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Reproductive and Hormonal Factors in Relation to Ovarian Cancer Risk and Survival

Amy L. Shafrir

A Dissertation Submitted to the Faculty of

The Harvard T.H. Chan School of Public Health

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Dissertation Advisor: Dr. Shelley Tworoger Amy L. Shafrir

Reproductive and Hormonal Factors in Relation to Ovarian Cancer Risk and Survival

Abstract

Ovarian cancer etiology is not fully elucidated and few modifiable risk factors have been identified. Ovarian cancer treatment has changed over time; however, survival is still poor. I investigated reproductive and hormonal factors in relation to ovarian cancer risk and survival. In the Nurses' Health Study (NHS), NHSII and New England Case-Control Study (NECC), I evaluated reproductive and hormonal factors and incidence of ovarian cancer characterized by estrogen receptor-α (ERα) and progesterone receptor (PR) status using polytomous logistic regression. In the NHSII, I investigated oral contraceptive (OC) use and ovarian cancer risk using Cox proportional hazards models. In the NECC, I evaluated the association of pre-diagnostic reproductive and hormonal factors with overall survival and platinum resistance among ovarian cancer cases using Cox proportional hazards models. Postmenopausal status was associated with an increased risk of PR- tumors (OR: 2.07; 95%CI: 1.15-3.75; p-heterogeneity=0.01) and age at natural menopause was inversely associated with PR- tumors (OR, per 5yr: 0.77; 95%CI: 0.61-0.96; p-het=0.01). Increasing duration of postmenopause was differentially associated by PR status (p-het=0.0009). In OC analyses, OC use of ≤6 months was associated with an increased risk of ovarian cancer (HR: 1.53; 95%CI: 1.00-2.35) and a suggestion of a decreased risk with ≥10 years of OC use (HR: 0.84; 95%CI: 0.51-1.39) compared to never use. The increased risk appeared to be driven by duration of mestranol use. In survival analyses, self-reported endometriosis was associated with a 29% (95%CI: 0.54-0.94) decreased risk of total death. Additionally, longer duration of hormone therapy (HT) was associated with a decreased risk of death (HR, ≥5yr vs. never: 0.70; 95%CI: 0.54-0.89). Further ≥5 years of HT

use was associated with a decreased risk of platinum resistance. While our results need to be confirmed in other studies, they suggest that the development of ovarian tumors through hormonal pathways differs by menopausal status, the association between OC use and ovarian cancer differs between younger and older birth cohorts, and that various reproductive and hormonal factors are associated with ovarian cancer survival. These results may help in further understanding ovarian cancer etiology and providing recommendations for ovarian cancer prevention and survival.

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Introduction

Ovarian cancer is the 11th most common cancer among women in the US, with 22,280 expected new cases in 2016, and is the 5th most common cause of cancer death among women, with 14,240 expected deaths in 2016 [1]. The overall 5-year survival for ovarian cancer is 46%; however, survival decreases with increasing stage and over 60% of cases are diagnosed with distant disease for which the 5-year survival is only 28% [2]. No effective early detection or screening exists for ovarian cancer and changes in treatment have not had a substantial impact on ovarian cancer mortality [1]. Thus, improving prevention recommendations and better understanding ovarian cancer etiology and survival are critical for reducing incidence and mortality from this deadly disease.

One of the challenges in ovarian cancer research is accounting for tumor heterogeneity, as risk factors may be differentially associated with ovarian cancer subtypes. For example, the association between reproductive risk factors and ovarian cancer has been shown to vary across ovarian cancer histologic subtypes [3-10]. Differential associations with reproductive risk factors and ovarian cancer were also observed for tumor behavior (borderline/invasive), dominance, and aggressiveness [4, 8, 11, 12]. The differential expression of other tumor markers such as estrogen receptor-α (ERα) and progesterone receptor (PR) are known to be important in the epidemiology and prognosis of breast cancer; [13-16] differential expression of these markers may also be important for ovarian tumors.

Several mechanisms have been used to describe the pathogenesis of ovarian cancer including ovulatory and hormonal factors. The 'incessant ovulation' hypothesis was the first hypothesis to

describe the pathogenesis of ovarian cancer [17]. During ovulation, ovarian surface epithelial (OSE) cells undergo wound healing and repair due to the trauma induced by ovulation. The decreased risk of ovarian cancer among long term users of oral contraceptives (OCs) provides evidence for the 'incessant ovulation' hypothesis as women with longer durations of OC use have on average fewer ovulatory cycles over a lifetime compared to never OC users [18]. However, as argued by Risch (1998), the reduction in risk due to one pregnancy is greater than the reduction in risk due to the missed ovulations during that pregnancy [19-21]. Additionally, during ovulation, OSE cells and the fallopian tubes are bathed in follicular fluid rich in hormonal and inflammatory factors. Therefore, Risch proposed that while ovulation may be involved in at least part of the etiology of ovarian cancer, other factors must be at play, namely hormonal factors including estrogen and progesterone [19].

As stated above, the majority of ovarian cancer cases are diagnosed at an advance stage when prognosis is poor. Approximately 80% of women will relapse after first-line platinum and taxane-based chemotherapy [2, 22], with relapse within 6 months of ending treatment (platinum resistance) associated with a worse outcome [23]. Demographic factors and tumor characteristics have been consistently associated with worse ovarian cancer survival; however, it is remains unclear how pre-diagnostic risk factors may influence survival from ovarian cancer [24-33]. Identifying reproductive and hormonal factors associated with survival and platinum resistance may elucidate mechanisms that lead to worse outcomes.

In the following three manuscripts, we sought to (1) understand the association between reproductive and hormonal factors and ovarian tumors classified based on ERα and PR status in

two prospective cohort studies, Nurses' Health Study (NHS) and NHSII, and one population-based case-control study, New England Case-Control Study (NECC); (2) investigate the relationship between OC use and ovarian cancer within the NHSII, a younger birth cohort than previous studies on OCs and ovarian cancer; and (3) assess the association between reproductive and hormonal factors and ovarian cancer survival and resistance to platinum-based chemotherapy in the NECC.

Part I: The association between reproductive and hormonal factors and ovarian cancer by estrogen-α and progesterone receptor status

Amy L. Shafrir[1,2], Megan S. Rice[2], Mamta Gupta[3], Kathryn L. Terry[1,4], Bernard A. Rosner[2,5], Rulla M. Tamimi[1,2], Jonathan L. Hecht[3]*, Shelley S. Tworoger[1,2]*

Affiliations:

[1]Department of Epidemiology, Harvard TH Chan School of Public Health, Boston, MA, USA

[2]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

[3]Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA

[4]Obstetrics and Gynecology Epidemiology Center, Department of Obstetrics and Gynecology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

[5]Department of Biostatistics, Harvard TH Chan School of Public Health, Boston, MA, USA

*These authors contributed equally to this work.

Abstract

Background: We assessed the association between reproductive and hormonal factors and incidence of ovarian cancer characterized by estrogen receptor-α (ERα) and progesterone receptor (PR) status.

Methods: Tissue microarrays were used to assess ERα and PR expression among 197 Nurses' Health Study (NHS), 42 NHSII and 76 New England Case-Control Study (NECC) epithelial ovarian cancer cases. NHS/NHSII cases were matched to up to 4 controls (n=954) on diagnosis date and birth year. NECC controls (n=725) were frequency matched on age. Cases were defined as receptor positive if at least 1% of tumor cells stained positive. Associations between risk

factors and ovarian cancer by ERα and PR status were assessed using polytomous logistic regression.

Results: 45% of ovarian tumors were PR+, 78% were ERα+ and 45% were ERα+/PR+, while 22% were ERα-/PR-. Postmenopausal status was associated with an increased risk of PR- tumors (OR: 2.07; 95%CI: 1.15-3.75; p-heterogeneity=0.01) and age at natural menopause was inversely associated with PR- tumors (OR, per 5yr: 0.77; 95%CI: 0.61-0.96; p-het=0.01). Increasing duration of postmenopause was differentially associated by PR status (p-het=0.0009). Number of children and tubal ligation were more strongly associated with ERα- versus ERα+ tumors (p-het=0.002 and 0.05, respectively). No differential associations were observed for oral contraceptive or hormone therapy use.

Conclusion: Postmenopausal women have an increased risk of developing PR- ovarian tumors compared to premenopausal women.

Impact: The associations observed for ovarian cancer differ from those seen for breast cancer suggesting that the biology for tumor development through ERα and PR pathways may differ.

Introduction

One challenge in understanding ovarian cancer etiology is accounting for tumor heterogeneity. Histologic subtypes of ovarian cancer have different gene and protein expression patterns in addition to morphological differences [34-37]. For example, p53 mutations are observed in most high-grade serous ovarian tumors; however, they are rarely observed in other histologies [34, 35].

Further, ovarian cancer risk factor associations differ by histology [3]. For example, the inverse association observed for parity and tubal ligation generally was stronger for endometrioid and clear cell ovarian tumors than for serous tumors [3-6, 8, 10, 21, 38, 39]. Differential associations for ovarian cancer risk factors with other tumor characteristics, including dominance and aggressiveness, have also been reported [11, 12]. An alternate approach is to characterize ovarian tumors by hormone receptor expression patterns, specifically estrogen receptor-α (ERα) and progesterone receptor (PR). It is well established that differences in the expression of ERα and PR are important in the epidemiology and prognosis of breast cancer [13-16]; however research on this topic is limited for ovarian tumors. We previously reported differential risk factor associations by ERα and PR status in a small study of 157 ovarian tumors in the Nurses' Health Study (NHS) [40]. Specifically, increasing age was positively associated with ERα+ tumors but inversely associated with ERα- tumors while postmenopausal versus premenopausal women had a decreased risk of PR+ but an increased risk of PR- ovarian cancer. In this study, we updated our previous analysis including 40 additional ovarian tumors from NHS, 42 tumors from NHSII and 76 tumors from the New England Case-Control (NECC) study, more than doubling our previous sample size.

Methods

Study population

The NHS began in 1976 when 121,700 US female nurses aged 30-55 completed baseline questionnaires collecting data on disease diagnoses and exposures. In 1989, NHSII began when 116,430 US female nurses, aged 25-42, completed baseline questionnaires. In both cohorts, follow-up questionnaires were mailed to study participants biennially to obtain updated

information on exposure and disease diagnoses. Incident cases of ovarian cancer were identified by biennial questionnaire, linkage to the National Death Index [41], the postal service or family members. Follow-up for both cohorts has been 85-90%. The institutional review board of the Brigham and Women's Hospital approved both studies.

The NECC is a population-based case-control study in Eastern Massachusetts and New Hampshire conducted in five waves between 1978 and 2008 [42, 43]. We included cases of epithelial ovarian cancer and controls from the final wave (2003-2008) recruited within Eastern Massachusetts. Cases were recruited through tumor registries of area hospitals and controls through town books. Exclusion criteria included: <18 years old, moved, had no phone, did not speak English, died, their physician declined permission to contact them (cases only), or had a prior bilateral oophorectomy (controls only). In the 2003-2008 wave, 68.3% of eligible cases and 51.2% of eligible controls enrolled [42, 43]. Cases and controls were frequency matched on age. The institutional review boards of the Brigham and Women's Hospital and Dartmouth Medical School approved the study.

Ovarian tumor block collection

We included cases diagnosed from 1976 to 2006 for NHS, 1989 to 2005 for NHSII and 2003 to 2008 for NECC. A gynecologic pathologist, blinded to exposure status, reviewed all pathology reports to confirm the diagnosis of epithelial ovarian cancer and classify tumors by behavior (invasive and borderline), histologic type (serous/poorly differentiated, mucinous, endometrioid, clear cell, other) and stage (I/II, III/IV). We requested representative paraffin-embedded tissue blocks of ovarian tumors for NHS and NHSII confirmed cases with a pathology report and

surgery and for NECC cases with an invasive tumor, no neoadjuvant chemotherapy, and surgery at Brigham and Women's Hospital, Boston, MA. Of the 1,083 confirmed NHS cases through 2006 and 201 NHSII confirmed cases through 2005, 73% and 62%, respectively, were eligible for tumor block collection. We received blocks for 314 NHS and 59 NHSII cases and had appropriate tissue on 27% and 37% of eligible cases, respectively, for tissue microarrays (TMAs). In NECC, 564 confirmed invasive cases were identified, of whom 40% were eligible for collection and 35% (n=78) of the eligible were included on the TMA. In NHS and NHSII, up to four controls (n=954) with no prior bilateral oophorectomy or menopause due to irradiation, no prior diagnosis of cancer (except non-melanoma skin cancer) and alive at the time of case diagnosis were matched to each case on year of birth. In NECC, controls recruited in Eastern Massachusetts from 20003-2008 (n=725) were included.

Assays

For each case, the pathologist (JH, MG) selected a primary tumor block, confirmed histology and behavior and assessed grade (I, II, III) [44], circled the area of tumor on the slide, and sent the block/slide to the Dana-Farber/Harvard Cancer Center Specialized Histopathology Services Core for TMA construction. Three cores per case were extracted using 0.6 mm (NHS/NHSII) or 1 mm (NECC) diameter hollow needles. Cores were transferred to a recipient paraffin-embedded block and sections were cut to create array slides, which were processed and stained within 2 weeks. Details of the immunohistochemistry process have been described previously [40]. Briefly, slides were soaked in Xylene overnight, deparaffinized and antigens were retrieved and stained with the primary antibodies: ERα (rabbit monoclonal; clone SP1; Neomarkers; dilution 1:40) and PR (mouse monoclonal; clone PgR 636; Dako; dilution 1:150). ERα was detected using the Leica

Bond III autostainer (Leica Biosystems) and PR was detected using biotin-free horseradish peroxidase enzyme-labeled polymer conjugated to anti-mouse secondary antibodies (Envision+ Systems; Dako). A gynecologic pathologist (JH) assessed the number of reactive vs. total cells (0%, 1-10%, 11-50%, 51-90%, >90%). The three cores for each case were scored independently. The maximum score of the three cores for each case was used in analyses [45]. Cores were designated not interpretable if tissue was missing from the slide or only a few cell clusters (<20 cells) were present.

Assessment of exposure and covariate information

Details of exposure assessment have been described previously [40, 46]. We assessed the following exposures and covariates by biennial self-reported questionnaires (NHS/NHSII) and in-person interviews (NECC): age at diagnosis, age at menarche, oral contraceptive use, tubal ligation, parity, menopausal status, age at menopause, hormone therapy (HT), and family history of breast/ovarian cancer. Calculation of age at natural menopause excluded women reporting a hysterectomy before menopause. Ovulatory years were calculated as age at natural menopause (or current age for premenopausal women) minus age at menarche with subtraction of 1 year for each pregnancy and duration of OC use. Duration of premenopause was calculated as current age, for premenopausal women, or age at natural menopause minus age at menarche, while duration of postmenopause was calculated as current age minus age at natural menopause (premenopausal women were coded as zero for this variable). Time since menopause was calculated among postmenopausal women only.

Statistical Analysis

As the distribution of ERα and PR was similar across the studies, we pooled the data for all primary analyses. Cases (and their associated matched controls in NHS/NHSII) were excluded from analyses if all three cores for that case had only non-tumor tissue (n=2 NHS) or non-interpretable ERα and PR stains (n=11 NHS, n=2 NHSII, n=1 NECC). In primary analyses, ERα and PR status was considered positive if $\geq$1% of cells stained positive and negative if 0% of cells stained positive based on the maximum score of the cores. The same cut point is used for breast cancer in clinical practice [47]. As in breast cancer, cases scored as ERα-/PR+ (n=7 NHS, n=2 NHSII, n=2 NECC) were excluded from analyses as ERα status is most likely misspecified among these cases [48]. The distribution of ERα and PR staining positivity by histologic subtype and stage were assessed using Chi-square test and grade was assessed using Chi-square test for trend. We calculated the Spearman's correlation between the maximum ERα and PR scores.

We assessed the relationship between reproductive and hormonal factors and ovarian cancer risk by receptor staining positivity using polytomous logistic regression (PLR) with a three category outcome (ERα+, ERα- and controls or PR+, PR- and controls). Analyses were also conducted using joint ERα/PR status (ERα+/PR+, ERα+/PR-, ERα-/PR-, and controls). Exposure and covariate information for an NHS and NHSII case and its matched control was taken from the questionnaire cycle prior to case diagnosis. For each exposure, we assessed the heterogeneity in the odds ratios (ORs) by ERα or PR expression status versus controls using a likelihood ratio test comparing a model where the association between the exposure of interest and ovarian cancer risk was allowed to vary by hormone receptor expression to another model where the association was not allowed to vary [49]. We adjusted for age at diagnosis and cohort, allowing the estimates

to vary by receptor status, as well as family history of breast and/or ovarian cancer, duration of OC use, number of pregnancies, and menopausal/HT status. Women missing OC duration were set to the median and a separate missing indicator was created.

To determine whether differential risk factor associations by ERα or PR status were explained by histology, we used an unconditional logistic regression model in case-case analyses, where receptor positive tumors were considered "cases" and receptor negative tumors were considered "controls". We controlled for the same covariates above as well as histology (serous, endometrioid, clear cell, other) and used methods of Jun *et al* (2010) to determine if histology explained a portion of the association between the exposure of interest and ERα or PR expression [50].

In sensitivity analyses, we used a 10% cutpoint for ERα and PR staining positivity, restricted to invasive cases only, and accounted for grade (grade I/II, grade III, unknown grade) in case-case analyses. Between-cohort heterogeneity was tested using a likelihood ratio test including interaction terms between the exposure of interest and indicators for cohort.

To assess the possibility of selection bias within our samples, we compared the distribution of the exposures of interest and tumor characteristics between all confirmed ovarian cancer cases, cases eligible for tumor block collection, cases with collected tumor blocks and cases included on the TMA.

Analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA.) or Stata version 11.0 (StataCorp LP, College Station, TX, USA). P-values were 2-sided and considered statistically significant if less than 0.05.

Results

Tumor characteristics

We had 315 cases (NHS=197, NHSII=42, NECC=76) and 1,679 controls (NHS=786, NHSII=168, NECC=725). 78% of ovarian tumors expressed ERα while 45% expressed PR (Table 1.1); 45% were ERα+/PR+, 32% were ERα+/PR- and 22% were ERα-/PR-. There was a modest correlation between ERα and PR expression (ρ=0.42; $p<0.0001$), which differed by histology ranging from 0.19 for serous to 0.64 for mucinous tumors. The distribution of ERα and PR expression varied significantly by histology ($p<0.0001$). A greater proportion of serous and endometrioid tumors expressed ERα and PR versus clear cell and mucinous tumors. The distribution of joint ERα and PR expression differed by histology; the majority of serous tumors were either ERα+/PR+ (50%) or ERα+/PR- (39%) while the majority of clear cell tumors were ERα-/PR- (84%) (Supplemental Table S1.1). PR expression also differed by grade ($p<0.0001$), with lower grade tumors more likely to be PR+ and Grade III tumors more likely to be PR-. More ERα+ tumors were classified as high stage compared to ERα- tumors ($p<0.0001$).

Table 1.1 Distribution of ovarian cancer cases by estrogen-α (ERα) and progesterone (PR) receptor status in the Nurses' Health Study, Nurses' Health Study II and New England Case-Control Study

	All cases	**ERα+[a]**	**ERα-**	**PR+[a]**	**PR-**
Total, n (%)[b]	315 (100%)	245 (78%)	70 (22%)	143 (45%)	172 (55%)
Histology, n (%)					
Serous	195 (62%)	174 (71%)	21 (30%)	98 (69%)	97 (56%)
Endometrioid	51 (16%)	43 (18%)	8 (11%)	30 (21%)	21 (12%)
Mucinous	16 (5%)	8 (3%)	8 (11%)	5 (4%)	11 (6%)
Clear cell	25 (8%)	4 (2%)	21 (30%)	1 (1%)	24 (14%)
Other	28 (9%)	16 (7%)	12 (17%)	9 (6%)	19 (11%)
P [c]			<0.0001		<0.0001
Grade, n (%)[d]					
I	26 (10%)	24 (12%)	2 (3%)	20 (17%)	6 (4%)
II	54 (20%)	40 (19%)	14 (22%)	32 (27%)	22 (14%)
III	190 (70%)	143 (69%)	47 (75%)	66 (56%)	124 (82%)
P [e]			0.14		<0.0001
Stage, n (%)					
I/II	117 (37%)	77 (31%)	40 (57%)	57 (40%)	60 (35%)
III/IV	198 (63%)	168 (69%)	30 (43%)	86 (60%)	112 (65%)
P			<0.0001		0.36

a. Ovarian tumors were classified as ERα+ and PR+ if 1% or greater of cells stained positive
b. Total N excludes 16 cases with unreadable ERα and PR staining and 10 cases scored as ERα-/PR+
c. P-value calculated using Chi-square test comparing ERα+ vs. ERα- and PR+ vs. PR-
d. 270 cases had data on grade
e. P-value calculated using Chi-square test for trend

Comparison to full sample

Confirmed NHS and NHSII cases, cases eligible for tumor block collection, cases with tumor blocks and cases on the TMA were similar with respect to reproductive/hormonal factors and tumor characteristics except that TMA cases were older at diagnosis, had lower stage cancer, and a shorter lapse time from date of diagnosis to date of tissue request (Supplemental Tables S1.2 and S1.3). A greater proportion of NHSII TMA cases were parous compared to confirmed cases (76% vs. 70%, respectively); however, the mean number of children among parous women was similar. All confirmed invasive NECC cases, cases eligible for block collection and TMA cases

were similar with respect to reproductive/hormonal factors and tumor characteristics, except that TMA cases were slightly older and had higher stage tumors (Supplemental Table S1.4).

Reproductive and hormonal factors by ERα expression

We observed significant heterogeneity between ERα+ and ERα- tumors for number of children and tubal ligation, with stronger protective associations for ERα- versus ERα+ tumors (Table 1.2). Each additional child was associated with a 28% decreased risk of ERα- (95%CI=0.60–0.87) compared to a non-significant 3% decreased risk of ERα+ tumors (95%CI=0.89–1.06; p-heterogeneity=0.002). Histology explained approximately 82% of the differential association between number of children and ERα status (p=0.03) (Supplemental Table S1.5). For tubal ligation, there was a stronger protective effect for ERα- (OR=0.25; 95%CI=0.09–0.69) versus ERα+ tumors (OR=0.65; 95%CI=0.43–0.97; p-het=0.05). Histology did not explain this differential association. ERα status was not associated with age at diagnosis (p=0.37). No significant heterogeneity by ERα status was observed for oral contraceptive use, HT use, age at menarche, or menopause-related variables (p-het≥0.07; Table 1.2).

Table 1.2 multivariate associations between reproductive and hormonal factors and ovarian cancer by estrogen-α and progesterone receptor status in the NHS, NHSII and NECC using 1% as the cutpoint for staining positivity[a]

			OR (95% CI)			OR (95% CI)		
	Controls	**Cases**	**ERα+**	**ERα-**	*P-hetero*	**PR+**	**PR-**	*P-hetero*
Parity								
Nulliparous	174	45	1.00 (Ref)	1.00 (Ref)	0.23	1.00 (Ref)	1.00 (Ref)	0.72
Parous	1504	269	0.59 (0.38, 0.91)	0.37 (0.19, 0.71)		0.56 (0.33, 0.94)	0.49 (0.30, 0.80)	
Nulliparous	174	45	1.00 (Ref)	1.00 (Ref)	0.01	1.00 (Ref)	1.00 (Ref)	0.73
1-2 children	732	136	0.62 (0.39, 0.98)	0.55 (0.29, 1.06)		0.60 (0.35, 1.04)	0.60 (0.36, 1.00)	
3-4 children	625	93	0.50 (0.31, 0.81)	0.16 (0.07, 0.38)		0.47 (0.26, 0.84)	0.34 (0.20, 0.60)	
5+ children	147	40	0.81 (0.46, 1.44)	0.26 (0.09, 0.78)		0.70 (0.34, 1.46)	0.59 (0.31, 1.15)	
Per child	1678	314	0.97 (0.89, 1.06)	0.72 (0.60, 0.87)	0.002	0.91 (0.81, 1.02)	0.93 (0.84, 1.03)	0.77
Oral Contraceptive (OC) use								
Never	650	154	1.00 (Ref)	1.00 (Ref)	0.28	1.00 (Ref)	1.00 (Ref)	0.60
Ever	1029	161	0.75 (0.56, 1.01)	0.55 (0.32, 0.93)		0.75 (0.52, 1.10)	0.66 (0.47, 0.94)	
Never[b]	650	154	1.00 (Ref)	1.00 (Ref)	0.41	1.00 (Ref)	1.00 (Ref)	0.81
>0-<1 year	187	34	0.79 (0.50, 1.26)	0.59 (0.25, 1.39)		0.86 (0.49, 1.53)	0.65 (0.37, 1.15)	
1-<5 years	372	72	0.97 (0.67, 1.39)	0.55 (0.27, 1.10)		0.99 (0.63, 1.56)	0.76 (0.49, 1.18)	
5-<10 years	246	32	0.57 (0.34, 0.93)	0.62 (0.28, 1.36)		0.55 (0.29, 1.03)	0.62 (0.35, 1.07)	
10+ years	192	14	0.30 (0.15, 0.61)	0.47 (0.18, 1.26)		0.33 (0.14, 0.79)	0.36 (0.17, 0.77)	
Per 5 years[b]	1647	306	0.68 (0.55, 0.85)	0.78 (0.56, 1.09)	0.51	0.72 (0.55, 0.92)	0.70 (0.55, 0.90)	0.91
Tubal ligation								
Never	1345	279	1.00 (Ref)	1.00 (Ref)	0.05	1.00 (Ref)	1.00 (Ref)	0.51
Ever	334	36	0.65 (0.43, 0.97)	0.25 (0.09, 0.69)		0.62 (0.37, 1.03)	0.49 (0.29, 0.83)	
Menopausal status at diagnosis[b]								
Premeno	480	69	1.00 (Ref)	1.00 (Ref)	0.23	1.00 (Ref)	1.00 (Ref)	0.01
Postmeno	1134	230	1.10 (0.68, 1.77)	1.93 (0.85, 4.37)		0.77 (0.43, 1.39)	2.07 (1.15, 3.75)	
Hormone therapy (HT) use[b,c]								
Never	574	83	1.00 (Ref)	1.00 (Ref)	0.78	1.00 (Ref)	1.00 (Ref)	0.35

Ever	511	130	1.62 (1.14, 2.31)	1.78 (0.98, 3.26)		1.39 (0.86, 2.25)	1.84 (1.25, 2.73)	
Never	574	83	1.00 (Ref)	1.00 (Ref)	0.74	1.00 (Ref)	1.00 (Ref)	0.44
>0-<5 years	272	54	1.16 (0.75, 1.81)	1.53 (0.76, 3.08)		1.18 (0.66, 2.10)	1.30 (0.81, 2.11)	
5+ years	238	76	2.20 (1.47, 3.31)	2.16 (1.05, 4.44)		1.68 (0.95, 2.96)	2.57 (1.64, 4.02)	
Per 5 years	1084	213	1.43 (1.24, 1.64)	1.17 (0.88, 1.54)	0.16	1.40 (1.17, 1.68)	1.36 (1.17, 1.58)	0.80
Estrogen-only HT use[b,c,d]								
Never	835	132	1.00 (Ref)	1.00 (Ref)	0.35	1.00 (Ref)	1.00 (Ref)	0.83
Ever	215	72	2.24 (1.53, 3.27)	1.59 (0.82, 3.11)		2.17 (1.29, 3.65)	2.02 (1.34, 3.06)	
Never	835	132	1.00 (Ref)	1.00 (Ref)	0.77	1.00 (Ref)	1.00 (Ref)	0.95
>0-<5 years	115	32	1.75 (1.06, 2.89)	1.41 (0.60, 3.32)		1.76 (0.89, 3.49)	1.61 (0.93, 2.77)	
5+ years	98	37	2.66 (1.64, 4.33)	1.96 (0.81, 4.71)		2.68 (1.38, 5.21)	2.38 (1.39, 4.07)	
Per 5 years	1048	201	1.48 (1.25, 1.74)	1.21 (0.86, 1.71)	0.25	1.49 (1.20, 1.85)	1.39 (1.16, 1.67)	0.57
Age at menarche								
Per year	1669	312	0.99 (0.90, 1.09)	1.16 (0.99, 1.35)	0.07	0.97 (0.86, 1.09)	1.08 (0.97, 1.20)	0.16
Age at natural menopause[b,c]								
Per 5 years	996	169	0.96 (0.77, 1.20)	0.74 (0.52, 1.04)	0.20	1.23 (0.89, 1.72)	0.77 (0.61, 0.96)	0.01
Duration of premenopause[b,e]								
Per 5 year	1466	236	1.08 (0.92, 1.27)	0.93 (0.72, 1.19)	0.29	1.14 (0.93, 1.41)	0.97 (0.81, 1.16)	0.19
Duration of postmenopause[b,e]								
Per 5 year	1466	236	0.99 (0.90, 1.12)	1.07 (0.90, 1.27)	0.40	0.85 (0.73, 1.00)	1.11 (0.98, 1.26)	0.001
Ovulatory years in quartiles[b,f]								
Quartile 1	359	31	1.00 (Ref)	1.00 (Ref)	0.28	1.00 (Ref)	1.00 (Ref)	0.05
Quartile 2	365	71	2.34 (1.39, 3.95)	2.10 (0.89, 4.96)		1.60 (0.85, 3.02)	3.19 (1.68, 6.08)	
Quartile 3	334	57	2.04 (1.17, 3.55)	2.01 (0.82, 4.91)		1.85 (0.95, 3.59)	2.41 (1.22, 4.76)	
Quartile 4	381	70	2.57 (1.50, 4.39)	1.19 (0.45, 3.12)		2.59 (1.37, 4.90)	2.03 (1.02, 4.03)	
Per 5 years	1439	229	1.19 (1.05, 1.36)	1.05 (0.84, 1.31)	0.33	1.28 (1.08, 1.52)	1.07 (0.92, 1.25)	0.10

a. Analyses were adjusted for age at diagnosis (per year), cohort (NHS/NHSII/NECC), family history of breast/ovarian cancer (yes/no), duration of OC use (per month), menopausal status (premenopausal/postmenopausal ever HT/postmenopausal never HT), and number of pregnancies (continuous)

b. Total N does not add up to 1994 due to missingness in the exposure (parity missing=2; age at menarche missing=13; OC missing=41; menopause missing=81; ever HT use missing=66; duration HT use miss=67; E-only HT use missing=110; duration E-only HT use missing=115; age at natural menopause missing=199; duration of post/pre-menopause missing=292; ovulatory years missing=326)

c. Among postmenopausal women
d. In ever/never analyses additionally adjusted for ever estrogen plus progestin HT use (yes/no), ever other HT use (yes/no) and in duration analyses additionally adjusted for duration of estrogen plus progestin HT use and duration of other HT use in duration analyses
e. Not adjusted for age at diagnosis
f. Ovulatory year was calculated as age at natural menopause (or age at diagnosis for premenopausal women) minus age at menarche with additional subtraction of one year of each pregnancy and OC duration

Reproductive and hormonal factors by PR expression

Factors relating to menopausal status were differentially associated by PR status (Table 1.2). Postmenopausal (versus premenopausal) women had a 2-fold increased risk of PR- tumors (OR=2.07; 95%CI=1.15–3.75) and a non-significant decreased risk of PR+ tumors (OR=0.77; 95%CI=0.43–1.39; p-het=0.01). Each five year increase in age at natural menopause was associated with a non-significant increased risk of PR+ tumors (OR=1.23; 95%CI=0.89–1.72), but a significant decreased risk of PR- tumors (OR=0.77; 95%CI=0.61–0.96; p-het=0.01). Duration of postmenopause was associated with a suggestively decreased risk of PR+ tumors and increased risk of PR- tumors (p-het=0.0009) while duration of premenopause was not differentially associated by PR status (p-het=0.19). Each five year increase in time since menopause was associated with a 20% (95%CI=0.67–0.96) decreased risk of PR+ tumors (p-het=0.02). A stronger positive association was observed for ovulatory years with PR+ versus PR- tumors (p-het=0.05). Accounting for histology did not significantly alter any of these risk estimates (Supplemental Table S1.5). In case-case analyses, age at diagnosis was differentially associated by PR status (p=0.04). Women aged $\geq$55 years versus <55 were 73% (95%CI=1.03–2.93) more likely to develop PR- versus PR+ tumors. No significant heterogeneity by PR status was observed for parity, oral contraceptive use, HT use, tubal ligation, or age at menarche (p-het>0.16).

ER/PR joint analyses

There was significant heterogeneity between number of children and joint ERα and PR expression (p-het=0.002; Table 1.3). Increasing number of children was associated with a significantly protective effect for ERα-/PR- tumors (OR=0.72; 95%CI=0.60–0.86), but not for

ERα+/PR+ or ERα+/PR- tumors. Significant heterogeneity was observed for menopausal status and age at natural menopause with joint ERα and PR expression (p-het=0.03 and 0.05, respectively). For example, postmenopausal status was positively associated with both ERα+/PR- tumors (OR=2.51; 95%CI=1.06–5.96) and ERα-/PR- tumors (OR=1.91; 95%CI=0.84–4.33) but with a non-significant decreased risk of ERα+/PR+ tumors. Each five year increase in duration of postmenopause was associated with a suggestive decreased risk of ERα+/PR+ tumors (OR=0.85; 95%CI=0.73–1.00), but a suggestive increased risk of ER α+/PR- tumors (OR=1.14; 95%CI=0.99–1.32; p-het=0.003).

Sensitivity analyses

Results obtained using the 10% cutpoint were similar to those using the 1% cutpoint for staining positivity (data not shown). Significant heterogeneity was observed between cohorts for HT, estrogen-only HT, and duration of postmenopause. The heterogeneity was driven by NECC and when considering NHS/NHSII only, the associations were similar to the main findings (data not shown). Adjustment for grade did not substantially alter the main findings (data not shown). Generally results were similar for invasive cases, except that age at menarche was differentially associated with ovarian cancer by ERα status (p=0.04), with an increased risk of ERα- tumors (OR=1.20; 95%CI=1.03–1.40) and there was significant heterogeneity for ovulatory years by PR status, with a stronger positive association for PR+ versus PR- tumors (OR=1.40; 95%CI=1.16-1.69 for PR+ vs. OR=1.06; 95%CI=0.91-1.24 for PR-; p-het=0.02 (data not shown).

Table 1.3 Association between reproductive and hormonal factors and ovarian cancer by joint ERα and PR status in the NHS, NHSII and NECC using 1% as the cutpoint for staining positivity[a]

		ERα+/PR+		ERα+/PR-		ERα-/PR-		
	Controls	**N**	**OR (95% CI)**	**N**	**OR (95% CI)**	**N**	**OR (95% CI)**	*P-hetero*
Parity								
Never	174	21	1.00 (Ref)	10	1.00 (Ref)	14	1.00 (Ref)	0.44
Ever	1504	122	0.56 (0.33, 0.94)	91	0.66 (0.33, 1.33)	56	0.37 (0.19, 0.71)	
Per child	1678	143	0.90 (0.80, 1.02)	101	1.05 (0.93, 1.18)	70	0.72 (0.60, 0.86)	0.002
Oral Contraceptive (OC) use								
Never	650	63	1.00 (Ref)	55	1.00 (Ref)	36	1.00 (Ref)	0.55
Ever	1029	80	0.75 (0.51, 1.10)	47	0.76 (0.49, 1.17)	34	0.55 (0.32, 0.93)	
Per 5 years[b]	1647	139	0.72 (0.55, 0.92)	98	0.63 (0.45, 0.90)	69	0.78 (0.56, 1.09)	0.69
Tubal ligation								
Never	1345	124	1.00 (Ref)	89	1.00 (Ref)	66	1.00 (Ref)	0.14
Ever	334	19	0.62 (0.37, 1.03)	13	0.71 (0.39, 1.30)	4	0.25 (0.09, 0.69)	
Menopausal status at diagnosis[b]								
Premeno	480	44	1.00 (Ref)	9	1.00 (Ref)	16	1.00 (Ref)	0.03
Postmeno	1134	89	0.77 (0.43, 1.39)	89	2.51 (1.06, 5.96)	52	1.91 (0.84, 4.33)	
Hormone therapy (HT) use[b,c]								
Never	574	35	1.00 (Ref)	29	1.00 (Ref)	19	1.00 (Ref)	0.63
Ever	511	45	1.39 (0.86, 2.25)	53	1.89 (1.16, 3.08)	32	1.78 (0.98, 3.26)	
Per 5 years	1084	80	1.40 (1.17, 1.68)	82	1.45 (1.22, 1.71)	51	1.17 (0.88, 1.54)	0.36
Estrogen-only HT use[b,c,d]								
Never	835	49	1.00 (Ref)	48	1.00 (Ref)	35	1.00 (Ref)	0.64
Ever	215	27	2.17 (1.29, 3.64)	31	2.31 (1.40, 3.81)	14	1.59 (0.82, 3.11)	
Per 5 years	1048	75	1.49 (1.21, 1.85)	77	1.46 (1.19, 1.79)	49	1.21 (0.86, 1.71)	0.50
Age at menarche								
Per year	1669	141	0.97 (0.86, 1.09)	101	1.02 (0.89, 1.17)	70	1.16 (1.00, 1.35)	0.23
Age at natural menopause[b,c,e]								
Per 5 years	950	65	1.21 (0.87, 1.69)	64	0.78 (0.59, 1.03)	35	0.72 (0.51, 1.03)	0.05

Duration premenopause[b,f]								
Per 5 years	1466	110	1.14 (0.93, 1.41)	72	1.00 (0.79, 1.26)	54	0.93 (0.72, 1.20)	0.38
Duration postmenopause[b,f]								
Per 5 years	1466	110	0.85 (0.73, 1.00)	72	1.14 (0.99, 1.32)	54	1.06 (0.89, 1.27)	0.003
Ovulatory years in quartiles[b,g]								
Quartile 1	359	18	1.00 (Ref)	5	1.00 (Ref)	8	1.00 (Ref)	0.09
Quartile 2	365	26	1.60 (0.84, 3.01)	27	4.95 (1.86, 13.1)	18	2.10 (0.89, 4.95)	
Quartile 3	334	25	1.85 (0.95, 3.59)	16	3.08 (1.10, 8.67)	16	2.01 (0.82, 4.91)	
Quartile 4	381	38	2.59 (1.37, 4.89)	21	3.35 (1.22, 9.22)	11	1.19 (0.45, 3.12)	
Per 5 years	1439	107	1.28 (1.08, 1.52)	69	1.09 (0.89, 1.34)	53	1.05 (0.84, 1.31)	0.27

a. Analyses were adjusted for age at diagnosis (per year), cohort (NHS/NHSII/NECC), family history of breast/ovarian cancer (yes/no), duration of OC use (per month), menopausal status (premenopausal/postmenopausal ever HT/postmenopausal never HT), and number of pregnancies (continuous)

b. Total N does not add up to 1994 due to missingness in the exposure

c. Among postmenopausal women

d. In ever/never analyses additionally adjusted for ever estrogen plus progestin HT use (yes/no), ever other HT use (yes/no) and in duration analyses additionally adjusted for duration of estrogen plus progestin HT use and duration of other HT use in duration analyses

e. Excludes NHSII cases and controls due to small sample size

f. Not adjusted for age at diagnosis

g. Ovulatory year was calculated as age at natural menopause (or age at diagnosis for premenopausal women) minus age at menarche with additional subtraction of one year of each pregnancy and OC duration

Discussion

In the current study we observed a modest correlation between ERα and PR expression for ovarian tumors, with 45% of the ovarian tumors expressing both ERα and PR; however the proportion greatly varied by histology. Factors relating to timing of menopause were differentially associated with ovarian cancer by PR expression. Overall, the associations suggested that postmenopausal women had an increased risk of PR- ovarian tumors compared to premenopausal women. Tubal ligation and number of children were differentially associated by ERα expression although the association for number of children appeared to be explained by histology, specifically variations in ERα expression by endometrioid and clear cell tumors.

The one previous study on this topic was conducted among 157 NHS ovarian cancer cases (also included in this analysis), using a 10% cutpoint to define positivity [40]. Overall, the results were similar for menopausal status, including the increased risk of PR- tumors among postmenopausal versus premenopausal women. However, the prior study observed a differential association of age by ERα expression. This difference may be due to the increased sensitivity of the ERα antibody used in the current study; while 94% of cores staining positive for ERα using the previous antibody also stained positive using the new antibody, 48% of cores that stained negative with the previous antibody were classified as ERα+ with the new antibody.

The distribution of ERα and PR expression within our study is similar to other studies, although the proportion of ERα+/PR+ ovarian tumors was slightly higher in our study [51, 52], possibly due to differences in the cutpoint used to define positivity and the antibodies used. One consistent finding across studies is the small proportion of clear cell ovarian tumors that express

either ERα or PR [40, 53-55]. Additionally, we observed that a higher proportion of PR- ovarian tumors were high grade compared to PR+ tumors, which is consistent with the finding that women with PR- tumors have worse survival compared to women with PR+ tumors [55]. In comparison to breast cancer, combined ERα+/ PR+ expression is lower among ovarian tumors; approximately 30-45% of ovarian tumors and 50-65% of breast tumors express both hormone receptors [40, 51, 52, 56-59]. PR expression is a downstream marker of ER activation by estrogen [60] and given that many ovarian tumors are PR-, this suggests that a large proportion of these tumors are not estrogen sensitive. Additionally, while basal-like breast tumors are molecularly similar to high-grade serous ovarian tumors, hormone receptor expression patterns differ between the two [61]. Between 55% and 85% of basal-like breast tumors are ERα-/PR- [62], while only 11% of high-grade serous tumors were ERα-/PR- in our study. Overall, this suggests that the underlying biology of tumor development related to hormone receptor pathways may differ between breast and ovarian cancers. In ovarian tumors, the loss of PR function may be an important marker of tumor progression. These results underline the importance of further investigating ERα and PR regulation in ovarian cancer.

Factors relating to the timing of menopausal status had differential associations by PR expression. Postmenopausal women were more likely to develop PR- and less likely to develop PR+ tumors compared to premenopausal women and later age at natural menopause was inversely associated PR- tumors, irrespective of ER expression. Increasing duration of postmenopause was associated with a greater risk of developing PR- versus PR+ tumors, particularly ER+/PR- tumors, suggesting that dysregulation of ER signaling and/or loss of PR may be important drivers in ovarian cancer development among postmenopausal women. These

results are in contrast with those observed for breast cancer in which premenopausal women tend to develop PR- tumors while postmenopausal women tend to develop PR+ tumors [15, 56-58, 63, 64] suggesting that the mechanism leading to PR loss or ER dysregulation differs between ovarian and breast cancer.

The biologic mechanism underlying the increase in PR- ovarian tumors among postmenopausal women is not clear. Ovarian cancer cell lines and primary tissue cultures tend not to express PR [65]. One hypothesis is that lower hormone levels among postmenopausal women may lead to ERα dysregulation leading to the loss of PR; however, conflicting results have been reported on the regulation of PR by estrogen in ovarian cancer cells. In mouse xenografts with PE04 (ERα+/PR+) ovarian cancer cells, administration of estrogen up-regulated PR expression [63, 64], but estradiol led to the downregulation of PR expression in two other ER+/PR+ ovarian cancer cell lines [66]. A second hypothesis is that lower hormone levels among postmenopausal women leads to less promotion of ERα+/PR+ tumors. Spillman *et al* (2010) observed that administration of estradiol versus vector in ovariectomized mice implanted with ERα+/PR+ PE04 ovarian cancer cells led to increased tumor growth [63]. This suggests that decreases in estradiol may slow the progression of ERα+/PR+ tumors among postmenopausal women. Finally, other changes that occur after menopause may affect PR expression of ovarian tumors including higher gonadotropin levels and lower progesterone levels. Future research should investigate the role of ERα dysregulation and PR loss in the development of ovarian tumors after menopause and the role of estrogen in promoting tumors in both the pre- and post-menopausal periods.

Only two reproductive factors were differentially associated with ovarian cancer by ERα status; number of children and tubal ligation. For both, we observed a stronger protective association for ERα- tumors compared to ERα+ tumors. However, the differential association observed for parity was likely due to the differential distribution of ERα expression by histology. The protective association for parity appears to be strongest for endometrioid and clear cell ovarian tumors, particularly endometrioid tumors [67-70]; however, clear cell tumors are predominantly ERα-, whereas endometrioid tumors are predominantly ERα+. The results for number of children and ERα expression were also the opposite as those noted for breast cancer, where parity is inversely associated with ERα+/PR+ tumors but either positively or not associated with ERα-/PR- tumors [14, 15, 71].

Interestingly, while tubal ligation has been most strongly associated with endometrioid and clear cell tumors (similar to parity), accounting for histology did not explain the stronger association of tubal ligation with preventing ERα- tumors. Endometrioid and clear cell cancers may develop from endometriosis precursors [34, 72] and tubal ligation may prevent ovarian cancer by blocking of endometriosis from reaching the ovary [3, 67, 73]. Akahane *et al* 2005 observed that endometriosis and atypical endometriosis that developed into clear cell cancer appeared to undergo a gradual reduction in ERα expression with malignant transformation [74]. The opposite was observed for endometrioid tumors in which there was up-regulation of ERα with malignant transformation. This suggests that loss of ERα may be an early step in the developmental process of clear cell cancer. Additionally, ovarian tumor development may be influenced by substances other than estrogen, such as inflammatory agents or oxidative injury, which may be prevented from reaching the ovarian surface with tubal ligation. These agents may interact with

ovarian cells to lead to the downregulation of ERα expression. Future research should investigate factors that may influence loss of ERα and the significance of such loss in clear cell tumor development.

Our study was based on a modest sample size, especially for the histology and joint ERα/PR analyses, limiting our power to detect associations and thus our results should be replicated in a larger study. As ERα and PR expression are continuous, the use of a dichotomous score may have been an oversimplification. However, we did observe similar results when using a 10% cutpoint. One major strength of our study is that exposure information in both the NHS and NHSII studies was collected prospectively. However, the NECC study could be subject to recall bias as cases and controls were asked to recall past exposures, although results were similar when considering the NHS/NHSII alone. A further strength was the use of tissue microarrays that allowed for the simultaneous staining of many cases, thus reducing variability in results due to differences assay variation over time.

The results of this study highlight that the development of ovarian and breast cancers through ERα and PR pathways is potentially different and future research should be directed at understanding how ERα and PR expression influence ovarian carcinogenesis. Additionally, our results suggest that the loss of PR function may be important in the development of ovarian tumors particularly among older, postmenopausal women. Future research should focus on the factor(s) driving the loss of PR expression among postmenopausal women, the possibility of ERα dysregulation in the development of ovarian cancer, particularly in clear cell tumors, and the possibility that ERβ as well as ERα may play a role in ovarian cancer development. This may

help elucidate the mechanisms through which different ovarian cancer histologic subtypes develop.

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Appendix S1

Supplemental Table S1.1 Histology distribution of ovarian cancer cases by ERα and PR expression in the NHS, NHSII and NECC[a]

	All cases	ERα+/PR+	ERα+/PR-	ERα-/PR-
Total, n (%)[b]	315 (100%)	143 (45%)	102 (32%)	70 (22%)
Histology, n (%)				
Serous	195 (62%)	98 (69%)	76 (75%)	21 (30%)
Endometrioid	51 (16%)	30 (21%)	13 (13%)	8 (11%)
Mucinous	16 (5%)	5 (4%)	3 (3%)	8 (11%)
Clear cell	25 (8%)	1 (1%)	3 (3%)	21 (30%)
Other	28 (9%)	9 (6%)	7 (7%)	12 (17%)
Grade, n (%)[c]				
I	26 (10%)	20 (17%)	4 (4%)	2 (3%)
II	54 (20%)	32 (27%)	8 (9%)	14 (22%)
III	190 (70%)	66 (56%)	77 (87%)	47 (75%)
Stage, n (%)				
I/II	117 (37%)	57 (40%)	20 (20%)	40 (57%)
III/IV	198 (63%)	86 (60%)	82 (80%)	30 (43%)

a. Ovarian tumors were classified as ERα+ and PR+ if 1% or greater of cells stained positive

b. Total N excludes 16 cases with unreadable ERα and PR staining and 10 cases scored as ERα-/PR+

c. 270 cases had data on grade

Supplemental Table S1.2 Comparison of characteristics between confirmed cases, cases eligible for block collection, cases with tumor blocks and cases included on the TMA for the NHS

Characteristic	Confirmed cases (1976-2006)[a]	Cases eligible for block collection (1976-2006)[b]	Cases with tumor blocks	Cases included on TMA
N(%)	1083 (100%)	793 (73%)	314 (29%)	217 (20%)
Age at diagnosis, mean (SD)	61.6 (9.7)	60.5 (9.7)	62.8 (9.1)	62.0 (8.9)
Lapse between date of diagnosis and date of tissue request, years, mean (SD) including request date		11.4 (6.9)[c]	8.5 (5.5)	8.7 (5.8)
Lapse between date of diagnosis and date of tissue request, n (%)				
0 to 5 years		187 (24%)	103 (33%)	80 (37%)
>5 to 10 years		191 (25%)	120 (38%)	68 (31%)
>10 to 27 years		388 (51%)	91 (29%)	69 (32%)
Parity, ever, N (%)[d]	971 (90%)	710 (90%)	289 (92%)	200 (93%)
Number of children among parous women, mean (SD)	3.1 (1.5)	3.1 (1.5)	3.1 (1.6)	3.1 (1.6)
Oral contraceptive use, ever, N (%)	419 (39%)	315 (40%)	138 (44%)	97 (45%)
Tubal ligation, ever, N (%)	122 (11%)	84 (11%)	42 (13%)	25 (12%)
Histology, N (%)				
Serous	725 (67%)	526 (66%)	203 (65%)	136 (63%)
Endometrioid	137 (13%)	108 (14%)	49 (16%)	34 (16%)
Mucinous	83 (8%)	67 (8%)	20 (6%)	15 (7%)
Clear cell	41 (4%)	35 (4%)	15 (5%)	13 (6%)
Other	97 (9%)	57 (7%)	27 (9%)	19 (9%)
Morphology, N (%)				
Borderline	105 (10%)	85 (11%)	33 (11%)	24 (11%)
Invasive	966 (89%)	705 (89%)	281 (89%)	193 (89%)
Unknown	12 (1%)	3 (<1%)	0 (0%)	0 (0%)
Disease Stage, N (%)[e]				
I, II	339 (33%)	255 (33%)	109 (35%)	79 (36%)
III, IV	674 (67%)	520 (67%)	202 (65%)	138 (64%)

a. Includes confirmed ovarian epithelial tumors, primary peritoneal tumors and fallopian tube tumors
b. Confirmed cases were eligible for block collection if we had received a valid pathology report
c. N=766
d. Missing parity: N=7 for confirmed cases, N=6 for eligible cases, N=1 for cases with tumor blocks, N=1 for cases on TMA
e. Missing disease stage: N=70 for confirmed cases, N=18 for eligible cases, N=3 for cases with tumor blocks

Supplemental Table S1.3 Comparison of characteristics between confirmed cases, cases eligible for block collection, cases with tumor blocks and cases included on the TMA for the NHS II

Characteristic	Confirmed cases (1989-2005)[a]	Cases eligible for block collection (1989-2005)[b]	Cases with tumor blocks	Cases included on TMA
N(%)	201 (100%)	124 (62%)	59 (29%)	46 (23%)
Age at diagnosis, mean (SD)	45.9 (5.9)	45.3 (6.2)	46.9 (6.2)	47.4 (6.0)
Lapse between date of diagnosis and date of tissue request, years, mean (SD) including request date		9.5 (4.2)	7.9 (3.7)	7.8 (3.9)
Lapse between date of diagnosis and date of tissue request, n (%)				
0 to 5 years		21 (17%)	14 (24%)	12 (26%)
>5 to 10 years		48 (39%)	29 (49%)	23 (50%)
>10 to 27 years		55 (44%)	16 (27%)	11 (24%)
Parity, ever, N (%)	140 (70%)	82 (66%)	41 (69%)	35 (76%)
Number of children among parous women, mean (SD)	2.1 (1.0)	2.1 (0.9)	2.0 (0.9)	2.0 (0.8)
Oral contraceptive use, ever, N (%)[c]	176 (88%)	107 (86%)	48 (81%)	39 (85%)
Tubal ligation, ever, N (%)	36 (18%)	18 (15%)	7 (12%)	6 (13%)
Histology, N (%)				
Serous	103 (51%)	63 (51%)	28 (47%)	23 (50%)
Endometrioid	34 (17%)	22 (18%)	10 (17%)	10 (22%)
Mucinous	23 (11%)	17 (14%)	10 (17%)	4 (9%)
Clear cell	16 (8%)	14 (11%)	7 (12%)	5 (11%)
Other	25 (12%)	8 (6%)	4 (7%)	4 (9%)
Morphology, N (%)				
Borderline	42 (21%)	35 (28%)	20 (34%)	10 (22%)
Invasive	154 (77%)	88 (71%)	38 (64%)	35 (76%)
Unknown	5 (2%)	1 (1%)	1 (2%)	1 (2%)
Disease Stage, N (%)[d]				
I, II	105 (56%)	72 (59%)	39 (66%)	27 (59%)
III, IV	82 (44%)	50 (41%)	20 (34%)	19 (41%)

a. Includes confirmed ovarian epithelial tumors, primary peritoneal tumors and fallopian tube tumors
b. Confirmed cases were eligible for block collection if we had received a valid pathology report
c. Missing oral contraceptive use: N=4 for confirmed cases, N=2 for time eligible cases, N=1 for cases with tumor blocks, N=0 for cases on TMA
d. Missing disease stage: N=14 for confirmed cases, N=2 for time eligible cases

Supplemental Table S1.4 Comparison of characteristics between confirmed invasive cases, cases eligible for block collection and cases included on the TMA for the New England case-control study

Characteristic	Confirmed invasive cases[a]	Cases eligible for block collection[b]	Cases included on TMA
N(%)	564 (100%)	224 (40%)	78 (14%)
Age at diagnosis, mean (SD)	56.8 (10.5)	57.3 (10.0)	58.6 (10.5)
Parity, ever, N (%)	388 (69%)	158 (71%)	56 (72%)
Number of children among parous women, mean (SD)	2.4 (1.3)	2.6 (1.4)	2.9 (1.5)
Oral contraceptive use, ever, N (%)	333 (59%)	129 (58%)	42 (54%)
Tubal ligation, ever, N (%)	64 (11%)	19 (8%)	6 (8%)
Histology, N (%)			
Serous	332 (60%)	143 (65%)	51 (65%)
Endometrioid	114 (21%)	44 (20%)	12 (15%)
Mucinous	28 (5%)	8 (4%)	2 (3%)
Clear cell	35 (6%)	13 (6%)	7 (9%)
Other	55 (10%)	16 (7%)	6 (8%)
Disease Stage, N (%)[c]			
I, II	234 (42%)	80 (36%)	24 (31%)
III, IV	328 (58%)	144 (64%)	54 (69%)

a. Confirmed invasive ovarian cancer cases from Eastern Massachusetts

b. Eligible cases that received surgery at Brigham and Women's Hospital, Boston, MA and had an invasive tumor and no neoadjuvant chemotherapy

c. Missing disease stage: N=2 confirmed invasive cases

Supplemental Table S1.5 Case-case analysis of the association between reproductive and hormonal factors and ovarian cancer by ERα and PR status using 1% as the cutpoint for staining positivity[a]

	ERα+ vs. ER-			PR+ vs. PR-		
	Multivariate Model[b]	**Multivariate Model + Histology[c]**	**Proportion of effect mediated by histology**	**Multivariate Model[b]**	**Multivariate Model + Histology[c]**	**Proportion of effect mediated by histology**
	OR (95% CI)	**OR (95% CI)**	**% (p)**	**OR (95% CI)**	**OR (95% CI)**	**% (p)**
Parity						
Never	1.00 (Ref)	1.00 (Ref)		1.00 (Ref)	1.00 (Ref)	
Ever	1.61 (0.77, 3.38)	0.71 (0.31, 1.62)	NA[d]	1.14 (0.57, 2.28)	0.75 (0.36, 1.60)	NA
Per child	1.26 (1.04, 1.53)	1.04 (0.86, 1.27)	82.0% (0.03)	0.99 (0.87, 1.14)	0.92 (0.79, 1.06)	NA
Oral Contraceptive (OC) use						
Never	1.00 (Ref)	1.00 (Ref)		1.00 (Ref)	1.00 (Ref)	
Ever	1.36 (0.75, 2.47)	1.10 (0.56, 2.17)	68.6% (0.43)	1.19 (0.71, 1.98)	1.05 (0.61, 1.81)	71.8% (0.56)
Per 5 years[e]	0.90 (0.58, 1.41)	1.03 (0.60, 1.58)	73.9% (0.70)	0.94 (0.66, 1.33)	0.97 (0.65, 1.44)	46.9% (0.81)
Menopausal status at diagnosis[e]						
Premeno	1.00 (Ref)	1.00 (Ref)		1.00 (Ref)	1.00 (Ref)	
Postmeno	0.53 (0.23, 1.37)	0.60 (0.19, 1.93)	21.1% (0.70)	0.39 (0.18, 0.84)	0.40 (0.17, 0.91)	2.2% (0.91)
Hormone therapy (HT) use[e,f]						
Never	1.00 (Ref)	1.00 (Ref)		1.00 (Ref)	1.00 (Ref)	
Ever	1.10 (0.51, 2.34)	0.97 (0.44, 2.12)	NA	0.79 (0.42, 1.47)	0.74 (0.39, 1.43)	NA
Per 5 years	1.35 (1.00, 1.83)	1.19 (0.89, 1.60)	41.6% (0.10)	1.06 (0.83, 1.34)	1.00 (0.78, 1.29)	96.4% (0.67)
Estrogen-only HT use[e,f,g]						
Never	1.00 (Ref)	1.00 (Ref)		1.00 (Ref)	1.00 (Ref)	
Ever	1.80 (0.79, 4.11)	1.57 (0.69, 3.55)	23.9% (0.41)	1.13 (0.60, 2.15)	1.05 (0.54, 2.04)	62.5% (0.73)
Per 5 years	1.40 (0.96, 2.02)	1.22 (0.84, 1.76)	40.4% (0.15)	1.09 (0.83, 1.42)	1.03 (0.78, 1.35)	70.1% (0.56)
Tubal ligation						
Never	1.00 (Ref)	1.00 (Ref)		1.00 (Ref)	1.00 (Ref)	

Ever	2.17 (0.72, 6.53)	2.79 (0.85, 9.14)	NA	1.26 (0.61, 2.39)	1.29 (0.62, 2.71)	NA
Age at menarche						
Per year	0.83 (0.67, 1.03)	0.84 (0.96, 1.05)	2.5% (0.93)	0.89 (0.75, 1.06)	0.90 (0.75, 1.07)	4.9% (0.83)
Age at natural menopause[e,f]						
Per 5 years	1.38 (0.90, 2.11)	1.56 (1.01, 2.42)	NA	1.92 (1.15, 3.19)	1.90 (1.16, 3.12)	1.1% (0.91)
Duration premenopause[e,h]						
Per 5 years	1.13 (0.82, 1.57)	1.19 (0.82, 1.74)	NA	1.22 (0.91, 1.63)	1.28 (0.94, 1.75)	NA
Duration postmenopause[e,h]						
Per 5 years	0.92 (0.72, 1.18)	0.87 (0.66, 1.16)	NA	0.82 (0.66, 1.03)	0.81 (0.64, 1.02)	NA
Ovulatory years[e,i]						
Per 5 years	1.15 (0.86, 1.54)	1.32 (0.96, 1.82)	NA	1.26 (0.97, 1.63)	1.39 (1.05, 1.86)	NA

a. OR shows the association for cases for being ERα+ vs. ERα- for each exposure. For example, for an exposure that has a positive association with ovarian cancer, an OR above 1 would suggest a stronger positive association for ERα+ as compared to ERα-

b. Analyses were adjusted for age at diagnosis (per year), cohort (NHS/NHSII/NECC), family history of breast/ovarian cancer (yes/no), duration of OC use (per month), menopausal status (premenopausal/postmenopausal ever HT/postmenopausal never HT/postmenopausal unknown HT), and number of pregnancies (continuous)

c. Analyses additionally adjusted for histology (serous/endometrioid/clear cell/other) except HT use and estrogen-only HT use were adjusted for serous vs. non-serous due to limited case numbers

d. Accounting for histology did not attenuate the association between the exposure and ERα or PR expression

e. Due to missingness in some of the exposures, the sample size for these analyses ranged from 169 cases for age at natural menopause to 314 cases for parity

f. Among postmenopausal women

g. Additionally adjusted for ever estrogen plus progestin HT use (yes/no), ever other HT use (yes/no) for ever/never analyses or duration of estrogen plus progestin HT use and duration of other HT use in duration analyses

h. Not adjusted for age at diagnosis

i. Ovulatory year was calculated as age at natural menopause (or age at diagnosis for premenopausal women) minus age at menarche with additional subtraction of one year of each pregnancy and OC duration

Part 2: A prospective cohort study of oral contraceptive use and ovarian cancer among women born from 1947 to 1964

Amy L. Shafrir[1,2], Helena Schock[3], Elizabeth M. Poole[2], Kathryn L. Terry[1,4], Rulla M. Tamimi[1,2], Susan E. Hankinson[1,2,5], Bernard A. Rosner[2,6], Shelley S. Tworoger[1,2]

Affiliations:

[1]Department of Epidemiology, Harvard TH Chan School of Public Health, Boston, MA, USA

[2]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

[3]Division of Cancer Epidemiology, German Cancer Research Center, Im Neuenheimer Feld 280, Heidelberg, Germany

[4]Obstetrics and Gynecology Epidemiology Center, Department of Obstetrics and Gynecology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

[5]Division of Biostatistics and Epidemiology, School of Public Health and Health Sciences, University of Massachusetts, Amherst, MA

[6]Department of Biostatistics, Harvard TH Chan School of Public Health, Boston, MA, USA

Abstract

Background: Oral contraceptives (OCs) have been consistently associated with a reduced risk of ovarian cancer; however, the majority of previous cohort studies have been conducted among women in older birth cohorts who used high-dose OC formulations.

Objective: To assess OC use, including estrogen and progestin type, dose and potency, and ovarian cancer risk among a younger birth cohort who used more recent OC formulations.

Methods: We included 110,930 eligible Nurses' Health Study II participants with 24 years of follow-up. In 1989, women recalled duration of OC use and OC brand used at each age from age

13 to baseline age. We categorized brands by estrogen and progestin type, dose, and potency. We used Cox proportional hazards models adjusted for age, calendar time, reproductive factors and body mass index to assess the association of ovarian cancer with measures of OC use and duration.

Results: Over 2,343,830 person-years of follow-up, we confirmed 293 ovarian cancer cases. At baseline, 83% of participants reported ever using OCs. Compared to never OC use, we observed an increased risk of ovarian cancer with six months or less of OC use (HR: 1.53; 95% CI: 1.00, 2.35) with a significant decreasing trend across categories of OC use (p-trend=0.03). The increased risk among short term OC users was restricted to duration of mestranol (HR: 1.54; 95% CI: 1.01, 2.34) and first generation progestin use (HR: 1.53; 95% CI: 1.04, 2.25). In cross-classified analyses, high cumulative estrogen and progestin dose was associated with a significant increased risk of ovarian cancer compared to low cumulative dose of both (HR: 1.50; 95% CI: 1.03, 2.18).

Conclusion: Among the young birth cohort of NHSII participants, the associations observed for total OC duration differ substantially from the results of previous cohort studies, which may be due to chance or possibly reflect changes in OC formulations over time.

Introduction

Ovarian cancer is the 11th most common cancer and the fifth most common cause of cancer death among women in the United States in 2016 [1]; therefore, it is important to identify modifiable risk factors for the prevention of ovarian cancer. Oral contraceptives (OC) are one of the strongest, potentially modifiable, preventive factors for ovarian cancer, with an approximately

30% decreased risk of ovarian cancer with ever OC use compared to never use reported in a recent pooled analysis of 45 studies [75].

In the United States, OCs were first marketed for contraception in the mid-1960s [76]. The first combination OC pills contained an estrogen, primarily mestranol, and a progestin, initially norethynodrel [77]. Since their introduction, the dose of estrogen has decreased from ~50 μg in the 1970s to ~35 μg in the 1980s and is as low as 20 μg in current combination OC pills; progestin only contraception also is available [76-78]. The progestin dose in combination OCs has slightly decreased over time [76].

The majority of cohort studies that assessed the relationship between OC use and ovarian cancer were conducted in older birth cohorts among women who primarily used higher dose formulations [75]; few data are available on associations of lower dose OC pills. Several case-control studies among younger birth cohorts observed an inverse association [79-84], and the only cohort study among a younger birth cohort observed a 40% (95% CI: 0.5-0.8) decreased risk of ovarian cancer with ever OC use compared to never use [85].

In this study, we assessed the association between oral contraceptives and ovarian cancer in the Nurses' Health Study II (NHSII), a prospective cohort study of women aged 25-42 in 1989. The reproductive years of NHSII participants spanned the transition from higher to lower dose combination OCs. Women reported their OC duration and OC brand from age 13 years until age at baseline, enabling us to examine not only duration of OC use but also estrogen and progestin dose and potency in relation to ovarian cancer risk.

Methods

Study population

The Nurses' Health Study II (NHSII) began in 1989 when 116,430 US female nurses, aged 25-42 completed a baseline questionnaire on health, lifestyle and disease diagnoses. Follow-up questionnaires were mailed to study participants biennially to obtain updated information on exposures and disease diagnoses, with a follow-up rate through June 2013 of 94% of the potential person-years. In the current study, we included oral contraceptive and covariate data from baseline, with follow-up of ovarian cancer cases through 2013. Women were excluded from analyses at baseline if they had a previous diagnosis of cancer except for non-melanoma skin cancer (n=1045), previous bilateral oophorectomy (n=2232), or menopause due to radiation (n=31).

Oral contraceptive use

Women were asked to report if they had ever used OCs and if they were currently using OCs at baseline. A life-events calendar was used to ascertain OC use starting at age 13 years. For each year of age, women were asked: (1) if they had used OCs for at least 2 months in that year, (2) if they had used OCs for at least 10+ months in that year, and (3) the brand of OC used during that year (women indicated the brand used the longest at each age if more than one brand was used). To aid in recall, a booklet with names and color photographs of all OC brands available during the relevant time period was sent with the questionnaire. Women reporting 2+ months, but not 10+ months, of OC use were categorized as having 6 months of OC use at that particular age, while women reporting both 2+ and 10+ months of OC use were categorized as having 12 months of OC use at that age. If women reported OC use and a pregnancy at the same age, we

classified them as having 6 months of use if they reported being pregnant <6 months at that age or 2 months of use if they reported being pregnant 6 or more months at that age. We only considered OC use during the premenopausal period.

Hunter and colleagues (1997) assessed the reproducibility of ever OC use and duration of OC use and evaluated the validity of OC formulation reporting in NHSII [86]. Women had excellent recall of ever use (sensitivity: 99%; specificity: 97%) and duration of use (spearman correlation: 0.94). Permission was obtained to assess medical records for 150 women reporting OC use before baseline. The OC brands reported on the 1989 questionnaire were compared to the medical records. The exact OC brand was in agreement 42% of the time but, when collapsed by estrogen and progestin type and dose, the percent agreement increased to 75%, suggesting that women were fairly good to recalling OC formulation.

Assessment of OC dose and potency

Richard P. Dickey's book Managing Contraceptive Pill/Drug Patients (5^{th}, 11^{th} and 14^{th} editions) was used to classify OC brands by estrogen type, dose and potency, and by progestin type, dose and potency [87-89]. The estrogenic potency (μg ethinyl estradiol equivalents per day) was based on mouse uterine assays, while the progestin potency (mg norethindrone equivalents per day) was based on the induction of glycogen vacuoles in human endometrium. For OC brands not included in the Managing Contraceptive Pill/Drug Patients book, information was obtained on estrogen and progestin type and dose from the Physician's Desk Reference, American Drug Index and other sources [90-94]. Of the 141 OC brands reported by women on the 1989 questionnaire, 29 were not included in Managing Contraceptive Pill/Drug Patients. These 29

brands were matched on estrogen type and dose and progestin type and dose to other brands included in the book to assign estrogen and progestin potency. For women reporting a known OC brand but unknown formulation, the dose of estrogen and progestin was set as the average of the known formulations within that brand; however, potency was left as missing.

We classified OC brands according to estrogen type: mestranol (ME) or ethinyl estradiol (EE). As eight different progestins were reported at baseline, the progestin types were grouped by generation. First generation progestins included norethindrone, norethynodrel, norethindrone acetate, ethynodiol diacetate, medroxyprogesterone acetate, and chlormadinone acetate, while second generation progestins included levonorgestrel and norgestrel [95]. Among women who reported the exact OC brand(s) they had used for at least 85% of their total duration (n=84,826 among 94,917 ever OC users), we calculated (1) duration of OC use by estrogen type and progestin generation and (2) cumulative dose and potency of estrogen and progestin. An additional 6,593 women were excluded from the cumulative estrogen potency analyses and 6,592 women were excluded from the cumulative progestin potency analyses as the potency of their OC brand was unknown. Further, we estimated average dose and potency over the entire period of prior OC use by dividing the cumulative total dose or potency by total duration of use.

Ascertainment of covariates

In 1989, women were asked to report several important lifestyle and reproductive factors including age at menarche, family history of breast cancer, use of other contraceptives including tubal ligation, intrauterine device (IUD), diaphragm, and male contraception, menopausal status, smoking status, race, physician-diagnosed migraines, and marital status. Women also reported

each age at which they had a pregnancy lasting 6 months or longer. In 1997, women reported on breastfeeding duration for each of their first four children and total duration of breastfeeding for additional pregnancies. This was used to define total breastfeeding duration in 1989. Additionally, women reported family history of ovarian cancer in 1993, and this was considered to reflect their status in 1989. Body mass index was calculated as kilograms (kg) per meter squared (m^2) using weight and height reported in 1989. Finally, endometriosis was reported in 1993, and status in 1989 was assigned based on the reported date of endometriosis diagnosis.

Ovarian cancer cases and deaths

Incident cases of epithelial ovarian cancer were identified on biennial questionnaires from 1989 to 2013 or, for deceased participants, from linkage to the National Death Index [96], the U.S. Postal Service, or family members. For all ovarian cancer cases we attempted to obtain pathology reports. A gynecological pathologist, blinded to exposure status, reviewed the reports to confirm the diagnosis of epithelial ovarian cancer and classify the cancers by behavior (invasive and borderline), histologic type (serous/poorly differentiated, mucinous, endometrioid, clear cell, and other) and stage. If medical records could not be obtained, pathology information was acquired through linkage with cancer registries.

Statistical analysis

We classified OC use using several different methods. For all analyses, person-years were calculated from 1989 to the date of ovarian or other cancer diagnosis (except for non-melanoma skin cancer), death, bilateral oophorectomy, menopause due to radiation, or end of study follow-up (June 2013). Cox proportional hazards models stratified by age in months and calendar time

were used to obtain hazard ratios (HR) and 95% confidence intervals (CI). Ever OC users who did not complete the life table at baseline (n=2191) were excluded. Analyses were adjusted for number of pregnancies (continuous), tubal ligation (yes/no), duration of breastfeeding (continuous in months, indicator for missing), and body mass index (continuous in kg/m^2). We additionally assessed the potential for confounding of the following covariates: laparoscopically-confirmed endometriosis (yes/no), use of IUD or diaphragm (yes/no), use of male contraception (yes/no), use of other contraceptives (yes/no), smoking status (past/current/never), race (Asian/White/Other), age at menarche (per year), and family history of breast and/or ovarian cancer (yes/no). None of these covariates changed the risk estimates substantially and as such were not included in the final models.

Total duration of OC use

Duration of use was categorized as never, 6 months or less, >6 months to 1 year, >1 to <5 years, 5 to <10 years, 10 or more years of OC use. Tests for trend were conducted by treating OC duration as an ordinal variable with values set to the median duration within each category and calculated using the Wald statistic. In sensitivity analyses, we stratified by median age at baseline and used a likelihood ratio test to assess effect modification. Additionally, we evaluated as our reference group, women who had never used OC pills and women who used OCs for 6 months or less, as some prior studies considered short term and never users together. Finally, we assessed total OC duration with follow-up starting in 1993 as some of the covariates were first assessed in 1993.

Duration of OC use by estrogen and progestin type

Duration for each estrogen (ME and EE) and progestin type (first generation and second generation) was categorized as never, 1 year or less, >1 to <5 years and 5 or more years of use. Each hormone type was analyzed in separate models. Hormone variables were not mutually exclusive, i.e. women who used both ME and EE were included in the analyses. For the duration by estrogen type, women who exclusively used progestin-only pills (n=138) were excluded. Additionally, women who were exclusive users of only one type of estrogen or progestin were excluded from analyses of the other type (n=42,430 exclusive users of EE; n=21,850 exclusive users of ME; n=48,274 exclusive users of 1st generation progestins; n=16,445 exclusive users of 2nd generation progestins).

Cumulative dose and potency

The cumulative dose and potency analyses were conducted among ever OC users with known estrogen (n=82,048) and progestin (n=82186) doses and known estrogen (n=75,784) and progestin (75,923) potencies, respectively. As these variables were highly correlated with total OC duration (Supplemental Table S2.1), we used the residuals from generalized linear models in our analyses of cumulative dose and potency. To calculate the residuals, we log transformed the cumulative dose and potency variables as well as total OC duration due right skewing. We then regressed the natural log of each of the cumulative dose and potency variables on the natural log of total OC duration. The residuals represent the difference between a woman's predicted cumulative dose/potency based on her total OC duration and her actual value. We cross-classified estrogen dose residuals below or at/above the median (0.03) and with the progestin dose residuals classified as below or at/above the median (0.20). A similar cross-classification

was used for the potency residuals (median estrogen potency residual=0.0025, median progestin potency residual=-0.034). We additionally adjusted these analyses for total duration of OC use (continuous in months). In sensitivity analyses, we excluded the top 5% of women from the cumulative dose and potency variables before creating the residuals to minimize the influence of outliers.

Average dose and potency

Secondary analyses among ever OC users with a known dose or potency considered average dose and potency for both estrogen and progestin, although the variability was limited. We created cross-classified variables for average dose and potency for estrogen and progestin, using cutpoints of 1117 µg estrogen per month and 21.4 mg progestin per month for dose and 799 µg EE equivalent per month and 22.8 mg norethindrone equivalent per month for potency. In sensitivity analyses, we restricted to women who had only used one or two known brands (n=68,084 for dose and 62,732 for potency), since average dose and potency variables were more likely to reflect long-term use of high dose/potency OCs or low dose/potency OCs among women who used only 1 or 2 brands.

For all analyses, we assessed potential differences in association by histologic subtype (serous vs. non-serous) using competing risk models [97]. In sensitivity analyses, we assessed time since last OC use among never OC users and past OC users at baseline. Time since last OC use was categorized as never OC use, <5 years since last OC use, 5-<10 since last OC use, 10-<15 years since last OC use and 15 or more years since last OC use at baseline. Additionally, we assessed the cross-classification of duration of OC use and time since last OC use categorized as never

use, ≤1 year OC use/<15 years since last use, >1 year OC use/<15 years since last use, ≤1 year OC use/15+ years since last use, >1 year OC use/15+ years since last use. All analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). P-values were 2-sided and considered statistically significant if less than 0.05.

Results

Study population characteristics

In 1989, 83% of women in NHSII had ever used OCs and 14% of ever users were currently using OCs (Table 2.1). Among ever OC users, most women had used OCs for either >1 to <5 years (39%) or 5 to <10 years (30%). Mean BMI was lower among those with longer OC duration. Never OC users and women with 10 or more years of OC use were the least likely to have children, 61% and 55%, respectively, were parous. However, among parous women, never OC users had a higher mean number of pregnancies lasting 6 months or more than women with 10 or more years of OC use. Additionally, duration of breastfeeding was lower among those with longer length of OC use. Further, never OC users were less likely to have a tubal ligation, experience migraines, or smoke cigarettes and were more likely to have never been married and used other forms of contraception compared to ever OC users.

Table 2.1 Characteristics of Nurses' Health Study II participants by duration of oral contraceptive (OC) use in 1989 (n=110,930)[1]

	Duration of oral contraceptive use					
	Never OC use (n=18,994)	**6 months or less OC use (n=9,770)**	**>6 months to 1 year OC use (n=9,065)**	**>1-<5 years OC use (n=36,027)**	**5-<10 years OC use (n=27,800)**	**10+ years OC use (n=9,274)**
Mean (Standard deviation)						
Age (years)[2]	34.4 (4.6)	35.3 (4.6)	35.1 (4.6)	34.7 (4.8)	34.4 (4.7)	35.5 (3.9)
Age at menarche (years)	12.4 (1.5)	12.5 (1.5)	12.4 (1.5)	12.4 (1.4)	12.4 (1.4)	12.4 (1.5)
Body mass index (kg/m^2)	24.5 (5.6)	24.1 (5.1)	24.0 (5.0)	24.1 (5.0)	23.9 (4.7)	23.7 (4.6)
Number of pregnancies ≥6 months[3]	2.2 (1.0)	2.2 (1.0)	2.2 (0.9)	2.1 (0.9)	1.9 (0.8)	1.7 (0.7)
Breastfeeding (months)[3,4]	14.7 (13.9)	13.3 (12.6)	12.8 (12.2)	12.5 (12.1)	10.9 (10.6)	8.0 (7.9)
Percentage						
Ever parous	61	75	75	77	73	55
Other current contraceptive method[5]						
IUD/diaphragm	17	16	15	13	11	7
Male contraception	32	33	33	32	28	21
Other contraception	23	20	19	17	14	12
Ever tubal ligation	10	16	15	18	18	12
Endometriosis[6]	3	4	5	4	4	4
Ever migraines	12	16	16	16	15	15
Family history of breast and/or ovarian cancer	8	8	7	7	7	7
Smoking status						
Never smoker	77	68	66	65	59	54
Current smoker	9	12	13	13	16	20
Past smoker	14	20	21	22	25	26
Race						
White	89	90	92	94	94	92
Asian	5	3	2	1	1	1

Other	6	7	6	5	5	7
Marital status[7]						
Married	73	80	82	84	83	74
Divorced/widowed	6	10	10	10	12	15
Never married	21	10	8	6	5	11

1. Values are standardized to the age distribution of the study population.
2. Value is not age adjusted
3. Among parous women
4. Missing breastfeeding duration among parous women (n=13,845)
5. IUD=Intrauterine device, male contraception includes vasectomy and male condoms, other contraception includes sponge, foam, rhythm method, and other contraception
6. Laparoscopically-confirmed endometriosis
7. Missing marital status (n=21,160)

Table 2.2 Oral contraceptive characteristics among ever OC users in the Nurses' Health Study II by total duration of OC use in 1989 (n=91,936)[1]

	6 months or less OC use (n=9,770)	>6 months to 1 year OC use (n=9,065)	>1-<5 years OC use (n=36,027)	5-<10 years OC use (n=27,800)	10+ years OC use (n=9,274)
Mean (Standard deviation)					
Estrogen[2,3]					
Cumulative dose	6,312 (2,961)	13,293 (5,402)	38,691 (20,002)	91,240 (37,301)	159,390 (61,984)
Cumulative potency	4,685 (2,613)	9,718 (4,006)	28,449 (13,615)	67,009 (24,041)	117,605 (41,729)
Average dose	1,132 (467)	1,131 (448)	1,123 (422)	1,123 (400)	1,088 (346)
Average potency	838 (414)	827 (330)	824 (282)	823 (243)	806 (225)
Progestin[4,5]					
Cumulative dose	124 (157)	255 (276)	714 (716)	1,647 (1,327)	2,880 (1,926)
Cumulative potency	146 (142)	303 (245)	876 (647)	2,041 (1,157)	3,603 (1,686)
Average dose	22 (27)	22 (23)	21 (20)	20 (16)	20 (12)
Average potency	26 (24)	26 (21)	25 (17)	25 (14)	25 (10)
Percentages					
Ever estrogen type use[6]					
Mestranol	31	36	41	48	55
Ethinyl estradiol	49	56	65	74	80
Missing type	20	14	10	7	7
Ever progestin type use[6]					
First generation[7]	58	65	71	76	80
Second generation[8]	23	28	36	42	47
Missing type	20	14	10	7	7
Number of OC brands used					
1 brand	80	68	52	37	21
2 brands	1	17	27	33	32
3 brands	0	0	7	13	20
4 or more brands	0	0	2	5	13

Unknown[9]	20	14	12	12	13

1. Values are standardized to the age distribution of the study population
2. N=9888 women missing cumulative and average estrogen dose and N=16,152 women missing cumulative and average estrogen potency
3. Units are as follows: cumulative estrogen dose: µg; cumulative estrogen potency: µg ethinyl estradiol equivalents; average estrogen dose: µg per month; average estrogen potency: µg ethinyl estradiol equivalents per month
4. N=9750 women missing cumulative and average progestin dose and N=16,013 women missing cumulative and average progestin potency
5. Units are as follows: cumulative progestin dose: mg; cumulative progestin potency: mg norethindrone equivalents; average progestin dose: mg per month; average progestin potency: mg norethindrone equivalents per month
6. Percentages can sum to more than 100% as women could have used both estrogen or progestin types
7. First generation progestin includes norethindrone, norethynodrel, norethindrone acetate, ethynodiol diacetate, medroxyprogesterone acetate, and chlormadinone acetate
8. Second generation progestin includes levonorgestrel and norgestrel
9. Women were included in the unknown number of brands category if they reported an ‘unknown OC brand’ at least once on the baseline questionnaire

Oral contraceptive use characteristics

Among ever OC users, 43% had ever used ME while 66% had ever used EE. The proportion of women using an OC brand containing ME was higher among those with longer total duration; these women were also more likely to have used multiple brands of OCs, while short term OC users were more likely to have used only 1 or 2 brands (Table 2.2). Additionally, women with short term OC use were less likely to know the specific OC brand they had used compared to women with longer durations. Ever OC users at baseline were more likely to have used first generation (72%) as opposed to second generation (37%) progestins. Cumulative dose ranged from 913 to 561,572 μg for estrogen and 4.11 to 45,994 mg for progestin, while cumulative potency ranged from 730 to 1,132,732 μg EE equivalent for estrogen and 18 to 45,994 mg norethindrone equivalent for progestin. Finally, there was relatively little variation in average dose and potency for estrogen and progestin across categories of duration.

Duration of total OC use

Compared to never use, 10+ years of OC use was not associated with a significant decreased risk of ovarian cancer (HR: 0.84; 95% CI: 0.51, 1.39; Table 2.3), although there was a significant trend across categories of OC duration (p-trend=0.03). Short term OC use of 6 months or less was associated with a significant 53% (95% CI: 1.00, 2.35) increased risk of ovarian cancer compared to never OC use in the full multivariate model. Among ever OC users, the risk of ovarian cancer decreased by 6% (95% CI 0.91, 0.98) for each year of use. In sensitivity analyses with duration of OC use stratified by the median age (35 years) at baseline, the increased risk among short term OC users was suggestively stronger for women aged ≥35 years at baseline (HR: 1.56; 95% CI: 0.98, 2.49 for ≤1 year vs. never use) compared to women aged <35 years at

baseline (HR: 1.32; 95% CI: 0.71, 2.46 for ≤1 year vs. never use); however this difference was not significant (p-interaction=0.81; Supplemental Table S2.2). Additionally, when we grouped the 6 month OC users with the never users, there was a significant 31% (95% CI: 0.49, 0.97) decreased risk of ovarian cancer with 5 to <10 years of OC use and a suggestion of a 28% (95% CI: 0.45, 1.15) decreased risk for 10 or more years of OC use compared to 'non-OC use', i.e. never and 6 months or less (Supplemental Table S2.3). Finally, starting follow-up in 1993 as opposed to 1989 did not substantially alter the risk estimates (data not shown).

Table 2.3 Association between duration of OC use in 1989 and ovarian cancer risk among Nurses' Health Study II participants (1989-2013)

			Model 1[1]		Model 2[2]		Model 3[3]	
	Cases	**Person-yrs**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
Duration of OC use								
Never	50	409,591	1.00	(Ref).	1.00	(Ref).	1.00	(Ref).
≤ 6 months	37	204,331	1.38	(0.90, 2.12)	1.49	(0.97, 2.29)	1.53	(1.00, 2.35)
>6 months – 1 year	30	189,930	1.25	(0.79, 1.96)	1.33	(0.85, 2.10)	1.38	(0.88, 2.19)
>1-<5 years	99	757,496	1.04	(0.74, 1.46)	1.13	(0.80, 1.59)	1.16	(0.82, 1.64)
5-<10 years	54	586,382	0.75	(0.51, 1.11)	0.78	(0.53, 1.14)	0.81	(0.55, 1.19)
≥10 years	23	193,020	0.88	(0.54, 1.45)	0.80	(0.49, 1.32)	0.84	(0.51, 1.39)
p-trend			*0.04*		*0.02*		*0.03*	

1. Adjusted for age (years) and calendar time
2. Adjusted for Model 1 variables + tubal ligation (yes/no), parity (number of pregnancies), breastfeeding duration (months), and missing breastfeeding duration
3. Adjusted for Model 2 variables + body mass index (kg/m^2)

OC duration by estrogen and progestin type

We further investigated the relationship between duration of OC use and ovarian cancer by estrogen and progestin type. The increased risk among women who used OCs for 6 months or less was most apparent for duration of ME and first generation progestins (Table 2.4). Women who used ME for one year or less had a significant 54% (95% CI: 1.01, 2.34) increased risk of ovarian cancer compared to never OC users, while women who used first generation progestins for one year or less had a significant 53% (95% CI: 1.04, 2.25) increased risk of ovarian cancer compared to never OC users. The comparable risk estimates for EE and second generation progestins was 1.09 and 1.33, respectively. Compared to never users, there was a suggestion of a 30% (95% CI: 0.44-1.07) decreased risk of ovarian cancer with 5 or more years of EE use, but no association for long term ME use. For both first and second generation progestins, we observed non-significant inverse associations with >5 years of OC use.

Cumulative dose and potency

To assess if risk differed by the estrogen or progestin content, we conducted a cross-classification analysis of cumulative estrogen and progestin dose and potency residuals (Table 2.5). When examining dose among ever OC users, we observed a significant 50% (95% CI: 1.03, 2.18) increased risk of ovarian cancer among women with estrogen and progestin dose residuals above the median compared to both below the median. Additionally, women with a cumulative estrogen dose residual above the median but progestin below the median had a non-significant increased risk of ovarian cancer (HR: 1.39; 95% CI: 0.88, 1.86). However, no association was observed for women with estrogen dose residuals below the median but progestin dose residuals above the median (HR: 1.13; 95% CI: 0.68, 1.86). The results for the potency residuals were not

consistent with the results for dose, with no significant associations. In sensitivity analyses excluding women within the top 5% of the cumulative dose or potency of estrogen and progestin the results were similar (data not shown).

Table 2.4 Associations between duration of OC use by estrogen and progestin type and ovarian cancer risk in the Nurses' Health Study II (1989-2013)[1]

			Multivariate-adjusted model[2]	
	Cases	**Person-yrs**	**HR**	**95% CI**
Never OC users	50	409,591	1.00	(Ref.)
Duration of mestranol use[3,4]				
≤1 year	42	226,455	1.54	(1.01, 2.34)
>1-<5 years	49	361,675	1.08	(0.72, 1.62)
≥5 years	30	234,097	0.93	(0.58, 1.47)
Duration of ethinyl estradiol use[3,5]				
≤1 year	35	293,787	1.09	(0.70, 1.69)
>1-<5 years	64	549,022	1.12	(0.77, 1.64)
≥5 years	34	435,899	0.69	(0.44, 1.07)
Duration of 1st generation progestin use[6,7]				
≤1 year	57	328,090	1.53	(1.04, 2.25)
>1-<5 years	78	577,347	1.19	(0.83, 1.71)
≥5 years	44	476,365	0.76	(0.50, 1.14)
Duration of 2nd generation progestin use[8,9]				
≤1 year	29	206,105	1.33	(0.83, 2.12)
>1-<5 years	34	314,532	0.99	(0.63, 1.54)
≥5 years	17	191,366	0.73	(0.42, 1.27)

1. Duration of OC use by type of estrogen and progestin is non-exclusive use, e.g. women with ethinyl estradiol use and mestranol use are included in both variables.
2. Adjusted for age (years), calendar time, parity (number of pregnancies), tubal ligation (yes/no), breastfeeding duration (months), missing breastfeeding duration (yes/no), body mass index (kg/m^2)
3. Excludes 138 women who used progestin-only pills exclusively
4. Excludes 42,430 women who exclusively used ethinyl estradiol
5. Excludes 21,850 women who exclusively used mestranol
6. First generation progestins include norethindrone, norethynodrel, norethindrone acetate, ethynodiol diacetate, medroxyprogesterone acetate, and chlormadinone acetate
7. Excludes 16,445 women who exclusively used 2nd generation progestins
8. Second generation progestins included levonorgestrel and norgestrel
9. Excludes 48,274 women who exclusively used 1st generation progestins

Average dose and potency

In secondary analyses, the results for average estrogen dose cross-classified with average progestin dose were similar to the results for the main residual analysis (Supplemental Table S2.4). Women with average estrogen and progestin doses above the median had a significant 51% (95% CI: 1.04, 2.19) increased risk of ovarian cancer compared to women with average estrogen and progestin doses below the median. The association for estrogen above the median and progestin below versus both below was similar but non-significant (HR: 1.39; 95% CI: 0.87, 2.22). The analyses for average estrogen and progestin potency were null. When analyses were restricted to women who used one or two OC brands, the results were qualitatively similar (data not shown).

Table 2.5 Association between residuals for cumulative estrogen and progestin dose and potency and ovarian cancer risk[1]

		Multivariate-adjusted models[2]				
	Cases	**HR**	**95% CI**	**Cases**	**HR**	**95% CI**
		Cumulative estrogen dose residual[3]				
		< Median			≥ Median	
Cumulative progestin dose residual						
< Median	52	1.00	(Ref.)	36	1.39	(0.88, 1.86)
≥ Median	22	1.13	(0.68, 1.86)	103	1.50	(1.03, 2.18)
		Cumulative estrogen potency residual				
		< Median			≥ Median	
Cumulative progestin potency residual						
< Median	45	1.00	(Ref.)	39	1.32	(0.85, 2.03)
≥ Median	44	1.31	(0.85, 2.00)	71	1.22	(0.82, 1.82)

1. Among ever OC users, with 82,048 women included in the dose residual analysis and 75,784 women included in the potency residual analysis
2. Adjusted for age (months), calendar time, parity (number of pregnancies), tubal ligation (yes/no), breastfeeding duration (months), missing breastfeeding (yes/no), body mass index (kg/m^2)
3. Median values are: 0.03 for cumulative estrogen dose residual, 0.204 for cumulative progestin dose residual, 0.0025 for cumulative estrogen potency residual, and -0.034 for cumulative progestin potency residual

Tumor heterogeneity

For each of the main analyses, we assessed differences in associations by tumor subtype (serous vs. non-serous). While we observed no significant differences by tumor heterogeneity, there was a suggestion of stronger associations for serous compared to non-serous tumors. For example, there was a suggestion of a stronger positive association for short term use (HR: 1.68; 95% CI: 0.93, 3.03 compared to HR: 1.37; 95% CI: 0.73, 2.57 for $\leq$6 months vs. never for serous vs. non-serous tumors, respectively; p-heterogeneity=0.65) and of a stronger inverse association for long term use (HR: 0.76; 95% CI: 0.45, 1.28 compared to HR: 0.88; 95% CI: 0.54, 1.44 for 5+ years vs. never for serous vs. non-serous tumors, respectively; p-heterogeneity=0.69). A similar pattern was observed for duration by EE and 2nd generation progestins as well as cross-classified analyses for dose residuals and average dose (data not shown).

Sensitivity analyses

Women with 15 or more years since their last OC use were 69% (95% CI: 1.15, 2.49) more likely to develop ovarian cancer compared to never OC users. The results were null for shorter time since last OC use and adjustment for duration of OC use did not substantial alter the risk estimates (data not shown). In cross-classified analyses, women with 1 year or less of OC use and 15 or more years since their last OC use were twice as likely to develop ovarian cancer as never OC users (HR: 2.04; 95% CI: 1.32, 3.15). In contrast, there was a non-significant 41% (95% CI: 0.90, 2.20) increased risk of ovarian cancer among women with an OC duration of greater than 1 year and 15 or more years since last OC use. The cross-classified results were null for women with less than 15 years since their last OC use (data not shown).

Discussion

In a prospective cohort study among a young birth cohort using more recent OC formulations with 24 years of follow-up, we observed that OC use of six months or less was associated with a significant increased risk of ovarian cancer compared to never use. Additionally, we did not observe the expected significant decreased risk of ovarian cancer with 10 or more years of use compared to never use, although there was a significant inverse trend with increasing duration among ever OC users. This is the first cohort study to assess duration of OC use and ovarian cancer by estrogen and progestin type. The increased risk of ovarian cancer for short term use was restricted to users of ME and first generation progestins. By considering cross-classification of estrogens and progestins, we observed that estrogen as opposed to the progestin content may be driving this association. Further, although the number of cases was small, there was a suggestion of a decreased risk of ovarian cancer among EE users of five or more years.

The ovulation hypothesis is a primary mechanism through which oral contraceptives are thought to decrease ovarian cancer risk [17]. During ovulation the ovary undergoes trauma and associated wound healing, exposing the ovaries and fallopian tubes to high levels of hormonal and inflammatory agents. It has been known since the 1950s that, during pregnancy, high levels of progesterone suppress ovulation, which led to the development of synthetic progestins to be used for contraception [98]. Interestingly, estrogen was included in OC pills initially by accident due to 'contamination' with ME [78]. Individually, high doses of estrogen decrease the mid-cycle follicular stimulating hormone (FSH) surge and moderate doses of progestin decrease the mid-cycle luteinizing hormone (LH) surge, both leading to inhibition of ovulation [78]. However, together lower doses of each can be used as estrogen and progestin have a synergistic

effect on ovulation inhibition [98, 99]. In addition to inhibiting ovulation, OCs decrease ovarian production of androgens, which may decrease ovarian cancer risk [19, 100]. Previous studies have reported that a portion of the protective effect of OCs on ovarian cancer risk is likely independent of ovulation suppression [101-103].

The majority of studies on OC use and ovarian cancer observed a substantial decreased risk of ovarian cancer with increasing duration of use [20, 21, 79, 80, 85, 104, 105]. A recent pooled analysis of 45 cohort and case-control studies reported an almost 45% decreased risk of ovarian cancer for 10 to 14 years of use compared to never users [75]. Of note, the decreased risk per 5 years of OC use was stronger among the 32 population- and hospital-based case-control studies compared to the 13 cohort studies (25.6%, 24.7%, and 16.2% per 5 years among ever OC users, respectively). We did not observe a significantly lower risk of ovarian cancer among long term users of OCs ($\geq$10 yrs) compared to never users, although we cannot rule out a modest effect. Among ever users of OCs, we did observe a 26% decreased risk for every 5 additional years of use, although this may have largely been driven by the higher risk for short term users observed in our study. Interestingly, this relationship differed by type of estrogen, with a 5% decreased risk for ME and 31% decreased risk for EE for every 5 years of use among ever users.

Importantly, most studies that assessed short term use of OCs observed no significant association between short term OC use and ovarian cancer, including the pooled analysis of 45 studies [75, 80, 83, 104, 106, 107]. However, some studies reported a suggestive [79, 82, 102] or significant [101, 108] decreased risk of ovarian cancer with short-term OC use. These results are in stark

contrast to the results observed in our study; we observed that short term (≤6 months) OC use was associated with a significant 53% increased risk of ovarian cancer.

Unique to our study, the NHSII includes women born between 1947 and 1964, while the majority of the previous cohort studies on ovarian cancer and OC use were conducted among older birth cohorts, with the median birth year ranging from before 1925 to approximately 1944 [75]. Importantly, many women in these latter studies were prescribed OCs when only higher dose OCs were available, while women in the NHSII were prescribed OCs during the transition from high dose to low dose formulations. Only one other study considered women from a similar birth cohort as NHSII. Kumle et al. (2004) assessed OC use and ovarian cancer among women born between 1942 and 1962 [85]. They observed no association with less than one year of use, but a 50% (95% CI: 0.3, 1.0) decreased risk of ovarian cancer with 10-14 years of OC use; however, they had a higher proportion of never OC users (27%) compared to our study population and the OC formulations used within the study population are unknown.

In addition to chance findings in our study, several possible reasons may explain the differing results in our study compared to prior work. First, in 1989, only 17% of NHSII participants had never used OCs, a much lower proportion than prior studies. For example, as noted above, the Kumle study had 27% never users, while in the Nurses' Health Study (NHS) 57% were never users [85, 104]. The higher proportion of ever users is consistent with trends of OC prescriptions in the U.S. during the 1970s and 1980s in which NHSII participants were using OCs and is consistent with changing laws in the US that by the early 1970s allowed all unmarried women in the US to access contraceptives [76]. As such, never users in our study may represent a highly

select group of individuals who differ from never users in prior research. For example, in two cohort studies among older birth cohorts, never OC users were more likely to be parous and have ever smoked compared to NHSII never users [104, 109]. As in previous cohort studies, we observed the expected decreased risk of ovarian cancer with increasing duration among ever OC users, further suggesting that NHSII never users may be a highly select group. In 2013 never OC users were asked to recall the reason that they had never used OCs. The majority of women had not used OCs due to fears about side effects or for other, unspecified reasons and only 4% of women had never used OCs for religious reasons.

A second challenge in comparing results across studies is differing definitions of never OC users. Although many studies do not explicitly define the reference group, at least a few studies have classified very short term OC users (generally <3 months) as never or 'non' OC users [83, 110-112]. In sensitivity analyses, we grouped the ≤6 month OC users with the never users and observed approximately a 30% lower risk for both 5 to <10 years and 10 or more years of use. This suggests that the definition of never or non OC use may impact the results. Careful classification of true never users in future studies, particularly from large consortia, may help clarify the association for short term use of OCs.

A potential biologic explanation for our findings, particularly among the short term users, is that the underlying hormonal milieu of women who use OCs for a short period time may influence their ovarian cancer risk. Notably, since a large majority of women ever used OCs, we have a substantial number (11% of ever users) who used for a very short period. In 2013, NHSII participants recalled the reason(s) they had stopped using OCs. Women who had used OCs for

only 6 months or less tended to stop for medical reasons, i.e. cardiovascular events and negative side effects, compared to women who used OCs for longer, who tended to stop due to pregnancy or switching to a different contraceptive (Supplemental Table S2.5). Prior reports support that negative side effects are a common reason for early OC discontinuation [113-116]. The most frequently reported side effects include breakthrough bleeding, nausea/vomiting, headaches, depression, weight gain, and breast tenderness [113-118]. Headaches, specifically migraines, have been associated with estrogen levels [119]. Decreases in estrogen levels, whether from endogenous or exogenous sources, have been linked to the occurrence of menstrual-related migraines, with larger drops in estrogen levels more likely to lead to a migraine [119]. Sulak et al. (2000) observed an increase in reports of headaches among OC users during the hormone-free week compared to the weeks when women were taking active OC pills, suggesting that the increased estrogen withdrawal while on OCs (compared to natural cycling) may lead to headaches in some women [120]. Little data are available on characteristics of women who discontinue OC use due to side effects compared to those who can tolerate and remain on OCs. These groups may respond differently to changes in hormonal levels induced by OCs or have a different underlying hormonal milieu, and such hormone differences may influence ovarian cancer risk. Future research should focus on differences in hormonal markers and other biologic characteristics of women who experience side effects while taking OCs compared to women who do not.

In our study, the increased risk for short term use of OCs appeared to be driven by use of ME, an estrogen found in older formulations of OCs. The biological mechanism for this association is unclear and these results contradict the findings of older studies conducted among women who

would have mostly used ME. However, one case-control study observed a weaker association for duration of ≥50 μg EE (HR: 0.95; 95% CI: 0.91-0.99 per year), likely to be a mixture of ME and EE OCs compared to duration of <35 μg EE (HR: 0.86; 95% CI: 0.79-0.94 per year), likely to be primarily EE OCs [83]. ME is a prodrug of EE, which has slowly replaced ME as the primary estrogen in more recent OC formulations [99]. ME is demethylated to EE primarily by the CYP2D6 enzyme in the liver [121]. Approximately 70% of the ME dose in an OC pill is converted into EE (Goldzieher 1993). However, for a given ingested ME or EE dose, there is substantial inter- and intra-individual variation in the levels of bioavailable EE, with the bioavailability of EE ranging from 25% to 65% of the ingested dose between individuals [122]. As mentioned above, short term OC users in NHSII were more likely to discontinue OC use because of side effects compared to women with longer durations. Short term users with side effects may receive a higher bioavailable EE dose for a given ingested EE or ME dose than what would be expected. While it is unclear if these women would also have higher endogenous estrogen levels, the relationship between circulating estrogens and ovarian cancer risk is complex. Two studies of postmenopausal women reported that circulating levels of estradiol or estrone were not related to ovarian cancer risk [123, 124]; however, both were based on small sample sizes. In the only study of premenopausal, pregnant women, those in the highest tertile of estradiol levels had an almost 3-fold increased risk of endometrioid ovarian cancer compared to pregnant women in the lowest tertile [125]. In the NHSII, we did not observe any significant differences by tumor histology for duration of ME or EE, although our power was limited. Larger studies that can assess the estrogenic environment in premenopausal women are needed to assess the association between estrogen levels and ovarian cancer risk. Additionally, future

research should focus on understanding if women who experience side effects compared to women who do not are more efficient at converting their ingested EE dose into bioavailable EE.

Our study had several limitations including the limited number of ovarian cancer cases and no information about OC use after baseline. The latter may have led to misclassification of our exposure, especially later in follow-up; however, the majority of ever OC users were past users (86%) at baseline and as such misclassification should have been limited. Additionally, any misclassification should be non-differential, biasing the results towards the null, which would not explain the increased risk with short term OC use that we observed. Further, the potency measurements are based on methods for which their relevance to humans is unknown and within humans there is wide inter- and intra-individual variation in bioavailability of EE. This calls into question the relevance of potency measurements of OCs. Additionally, the range of potency levels, particularly for progestins, was very limited in our study. Finally, we have controlled for confounding as much as possible; however, we cannot rule out that there may be some residual confounding.

Our study also has a number of strengths that should be highlighted. Although we have a limited number of ovarian cancer cases, we had 24 years of follow-up and are one of the only two cohort studies with a younger birth cohort that has reasonable power to assess OC use and ovarian cancer. This enabled us to assess OC use that includes newer OC formulations. Additionally, we have detailed information on OC use including the OC brand that women used at each age before baseline. Finally, the NHSII is a prospective study which allows us to assess OC use and ovarian cancer without differential misclassification due to recall bias.

Conclusion

In this study, we observed a significant increased risk of ovarian cancer with short term OC use and a non-significant inverse association with ten or more years of OC use. Further research into patterns of OC use in the US population during this time period, including characteristics of never users, as well as reanalysis of prior studies to separate never and short term use may help clarify our results. Further, better understanding the underlying hormonal milieu among women who cannot tolerate OCs may elucidate potential mechanisms that could influence ovarian cancer development. Future research among young birth cohorts using both high and low OC formulations are needed to confirm if our results are unique to our dataset or reflect the association of OCs and ovarian cancer among younger birth cohorts who used newer formulations.

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Appendix S1

Supplemental Table S2.1 Correlations between cumulative dose and potency for estrogen and progestin and total OC duration[1,2]

	OC duration				Cumulative estrogen		Cumulative progestin		Average estrogen		Average progestin	
	ME	**EE**	**1st gen**	**2nd gen**	**Dose**	**Potency**	**Dose**	**Potency**	**Dose**	**Potency**	**Dose**	**Potency**
OC duration[3]												
Total	0.50	0.69	0.73	0.44	0.88	0.90	0.64	0.78	-0.03	-0.03	-0.04	-0.03
ME		-0.28	0.68	-0.20	0.72	0.55	0.52	0.44	0.44	0.17	0.15	0.06
EE			0.24	0.65	0.37	0.53	0.27	0.49	-0.40	-0.16	-0.17	-0.08
1st gen				-0.29	0.76	0.71	0.70	0.61	0.16	0.08	0.16	0.03
2nd gen					0.23	0.32	-0.03	0.28	-0.25	-0.13	-0.27	-0.07
Cumulative estrogen												
Dose						0.93	0.69	0.76	0.35	0.21	0.08	0.06
Potency							0.65	0.72	0.22	0.29	0.02	-0.01
Cumulative progestin												
Dose								0.92	0.20	0.09	0.53	0.46
Potency									0.13	0.00	0.38	0.43
Average estrogen												
Dose										0.61	0.33	0.24
Potency											0.20	0.03
Average progestin												
Dose												0.91

1. Correlations are presented as Pearson Correlation Coefficients (ρ)
2. Sample sizes ranged from 75,784 to 82,186
3. ME=mestranol, EE=ethinyl estradiol, 1st gen=first generation progestins (norethindrone, norethynodrel, norethindrone acetate, ethynodiol diacetate, medroxyprogesterone acetate, and chlormadinone acetate), 2nd gen=second generation progestins (levonorgestrel and norgestrel)

Supplemental Table S2.2 Association between duration of OC use in 1989 and ovarian cancer risk stratified by median age (35) at baseline among Nurses' Health Study 2 participants (1989-2013)

	Age <35 years			Age ≥35 years			
	Cases	**HR**	**95% CI**	**Cases**	**HR**	**95% CI**	*P-int*[1]
Duration of OC use[2]							
Never	21	1.00	(Ref).	29	1.00	(Ref).	0.81
≤1 year	20	1.32	(0.71, 2.46)	47	1.56	(0.98, 2.49)	
>1-<5 years	33	1.07	(0.61, 1.87)	66	1.22	(0.78, 1.90)	
≥5 years	26	0.76	(0.42, 1.35)	51	0.86	(0.54, 1.36)	

1. P-int=P-value for interaction, calculated using the likelihood ratio test
2. Analyses adjusted for age (years), calendar time, tubal ligation (yes/no), parity (number of pregnancies), breastfeeding duration (months), missing breastfeeding duration, and body mass index (kg/m^2)

Supplemental Table S2.3 Association between duration of OC use with ≤6 months of OC use grouped with never OC users in 1989 and ovarian cancer risk among Nurses' Health Study II participants (1989-2013)

			Multivariate adjusted model[1]	
	Cases	**Person-yrs**	**HR**	**95% CI**
Duration of OC use				
Never or ≤ 6 months	87	613,922	1.00	(Ref).
>6 months – 1 year	30	189,930	1.18	(0.77, 1.78)
>1-<5 years	99	757,496	0.99	(0.74, 1.32)
5-<10 years	54	586,382	0.69	(0.49, 0.97)
≥10 years	23	193,020	0.72	(0.45, 1.15)

1. Adjusted for age (years) and calendar time tubal ligation (yes/no), parity (number of pregnancies), breastfeeding duration (months), and missing breastfeeding duration body mass index (kg/m^2)

Supplemental Table S2.4 Association between average estrogen and progestin dose/potency and ovarian cancer risk (1989-2013)

		Multivariate-adjusted models[2]				
	Cases	**HR**	**95% CI**	**Cases**	**HR**	**95% CI**
			Average estrogen dose[3]			
		< Median			≥ Median	
Average progestin dose						
< Median	53	1.00	(Ref.)	35	1.39	(0.87, 2.22)
≥ Median	23	1.15	(0.71, 1.88)	102	1.51	(1.04, 2.19)
			Average estrogen potency			
		< Median			≥ Median	
Average progestin potency[4]						
< Median	68	1.00	(Ref.)	49	1.04	(0.72, 1.51)
≥ Median	22	0.78	(0.48, 1.27)	60	1.03	(0.71, 1.49)

1. Among ever OC users with 82,048 women included in the average dose analysis and 75,784 women included in the average potency analysis

2. Adjusted for age (months), calendar time, parity (number of pregnancies), tubal ligation (yes/no), breastfeeding duration (months), missing breastfeeding (yes/no), body mass index (kg/m^2)

3. Median values are: 1117 µg per month for average estrogen dose, 21.4 mg per month for average progestin dose, 799 µg EE equivalent per month for average estrogen potency, and 22.8 mg norethindrone equivalent per month for average progestin potency

Supplemental Table S2.5 Reasons for stopping OC use reported in 2013 by duration of OC use in 1989 among ever OC users (n=91936)

	6 months or less OC use (n=9770)	>6 months to 1 year OC use (n=9065)	>1-<5 years OC use (n=36027)	5-<10 years OC use (n=27800)	10+ years OC use (n=9274)
Percentages					
Reasons for discontinuing OC use[1]					
Medical Reasons[2]	23	19	12	7	4
Pregnancy/switch methods[3]	24	34	46	50	46
Other reasons[4]	14	12	10	10	14
Missing	43	40	37	37	39

1. Percentages can add up to more than 100% as women could report more than one reason for stopping OC use
2. Includes stopping OC use because of a cardiovascular event, worse premenstrual syndrome, migraines, and/or other side effects
3. Includes stopping OC use because of pregnancy or due to switching to a different form of contraception
4. Includes stopping OC use for other, unspecified reasons

Part 3: Reproductive and hormonal factors in relation to survival and platinum resistance among ovarian cancer cases

Amy L. Shafrir[1,2], Ana Babic[3], Rulla M. Tamimi[1,2], Bernard A. Rosner[2,4], Shelley S. Tworoger[1,2], Kathryn L. Terry[1,5]

Affiliations:

[1]Department of Epidemiology, Harvard TH Chan School of Public Health, Boston, MA, USA

[2]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

[3]Department of Medical Oncology, Dana–Farber Cancer Institute and Harvard Medical School, Boston, MA

[4]Department of Biostatistics, Harvard TH Chan School of Public Health, Boston, MA, USA

[5]Obstetrics and Gynecology Epidemiology Center, Department of Obstetrics and Gynecology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

Abstract

Background: Ovarian cancer survival is poor, particularly for women with platinum-resistant disease.

Objective: We evaluated the association of pre-diagnostic reproductive and hormonal factors with overall survival and, secondarily, platinum resistance among ovarian cancer patients.

Methods: We included 1,649 cases of invasive epithelial ovarian cancer from a population-based case-control study conducted in Eastern Massachusetts and New Hampshire from 1992-2008. Reproductive and hormonal factors were assessed during in-person interviews and mortality via linkage to the National Death Index. Invasive ovarian cancer cases with medical record data on platinum chemotherapy (n=446) were included in the analyses on platinum resistance, which was

defined as a recurrence within the first six months after the end of platinum-based chemotherapy. We used Cox proportional hazards models to calculate risk estimates controlling for age, enrollment phase, and reproductive, tumor, and treatment characteristics.

Results: We observed 911 deaths during 12,552 person-years of follow-up. Self-reported endometriosis was associated with a 29% (95% CI: 0.54-0.94) decreased risk of total death. Older age at natural menopause was associated with a poor prognosis (HR, ≤50 vs. >50yr: 1.22; 95% CI: 1.02-1.45), while longer duration of hormone therapy (HT) was associated with improved survival (HR, ≥5yr vs. never: 0.70; 95% CI: 0.54-0.89). Further, older age at menarche was associated with worse survival. We observed no association between oral contraceptive use, parity, tubal ligation, and ovulatory years and overall survival. We identified 45 platinum-resistant ovarian cancer cases. Five or more years of HT use was associated with a decreased risk of platinum resistance (HR: 0.52).

Conclusion: Among ovarian cancer cases, increasing age at menarche and age at menopause were associated with worse overall survival, while longer HT use and endometriosis were associated with improved survival. These results suggest that pre-diagnosis exposures can influence tumor aggressiveness and survival.

Introduction

Ovarian cancer is the deadliest gynecologic malignancy, accounting for an estimated 14,240 deaths in the United States in 2016 [1]. Among women with ovarian cancer, only 46% will survive for five years after diagnosis, primarily because 60% of women are diagnosed at an advanced disease stage [2]. Further, approximately 80% of women will eventually relapse after first-line platinum and taxane-based chemotherapy [2, 22], with relapse within 6 months of

ending treatment (platinum resistance) being associated with a worse outcome [23]. Thus, identifying factors associated with survival and chemo-resistance may elucidate mechanisms that lead to poor survival and possibly help to identify women who may benefit from alternative therapies.

Increasing age, advanced disease stage and poor tumor differentiation have been consistently associated with worse ovarian cancer survival [24, 25]. However, the literature has been mixed on the relationship between reproductive ovarian cancer risk factors and overall survival. One recent study noted improved survival for ovarian cancer patients with endometriosis [26]; however, a 2014 meta-analysis of smaller studies reported no association after adjustment for covariates [27]. Additionally, tubal ligation has been associated with an increased risk of ovarian cancer death in two small studies [28, 29], but not in other larger studies [30-32]. Finally, use of menopausal hormone therapy (HT) before diagnosis has been associated with a survival advantage in one study [33], but was not associated with ovarian cancer survival in other smaller studies [30, 31, 126, 127]. Inconsistencies across studies may be driven by low sample sizes.

Thus, in this study, we used the large, population-based New England Case-Control study to examine the association between reproductive and hormonal factors and overall survival among 1,649 ovarian cancer cases. We had detailed characterization of many pre-diagnosis exposures as well as data on tumor characteristics and treatment. Secondarily, given the particularly poor survival among patients with platinum resistance, we evaluated associations of reproductive and hormonal factors with platinum resistance among 446 ovarian cancer cases who were treated with platinum therapy and had information on relapse during the study interval.

Methods

Study population

The New England Case-Control Study (NECC) is a population-based study of ovarian cancer conducted in Eastern Massachusetts and New Hampshire. Data included in this analysis come from three enrollment phases (1992-1997, 1998-2002, 2003-2008). Details of enrollment into the three study phases have been described previously [128]. Briefly, 3,957 women diagnosed with incident ovarian cancer in Eastern Massachusetts or New Hampshire were identified through hospital tumor boards and statewide cancer registries. Exclusion criteria for the ovarian cancer cases included: <18 years old, moved, had no phone, did not speak English, died, or their physician declined permission to contact them. Of the ovarian cancer cases identified, 78% were eligible and 2,203 agreed to enroll in the study. Enrolled controls (n=2,100) were not included in this analysis. Each participant provided a signed informed consent and the study was approved by the Institutional Review Boards at Brigham and Women's Hospital and Dartmouth Medical School.

Case outcomes

A gynecologic pathologist reviewed the pathology reports for all cases to classify ovarian tumors by histology (serous, endometrioid, mucinous, clear cell, other), grade (borderline, I, II, III) and stage (I, II, III, IV). Among a subset of women (n=881) diagnosed at Brigham and Women's Hospital, Boston, MA, USA and Massachusetts General Hospital, Boston, MA, USA, we abstracted data on chemotherapy, residual disease and ovarian cancer recurrence. Information was collected on first-line adjuvant chemotherapy, including each chemotherapy drug the woman received, the number of cycles for each drug, and the start and end dates. Medical records up to

July 2015, when available, were searched for evidence of ovarian cancer recurrence. Information was also collected on the size of residual disease after the initial debulking surgery and response to chemotherapy (complete, partial, poor/limited, stable disease, unknown/other). Platinum-resistance was defined as an ovarian cancer recurrence within the first six months of ending platinum-based chemotherapy among women with a complete response to platinum-based chemotherapy. Ovarian cancer cases with a complete response to platinum-based chemotherapy that had a recurrence more than six months after completing their chemotherapy were considered platinum-sensitive. The date of death for cases was collected primarily through the Social Security Death Index and also from voter and census data and obituaries.

Assessment of exposure and covariate information

Detailed information on lifestyle, reproductive factors, medical history, and body size at least one year before diagnosis were obtained for each participant through in-person interviews. Cases were asked to recall each pregnancy, the outcome of each pregnancy including induced abortions and miscarriages, and the number of months each child was breastfed. In analyses on parity, pregnancies lasting six months or longer were included. Age at first birth was calculated using the date of the first pregnancy minus date of birth, while age at last birth was calculated using the date of the last pregnancy. Women reported if they ever had a tubal ligation, used an intrauterine device (IUD), and reported their age at menarche and how long it took for their menstrual cycles to become regular. Self-reported endometriosis, including endometriosis discovered as a cause of infertility, was collected. Additionally, cases recalled their oral contraceptive (OC) use including the start and end dates of each OC they used. Women were classified as postmenopausal before their diagnosis if (1) their periods had stopped naturally or (2) they had a hysterectomy or

medical menopause and were older than 60 years. Women were classified as having uncertain menopause status if they reported they had a hysterectomy without an oophorectomy and were not yet 60 years old or reported that their periods were still occurring due to HT use. Age at menopause was considered only for women who reported that their periods had stopped naturally. Ovulatory years were calculated as the age at natural menopause (or current age for premenopausal women) minus age at menarche and additional subtraction of one year for each pregnancy and OC duration. Women reported each menopausal HT they had used including the type and duration of use, except in phase 1 of the study, which was excluded from this analysis. Women were classified as smokers if they had smoked at least 100 cigarettes. Women reported their weight one year before diagnosis and their current height. Body mass index (BMI) was then calculated as weight in kilograms divided by height in meters squared.

Statistical analyses

Ovarian cancer cases with non-epithelial ovarian cancer (n=127), borderline ovarian cancer (n=426) and missing information on date of death (n=1) were excluded from the overall survival analyses leaving 1,649 invasive cases in the current analysis. Of the 881 women who had medical record information, 783 had used platinum-based chemotherapy in their first-line adjuvant treatment and 541 had a complete response to their platinum therapy, i.e. there was no evidence of their original ovarian tumor. Of the 541 with a complete chemotherapy response, we further excluded women with missing therapy end dates (n=81) and missing recurrence information (n=14), leaving 446 in the final platinum-resistance analysis. Cox proportional hazards model was used to calculate hazard ratios (HR) and 95% confidence intervals (CI) for the association of each reproductive and hormonal factor with overall death or platinum relapse.

The time scales were years since ovarian cancer diagnosis or months since the end of platinum chemotherapy, respectively. For the overall survival analysis, cases contributed person-time from the date of ovarian cancer diagnosis until death or the end of follow-up in February 2015. For the platinum resistance analysis, cases contributed person time from the date they ended their platinum therapy until six months after they ended platinum therapy, date of death, date of recurrence, or date of last follow-up (July 2015).

Our initial statistical models adjusted for age at diagnosis (years), study phases (1, 2, 3), state of residence (MA, NH), menopausal status (premenopausal, postmenopausal, dubious menopause), smoking status (current, past, never), BMI (kg/m^2), and parity (ever, never). Then we evaluated a second model additionally adjusting for stage (I/II, III/IV), grade (I, II, III, unknown), histology (serous, endometrioid, mucinous, clear cell, other), and debulking status (optimal debulking, non-optimal debulking, unknown). The fully-adjusted models further accounted for chemotherapy (no chemotherapy, platinum and taxol chemotherapy, chemotherapy other than platinum and taxol, unknown chemotherapy type) in the overall survival analysis. In the platinum analysis, fully-adjusted models included maintenance chemotherapy use (yes, no) and calendar time (before 2000, 2000 and later).

For the overall survival analysis, we performed a number of sensitivity analyses including restricting to high-grade serous cases, excluding cases with stage IV disease, and restricting to cases with stage III disease. As the cause of death was not known for the majority of women, we also restricted to the first 5-years of follow-up since most deaths are likely to be due to ovarian cancer during this time period. Of the 554 deaths in the European Prospective Investigation into

Cancer and Nutrition (EPIC), 92% were due to ovarian cancer over a mean follow-up of 3.6 (SD=3.2) years [33]. As we may be missing the more aggressive cases who would have died quickly after diagnosis, we restricted analyses to women interviewed within one year of diagnosis. We tested for effect modification by study phase, age at diagnosis ($\leq$50 yrs vs. >50 yrs), and histology (serous vs. non-serous) using a likelihood ratio test. Additionally, since we observed a strong association between endometriosis and overall survival, we stratified by tumor histology, grade and stage. Finally, we assessed the proportional hazards assumption using a likelihood ratio test comparing a model with an interaction term between the exposure of interest and time since diagnosis to a model without the interaction term.

All analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). P-values were 2-sided and considered statistically significant if less than 0.05.

Results

Study population

During 12,522 person-years of follow-up, 911 deaths occurred among 1,649 invasive epithelial ovarian cancer cases. The average age at diagnosis among cases that died was 58 years compared to 52 years among cases who did not die during follow-up (Table 3.1). Cases who died were less likely to report ever OC use (47% vs. 59%) and more likely to be parous (75% vs. 64%). Additionally, women who died were more likely to be diagnosed with high grade (72% vs. 42%) and serous (76% vs. 37%) tumors compared to women who did not die during follow-up.

Among the 446 ovarian cancer cases included in the platinum-resistance analysis (Table 3.1), 45 (10.1%) had a recurrence within the first six months of ending platinum-based chemotherapy (platinum resistant). The majority of platinum-resistant cases, 78%, had a serous tumor compared to only 54% of platinum-sensitive cases. Additionally, platinum-resistant cases were more likely to be older, have a high-grade tumor, be parous and be postmenopausal at diagnosis compared to platinum-sensitive cases.

Table 3.1 Characteristics of NECC invasive epithelial ovarian cancer cases for the survival analysis (N=1649) and the platinum-resistance analysis (N=446)

	Overall Survival				**Platinum-resistance analysis**			
	Alive (n=738)		**Deaths (n=911)**		**Platinum sensitive (n=401)**		**Platinum resistant (n=45)**	
	N	**(%)**	**N**	**(%)**	**N**	**(%)**	**N**	**(%)**
Ever OC use	432	(59)	429	(47)	223	(56)	28	(62)
Ever parous	470	(64)	687	(75)	251	(63)	35	(78)
Tubal ligation	76	(10)	142	(16)	42	(10)	9	(20)
Ever smoked	228	(31)	385	(42)	107	(27)	14	(31)
Postmenopausal	355	(48)	623	(68)	218	(54)	34	(76)
Dubious menopause	57	(8)	60	(7)	28	(7)	4	(9)
Ever breastfed[1]	234	(32)	296	(32)	131	(33)	18	(40)
Endometriosis	102	(14)	57	(6)	55	(14)	2	(4)
Ever HT use[2]	134	(38)	213	(34)	86	(39)	15	(44)
Grade								
I	171	(23)	47	(5)	44	(11)	0	(0)
II	167	(23)	146	(16)	83	(21)	7	(16)
III	313	(42)	657	(72)	232	(58)	36	(80)
Unknown	72	(10)	51	(6)	40	(10)	2	(4)
Missing	15	(2)	10	(1)	2	(1)	0	(0)
Histology								
Serous	273	(37)	695	(76)	215	(54)	35	(78)
Endometrioid	245	(33)	81	(9)	99	(25)	2	(4)
Mucinous	74	(10)	21	(2)	15	(4)	1	(2)
Clear cell	83	(11)	31	(3)	38	(9)	3	(7)
Other	63	(9)	83	(9)	34	(8)	4	(9)
Chemotherapy[3]								
No chemotherapy	132	(18)	91	(10)	-	(-)	-	(-)

Platinum + Taxol	272	(37)	413	(45)	368	(92)	42	(93)
Other chemotherapy	34	(5)	98	(11)	33	(8)	3	(7)
Unknown chemo type	300	(41)	309	(34)	-	(-)	-	(-)
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age at diagnosis	52.3	11.2	58.0	10.4	54.1	11.2	57.6	10.0
Length of follow-up[4]	12.3	4.5	3.8	3.7	8.6	4.6	3.0	2.5
Years of OC use[5]	4.7	4.8	3.8	4.2	3.9	4.1	3.7	4.6
Number of children[1]	2.3	1.2	2.7	1.3	2.4	1.3	2.5	0.9
Age at natural menopause	48.7	5.0	49.0	5.2	49.0	4.8	49.9	5.7
Age at menarche	12.5	1.5	12.6	1.5	12.4	1.3	12.5	1.5
Ovulatory years	28.7	8.3	31.3	6.9	30.0	7.5	32.6	8.6
Years of HT use[6]	8.4	15.9	8.3	12.7	9.5	13.8	5.0	5.0

1. Among parous
2. Among postmenopausal women
3. Other chemotherapy category includes platinum without taxol
4. Mean number of years until death or censoring in overall survival analysis and mean number of months until recurrence or censoring in platinum-resistance analysis
5. Among OC users
6. Among ever HT users

Overall survival

Parity and tubal ligation were significantly positively associated with overall survival before adjusting for tumor characteristics; however after adjustment for tumor histology in particular, the associations were substantially attenuated (Table 3.2). Further, induced abortion was not significantly associated with survival, but women with at least one miscarriage had an 18% (95% CI: 1.01-1.38) increased risk of death compared to women who never reported a miscarriage. In the fully-adjusted analyses, increasing age at menarche was associated with an increased risk of death with a HR of 1.23 (95% CI: 1.05-1.43) for an age at menarche of 13 years vs. <13 years. Additionally, women who were older than 50 years at natural menopause had a 22% (95% CI: 1.02, 1.45) increased risk of death compared to women who were 50 years or younger at menopause. Further, there was a borderline significant relationship between never having regular

menstrual cycles and survival (HR: 0.73; 95% CI: 0.54-0.99). Postmenopausal use of HT was not associated with overall death (HR: 0.88; 95% CI: 0.72-1.08), although longer duration of HT use was associated with a decreased risk of death. Cases who had used HT for 5 or more years before diagnosis were 30% (95% CI: 0.54-0.89) less likely to die compared to never users of HT. No association was observed for breastfeeding, oral contraceptive use, or ovulatory years ($p>0.17$) as well as age at first and last birth and IUD use (data not shown). Women who reported ever having endometriosis were 29% (95% CI: 0.54-0.94) less likely to die after their ovarian cancer diagnosis compared to women without endometriosis. The reduced risk of death among women with endometriosis was consistent across tumor histology/grade (p-heterogeneity=0.31) and stage (p-heterogeneity=0.11), although not all of the point estimates reached statistical significance due to limited power for some subtypes (Table 3.3).

Table 3.2 Association between reproductive and hormonal factors and overall survival among invasive ovarian cancer cases in the NECC (N=1649)

			Reproductive characteristics model[1]		Reproductive and tumor characteristics model[2]		Reproductive, tumor and treatment characteristics model[3]	
	Deaths	**Person-yrs**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
Parity								
Nulliparous	224	4,276	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Parous	687	8,246	1.19	(1.01-1.39)	0.90	(0.77-1.06)	0.89	(0.76-1.05)
Nulliparous	224	4,276	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
1-2 children	370	5,072	1.16	(0.98-1.38)	0.90	(0.76-1.07)	0.89	(0.75-1.06)
3-4 children	254	2,622	1.21	(1.00-1.47)	0.89	(0.73-1.08)	0.88	(0.73-1.07)
5+ children	63	551	1.34	(1.00-1.80)	0.99	(0.74-1.34)	0.99	(0.73-1.34)
Breastfeeding[4]								
Never	390	4,528	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	296	3,708	1.03	(0.88-1.20)	0.99	(0.85-1.16)	1.00	(0.85-1.17)
Oral contraceptive use								
Never	482	5,780	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	429	6,742	1.01	(0.88-1.17)	1.05	(0.91-1.22)	1.05	(0.90-1.21)
Never	482	5,780	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Less than 1 yr	104	1,433	1.07	(0.86-1.33)	1.18	(0.95-1.48)	1.17	(0.93-1.46)
1 to <5 yrs	190	2,849	1.06	(0.89-1.27)	1.07	(0.89-1.28)	1.08	(0.90-1.29)
5 to <10 yrs	96	1,511	1.00	(0.79-1.26)	1.01	(0.80-1.27)	0.99	(0.79-1.26)
10+ yrs	39	950	0.75	(0.54-1.05)	0.82	(0.59-1.15)	0.82	(0.59-1.15)
Tubal ligation								
Never	769	11,077	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	142	1445	1.24	(1.03-1.49)	1.01	(0.84-1.22)	1.02	(0.85-1.24)
Age at menarche[5]								
<13 years	422	6,292	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
13 years	290	3,652	1.14	(0.98-1.33)	1.24	(1.07-1.45)	1.23	(1.05-1.43)

>13 years	194	2,508	1.09	(0.91-1.29)	1.21	(1.01-1.44)	1.19	(1.00-1.42)
Menopausal status at diagnosis[6]								
Premenopausal	228	5,257	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Postmenopausal	623	6,365	1.22	(0.97-1.54)	1.04	(0.82-1.32)	1.04	(0.82-1.32)
Age at natural menopause[7]								
≤50 years	319	3,732	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
>50 years	235	2,066	1.23	(1.03-1.46)	1.22	(1.03-1.46)	1.22	(1.02-1.45)
Ovulatory years[8]								
<26.5 years	150	3,425	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
26.5-<32 years	203	2,706	1.19	(0.96-1.49)	1.10	(0.88-1.37)	1.11	(0.89-1.39)
32-35 years	183	2,255	1.16	(0.92-1.47)	1.08	(0.86-1.37)	1.09	(0.86-1.38)
>35 years	240	2,593	1.20	(0.95-1.51)	1.14	(0.90-1.44)	1.15	(0.91-1.45)
Hormone therapy use[9]								
Never	265	2,750	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	174	1,967	0.90	(0.74-1.10)	0.88	(0.72-1.08)	0.88	(0.72-1.08)
Never	265	2,750	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
<5 years	85	871	1.04	(0.81-1.33)	1.20	(0.93-1.55)	1.21	(0.94-1.56)
≥5 years	89	1,096	0.80	(0.63-1.02)	0.69	(0.54-0.89)	0.70	(0.54-0.89)
Miscarriage and induced abortion								
Never	588	8,673	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	323	3,850	1.14	(0.99-1.31)	1.12	(0.98-1.29)	1.11	(0.97-1.28)
Menstrual cycles								
Regular	866	11,701	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Never regular	45	821	0.81	(0.60-1.09)	0.75	(0.55-1.01)	0.73	(0.54-0.99)
Endometriosis								
Never	854	11,068	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	57	1,454	0.59	(0.45-0.78)	0.72	(0.55-0.94)	0.71	(0.54-0.94)

1. Adjusted for age at diagnosis, study phase, study center (NH, MA), menopause status (pre, post, dubious), smoking status (never, current, former), parity (ever, never), OC use (ever, never), BMI (kg/m2)
2. Adjusted for same variables as reproductive characteristics model plus disease stage (I/II, III/IV), grade (I, II, III, missing), histology (serous, endometrioid, mucinous, clear cell, other), debulking status (not optimal, optimal, unknown)

3. Adjusted for same variables as the reproductive and tumor characteristics model plus chemotherapy (no chemotherapy, platinum + taxol, other chemotherapy, unknown chemotherapy type)
4. Among parous women (n=1152 for age at last and first birth, n=1155 for breastfeeding)
5. Among women with known age at menarche (n=1642)
6. Among women with known menopausal status (n=1532)
7. Among women with a natural menopause (n=878)
8. Calculated as age at menopause (or current age, if premenopausal) minus age at menarche with additional subtraction of 1 year for each pregnancy and duration of OC use (n=1424)
9. Among postmenopausal women in the last two study phases (n=753)

Table 3.3 Association between endometriosis and overall survival among invasive ovarian cancer cases by tumor histology and stage in the NECC (N=1649)[1]

				Reproductive and tumor characteristics model[2]		
		Deaths	**Person-yrs**	**HR**	**95% CI**	**p-het[3]**
Endometriosis						
			All Invasive Tumors (n=1649)			
	Never	854	11,068	1.00	(ref.)	
	Ever	57	1,454	0.71	(0.54-0.94)	
Endometriosis						
			High-Grade Serous Invasive Tumors (n=738)			0.31
	Never	517	3,685	1.00	(ref.)	
	Ever	32	325	0.78	(0.54-1.12)	
			Low-Grade Serous Invasive Tumors (n=167)			
	Never	101	1,034	1.00	(ref.)	
	Ever	8	177	0.40	(0.19-0.84)	
			Endometrioid and Clear Cell Invasive Tumors (n=440)			
	Never	99	3,905	1.00	(ref.)	
	Ever	13	813	0.66	(0.36-1.22)	
Endometriosis						
			Stage I/II Tumors (n=704)			0.11
	Never	176	6,556	1.00	(ref.)	
	Ever	12	995	0.56	(0.30-1.03)	
			Stage III/IV Tumors (n=939)			
	Never	675	4,494	1.00	(ref.)	
	Ever	45	443	0.75	(0.55-1.02)	

1. Analyses by stage, excluded 6 cases who were missing stage and analyses by histology exclude 95 mucinous cases, 63 serous tumors with unknown grade, and 146 other histology cases
2. Adjusted for age at diagnosis, study phase, study center (NH, MA), menopause status (pre, post, dubious), smoking status (never, current, former), parity (ever, never), OC use (ever, never), BMI (kg/m2), disease stage (I/II, III/IV), grade (I, II, III, missing), histology (serous, endometrioid, mucinous, clear cell, other), and optimal debulking (not optimal, optimal, unknown)
3. P-value for heterogeneity calculated using a likelihood ratio test for the interaction between histology/stage and endometriosis

Sensitivity analyses

In sensitivity analyses, there were no significant interactions between any of the exposures and study phase (data not shown). There was a significant interaction between menopausal status and tumor histology (p-heterogeneity=0.02), although menopausal status was not significantly associated with risk for serous or non-serous tumors. Additionally, we observed significant interactions of age at diagnosis with parity, menopausal status and regularity of menstrual cycles (p-heterogeneity<0.04). For example, there was a significant inverse association with death for parity among women aged >50 years (HR: 0.81; 95% CI: 0.67-0.98), but not ≤50 years (HR: 1.08; 95% CI: 0.79, 1.47). Conversely, the inverse association for never regular menstrual cycles was only observed among women aged ≤50 years (data not shown).

Overall, associations were slightly stronger among women interviewed within one year of diagnosis, expect for endometriosis, age at natural menopause, and five or more years of HT use, which were in the same direction as the full analysis but no longer significant. Similarly, results were generally stronger in analyses restricted to the first five years of follow-up (data not shown), including a significant association for ovulatory years (HR, top vs. bottom quartile: 1.31; 95% CI: 1.00-1.72). Results were substantially unchanged when excluding stage IV disease or restricting to stage III tumors, but slightly attenuated, although in the same direction, when restricting to high-grade serous tumors (data not shown). Finally, no significant violations of the proportional hazards assumption were observed except for HT use, where the inverse association appeared to attenuate with increasing time since diagnosis (data not shown).

Platinum resistance

Among women who had a complete response to platinum-based chemotherapy, no significant associations were observed with platinum resistance except for long duration of HT use (Table 3.4). HT use for five years or longer was associated with a significant decreased risk of platinum resistance (HR: 0.52; 95% CI: 0.32-0.85). Additionally, although non-significant, hazard ratios for age at menarche (HR, ≥13 vs. <13yr: 1.29; 95% CI: 0.70-2.36), age at natural menopause (HR, >50 vs. ≤50yr: 1.44; 95% CI: 0.68-3:08), and endometriosis (HR: 0.33; 95% CI: 0.08-1.41) are all in the same direction as the risk estimates for the overall survival analysis.

Table 3.4 Association between reproductive and hormonal factors and platinum-resistance among invasive ovarian cancer cases in the NECC (n=446)

			Reproductive characteristics model[1]		Reproductive and tumor characteristics model[2]		Reproductive, tumor and treatment characteristics model[3]	
	Platinum-resistant	**Person-months**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
Parity								
Nulliparous	10	936	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Parous	35	1,668	1.61	(0.76-3.38)	1.34	(0.61-2.93)	1.38	(0.63-3.03)
Breastfeeding[4]								
Never	17	796	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	18	866	1.05	(0.52-2.11)	1.35	(0.66-2.76)	1.35	(0.66-2.76)
Oral contraceptive use								
Never	17	1,144	1.00	(ref.)	1.00	(ref.)	1	(ref.)
Ever	28	1,460	1.41	(0.75-2.66)	1.48	(0.76-2.88)	1.47	(0.75-2.85)
Tubal ligation								
Never	36	2,310	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	9	293	1.72	(0.78-3.76)	1.36	(0.61-3.02)	1.37	(0.62-3.05)
Age at menarche[5]								
<13 years	22	1,377	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
≥13 years	23	1,221	1.20	(0.66-2.17)	1.32	(0.72-2.41)	1.29	(0.70-2.36)
Menopausal status at diagnosis[6]								
Premenopausal	7	963	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Postmenopausal	34	1,457	2.64	(0.83-8.38)	2.25	(0.70-7.29)	2.23	(0.69-7.25)
Age at natural menopause[7]								
≤50 years	14	792	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
>50 years	16	536	1.59	(0.77-3.28)	1.48	(0.70-3.13)	1.44	(0.68-3.08)

Ever HT use[8]								
Never	76	903	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	51	698	0.82	(0.57-1.18)	0.75	(0.51-1.10)	0.74	(0.50-1.08)
Miscarriage and induced abortion								
Never	24	1,768	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	21	836	1.57	(0.86-2.88)	1.64	(0.88-3.04)	1.66	(0.90-3.08)
Menstrual cycles								
Regular	43	2,506	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Never regular	2	98	1.66	(0.39-7.13)	1.54	(0.35-6.78)	1.43	(0.32-6.38)
Endometriosis								
Never	43	2,264	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Ever	2	339	0.27	(0.06-1.12)	0.33	(0.08-1.45)	0.33	(0.08-1.41)

1. Adjusted for age at diagnosis, calendar time (<2000, 2000+), study center (NH, MA), menopause status (pre, post, dubious), smoking status (never, current, former), parity (ever, never), OC use (ever, never), BMI (kg/m2)
2. Adjusted for same variables as reproductive characteristics model plus disease stage (I/II, III/IV), grade (I, II, III, missing), histology (serous, endometrioid, mucinous, clear cell, other), optimal debulking (optimal, not optimal, unknown)
3. Adjusted for same variables as the reproductive and tumor characteristics model plus maintenance therapy (yes, no)
4. Among parous women (n=286 for age at first and last birth, n=285 for breastfeeding)
5. Among women with known age at menarche (n=445)
6. Among women with known menopausal status (n=414)
7. Among women with a natural menopause (n=230)
8. Among postmenopausal women in the last two study phases (n=236)

Discussion

In one of the largest studies to date, we investigated the association between reproductive and hormonal factors and overall survival among 1,649 ovarian cancer cases in the New England Case-Control study with careful control for reproductive and tumor characteristics and treatment. Endometriosis, never having regular menstrual cycles, and a longer duration of HT use were associated with improved survival whereas spontaneous miscarriage, older age at menarche, and older age at natural menopause were associated with a worse prognosis. Consistent with the majority of previous research we did not observe any associations between survival and OC use, parity, breastfeeding, tubal ligation, and ovulatory years [29-33, 129-131]. In the first analysis of its kind, we observed a significant decreased risk of platinum resistance for long-term HT use, although power was limited by a smaller sample size of only 446 ovarian cancer cases with complete response to platinum.

Endometriosis has been consistently associated with an increased risk of ovarian cancer, particularly for endometrioid and clear cell tumors [132]. Interestingly, in our study, we observed a 29% decreased risk of death among ovarian cancer cases with self-reported endometriosis before diagnosis. Although endometriosis-associated ovarian cancers (endometrioid, clear cell and low grade serous) are less aggressive than high-grade serous disease, our results suggested that the association was independent of tumor histology and disease stage, and remained after adjustment for debulking, tumor characteristics, and chemotherapy. Prior research in this area has been mixed. A recent meta-analysis of seven studies observed improved survival for women with endometriosis in univariate analyses, but the association was attenuated among the four studies that controlled for age and grade [27]. Similarly, more recent studies observed improved

survival for women with endometriosis in univariate analyses, but not after adjusting for confounders [133-135]; however, the majority of these studies had small sample sizes, limiting power particularly in multivariate analyses. The largest study to date of endometriosis and survival was based within a Swedish registry and reported an improved prognosis among ovarian cancer patients with endometriosis (HR: 0.81; 95% CI: 0.65-1.01) [26]. However, this association was attenuated with adjustment for tumor histology and stage among a small subset of the cases with tumor characteristics data (n=218). With 1,649 ovarian cancer cases, ours is the largest study to date to assess endometriosis and survival with adjustment for tumor characteristics. It is likely that inconsistencies for this association are largely driven by small sample sizes, supporting a consortial approach.

The biological mechanism underlying the potential association between endometriosis and ovarian cancer survival is unclear, but may involve differences in the immunologic milieu microenvironment within the peritoneal cavity of women with endometriosis [136]. Additionally, ovarian tumors among women with endometriosis may be more likely to express progesterone receptors (PR) as endometriosis lesions have been reported to express PR [137]. The expression of PR has been associated with improved survival particularly among endometrioid and high-grade serous ovarian cancer cases [138]. However, the expression of PR does not explain the decreased risk associated with endometriosis among clear cell tumors, which are generally PR negative [40]. Finally, endometriosis may affect survival through improved treatment outcomes. Although the association did not reach statistical significance, most likely due to a small sample size, we did observe a non-significant decreased risk of platinum resistance among women who

reported endometriosis. Future research should investigate the potential mechanisms through which endometriosis improves survival from ovarian cancer.

A recent pooled analysis of 52 epidemiologic studies observed a significant increased risk of ovarian cancer with current HT use, particularly for serous and endometrioid tumors [139]. While HT increases risk of certain tumor subtypes, it may lead to less aggressive tumors, as we observed that longer duration of pre-diagnosis HT use was associated with a decreased risk of death. These results are similar to those observed in a large European cohort study [33] that observed a 30% (95% CI: 0.50-0.99) decreased risk of death for 5 or more years of HT use. Additionally, a smaller study noted a decreased risk of death with HT use among ovarian cancer patients who had optimal tumor debulking [140]. In contrast, most smaller studies did not observe an association between pre-diagnostic HT use and survival, although they had limited power and few cases with longer duration of use [29-31, 126, 127]. Interestingly, similar associations of improved survival with HT use have been noted for breast cancer, with potential mechanisms including hormonal influences [141, 142]. Ovarian tumors that develop among long-term users of HT may be more likely to express PR, which as noted above has been associated with improved survival [138]. Additionally, prior HT use may lead to tumors that are less likely to be platinum-resistant as we observed that five or more years of HT use was associated with a 48% decreased risk of platinum resistance. Further, we cannot exclude the possibility that long-term HT users may have been diagnosed with ovarian cancer at an earlier stage due to their increased access to health care settings compared to never HT users. Better understanding the interplay between hormone exposure and ovarian tumor development may elucidate novel mechanistic pathways in survival.

In our study, cases with an older age at menarche or an older age at natural menopause had worse survival. Contrary to our findings, two studies have reported improved survival with older age menarche [32, 130], while the majority of studies observed no association [29-31, 33, 129, 131]. Additionally, all other studies that have assessed age at menopause and survival among ovarian cancer cases have reported null findings [30, 33, 129, 131]. Given the overall weight of the evidence, it is likely that our results were due to chance.

Only one other study has assessed abortion and survival among ovarian cancer cases [129]. In contrast to our findings of an increased risk of death with at least one spontaneous miscarriage, Jacobsen et al 1993 reported a protective effect on survival for two or more abortions. However, this study did not distinguish between induced and spontaneous abortions making it difficult to directly compare to our results. Additionally, we are the only study, as far as we know, that has assessed menstrual cycle regularity and overall survival. We observed a better prognosis among women who reported never having regular menstrual cycles. Future research is needed to validate both the miscarriage and never regular menstrual cycle findings in our analysis.

Our platinum-resistance analyses were limited by a small sample size. Only 10% of ovarian cancer cases with a complete response to platinum-based chemotherapy had a recurrence within 6 months of ending treatment. However, the observed direction of effects, including a significant association with longer duration of HT use, were similar to those observed in the analysis of overall survival. While this may be due, in part, to the worse outcomes for patients with platinum resistance, such research, in large datasets with detailed treatment and recurrence data, is crucial

to identify the underlying pathways through which pre-diagnosis exposures may influence tumor aggressiveness and hence survival.

One limitation of our study is that we did not have information on cause of death; however prior studies have shown that most deaths at least within the first three to five years after diagnosis are due to ovarian cancer [11, 33]. In analyses restricted to the first five years of follow-up, we observed similar results to the main findings. Additionally, cases were interviewed a median of 8.9 months (min 0.8 months, max 45.4 months) after ovarian cancer diagnosis, which means women with rapidly fatal cancers would be less likely to be included. However, when we restricted analyses to women interviewed within one year of diagnosis the results were similar. Further, there were a limited number of ovarian cancer cases who had a complete response to platinum-based chemotherapy thereby limiting power to observe associations between reproductive factors and platinum-resistance. While all exposures, including endometriosis, were self-reported, any misclassification should be non-differential which would have attenuated our observed associations. Finally, a large number of statistical tests were performed in this study, therefore there is the possibility that at least some of the results we have observed may be due to chance.

Our study was one of the largest studies to date to assess reproductive and hormonal factors and overall survival. The large sample size allowed us to assess effect modification by histology and age at diagnosis. Additionally, we had detailed exposure and covariate information collected through in-person interviews and pathology reports to abstract tumor characteristics. Further, for over half of the study population we had access to detailed medical information including

chemotherapy, debulking status and response to chemotherapy enabling us to control for chemotherapy use and assess predictors of platinum resistance.

Conclusion

Among ovarian cancer cases we observed improved overall survival among women with endometriosis and long-term users of HT, which may be through changes in the hormonal or immunologic milieu of these women. Additionally, long-term use of HT may be associated with a reduced risk of platinum resistance. Future research should focus on understanding the mechanisms through which endometriosis and HT may improve survival after an ovarian cancer diagnosis. Additionally, further research is needed to validate our findings for spontaneous miscarriage and menstrual cycle irregularity.

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Overall Conclusion

Our results suggest that development of ovarian tumors through hormonal pathways may differ by menopausal status, with postmenopausal women more likely to develop PR- tumors compared to premenopausal women. Future research should focus on the drivers for PR loss among postmenopausal women and how loss of PR may lead to the development of ovarian cancer. Additionally, we observed that the association between OC use and ovarian cancer differed in the NHSII, a young birth cohort, compared to the findings from previous studies, the majority of which were conducted among older birth cohorts. While these findings may be due to chance and need to be confirmed in other young birth cohorts, future studies should focus on understanding how risk factors and hormone levels may differ between women who can tolerate OCs and women who cannot. Finally, we observed that various reproductive and hormonal factors including endometriosis and HT use were associated with ovarian cancer survival. Future research should focus on understanding the hormonal and immunologic pathways through which pre-diagnostic endometriosis and HT use could impact on ovarian cancer survival. All together these results provide valuable information on the etiology of ovarian cancer through hormonal pathways and on potential recommendations for the prevention of ovarian cancer development and mortality.

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