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Endogenous androgens and risk of epithelial invasive ovarian cancer by tumor characteristics in the European Prospective Investigation into Cancer and Nutrition

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Abbreviations: AR: androgen receptor; BMI: body mass index; B-RAF: serine/threonine-protein kinase B-Raf; CI: confidence interval; DHEAS: dehydroepiandrosterone sulfate; DHT: dihydrotestosterone; DSL: Diagnostic System Laboratories; EOC: epithelial invasive ovarian cancer; EPIC: European Prospective Investigation into Nutrition and Cancer; ER: estrogen receptor; HEK293: human embryonic kidney; HepG2: human hepatocytes; HRT: hormone replacement therapy; IARC: International Agency for Research on Cancer; ICD: International Classification of Diseases for Oncology; K-RAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; NOS: not otherwise specified; OC: oral contraceptive; OR: odds ratio; PCOS: polycystic ovarian syndrome; PTEN: Phosphatase and tensin homolog; r: pearson partial rank correlation; RIA: radioimmunosorbent assay; SAS: Statistical Analysis Software; SD: standard deviation; SHBG: sex hormone binding globulin

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The role of endogenous androgens and sex hormone-binding globulin (SHBG) in ovarian carcinogenesis is poorly understood. Epithelial invasive ovarian cancer (EOC) is a heterogeneous disease and there are no prospective data on endogenous androgens and EOC risk by tumor characteristics (histology, grade, stage) or the dualistic model of ovarian carcinogenesis (*i.e.* type I *vs.* type II, leading to less or more aggressive tumors). We conducted a nested case–control study in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort evaluating androgens and SHBG and invasive EOC risk by tumor characteristics. Female participants who provided a blood sample and were not using exogenous hormones at blood donation were eligible ($n = 183{,}257$). A total of 565 eligible women developed EOC; two controls ($n = 1{,}097$) were matched per case. We used multivariable conditional logistic regression models. We observed no association between androgens, SHBG and EOC overall. A doubling of androstenedione reduced risk of serous carcinomas by 21% (odds ratio (OR)log2 = 0.79, 95% confidence interval [CI] = [0.64–0.97]). Moreover, associations differed for low-grade and high-grade carcinomas, with positive associations for low-grade and inverse associations for high-grade carcinomas (*e.g.* androstenedione: low grade: $OR_{log2} = 1.99$ [0.98–4.06]; high grade: $OR_{log2} = 0.75$ [0.61–0.93], $p_{het} \leq 0.01$), similar associations were observed for type I/II tumors. This is the first prospective study to evaluate androgens, SHBG and EOC risk by tumor characteristics and type I/II status. Our findings support a possible role of androgens in ovarian carcinogenesis. Additional studies exploring this association are needed.

Epidemiology

What's new?

There appear to be several types of epithelial invasive ovarian cancer (EOC), and hormone-related risk factors are poorly understood. In this study, the authors found that the impact of endogenous androgens on the risk of developing EOC differed depending upon tumor characteristics. Androgen concentrations were positively associated with the risk of low-grade and type-I carcinomas, but the study found an inverse association for high-grade tumors. These findings support a possible role for androgens in ovarian carcinogenesis, and emphasize the need for additional research.

The ovary is a hormone-producing organ and it is speculated that endogenous androgens may play a significant role in ovarian pathogenesis.[1] Several lines of evidence from *in vitro* and animal studies demonstrate a fundamental role for hormones in cell development by controlling proliferation, differentiation and apoptosis.[1,2] Androgens are associated with proliferation of Müllerian epithelial cells and invasion of EOC cells *in vitro* and *in vivo*.[3] Well-established epidemiologic associations between hormonally related exposures or risk factors (*e.g.* parity, oral contraceptive [OC] use, breast feeding, hormone replacement therapy [HRT] polycystic ovary syndrome [PCOS]) and ovarian cancer risk[1] further support the possible involvement of endogenous hormones in the development of ovarian tumors.

To date, EOC has largely been investigated as a single disease. However, ovarian cancer is increasingly recognized to be a collection of up to five distinct disease entities, including high-grade serous, low-grade serous, endometrioid, clear cell and mucinous carcinomas.[4] In addition, some propose that the majority of "ovarian" carcinomas originate outside the ovary and involve it secondarily.[5] Recent molecular pathology and genetic studies suggest that these diseases may develop through two major pathways of carcinogenesis: type I (leads to less aggressive tumors) and type II (leads to more aggressive tumors). The best evidence supporting this hypothesis is available for serous tumors.[6] Type I tumors (*e.g.* low-grade serous tumors) are thought to arise in a step-wise manner from atypical proliferative (borderline) tumors or endometriosis,[7] whereas the cell of origin of high-grade serous carcinomas (type II) may reside in occult tubal intraepithelial carcinoma that implants secondarily in the ovary.[8] Recent epidemiologic research further supports important differences by ovarian tumor subtype, as hormone-related risk factors may be differentially associated with ovarian tumor characteristics and type I/II status.[9,10]

Prior prospective studies on the associations of androgens and sex hormone-binding globulin (SHBG) with risk of developing EOC have been small (largest prior study case $n = 224$;[11]) and results were inconclusive.[11–14] However, no prior study has evaluated the association between androgens, SHBG and EOC by tumor characteristics (histology, grade, stage, type I/type II). Given the heterogeneity between ovarian cancer subtypes, we conducted the first prospective study to address the role of androgens and SHBG in ovarian carcinogenesis by tumor characteristics (histology, grade, stage) and by type I/type II tumors within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

Study Population and Methods

The EPIC cohort

EPIC is an ongoing multicenter prospective cohort study designed to investigate the relationship between diet, nutrition and metabolic factors with cancer. Descriptions of study design, population and baseline data collection of the cohort have been reported in detail previously.[15,16] In brief, 519,978 participants (366,521 women) aged 25 to 70 years were enrolled from 1992 to 2000 in 23 centers in 10 European countries: Denmark, France, Germany, Greece, Italy, Norway, the Netherlands, Spain, Sweden, and the United Kingdom. Data on diet, reproductive and menstrual factors, current and past use of exogenous hormones (OC and HRT), disease history, smoking, and anthropometric measures were collected at baseline. A total of 385,747 study participants (226,673 women and 159,074 men) also provided a baseline blood sample.

All subjects gave written informed consent to use their data for future analyses. The Ethical Review Board of the International Agency for Research on Cancer (IARC) and the Institutional Review Board of each EPIC center approved these analyses.

Blood sample collection and storage

Details of blood sample collection and storage have been published elsewhere.[16] Briefly, for each participant 30 mL of blood was drawn, and after centrifugation blood fractions (serum, plasma, buffy coat, and red blood cells) were aliquoted in 28 plastic straws, which were heat sealed and stored. For all centers except Sweden and Denmark, samples are stored under liquid nitrogen (−196 C). Samples from Sweden are stored locally at −70 C; samples from Denmark are locally stored in 1 mL tubes in liquid nitrogen vapor (−150 C).

Determination of menopausal status and phase of menstrual cycle at blood donation

Women were considered as premenopausal when they reported regular menstrual cycles over the 12 months prior to blood donation. If this information was missing, women were considered to be premenopausal if they were less than 42 years at blood donation. Women were considered postmenopausal if they reported not having any menses over the past 12 months or when they were >55 years of age. Women between 42 and 55 years of age with missing or incomplete questionnaire data, irregular menstrual cycles in the past 12 months, or who reported previous hysterectomy (without oophorectomy) were classified as being peri-menopausal/having unknown menopausal status.

The determination of the phase of menstrual cycle in premenopausal women in EPIC has been reported previously.[17,18] Briefly, two different dating methods were used: "forward" dating counted forward from the woman's reported date of the start of her last menses, whereas "backward" dating counted backward from the date of the start of her next menses after blood donation. When both dating methods were available, the backward dating method was used to determine the menstrual cycle phase as it is known to be more accurate than forward dating.[19,20]

Follow-up for cancer incidence and vital status

In all countries with the exception of France, Germany and Greece, follow-up was based on employing record linkage

with cancer and pathology registries and the end of follow-up was the date of last complete follow-up for both cancer incidence and vital status, which ranged between 2003 and 2006, depending on the study center. In France, Germany, Greece, and Naples, participant follow-up and cancer outcome was verified with health insurance records, cancer and pathology registries and active follow-up through study participants and their next of kin. Vital status was collected from mortality registries at the regional or national level, which was combined with health insurance data or data collected by active follow-up. End of follow-up for these centers was the last contact, date of diagnosis, or date of death, whichever occurred first. The end of follow-up for these centers ranged from 2005 (France) to 2008 (Germany).

Selection of case and control subjects

After *a priori* exclusion of women with history of cancer prior to recruitment (n = 19,707), incomplete data on follow-up (n = 2,209) lifestyle (n = 526) and/or diet (n = 2,713), and women with bilateral oophorectomy at baseline (n = 10,500) a total of 344,754 women were evaluated for eligibility. Participants not using exogenous hormones (OC or HRT) at time of blood donation were eligible for the present analysis (n = 183,257). Case subjects were selected among women who developed incident epithelial invasive ovarian (C569), fallopian tube (C570) or peritoneal cancer (C480, C481, C482, C488) after recruitment into the EPIC study according to the International Classification of Diseases for Oncology (ICD) 0–3 and with data on tumor histology. We excluded women with a previous cancer diagnosis (except non-melanoma skin cancer) and women with non-epithelial or borderline tumors, as these are less frequently observed and may have unique etiology. We identified 760 invasive cases with blood sample and with data on OC/HRT use at blood donation. We excluded 189 women using OC/HRT at blood donation, two cases with missing histology data; four case–control sets were excluded due to missing hormone measurements for either the case or both matched controls. Thus, a total of 565 eligible cases are included in this analysis (528 ovarian, 22 fallopian tube and 15 peritoneal) along with their 1,097 matched controls (532 complete sets: one case, two controls). For the present analysis we included 201 cases and 372 matched controls from a prior analysis on endogenous androgens and ovarian cancer risk in EPIC (study phase 1[12]) and an additional 364 incident EOC cases diagnosed during more recent rounds of follow-up along with an additional 725 matched controls (study phase 2).

For each case subject up to two controls were randomly selected among appropriate risk sets consisting of all female cohort members with a blood sample, alive and free of cancer at the time of diagnosis of the index case. An incidence density sampling protocol was used, such that controls could include subjects who became a case later in time and each control could be sampled more than once. Cases and controls in both study phases were matched on: study recruitment center, age at blood donation (±6 months), time of the day of blood collection (±1 h), fasting status (<3 h, 3–6 h, >6 h), and menopausal status at blood collection (premenopausal, perimenopausal, postmenopausal), as well as menstrual cycle phase for premenopausal women ("early follicular" (days 0–7 of the cycle), "late follicular" (days 8–11), "peri-ovulatory" (days 12–16), "mid-luteal" (days 20–24), and "other luteal" (days 17–19 or days 25–40). Cases missing data on phase of menstrual cycle were matched to controls with missing information on menstrual cycle phase.

Information on tumor characteristics (histologic subtype [serous, endometrioid, clear cell, mucinous, not otherwise specified (NOS), grade [well, moderately or poorly/undifferentiated] and stage [local, regional, metastatic]) was available from pathology reports and from cancer registries. A total of 53% of tumors were of serous histology (n = 302), 18% not otherwise specified (NOS) (n = 99), 12% endometrioid (n = 66), 7% mucinous (n = 41), 5% other (malignant epithelial neoplasms, carcinoma, malignant mixed Müllerian or malignant Brenner tumors; n = 29), and 5% clear cell (n = 28). Information on tumor grade and stage was 60% and 88% complete, respectively. Well differentiated tumors were classified low grade; moderately and poorly/undifferentiated tumors were classified high grade. We classified cases with local disease as low stage and cases with regional or metastatic disease as high stage.

We additionally sub-classified tumors based on the dualistic model of ovarian carcinogenesis, as put forward by Shih and Kurman[21] (Fig. 1). Type I tumors include low-grade serous, endometrioid and mucinous carcinomas, and Brenner tumors. Type II tumors consist mainly of high-grade serous carcinomas, but also high-grade endometrioid, undifferentiated and malignant mixed Müllerian tumors. Clear cell carcinomas (n = 28) were excluded from type I/II analyses, as they show a unique clinical behavior and demonstrate features of both type I and type II tumors so that their proper classification remains controversial.[22,23]

Laboratory assays

Pre-diagnostic circulating concentrations of testosterone (nmol/L), dehydroepiandrosterone sulfate (DHEAS; μmol/L), androstenedione (nmol/L), and SHBG (nmol/L) for cases and matched controls were analyzed within the same analytical batch by laboratory technicians blinded to case–control status.

Laboratory assays were conducted at IARC (study phase 1) and at the specialized hormonal laboratory of the Division of Cancer Epidemiology at the German Cancer Research Center (study phase 2) using the same commercially available immunoassays. Testosterone and DHEAS were measured by direct radioimmunosorbent arrays (RIA; Immunotech, Marseille, France; Beckman Coulter, Brea, CA). Androstenedione was measured by direct RIA (Diagnostic System Laboratories (DSL), Webster, TX; Beckman and Coulter, Brea, CA). SHBG

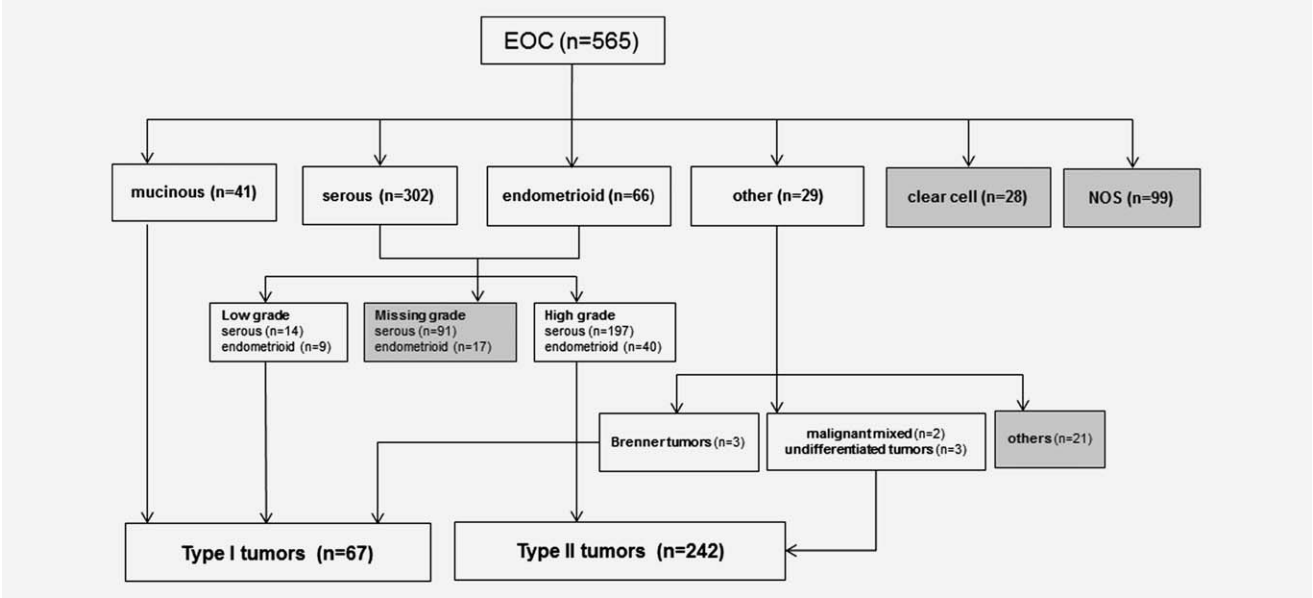

Figure 1. Algorithm for classification of EOC cases in EPIC into type I/type II based on the dualistic model. Marked in grey: exclusion of non-eligible tumors for type I/type II classification ($n = 256$; serous/endometrioid tumors without information on grade; NOS tumors, clear cell or not eligible morphologies from the category other).

was analyzed with direct 'sandwich' immunoradiometric assay (CIS-Bio, Gif-sur-Yvette, France).

Inter-batch CVs ranged from 8% (DHEAS) to 12% (testosterone), with exception of one batch where the interbatch CV for androstenedione was 21.42%. Serum concentrations of free testosterone (*i.e.* the fractions of hormones not linked to binding proteins in the blood) were calculated assuming a constant serum albumin concentration of 43 g/L.[24] The validity of this approach has been confirmed previously.[25,26]

Statistical analyses

Hormone measurements were log2 transformed to achieve approximate normality and centered on the mean value of zero independently for study phase 1 and study phase 2. Case and control differences across baseline characteristics were assessed using conditional logistic regression. We used Pearson partial rank correlation coefficients (r) adjusted for age at blood donation and EPIC recruitment center to assess correlations between reproductive variables and endogenous androgens, as well as between endogenous androgens.

Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated using conditional logistic regression models. The risk associated with serum concentrations of androgens, and SHBG was examined in tertiles with phase-specific cutoff points, based on the distribution in all study controls. To test for trends in ovarian cancer risk as a function of increasing hormone concentrations, models were fitted for hormone concentrations on the log2-transformed continuous scale.

Covariates changing the OR by more than 10% (*i.e.*, by a factor 1.10 or its reciprocal) were retained in the multivariate logistic regression models.[27] The final adjusted model included body mass index (BMI; continuous), ever full term pregnancy (never/ever) and past HRT use (never/ever). Missing values (<6%) were accounted for by creating an extra category in each covariate. Additional covariates that were evaluated but which were not included in the final model were age at menarche (continuous), age at menopause (continuous), age at first pregnancy (nulliparous, continuous), number of full term pregnancies (0, 1, 2, $\leq$3), duration of past OC use (never; <5, 5–10, >10 years), duration of past HRT use (never; <5, 5–10, >10 years), average lifetime alcohol consumption level (0, >0–<3 g/d, 3–19 g/d, and >19 g/d), physical activity (active, moderately active, moderately inactive, and inactive) and height (cm, continuous). Heterogeneity in the associations between androgens and EOC by tumor characteristics was assessed using likelihood-ratio tests for the comparison of the model fit for logistic regression models with and without corresponding interaction terms.[28]

Sensitivity analyses included stratification by menopausal status at blood draw and age at diagnosis (<55 and $\geq$55); exclusion of women providing a blood sample <2 years prior to diagnosis and women who had a prior hysterectomy ($n = 118$). In sensitivity analyses, women above the limit of detection for SHBG were set to the upper limit of detection ($n = 13$; 178 nmol/L: highest value that could be extrapolated from the assay's standard curve). In analyses stratified by menopausal status at blood donation, we combined postmenopausal and perimenopausal women as circulating concentrations of androgens and SHBG gradually change with age, but do not further vary by menopausal status.[29]

All statistical tests were two-tailed and significant at the $p < 0.05$ level. SAS v. 9.2 (SAS Institute Inc., Cary, NC) was used for all statistical analysis.

Table 1. Selected characteristics of EOC cases in the EPIC study—overall cases and by histologic subtypes [median (range); number (percentage)]

	All cases (n = 565)	Serous (n = 302)	Mucinous (n = 41)	Endometrioid (n = 66)	Clear cell (n = 28)	NOS (n = 99)	Other (n = 29)	p_{het}[1]
Age at blood donation	57 (33.6–80.7)	56.3 (33.6–73.3)	54.5 (35.9–66.6)	55.8 (36.1–71.5)	54.8 (35.2–64.8)	59.7 (42.4–77.3)	63.4 (40.5–80.7)	
Age at diagnosis	63.6 (37.4–86.5)	63.6 (41.2–82.6)	60.2 (37.4–76.6)	61.2 (42–78.4)	60.7 (45.6–71.8)	66 (47.8–86.5)	66.4 (46.2–86.4)	<0.01
Lagtime	6.7 (0–16)	7 (0–16)	5.6 (0.2–16)	5.4 (0.3–13.8)	8 (0.4–13.4)	6.8 (0.1–14.1)	6.1 (1–15.6)	0.37
Stage[a]								<0.01
Localized	76 (15%)	28 (10%)	15 (45%)	18 (30%)	7 (27%)	7 (10%)	1 (5%)	
Regional	83 (17%)	41 (14%)	5 (15%)	15 (25%)	13 (50%)	7 (10%)	2 (9%)	
Metastatic	337 (68%)	215 (76%)	13 (39%)	27 (45%)	6 (23%)	57 (80%)	19 (86%)	
Grade[2]								<0.01
Well differentiated	35 (10%)	14 (7%)	10 (43%)	9 (18%)	1 (10%)	1 (3%)		
Moderately differentiated	121 (35%)	77 (36%)	10 (43%)	16 (33%)	4 (40%)	10 (28%)	4 (29%)	
Poorly or undifferentiated	187 (55%)	120 (57%)	3 (13%)	24 (49%)	5 (50%)	25 (69%)	10 (71%)	
Kurman[2]								<0.01
Type I	67 (22%)	14 (7%)	41 (100%)	9 (18%)	–	–	3 (38%)	
Type II	242 (78%)	197 (93%)		40 (82%)	–	–	5 (63%)	

[1]Heterogeneity between histologic subtypes was assessed by general linear regression models.
[2]Percentages are presented for women with available data.

Results

Baseline characteristics of ovarian cancer cases by tumor characteristics are presented in Table 1. Median age at blood donation was 57 years (range 33.6–80.7 years), but differed by ovarian tumors histologic subtypes (*e.g.*, 54.5 years for mucinous tumors *vs.* 63.4 years for tumors classified as "other"; $p \leq 0.01$). The median age at diagnosis for cases was 63.6 years (range: 37.4–86.5 years), and varied between tumors of different histologic subtypes (*e.g.*, 60.2 years for mucinous *vs.* 63.6 years for serous and 66.4 years for "other" tumors; $p \leq 0.01$). The median time between blood donation and cancer diagnosis was 6.7 years (range 0–16 years) (Table 1). We observed significant differences in tumor grade, stage, and type I/type II status between the histologic subtypes. For example, a higher proportion of mucinous than serous tumors were locally limited at diagnosis (45% *vs.* 10%, $p < 0.01$) and mucinous tumors were more likely to be well differentiated at diagnosis, relative to the other subtypes ($p < 0.01$). Tumors were predominantly type II in all histologic subtypes, with the exception of mucinous carcinomas, which are type I by definition.

We evaluated the associations between risk factors for ovarian cancer by tumor characteristics among cases and their matched controls. We observed statistically significant differences for parity (cases with serous ($p = 0.03$), mucinous ($p = 0.03$), and NOS ($p < 0.01$) tumors were less likely to be parous than their matched control subjects) and past use of oral contraceptives (less frequent among cases with serous ($p < 0.01$) and NOS ($p < 0.01$) tumors compared to their matched controls). Cases with endometrioid carcinomas were more frequently past HRT users as compared to their matched control subjects ($p = 0.02$) and cases with clear cell ($p = 0.04$) and NOS ($p = 0.02$) tumors had significant higher BMI than their matched controls.

Geometric mean concentrations were not significantly different among cases compared with controls overall or within study phases (data not shown). Androstenedione concentrations were higher among cases with mucinous tumors ($p = 0.05$) and lower for cases with serous tumors ($p = 0.03$), relative to their matched controls. We observed no other case–control differences in hormone concentrations (Table 2).

In conditional logistic regression models, none of the hormone measurements showed any significant association with the overall risk of EOC (all subtypes combined; Table 3), and there was no significant association when comparing top *vs.* bottom tertiles of the hormone measurements. Examining EOC by subtype, however, androstenedione concentrations were found to be significantly and inversely associated with risk of developing serous tumors ($OR_{log2} = 0.79$ [0.64–0.97]). We observed statistically significant heterogeneity for androstenedione across tumor grade categories (low-grade tumors ($OR_{log2} = 1.99$ [0.98–4.06]; high-grade tumors ($OR_{log2} = 0.75$ [0.61–0.93]; $p_{het} < 0.01$). Furthermore, a doubling of androstenedione concentration was associated with a 99% increase

Table 2. Selected baseline characteristics of EOC cases and matched controls at enrolment in the EPIC study overall and by histologic subtypes [median (range) or number (percentage)]

	Controls (n = 1,097)	All cases (n = 565)	Serous (n = 302)	Mucinous (n = 41)	Endometrioid (n = 66)	Clear cell (n = 28)	NOS (n = 99)	Other (n = 29)
Age at blood donation[1]	56.9 (33.6–79.3)	57 (33.6–80.7)						
Menopausal status[1]								
Pre	219 (20%)	112 (20%)	64 (21%)	12 (29%)	16 (24%)	4 (14%)	9 (9%)	7 (24%)
Post	878 (80%)	453 (80%)	238 (79%)	29 (71%)	50 (76%)	24 (86%)	90 (91%)	22 (76%)
Age at menopause[2,3]	50 (30–59)	**50 (32–60)****	50 (32–60)	50 (45–59)	52 (36–60)	50.5 (42–56)	51 (34–59)	50 (39–55)
Ever fullterm pregnancy[3]								
No	124 (12%)	**95 (17%)***	**47 (16%)****	**11 (28%)****	13 (20%)	5 (19%)	15 (16%)	4 (14%)
Yes	935 (88%)	**448 (83%)***	**243 (84%)****	**28 (72%)****	51 (80%)	21 (81%)	80 (84%)	25 (86%)
Ever OC use[3]								
Never	594 (54%)	**349 (62%)****	**184 (61%)****	21 (51%)	31 (47%)	16 (57%)	**74 (76%)***	23 (79%)
Ever	498 (46%)	**214 (38%)****	**117 (39%)****	20 (49%)	35 (53%)	12 (43%)	**24 (24%)***	6 (21%)
Ever HRT use[3,4]								
Never	867 (86%)	452 (87%)	239 (87%)	32 (94%)	**45 (78%)****	24 (89%)	88 (90%)	24 (86%)
Ever	145 (14%)	69 (13%)	37 (13%)	2 (6%)	**13 (22%)****	3 (11%)	10 (10%)	4 (14%)
BMI[3]	25.3 (15.5–50.6)	**26 (17.8–44.9)***	25.4 (17.8–41.5)	26.2 (18.9–44.2)	25.4 (18.9–40.8)	**26.9 (20.1–44.5)****	**27.3 (19.3–44.6)****	25.5 (20.3–44.9)
Smoking[3]								
Never	644 (59%)	313 (56%)	175 (58%)	16 (39%)	40 (61%)	15 (54%)	48 (50%)	19 (66%)
Former	245 (22%)	128 (23%)	61 (20%)	10 (24%)	14 (21%)	8 (29%)	29 (30%)	6 (21%)
Current	201 (18%)	120 (21%)	65 (22%)	15 (37%)	12 (18%)	5 (18%)	19 (20%)	4 (14%)
Hormones[5]								
Testosterone (nmol/L)[5]	1.12 (1.08–1.15)	1.12 (1.07–1.16)	1.08 (1.02–1.14)	1.27 (1.11–1.45)	1.17 (1.05–1.30)	1.19 (1.04–1.37)	1.07 (0.96–1.18)	1.32 (1.06–1.65)
Free testosterone (pmol/L)[5]	14.93 (14.37–15.52)	15.12 (14.34–15.94)	14.42 (13.42–15.49)	18.23 (15.19–21.88)	15.60 (13.47–18.07)	18.75 (15.21–23.11)	14.55 (12.70–16.68)	16.53 (12.48–21.89)
Androstendione (nmol/L)[5]	2.48 (2.39–2.57)	2.45 (2.33–2.58)	**2.25 (2.10–2.42)****	**3.04 (2.50–3.71)****	2.89 (2.52–3.32)	2.86 (2.32–3.53)	2.35 (2.10–2.62)	2.93 (2.38–3.59)
DHEAS (nmol/L)[5]	1.35 (1.30–1.40)	1.34 (1.27–1.41)	1.27 (1.18–1.36)	1.69 (1.41–2.02)	1.47 (1.27–1.70)	1.64 (1.35–2.00)	1.24 (1.09–1.41)	1.42 (1.11–1.83)
SHBG (nmol/L)[5]	46.53 (44.98–48.14)	45.81 (43.69–48.02)	47.43 (44.51–50.54)	42.94 (35.76v51.56)	47.45 (41.28v54.53)	36.99 (29.70–46.07)	42.37 (37.60–47.75)	51.46 (42.38–62.48)

[1]Matching factor
[2]Among menopausal women only
[3]Significant differences between cases and matched controls based on conditional logistic regression in bold
[4]Among postmenopausal women only (*$p < 0.01$, **$p < 0.05$)
[5]Significant differences in hormone concentrations between cases and matched controls based on geometric means (95% confidence intervals), significant differences in bold (*$p < 0.01$, **$p < 0.05$).

Table 3. Odds ratios (95% CI) for ovarian cancer by tertile concentrations and for doubling in circulating serum testosterone, free testosterone, DHEAS, androstenedione and SHBG by cancer characteristics[1]

	Tertiles					
	1	2	3	OR_{log2} (95% CI)	p_{trend}[2]	p_{het}[3]
Testosterone						
Overall (565 sets)	Ref.	1.25 (0.96–1.62)	1.01 (0.75–1.36)	0.99 (0.83–1.17)	0.88	
Histology						
Serous (302 sets)	Ref.	1.37 (0.96–1.98)	1.08 (0.72–1.61)	0.91 (0.72–1.16)	0.45	0.07[4]
Grade						
Low Grade (35 sets)	Ref.	0.68 (0.22–2.10)	1.61 (0.49–5.26)	1.24 (0.59–2.61)	0.57	
High Grade (306 sets)	Ref.	1.16 (0.81–1.66)	0.95 (0.63–1.42)	0.87 (0.69–1.11)	0.27	0.40
Stage						
Low Stage (76 sets)	Ref.	1.24 (0.61–2.51)	0.80 (0.33–1.91)	1.04 (0.60–1.81)	0.88	
High Stage (419 sets)	Ref.	1.32 (0.97–1.80)	0.98 (0.70–1.39)	0.96 (0.79–1.17)	0.69	0.46
Type I/Type II						
Type I (67 sets)	Ref.	1.31 (0.58–2.93)	1.67 (0.65–4.30)	1.41 (0.79–2.51)	0.25	
Type II (242 sets)	Ref.	1.23 (0.82–1.85)	1.00 (0.64–1.57)	0.89 (0.68–1.15)	0.37	0.19
Free testosterone						
Overall (565 sets)	Ref.	1.03 (0.79–1.34)	0.81 (0.60–1.08)	0.97 (0.85–1.11)	0.68	
Histology						
Serous (302 sets)	Ref.	1.12 (0.79–1.59)	0.67 (0.45–1.00)	0.90 (0.75–1.08)	0.25	**0.02**[4]
Grade						
Low Grade (35 sets)	Ref.	0.91 (0.27–3.10)	1.00 (0.32–3.17)	1.19 (0.65–2.16)	0.57	
High Grade (306 sets)	Ref.	1.10 (0.78–1.56)	0.65 (0.44–0.98)	0.87 (0.73–1.05)	0.16	0.30
Stage						
Low Stage (76 sets)	Ref.	1.47 (0.70–3.11)	1.08 (0.49–2.41)	1.20 (0.81–1.78)	0.35	
High Stage (419 sets)	Ref.	0.98 (0.72–1.33)	0.77 (0.55–1.09)	0.93 (0.80–1.09)	0.35	0.28
Type I/Type II						
Type I (67 sets)	Ref.	1.65 (0.71–3.85)	1.59 (0.66–3.81)	1.25 (0.80–1.94)	0.33	
Type II (242 sets)	Ref.	1.10 (0.74–1.63)	0.64 (0.41–1.00)	0.88 (0.71–1.09)	0.25	0.15
DHEAS						
Overall (565 sets)	Ref.	1.03 (0.79–1.35)	0.99 (0.75–1.32)	0.98 (0.86–1.10)	0.70	
Histology						
Serous (302 sets)	Ref.	1.03 (0.72–1.48)	0.88 (0.60–1.29)	0.89 (0.75–1.05)	0.16	**0.03**[4]
Grade						
Low Grade (35 sets)	Ref.	1.12 (0.31–3.98)	1.67 (0.53–5.22)	1.17 (0.70–1.96)	0.56	
High Grade (306 sets)	Ref.	0.95 (0.66–1.35)	0.77 (0.52–1.14)	0.87 (0.74–1.02)	0.08	0.15
Stage						
Low Stage (76 sets)	Ref.	0.86 (0.40–1.86)	1.00 (0.45–2.21)	1.05 (0.72–1.52)	0.81	
High Stage (419 sets)	Ref.	1.08 (0.80–1.47)	0.94 (0.68–1.32)	0.95 (0.82–1.09)	0.43	0.37
Type I/Type II						
Type I (67 sets)	Ref.	1.15 (0.48–2.72)	1.68 (0.67–4.21)	1.41 (0.93–2.16)	0.11	
Type II (242 sets)	Ref.	0.90 (0.60–1.36)	0.75 (0.48–1.16)	0.86 (0.71–1.02)	0.09	**0.01**
Androstenedione						
Overall (565 sets)	Ref.	0.81 (0.62–1.05)	0.83 (0.61–1.13)	0.95 (0.81–1.10)	0.48	
Histology						
Serouvs (302 sets)	Ref.	0.63 (0.43–0.91)	0.69 (0.45–1.06)	**0.79 (0.64–0.97)**	0.03	< **0.01**

Table 3. Odds ratios (95% CI) for ovarian cancer by tertile concentrations and for doubling in circulating serum testosterone, free testosterone, DHEAS, androstenedione and SHBG by cancer characteristics (Continued)

	Tertiles					
	1	2	3	OR_{log2} (95% CI)	p_{trend}[2]	p_{het}[3]
Grade						
Low Grade (35 sets)	Ref.	3.13 (0.74–13.3)	4.52 (0.96–21.4)	1.99 (0.98–4.06)	0.06	
High Grade (306 sets)	Ref.	0.62 (0.43–0.90)	0.62 (0.41–0.94)	**0.75 (0.61–0.93)**	< 0.01	< **0.01**
Stage						
Low Stage (76 sets)	Ref.	0.60 (0.28–1.28)	0.84 (0.38–1.85)	0.90 (0.60–1.35)	0.62	
High Stage (419 sets)	Ref.	0.86 (0.63–1.17)	0.70 (0.49–1.02)	0.89 (0.74–1.07)	0.21	0.77
Type I/Type II						
Type I (67 sets)	Ref.	1.46 (0.59–3.64)	2.51 (0.94–6.75)	**1.99 (1.18–3.35)**	0.01	
Type II (242 sets)	Ref.	0.52 (0.34–0.79)	0.62 (0.39–0.99)	**0.71 (0.57–0.90)**	< 0.01	<**0.01**
			SHBG			
Overall (565 sets)	Ref.	0.77 (0.59–1.00)	1.08 (0.82–1.43)	1.04 (0.90–1.20)	0.59	
Histology						
Serous (302 sets)	Ref.	0.81 (0.56–1.17)	1.25 (0.85–1.83)	1.14 (0.93–1.39)	0.19	<0.01[4]
Grade						
Low Grade (35 sets)	Ref.	0.96 (0.32–2.86)	0.78 (0.24–2.48)	0.93 (0.53–1.63)	0.80	
High Grade (306 sets)	Ref.	0.77 (0.53–1.11)	1.36 (0.93–1.99)	1.17 (0.95–1.44)	0.14	0.53
Stage						
Low Stage (76 sets)	Ref.	0.60 (0.27–1.34)	0.69 (0.32–1.51)	0.85 (0.57–1.28)	0.45	
High Stage (419 sets)	Ref.	0.81 (0.59–1.10)	1.10 (0.79–1.52)	1.08 (0.91–1.28)	0.38	0.47
Type I/Type II						
Type I (67 sets)	Ref.	0.65 (0.28–1.50)	0.84 (0.36–1.92)	0.98 (0.64–1.50)	0.92	
Type II (242 sets)	Ref.	0.77 (0.51–1.16)	1.43 (0.92–2.21)	1.18 (0.93–1.49)	0.17	0.42

[1]Matched for study center, age at blood donation, menopausal status, time of the day of blood collection, fasting status and phase of the menstrual cycle and additional adjusted for BMI (continuous scale), ever full term pregnancy (never/ever), HRT use (never/ever)
[2]Linear trends for OR estimates using log2 transformed endogenous hormones and SHBG
[3]Statistical tests for heterogeneity were based on likelihood-ratio test, comparing the model fit for logistic regression models with and without corresponding interaction term
[4]Subgroups of endometrioid, mucinous, clear cell, NOS and other tumors are presented in Supporting Information Table 1, p_{het} is presented for all histological subtypes.

in risk of type I tumors ($OR_{log2} = 1.99$ [1.18–3.35]), and a 29% decrease in risk of type II tumors ($OR_{log2} = 0.71$ [0.57–0.90]; $p_{het} < 0.01$). The same pattern was observed for DHEAS with statistical significant heterogeneity ($OR_{log2} = 1.41$ [0.93–2.16]; for type I tumors; $OR_{log2} = 0.86$ [0.71–1.02]; for type II tumors; $p_{het} = 0.01$). None of the other androgens showed significant associations with any of the other ovarian tumor characteristics, as defined by histology, grade and stage or type I/II classification (Table 3; Supporting Information Table 1). We observed statistically significant heterogeneity between histological subtypes, tumor grade and type I/type II tumors for several hormones although heterogeneity was most evident for androstendione (Table 3).

Subgroup analyses showed similar associations between endogenous androgens and EOC risk for women by age at diagnosis (<55 *vs.* ≥55) or women who were either pre- or postmenopausal at blood collection, with the exception of androstenedione and serous tumors by menopausal status at blood collection (premenopausal ($OR_{log2} = 0.96$ [0.55–1.67]; postmenopausal ($OR_{log2} = 0.76$ [0.61–0.95]; $p_{het} = 0.37$; data not shown). Further, we observed some heterogeneity for risk associations with SHBG by menopausal status at blood collection. While the heterogeneity was not statistically significant, SHBG was positively associated with EOC in women premenopausal at blood collection but not associated with EOC in women postmenopausal at blood collection (premenopausal: $OR_{log2} = 1.43$ [1.03–1.97]; postmenopausal: $OR_{log2} = 0.95$ [0.81–1.12], $p_{het} = 0.08$). A similar pattern was observed for the serous histologic subgroup stratified by menopausal status at blood collection (premenopausal: $OR_{log2} = 1.88$ [1.17–3.04]; postmenopausal: $OR_{log2} = 1.01$ [0.81–1.26], $p_{het} = 0.04$). Also, among serous tumors, we observed significant heterogeneity in the strength of associations of SHBG with risk by age at diagnosis (<55 *vs.* ≥55), with 64% increase in risk in women age <55 years of age at diagnosis and no association in women older than age ≥55

at diagnosis (age at diagnosis: <55: $OR_{log2} = 1.64$ [1.03–2.62]; age at diagnosis ≥55: $OR_{log2} = 1.03$ [0.83–1.29]; $p_{het} = 0.02$), data not shown.

Results from sensitivity analyses excluding women with hysterectomy (without bilateral oophorectomy) were not markedly different from overall results. Results were similar after excluding women diagnosed within 2 years after blood donation, with the exception of the association between DHEAS and serous tumors. This association was strengthened after excluding women diagnosed in the 2 years after blood donation (including women diagnosed <2 years after blood donation: $OR_{log2} = 0.89$ [0.75–1.05]; excluding women diagnosed <2 years after blood donation <2 years: OR_{log2}: 0.80 [0.67–0.96]). Overall, risk estimates were similar when analyses were restricted to phase 2 participants. Exceptions were free testosterone in NOS tumors (phase 1 *vs.* 2: $p = 0.01$) and DHEAS among women premenopausal at blood donation ($p \leq 0.01$) or age <55 at diagnosis ($p \leq 0.01$)); however, case numbers were limited (case *n* range: 35–71) and individual effect estimates were not statistically significant except for free testosterone and NOS tumors (phase 1, case $n = 35$, OR: 0.50 [0.27–0.92]; phase 2, case $n = 57$, OR: 1.54 [1.0–2.37]; $p = 0.01$). Tumor characteristics were consistent across study phases ($p \geq 0.19$).

Finally, while androgen concentrations were stable across storage methods/temperature (range: −70 to −196°C; $p > 0.11$), SHBG concentrations differed (geometric means of SHBG: −196°C = 56.6 nmol/L; −150°C: 69.6 nmol/L; −70°C: 54.4 nmol/l; $p < 0.01$). While we matched on center, in practice matching for storage conditions, we excluded participants ($n = 83$ cases/148 controls) whose samples had been stored at −150°C in sensitivity analyses, given that SHBG was higher in this subgroup. Results after this exclusion were similar to overall results (*e.g.* SHBG in premenopausal women: overall: $OR_{log2} = 1.43$ [1.03–1.97], excluding samples stored at −150°C: $OR_{log2} = 1.27$ [0.92–1.77] *p* for difference = 0.61).

Discussion

With a total of 565 cases and 1,097 matched controls, this is the largest prospective study to date on the relationship between pre-diagnostic endogenous androgens and SHBG and EOC, and the first to examine the relationship by tumor characteristics (histology, grade and stage) and following the dualistic model of ovarian carcinogenesis according to type I/type II classification. We found no overall association between pre-diagnostic androgens and SHBG concentrations with risk of overall EOC. However, in analyses stratified by histologic subtype and clinical tumor characteristics, androstenedione was inversely associated with risk of serous tumors, high-grade tumors, and type II tumors and positively associated with low-grade tumors and type I tumors.

Four prospective studies[11–14] including a prior analysis within the EPIC cohort[12] have addressed the association of circulating androgens with risk of EOC overall, with only one observing significant associations between androgens and ovarian cancer risk (case $n = 31$;[14]). Of the three studies with data by menopausal status at blood collection,[11–13] two, including our own previous analysis,[12] showed non-significant inverse associations between androstenedione and EOC in postmenopausal women and one showed direct associations between androstenedione and EOC among premenopausal women.[13] However, in a US study of cases and controls identified in the combined Nurses' Health Study cohorts (NHS) I and II and the Women's Health Study (WHS) no associations between circulating androgens and overall ovarian cancer risk were observed, although for androstenedione and DHEAS there was a suggestive inverse association, particularly for women diagnosed with EOC at age ≥55 or older (case $n = 224$;[11]). A statistically significant association with free testosterone was observed in postmenopausal women in the previous EPIC study but not in any of the other studies.[12] Inconsistency of results from previous studies may be due to heterogeneous inclusion criteria with respect to major tumor characteristics and risk factors (*i.e.* inclusion of non-epithelial tumors,[14] invasive and borderline tumors[11,12] and additional peritoneal cancers,[11] restricting to ovarian tumors[14] or invasive tumors,[13] exclusion of women with previous hysterectomy or unilateral oophorectomy[12]). Moreover, the previously unaddressed heterogeneity of EOC may contribute to inconsistency in prior findings, as so far EOC has been analyzed exclusively as a single disease.

Recently, the paradigm of the origin of ovarian cancer has shifted to one of five distinct diseases (high-grade serous, low-grade serous, endometrioid, clear cell and mucinous carcinomas) developing along two pathways (type I/II) and with hypothesized extra-ovarian origin for the majority of tumors (*i.e.* fallopian tube fimbria and endometrium[6,8]). Type I tumors (low-grade serous, low-grade endometrioid, mucinous and malignant Brenner tumors) have been claimed to develop in a step-wise manner from borderline tumors or endometriosis and are characterized by specific mutations including *ARID1A,* KRAS, BRAF, *PIK3CA,* PTEN, and *RNF43* with relative genetically stability.[8] In contrast, type II tumors (the majority of which are high-grade serous carcinomas) are aggressive and typically present at advanced stage. These tumors are characterized by genetically instability and a very high frequency of TP53 mutations, but only occasionally they harbor the mutations characteristic of type I tumors.[30]

Our results suggest a positive association between androstenedione (an intermediate in the testosterone synthesis pathway) in low-grade/type I tumors, and an inverse association for serous and high-grade/type II tumors. However, if androgenic effects were of major importance for ovarian carcinogenesis, we would expect stronger associations with total and/or free testosterone and EOC, as those are the most potent androgens. Explaining the observed associations of androstenedione, but not any other androgens with EOC risk is challenging. Androgen action involves a complex interaction of biosynthesis, metabolism and receptor activation. Androstenedione is of both adrenal and ovarian origin (premenopausal women: 50% ovarian; postmenopausal women: 20% ovarian), whereas DHEAS is an adrenal derived pre-androgen and testosterone is

predominantly produced in the ovary. Androgen receptors (AR) and estrogen receptors (ER) are expressed throughout the reproductive system including ovaries, the fallopian tubes and endometrium.[31,32] Androstenedione and DHEAS have low binding affinity for AR while testosterone and dihydrotestosterone (DHT) have highest AR affinity and androgenic activity. Prior research suggests EOC tumors express the AR with differences by histological subtype.[33] Research in serous cancer cell lines suggests that stimulation of the AR leads to an increase in proliferation and a decrease in apoptosis in the ovarian surface epithelium[34] suggesting more potent androgens (*e.g.* testosterone, DHT) may play a role in ovarian carcinogenesis.[31] However, AR expression in serous carcinomas was associated with improved survival in one study ($n = 90$).[35]

Androgens may have a direct impact on ovarian carcinogenesis, or act via their function as precursors to estrogens. Androstenedione is the preferential substrate for aromatase, and higher androstenedione synthesis may lead to an increased synthesis of estrogens.[31] The aromatase inhibitor letrozole, has shown to inhibit the progression ERα positive ovarian cancer in mice.[36] It has been demonstrated *in vitro* that androstenedione is the DHEA metabolite with the greatest ERα agonist activity in embryonic kidney cells and ERß in human hepatocytes.[37] To our knowledge, there is no data in ovarian cancer cells. Given its affinity for the ER, one might speculate that androstendione affects EOC risk *via* these receptors. The expression of ER in general[38] and especially ERα has been associated with longer overall survival in ovarian cancer patients,[39] whereas the expression of ERβ2 in advanced serous ovarian cancer has been associated with an unfavorable prognosis.[40] A recently published pooled analysis on the role of hormone receptors in ovarian cancer survival[38] evaluating tumors by histologic subtype showed no association between ER expression and survival in mucinous or low-grade serous carcinomas, both type I by definition. Recently it has been shown that ERα was expressed in both type I and type II tumors. However, the ERα signaling pathway may be defective in a proportion of type II tumors.[41]

There are no previous data on androgens and EOC by tumor histology, grade or type I/II status. However, prospective data suggest a positive association between serum androgen concentrations in pregnant women and risk of non-epithelial sex cord stromal tumors (SCST); these tumors are of intra-ovarian origin with the majority of SCST diagnosed between ages 30 and 50[42] similarly to low grade/type I tumors in our study (diagnosed between the ages of 37 and 76 in our study population). Recently, the role of androgenic stimulation has been hypothesized for Brenner tumors, also type I tumors, based on the epithelial and stromal expression of AR together with expression of markers of steroidogenesis (calretinin, inhibin, and steroidogenic factor 1) in stromal cells surrounding the epithelial nests.[43]

While circulating SHBG has been proposed to play a role in ovarian carcinogenesis,[44] previous prospective studies have not shown associations between SHBG and ovarian cancer risk.[12,13] We observed a significant positive association between SHBG and EOC among women who were premenopausal at blood collection. Stratified analyses by BMI showed increased risk for women with BMI < 25, whereas the opposite was observed for women with BMI ≥ 25. To avoid possible bias due to weight loss shortly before cancer diagnosis we excluded women diagnosed within 2 years after study recruitment in sensitivity analyses; results were similar after this exclusion. Given the inverse correlation between SHBG and free testosterone (the majority of testosterone is bound to SHBG); we hypothesized an inverse association between SHBG and EOC risk. The observed positive associations warrant further investigation.

Although this is the largest prospective study to date on androgens, SHBG and ovarian cancer risk, and the first to evaluate risk of EOC by important subtypes, case numbers are small for many tumor subtypes and we restricted our analyses to invasive cases. Further, we included cases and controls from a previous study in EPIC.[12] Different assays were used for androgens in the two study phases, however to allow for differences in phases we used phase-specific cut off points for tertiles. We standardized biomarker concentrations to a mean of zero, which may obscure phase-specific differences in distributions. However, we compared results for doubling of biomarker concentrations in phases 1 and 2 and observed similar results. The possibility of chance findings for androstenedione cannot be excluded. However, analyses were hypothesis driven. A further limitation of this and previous studies is that participants provided a single blood sample. The stability of hormone measurements over time has been shown previously for a period over at least 2 to 3 years, for both premenopausal[45,46] and postmenopausal women,[47] however, a single measurement may not accurately reflect a woman's average blood concentration over longer time periods. Circulating hormone concentrations may not adequately reflect concentrations at the tissue level and paracrine exposure may be of specific importance for ovarian cancer because the ovarian surface epithelium is not vascular.[1] However, in postmenopausal women (which comprise the majority of our study population (80%)) hormone concentrations in the homogenates of ovarian tissue correlate with circulating testosterone ($r = 0.79$, $p \leq 0.01$) and androstenedione ($r = 0.30$, $p \leq 0.01$).[48] To our knowledge, there are no data on the association between circulating and ovarian tissue androgens in premenopausal women. Finally, it is plausible that the observed inverse association between androstendione and high grade/type II tumors is due to underlying ovarian pathology altering androstenedione synthesis. However, results excluding women diagnosed within 2 years of blood donation were essentially unchanged.

In this study we observed increased risk of low grade or type I tumors and decreased risk of serous, and high-grade or type II tumors (predominantly of serous histology) with higher concentrations of circulating androstenedione. These data support current hypotheses on distinct etiopathogenesis by tumor subtype. Larger pooled studies are needed to confirm our findings. Collecting tumor tissue in order to correlate circulating hormone concentrations with receptor status with EOC risk might be of further importance to explore androgen-related carcinogenesis in EOC subtypes.

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