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Inflammation, Anti-inflammatory Drug Use and Risk of Ovarian Cancer

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Inflammation, Anti-inflammatory Drug Use and Risk of Ovarian Cancer

Abstract

Ovarian cancer is a highly fatal malignancy. Advancing knowledge of ovarian cancer etiology to improve prevention strategies is essential to reducing ovarian cancer mortality. My research investigated the role of inflammation in ovarian cancer etiology. First, I evaluated if non-steroidal anti-inflammatory drug (NSAID) use is associated with risk of ovarian cancer in the Nurses' Health Study (NHS) and NHSII cohorts. Using Cox proportional hazards models, I evaluated associations for aspirin and non-aspirin NSAIDs, considering timing, frequency, quantity, dose, and duration. Then, to investigate the prostaglandin synthesis pathway as a possible mechanism underlying the association between NSAID use and ovarian cancer, I conducted two additional studies. In a case-control study nested in the NHS, NHSII, and Shanghai Women's Health Study, I estimated the association between pre-diagnosis urinary PGE-M, a marker of prostaglandin E2, and ovarian cancer using unconditional logistic regression. In a case-control study based in the NHS, NHSII and New England Case-Control study, I used polytomous logistic regression to evaluate if the association between NSAID use and ovarian cancer differed by COX1 and COX2 expression, or infiltration with tumor-associated macrophages (TAMs). There was no evidence of an association between aspirin use and ovarian cancer risk when all aspirin products were evaluated together; however, the hazard ratio (HR) for current versus non-use of low-dose aspirin was 0.77 (95%CI=0.61-0.96). We observed a positive association for ≥10 years of non-aspirin NSAID use versus non-use (HR=1.34, 95%CI=1.06-1.70; p-trend=0.03), and a positive trend for tablets per week (p=0.03). Pre-diagnosis urinary PGE-M was not associated with risk of ovarian cancer (odds ratio [OR]=

1.09, 95%CI=0.71-1.66; p-trend=0.48). Additionally, the association between NSAIDs and ovarian cancer did not differ by tumor COX1 or COX2 expression (p-heterogeneity>0.05). We observed significant heterogeneity for current aspirin use (p-heterogeneity<0.001) and NSAID use (p-heterogeneity<0.001) with risk of ovarian cancer by infiltration with pro-tumorigenic M2-type TAMs. Overall, our results suggested a lower risk of ovarian cancer among low-dose aspirin users and an increased risk of ovarian cancer among women with heavy use of non-aspirin NSAIDs. There was no evidence that the prostaglandin synthesis pathway influences ovarian carcinogenesis, but immune function may be relevant.

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Introduction

Ovarian cancer is the fifth most common cause of cancer death among U.S. women and the seventh leading cause worldwide (1, 2). Despite advances in treatment, five-year survival is only 46% (2). No screening mechanisms have been developed that effectively reduce ovarian cancer mortality (3, 4), so reducing death from ovarian cancer is contingent on understanding the etiology of the disease and developing prevention strategies.

Inflammation, immune function, and hormones are hypothesized to be key contributors to ovarian cancer etiology (5). My research focused on inflammation and immune function. There is prior evidence of a role for inflammation in ovarian tumorigenesis (6, 7). Systemic inflammatory markers, including C-reactive protein and tumor necrosis factor α have been associated with an increased risk of ovarian cancer (8-13). Greater lifetime ovulatory cycles have also been associated with increased ovarian cancer risk (14-17), and the association may be mediated by pro-inflammatory events that occur with the monthly wounding and repair of the ovarian surface epithelium (18, 19). Here, I assessed the role of inflammation in ovarian carcinogenesis by evaluating the association between anti-inflammatory drug use and risk of ovarian cancer and considering inflammatory and immune pathways that may underlie that association.

Aspirin and non-aspirin non-steroidal anti-inflammatory drugs (NSAIDs) are hypothesized to influence risk of ovarian cancer. Overall, past research supported a modest association between NSAID use and reduced risk of ovarian cancer; however, the results of individual studies were inconsistent (20-28). A key goal of my research was to investigate explanations for the inconsistencies across studies by considering how variations in timing, frequency, dose, quantity, and duration of NSAID use affect risk.

My research also considered biologic pathways by which NSAID use could influence ovarian carcinogenesis, focusing on the prostaglandin synthesis pathway. NSAIDs are anti-inflammatory agents that influence inflammatory processes by blocking COX1 and COX2, important enzymes in the prostaglandin synthesis pathway (29-32). Prostaglandin E2 (PGE2), COX1 and COX2 are over-expressed in ovarian cancer compared to normal ovarian tissue (33-38), and tumor expression of COX2 has been associated with poorer prognosis (39-41). The primary role of prostaglandins is the modulation of inflammation; however, these bioactive molecules have also been reported as influential in ovulation, wound healing and the differentiation of monocytes into tumor-associated macrophages (42-49).

The research included here addresses three questions. (1) Are aspirin and non-aspirin NSAID use associated with risk of ovarian cancer, and do the associations vary by timing, frequency, quantity, dose, or duration of use? (2) Are pre-diagnosis prostaglandin levels, as measured by urinary PGE-M, associated with risk of ovarian cancer? (3) Does the association between NSAID use and ovarian cancer differ by tumor expression of COX1 and COX2, or by infiltration with tumor-associated macrophages? I addressed these questions in the context of three prospective cohort studies, the Nurses' Health Study, the Nurses' Health Study II and the Shanghai Women's Health Study, and one population-based study, the New England Case-Control Study.

Part 1: Analgesic Use and Risk of Ovarian Cancer in the Nurses' Health Studies

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Abstract

Background: Localized inflammation occurs with ovulation and may contribute to ovarian tumorigenesis. Therefore we hypothesized that regular use of nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, may reduce ovarian cancer risk.

Methods: We prospectively followed 93,664 Nurses' Health Study (NHS) participants and 111,834 NHSII participants who provided biennially updated data on use of aspirin, non-aspirin NSAIDs and acetaminophen over 26-34 years. Of these women, 1,054 developed epithelial ovarian cancer. We used Cox proportional hazards models to estimate hazard ratios (HR) and 95% confidence intervals (CI) for associations of aspirin, non-aspirin NSAID, and acetaminophen use, including timing, dose, duration, frequency, and number of tablets, with risk of ovarian cancer. All statistical tests were two-sided, with a significance level of 0.05.

Results: We did not observe significant associations between aspirin and ovarian cancer risk when all aspirin products (low- [≤100mg] and standard-dose [325mg]) were evaluated together (HR, current versus non-use=0.99 [95%CI=0.83-1.19]). However, the comparable HR for low-dose aspirin was 0.77 (95%CI=0.61-0.96), while no association was observed for standard-dose aspirin (HR=1.17, 95%CI=0.92-1.49). We observed a positive association for ≥10 years duration of non-aspirin NSAIDs ≥2 times per week compared to non-use (HR=1.34, 95%CI=1.06-1.70; p-trend=0.03), and a positive trend for NSAID tablets per week (p=0.03). There were no clear associations for acetaminophen.

Conclusions: Our results are consistent with a possible reduced risk of ovarian cancer among frequent (≥5 days/week) low-dose aspirin users. We observed an increased risk of ovarian cancer with long-term high-quantity use of analgesics, particularly non-aspirin NSAIDs, though this finding requires confirmation.

Introduction

Ovarian cancer is the fifth most common cause of cancer-related death among US women (50). There is growing evidence to support a role for inflammation in ovarian cancer etiology (6, 7, 13, 18, 19). Localized inflammation, as occurs with ovulation, may contribute to ovarian tumorigenesis (18), and epidemiologic studies have consistently observed positive associations between a higher number of lifetime ovulatory cycles and risk of ovarian cancer (14-17). Systemic inflammation has also been associated with increased risk of ovarian cancer. For example, meta-analyses of circulating C-reactive protein (CRP), a marker of systemic inflammation, reported a nearly two-fold increased risk of ovarian cancer for those with high versus low CRP levels (12, 13).

Anti-inflammatory agents, including aspirin and non-aspirin non-steroidal anti-inflammatory drugs (NSAIDs), are common medications that may influence ovarian cancer development; however, results of prior studies have been mixed. A pooled analysis of 12 case-control studies reported a modest, though statistically significant, lower ovarian cancer risk among aspirin users and a suggestion of a lower risk among non-aspirin NSAID users (27). In contrast, past analyses in prospective cohort studies, including ours, did not observe clear associations with regular use of aspirin or non-aspirin NSAIDs (21, 22, 24-26, 51-53). Possible explanations for these mixed results include evaluation of prospective versus retrospective data, differences in age distributions across study designs, and heterogeneity in the measurement and definition of medication use across studies.

Previously, no prospective cohort studies had both adequate power and sufficiently detailed exposure data to evaluate whether timing and patterns of analgesic use are associated with ovarian cancer risk. We used prospectively collected data from the Nurses' Health Studies

(NHS) on type, timing, frequency, quantity, dose, and duration of analgesic use to conduct a detailed analysis of aspirin, non-aspirin NSAID, and acetaminophen use and risk of ovarian cancer. We included acetaminophen because it is an analgesic with weaker anti-inflammatory properties than aspirin and non-aspirin NSAIDs (54), and therefore an informative comparator.

Methods

Study population

The NHS and NHSII are large, prospective cohort studies. The NHS enrolled 121,700 female registered nurses, ages 30-55, in 1976. In 1989, the NHSII enrolled a similar cohort of 116,429 women, aged 25-42. All women in the NHS/NHSII have been followed biennially to collect data on lifestyle, health factors, and disease outcomes, including analgesic use and ovarian cancer diagnosis. Study protocols for NHS/NHSII were approved by the institutional review board at Brigham and Women's Hospital.

Assessment of analgesic use

The NHS collected biennial information on aspirin use beginning in 1980 when women were asked if they used aspirin most weeks, and those who responded affirmatively were asked to report the number of aspirin tablets used per week and duration of use. Data on current regular aspirin use were collected biennially thereafter (except 1986), and information on frequency of aspirin use was first collected in 1984 and updated biennially beginning in 1988. The number of aspirin tablets taken during a typical week was first queried in 1994 with instructions to count 4 baby aspirin as 1 regular 325mg tablet, and beginning in 2000, women reported the number of tablets separately for regular (325mg) and low-dose (≤100mg) aspirin. NHSII first collected data

on current aspirin use in 1989. Questions on current regular use and frequency of use were repeated biennially beginning in 1993, and questions on the number of tablets per week were added in 1999. Beginning in 2001, women reported the number of tablets separately for regular and low-dose aspirin.

Non-aspirin NSAIDs and acetaminophen (current use and frequency) were queried biennially in the NHS starting in 1990. Information on tablets per week was collected biennially beginning in 1998. NHSII queried current use of non-aspirin NSAIDs and acetaminophen starting in 1989. Current use and frequency were queried biennially beginning in 1995 for both drugs, and questions on tablets per week were added in 1999.

Identification of ovarian cancer cases

We identified incident cases of epithelial ovarian cancer by self-report on biennial questionnaires, or when a participant was reported deceased by a family member or the US postal service, with cause of death identified via linkage to the National Death Index. We requested permission to review medical records from all participants with a reported diagnosis of ovarian or primary peritoneal cancer. A gynecologic pathologist, who was blinded to exposure status, reviewed the reports to confirm the ovarian cancer diagnosis and classify the cancer by grade, morphology, and histotype. When pathology reports could not be obtained, information on the diagnosis was accessed via linkage to cancer registries.

Assessment of covariates

We regularly collected data on ovarian cancer risk factors, including age, menopausal status, parity, oral contraceptive (OC) use, hormone therapy (HT) use, tubal ligation,

hysterectomy, family history of breast or ovarian cancer, and body mass index (BMI). Most factors were queried every 2-4 years. We also asked about markers of health care utilization (e.g., physical exams, mammography screening), health-related behaviors (e.g., physical activity, smoking), medication use (e.g., anti-hypertensives) and comorbidities (e.g., cardiovascular disease, hypertension, diabetes, gout, rheumatoid arthritis, osteoarthritis, multiple sclerosis, systemic lupus erythematosus, ulcerative colitis).

Statistical analysis

Follow-up for this analysis began in 1980 (NHS) and 1989 (NHSII). We excluded participants at baseline if they reported a prior diagnosis of cancer (other than non-melanoma skin cancer), a bilateral oophorectomy, or menopause due to pelvic irradiation. After exclusions, 93,664 NHS participants and 111,834 NHSII participants were eligible for the analysis and followed until the first of the following events: diagnosis of ovarian cancer, diagnosis of other cancer (except non-melanoma skin cancer), bilateral oophorectomy, pelvic irradiation, death, loss to follow-up, or end-of-study (June 2014 NHS; June 2015 NHSII).

We used Cox proportional hazards models with time-updated exposures and covariates to estimate hazard ratios (HR) and 95% confidence intervals (CI) for the associations between analgesic use and ovarian cancer. Participants contributed person-time when they had responded to questions on analgesic use within the past four years. Since pain and abdominal discomfort are commonly reported in the year leading up to ovarian cancer diagnosis (55), we incorporated a latency period of 2-4 years to limit the potential for reverse causation. For example, aspirin use in 1980 was used when evaluating ovarian cancer incidence from 1982-1984, aspirin use in 1982 was used when evaluating incidence from 1984-1986, and so on.

Our analyses separately evaluated on-study, regular use (≥ 2 times per week) of aspirin, non-aspirin NSAIDs, and acetaminophen. We evaluated total aspirin use in the main analysis (1980-2015) and assessed low- and standard-dose aspirin separately in additional analyses (2000-2015). Non-aspirin NSAIDs and acetaminophen were considered from 1989-2015. For each drug type, we evaluated regular use and duration of use over study follow-up. Frequency was evaluated using time-updated frequency data (i.e., simple update) and frequency data averaged across all previous questionnaires (i.e., cumulative average) to better capture long-term patterns of use. Tablets per week were similarly evaluated. In a post-hoc analysis, we combined duration of use and tablets per week to create a composite variable, cumulative tablet-days, that captured more extreme patterns of analgesic use. Tests for trend were conducted by linearly modeling category medians.

We stratified all models by age, calendar year and cohort to account for potential differences in baseline hazards. In multivariate analyses we further adjusted for menopausal status (pre/post), parity (ever/never and number of children), duration of OC use (never, <1, 1-5, >5 years), duration of estrogen, estrogen-plus-progestin or other postmenopausal HT by type (years), history of tubal ligation (yes/no), history of hysterectomy (yes/no), family history of breast cancer or ovarian cancer (yes/no), and BMI (<20, 20-<25, 25-<30, 30+). We evaluated the impact of mutually adjusting for other analgesics, and considered the potential for confounding by healthcare utilization, health-related behaviors, other medication use, and comorbidities. To test the proportional hazards assumption we compared models with and without interactions between calendar time and exposure, and between age and exposure.

We hypothesized a priori that premenopausal aspirin and non-aspirin NSAID use may be more strongly associated with risk of ovarian cancer, so we separately evaluated analgesic use

during the pre- and postmenopausal time periods using the same approach as described above. We evaluated premenopausal analgesic exposure and risk of pre- and postmenopausal ovarian cancer, and postmenopausal analgesic exposure and risk of postmenopausal ovarian cancer. Finally, we used competing risks regression (56, 57) to evaluate whether analgesic use was differentially associated with risk of serous versus non-serous ovarian cancer, or risk of rapidly fatal (fatal within three years) versus non-rapidly fatal ovarian cancer. For example, to evaluate risk of serous versus non-serous ovarian cancer we used a likelihood ratio test to compare a model that constrained the association to be the same across histotypes to one that allowed different associations.

All analyses were conducted using SAS version 9.4 (Cary, NC, USA). All statistical tests were two-sided, and p-values less than 0.05 were considered statistically significant.

Results

Over 1,779,572 person-years (PY) in NHS and 1,884,999 PY in NHSII, we observed 1054 incident cases of epithelial ovarian cancer. In both studies, current aspirin users were more likely to use other medications, including OCs and HT (Table 1.1). Current aspirin users were also more likely to report cardiovascular events, hypertension and chronic inflammatory diseases. In the NHS cohort, current aspirin users were less likely to report a history of gastric or duodenal ulcer

Table 1.1 Age-Standardized characteristics (% or mean (SD)) of person-years contributed by women in the Nurses' Health Studies (1980-2015)

	Nurses' Health Study			Nurses' Health Study II		
	No Aspirin (298,000 PY)	Past Aspirin (440,187 PY)	Current Aspirin (1,041,385 PY)	No Aspirin (1,221,705 PY)	Past Aspirin (275,753 PY)	Current Aspirin (387,542 PY)
Current NSAIDs, %	22.7	46.1	35.6	36.5	50.6	50.3
Current acetaminophen, %	28.6	37.9	29.8	22.3	26.5	37.1
Age, years*	51.0 (9.8)	61.7 (10.0)	58.7 (11.3)	41.0 (7.6)	48.8 (6.8)	47.9 (8.2)
Postmenopausal, %	68.8	68.4	68.4	18.5	19.0	19.9
Tubal ligation, %	13.5	23.4	19.0	22.8	22.8	24.7
Hysterectomy, %	20.0	20.4	20.3	7.0	7.9	9.2
Family history of breast or ovarian cancer, %	12.9	15.4	14.0	11.3	12.3	11.2
Parous, %	94.4	95.1	95.0	80.6	77.3	75.8
Number of children†	3.2 (1.6)	3.1 (1.4)	3.2 (1.5)	2.3 (1.0)	2.2 (0.9)	2.2 (0.9)
Ever used OCs, %	40.3	57.6	50.9	85.9	89.2	89.0
Duration of OC use (years)‡	4.2 (3.6)	4.0 (3.8)	4.0 (3.9)	5.1 (4.6)	6.0 (5.2)	5.5 (4.9)
Estrogen HT use, %§	20.6	24.1	23.1	7.9	8.9	9.0
Estrogen HT use (years)‖	6.9 (8.9)	6.8 (5.8)	6.5 (6.1)	4.1 (5.5)	4.3 (3.0)	4.2 (2.8)
Estrogen-progestin HT use, %§	14.7	32.4	25.8	29.4	29.8	31.5
Estrogen-progestin HT use (years)‖	5.6 (5.9)	5.4 (3.7)	5.6 (4.0)	3.5 (3.8)	3.6 (2.3)	3.6 (2.2)
Other HT use, %§	19.1	21.3	21.3	9.7	10.9	9.3
Other HT use (years)‖	3.3 (4.7)	3.0 (2.6)	3.1 (2.9)	2.0 (2.3)	2.1(1.3)	2.1 (1.3)
Body mass index (kg/m^2)	25.0 (4.7)	26.1 (5.3)	25.9 (5.1)	25.7 (5.8)	26.4 (6.2)	26.7 (6.5)
Physical activity (MET-hrs/week)	16.9 (22.5)	16.4 (16.2)	16.8 (16.6)	22.0 (25.8)	22.5 (23.9)	22.4 (26.4)
Recent physical exam, %	82.7	84.9	83.7	83.1	81.8	85.0
Beta blockers, %	10.5	11.0	15.2	3.3	4.4	7.1
Thiazide diuretics, %	10.8	11.2	13.0	3.4	4.5	6.5
Calcium channel blockers, %	6.2	6.4	8.8	2.1	2.5	4.8
History of hypertension, %	28.2	34.8	36.8	12.4	16.7	21.8
History of high cholesterol, %	28.2	41.1	38.1	23.7	29.5	32.9

Table 1.1 (Continued)

MI or angina, %	4.8	5.0	8.1	1.0	1.7	2.9
Stroke, %	1.1	1.2	1.5	0.3	0.7	1.3
History of diabetes, %	4.4	5.6	6.5	1.8	2.6	4.8
Rheumatoid arthritis, %	6.5	7.8	6.7	1.9	2.8	2.6
Other arthritis, %	28.2	35.9	33.4	13.3	16.4	17.7
Non-GI autoimmune disease, %	7.3	10.3	8.4	3.3	4.4	4.5
Ulcerative colitis, %	1.6	1.6	1.3	1.5	1.7	1.6
History of gastric or duodenal ulcer, %	10.8	9.0	6.6	4.0	5.3	5.2

* Value is not age-adjusted
† Number of children among parous women
‡ Duration of OC use among past and current OC users
§ HT use among postmenopausal women
|| Duration of HT use among past and current HT users

We did not observe an association for total (low- and standard-dose) current aspirin use (HR=0.99, 95%CI=0.83-1.19), years of aspirin use ($HR_{\geq 15v.<1}$=1.13, 95% CI=0.91-1.39; p-trend=0.22), or cumulative average tablets/week ($HR_{\geq 10v.<1}$=1.02, 95% CI=0.78-1.33; p-trend=0.65) with ovarian cancer risk (Table 1.2). There was a non-significant lower risk of ovarian cancer among women with high cumulative average frequency (≥5 days/week) of aspirin use compared to non-users (HR=0.86, 95%CI=0.66-1.12; p-trend=0.52). A post-hoc analysis of cumulative tablet-days showed no evidence of an association for moderate aspirin intake (data not shown); however, we observed a positive association for those with ≥2500 cumulative aspirin tablet-days compared to those without regular aspirin use (HR=1.59, 95%CI=1.09-2.30; p-trend=0.019). The associations between aspirin use and risk of ovarian cancer did not differ for premenopausal versus postmenopausal use (Supplemental Table 1.1).

We evaluated low-dose and standard-dose aspirin separately from 2000-2015 (Table 1.3). Current low-dose aspirin use was associated with a lower risk of ovarian cancer when compared to non-use (HR=0.77, 95%CI=0.61-0.96), while current standard-dose aspirin use was not associated (HR=1.17, 95%CI=0.92-1.49). We did not observe an association between longer duration of low-dose aspirin use and risk of ovarian cancer ($HR_{\geq 5v.<1year}$=0.92, 95% CI=0.57-1.48; p-trend=0.41); however, we observed a positive association for standard-dose aspirin ($HR_{\geq 5v.<1year}$=1.77, 95%CI=1.13-2.77; p-trend=0.004). Results for cumulative average frequency and tablets were non-significant for both low-dose and standard-dose aspirin. We observed an inverse association for ≥2500 tablet-days of low-dose aspirin compared to no regular use (HR=0.77, 95%CI=0.50-1.19; p-trend=0.05), and a positive association for ≥2500 tablet-days of standard-dose aspirin compared to no regular use (HR=1.58, 95%CI=1.00-2.48; p-trend=0.021; Table 1.4).

Table 1.2 Analgesic use and risk of epithelial ovarian cancer in the Nurses' Health Studies

	Aspirin (1980-2015)		NSAIDs (1989-2015)		Acetaminophen (1989-2015)	
	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*
Current regular use						
None	259	1.00 (ref)	273	1.00 (ref)	313	1.00 (ref)
Past	263	0.95 (0.78, 1.16)	209	1.07 (0.88, 1.30)	274	1.05 (0.88, 1.25)
Current	532	0.99 (0.83, 1.19)	381	1.19 (1.00, 1.41)	254	1.02 (0.86, 1.21)
Duration (years)						
<1	316	1.00 (ref)	313	1.00 (ref)	366	1.00 (ref)
1-4	158	1.00 (0.82, 1.22)	251	1.22 (1.03, 1.45)	247	1.03 (0.87, 1.21)
5-9	174	1.18 (0.96, 1.44)	186	1.20 (0.99, 1.47)	193	1.21 (1.00, 1.45)
10-15†	104	1.11 (0.87, 1.42)	132	1.34 (1.06, 1.70)	79	1.12 (0.87, 1.46)
≥15	218	1.13 (0.91, 1.39)	--	--	--	--
p-trend		0.22		0.022		0.12
Days per week (cumulative average)‡						
<2	631	1.00 (ref)	637	1.00 (ref)	715	1.00 (ref)
3-4	255	1.03 (0.88, 1.20)	136	1.02 (0.85, 1.24)	86	1.00 (0.79, 1.25)
≥5	64	0.86 (0.66, 1.12)	61	1.18 (0.90, 1.54)	21	0.92 (0.60, 1.43)
p-trend		0.52		0.29		0.79
Tablets per week (cumulative average)‡						
<1	403	1.00 (ref)	171	1.00 (ref)	220	1.00 (ref)
1-3	324	1.15 (0.99, 1.34)	79	1.16 (0.88, 1.53)	80	1.14 (0.88, 1.48)
4-5	113	1.31 (1.06, 1.63)	45	1.21 (0.86, 1.69)	38	1.28 (0.90, 1.82)
6-9	82	1.04 (0.81, 1.33)	37	1.27 (0.87, 1.83)	22	1.14 (0.73, 1.78)
≥10	66	1.02 (0.78, 1.33)	76	1.35 (1.02, 1.79)	43	1.22 (0.88, 1.71)
p-trend		0.65		0.031		0.17

*Stratified by cohort, age in months and calendar years, and adjusted for age, menopausal status, parity (ever/never and number of children), duration of oral contraceptive use (never, <1, 1 to 5, >5 years), duration of postmenopausal hormone use by type, history of tubal ligation (yes, no), history of hysterectomy (yes, no), family history of breast cancer or ovarian cancer (yes, no), BMI (<20, 20 to <25, 25 to <30, ≥30)

† Top category of duration analysis for NSAIDs and acetaminophen is ≥10 years

‡ Aspirin days per week follow-up 1984-2015; NSAID and acetaminophen tablets per week follow-up 2000-2015

Table 1.3 Aspirin use and risk of epithelial ovarian cancer in the Nurses' Health Studies, by aspirin dose, follow-up 2000-2015

		Standard Dose Aspirin		Low Dose Aspirin	
		Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*
Current regular use					
	None	276	1.00 (ref)	287	1.00 (ref)
	Past	57	1.26 (0.93, 1.70)	22	0.62 (0.40, 0.98)
	Current	91	1.17 (0.92, 1.49)	122	0.77 (0.61, 0.96)
Duration (years)					
	<1	328	1.00 (ref)	324	1.00 (ref)
	1-4	88	1.27 (0.99, 1.64)	71	0.81 (0.62, 1.07)
	≥5	24	1.77 (1.13, 2.77)	22	0.92 (0.57, 1.48)
	p-trend		0.004		0.41
Days per week (cumulative average)					
	<2	355	1.00 (ref)	362	1.00 (ref)
	3-4	45	1.12 (0.82, 1.54)	57	0.86 (0.65, 1.15)
	≥5	36	1.17 (0.83, 1.66)	47	0.78 (0.57, 1.06)
	p-trend		0.29		0.08
Tablets per week (cumulative average)					
	<1	316	1.00 (ref)	320	1.00 (ref)
	1-3	38	1.14 (0.81, 1.61)	41	0.97 (0.69, 1.36)
	4-5	21	1.19 (0.76, 1.86)	30	0.93 (0.64, 1.37)
	6-9	21	2.00 (1.27, 3.15)	26	1.01 (0.66, 1.53)
	≥10	27	1.05 (0.70, 1.56)	30	0.70 (0.48, 1.03)
	p-trend		0.17		0.11

*Stratified by cohort, age in months and calendar years, and adjusted for age, menopausal status, parity (ever/never and number of children), duration of oral contraceptive use (never, <1, 1 to 5, >5 years), duration of postmenopausal hormone use by type, history of tubal ligation (yes, no), history of hysterectomy (yes, no), family history of breast cancer or ovarian cancer (yes, no), BMI (<20, 20 to <25, 25 to <30, ≥30)

Table 1.4 Cumulative tablet-days of analgesic use and risk of epithelial ovarian cancer in the Nurses' Health Studies, follow-up 2000-2015

	Low-dose Aspirin		Standard-dose Aspirin		NSAIDs		Acetaminophen	
	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*
None	287	1.00 (ref)	276	1.00 (ref)	199	1.00 (ref)	293	1.00 (ref)
1-499	21	1.03 (0.66, 1.61)	33	1.11 (0.77, 1.60)	59	1.22 (0.90, 1.64)	86	1.24 (0.97, 1.59)
500-999	6	1.16 (0.51, 2.63)	6	0.97 (0.43, 2.19)	18	1.06 (0.65, 1.74)	23	1.34 (0.87, 2.07)
1000-2499	61	0.75 (0.57, 1.00)	53	1.26 (0.93, 1.70)	65	1.15 (0.86, 1.53)	55	1.09 (0.81, 1.46)
≥2500	27	0.77 (0.50, 1.19)	21	1.58 (1.00, 2.48)	58	1.65 (1.19, 2.28)	39	1.41 (0.99, 2.00)
p-trend		0.05		0.021		0.006		0.10

* Stratified by cohort, age in months and calendar years, and adjusted for age, menopausal status, parity (ever/never and number of children), duration of oral contraceptive use (never, <1, 1 to 5, >5 years), duration of postmenopausal hormone use by type, history of tubal ligation (yes, no), history of hysterectomy (yes, no), family history of breast cancer or ovarian cancer (yes, no), BMI (<20, 20 to <25, 25 to <30, ≥30)

Current non-aspirin NSAID use was positively associated with risk of ovarian cancer compared to non-use (HR=1.19, 95%CI=1.00-1.41; Table 1.2). Further, we observed significant positive associations for ≥10 years duration of NSAID use compared to no regular use (HR=1.34, 95%CI=1.06-1.70; p-trend=0.022), greater cumulative average tablets per week ($HR_{\geq 10v.<1}$=1.35, 95%CI=1.02-1.79; p-trend=0.031), and ≥2500 tablet-days (HR=1.65, 95%CI=1.19-2.28, p-trend=0.006; Table 1.4). Cumulative average frequency of non-aspirin NSAID use was not associated with ovarian cancer risk ($HR_{\geq 5v.<2days/week}$=1.18, 95%CI=0.90-1.54; p-trend=0.29). We did not observe consistent differences for premenopausal versus postmenopausal use of non-aspirin NSAIDs (Supplemental Table 1.1).

There was no evidence of an association for regular acetaminophen use (Table 1.2). For example, the association between current acetaminophen use and ovarian cancer risk was 1.02 (95%CI=0.86-1.21). We observed a positive, but non-significant, association for heavy acetaminophen use (≥2500 tablet-days) compared to no regular use (HR=1.41, 95%CI=0.99-2.00, p-trend=0.10; Table 1.4), and we did not observe any consistent differences for premenopausal versus postmenopausal use of acetaminophen (Supplemental Table 1.1).

Results for all three analgesic types were robust to mutual adjustment for use of the other two analgesic types and to further adjustment for healthcare utilization, health behaviors, and comorbidities (data not shown). We did not observe substantial changes in associations when we restricted our analyses to individuals without each of the following: myocardial infarction, angina, stroke, arthritis, non-GI autoimmune disease or history of GI ulcer (data not shown). Results remained similar when the latency period was reduced to 0-2 years or lengthened to 4-6 years. When we lengthened the latency period to 8-10 years, the results for non-aspirin NSAIDs remained similar while associations for aspirin and acetaminophen were weaker in magnitude

(data not shown). Results for simple updated frequency and tablets were similar to the results for cumulative averages (data not shown).

We evaluated associations by tumor type and observed similar associations for rapidly fatal and non-rapidly fatal ovarian cancer (data not shown). We also considered heterogeneity by tumor histotype and observed that results were somewhat stronger for the serous histotype, although tests for heterogeneity for the serous and non-serous subtypes were not significant (Table 1.5, Supplemental Table 1.2). For example, those with cumulative average frequency of aspirin use ≥5 versus <2 days per week had HR=0.80 (95%CI=0.58-1.11) for risk of serous ovarian cancer and HR=1.06 (95%CI=0.61-1.82) for risk of non-serous ovarian cancer. Similarly the positive association for ≥10 years versus <1 year of non-aspirin NSAID use was significantly positive for serous (p-trend=0.007), but not non-serous (p-trend=0.66) tumors.

Table 1.5 Aspirin or NSAID use and risk of ovarian cancer in the Nurses' Health Studies, by tumor histotype

	Aspirin (1980-2015)				NSAIDs (1989-2015)			
	Serous		Non-serous		Serous		Non-serous	
	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*
Current regular use								
None	141	1.00 (ref)	82	1.00 (ref)	160	1.00 (ref)	65	1.00 (ref)
Past	155	0.83 (0.64, 1.07)	59	1.12 (0.76, 1.65)	126	1.16 (0.91, 1.48)	42	0.99 (0.65, 1.52)
Current	325	0.91 (0.73, 1.14)	122	1.11 (0.80, 1.54)	225	1.23 (1.00, 1.52)	92	1.16 (0.81, 1.64)
p-heterogeneity				0.45				0.82
Duration (years)								
<1	174	1.00 (ref)	99	1.00 (ref)	186	1.00 (ref)	72	1.00 (ref)
1-4	88	0.95 (0.73, 1.23)	44	1.04 (0.72, 1.51)	136	1.18 (0.95, 1.47)	68	1.32 (0.93, 1.87)
5-9	95	1.01 (0.77, 1.31)	46	1.42 (0.96, 2.10)	115	1.31 (1.03, 1.66)	39	1.09 (0.71, 1.66)
10-15†	63	0.99 (0.73, 1.35)	23	1.34 (0.81, 2.21)	83	1.48 (1.11, 1.98)	26	1.25 (0.74, 2.09)
≥15	135	1.07 (0.83, 1.38)	42	1.26 (0.82, 1.92)	--	--	--	--
p-trend		0.54		0.20		0.007		0.66
p-heterogeneity				0.44				0.34
Days per week (cumulative average)‡								
<2	367	1.00 (ref)	155	1.00 (ref)	378	1.00 (ref)	140	1.00 (ref)
3-4	155	0.95 (0.79, 1.15)	57	1.28 (0.93, 1.76)	81	1.04 (0.82, 1.32)	36	1.14 (0.78, 1.66)
≥5	41	0.80 (0.58, 1.11)	15	1.06 (0.61, 1.82)	37	1.24 (0.89, 1.72)	9	0.78 (0.39, 1.54)
p-trend		0.21		0.34		0.24		0.87
p-heterogeneity				0.15				0.46
Tablets per week (cumulative average)‡								
<1	238	1.00 (ref)	97	1.00 (ref)	101	1.00 (ref)	33	1.00 (ref)
1-3	200	1.10 (0.91, 1.33)	74	1.41 (1.02, 1.94)	48	1.33 (0.95, 1.87)	17	1.14 (0.62, 2.08)
4-5	68	1.24 (0.96, 1.62)	26	1.50 (0.96, 2.33)	26	1.26 (0.82, 1.93)	12	1.45 (0.74, 2.85)
6-9	51	1.01 (0.75, 1.36)	14	0.97 (0.54, 1.73)	21	1.33 (0.83, 2.12)	10	1.70 (0.81, 3.56)
≥10	39	1.01 (0.73, 1.40)	19	1.24 (0.75, 2.05)	46	1.48 (1.05, 2.09)	16	1.28 (0.69, 2.37)
p-trend		0.80		0.50		0.032		0.28
p-heterogeneity				0.66				0.89

* Stratified by cohort, age in months and calendar years, and adjusted for age, menopausal status, parity (ever/never and number of children), duration of oral contraceptive use (never, <1, 1 to 5, >5 years), duration of postmenopausal hormone use by type, history of tubal ligation (yes, no), history of hysterectomy (yes, no), family history of breast cancer or ovarian cancer (yes, no), BMI (<20, 20 to <25, 25 to <30, ≥30)

† Top category of duration analysis for NSAIDs is ≥10 years

‡ Aspirin days per week follow-up 1984-2015; NSAID tablets per week follow-up 2000-2015

Discussion

This prospective study is among the first to evaluate, in detail, the associations between type, timing, frequency, quantity, and duration of analgesic use with risk of ovarian cancer, using regularly updated exposure information. We did not observe associations between total aspirin use and ovarian cancer risk. However, when we evaluated low-dose and standard-dose aspirin separately, the results suggested an inverse association between low-dose aspirin use and ovarian cancer risk, but no inverse association for standard-dose aspirin. Non-aspirin NSAIDs and acetaminophen were not inversely associated with risk of ovarian cancer, and our results suggested that heavy use of these medications may be associated with an increased risk.

Aspirin is thought to lower risk of ovarian cancer by reducing inflammation, a process that may contribute to ovarian carcinogenesis (6). While multiple plausible mechanisms of action have been identified, suppression of prostaglandin synthesis via inhibition of the cyclooxygenase (COX)-1 and COX-2 enzymes is the most widely accepted mechanism by which standard-dose aspirin use may influence risk (29-32). Use of low-dose aspirin inhibits COX-1 (58) and may also influence carcinogenesis by reducing platelet activation and recruitment (59).

Non-aspirin NSAIDs and acetaminophen are analgesics that share some, but not all, mechanisms of action with aspirin (29-32, 54). For example while aspirin, non-aspirin NSAIDs and acetaminophen can block COX enzymes, the extent to which they each block COX-1 and COX-2 differs (29, 54). In this analysis, we observed a significant, positive association between high quantity or long-term use of non-aspirin NSAIDs and risk of ovarian cancer and a suggestion of a positive association for acetaminophen use. This may be due to residual confounding, though our findings were robust to adjustment for a number of health factors, or a true effect of long-term regular analgesic use. While our results differed from prior case-control

and prospective studies observing no association for regular non-aspirin NSAID or acetaminophen use, the two population-based studies that previously evaluated long-term non-aspirin NSAID use for ≥10 years also observed positive associations with ovarian cancer risk (60, 61). For acetaminophen, of the two population-based studies that previously evaluated use for ≥10 years, one reported a positive association (60) and the other reported an inverse association (62). This is the first prospective, population-based study to assess acetaminophen use for ≥10 years.

This is the first prospective study with adequate power and sufficiently detailed exposure data to evaluate how timing and patterns of analgesic use are associated with risk of ovarian cancer. Participants provided biennially updated exposure information that captured current type, frequency, quantity, dose (aspirin only), and duration of analgesic use. This level of detail allowed us to consider the patterns of aspirin use that may be most relevant to ovarian cancer risk and prevention (28). Further, the availability of frequently updated exposure data in conjunction with the prospective study design limited the potential for exposure misclassification and recall bias, and allowed us to incorporate a 2-4 year latency period between the timing of the exposure measurement and evaluation for ovarian cancer onset, thereby lowering the possibility of reverse causation.

Our study had several limitations. This study is one of the most detailed evaluations of on-study analgesic use and ovarian cancer risk; however, since follow-up began when women were ages 25-59, it is likely that we underestimated premenopausal use and overall duration of use for women who were older at baseline. We also had limited power to consider medication quantity and dose. Another limitation was that we did not query indication for use. We addressed this by running sensitivity analyses among those without common indications for analgesic use

for which we had collected data (e.g., cardiovascular disease and arthritis); results were similar to those from our primary analysis. Further, we were able to consider the distribution of indications reported among approximately 8,000 NHS/NHSII participants in a sub-study (63), and, despite differences in the distribution of indications (data not shown), we observed a similar increased risk of ovarian cancer among heavy users of all analgesic types. Finally, our study was conducted primarily among white women, though there is no evidence to suggest the association between analgesic use and ovarian cancer risk differs by race or ethnicity (23).

In summary, our results cannot rule out a reduced risk of ovarian cancer among frequent (≥5 days/week) aspirin users, and we observed a significantly lower risk for current (2-4 years before diagnosis) low-dose aspirin use. Our results also suggest an increased risk of ovarian cancer among long-term, high-quantity users of analgesics. Further exploration is warranted to evaluate the mechanisms by which heavy use of aspirin, non-aspirin NSAIDs and acetaminophen may contribute to ovarian cancer etiology, and to replicate our findings. Meanwhile, our results suggest that the 2016 USPTF recommendation advising adults aged 50-59 with a 10-year risk of CVD >10% to initiate low-dose aspirin (64) is unlikely to increase risk of ovarian cancer.

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ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The authors take full responsibility for the analysis and interpretation of data.

Appendix

Supplemental Table S.1.1 Analgesic use and risk of ovarian cancer in the Nurses' Health Studies, by menopausal status at the time of analgesic assessment

	Aspirin (1980-2015)				NSAIDs (1989-2015)				Acetaminophen (1989-2015)			
	Premenopausal		Postmenopausal*		Premenopausal		Postmenopausal*		Premenopausal		Postmenopausal*	
	Cases	Adjusted HR (95% CI)†	Cases	Adjusted HR (95% CI)†	Cases	Adjusted HR (95% CI)†	Cases	Adjusted HR (95% CI)†	Cases	Adjusted HR (95% CI)†	Cases	Adjusted HR (95% CI)†
Duration (years)												
<1	358	1.00 (ref)	359	1.00 (ref)	155	1.00 (ref)	298	1.00 (ref)	197	1.00 (ref)	458	1.00 (ref)
1-4	153	1.01 (0.83, 1.23)	94	1.14 (0.90, 1.44)	143	1.08 (0.85, 1.36)	193	1.70 (1.41, 2.04)	135	0.93 (0.74, 1.16)	65	1.07 (0.80, 1.44)
5-9	101	1.15 (0.90, 1.45)	119	1.39 (1.13, 1.72)	85	1.15 (0.86, 1.54)	129	1.65 (1.33, 2.04)	67	1.12 (0.84, 1.50)	25	0.97 (0.62, 1.51)
10-15‡	30	0.90 (0.61, 1.32)	89	1.40 (1.11, 1.78)	37	1.20 (0.80, 1.80)	88	1.80 (1.39, 2.33)	21	1.12 (0.70, 1.80)	--	--
≥15	93	1.35 (1.05, 1.72)	189	1.42 (1.18, 1.71)	--	--	--	--	--	--	--	--
p-trend		0.04		<0.01		0.32		<0.01		0.40		0.89
Days per week (cumulative average)§												
<2	439	1.00 (ref)	350	1.00 (ref)	261	1.00 (ref)	447	1.00 (ref)	305	1.00 (ref)	499	1.00 (ref)
3-4	91	1.13 (0.90, 1.42)	221	1.19 (1.00, 1.41)	70	1.12 (0.85, 1.46)	97	1.09 (0.87, 1.37)	39	1.11 (0.79, 1.55)	78	1.13 (0.89, 1.44)
≥5	29	1.14 (0.78, 1.67)	99	0.92 (0.73, 1.15)	24	1.41 (0.92, 2.16)	57	1.21 (0.91, 1.60)	7	1.21 (0.57, 2.58)	20	0.82 (0.52, 1.28)
p-trend		0.27		0.97		0.11		0.16		0.45		0.93
Tablets per week (cumulative average)§												
<1	330	1.00 (ref)	234	1.00 (ref)	44	1.00 (ref)	140	1.00 (ref)	72	1.00 (ref)	164	1.00 (ref)
1-3	159	1.14 (0.93, 1.39)	276	1.32 (1.10, 1.57)	18	0.61 (0.35, 1.07)	55	1.32 (0.96, 1.83)	28	1.34 (0.83, 2.14)	65	1.33 (0.97, 1.81)
4-5	47	0.99 (0.72, 1.34)	84	1.27 (0.98, 1.63)	14	0.73 (0.40, 1.34)	37	1.54 (1.06, 2.24)	9	0.78 (0.33, 1.82)	29	1.21 (0.77, 1.89)

Supplemental Table S.1.1 (Continued)

6-9	36	1.13 (0.80, 1.61)	74	1.24 (0.95, 1.62)	18	1.77 (1.00, 3.12)	20	1.16 (0.72, 1.88)	4	1.19 (0.43, 3.33)	19	1.29 (0.79, 2.10)
≥10	64	1.22 (0.93, 1.60)	56	0.91 (0.68, 1.23)	32	1.32 (0.82, 2.13)	59	1.44 (1.05, 1.97)	7	0.49 (0.15, 1.59)	39	1.45 (1.00, 2.11)
p-trend		0.18		0.76		0.03		0.03		0.34		0.05

*Analysis restricted to postmenopausal cases

† Stratified by cohort, age in months and calendar years, and adjusted for age, menopausal status, parity (ever/never and number of children), duration of oral contraceptive use (never, <1, 1 to 5, >5 years), duration of postmenopausal hormone use by type, history of tubal ligation (yes, no), history of hysterectomy (yes, no), family history of breast cancer or ovarian cancer (yes, no), BMI (<20, 20 to <25, 25 to <30, ≥30)

‡ Top category of duration analysis for NSAIDs and acetaminophen is ≥10 years

§ Cumulative averages were calculated using premenopausal or postmenopausal person-years only; aspirin cumulative average days per week follow-up 1984-2015 and NSAID cumulative average tablets per week follow-up 2000-2015

Supplemental Table S.1.2 Acetaminophen and risk of ovarian cancer in the Nurses' Health Studies, by tumor histotype

	Serous		Non-serous	
	Cases	Multivariate-adjusted HR (95% CI)*	Cases	Multivariate-adjusted HR (95% CI)*
Current regular use				
None	176	1.00 (ref)	82	1.00 (ref)
Past	167	1.14 (0.92, 1.42)	58	0.94 (0.65, 1.36)
Current	152	1.10 (0.89, 1.36)	54	0.88 (0.61, 1.26)
p-heterogeneity				0.52
Duration (years)				
<1	210	1.00 (ref)	95	1.00 (ref)
1-4	145	1.09 (0.89, 1.34)	59	0.94 (0.67, 1.31)
5-9	121	1.30 (1.03, 1.63)	39	1.00 (0.68, 1.48)
≥10	47	1.16 (0.84, 1.61)	13	0.87 (0.47, 1.60)
p-trend		0.11		0.76
p-heterogeneity				0.30
Days per week (cumulative average)				
<2	428	1.00 (ref)	162	1.00 (ref)
3-4	50	0.97 (0.73, 1.29)	17	0.89 (0.54, 1.49)
≥5	12	0.93 (0.54, 1.59)	4	0.85 (0.31, 2.30)
p-trend		0.75		0.60
p-heterogeneity				0.76
Tablets per week (cumulative average)†				
<1	126	1.00 (ref)	57	1.00 (ref)
1-3	51	1.22 (0.88, 1.68)	14	0.81 (0.44, 1.47)
4-5	21	1.25 (0.80, 1.95)	5	0.73 (0.29, 1.83)
6-9	15	1.30 (0.76, 2.19)	3	0.73 (0.22, 2.39)
≥10	27	1.36 (0.91, 2.04)	7	0.77 (0.34, 1.72)
p-trend		0.09		0.39
p-heterogeneity				0.12

*Stratified by cohort, age in months and calendar years, and adjusted for age, menopausal status, parity (ever/never and number of children), duration of oral contraceptive use (never, <1, 1 to 5, >5 years), duration of postmenopausal hormone use by type, history of tubal ligation (yes, no), history of hysterectomy (yes, no), family history of breast cancer or ovarian cancer (yes, no), BMI (<20, 20 to <25, 25 to <30, ≥30)

† Cumulative average tablets per week follow-up 2000-2015

Part 2: Urinary PGE-M levels and risk of ovarian cancer

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Abstract

Background: Regular aspirin use may lower ovarian cancer risk by blocking the cyclooxygenase enzymes, resulting in lower expression of prostaglandins, including prostaglandin E2 (PGE2). We evaluated whether higher pre-diagnosis PGE-M (a urinary biomarker of PGE2) is associated with increased risk of ovarian cancer in three prospective cohorts.

Methods: We conducted a nested case-control study among participants in the Nurses' Health Study (NHS), NHSII and Shanghai Women's Health Study (SWHS) with a baseline urine specimen available. Our analyses included 304 cases of epithelial ovarian cancer diagnosed from 1996-2015 and 600 matched controls. We measured PGE-M using LC/MS methods with normalization to urinary creatinine. We estimated odds ratios (OR) and 95% confidence intervals (CI) using unconditional logistic regression, with PGE-M levels modeled in study-specific quartiles. All models were adjusted for matching factors, and multivariate models were further adjusted for ovarian cancer risk factors.

Results: There was no evidence of an association between urinary PGE-M levels and ovarian cancer risk for women with PGE-M levels in the top versus bottom quartile (OR=1.09, 95%CI=0.71-1.66; p-trend=0.48). We did not observe heterogeneity by histotype (p=0.81), and there was no evidence of effect modification by BMI (p-interaction=0.70) or non-steroidal anti-inflammatory drug use (p-interaction=0.73).

Conclusions: Pre-diagnosis urinary PGE-M levels were not significantly associated with ovarian cancer risk. It is unlikely that systemic prostaglandin levels are strongly associated with risk of ovarian cancer. Further research with larger sample sizes is needed to evaluate the potential for a more modest association, and to consider associations for specific tumor subtypes.

Introduction

Chronic inflammation may contribute to the etiology of epithelial ovarian cancer. There is increasing evidence that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a modestly lower risk of ovarian cancer; however the underlying biologic mechanisms remain poorly understood (20, 23, 27, 28, 65-67). Aspirin and non-aspirin NSAIDs down-regulate the prostaglandin synthesis pathway via inhibition of the cyclooxygenase (COX) enzymes (29-32). COX1 and COX2 are overexpressed in ovarian tumor tissue relative to normal tissue (34, 35, 38, 68), and greater COX1 and COX2 expression have been associated with poorer prognosis (33, 39-41, 68), suggesting that NSAIDs may influence risk through the prostaglandin pathway.

COX1 and COX2 promote the conversion of arachidonic acid into bioactive prostaglandins, the most common of which is prostaglandin E2 (PGE2) (69, 70). PGE2 is the most abundant prostaglandin; however, endogenous levels of circulating PGE2 cannot be reliably measured in humans (71, 72). Prior studies of prostaglandins and cancer have measured 11 alpha-hydroxy,9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M), the primary urinary metabolite of PGE2, in urine to approximate systemic levels of prostaglandins (71, 73). In these studies, PGE-M was associated with an increased risk of colorectal cancer (74, 75), gastric cancer (76, 77), small cell lung cancer (78), pancreatic cancer (79, 80), and postmenopausal breast cancer (81, 82).

Here, we evaluated the association between pre-diagnosis urinary PGE-M levels and ovarian cancer risk in a case-control study nested in three prospective cohort studies, the Nurses' Health Study (NHS), NHSII, and the Shanghai Women's Health Study (SWHS). We

hypothesized that higher PGE-M levels would be associated with increased ovarian cancer risk.

Methods

Nurses' Health Studies

The NHS was established in 1976 with the enrollment of 121,700 female registered nurses, aged 30-55. The NHSII was established in 1989 with the enrollment of 116,429 female registered nurses, aged 25-42. NHS/NHSII participants completed a questionnaire on lifestyle factors, medication use and disease status at the time of enrollment, and provided updated lifestyle and health information biennially, by mailed questionnaire. Cases of epithelial ovarian cancer were identified on return of biennial questionnaires or via linkage to the National Death Index. An expert gynecologic pathologist confirmed cases by medical record review and, when records were not available, cases were confirmed by linkage to cancer registries. Tumor behavior and histopathology characteristics were abstracted for confirmed cases.

A subset of women in the NHS/NHSII provided a urine sample (described in (12, 83)). In brief, 18,743 NHS participants aged 53-80 sent spot urine specimens by overnight mail (with an icepack) between 2000 and 2002 (93% first morning urine), where it was aliquoted without a preservative and stored in liquid nitrogen freezers at ≤-130°C. Similarly, 29,611 NHSII participants aged 32-54 years provided spot urine specimens between 1996 and 1999 (80% first morning urine). Of these, 18,521 premenopausal women provided a urine sample 7-9 days prior to the anticipated start of their next menstrual cycle (luteal phase). The other 11,090 participants provided an untimed specimen. All participants completed a biospecimen collection questionnaire asking about time of urine collection, whether it was a first morning urine,

medication use, and weight, among other characteristics. NHS/NHSII study protocols were approved by the Institutional Review Board at Brigham and Women's Hospital.

Shanghai Women's Health Study

The SWHS conducted baseline interviews from 1996-2000, capturing data from 74,942 Chinese women, aged 40-70, living in urban communities in Shanghai. Women were approached by a trained interviewer and, after providing informed consent, were asked to complete a self-administered questionnaire and in-person interview to collect data on lifestyle factors, medication use, and disease outcomes. In-person follow-up surveys have been conducted every 2 to 6 years to obtain information on lifestyle factors and disease outcomes. Ovarian cancer cases were identified with a combination of record linkage to the Shanghai Cancer Registry or Shanghai Vital Statistics Unit and in-person follow-up surveys. Diagnoses were confirmed by medical record review, and assigned to a histologic type using International Classification of Diseases for Oncology codes.

Between 1997 and 2000, 65,754 SWHS women provided a spot urine sample, as described previously (84). In brief, each participant answered questions related to the urine collection and provided a urine sample using a sterilized 100mL cup with 125mg of ascorbic acid. The sample was transported to the laboratory, with an ice pack, within 6 hours of collection and stored at ≤-70°C.

The Institutional Review Boards of all relevant institutions in the United States and the People's Republic of China approved this study.

Study design

We conducted a case-control study nested within the NHS/NHSII/SWHS biospecimen cohorts. Two controls were matched to each case using incidence density sampling, within cohort, on year of birth (+/- 1 year for NHS/NHSII; +/- 2 years for SWHS), date (+/- 1 month) and time (+/- 2hr for NHS/NHSII; morning vs. afternoon for SWHS) of collection, and menopausal status (premenopausal, postmenopausal, unknown) at urine collection. NHS/NHSII additionally matched on menopausal status at diagnosis (premenopausal, postmenopausal, unknown), hormone therapy (HT) use at collection (yes/no), and luteal day (NHSII women only; +/- 1 day); NHS cases from before 2004 were not matched on time of day, hormone therapy, or fasting status. SWHS also matched on antibiotic use at collection. Covariate data were assessed from questionnaires or interviews at or near the time of sample collection, including parity, oral contraceptive (OC) use, intrauterine device (IUD) use, tubal ligation, hysterectomy, family history of ovarian cancer, smoking, weight and height (for calculation of BMI in kg/m^2), and use of anti-inflammatory drugs.

Laboratory assays

The Eicosanoid Core Laboratory (PI: Ginger Milne) at Vanderbilt University measured PGE-M levels in the NHS/NHSII samples using a liquid chromatography-mass spectrometry (LC/MS) method that has been described previously (78, 81, 85, 86). Briefly, PGE-M in each 0.5mL urine specimen was stabilized by conversion to the *O*-methyloxime derivative and purified by C18 solid phase extraction with subsequent addition of the *O*-methyloxime derivatized deuterium-labeled internal standard (8ng, custom synthesis). LC was performed on an Acquity BEH C18 column (2.0 × 50 mm, 1.7μm particle, Waters Corporation, Milford, MA,

USA) connected to a Waters Acquity I-Class UPLC system. Mobile phase A was 95:4.9:0.1 (v/v/v) 5 mM ammonium acetate: acetonitrile: acetic acid, and mobile phase B was 10.0:89.9:0.1 (v/v/v) 5 mM ammonium acetate: acetonitrile: acetic acid. LC flow was delivered to a Waters Xevo TQ-S Micro triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The lower limit of detection of PGE-M was 0.05 ng/mL, substantially lower than levels typically detected in human urine. Results from a pilot study of 40 NHS/NHSII participants with urine collected two years apart, reported good within person stability of PGE-M (intraclass correlation=0.62).

For SWHS samples, levels of PGE-M were measured at the Shanghai Institutes for Biological Sciences (PI: Huiyong Yin) using a similar LC/MS method. Briefly, D6-PGE-M internal standard (2 ng, Cayman Chemical, Ann Arbor, MI USA) was added to 0.75 ml urine and acidified to pH 3 with HCl. Endogenous PGE-M was then converted to the *O*-methyloxime derivative by treatment with methoxyamine HCl. The methoximated PGE-M was extracted, applied to a C18 Sep-Pak, and eluted with ethyl acetate. LC was performed on a Phenomenex Kinetex-C18 column (2.6 μm, 2.1 mm × 50.0 mm) attached to a CTC-HTS autosampler and Shimadzu LC-10 A VP system (Kyoto, Japan).

Case-control pairs were assayed together, and quality control (QC) samples were included to assess analytic error. The analytical personnel were blinded to case, control, and QC status. The intra-assay coefficients of variation (CVs) were <4% and the inter-assay CVs were <9% in our NHS/NHSII samples and among prior PGE-M measures of SWHS samples at the Yin laboratory (81). Urinary creatinine was measured to standardize levels of PGE-M to account for variation in urine concentrations (ng PGE-M/mg creatinine).

Statistical analysis

We log-transformed ng PGE-M/mg creatinine and identified statistical outliers using the generalized extreme studentized deviate many-outlier procedure (87). One outlier with high PGE-M levels was detected in the SWHS dataset and excluded from analyses. We estimated odds ratios (OR) and 95% confidence intervals (CI) using unconditional logistic regression, as early case-control groups in the NHS were matched differently from subsequent groups. To avoid linearity assumptions and acknowledge the different distributions of PGE-M in NHS and NHSII versus SWHS, we evaluated PGE-M in study-specific quartiles (NHS/NHSII, SWHS) with cut-points determined using the distribution of PGE-M values among controls from each study, and we tested for trend using study-specific quartile medians. We first meta-analyzed the results from NHS/NHSII and SWHS and tested for heterogeneity between studies. In absence of heterogeneity ($p>0.10$ for all estimates), we pooled data from all three studies and used ordinal tests for trend in pooled analyses. To evaluate the possibility of a non-linear association, we fit a restricted cubic spline function with three knots and tested for non-linearity using a likelihood ratio test that compared the model with only a linear term for PGE-M to one with linear and cubic spline terms ($p=0.93$). Models were adjusted for matching factors (except antibiotic use) and multivariate models were further adjusted for parity (nulliparous, 1, 2, 3, >3), OC use (never, <1 year, 1-5 years, ≥5 years), IUD use (ever, never), tubal ligation (yes, no), hysterectomy (yes, no), family history of ovarian cancer (yes, no), smoking (current, past, never), and BMI (continuous). Pooled analyses further adjusted for cohort and included an interaction term between cohort and IUD use given prior work suggesting different associations with this exposure between the cohorts (88, 89).

We considered different associations by tumor histotype (serous/poorly differentiated, non-serous [endometrioid, clear cell, mucinous], other/unknown histotype) using polytomous logistic regression. The p-value for heterogeneity was calculated using a likelihood ratio test comparing the polytomous model to a model with constant associations for serous and non-serous cancers. We evaluated multiplicative effect modification by BMI and anti-inflammatory drug use using unconditional logistic regression adjusted for matching factors and covariates by including cross-product terms between each of these variables and PGE-M in our models and conducting a likelihood ratio test. Per the World Health Organization, the definition of overweight differs by race/ethnicity, so SWHS women (Chinese) were classified as overweight for BMI $\geq$24 kg/m^2, and NHS/NHSII (primarily non-Hispanic white) were classified as overweight if BMI $\geq$25 kg/m^2 (90). We considered use of a common cutpoint, BMI $\geq$25 kg/m^2, in sensitivity analyses. Anti-inflammatory drug use was defined as use within 1 week of urine collection or report of regular use of aspirin or non-aspirin NSAIDs over the past two years.

We used SAS 9.4 by SAS Institute Inc., Cary, NC, USA for all analyses. Statistical tests assumed 2-sided p-values with α=0.05.

Results

In total, we included 304 cases of epithelial ovarian cancer and 600 matched controls with PGE-M measures. Our study populations differed with respect to PGE-M levels, age, menopausal status, hysterectomy, OC use, IUD use, and regular use of aspirin-based medications (Table 2.1). Smoking prevalence was low in all three studies. Overall, parity and OC use were less common among ovarian cancer cases compared to controls, while family history of ovarian cancer was more common. Among controls, IUD use was associated with lower urinary PGE-M,

while BMI, smoking, OC use and hysterectomy were all associated with higher urinary PGE-M levels (Supplemental Table 2.1).

Table 2.1 Age-standardized characteristics of ovarian cancer cases and controls at the time of urine collection (NHS 2000-2002, NHSII 1996-1999, SWHS 1997-2000)

	NHS		NHSII		SWHS	
	Control (n=247)	Case (n=123)	Control (n=141)	Case (n=71)	Control (n=212)	Case (n=110)
PGEM ng/ creatinine mg	4.1(1.8)	3.7(1.8)	3.7(2.0)	3.7(2.0)	10.0(1.6)	10.0(1.6)
Age at specimen collection*†	68.0(6.5)	67.9(6.4)	44.8(4.5)	44.6(4.5)	53.1(8.1)	53.0(8.0)
Years from collection to diagnosis*	--	5.9(3.9)	--	7.6(5.0)	--	5.5(2.9)
BMI	25.7(4.0)	25.6(4.5)	26.2(6.2)	27.7(7.2)	24.0(3.6)	24.6(3.7)
Menopausal status and hormone therapy (HT) ‡, %						
- Premenopausal, %	1.2	0.8	80.5	82.4	47.9	45.2
- Postmenopausal/no HT, %	37.7	31.8	5.8	5.4	45.6	45.7
- Postmenopausal/HT use, %	59.9	67.1	5.8	5.5	6.5	9.1
- Unknown, %	1.2	0.3	8.0	6.7	0.0	0.0
Parity, %						
- Nulliparous, %	3.2	4.1	20.7	23.7	2.7	4.6
- 1 child, %	6.1	5.7	8.5	16.9	12.3	15.1
- 2 children, %	27.9	32.9	44.0	45.5	29.4	22.5
- 3 children, %	25.1	27.3	19.0	8.3	26.0	27.0
- 4+ children, %	37.7	30.1	7.8	5.6	29.5	30.8
Ever IUD use, %	5.7	4.2	3.5	4.2	57.7	53.5
Oral contraceptive (OC) use, %						
- Never, %	53.5	47.3	13.4	18.4	76.6	78.6
- <1 year, %	12.1	15.6	9.2	12.5	6.1	10.3
- 1-5 years, %	19.4	21.5	40.4	43.5	7.0	6.7
- 5+ years, %	15.0	15.6	37.0	25.6	10.3	4.4
Tubal ligation, %	22.2	18.4	29.2	11.3	11.9	17.0
Hysterectomy, %	28.4	35.9	12.3	15.1	0.5	0.0
Family history of ovarian cancer§, %	2.8	5.7	1.4	4.3	0.0	0.0
Smoking status, %						
- Never, %	47.8	50.7	70.2	63.6	97.6	94.8
- Past, %	47.4	45.1	22.7	26.6	0.5	0.9
- Current, %	4.8	4.1	7.1	9.8	1.9	4.2
Regular aspirin use, %	44.6	52.2	13.6	18.2	1.8	4.4

Values are means (SD) or percentages and are standardized to the age distribution of the study population.
Values of polytomous variables may not sum to 100% due to rounding
*Value is not age adjusted
†Matching factor in NHS, NHSII, SWHS
‡Menopausal status was a matching factor for all studies; HT use was a matching factor in NHS (2005-2015) and NHSII only
§No SWHS participants in this nested case-control study reported a family history of ovarian cancer

With respect to risk of ovarian cancer, in NHS/NHSII there was no evidence of an association between PGE-M levels and risk in multivariate models (top versus bottom quartile OR=0.99, 95%CI=0.58-1.70; p-trend=0.70; Table 2.2). Results were similar in SWHS (OR=1.22, 95%CI=0.58-2.59; p-trend=0.71) and when the cohorts were combined (OR=1.09, 95% CI=0.71-1.66; p-trend=0.48). The p-heterogeneity comparing the NHS/NHSII and SWHS was 0.57.

Table 2.2 Urinary PGE-M levels and risk of epithelial ovarian cancer in NHS/NHSII and SWHS

	PGE-M (ng/mg creatinine) quartiles				
	Q1 (low)	Q2	Q3	Q4 (high)	p-trend*
NHS/NHSII					
Cases/controls	44/97	48/97	59/97	43/97	
†Model 1 OR (95% CI)	(ref)	1.17 (0.71-1.95)	1.35 (0.82-2.22)	1.05 (0.63-1.77)	0.93
‡Model 2 OR (95% CI)	(ref)	1.05 (0.62-1.78)	1.20 (0.72-2.01)	0.99 (0.58-1.70)	0.70
SWHS					
Cases/controls	22/53	24/54	36/53	28/52	
†Model 1 OR (95% CI)	(ref)	1.18 (0.58-2.43)	1.79 (0.89-3.59)	1.44 (0.70-2.98)	0.39
‡Model 2 OR (95% CI)	(ref)	1.11 (0.53-2.33)	1.72 (0.84-3.53)	1.22 (0.58-2.59)	0.71
Pooled					
Cases/controls	66/150	72/151	95/150	71/149	
†Model 1 OR (95% CI)	(ref)	1.14 (0.75-1.71)	1.45 (0.98-2.16)	1.14 (0.75-1.72)	0.33
‡Model 2 OR (95% CI)	(ref)	1.10 (0.72-1.67)	1.37 (0.92-2.06)	1.09 (0.71-1.66)	0.48

*Tests for trend use quartile medians for NHS/NHSII and SWHS, and ordinal for the pooled analysis.
†Unconditional logistic regression adjusting for matching factors. Cohort was included as a matching factor in the pooled analysis.
‡Previous model further adjusted for parity, OC use, IUD use, tubal ligation, hysterectomy, family history of ovarian cancer, smoking, and BMI. Hysterectomy and family history of ovarian cancer were not adjusted for in the analysis of SWHS only, since prevalence of these factors was very low.

There was no heterogeneity in the PGE-M association by histotype (p=0.81; Table 2.3). A positive association between PGE-M level and risk of non-serous (endometrioid, clear cell, mucinous) tumors was suggested for quartile 3 versus quartile 1 (OR=2.05, 95%CI=1.04-4.02), but this was attenuated after multivariable adjustment (OR=1.85, 95%CI=0.94-3.68).

Table 2.3 Urinary PGE-M levels and risk of ovarian cancer in NHS, NHSII and SWHS, by tumor histotype

	PGE-M (ng/mg creatinine) quartiles					
	Q1 (low)	Q2	Q3	Q4 (high)	p-trend*	p-het
Controls	150	151	150	149		
Serous						
Cases	34	39	36	42		
†Model 1 OR (95% CI)	(ref)	1.17 (0.70-1.97)	1.05 (0.62-1.78)	1.27 (0.75-2.12)	0.47	
‡Model 2 OR (95% CI)	(ref)	1.17 (0.69-1.99)	1.00 (0.59-1.72)	1.25 (0.74-2.12)	0.55	
Endometriod, clear cell and mucinous						
Cases	16	17	29	15		
†Model 1 OR (95% CI)	(ref)	1.08 (0.52-2.25)	2.05 (1.04-4.02)	1.09 (0.51-2.34)	0.37	
‡Model 2 OR (95% CI)	(ref)	1.04 (0.49-2.18)	1.85 (0.94-3.68)	0.97 (0.45-2.12)	0.57	
Other and unknown						
Cases	16	16	30	14		
†Model 1 OR (95% CI)	(ref)	1.10 (0.52-2.31)	1.73 (0.89-3.37)	0.85 (0.39-1.85)	0.90	
‡Model 2 OR (95% CI)	(ref)	1.01 (0.47-2.17)	1.53 (0.77-3.03)	0.74 (0.34-1.65)	0.82	
						0.81

*Ordinal test for trend
†Unconditional polytomous logistic regression adjusting for matching factors.
‡Model 1, further adjusted for parity, OC use, IUD use, tubal ligation, hysterectomy, smoking, and BMI.

We hypothesized a priori that the association between pre-diagnosis urinary PGE-M and ovarian cancer risk would differ by levels of inflammatory exposures, including anti-inflammatory drug use and BMI. Results did not differ by anti-inflammatory drug use (p-interaction=0.73; Table 2.4). For example, among women who reported recent or regular use of anti-inflammatory drugs the OR comparing the top versus bottom quartile was 1.02 (95%CI=0.55-1.90; p-trend=0.80) and among women who did not report anti-inflammatory drug use the OR was 1.13 (95%CI=0.59-2.15; p-trend=0.47). Similar results were observed when stratifying by BMI (p-interaction=0.70), though point estimates suggested positive associations for quartiles 2 versus 1 (OR=1.80, 95%CI=0.98-3.30) and 3 versus 1 (OR=2.15, 95%CI=1.20-3.85) among normal weight individuals, and no associations or inverse associations for quartiles

2 versus 1 (OR=0.66, 95%CI=0.35-1.26) and 3 versus 1 (OR=0.88, 95%CI=0.48-1.62) among overweight and obese individuals. The results were unaltered when we used a common BMI cutpoint (≥25 kg/m^2) across cohorts.

Table 2.4 Association of urinary PGE-M levels and risk of ovarian cancer in NHS, NHSII and SWHS, stratified by inflammatory exposures

	PGE-M (ng/mg creatinine) quartile					
	Q1 (low)	Q2	Q3	Q4 (high)	p-trend*	p-int
No recent or regular NSAID use						
Cases/controls	26/65	36/86	58/94	41/87		
†Multivariate-adjusted		0.98	1.40	1.13		
OR (95% CI)	(ref)	(0.52-1.86)	(0.77-2.54)	(0.59-2.15)	0.47	
Recent or regular NSAID use						
Cases/controls	40/85	36/65	37/56	30/62		
†Multivariate-adjusted		1.14	1.33	1.02		
OR (95% CI)	(ref)	(0.63-2.08)	(0.72-2.46)	(0.55-1.90)	0.80	
						0.73
Normal weight‡						
Cases/controls	32/94	41/75	53/74	28/71		
†Multivariate-adjusted		1.80	2.15	1.08		
OR (95% CI)	(ref)	(0.98-3.30)	(1.20-3.85)	(0.57-2.06)	0.54	
Overweight‡						
Cases/controls	34/56	31/76	42/76	43/78		
†Multivariate-adjusted		0.66	0.88	1.03		
OR (95% CI)	(ref)	(0.35-1.26)	(0.48-1.62)	(0.56-1.91)	0.63	
						0.70

*Ordinal tests for trend
†Unconditional logistic regression model adjusted for matching factors, parity, OC use, IUD use, tubal ligation, hysterectomy, family history of ovarian cancer, smoking, and BMI (NSAID analysis only).
‡Per the World Health Organization, definition of overweight differs by race/ethnicity, so SWHS women (Chinese) are classified as overweight for BMI ≥24 kg/m^2, and NHS/NHSII (primarily non-Hispanic white) are classified as overweight if BMI ≥25 kg/m^2.

NHS/NHSII collected first morning urine specimens from the majority of study participants, and results were robust in the subset of the population who provided first morning urine (top versus bottom quartile OR=0.99, 95%CI=0.56-1.77, p-trend=0.69). We also observed consistent results when we removed cases diagnosed within one year of urine collection from our analyses (top versus bottom quartile OR=1.15, 95%CI=0.74-1.78, p-trend=0.38).

Discussion

In this large, prospective nested case-control study including primarily non-Hispanic Caucasian and Chinese women, we observed that higher pre-diagnosis urinary PGE-M levels were not significantly associated with an increased risk of ovarian cancer. Despite this, there was a suggestive, though non-significant positive association between urinary PGE-M and risk of serous ovarian cancer, although this was based on only 151 cases. No associations were observed for other histotypes. Further, we did not observe any significant interactions by recent NSAID use or BMI, which are key inflammatory factors that may interact with the prostaglandin synthesis pathway (72, 78, 91-95). Consistent with existing literature, we observed a positive association between urinary PGE-M and inflammatory factors, including smoking (86, 96) and BMI (82).

Research on ovarian cancer biology supports a role for prostaglandins in ovarian cancer etiology. One in vitro study reported that the COX2 inhibitor NS-398 reduced PGE2 in ovarian cancer cells (33), and research by the same group and others observed PGE2 in the ascites of ovarian cancer patients (33), COX2 expression in ovarian tumors (33-38, 68), and poorer survival among patients with COX2+ ovarian cancer (33, 39-41, 68). In our recent work, we observed COX1 expression, COX2 expression, or both in many ovarian cancer cases (68, 97); however, when we evaluated the associations between aspirin or non-aspirin NSAID use and ovarian cancer risk by tumor expression of these markers, there was no evidence of heterogeneity (97). This finding, in conjunction with the results presented here, does not support that aspirin or non-aspirin NSAIDs may influence ovarian cancer risk by blocking the cyclooxygenase enzymes and lowering expression of prostaglandins.

Epidemiologic research has reported positive associations between PGE-M levels and risk of colorectal cancer (74, 75), gastric cancer (76, 77), small cell lung cancer (78), and pancreatic cancer (79). These associations varied substantially in magnitude, from a 5.6-fold (95%CI=2.4-13.5) higher risk of colorectal cancer for women comparing PGE-M levels in the highest versus lowest quartile to a 1.63-fold (95%CI=1.01-2.63) increased risk of pancreatic cancer comparing the highest versus lowest tertile. Our results were more similar to studies of postmenopausal breast cancer, which did not report a positive association overall; however, associations were observed among specific, albeit small (n<150), subgroups (e.g., those with low BMI (81) or non-regular users of NSAIDs (82)). Similar to this, we observed a possible positive or U-shaped association between urinary PGE-M and ovarian cancer among normal-weight women, but no association among women with higher BMI. Multiple obesity pathways are related to prostaglandin synthesis or signaling (91-93) and any modest effects of prostaglandins on ovarian cancer may be eclipsed by more extensive dysregulation of inflammation among those with high BMI, so this finding warrants further exploration in future studies.

Urinary PGE-M may also reflect anti-inflammatory factors, including use of aspirin and non-aspirin NSAIDs. Use of NSAIDs, including aspirin (72, 94, 95), ibuprofen (78), and indomethacin (95), have been associated with lower levels of PGE-M. Our three study populations reported very different patterns of NSAID use. Aspirin or NSAID use was common to nearly 50% of NHS participants, approximately 15% of NHSII participants, and fewer than 5% of SWHS participants. Despite these differences in usage patterns, we observed similar associations between PGE-M levels and ovarian cancer in NHS/NHSII and SWHS. Further, unlike studies of postmenopausal breast cancer, we observed no evidence of effect modification by recent or regular use of any NSAIDs.

This study had several strengths, including pre-diagnosis urine collection and collection of detailed covariate information that allowed us to control for important confounders and evaluate effect modification by inflammatory exposures. Further, the inclusion of study populations with different racial/ethnic backgrounds and different NSAID usage patterns added validity to the observed associations. We also recognize several important limitations of our study. Biomarker validity is one potential concern in this study. While urinary PGE-M reflects systemic PGE2 levels (71, 73), it is unclear if urinary PGE-M levels are reflective of PGE2 exposure in the peritoneal cavity. However, other systemic markers (e.g., C-reactive protein, androgens) have been associated with ovarian cancer risk (12, 13, 98). It is also possible that there was degradation of PGE-M with long-term storage or during sample processing. We minimized the impact of this by matching on urine collection characteristics and handling case-control groups together during analysis. Further, we minimized the potential for reverse causation by collecting the urine biospecimen prior to ovarian cancer diagnosis, and conducting a sensitivity analysis excluding case diagnoses within one year of specimen collection. While we pooled three cohorts, our sample size was limited with respect to detecting modest associations, examining associations with specific histotypes, and detecting effect modification.

In summary, we observed no evidence of an association between pre-diagnosis urinary PGE-M levels and risk of ovarian cancer, despite the modest inverse association of aspirin and non-aspirin NSAIDs in these and other populations (20, 23, 27, 28, 65-67). Overall, and particularly when considered in conjunction with our finding that the associations between NSAID use ovarian cancer risk do not differ by tumor expression of COX1 or COX2 (97), the results of this study suggest that regulation of the prostaglandin synthesis pathway may not be an important link between NSAID use and ovarian carcinogenesis.

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Appendix

Supplemental Table S.2.1 Age-standardized characteristics of controls by study-specific quartile of PGE-M (ng/mg creatinine)

	Quartile 1 (n=151)	Quartile 2 (n=149)	Quartile 3 (n=149)	Quartile 4 (n=151)
Age at specimen collection*	60.2(11.7)	57.5(11.5)	55.5(11.7)	56.1(11.2)
Cohort*				
- NHS, %	52.3	43.0	36.2	33.1
- NHSII, %	12.6	21.5	28.9	31.1
- SWHS, %	35.1	35.6	34.9	35.8
BMI	24.0(3.9)	25.1(4.6)	25.5(4.6)	26.3(4.7)
Menopausal status/use of hormone therapy (HT), %				
- Premenopausal, %	36.2	32.3	39.8	37.2
- Postmenopausal/no HT, %	31.0	40.5	33.8	30.8
- Postmenopausal/HT use, %	31.4	26.3	25.7	30.9
- Unknown, %	1.4	0.8	0.7	1.0
Parity, %				
- Nulliparous, %	6.1	9.0	5.8	6.8
- 1 child, %	10.9	7.8	7.7	9.4
- 2 children, %	28.5	35.2	35.4	27.9
- 3 children, %	26.7	19.1	26.2	25.5
- 4+ children, %	27.8	28.8	24.9	30.4
Ever IUD use, %	25.9	24.1	22.8	22.8
Oral contraceptive (OC) use, %				
- Never, %	57.8	54.3	49.0	49.0
- <1 year, %	10.7	7.5	9.7	9.7
- 1-5 years, %	17.0	19.7	23.2	21.2
- 5+ years, %	14.6	18.6	18.1	20.0
Tubal ligation, %	17.3	20.6	18.7	25.3
Hysterectomy, %	9.2	15.7	14.6	22.3
Family history of ovarian cancer, %	1.8	1.3	1.7	1.1
Smoking status, %				
- Never, %	76.2	70.5	69.8	68.0
- Past, %	21.5	24.8	25.7	27.5
- Current, %	2.2	4.7	4.5	4.5
Regular aspirin use, %	31.1	19.0	16.7	18.0

Values are means (SD) or percentages and are standardized to the age distribution of the study population.
Values of polytomous variables may not sum to 100% due to rounding
*Value is not age adjusted

Part 3: Anti-inflammatory drug use and ovarian cancer risk by COX1/COX2 expression and infiltration of tumor-associated macrophages

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Abstract

Background: NSAID use may affect risk of ovarian cancer via prostaglandin synthesis and tumor-associated macrophage (TAM) infiltration. We evaluated if associations between aspirin or non-aspirin NSAID use and ovarian cancer risk differed by tumor expression of prostaglandin-related (COX1, COX2) and TAM-related (CD68, CD163) markers.

Methods: We evaluated cases and matched controls from the Nurses' Health Study (NHS), NHSII, and New England Case Control Study (NECC). Cases with immunohistochemistry data on COX1 and COX2 (n=532) or CD68 and CD163 (n=530) were included. We used polytomous logistic regression, adjusted for ovarian cancer risk factors, to estimate odds ratios (OR) for NSAID use and ovarian cancer risk by marker level.

Results: Current aspirin use had a non-significant inverse association and current non-aspirin NSAID use had no association with ovarian cancer risk. NSAID use was not differentially associated with ovarian cancer by COX1 or COX2 expression. However, current aspirin use was associated with lower ovarian cancer risk for high (OR=0.54, 95%CI=0.37-0.78), but not low (OR=1.50, 95%CI=0.97-2.31), CD163 density (p-heterogeneity<0.001). Similar results were observed for duration and tablets of aspirin use and for current NSAID use. Results were not clearly different by macrophage density defined by the less specific macrophage marker, CD68.

Conclusions: NSAID use was inversely associated with risk of ovarian cancer with high density of CD163, a marker for M2-type, immunosuppressive macrophages. However, the relationship did not differ by markers of prostaglandin synthesis. Future research should explore prostaglandin-independent mechanisms for the association between NSAID use and ovarian cancer risk, including immune mechanisms.

Introduction

Inflammation may play a role in the etiology of epithelial ovarian cancer. Women with elevated circulating C-reactive protein, a marker of systemic inflammation, have an increased ovarian cancer risk (12, 13), and there is growing evidence of an association between anti-inflammatory drug use and risk (20, 23, 27, 28, 65, 66). Recent studies reported a lower risk of ovarian cancer among regular users of aspirin that was strongest among those with frequent aspirin use or low-dose aspirin use (20, 23, 27, 28, 66). Our work additionally observed an increased risk of ovarian cancer among long-term, regular users of anti-inflammatory drugs, particularly non-aspirin NSAIDs (66).

A key mechanism of action for NSAIDs is down-regulation of prostaglandin synthesis via inhibition of the cyclooxygenase (COX) enzymes, COX1 and COX2 (29-32). This mechanism may be relevant to the influence of NSAID use on cancer risk. Prior work showed a strong inverse association between aspirin use and colorectal cancer risk that was only evident for COX2+ tumors (99). In contrast, the association between aspirin use and breast cancer did not differ by COX2 status, suggesting different mechanistic pathways across cancer sites (100).

In addition to promoting inflammation, prostaglandins may modulate immune function, in part by inducing activation and polarization of macrophages (44, 101, 102). Macrophages are important inflammatory regulators in cancer (103-108). Tumor-associated macrophages (TAMs) frequently activate and polarize to the M2 phenotype in response to inflammatory signaling (44, 101, 103, 109, 110). Once activated, they alter the inflammatory response, inhibit Type I T-helper (Th1) adaptive immunity, contribute to matrix remodeling, and promote cell proliferation and angiogenesis (105, 111-114). M2-type TAM infiltration was associated with worse prognosis in breast cancer, while results were mixed for ovarian cancer (115-117). Most ovarian

cancer studies used CD68 as a total macrophage marker and CD163 as an M2-type marker (115-121).

Here, we evaluate if the associations between NSAID use and risk of ovarian cancer differ by COX1 or COX2 expression or by infiltration of M2-type TAMs. We hypothesized that the inverse association between anti-inflammatory drug use and ovarian cancer would be strongest for tumors that expressed higher levels of COX1 and COX2 receptors, a greater absolute number of M2-type macrophages (high CD163 density), or a greater ratio of M2-type to total macrophages (CD163:CD68).

Methods

Study population

The Nurses' Health Study (NHS) is a prospective cohort study that enrolled 121,700 female registered nurses aged 30-55 in 1976. The NHSII is a similar cohort of 116,429 female registered nurses aged 25-42 in 1989. Women completed a baseline questionnaire on lifestyle factors, reproductive factors, medication use, and disease outcomes. Updated questionnaires were administered biennially thereafter. Incident cases of epithelial ovarian cancer were identified from questionnaires, reports from family, or linkage to the National Death Index. Cases were confirmed by medical record review or through cancer registry linkage. To facilitate pooling with the New England Case Control (NECC) study, we matched four controls per case on year of birth and questionnaire completion at the time of case diagnosis. Women were ineligible for selection as controls if they had experienced any of the following prior to the case index date: bilateral oophorectomy, pelvic irradiation, or history of cancer, except non-melanoma

skin cancer. The Institutional Review Board at Brigham and Women's Hospital approved the NHS/NHSII study protocols.

The NECC is a population-based case control study (detailed elsewhere (122, 123)). Briefly, 1,513 cases of epithelial ovarian cancer were identified from statewide cancer registries and tumor boards in Eastern Massachusetts and New Hampshire. Cases were interviewed a median of 8.5 months after diagnosis. Controls were identified via drivers' license registries and town resident lists, and frequency matched to cases by age and state of residence. Of 4,366 potential controls, 1,426 did not meet eligibility criteria, 1,362 declined to participate, and 1,578 were enrolled as controls. Women were ineligible if they were younger than 18 years of age, did not have a phone, did not speak English, moved, died, had a prior bilateral oophorectomy, or their physician declined permission to contact (cases). The Institutional Review Boards at Brigham and Women's Hospital and Dartmouth Medical School approved the study protocols.

We included cases diagnosed from 1976 to 2012 in NHS/NHSII and 1998 to 2008 in NECC. An expert gynecologic pathologist (JLH) who was blinded to exposure status reviewed case medical records, confirming the diagnosis and recording tumor morphology (invasive, borderline), histology (serous, mucinous, endometrioid, clear cell, other), grade (I, II, III), and stage (I, II, III, IV).

Assessment of anti-inflammatory drug use and covariates

NHS/NHSII assessed aspirin and non-aspirin NSAID use via self-report on biennial questionnaires (66). Women in NHS reported current regular use of aspirin on all biennial questionnaires except 1986. Data on the number of aspirin tablets used per week was collected in 1980, 1982 and biennially beginning in 1994. Current regular use of non-aspirin NSAIDs was

queried biennially starting in 1990, and the number non-aspirin NSAID tablets used per week was collected biennially beginning in 1998. In NHSII, current regular use of aspirin and NSAIDs was queried in 1989, 1993, and biennially thereafter. Questions on number of tablets per week were added in 1999 and repeated biennially. Data on the majority of ovarian cancer risk factors, including menopausal status, parity, oral contraceptive (OC) use, postmenopausal hormone therapy (HT) use, tubal ligation, hysterectomy, family history of breast or ovarian cancer, and weight (used in calculation of BMI) were self-reported on questionnaires every 2-4 years.

The NECC assessed anti-inflammatory drug use by in-person interview. The interviewer asked women to recall the time period from childhood up to one year before diagnosis for cases or up to one year before the interview date for controls, and report if they had ever used aspirin, ibuprofen or other analgesics continuously for six months or longer. For each drug type, women were asked to report age at first use, duration of use, and usual dose for every non-consecutive period of use lasting at least six months. Women were also asked about menopausal status, parity, OC use, HT use, tubal ligation, hysterectomy, family history of breast or ovarian cancer, height and weight.

Tumor block collection and creation of tissue microarrays

NHS/NHSII requested paraffin-embedded tissue blocks containing representative tumor samples from cases with a pathology report. Tumors (n=450) were collected. The primary reasons tumor blocks were not collected were that the tissue had been destroyed, the patient was deceased, or the hospital was not able to send a sample (124). The NECC accessed tumor blocks from cases (n=157), most of whom were diagnosed at Brigham and Women's Hospital (n=119). In NECC, funding was available to obtain tissue blocks for only a subset of cases, oversampling

high-grade serous tumors. Tissue blocks were reviewed to verify histology and grade and make tissue microarrays (TMAs). TMAs were arrayed at the Dana-Farber/Harvard Cancer Center (DF/HCC) Specialized Histopathology Core by taking three core biopsies with a 1.0mm (NECC) or 0.6mm (NECC/NHS/NHSII) diameter from ovarian cancer tissue blocks and re-embedding the cores into a single block (125, 126).

Immunohistochemistry

Slides were cut from TMA blocks and, within two weeks, stained for a single marker and counterstained for hematoxylin at the DF/HCC Specialized Histopathology Core. Staining was performed on the Leica Bond III staining platform using the Bond Polymer Refine Detection Kit (Leica Biosystems). Primary antibodies, dilutions, and antigen retrieval are in Supplemental Table 3.1.

Staining was evaluated in a quantitative or semi-quantitative manner by one of two gynecologic pathologists (JLH, MG). COX1 was evaluated in four categories: no staining, weak intensity staining in any cell, moderate intensity staining in ≥10% of cells, and high intensity staining in ≥10% of cells. COX2 was evaluated in five categories based on percent staining positive: 0, >0-5, >5-25, >25-75, >75. CD68 and CD163 density were scored separately for tumor stroma and epithelium as: none, low (< 10% of cells, scattered), moderate (< 10% of cells, with aggregation - at least three aggregates of three macrophages), high (> 10% of cells macrophages or an area of confluent macrophages). Stromal and epithelial scores were summed to reflect total TAM infiltration. The intraclass correlation coefficients (ICCs) across the three cores were: COX1, 0.81; COX2, 0.72; CD163, 0.71; and CD68, 0.67.

We dichotomized stain scores at the median. Specifically, positive staining was defined as the following: COX1+, moderate to high intensity staining of ≥10% of cells, and COX2+, >5% of cells stained; otherwise tumors were coded as stain negative. CD68 staining was used to estimate total macrophage density and CD163 was stained as a marker of M2-type macrophages (107, 115, 116, 127). Tumors were classified as high density (i.e., CD68 high or CD163 high) when the sum of the epithelium and stromal scores was greater than 4. The ratio of CD163/CD68 was calculated using the summed scores.

Statistical analysis

We created two analytic datasets: one examining COX1 and COX2, and one examining CD163 and CD168. For each dataset, we excluded cases with missing data on one or both of the relevant markers. We had 532 cases for analyses of COX1 and COX2 and 530 cases for analyses of CD163 and CD168. We then excluded participants with missing data on the exposure of interest (n=80 aspirin and n=109 NSAIDs for COX analyses, and n=79 aspirin and n=106 NSAIDs for TAM analyses).

Aspirin and non-aspirin NSAID use were harmonized and evaluated using three metrics: current use (current, past, non-use), duration of use (<1, 1 to <5, 5 to <10, and ≥10 years), and tablets used per week (<1, 1 to <6, and ≥6 tablets per week). We captured on-study use (minimum age of 25) for NHS/NHSII and use after age 25 for NECC. Current use was defined as use during the questionnaire cycle prior to case diagnosis for NHS/NHSII and 1-year prior to the case index date in NECC. Duration of use was assessed in years. Tablets per week reflected cumulative average tablets per week in NHS/NHSII and tablets per week for the longest continuous period of anti-inflammatory drug use for NECC.

We evaluated the correlation among tumor markers using Spearman correlations. We fit logistic regression models to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for NSAID use and ovarian cancer risk in the full study population, and used polytomous logistic regression to estimate ORs and 95% CIs for ovarian cancer defined by marker status, COX1(+/-), COX2(+/-), CD163(high/low) and CD68(high/low), and by the ratio of CD163 to CD68 (<1/≥1). We adjusted for matching factors and for ovarian cancer risk factors in multivariate models. Covariates not associated with the tumor markers were constrained to have the same estimate for all tumor subtypes. These included cohort (NHS, NHSII, NECC); age (continuous in years); menopausal status (premenopausal/unknown, postmenopausal) parity (nulliparous, 1, 2, 3, >3 children); estrogen, estrogen plus progestin, and other HT use separately (ever/never); tubal ligation (yes/no); family history of breast or ovarian cancer (yes/no); and BMI (<20, 20-25, 25-30, 30+ kg/m^2). Hysterectomy (yes/no) and OC use (<1, 1-5, 5-10, 10+ years) were differentially associated by tumor marker status for multiple markers, so they were modeled as unconstrained variables with different estimates for each tumor type. The distribution of tumor markers was similar for NHS/NHSII and NECC (data not shown) and there was no evidence of heterogeneity across studies ($p>0.05$), so data were pooled.

We conducted a planned sensitivity analysis restricting to invasive epithelial ovarian cancer. Another sensitivity analysis assessed low-dose aspirin and standard dose aspirin separately. This analysis was restricted by data availability to NHS/NHSII participants with a case index date between 2001 and 2012, when low-dose aspirin was assessed. We conducted a third sensitivity analysis to evaluate the association of analgesic use and risk of COX1+ and COX1- tumors accounting for COX2 status and vice versa, as well as COX68-low and CD68-high tumors accounting for CD163 levels and vice versa (128). All analyses were conducted

using SAS statistical software version 9.4 (SAS Institute, Cary, NC, USA) or Stata statistical software version 12.1 (StataCorp, College Station, TX, USA). Statistical tests were two-sided with p-values <0.05 considered statistically significant.

Results

Cases with tissue included on TMAs were more likely to be postmenopausal, slightly less likely to use OCs, and had greater parity than observed in the full population of cases from NHS/NHSII and NECC (Supplemental Table 3.2). Among cases with tissue and matched controls, we did not observe a significant association between aspirin use and risk of ovarian cancer, although there was a suggestion of an inverse association (e.g., OR, current vs. non-use=0.78, 95%CI: 0.55-1.09; Supplemental Table 3.3). The association between non-aspirin NSAID use and risk of ovarian cancer was generally null, though women with ≥10 years of NSAID use had a 1.8-fold increased risk of ovarian cancer (95%CI: 1.17-2.77) relative to those with <1 year of NSAID use.

<u>COX1 and COX2</u>

COX1+ ovarian cancers were more likely to be serous, high grade, and high stage than COX1- cancers, while the distribution of histopathologic features was similar for COX2+ versus COX2- ovarian cancers (Table 3.1). Further, women with COX1+ cancer were more likely to be postmenopausal, had shorter durations of oral contraceptive use and reported greater estrogen plus progestin HT use than women with COX1- cancer (Supplemental Table 3.4). Ovarian cancer cases that were COX2+ versus COX2- had a higher prevalence of tubal ligation as well as

longer duration of oral contraceptive and estrogen plus progestin HT use. The spearman correlation between COX1 and COX2 levels was 0.07 (Table 3.2).

Table 3.1 Distribution of ovarian cancer histopathology by tumor marker in the Nurses' Health Studies and the New England Case Control Study

	COX1*		**COX2†**		**CD163‡**		**CD68‡**	
	-	+	-	+	low	high	low	high
Total, n	378	154	353	179	212	318	183	347
Histology, (%)								
Serous	65.1	83.8	70.0	71.5	53.8	82.1	60.1	76.4
Mucinous	5.3	0.0	1.7	7.8	9.0	0.9	8.7	1.7
Endometrioid	17.5	7.1	15.6	12.3	20.8	9.1	16.4	12.4
Clear cell	7.1	6.5	8.5	3.9	11.3	4.1	9.8	5.5
Other	5.0	2.6	4.2	4.5	5.2	3.8	4.9	4.0
Grade, (%)								
Borderline	12.4	7.1	9.9	12.8	23.1	3.5	23.0	5.2
1	9.5	3.9	7.9	7.8	13.2	4.7	13.1	5.5
2	6.1	2.6	4.8	5.6	8.0	2.8	6.0	4.3
3	70.1	85.1	76.2	70.9	53.8	86.8	57.4	82.1
Unknown	1.9	1.3	1.1	2.8	1.9	2.2	0.5	2.9
Stage, (%)								
1	29.1	19.5	24.4	30.2	40.1	17.0	36.6	20.7
2	9.8	4.5	9.6	5.6	9.9	6.9	7.7	8.4
3	51.1	63.6	55.5	53.1	40.6	64.8	45.4	60.2
4	4.0	3.9	4.0	3.9	2.4	5.0	2.7	4.6
Unknown	6.1	8.4	6.5	7.3	7.1	6.3	7.7	6.1

*Tumors were classified as COX1- when there was no evidence of staining or only weak intensity staining, and COX1+ when there was moderate to high intensity staining in ≥10% of cells.
†Tumors were classified as COX2- when <5% of cells stained, and COX2+ when there was staining in ≥5% of cells.
‡CD68 and CD163 were scored as low when <10% of cells stained and staining was scattered. CD68 and CD163 were scored as high when <10% of cells stained, with aggregation, or when ≥10% of cells stained.

Table 3.2 Correlations* among tumor markers in the Nurses' Health Studies and the New England Case Control Study

	COX1	COX2	CD68	CD163	CD68/CD163
COX1	1.00	0.07	0.13	0.19	0.10
COX2		1.00	0.09	0.13	0.08
CD68			1.00	0.81	-0.13
CD163				1.00	0.45
CD68/CD163					1.00

*Spearman correlations were calculated among the 513 cases with data on all 4 tumor markers (COX1, COX2, CD68 and CD163). Correlations with an absolute value ≥0.146 are statistically significant, assuming a two-sided test with α=0.05.

We observed no evidence of heterogeneity for the association between aspirin or non-aspirin NSAID use and ovarian cancer risk by COX1 or COX2 receptor status, for regular use, duration, and tablets/week (p-heterogeneity≥0.22; Table 3.3). For example, the OR for current (versus never) use of aspirin and ovarian cancer was 0.71 (95%CI=0.50-1.01) for COX1- and 0.87 (95%CI=0.54-1.38) for COX1+ tumors (p-heterogeneity=0.72). Similarly, regular non-aspirin NSAID use was not associated with risk of ovarian cancer for COX1- (OR=0.96, 95%CI=0.68-1.34), COX1+(OR=1.05, 95%CI=0.66-1.68), COX2- (OR=1.00, 95%CI=0.70-1.41) and COX2+ (OR=1.00, 95%CI=0.65-1.53) cases.

When we cross-classified tumors by COX1 and COX2 status, there was no significant evidence of an association between current aspirin use and any of the four tumor types. For example, the OR for current aspirin use and risk of COX1-/COX2- ovarian cancer was 0.75 (95%CI=0.50-1.12); while the OR for current aspirin use and risk of COX1+/COX2+ ovarian cancer was 0.74 (95%CI=0.38-1.44). The ORs for current non-aspirin NSAID use and risk of COX1-/COX2- and COX1+/COX2+ cancers were similar (OR=0.90, 95%CI=0.60-1.34; OR=0.77, 95%CI=0.37-1.60, respectively).

Table 3.3 Associations between aspirin and non-aspirin NSAID use by COX1/COX2 level in the Nurses' Health Studies and the New England Case Control Study

	Controls (n)	Cases (n)	OR (95% CI) COX1-	OR (95% CI) COX1+	P-het	OR (95% CI) COX2-	OR (95% CI) COX2+	P-het
Aspirin								
Regular use								
No regular use	1552	197	(ref)	(ref)		(ref)	(ref)	
Past use	280	92	0.74 (0.49, 1.11)	0.87 (0.50, 1.49)		0.79 (0.52, 1.19)	0.78 (0.47, 1.28)	
Current use	605	163	0.71 (0.50, 1.01)	0.87 (0.54, 1.38)		0.82 (0.57, 1.17)	0.68 (0.44, 1.06)	
					0.72			0.73
Duration								
<1 year	1616	212	(ref)	(ref)		(ref)	(ref)	
1 to <5 years	206	39	0.68 (0.43, 1.09)	0.72 (0.36, 1.44)		0.67 (0.41, 1.09)	0.75 (0.40, 1.38)	
5 to <10 years	197	62	0.95 (0.62, 1.45)	0.96 (0.53, 1.77)		1.14 (0.75, 1.73)	0.67 (0.36, 1.24)	
10+ years	347	109	0.76 (0.51, 1.13)	0.95 (0.57, 1.59)		0.92 (0.62, 1.37)	0.70 (0.42, 1.17)	
p-trend			0.34	0.92		0.91	0.22	
					0.46			0.22
Tablets								
<1 tablet/week	1728	260	(ref)	(ref)		(ref)	(ref)	
1 to <6 tablets/week	395	131	0.91 (0.66, 1.25)	0.94 (0.61, 1.46)		0.90 (0.65, 1.24)	1.01 (0.67, 1.53)	
6+ tablets per week	292	62	0.76 (0.52, 1.13)	0.87 (0.51, 1.49)		0.82 (0.56, 1.22)	0.79 (0.46, 1.34)	
p-trend			0.17	0.62		0.31	0.40	
					0.69			0.95
NSAIDs								
Regular use								
No regular use	1600	208	(ref)	(ref)		(ref)	(ref)	
Past use	247	105	1.54 (1.08, 2.20)	1.50 (0.91, 2.47)		1.71 (1.19, 2.44)	1.27 (0.79, 2.04)	
Current use	460	110	0.96 (0.68, 1.34)	1.05 (0.66, 1.68)		1.00 (0.70, 1.41)	1.00 (0.65, 1.53)	
					0.92			0.55

Table 3.3 (Continued)

Duration							
<1 year	1680	226	(ref)	(ref)		(ref)	(ref)	
1 to <5 years	347	84	0.92 (0.64, 1.31)	1.03 (0.63, 1.68)		1.12 (0.79, 1.60)	0.67 (0.40, 1.11)	
5 to <10 years	206	72	1.27 (0.86, 1.88)	1.21 (0.70, 2.11)		1.38 (0.93, 2.06)	1.09 (0.65, 1.83)	
10+ years	111	45	1.75 (1.08, 2.82)	1.92 (0.99, 3.72)		1.59 (0.95, 2.68)	2.24 (1.28, 3.93)	
p-trend			0.010	0.05		0.02	0.006	
					0.93			0.51
Tablets								
<1 tablet/week	1478	184	(ref)	(ref)		(ref)	(ref)	
1 to <6 tablets/week	164	50	0.96 (0.59, 1.54)	1.45 (0.78, 2.70)		0.87 (0.51, 1.48)	1.49 (0.87, 2.55)	
6+ tablets per week	225	48	1.03 (0.65, 1.61)	1.07 (0.55, 2.09)		1.01 (0.63, 1.64)	1.08 (0.60, 1.92)	
p-trend			0.97	0.77		0.95	0.71	
					0.82			0.74

Models are adjusted for: cohort, age, menopausal status (pre/post), parity (nulliparous, 1, 2, 3, >3), oral contraceptive use (<1, 1-5, 5-10, 10+ years), estrogen, estrogen+progestin and other HT use (ever/never), tubal ligation (yes/no), hysterectomy (yes/no), family history of breast or ovarian cancer (yes/no), and BMI (<20, 20-25, 25-30, 30+).

TAM markers: CD68 and CD163

CD163 and CD68 were strongly correlated (ρ=0.81; Table 3.2). Tumors with high density CD163 or CD68 were more likely to be serous, high grade and stage III (Table 3.2). When we considered the distribution of ovarian cancer risk factors by density of CD163 and CD68, OC use, tubal ligation, and greater parity were more common for cases with high density of CD163 or CD68 (Supplemental Table 3.4).

We observed consistent evidence of a differential association for all metrics of aspirin use and risk of ovarian cancer by CD163 density (Table 3.4). Current aspirin use (vs. non-use) was suggestively associated with a higher risk of ovarian cancer with low CD163 density (OR=1.50, 95%CI=0.97-2.31) and a lower risk of ovarian cancer with high CD163 density (OR=0.54, 95%CI=0.37-0.78; p-heterogeneity<0.001). We observed similar differences for duration of aspirin use (p-heterogeneity=0.012), and tablets per week (p-heterogeneity<0.001). The comparable associations by CD68 density were not significantly different (e.g., current use vs. non-use $OR_{CD68low}$=0.99, 95%CI=0.63-1.57; $OR_{CD68high}$=0.71, 95%CI=0.49-1.01; p-heterogeneity=0.17). No heterogeneity was observed by the ratio of CD163/CD68 (p>0.05).

A subset of associations between non-aspirin NSAIDs and risk of ovarian cancer also differed by CD163 density and CD68 density. For example when we compared those with current vs. non-use of NSAIDs, we observed a 2.00-fold higher risk (95%CI=1.32-3.05) of ovarian cancer with low CD163, and a 0.65 times lower risk (95%CI=0.45-0.93) of ovarian cancer with high CD163 (p-heterogeneity<0.001). When we evaluated tablets per week, associations were similar with a positive association for CD163 low cancer and a possible inverse association for CD163 high cancer, but they were not significantly different (p-heterogeneity=0.35). There was no evidence of an association for tablets per week and risk of

ovarian cancer by CD68 level (p-heterogeneity=0.73). For NSAID duration, we observed a significant difference for risk of ovarian cancer with low CD163 density versus high CD163 density (p-heterogeneity=0.05), but no difference was evident by CD68 (p-heterogeneity=0.62).

We also considered the cross-classification of CD68 and CD163 status. Current aspirin use was most strongly associated with a lower risk of ovarian cancer with high levels of CD68 and CD163 (OR=0.59, 95% CI=0.40, 0.86), though the association between current aspirin use and lower risk of CD68 low/CD163 high tumors was also significant (OR=0.18, 95% CI=0.05-0.62). Associations for the other two tumor types were in the opposite direction, but non-significant (data not shown). Current NSAID use was positively associated with risk of ovarian cancer with low levels of CD68 and CD163 (OR=2.10, 95%CI=1.27-3.49), but inversely associated with risk of ovarian cancer with high levels of CD68 and CD163 (OR=0.68, 95%CI=0.47-0.99).

Table 3.4 Associations between aspirin and non-aspirin NSAID use by levels of CD163, CD68, and their ratio in the Nurses' Health Studies and the New England Case Control Study

			OR (95% CI)			OR (95% CI)			OR (95% CI)		
	Control (n)	Case (n)	CD163 low	CD163 high	P-het	CD68 low	CD68 high	P-het	CD163/CD68<1	CD163/CD68≥1	P-het
Aspirin											
Regular use											
No regular use	1552	197	(ref)	(ref)		(ref)	(ref)		(ref)	(ref)	
Past use	280	91	1.43 (0.88, 2.35)	0.56 (0.37, 0.87)		1.12 (0.67, 1.87)	0.67 (0.44, 1.02)		0.95 (0.54, 1.67)	0.74 (0.50, 1.11)	
Current use	605	163	1.50 (0.97, 2.31)	0.54 (0.37, 0.78)		0.99 (0.63, 1.57)	0.71 (0.49, 1.01)		1.20 (0.75, 1.91)	0.67 (0.47, 0.95)	
					<0.001			0.17			0.07
Duration											
<1 year	1616	213	(ref)	(ref)		(ref)	(ref)		(ref)	(ref)	
1 to <5 years	206	39	1.07 (0.60, 1.92)	0.54 (0.33, 0.90)		0.86 (0.46, 1.60)	0.63 (0.39, 1.02)		0.75 (0.36, 1.58)	0.69 (0.43, 1.08)	
5 to <10 years	197	62	1.70 (1.02, 2.83)	0.67 (0.42, 1.07)		1.06 (0.60, 1.88)	0.91 (0.59, 1.40)		1.73 (1.00, 2.99)	0.74 (0.47, 1.16)	
10+ years	347	107	1.25 (0.78, 2.02)	0.64 (0.42, 0.96)		1.03 (0.63, 1.69)	0.72 (0.48, 1.08)		0.92 (0.52, 1.60)	0.80 (0.54, 1.18)	
p-trend			0.21	0.07		0.71	0.25		0.81	0.37	
					0.012			0.24			0.42
Tablets											
<1 tablet/week	1728	262	(ref)	(ref)		(ref)	(ref)		(ref)	(ref)	
1 to <6 tablets/week	395	127	1.62 (1.09, 2.40)	0.63 (0.44, 0.89)		1.17 (0.77, 1.79)	0.79 (0.57, 1.10)		1.20 (0.77, 1.87)	0.81 (0.59, 1.12)	
6+ tablets per week	292	64	1.58 (1.00, 2.48)	0.53 (0.34, 0.81)		1.15 (0.70, 1.89)	0.69 (0.47, 1.03)		1.17 (0.68, 1.99)	0.71 (0.48, 1.04)	
p-trend			0.04	0.001		0.54	0.06		0.52	0.07	
					<0.001			0.09			0.10

Table 3.4 (Continued)

NSAIDs										
Regular use										
No regular use	1600	208	(ref)	(ref)		(ref)	(ref)		(ref)	(ref)	
Past use	247	105	2.65 (1.69, 4.16)	1.16 (0.80, 1.69)		2.35 (1.45, 3.81)	1.31 (0.91, 1.88)		1.97 (1.21, 3.19)	1.40 (0.98, 2.00)	
Current use	460	111	2.00 (1.32, 3.05)	0.65 (0.45, 0.93)		1.63 (1.03, 2.58)	0.81 (0.58, 1.14)		1.33 (0.84, 2.11)	0.88 (0.63, 1.24)	
					<0.001			0.02			0.26
Duration										
<1 year	1680	225	(ref)	(ref)		(ref)	(ref)		(ref)	(ref)	
1 to <5 years	347	81	1.66 (1.07, 2.58)	0.64 (0.43, 0.96)		1.53 (0.96, 2.45)	0.73 (0.50, 1.06)		1.16 (0.69, 1.94)	0.85 (0.60, 1.22)	
5 to <10 years	206	71	2.14 (1.33, 3.46)	0.92 (0.60, 1.41)		1.99 (1.19, 3.32)	1.03 (0.68, 1.54)		1.82 (1.08, 3.08)	1.10 (0.73, 1.64)	
10+ years	111	48	2.79 (1.55, 5.02)	1.60 (0.98, 2.62)		1.86 (0.91, 3.80)	1.96 (1.24, 3.08)		2.96 (1.61, 5.45)	1.59 (0.98, 2.58)	
p-trend			<0.001	0.10		0.01	0.006		<0.001	0.06	0.06
					0.05			0.62			
Tablets										
<1 tablet/week	1478	185	(ref)	(ref)		(ref)	(ref)		(ref)	(ref)	
1 to <6 tablets/week	164	51	2.03 (1.13, 3.64)	0.84 (0.51, 1.39)		1.48 (0.76, 2.85)	1.04 (0.66, 1.66)		1.70 (0.92, 3.12)	0.95 (0.59, 1.54)	
6+ tablets per week	225	48	1.27 (0.67, 2.42)	0.96 (0.61, 1.51)		1.12 (0.56, 2.26)	1.00 (0.64, 1.56)		0.80 (0.38, 1.72)	1.11 (0.72, 1.72)	
p-trend			0.39	0.72		0.71	0.97		0.73	0.73	
					0.35			0.73			0.62

Models are adjusted for: cohort, age, menopausal status (pre/post), parity (nulliparous, 1, 2, 3, >3), oral contraceptive use (<1, 1-5, 5-10, 10+ years), estrogen, estrogen+progestin and other HT use (ever/never), tubal ligation (yes/no), hysterectomy (yes/no), family history of breast or ovarian cancer (yes/no), and BMI (<20, 20-25, 25-30, 30+).

Sensitivity analyses

Results were similar when we restricted our analyses to invasive epithelial ovarian cancer or high-grade serous ovarian cancer only (data not shown). Notably, the differential association for anti-inflammatory drug use and risk of ovarian cancer by CD163 density remained statistically significant for all measures of aspirin use, and for current non-aspirin NSAID use. When we evaluated ever versus never low-dose aspirin use, we observed no evidence of heterogeneity (all p-heterogeneity>0.30), though power was limited (n=161 cases; data not shown). Results were similar when we evaluated COX1+ and COX1- tumors accounting for COX2 status and vice versa (Supplemental Table 3.5). When we accounted for CD68 status, associations for aspirin and NSAID use with risk of CD163 low ovarian cancer remained positive (e.g. OR=1.68, 95%CI=1.00-2.82 for current versus no aspirin use), while associations with risk of CD163 high ovarian cancer became more inverse (e.g. comparable OR=0.39, 95%CI=0.23-0.66). Accounting for CD163 status, we observed an inverse association for current aspirin and risk of CD68 low ovarian cancer, but no association for CD68 high ovarian cancer. The association for current NSAID use and risk of CD68 low ovarian cancer changed from significantly positive to inverse, while the association for current NSAID use and risk of CD68 high ovarian cancer did not change substantially (Supplemental Table 3.5).

Discussion

In this study, we examined the association of anti-inflammatory drug use with risk of ovarian cancer by markers of increased prostaglandin synthesis and macrophage infiltration. Associations of NSAID use with risk of ovarian cancer were consistent with our previous work (66); however, there was heterogeneity for the association between aspirin use and ovarian

cancer risk by density of M2-type macrophage infiltration. Our analyses suggested no evidence of heterogeneity by COX1/COX2 expression. Notably, regular aspirin use for any duration, or at any level of tablet intake, was associated with a lower risk of ovarian cancer with high infiltration of M2-type macrophages (CD163+), but higher risk of ovarian cancer with low infiltration of M2-type macrophages. We did not observe a difference by overall macrophage levels (CD68), though the results of our sensitivity analysis of CD68, accounting for CD163, suggested that the association between anti-inflammatory drug use and risk of ovarian cancer by level of M1-type macrophages may be in the opposite direction of the associations by level of M2-type macrophages. Additional research is needed to confirm our findings and more thoroughly evaluate associations between aspirin use and ovarian cancer subtypes defined by these and other immune markers.

The primary mechanism of action through which regular intake of aspirin or non-aspirin NSAIDs are thought to influence carcinogenesis is down-regulation of the prostaglandin synthesis pathway by inhibition of the cyclooxygenase enzymes, COX1 and COX2 (29-32). Two prior studies evaluated the associations of aspirin and non-aspirin NSAIDs with risk of cancer by the expression of cyclooxygenase enzymes (99, 100). One study reported that the inverse association between long-term, regular aspirin use and risk of colorectal cancer was restricted to tumors overexpressing COX2 (99). The second study conducted a similar analysis for breast cancer, and found no associations overall or by COX2 expression (100). Our study observed a non-significant inverse association for aspirin use and a positive association for long durations of non-aspirin NSAID use, a finding consistent with our prior study of the full NHS/NHSII cohorts (66) and with a larger study in the Ovarian Cancer Cohort Consortium (129). Similar to breast cancer, we observed no evidence of heterogeneity by COX1 or COX2 expression. This lack of

heterogeneity could be real or could, in part, be due to limited case numbers to examine low-dose aspirin, which has previously been more strongly related to ovarian cancer risk (27, 66).

These results do not support prostaglandin synthesis as the primary mechanism by which NSAIDs influence ovarian cancer risk, so other mechanisms should be considered. We observed significant heterogeneity by M2-type macrophage density for both aspirin and non-aspirin NSAIDs, suggesting that aspirin and other NSAIDs may work by reducing differentiation of macrophages to the immunosuppressive M2 type. While prostaglandins promote macrophage differentiation (44, 101, 102), other molecules and pathways are also involved in the activation, differentiation, and tumor-promoting activity of this immune cell population. For example, monocyte chemoattractant protein 1 (MCP-1/CCL2) is lower in breast and pancreatic cancer cells treated with aspirin, and also affects macrophage infiltration in ovarian cancer (130-132). Further research should consider the effects of aspirin on MCP-1 and other factors that may regulate immune cell recruitment and differentiation in ovarian cancer (133). If associations between NSAID use and ovarian cancer risk are not fully explained by immune mechanisms, they may also reflect NSAID-driven modifications in gene expression. For example, a study of PC3 human prostate cancer cells reported that expression of genes involved in DNA repair, cell growth, and cell proliferation was altered in cells treated with high, but clinically relevant concentrations of multiple NSAIDs (134). Dysregulation of many of these same genes has been reported in ovarian cancer (5), and may be an intermediate step by which NSAIDs influence of ovarian cancer risk.

Strengths of our study included the large study population from two prospective studies, and a population-based case-control study. All studies collected detailed exposure and confounder data, including multiple metrics of anti-inflammatory drug use for both aspirin and

non-aspirin NSAIDs. Additionally, measures of the tissue markers were reproducible across cores and TMAs were cut and stained by the same laboratories across all three studies, reducing assay variability.

We also acknowledge some important limitations of this research. It is possible that bias arose in the identification of cases for inclusion (i.e., cases with available tissue blocks). However, cases for whom we had tumor tissue blocks had similar distributions of NSAID use to the full case population (Supplemental Table 3.2). Further the results of this analysis were similar to those reported in a prospective cohort analysis of the NHS/NHSII (66). We also recognize that information from NECC, a retrospective study, was affected by recall bias, though the results from the NECC were not substantially different from the NHS/NHSII. Finally, we gained substantial power by pooling the NHS/NHSII/NECC, but data harmonization resulted in the loss of some metrics of medication use (i.e., frequency), limited the number of categories we could consider for the exposure and covariates, and precluded a detailed evaluation of dose-response relationships for duration and tablets of anti-inflammatory drug use.

In summary, we observed that the associations between aspirin or non-aspirin NSAID use and risk of ovarian cancer did not differ by levels of COX1 or COX2 expression, suggesting that if associations between anti-inflammatory drug use and ovarian cancer risk are causal, they may act through a prostaglandin-independent biologic pathway. However, given that we saw very strong differences in association of NSAID use by density of M2-type macrophages, which have an immunosuppressive effect on the tumor, there may be alternate mechanisms by which these drugs, particularly aspirin, influence ovarian carcinogenesis. Recent large studies have consistently shown a modest inverse association of daily or low-dose aspirin with ovarian cancer risk, thus elucidating potential mechanisms by which NSAIDs can alter the tumor

microenvironment, particularly with respect to cellular factors such as immunity, is crucial to determining whether aspirin use may prevent ovarian cancer. Further research should leverage both larger population-based studies as well as experimental models, considering the complex distribution of immune cell types found in epithelial ovarian cancers.

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Appendix

Supplemental Table S.3.1 Antibodies and retrieval methods

Antibody	Catalog # (Company)	Dilution	Retrieval
COX1	HPA002834 (Sigma Aldrich)	1:50	H2 (20 minutes)
COX2	RM-9121-R7 (Neomarkers)	Ready To Use	H1 (40 minutes)
CD68	M0876 (Dako)	1:200	H1 (30 minutes)
CD163	VP-C374 (Vector)	1:250	H2 (30 minutes)

*Antigen retrieval was performed online using Bond Epitope Retrieval 1 ("H1") pH 6.0, or Bond Epitope Retrieval 2 ("H2") pH 8.0 at 100°C for the time indicated.

Supplemental Table S.3.2 Age-standardized case characteristics for cases on TMAs and total cases in the Nurses' Health Studies and the New England Case Control Study

	Cases on TMAs		All Cases	
	NHS/NHSII (n=417)	NECC (n=157)	NHS/NHSII (n=1667)	NECC (n=1496)
Age, years[*]	62.6(8.7)	58.5(9.7)	61.8(9.0)	54.3(11.5)
Aspirin Use[†]				
- Past aspirin, %	27.1	2.5	21.2	3.6
- Current aspirin, %	51.3	8.8	53.5	5.0
NSAID Use[†]				
- Past NSAIDs, %	34.7	3.3	31.0	5.1
- Current NSAIDs, %	37.5	4.0	36.9	8.0
Postmenopausal, %	74.8	79.9	58.8	39.7
Tubal ligation, %	13.9	15.4	16.2	13.3
Hysterectomy, %	30.7	13.8	16.6	8.6
Parous, %	89.9	75.6	85.4	64.9
Number of children[‡]				
- 1, %	11.6	12.5	13.8	23.5
- 2, %	38.1	36.4	39.9	42.0
- 3, %	26.8	27.6	25.9	21.0
- >3, %	23.5	23.5	20.4	13.5
Ever used oral contraceptives, %	56.3	50.9	58.0	63.6
Duration of use (yrs) [§]				
- <1 year, %	28.9	19.9	33.9	19.2
- 1-<5, %	41.0	48.4	41.1	34.7
- 5-<10, %	20.7	16.2	16.2	25.8
- 10+, %	8.7	15.4	8.8	20.3
Postmenopausal estrogen use[‖], %	33.5	11.9	29.2	15.2
Postmenopausal estrogen and progesterone[‖], %	29.3	24.9	28.4	24.7
Other postmenopausal hormones[‖], %	21.9	8.7	22.1	4.3
Body mass index kg/m2				
- <20, %	7.9	4.6	8.6	6.3
- 20-<25, %	41.3	45.1	44.1	41.1
- 25-<30, %	29.9	29.2	29.7	29.7
- 30+, %	21.0	21.1	17.5	22.9
Family history of breast or ovarian	18.7	22.8	16.3	21.4

Values are means(SD) or percentages and are standardized to the age distribution of the study population

Values of polytomous variables may not sum to 100% due to rounding

[*] Value is not age adjusted

[†] In NHS/NHSII 53.2% did not have data on aspirin use and 63.9% did not have data on NSAID use; in NECC 0.2% did not have data on aspirin use and 0.9% did not have data on NSAID use. The proportions without data were lower for cases on TMAs (NHS/NHSII 20.4% for aspirin, 28.8% for NSAIDs; NECC 0.0% for aspirin, 0.4% for NSAIDs).

[‡] Number of children among parous women

[§] Duration of oral contraceptive (OC) use among past and current OC users

[‖] Hormone Therapy (HT) among postmenopausal women

Supplemental Table S. 3.3 Associations between aspirin and non-aspirin NSAID use among women in the Nurses' Health Studies and the New England Case Control Study with tumor marker data

	NHS/NHSII			NECC			Combined		
	Controls (n)	**Cases (n)**	**OR (95% CI)**	**Controls (n)**	**Cases (n)**	**OR (95% CI)**	**Controls (n)**	**Cases (n)**	**OR (95% CI)**
Aspirin									
Regular use									
No regular use	171	57	(ref)	1381	132	(ref)	1552	189	(ref)
Past use	242	88	0.89 (0.57, 1.39)	38	3	0.72 (0.21, 2.43)	280	91	0.83 (0.56, 1.21)
Current use	467	146	0.89 (0.58, 1.36)	138	10	0.56 (0.28, 1.11)	605	156	0.78 (0.55, 1.09)
Duration									
<1 year	229	72	(ref)	1387	132	(ref)	1616	204	(ref)
1 to <5 years	127	31	0.78 (0.47, 1.28)	79	7	0.73 (0.32, 1.65)	206	38	0.72 (0.47, 1.09)
5 to <10 years	155	57	1.13 (0.72, 1.78)	42	4	0.72 (0.24, 2.14)	197	61	1.00 (0.68, 1.49)
10+ years	302	103	0.96 (0.62, 1.49)	45	1	0.17 (0.02, 1.24)	347	104	0.84 (0.58, 1.22)
p-trend			0.89			0.05			0.59
Tablets									
<1 tablet/week	347	119	(ref)	1381	132	(ref)	1728	251	(ref)
1 to <6 tablets/week	357	122	0.97 (0.70, 1.34)	38	3	0.65 (0.19, 2.20)	395	125	0.93 (0.69, 1.26)
6+ tablets per week	158	51	0.96 (0.63, 1.44)	134	9	0.52 (0.25, 1.07)	292	60	0.82 (0.58, 1.15)
p-trend			0.83			0.07			0.25
NSAIDs									
Regular use									
No regular use	277	70	(ref)	1323	131	(ref)	1600	201	(ref)
Past use	183	95	1.79 (1.21, 2.64)	64	6	1.02 (0.43, 2.45)	247	101	1.54 (1.11, 2.14)
Current use	297	98	1.17 (0.80, 1.71)	163	7	0.48 (0.22, 1.05)	460	105	0.96 (0.71, 1.31)
Duration									
<1 year	330	86	(ref)	1350	132	(ref)	1680	218	(ref)
1 to <5 years	248	73	1.06 (0.73, 1.55)	99	6	0.68 (0.29, 1.60)	347	79	0.92 (0.67, 1.28)
5 to <10 years	166	66	1.43 (0.95, 2.15)	40	3	0.81 (0.24, 2.71)	206	69	1.25 (0.87, 1.79)
10+ years	53	42	2.55 (1.49, 4.37)	58	2	0.39 (0.09, 1.65)	111	44	1.80 (1.17, 2.77)
p-trend			<0.001			0.16			0.003

Supplemental Table S.3.3 (Continued)

Tablets									
<1 tablet/week	155	46	(ref)	1323	131	(ref)	1478	177	(ref)
1 to <6 tablets/week	88	44	1.37 (0.77, 2.45)	76	4	0.55 (0.19, 1.55)	164	48	1.12 (0.73, 1.72)
6+ tablets per week	79	39	1.27 (0.70, 2.33)	146	8	0.62 (0.29, 1.30)	225	47	1.04 (0.69, 1.57)
p-trend			0.51			0.15			0.86

Multivariate model adjusts for: menopausal status (pre/post), parity (nulliparous, 1, 2, 3, >3), oral contraceptive use (<1, 1-5, 5-10, 10+ years), estrogen, estrogen+progestin and other HT use (ever/never), tubal ligation (yes/no), hysterectomy (yes/no), family history of breast or ovarian cancer (yes/no), and BMI (<20, 20-25, 25-30, 30+).

Supplemental Table S.3.4 Age-standardized case characteristics by tumor marker expression in the Nurses' Health Studies and the New England Case Control Study

	COX1		COX2		CD163		CD68	
	- (n=378)	+ (n=154)	- (n=353)	+ (n=179)	low (n=212)	high (n=318)	low (n=183)	high (n=347)
Age, years*	61.3(9.4)	62.0(8.6)	61.5(9.2)	61.5(9.3)	61.0(8.9)	61.8(9.4)	61.3(9.0)	61.6(9.3)
Aspirin Use								
- No regular aspirin, %	37.5	32.8	34.8	40.2	24.0	46.1	28.9	41.4
- Past aspirin, %	17.0	18.0	16.4	19.0	19.6	15.1	18.9	16.1
- Current aspirin, %	29.7	32.8	31.6	30.3	36.9	26.9	30.4	31.1
- Unknown, %	15.8	16.4	17.2	10.6	19.4	11.9	21.9	11.4
NSAID Use								
- No regular NSAIDs, %	38.8	38.8	37.8	42.4	25.2	48.7	26.4	46.0
- Past NSAIDs, %	20.2	17.8	20.8	17.0	22.8	18.1	20.8	19.3
- Current NSAIDs, %	20.5	20.4	19.9	22.4	27.1	16.4	23.0	19.9
- Unknown, %	20.4	23.0	21.5	18.2	24.9	16.8	29.8	14.8
Postmenopausal, %	73.9	82.6	76.1	75.3	76.0	75.7	76.8	75.4
Tubal ligation, %	13.8	13.5	11.5	18.9	10.5	15.5	12.3	14.1
Hysterectomy, %	26.1	30.0	28.2	23.4	30.6	23.8	30.2	24.5
Parous, %	85.0	86.6	85.9	84.5	82.0	87.0	84.2	85.4
Number of children†								
- 1, %	12.8	10.0	13.0	9.8	11.8	13.3	10.3	13.6
- 2, %	39.9	29.3	37.3	36.2	40.5	34.7	41.8	34.9
- 3, %	24.8	31.5	23.4	34.6	28.5	25.4	28.8	25.3
- >3, %	22.4	29.2	26.3	19.4	19.2	26.6	19.0	26.3
Ever used oral contraceptives, %	58.5	61.5	57.6	64.6	59.9	62.4	57.8	63.6
Duration of use (yrs)‡								
- <1 year, %	26.2	24.7	27.6	23.5	24.7	25.2	29.3	23.0
- 1-<5, %	41.8	48.7	44.3	45.2	42.7	44.1	39.1	46.1
- 5-<10, %	21.6	12.3	17.2	20.0	20.6	20.5	18.4	20.8
- 10+, %	10.5	10.4	10.2	11.3	11.2	10.2	12.0	10.0
Postmenopausal estrogen use§, %	28.4	27.7	29.1	27.5	29.1	28.4	30.8	27.6

Supplemental Table S.3.4 (Continued)

Postmenopausal estrogen and progesterone[§], %	26.6	31.5	23.6	38.2	27.9	29.0	24.1	30.5
Other postmenopausal hormones[§], %	19.2	18.9	19.9	17.3	18.8	18.4	18.5	18.8
Body mass index kg/m2								
- <20, %	8.6	4.9	7.8	6.7	7.9	7.3	7.1	7.5
- 20-<25, %	40.5	46.5	41.2	43.9	41.4	43.9	45.3	41.9
- 25-<30, %	30.5	30.0	28.9	32.6	30.0	29.1	28.5	29.6
- 30+, %	20.5	18.6	22.1	16.8	20.6	19.7	19.1	21.0
Family history of breast or ovarian cancer, %	20.1	18.2	18.9	23.4	18.9	20.8	19.2	20.5

Values are means(SD) or percentages and are standardized to the age distribution of the study population

Values of polytomous variables may not sum to 100% due to rounding

[*] Value is not age adjusted

[†] Number of children among parous women

[‡] Duration of oral contraceptive (OC) use among past and current OC users

[§] Hormone Therapy (HT) among postmenopausal women

Supplemental Table S.3.5 Associations between aspirin and non-aspirin NSAID use by mutually adjusted* tumor marker levels in the Nurses' Health Studies and the New England Case Control Study

	Controls (n)	Cases (n)	OR (95% CI)		p-het	OR (95% CI)		p-het
			COX1-	COX1+		COX2-	COX2+	
Aspirin								
No regular use	1552	197	(ref)	(ref)		(ref)	(ref)	
Past use	280	92	0.76 (0.50, 1.15)	0.87 (0.50, 1.51)	0.62	0.80 (0.52, 1.22)	0.78 (0.47, 1.29)	0.94
Current use	605	163	0.71 (0.50, 1.02)	0.88 (0.55, 1.42)	0.37	0.81 (0.56, 1.17)	0.68 (0.43, 1.06)	0.45
NSAIDs								
No regular use	1600	208	(ref)	(ref)		(ref)	(ref)	
Past use	247	105	1.55 (1.09, 2.22)	1.48 (0.90, 2.45)	0.86	1.69 (1.18, 2.44)	1.26 (0.78, 2.03)	0.26
Current use	460	110	0.95 (0.68, 1.34)	1.03 (0.64, 1.66)	0.76	0.98 (0.69, 1.39)	0.97 (0.63, 1.50)	0.98
			CD163 low	CD163 high		CD68 low	CD68 high	
Aspirin								
No regular use	1552	197	(ref)	(ref)		(ref)	(ref)	
Past use	280	91	1.62 (0.89, 2.93)	0.61 (0.37, 1.00)	0.004	0.93 (0.48, 1.82)	0.88 (0.56, 1.41)	0.89
Current use	605	163	1.68 (1.00, 2.82)	0.39 (0.23, 0.66)	<0.001	0.40 (0.18, 0.90)	0.93 (0.63, 1.40)	0.04
NSAIDs								
No regular use	1600	208	(ref)	(ref)		(ref)	(ref)	
Past use	247	105	2.55 (1.53, 4.22)	1.33 (0.87, 2.05)	0.03	2.14 (1.18, 3.91)	1.54 (1.04, 2.27)	0.31
Current use	460	111	1.79 (1.10, 2.91)	0.51 (0.28, 0.92)	<0.001	0.64 (0.25, 1.63)	0.97 (0.67, 1.41)	0.40

Models are adjusted for: menopausal status (pre/post), parity (nulliparous, 1, 2, 3, >3), oral contraceptive use (<1, 1-5, 5-10, 10+ years), estrogen, estrogen+progestin and other HT use (ever/never), tubal ligation (yes/no), hysterectomy (yes/no), family history of breast or ovarian cancer (yes/no), and BMI (<20, 20-25, 25-30, 30+).

*COX1 is adjusted for COX2 and vice-versa; CD68 is adjusted for CD163 and vice versa

Conclusion:

The goal of my research was to evaluate the role of inflammation in ovarian cancer etiology. With input from my research committee and collaborators, I approached this work in two stages. First, I evaluated the association between anti-inflammatory drug use and risk of ovarian cancer, finding a possible inverse association between low-dose aspirin use and ovarian cancer risk, but a positive association for heavy use of non-aspirin NSAIDs and, to a lesser extent, aspirin and acetaminophen (an analgesic with limited anti-inflammatory properties (54)). Next, I leveraged molecular epidemiology to evaluate if regulation of the prostaglandin synthesis pathway could be the biologic mechanism underlying the associations between analgesic use and ovarian cancer risk. My work suggested that regulation of this pathway is not relevant to ovarian carcinogenesis, but regulation of immune function may be highly relevant.

The results of my work opened three areas of research for further investigation. First, the mechanism underlying the association between low-dose aspirin and a reduced risk of ovarian cancer merits further evaluation. My research on analgesic use and risk of ovarian cancer in the Nurses' Health Studies was one of several recent studies to describe a possible, inverse association between low-dose aspirin use and risk of ovarian cancer (20, 23, 67); however, my follow-up assessment of the mechanism underlying this association indicated that it may not be driven by regulation of pro-inflammatory prostaglandins. An alternate mechanism of action for low-dose aspirin that is less relevant for regular dose aspirin and non-aspirin NSAIDs is the irreversible inhibition of COX1 (29). In addition to affecting prostaglandin synthesis, inhibition of COX1 can regulate platelet activation and angiogenesis (29, 30). Basic biology has recognized platelet activation as a possible pro-tumorigenic factor in ovarian cancer (135), and there is evidence that platelets contribute to venous thromboembolism (136), a disease that has been

associated with a 7- to 13-fold increased risk of ovarian cancer (137, 138). The effects of COX1 on angiogenesis also merit further investigation, as angiogenesis is a necessary process in ovarian tumor growth, invasion and metastasis (139).

A second question to address is: what is driving the association between heavy use of non-aspirin NSAIDs and risk of ovarian cancer? My assessment of analgesic use and ovarian cancer risk resulted in the unexpected finding of a positive association between heavy use of non-aspirin NSAIDs and risk of ovarian cancer, a finding that may also extend to heavy use of aspirin or acetaminophen. This finding was observed in another recent study (67); however, there is still question as to whether it reflects chance, confounding by indication, or a true, causal association. Replication studies will be useful in evaluating chance and, with data on indication for analgesic use, could also evaluate the potential for confounding by indication. Meanwhile, the potential for a causal association should also be considered. Studies in prostate cancer cell lines have reported a positive association between NSAID exposure at high, yet clinically relevant, doses and dysregulation of DNA repair pathways (134). Genes involved in DNA repair are commonly dysregulated in ovarian cancer (e.g., *TP53*, *BRCA1*, *BRCA2*) (5), so future work could evaluate if high doses and long durations of NSAID use are differentially associated with risk of ovarian cancer by gene expression profile.

The third area of research that merits further investigation is how aspirin and non-aspirin NSAIDs affect the immune response to ovarian cancer. My research reported a differential association of aspirin and non-aspirin NSAID use with risk of high- versus low-infiltration of immunosuppressive, M2-type macrophages. Macrophages are one of several immune cell types that influence ovarian cancer prognosis, so an important next step in this research is to evaluate if aspirin and non-aspirin NSAIDs are associated with ovarian cancer subtypes defined by

infiltration with other immune cell types, including CD8+ tumor infiltrating lymphocytes and FOXP3+ regulatory T cells (140, 141). This work could inform immune mechanisms by which aspirin and non-aspirin NSAID use influence ovarian cancer risk and, if extended to analyses of ovarian cancer survival, could begin to inform the use of aspirin and non-aspirin NSAIDs during ovarian cancer treatment, including treatment with immunotherapies.

Overall, the results of my research support inflammation and immune function as important pathways in the development of epithelial ovarian cancer. Further evaluation of inflammatory pathways and immune mechanisms is needed to inform the etiology of ovarian cancer subtypes and to improve prevention and early detection of this highly fatal malignancy.

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