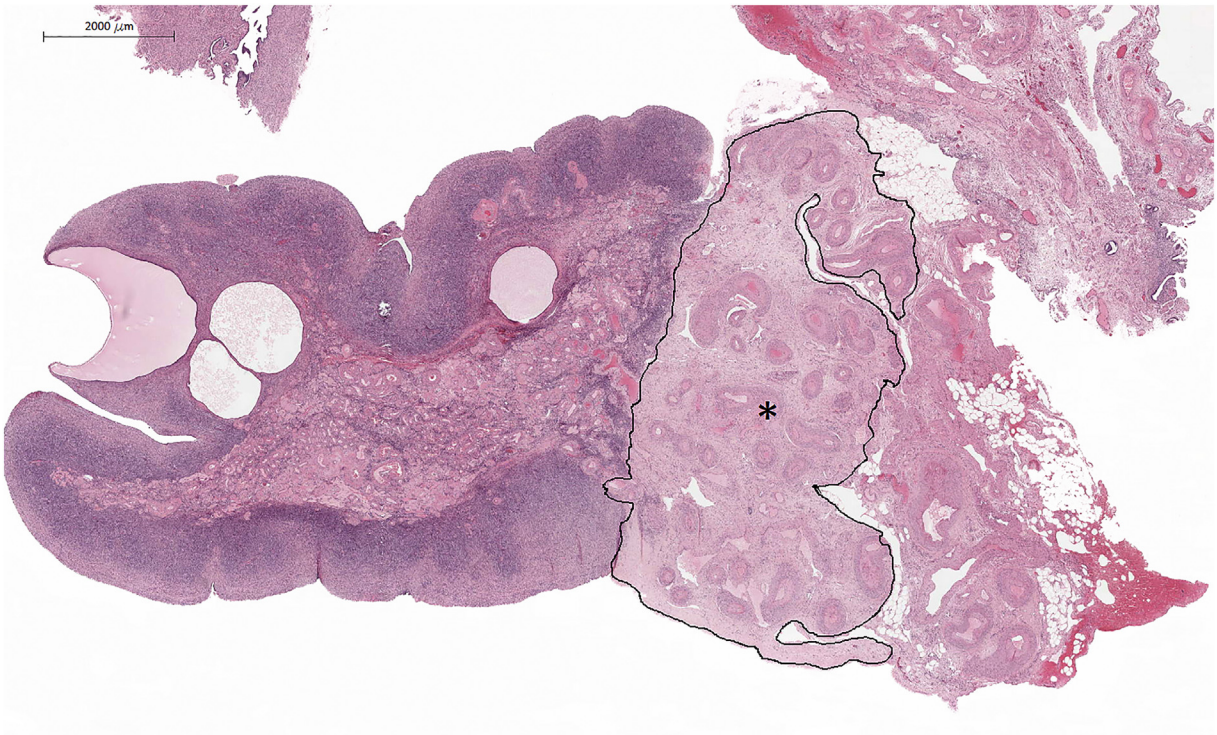

Fig. 1 Transversal section of the ovary, including the hilus and mesovarian fatty tissue. The black line indicates the border of the ovarian hilus. The hilus (marked with *) is delineated by the corticohilar border, the hilar serosa, and the mesovarian fatty tissue surrounding the hilar vessel structures.

Switzerland). Androstenedione and testosterone were measured with an in-house developed liquid chromatography–tandem mass spectrometry method [28]. For control patients, serum was not available owing to the retrospective character of the study.

2.5. Statistical analysis

Relationships between the presence of Leydig cells, stromal hyperplasia, and the subgroups were analyzed using the Pearson χ^2. The Mann-Whitney U test was used to compare the hilar surface area, numbers of Leydig cells, Leydig cell density, and serum sex steroid levels between subgroups. The correlation between hormone receptor expression and the presence of Leydig cells and stromal hyperplasia was analyzed using the Pearson χ^2. Spearman ρ was used to calculate the correlation between the absolute number of Leydig cells and the Leydig cell density. The correlation between the presence of EEC and risk factors was estimated with univariable and multivariable logistic regression analysis. Factors identified by univariable regression analysis with $P < .20$ were used for multivariable regression analysis. All tests were 2 sided, and P values of <.05 were considered significant. Statistical analyses were performed using SPSS 22.0 for Microsoft Windows (SPSS, Chicago, IL).

3. Results

A total of 88 patients were identified, of whom 11 patients were excluded: 3 EC patients were premenopausal; 2 EC patients had ovarian metastases; in 3 EC patients, ovarian histology samples were absent; and 3 control patients revealed endometrial hyperplasia after revision. The total study group consisted of 77 patients: 36 EEC patients, 19 NEEC patients, and 22 control patients. Baseline characteristics of all patients are shown in Table 1. The subgroups were comparable with respect to age and body mass index (BMI). The gynecologic diagnoses of patients with a benign uterine condition included uterine leiomyoma (n = 16), genital prolapse (n = 2), and atrophic postmenopausal bleeding (n = 4). After histopathological evaluation by a pathologist (J. B.), all control patients were confirmed to have inactive endometrium.

3.1. Distribution of Leydig cells

An example of a focus of Leydig cells is shown in Fig. 2. Leydig cells were found in 53.2% of patients (n = 41): 26 EEC patients, 8 NEEC patients, and 7 control patients. In none of the ovaries, an exclusively extrahilar localization of Leydig cells was present. The foci of Leydig cells were limited to the

Table 1 Clinicopathological features of all included patients (n = 77)

	EEC (n = 36)	NEEC (n = 19)	Control (n = 22)
Age (y), median (range)	67.0 (46.0-85.0)	70.0 (54.0-88.0)	64.0 (50.0-74.0)
BMI (kg/m^2), median (range)	27.5 (18.0-50.5)	25.1 (18.3-37.2)	24.4 (21.0-31.5)
Grade			NA
1	5 (13.9%)		
2	23 (63.9%)		
3	8 (22.2%)	19 (100.0%)	
FIGO stage [a]			NA
Early	25 (69.4%)	6 (31.6%)	
Advanced	11 (30.6%)	13 (68.4%)	
Myometrial invasion			NA
<50%	10 (27.8%)	10 (52.6%)	
>50%	26 (72.2%)	9 (47.4%)	
Lymph nodes			NA
Positive	4 (11.1%)	5 (26.3%)	
Negative	12 (33.3%)	10 (52.6%)	
Unknown	20 (55.6%)	4 (21.1%)	
Recurrence			NA
Yes	12 (33.3%)	9 (47.4%)	
Distant	6 (16.7%)	5 (26.3%)	
No	24 (66.7%)	10 (52.6%)	
Death			NA
Yes	13 (36.1%)	10 (52.6%)	
By EC	10 (27.8%)	7 (36.8%)	
No	23 (63.9%)	9 (47.4%)	

Abbreviation: NA, not applicable.

[a] FIGO stages 1 and 2 were classified as early stage; FIGO stages 3 and 4 were classified as advanced stage.

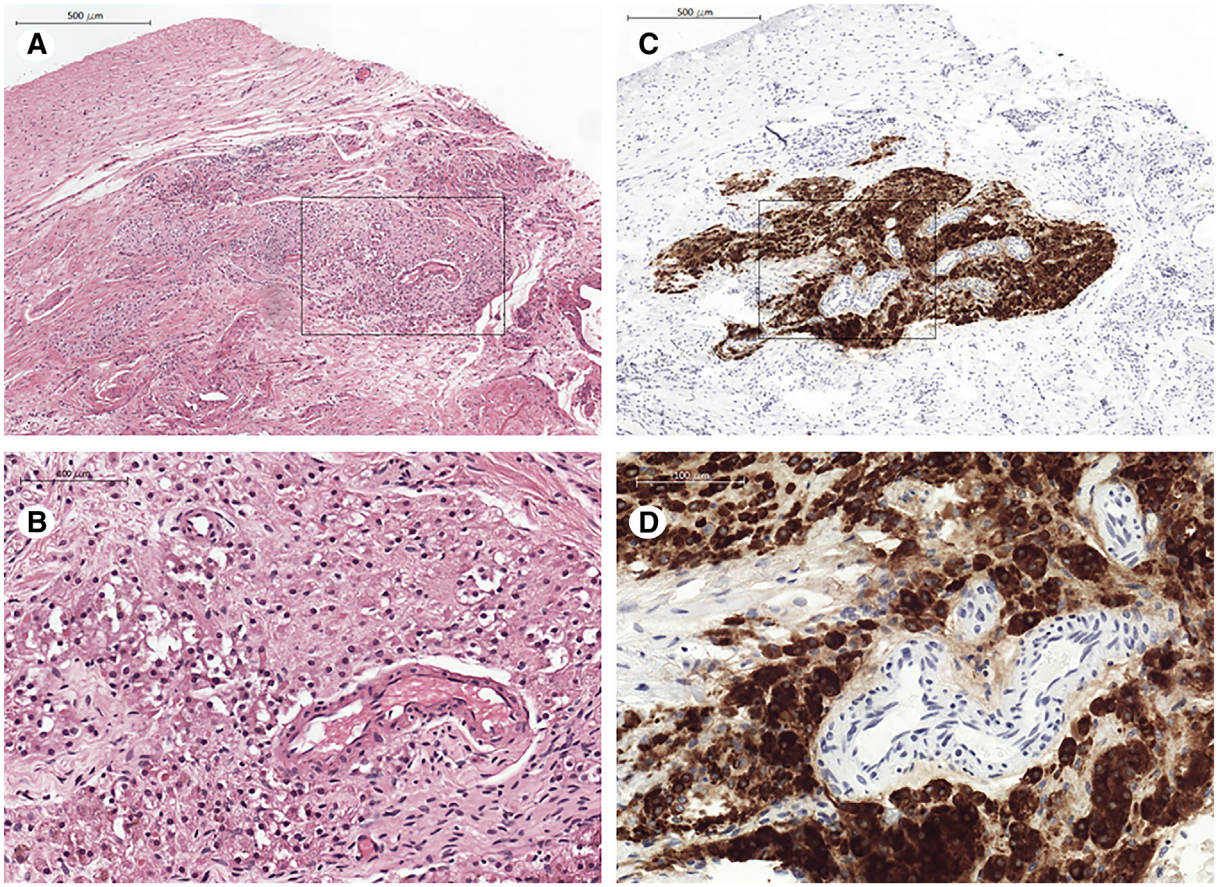

Fig. 2 Example of a Leydig cell focus. A and B, Hematoxylin and eosin section (original magnifications ×5 [A] and ×20 [B]). C-D. α-inhibin staining of a Leydig cell focus (original magnifications ×5 [C] and ×20 [D]). The Leydig cells are recognized as medium-sized cuboidal cells with abundant eosinophilic cytoplasm with round, slightly hyperchromatic nuclei with 1 or 2 small nucleoli.

ovarian hilus in most patients, except in 2 EEC patients and 1 control patient: in 2 patients, the Leydig cells were colocalized in the ovarian cortex, and 1 EEC patient showed colocalization in the left fallopian tube. In 17 patients, there was only a single focus of Leydig cells (8 EEC patients, 4 NEEC patients, 5 control patients), and in 24 patients, Leydig cells were present in multiple foci (18 EEC patients, 4 NEEC patients, 2 control patients). In 23 patients, Leydig cells were present in only one ovary (12 EEC patients, 6 NEEC patients, 5 control patients), and in 18 patients, Leydig cells were present in both ovaries (14 EEC patients, 2 NEEC patients, 2 control patients).

3.2. Quantification of ovarian Leydig cells

The numbers of Leydig cells were manually counted, and Leydig cell densities were calculated. The hilar surface area was comparable between patients with and without Leydig cells: 77.7 mm^2 (range, 9.3-235.6 mm^2) versus 77.8 mm^2 (range, 2.4-205.3 mm^2; $P = .819$). Furthermore, the hilar surface area was comparable between EEC, NEEC, and control patients (Table 2). Correlation between the total number of Leydig cells and the Leydig cell density was significant (Spearman $\rho = 0.838$, $P < .001$). Patients with Leydig cells

Table 2 Number of Leydig cells and Leydig cell density (patient-based numbers)

	EEC (n = 36)	NEEC (n = 19)	Control (n = 22)
No. Leydig cells	282.8 (±446.2) [a,b]	76.3 (±117.1) [b]	66.4 (±111.7) [a]
Leydig cell density (cells/mm^2)	3.3 (±4.7) [c,d]	0.9 (±1.4) [c]	0.7 (±1.2) [d]
Total hilar surface area (mm^2)	73.3 (2.4-219.5)	77.6 (44.8-237.6)	74.2 (28.4-221.7)

Values are represented as mean (standard deviation).
[a] $P = .008$.
[b] $P = .034$.
[c] $P = .026$.
[d] $P = .008$.

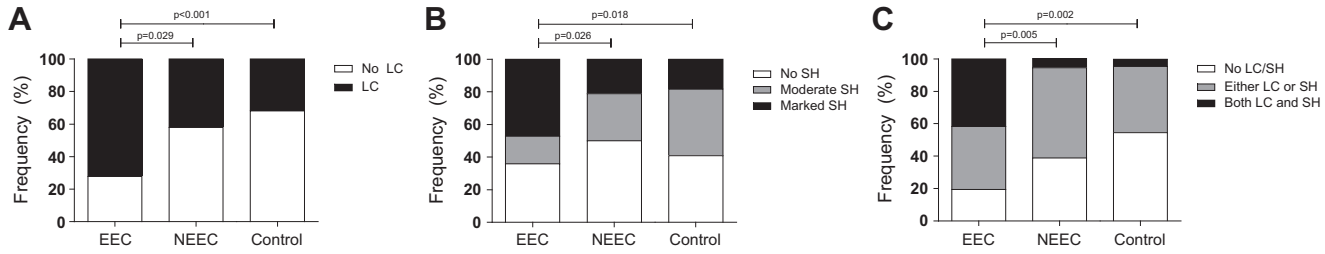

Fig. 3 The presence of Leydig cells and stromal hyperplasia in EEC, NEEC, and control patients. A, The presence of Leydig cells. B, The presence of stromal hyperplasia. C, The presence of combined presence of Leydig cells and marked stromal hyperplasia. χ^2 Test was indicated to compare groups. LC, Leydig cells; SH, stromal hyperplasia.

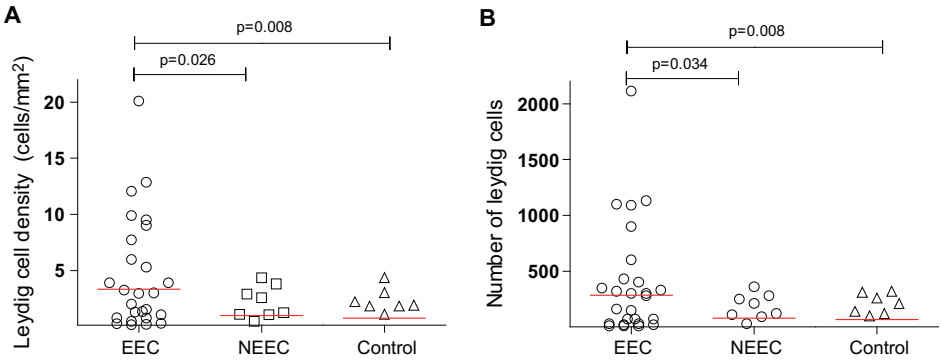

Fig. 4 Leydig cell density (in cells per millimeter squared) and total number of Leydig cells in EEC, NEEC, and control patients. A, Leydig cell density (in cells per millimeter squared). B, Total numbers of Leydig cells. Red horizontal line represents mean. The numbers displayed are greater than 0.1 cells/mm^2 for Leydig cell density and greater than 1 cell for total number of Leydig cells; however, all patients have been included in analysis.

did not significantly differ from patients without Leydig cells regarding BMI (median, 24.1 [18.0-47.5] kg/m^2 versus 26.9 [18.4-50.5] kg/m^2, P = .191) and advanced FIGO stage (38.2% versus 50.0%, P = .39).

Leydig cells were present in 72.2% (n = 26) of EEC patients compared with 42.1% (n = 8) of NEEC patients (P = .029) and 31.8% (n = 7) of control patients (P < .001; Fig. 3A). The Leydig cell densities and absolute number of Leydig cells are shown in Table 2 and Fig. 4. In EEC patients, the mean Leydig cell density was 3.3 cells/mm^2 compared with 0.9 cells/mm^2 in NEEC patients (P = .026) and 0.7 cells/mm^2 in control patients (P = .008). In EEC patients, mean number of Leydig cells was 282.8 cells compared with 76.3 cells in NEEC patients (P = .034) and 66.4 cells in control patients (P = .008). Leydig cell densities greater than 5.0 cells/mm^2 were only seen in EEC patients: maximum Leydig cell density was 20.1 cells/mm^2 compared with 4.4 cells/mm^2 in NEEC patients and 4.4 cells/mm^2 in control patients. In absolute numbers, maximum number of Leydig cells was 2112 cells in EEC patients compared with 363 cells in NEEC patients and 320 cells in control patients.

Based on the above-mentioned mean values, a cutoff value for LCH could be proposed at a total of 300 Leydig cells bilaterally, examining a representative cross section of both hili.

Table 3 Serum levels of DHEAS, testosterone, androstenedione, and estradiol in patients with and without Leydig cells and stromal hyperplasia

	DHEAS (μmol/L)	Testosterone (nmol/L)	Androstenedione (nmol/L)	Estradiol (pmol/L)
No LC (n = 18)	1.86 (0.41-3.05) [a]	0.54 (0.20-1.17) [b]	1.70 (0.70-7.25)	47.00 (9.0-180.0)
LC (n = 30)	2.61 (0.27-6.33) [a]	0.79 (0.13-1.63) [b]	1.86 (0.17-7.81)	56.00 (9.0-170.0)
No SH (n = 31)	2.22 (0.27-6.33)	0.57 (0.13-1.21)	1.61 (0.17-4.98)	44.00 (9.00-170.00)
SH (n = 17)	2.66 (0.47-6.81)	0.81 (0.20-1.63)	2.11 (0.51-7.81)	67.00 (9.00-130.00)
LC, SH, or none (n = 35)	1.97 (0.27-6.33) [c]	0.56 (0.13-1.21) [c]	1.58 (0.17-7.25) [c]	44.00 (9.00-170.0) [c]
LC and SH (n = 13)	2.69 (0.70-5.80) [c]	0.87 (0.23-1.63) [c]	2.15 (1.38-7.81) [c]	68.00 (9.00-130.0) [c]

NOTE. Values are presented as median (range). In 6 patients, serum steroid levels could not be determined because of technical issues.
Abbreviations: LC, Leydig cells; SH, stromal hyperplasia.
[a] P = .020.
[b] P = .030.
[c] P = .001.

Table 4 ER, PR, and AR expression in patients with and without Leydig cells and stromal hyperplasia

	EC			EEC			NEEC		
	ER (%) [a]	PR (%) [a]	AR (%) [a]	ER (%)	PR (%)	AR (%)	ER (%)	PR (%)	AR (%)
No LC (n = 19)	52.6	52.6	30.0	70.0	90.0	40.0	45.5	72.7	18.2
LC (n = 32)	78.1	59.4	38.7	91.7	79.2	43.5	37.5	100.0	25.0
No SH (n = 33)	61.8	41.2 [b]	35.3	83.3	72.2	50.0	41.2	11.8	17.6
SH (n = 18)	81.8	77.3 [b]	36.4	89.5	89.5	38.9	50.0	25.0	25.0
LC, SH, or none (n = 36)	58.3 [c]	47.2 [d]	35.1	80.0	80.0	50.0	38.9	16.7	16.7
LC and SH (n = 15)	93.3 [c]	78.6 [d]	35.7	92.9	85.7	30.8	100.0	0.0	100.0

NOTE. In 4 patients, hormone receptor status could not be determined because of technical issues.
Abbreviations: LC, Leydig cells; SH, stromal hyperplasia.
[a] Values for ER, PR, and AR expression represent the percentage of ECs showing positive expression.
[b] $P = .008$.
[c] $P = .014$.
[d] $P = .031$

3.3. Combined presence of Leydig cells and marked stromal hyperplasia

The presence of ovarian stromal hyperplasia in the 3 groups is presented in Fig. 3B. In 47.2% (n = 17) of EEC patients, marked stromal hyperplasia was found compared with 21.1% (n = 4) of NEEC patients ($P = .026$) and 18.2% (n = 4) of control patients ($P = .018$). The combined presence of Leydig cells and marked stromal hyperplasia in the 3 groups is presented in Fig. 3C. A combination of both was found in 41.7% (n = 15) of EEC patients, 5.6% (n = 1) of NEEC patients ($P = .005$), and 4.5% (n = 1) of control patients ($P = .002$).

3.4. Correlation with serum sex steroid levels

In all patients with EC, serum levels of DHEAS, testosterone, androstenedione, and estradiol were determined. The correlation between serum sex steroid levels and the presence of Leydig cells, marked stromal hyperplasia, and combined presence is shown in Table 3. Combined presence of Leydig cells and marked stromal hyperplasia was correlated with higher serum levels of all hormones. Presence of Leydig cells alone was correlated with higher serum levels of DHEAS and testosterone. Serum sex steroid levels tended to be higher in the presence of marked stromal hyperplasia. Also, correlations between histologic subtype and BMI were explored. BMI greater than 25 kg/m^2 was significantly correlated with higher serum estradiol levels, but not with higher serum androgen levels (data not shown). There were no correlations between histologic subtype and serum sex steroid levels.

3.5. Correlation with hormone receptor status

The correlation between hormone receptor status in the tumor and the presence of Leydig cells, marked stromal hyperplasia, and combined presence is shown in Table 4. In patients with combined presence of Leydig cells and marked stromal hyperplasia, ER expression was seen in 93.3% compared with 58.3% in patients without combined hyperplasia ($P = .014$); PR expression was seen in 80.0% of patients with combined presence of Leydig cells and marked stromal hyperplasia compared with 47.2% in patients without ($P = .031$). There were no significant differences in the AR expression between the subgroups. Separate analyses for EEC and NEEC showed no significant differences concerning hormone receptor expression (Table 4).

3.6. Logistic regression analysis

The correlation between the presence of EEC and risk factors, including BMI, age, the presence of Leydig cells, and the presence of marked stromal hyperplasia, was explored

Table 5 Univariable and multivariable logistic regression for EEC correlated with age, BMI, and the presence of Leydig cells

EEC	Univariable		Multivariable	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (y) [a]	1.00 (0.96-1.05)	.862	–	
BMI (kg/m^2) [a]	1.09 (1.01-1.17)	.030	1.07 (0.98-1.17)	.138
Leydig cells, yes-no	4.10 (1.59-10.58)	.004	4.20 (1.44-12.24)	.008
Marked SH, yes-no	5.07 (1.90-13.54)	.001	4.61 (1.41-15.07)	.011

Abbreviations: CI, confidence interval; OR, odds ratio; SH, stromal hyperplasia.
[a] Analyzed as continuous values.

(Table 5). Multivariable analysis showed that both the presence of Leydig cells and marked stromal hyperplasia were independently correlated with an increased risk for EEC.

4. Discussion

It was hypothesized that Leydig cells and stromal hyperplasia in the ovaria of postmenopausal women might contribute to the development of EEC by the production of androgens [3,5-7,10,12-17]. Up to date, there are no histologic criteria for quantification of LCH in the ovary [19,20,29]. In an attempt to systematically explore possible correlations with the presence of EEC, we quantified the distribution and number of Leydig cells in the ovarian hilus of women with known EEC, women with NEEC, and controls. We manually quantified the number of Leydig cells in the hilus of both ovaries and demonstrated that Leydig cells were observed not only more frequently but also more extensively in EEC patients compared with NEEC and control patients. Based on our results, we propose a cutoff value for LCH at a total of 300 cells bilaterally, examining a representative cross section of both hili. The same cutoff value is proposed in case only one ovary is available for histologic examination, because in our study, unilateral Leydig cells were encountered in 23 (56%) of a total of 41 cases. Leydig cells can easily be missed, and examination with high-magnification view (minimal ×100 or ×200) is helpful in tracing them. In addition, the presence of multiple nodules of Leydig cells can be a clue in detecting LCH, as in our study, multiple nodules of at least 20 cells were present in all cases with LCH. Therefore, when at least 2 nodules of 20 cells are found at first screening, we recommend additional counting of the total hilar area. Within this study, the additional value of inhibin-α staining for quantification of Leydig cells was explored in a subset of cases; however, we could not demonstrate a benefit because it was difficult to distinguish between individual cells (data not shown).

Despite standardized processing of the ovaries, differences in the total embedded hilar surface were expected. Therefore, a surface-adapted measuring unit (density, in cells per millimeter squared) was used to correct for the differences in embedded ovarian hilar surfaces with good correlation between Leydig cell density and total number of Leydig cells. Comparable mean ovarian hilar surface areas in patients with and without Leydig cells supports that sampling bias is limited.

Established criteria for the quantification of stromal hyperplasia were used, and marked ovarian stromal hyperplasia was present more often in EEC patients than in NEEC or control patients, which is in line with previous studies. Jongen et al [18] found marked stromal hyperplasia in 45.5% of EEC patients, which is comparable with our results (47.2%) [10]. Interestingly, a combination of both Leydig cells and marked stromal hyperplasia was found almost exclusively in EEC patients, supporting a synergistic effect of androgen production by both ovarian components on the development of EEC. This seems to be supported by higher serum levels of androgens and estradiol in EC patients demonstrating both Leydig cells and stromal hyperplasia. The observed increased estradiol levels in patients with both presence of Leydig cells and marked stromal hyperplasia could be explained by an increased peripheral conversion from androgens to estradiol, mediated by aromatase. Multivariable logistic regression showed that the presence of Leydig cells and marked stromal hyperplasia were correlated with increased risk of EEC, adjusted for possible confounders (age and BMI). In addition, EC patients with combined Leydig cells and marked stromal hyperplasia demonstrated increased ER and PR expression, which implies indeed a hormone-dependent carcinogenesis in these patients. In contrast, there was no increased expression of AR in EC patients demonstrating LCH and/or stromal hyperplasia. Furthermore, expression of AR was not correlated with higher serum levels of androgens. Existing data support that an alteration in intracrinologic metabolism of androgens in EC favors an estrogenic pathway instead of an androgenic pathway: Testosterone is formed from androstenedione by the action of 17β-hydroxysteroid dehydrogenase 5 and can be activated to DHT by the action of 5α-reductase [30]. DHT is the most potent endogenous AR agonist and thus a prerequisite for substantial activation of AR-dependent signaling [31]. On the other hand, androstenedione can be also converted into estrone and estradiol by the action of aromatase [32,33]. Estradiol is the most potent endogenous ER agonist and thus a prerequisite for substantial activation of ER-dependent signaling. Interestingly, studies have shown a decrease in both 5α-reductase and 17β-hydroxysteroid dehydrogenase 5 expression in EC, which would lead to a decrease of DHT activation and thus decreased AR-dependent signaling [34,35]. Also, an increased conversion from testosterone to androstenedione was found [33]. The latter has been shown to upregulate aromatase messenger RNA expression. In summary, these data suggest that intracrinologic metabolism of androgens favors an estrogenic pathway that may explain the low percentage of AR expression, but the high ER and PR expression. However, the exact role of AR in the development of EC needs to be further explored.

To our knowledge, this is the first study systematically quantifying the frequency and number of Leydig cells in patients with and without EC. However, this retrospective study has some limitations that need to be addressed. Serum sex steroid levels were determined in samples obtained by peripheral venapuncture. Because in previous studies higher circulating sex steroid levels were observed in the ovarian vein when compared with peripheral circulation, differences in sex steroid concentrations in relation to Leydig cell density might even be larger when sampling the ovarian vein [18]. Second, although we were able to correct for confounders in multivariable analysis, our sample size is limited, possibly limiting generalizability of our results including proposed cutoff value.

Concluding, we have systematically quantified the presence of Leydig cells and propose a cutoff value to define LCH. Combined presence of Leydig cells and marked stromal hyperplasia was observed more frequently in EEC patients and

was associated with higher serum sex steroid levels and hormone receptor–positive EEC. The correlation between sex hormone levels and LCH may support endometrial pathology screening in these women. Structural examination by high-magnification view for the presence of Leydig cells and stromal hyperplasia by the pathologist can guide the gynecologist for subsequent endometrial evaluation.

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