A Prospective Study of Endometriosis and Breast Health: Findings From the Nurses' Health Study II

A PROSPECTIVE STUDY OF ENDOMETRIOSIS AND BREAST HEALTH: FINDINGS FROM THE NURSES' HEALTH STUDY II

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Abstract

Endometriosis is a chronic gynecologic disease affecting approximately ten percent of women in the United States. Endometriosis lesions depend on estrogen for growth and maintenance and it is hypothesized that women with endometriosis have an altered hormonal and inflammatory state. Emerging evidence suggests that women with endometriosis may be at increased risk of breast cancer. Using data from the Nurses' Health Study II, a prospective cohort of 116,430 women, this thesis investigates endometriosis and breast health. Specifically we investigated whether endometriosis influences risk of breast cancer and benign breast disease and alters mammographic density. Lastly, we investigated whether breastfeeding duration influenced endometriosis risk.

Across all analyses, endometriosis was confirmed using laparoscopy, considered the clinical diagnostic gold standard. Information on breast cancer and benign breast disease was collected every two years and confirmed by medical record or pathology slides respectively. Mammographic density was measured from mammograms of a subset of participants without breast cancer using a computer assisted thresholding technique. Detailed breastfeeding information was collected between 1997-2001. Cox proportional hazard models were used to calculate hazard ratios (HR) and 95% confidence intervals (CI) and linear regression using

generalized estimating equations was used to estimate difference in mammographic density measurements.

We found that while women with endometriosis were not at increased risk of overall breast cancer (HR:1.05, CI:0.95-1.16), they did appear to be at an increased risk of ER+/PR- tumors (HR:1.72, CI:1.27-2.32). Endometriosis moderately increased risk of biopsy confirmed benign breast disease, both proliferative (HR:1.23, CI:1.01-1.51) and non-proliferative lesions (HR:1.25, CI:0.93-1.69). Endometriosis did not significantly alter mammographic density. History of breastfeeding was inversely associated with endometriosis (P-value, test for linear trend: <0.0001), which was partially, but not fully mediated through postpartum amenorrhea.

Our findings report novel associations with endometriosis and ER+/PR- breast tumors and benign breast disease lesions and no difference in mammographic density. This may elucidate avenues of research on how endometriosis lesions may alter chronic disease risk. Given the debilitating symptoms and few known modifiable risk factors of endometriosis, our findings of an inverse relationship with breastfeeding and endometriosis may inform treatments and prevention strategies for endometriosis in the future.

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An Introduction to:

A Prospective Study of Endometriosis and Breast Health:

Findings from the Nurses' Health Study II

Endometriosis occurs when endometrial tissue, usually found in the endometrium lining the walls of the uterus, is present outside of the uterus[1]. Most endometriosis lesions appear close to the uterus, for example on the ovaries, fallopian tubes, vagina, and ligaments supporting the uterus, however the disease has been found in distant locations throughout the body[2]. The main symptoms of endometriosis include infertility, dysmenorrhea (painful menstrual cramping), dyspareaunia (painful intercourse), dysuria (painful urination), dyschezia (painful defecation), or pelvic pain not associated with menses[2]. There are four stages of endometriosis lesions: Stage I (minimal), Stage II (mild), Stage III (moderate), and Stage IV (severe)[3]. Staging is based on extent of lesion development, scarring, and adhesions within the uterine cavity that is visualized during laparoscopy. However current staging has not been shown to be correlated with pain severity symptoms or risk of infertilty[4].

The pathogenesis of endometriosis is complex and multifactorial. The earliest and most accepted etiologic theory was established in 1927. Dubbed "Sampson's Theory," it posits that retrograde menstruation and subsequent implantation and growth of endometriotic tissue on extra-uterine structures is the primary mechanism that gives rise to endometriosis[5]. This theory is supported by studies demonstrating clustering of endometriotic lesions around the distal ends of the fallopian tubes, the presence of viable endometrial cells in peritoneal fluid[6], and risk factors associated with increased exposure to menstruation (earlier age at menarche, shorter menstrual cycle length, heavy flow[2, 7, 8]) increasing endometriosis risk. However, the majority of women

experience retrograde menstruation in some capacity. One study estimated that approximately 90% of women experience some level of retrograde menstruation[9], suggesting that the true differences between women with and without the disease may be due to varying rates of implantation of endometrial cells and not the occurrence of retrograde menstruation itself. Factors that influence adherence, proliferation, and maintenance of the cells and lesions (such as hormonal milieu, immunological factors, and angiogenic processes) have been implicated.

Certain hormonal environments, in particular exposure to estrogen, may facilitate the proliferation and survival of endometriotic tissue[10, 11]. There is a wide range of circumstantial evidence that shows that endometriosis risk factors are also associated with hormone levels. For example, the association between endometriosis and age at menarche[7], body mass index (BMI)[12], and oral contraceptive use[13], as well as the prevalence of the disease among reproductive aged women, all suggest a hormonal association[2]. Additionally, early work has shown that endometriosis plaques have estrogen, progesterone, and androgen receptors and grow in the presence of estrogen but atrophy when exposed to androgens [11, 14-16].This theory is not inherently independent of the retrograde menstruation theory, since hormone levels may influence the volume of retrograde menstruation or the promotion and survival of endometrial implants outside of the uterus.

In addition, there is evidence to suggest that endometriosis is associated with immunologic and inflammatory responses[17]. Women with compromised immune systems may have more endometrial plaques outside of the uterus than women with normal immune function. Case-control studies have observed abnormal levels and function of growth factors, macrophages, and pro-inflammatory cytokines in the peritoneal fluid and serum of women with

endometriosis[14, 15, 18-20]. Several case reports and small studies suggest an increased risk of autoimmune diseases among women with endometriosis[21-27].

The impetus of this dissertation work arose from an interest in the overlapping risk factors shared between the two conditions[7, 28, 29]. Early age at first menstrual period, short menstrual cycle length, nulliparity, and lean early life and premenopausal body size are risk factors which are thought to be associated with increased risk of both endometriosis and types of breast cancer[2, 7, 29]. Additionally breast cancer and endometriosis are both hypothesized to be influenced by circulating hormone levels. While previous research has reported on the relationship between endometriosis and breast cancer[30], research has been confined to mostly case-control or medical record based studies which a) may represent the most severe endometriosis cases and b) had limited information confounding and mediating factors. We were interested in better understanding how endometriosis could be associated with breast cancer risk within a study setting that was able to control for these overlapping shared risk-factors. Additionally there is a growing body of evidence that women with endometriosis may be at risk for other chronic disease conditions including cardiovascular disease, ovarian cancer, and skin cancer[30]. Benign breast disease and high mammographic density are both considered strong, independently associated risk factors for breast cancer[31, 32].

Given the shared risk factors between endometriosis and breast cancer and the overlapping hormonal etiology, this dissertation will focus on the potential relationship between endometriosis and breast health. It will be divided in to four aims:

1) To prospectively investigate the relationship between endometriosis and breast cancer with a focus on breast cancer heterogeneity

2) To prospectively investigate the relationship between endometriosis and biopsy confirmed benign breast disease
3) To cross-sectionally investigate the relationship between endometriosis and measurements of mammographic density
4) To prospectively investigate the relationship between breastfeeding history and duration and risk of incident endometriosis

Our Contribution

By investigating the heterogeneity in breast cancer tumors, as well as understanding how endometriosis influences benign breast disease and mammographic density we hope our research can advance the state of knowledge on endometriosis and breast health. To our knowledge, our study is the first longitudinal cohort study of endometriosis and breast cancer to control for potential confounding and mediating factors and to investigate heterogeneity in breast cancer lesions by tumor hormone receptor status. Additionally, we are the first to investigate the role of endometriosis on benign breast disease risk and mammographic density. Through comprehensively investigating whether endometriosis alters risk for breast cancer and independent risk factors for breast cancer, we hope that this research will help guide further investigation into the potential mechanism and shared etiology. Breastfeeding may be an important modifiable risk factor for endometriosis risk. Our analyses investigating the relationship between breastfeeding duration and risk of endometriosis, builds off of the previous limited research, by quantifying duration of breastfeeding necessary to decrease risk and separating the role of postpartum amenorrhea from breastfeeding on risk of endometriosis.

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Aim 1: A Prospective Study of Endometriosis and Risk of Breast Cancer

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Abstract:

Background: Endometriosis, which affects approximately 10% of reproductive aged women, has been associated with altered hormonal and inflammatory environments. Previous studies of endometriosis and breast cancer report mixed results, potentially due to limited data on confounding and mediating factors. Additionally, no study has addressed heterogeneity across breast cancer hormone receptor types.

Methods: We evaluated the association between laparoscopically-confirmed endometriosis and breast cancer among participants in the Nurses' Health Study II cohort. Multivariable Cox proportional hazard models were used to calculate hazard ratios (HR) and 95% confidence intervals (CI). Breast cancer was further classified by menopausal status and tumor hormone receptor status.

Results: Within this cohort, there were 12,282 women with endometriosis. Over >20 years of follow-up, 4,979 incident breast cancer cases were observed among 2,544,923 person years. Women with endometriosis were not at higher risk for overall (HR:1.05, 95% CI: 0.95-1.16), premenopausal (HR:1.07, 95% CI: 0.91-1.26), or postmenopausal breast cancer (HR:0.98, 95% CI: 0.85-1.13). However, associations varied significantly by tumor hormone receptor status (P-value, test for heterogeneity: 0.002). While women with endometriosis were not at increased risk of estrogen/progesterone receptor positive (ER+/PR+) tumors (HR:1.00, 95% CI:0.87-1.15) or ER-/PR- tumors(HR:0.82, 95% CI:0.60-1.11), endometriosis was associated with significant increased risk of ER+/PR- breast cancers in crude and multivariable adjusted models (HR:1.72, 95% CI: 1.27-2.32).

Discussion: Endometriosis was not found to be associated with overall breast cancer risk or with ER+/PR+ and ER-/PR- tumors. Women with endometriosis have an altered hormonal and inflammatory state which may contribute to increased risk in ER+/PR- breast cancers. Future work should focus on disease heterogeneity and confirm these relationships.

Introduction

Endometriosis is a painful and debilitating gynecologic condition defined by the presence of endometrial-like tissue outside the uterus. This chronic disorder affects 6-10% of women of reproductive age in the United States[1] and it is the third leading cause of gynecologic hospitalization. Yet its etiology and relationship to the risk of other diseases, including breast cancer, remain unclear[2, 3].

Endometriosis and breast cancer share several pathophysiologies such as immune dysfunction, chronic inflammation, and an abnormal steroid hormonal environment, as well as many risk factors such as body size, age at menarche, and parity. Additionally, many of the treatments for endometriosis, including oral contraceptives, analgesic use, and oophorectomy, may alter breast cancer risk[4-8]. The literature are mixed regarding the relationship between endometriosis and breast cancer[9]. Most studies have suggested a modest positive association between endometriosis and the risk of breast cancer, although many lacked statistical significance[10-15], while four studies showed no clear association[16-19], and four studies reported an inverse relationship[20-22]. However, the studies conducted to date are limited by inadequate or minimal control for confounding and assessment of potential mediators. Additionally, in breast cancer epidemiology, there has been an emphasis on understanding how risk factor relationships differ by tumor hormone receptors and menopausal status at time of cancer. No study has investigated heterogeneity in the association of endometriosis across breast cancers by tumor hormone receptor status.

To address these limitations, we investigated the relationship between endometriosis and subsequent breast cancer risk in the Nurses' Health Study II, a prospective cohort with detailed information on endometriosis, breast cancer, and potential confounders and mediators. We

investigated heterogeneity in breast cancer tumors by hormone receptor status and menopausal status at diagnosis of breast cancer.

Methods

The Nurses' Health Study II (NHSII) is a prospective cohort study that began in 1989 when 116,430 registered nurses, 25-42 years old, returned a mailed questionnaire on their health and lifestyle. Follow-up questionnaires, that collect information on environmental, dietary, and lifestyle risk factors, have been sent biennially. The cumulative follow-up rate from the original cohort is ≥90%. The study was approved by the Institutional Review Board of Brigham and Women's Hospital.

Exposure definition

On the 1993 questionnaire, women were first asked if they had ever had "physician diagnosed endometriosis." If they answered "yes," they were asked to report when the diagnosis had occurred and if their disease had been confirmed by laparoscopy. Endometriosis diagnosis has been assessed on every questionnaire subsequently allowing women to become exposed through follow-up.

Self-reported endometriosis was validated previously among 200 participants using medical records[1]. Among women who reported laparoscopic confirmation, a laparoscopic diagnosis of endometriosis was confirmed in 96% of medical records. Conversely, among those women without laparoscopic confirmation, evidence of clinical diagnosis was found in only 54% of medical records. Thus, to reduce the magnitude of misclassification, endometriosis exposure was restricted to those women with laparoscopic confirmation. Women who reported endometriosis diagnosis without laparoscopic confirmation who later had a confirmation by

laparoscopy were assigned to the endometriosis exposed group at the time of the initial clinical diagnosis. Those who reported endometriosis diagnosis but never laparoscopic confirmation were censored at the report of clinical diagnosis.

Outcome definition

Self-reported cancer diagnoses have been reported on every questionnaire. Women who reported a new breast cancer diagnosis were asked to indicate the diagnosis date. Permission to obtain the pertinent medical records was then acquired, with repeat mailings and telephone outreach used to contact non-respondents. For deceased respondents, next-of-kin were contacted for permission to review medical records or data were linked from the state cancer registries. Information on histopathology, size, invasiveness, grade, node status, and hormone receptor status was recorded. For our primary analysis, we combined in situ and invasive breast cancer diagnoses.

For our analyses of hormone receptors status, we combined information on estrogen and progesterone status of the tumor from medical records and tissue microarrays (TMAs) to create three categories: estrogen /progesterone receptor positive (ER+/PR+) breast cancer, ER+/PR- breast cancer, and ER-/PR- breast cancer, since previous work has indicated that ER-/PR+ is not a reproducible subtype[23]. As described in detail previously[24, 25], tissue blocks were obtained from approximately 60% of NHSII cases reported through 2006. TMA sections were stained for a panel of immunohistochemical markers and scored. TMAs were used as the primary categorization of hormone receptor status and medical/pathology reports were used to determine values for women without TMA information.

Statistical Methods

Women who reported a diagnosis of breast or other cancer (other than non-melanoma skin cancer) prior to June 1989 were excluded from our analysis. Women were followed from 1989

until return of the 2013 questionnaire. Person-months at risk were calculated from entry into the cohort until confirmed i) death ii) breast cancer diagnosis, or iii) other cancer (other than non-melanoma skin cancer).

Cox proportional hazard models stratified by calendar time with age (months) as the time metameter were used to calculate the hazard ratios (HR) and 95% confidence intervals (CI) of breast cancer (Model 1). The proportional hazard assumption was tested and met using a likelihood ratio test for the interaction between exposure and time. Subsequent analyses were further stratified by menopausal status and mechanism of menopausal transition (natural vs. surgical menopause) among postmenopausal women.

Known a priori risk factors for endometriosis and breast cancer were adjusted for and time-varying covariates were updated biennially at every questionnaire cycle (Table2 and Table 3: Model 2). Some treatments for endometriosis such as hysterectomy, oophorectomy, hormone therapy use (HT), and analgesic use may be on the causal pathway between endometriosis and breast cancer. We therefore additionally considered these covariates as potential mediators, updated them during each questionnaire cycle, and included them in our final model 3 (Table 2 and Table 3: Model 3).

For analyses of tumor hormone receptor status, we accounted for competing risks in Cox regression by using the Lunn and McNeil approach[26, 27], which allows for some covariates to have the same regression coefficient across tumor types, while other covariates can have different regression coefficients across tumor types based on previously published relationships (footnote, table 3)[4]. In sensitivity analyses all covariate relationships were allowed to vary across tumor types. Likelihood ratio tests were used to assess heterogeneity for hormone receptor subtypes between the three groups overall and for pair-wise comparisons.

Sensitivity Analyses

Since infertility history and cause of menopausal transition (natural or surgical) may influence breast cancer risk and are each correlated with endometriosis, we examined whether the effect of endometriosis on breast cancer risk differed by these covariates and tested the significance of interactions by likelihood ratio tests.

Several additional *a priori* sensitivity analyses were conducted to investigate the magnitude of potential biases. 1) To investigate the possible role of selection bias, we excluded prevalent endometriosis cases diagnosed before cohort enrollment (1989). 2) In studies of endometriosis, there is concern about a lengthy diagnostic delay between symptoms onset and surgical diagnosis. In the NHSII, the average diagnostic delay from symptom onset to disease diagnosis was approximately 4 years[1], while international multicenter studies have observed an average delay of 7 years[28]. To investigate the effect of this temporal misclassification of exposure, the diagnostic date of endometriosis was set earlier by 4, 6, and 8 years. 3) In sensitivity analyses for our exposure definition, we expanded our endometriosis definition to include disease diagnoses with and without laparoscopic confirmation. 4) In sensitivity analyses for our outcome definition, we restricted analyses to only invasive breast cancer. 5) In sensitivity analyses of the competing risk models for hormone receptor types, covariate relationships were allowed to vary across tumor types.

Results

In 1989, women with endometriosis had lower BMI (kg/m^2) in adulthood and at age 18, were more likely to be nulliparous, have biopsy confirmed diagnosis of BBD, and earlier age at first menstrual period (**Table 1.1**). Women with endometriosis were also more likely than women

Table 1.1 Characteristics of women in the Nurses' Health Study II at baseline in 1989 by endometriosis status, adjusted for age

	Endometriosis	
	No (n=109,936)	Yes (n=5,389)
	Mean (SD)	
Age in 1989*	34.3 (4.7)	35.6 (4.2)
Height (meters)	1.65 (0.07)	1.65 (0.07)
Body Mass Index (kg/m2)	24.1 (5.1)	23.7 (4.6)
BMI at age 18 (kg/m2)	21.3 (3.4)	20.8 (3.2)
Alcohol Intake (gms/day)	3.1 (6.1)	2.9 (5.8)
Number of pregnancies among parous women	2.1 (0.9)	1.8 (0.8)
	%	
Family history of breast cancer	6	7
Biopsy confirmed diagnosis of BBD	8	11
Caucasian	92	94
Age at first menstrual period		
- <12 years	24	29
- 12 years	30	30
- 13 years	28	26
- 14 years	10	9
- >14 years	8	7
Nulliparous	30	42
Current oral contraceptive use		
- Never	17	11
- Past	70	80
- Current	13	10
Smoking history		
- Never	65	64
- Past	21	21
- Current	13	14
Recent health seeking behavior†	95	98
Analgesic 2+ times per week	41	52
Hysterectomy	4	22
Oophorectomy		
- No procedure	98	83
- Unilateral	1	4
- Bilateral	1	14
Hormone Therapy use		
- Never User	90	68
- Past User	7	16
- Current User	3	17

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
† health seeking behavior defined by whether participant sought recent medical evaluation for screening or for symptoms
* Value is not age adjusted

without an endometriosis diagnosis to be current or past users of oral contraceptives (OCs), to use analgesics regularly, have had a hysterectomy or oophorectomy, to have used HT or to demonstrate recent health seeking behavior (recent medical evaluation for screening/symptoms of a variety of chronic diseases including mammography and breast exam, pap smear, diabetes, and hypertension screening).

In crude and fully adjusted models (**Table 1.2,** Model 3), no association was found between endometriosis and risk of breast cancer overall (HR:1.05, 95% CI: 0.95-1.16) (Table 2) or invasive breast cancer (HR:1.05, 95% CI:0.94-1.17). When stratifying by menopausal status of the women, women with endometriosis were not at higher risk for premenopausal (HR:1.07, 95% CI:0.91-1.26) or postmenopausal breast cancer (HR:0.98, 95% CI:0.84-1.14). These results did not change significantly among postmenopausal women when stratified by mode of menopause transition (natural menopause HR:1.03, 95% CI:0.80-1.33; surgical menopause HR:0.89, 95% CI:0.74-1.08).

The association between endometriosis and breast cancer varied significantly by tumor hormone receptor status (P-value, test for heterogeneity: 0.002) (**Table 1.3**). Women with endometriosis were not at increased risk of estrogen/progesterone receptor positive (ER+/PR+) tumors (HR:1.00, 95% CI:0.87-1.15) or ER-/PR- tumors (HR:0.82, 95% CI:0.60-1.11) (P-value, test for heterogeneity for pair-wise comparison between ER+/PR+ and ER-/PR-: 0.21). However, women with endometriosis had a significantly increased risk for ER+/PR- breast cancer in crude models (HR: 2.17, 95% CI:1.65-2.85) and models adjusted for potential confounding and mediating factors (HR: 1.72, 95% CI:1.27-2.32). While remaining statistically significantly associated, the effect estimate between model 1 and model 2 was attenuated by parity, age at first

Table 1.2: Laparoscopically confirmed endometriosis in relation to breast cancer risk, in the Nurses' Health Study II

Endometriosis	Cases/Person-years	Model 1	Model 2	Model 3
		Hazard Ratio (95% CI)		
		Breast Cancer Overall		
No	4479/ 2,329,489	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	500/ 215,434	1.07 (0.97-1.17)	0.96 (0.88-1.06)	1.05 (0.95-1.16)
		Breast Cancer by Menopausal Status		
		Premenopausal women		
No	2,258/ 1,356,591	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	167/ 82,517	1.16 (0.99-1.35)	1.05 (0.89-1.23)	1.07 (0.91-1.26)*
		Postmenopausal women		
No	1,419/ 501,465	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	235/ 97,068	0.98 (0.85-1.13)	0.93 (0.80-1.07)	0.98 (0.84-1.14)
		Stratified by mode of menopause transition among postmenopausal women		
		Natural Menopause		
No	1035/ 352,180	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	66/ 20,196	1.17 (0.91-1.50)	1.06 (0.82-1.36)	1.03 (0.80-1.33)†
		Any Surgery (Hysterectomy, oophorectomy)		
No	384/ 153,531	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	169/ 76,873	0.98 (0.82-1.18)	0.90 (0.75-1.09)	0.89 (0.74-1.08)†

Model 1: age and calendar time adjusted
Model 2: adjusted for Model 1 + family history of breast cancer in a mother or sister, age at menarche, BMI (kg/m^2), BMI at 18, smoking history, biopsy confirmed benign breast disease, alcohol intake, recent health seeking behavior , height, oral contraceptive use history, parity + age, total breast feeding, and birth weight
Model 3: Model 2+ hormone therapy use, hysterectomy, oophorectomy, any analgesic use greater than 2+ times per week
*Model 3: Model 2+ hormone therapy use, hysterectomy, unilateral oophorectomy, any analgesic use greater than 2+ times per week
†Model 3: Model 2+ hormone use, any analgesic use greater than 2+ times per week

Table 1.3 Laparoscopically confirmed endometriosis in relation to breast cancer risk by tumor hormone receptor status, in the Nurses' Health Study II

Tumor hormone receptor status	Cases /person years without endometriosis	Cases /person years with endometriosis	Model 1	Model 2	Model 3	
			Hazard Ratio (95% CI)			P-value[†]
ER+/PR+	2,333 /2,331,509	246 /215,657	1.14 (1.00-1.30)	1.00 (0.87-1.14)	1.00 (0.87-1.15)	0.002
ER+/PR-	309 /2,333,332	62/ 215,826	2.17 (1.65-2.85)	1.90 (1.44-2.50)	1.72 (1.27-2.32)	
ER-/PR-	528/ 2,333,127	49 /215,834	1.00 (0.75-1.34)	0.90 (0.67-1.21)	0.82 (0.60-1.11)	

Model 1: age and calendar time adjusted
Model 2: Model 1 + family history of breast cancer, age at menarche, BMI*, BMI at 18*, smoking, bbd biopsy, alcohol use, Check-up , birth weight, parity + age at pregnancy*, breastfeeding*, oral contraceptive use*, height
Model 3: adjusted for model 2 + HT use*, hysterectomy + oophorectomy*, analgesic use
*Association between the outcome and the covariate is allowed to vary by ER/PR status
[†]Likelihood Ratio P-value for test of heterogeneity across hormone receptor status-defined tumor types

birth, and history of benign breast disease while hysterectomy and oophorectomy use altered the effect estimate in Model 2 compared to Model 3.

In sensitivity analyses, restricting to incident endometriosis cases, predating endometriosis diagnosis by 4, 6, or 8 years, expanding the endometriosis exposure definition to include endometriosis diagnosed without laparoscopy, restricting the outcome definition include only invasive breast cancer, and stratification by infertility history resulted in similar effect estimates for overall breast cancer, breast cancer stratified by menopausal status, and tumor hormone receptor status analyses (data not shown). For tumor hormone receptor status analysis, sensitivity analyses were also conducted which allowed covariate relationships to vary across outcomes for the competing risk models and this did not significantly change effect estimates (data not shown).

Discussion

Endometriosis diagnosis was not associated with overall breast cancer risk, nor did endometriosis appear to be associated with increased risk of pre-menopausal or post-menopausal breast cancer. We observed significant heterogeneity across tumor hormone receptor status of breast cancers. While women with endometriosis were not at higher risk of ER+/PR+ or ER-/PR- tumors, they were at an approximately two-fold increased risk of ER+/PR- breast cancer.

Our finding, that women with endometriosis were not at increased risk for overall breast cancer, is consistent with the literature in which the majority of studies have reported no clear statistically significant association with overall breast cancer risk[9].

We did not observe differential associations with breast cancer risk by menopausal status. Previous studies that have investigated breast cancer by menopausal status have reported mixed

results. One small case-control study among premenopausal women reported an increased risk of breast cancer in women with endometriosis (OR 1.99; 95% CI: 1.0-4.0) [19]. This observation was also consistent with evidence demonstrating a greater risk of both endometriosis and premenopausal breast cancer among lean women, contrary to the pathophysiology elucidated for many hormonally and inflammatory dependent disease processes[29-32]. However, subsequent studies have not replicated this finding[14] nor does ours. Our study is consistent with results in previous work that endometriosis does not appear to be associated with overall breast cancer risk in naturally postmenopausal women[14, 17]. Since treatment for endometriosis may involve surgical menopause, the relationship between endometriosis and surgically postmenopausal women is more complex. While one small study found increased risk of breast cancer among women who underwent hysterectomy or oophorectomy as treatment for endometriosis (RR: 3.2, 95% CI: 1.2-2.8)[15], they lacked information on exogenous hormone usage -- which is common among these women and may alter breast cancer risk. In our analyses we used detailed information on hysterectomy, oophorectomy, and HT to consider these covariates as potential mediators and found no significant change after adjustment (Model 3) indicating that in our population they were neither important confounders nor mediators after adjusting for a priori potential confounding factors.

No previous study has investigated the association of endometriosis with breast cancer by tumor hormone receptor status, although there is well-documented disease heterogeneity occurring for other important risk factors[4, 33-36]. We found that women with endometriosis were at significant increased risk of ER+/PR- breast cancer, but not ER+/PR+ or ER-/PR- breast cancer. Adult BMI[4, 33, 35, 36], age at menarche[33], and time between menarche and first birth[4] are risk factors for which stronger relationships have been observed with ER+/PR- tumors than with

other tumors types. Interestingly, these risk factors have also been found to be consistently related to endometriosis risk[1, 37, 38]. The relationship we report with endometriosis differs in that we see an association with ER+/PR- tumors, but no association with other tumors. While this pattern is unique, previous research has reported associations with ER+/PR- tumors and risk factors (family history of breast cancer, age at menopause) that differ from other tumors in the direction of the effect estimate, however these findings have not been consistent [36]. Our finding may support the mounting evidence that ER+/PR- tumors differ biologically and in their risk factor profile from other tumors[4, 36, 39, 40].

It is well established that sex steroid hormones play an important role in endometriosis and breast cancer etiology. Hormones may influence endometriosis risk and hormones may be altered by an endometriosis lesion such as an endometrioma. Endometriosis implants depend on circulating estrogen for growth and maintenance[41]. Besides high estrogen levels, endometriosis is also characterized by low localized progesterone levels. It is hypothesized that the low levels of progesterone in endometriosis may limit its biologic ability to block matrix metallopeptidase expression and enhance endometrial cell apoptosis[42, 43]. Both hyper-estrogenic and hypo-progesteronic environments have been proposed in the pathogenesis of both specific breast cancer tumor types and endometriosis[44]. Chronic inflammation is another pathway by which endometriosis may influence breast cancer risk[45-47]. Increased levels of inflammatory markers have been found in both the peritoneal fluid and peripheral blood of women with endometriosis [48, 49]. Thus, a shared altered hormonal and inflammatory environment may be consistent with an environment in which endometriosis and ER+/PR- breast cancer tumors may thrive.

Despite this study's many strengths including its large sample size, validated exposure and outcome definitions, ability to adjust for time-varying confounding and assess possible mediating factors, and ability to assess heterogeneity in outcome, there also are some limitations.

Due to the possible delay in endometriosis diagnosis, some members of our cohort may have asymptomatic disease or disease that has not yet been diagnosed. Since the prevalence of endometriosis is ~10%, the inclusion of undiagnosed endometriosis cases in the unexposed group would be likely to have a limited effect[50] among the large truly unexposed women in this cohort, and while this misclassification still may bias our estimates, the bias would most likely attenuate our findings. A bias by diagnostic delay was explored more thoroughly through the sensitivity analyses and did not significantly alter results. As discussed previously, hormonal treatments for endometriosis may act as mediators along the causal pathway between endometriosis and breast cancer. While our participants contributed information on hormone therapy, we did not have sufficiently detailed information on other hormonal treatments used for endometriosis such as Danazol and Lupron. These hormones are thought to be protective against breast cancer: Lupron has been shown to reduce breast density and has been used as a breast cancer treatment[51, 52]. However, Danazol treatment was most popular before cohort initiation, and in sensitivity analyses our results did not change significantly after restricting to incident endometriosis diagnoses after 1989.

The long-term follow up of this cohort with detailed and validated exposure and outcome measures enabled a unique analysis of the temporal relationship between endometriosis and breast cancer. While the participants are not a random sample of all US women, it is unlikely that the biologic relationships that we analyzed would differ between this cohort of female nurses and women in general. The level of health education in this cohort allows for high quality

information to be collected by self-report and reduces confounding by education and socio-economic status.

In sum, while endometriosis may not increase women's risk of overall breast cancer, women with endometriosis may be at increased risk for a specific type of tumor, ER+/PR- breast cancer, which should be confirmed in new studies. Additionally, further research into the potential relationship between endometriosis and breast cancer should focus on disease heterogeneity especially hormone receptor status of the breast cancer tumors.

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Aim 2: A Prospective study of Endometriosis and Risk of Benign Breast Disease

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Abstract:

Background: Endometriosis is a chronic gynecologic disorder that affects ~10% of U.S. women. Given the altered hormonal and inflammatory environment of women with endometriosis, several studies have suggested a relationship between endometriosis and breast cancer. This is the first study to investigate the relationship between endometriosis and benign breast disease (BBD), benign lesions that are associated with an increased risk of breast cancer.

Methods: Among women in the Nurses' Health Study II followed from 1991-2003, we assessed the association between laparoscopically confirmed endometriosis, the clinical gold standard for diagnosis, and biopsy confirmed BBD. Cox proportional hazard models, adjusted for *a priori* potential confounding factors, were used to calculate hazard ratios (HR) and 95% confidence intervals (CI). BBD was further classified by study pathologists as proliferative or nonproliferative disease. Effect modification by infertility and use of screening mammography was investigated.

Results: Endometriosis was associated with a modest increased risk of biopsy confirmed BBD in crude and multivariable adjusted models (HR:1.34, 95% CI:1.13-1.58; HR:1.24, 95% CI:1.05-1.47, respectively). When evaluating subtypes of BBD, we did not find heterogeneity between nonproliferative (n=675) or proliferative (n=1336) BBD lesions, as endometriosis was associated with a modest increased risk for both (HR nonproliferative:1.25, 95% CI:0.93-1.69; HR proliferative:1.23, 95% CI:1.01-1.51). The relationship between endometriosis and risk of proliferative BBD appeared strongest among women who had experienced infertility (HR: 1.37, 95% CI:1.01-1.85; P-value, test for heterogeneity=0.05). Sensitivity analyses investigating screening behaviors between those with and without endometriosis did not significantly attenuate results.

Discussion: Endometriosis may confer higher risk of BBD, with the strongest relationship among infertile women, although future work should replicate this novel finding.

Introduction

Breast cancer is the most common cancer for women, with approximately 300,000 women in the United States diagnosed with the disease in 2013 alone[1]. Benign breast disease (BBD) confers an increased risk of breast cancer and may represent an earlier stage of breast carcinogenesis. BBD is a heterogeneous condition with several histologic subtypes; research has consistently found that proliferative BBD without atypia moderately (1.3-1.9 fold) increases risk of breast cancer later in life, while proliferative BBD with atypical hyperplasia confers between 4 and 6-fold increased risk of breast cancer[2-6].

Endometriosis is a chronic gynecologic disorder that affects approximately 10% of women nationwide[7-9] and is characterized by the presence of endometrial tissue outside the uterine cavity[7]. Symptoms for endometriosis include chronic pelvic pain, dysmenorrhea (painful periods), dyspareunia (painful intercourse), dysuria (painful urination), infertility, and dyschezia (painful defecation)[8, 10]. Recent research has indicated that women with endometriosis may be at increased risk for breast cancer[11], with several studies suggesting a modest positive association, although many lacked statistical significance[12-17]. To our knowledge, no previous study has investigated endometriosis in relation to risk of BBD, a breast cancer risk factor and possible preliminary marker of carcinogenesis.

In this manuscript, we prospectively investigated the relationship between endometriosis and BBD within the Nurses' Health Study II (NHSII), a longitudinal cohort study of over 100,000 women. Given the altered hormonal and inflammatory environment of women with endometriosis, we hypothesized that women with endometriosis would be at increased risk of proliferative BBD compared to women without endometriosis.

Methods

The NHSII is a prospective cohort study that began in 1989 when 116,430 female registered nurses, 25-42 years old, returned a mailed questionnaire on their health and lifestyle. Follow-up questionnaires that collect information on environmental, dietary, and lifestyle risk factors have been sent biennially. The cumulative follow-up rate on each questionnaire cycles is over 90%. This study was approved by the Institutional Review Board of Brigham and Women's Hospital.

Exposure definition

On the 1993 questionnaire, women were first asked if they had ever had "physician diagnosed endometriosis." If they answered "yes," they were asked to report when the diagnosis had occurred and if their disease had been confirmed by laparoscopy. Endometriosis diagnosis has been assessed on every questionnaire subsequently allowing women to become exposed throughout cohort follow-up.

Self-reported endometriosis was validated previously among 200 participants using medical records[9]. Among women who reported laparoscopic confirmation, a laparoscopic diagnosis of endometriosis was confirmed in 96% of medical records. Conversely, among those women without laparoscopic confirmation, evidence of clinical diagnosis was found in only 54% of medical records. Thus, to reduce the magnitude of misclassification, endometriosis exposure was restricted to those women with laparoscopic confirmation. Women who reported endometriosis diagnosis without laparoscopic confirmation who later had a confirmation by laparoscopy were assigned to the endometriosis exposed group at the time of the initial clinical diagnosis. Those who reported endometriosis diagnosis but never laparoscopic confirmation were censored at the report of clinical diagnosis.

Outcome definition

In 1989, women were first asked whether or not they had received a diagnosis of fibrocystic or other benign breast disease. On each subsequent questionnaire, women reported whether they received a physician diagnosis of benign breast disease and whether the diagnosis was confirmed by biopsy and/or aspiration. If women reported a biopsy confirmed benign breast disease between 1993 and 2003, they were contacted to ask permission to obtain biopsy specimens and seek confirmation of their diagnosis. Collection of these samples has been described previously[18]. A total of 3,588 women reported a first diagnosis of biopsy-confirmed BBD, among whom 2,643 gave permission to review their biopsy records and pathology slides. Pathology material was obtained and reviewed for 2,313 women (87.5% of those who had given their permission); and 2,208 women had valid biopsy information. The main reasons for exclusion included that the pathology specimen did not contain breast tissue or that the biopsy date was before 1989.

Benign breast biopsy slides were obtained and reviewed by one of three pathologists who were blinded to the participants' endometriosis status (L.C. Collins, S. J. Schnitt, J. L. Connolly). Dupont and Page criteria were used to classify benign breast disease into three categories: nonproliferative, proliferative without atypia, and atypical hyperplasia.[6] Biopsy samples that showed atypia or questionable atypia were jointly reviewed by two of our pathologists for confirmation or to reach consensus. Samples with intraductal papilloma, radial scar, sclerosing adenosis, fibroadenoma, fibroadenomatous change, or moderate to florid usual ductal hyperplasia in the absence of atypical hyperplasia were classified as proliferative without atypia.

Statistical Methods

Women who reported a diagnosis of prior BBD or cancer (other than non-melanoma skin cancer) prior to June 1991 were excluded from our analysis. Women contributed person-time from 1991 until return of the 2003 questionnaire. Women were followed from entry into the cohort sample until the confirmed minimum of confirmed i) death, ii) BBD, or iii) cancer (other than non-melanoma skin cancer).

Cox proportional hazard models stratified by calendar time with age (months) as the time scale were used to calculate the hazard ratios (HR) and 95% confidence intervals (CI) of BBD (Model 1). The proportional hazard assumption was assessed using a likelihood ratio test for the interaction between exposure and age (months); the assumption was not violated ($p>0.05$). Known risk factors for endometriosis and BBD were adjusted for, and time-varying covariates were updated biennially at every questionnaire cycle (Model 2). Some treatments for endometriosis, such as hysterectomy, oophorectomy, post-menopausal hormone therapy use (HT), and analgesic use, may be on the causal pathway between endometriosis and BBD. We therefore additionally considered these covariates as potential mediators and updated them during each questionnaire cycle (Model 3).

To compare subtypes of BBD, we accounted for competing risks in Cox regression by using the Lunn and McNeil approach.[19, 20] Likelihood ratio tests were used to assess heterogeneity of effects between proliferative and nonproliferative lesions.

History of infertility may influence the relationship between endometriosis and BBD, therefore we examined whether the effect of endometriosis on BBD differed by whether or not women ever experienced infertility (12 months of trying to conceive without success), and tested the significance of interactions by likelihood ratio tests. Additionally, we investigated effect

modification by health-seeking behavior/connection with the medical system, we assessed whether the effect of endometriosis on BBD differed by history of mammography or breast exam for screening purposes.

Sensitivity Analyses

We conducted sensitivity analyses to both restrict our outcome definition of BBD. Proliferative BBD lesions were further divided into groups with and without atypia, since historically these types have different associations with breast cancer.

Several additional *a priori* sensitivity analyses were conducted to investigate the presence and quantify the magnitude of potential biases. 1) We excluded prevalent endometriosis cases diagnosed before cohort enrollment (1989). 2) In studies of endometriosis, there is concern about a lengthy diagnostic delay between symptom onset and surgical diagnosis. In the NHSII, the average diagnostic delay from symptom onset to disease diagnosis was approximately 4 years[9], while international multicenter studies have observed an average delay of 7 years[21]. To investigate the effect of this temporal misclassification of exposure, the diagnostic date of endometriosis was set earlier by 2, 4, and 6 years. 3) We expanded our endometriosis definition to include disease diagnoses with and without laparoscopic confirmation.

Results

Compared to women without endometriosis in 1991, women with the disease were leaner at age 18 and in 1991, were more likely to be nulliparous, to report screening mammograms, to report clinical breast exams, to have had a hysterectomy or oophorectomy, and to report use of HT and oral contraceptives. Between 1991 and 2003, there were 2,009 women with biopsied BBD information (1,334 with proliferative and 675 with nonproliferative BBD) (**Table 2.1**).

Women with endometriosis had a modest significant increased risk of proliferative BBD in crude (HR:1.35, 95% CI: 1.11-1.66) and multivariable adjusted models (HR:1.23, 95% CI: 1.01-1.51)(**Table 2.2**). Attenuation with adjustment was driven by a combination of age at first birth, family history of breast cancer, BMI, and recent health seeking behavior (mammogram or screening test). We did not find a significant difference in associations between endometriosis and proliferative and nonproliferative BBD lesions (heterogeneity P-value: 0.80). Endometriosis also was associated with increased risk of nonproliferative BBD in multivariate adjusted models (HR: 1.25, 95% CI: 0.93-1.69).When proliferative and nonproliferative BBD cases were combined, endometriosis increased risk for BBD (HR: 1.24, 95% CI: 1.05-1.4) (**Table 2.2**).

In age and calendar time adjusted models due to small sample size, endometriosis was significantly associated with an increased risk of proliferative BBD without atypia (n=1,226) (HR Proliferative BBD without atypia: 1.37, 95% CI: 1.11-1.69) and the effect estimate was similar although not statistically significant for proliferative BBD with atypia (n=108) (HR Proliferative BBD with atypia: 1.14, 95% CI: 0.53-2.45).

When analyses were stratified by infertility history as defined by trying to conceive for 12 months or more without success, we observed a suggestion of heterogeneity in the association of endometriosis with proliferative BBD between women with and without infertility (test for heterogeneity of proliferative BBD p-value: 0.05)(**Table 2.3**). Endometriosis was associated with increased risk of proliferative BBD among women who had ever experienced infertility (HR: 1.37, 95% CI:1.01-1.85), but not among women who had never experienced infertility (HR:0.95, 95% CI: 0.66-1.36). We did not observe significant heterogeneity by infertility history for nonproliferative BBD (P-value:0.78).

Table 2.1 Characteristics of women in the Nurses' Health Study II at baseline in 1991 by endometriosis status

	Laparoscopically Confirmed Endometriosis	
	No (n=72,995)	Yes (n=3,398)
	Mean (SD)	
Age (years)*	35.5(4.7)	36.4(4.3)
Height (meters)	1.6(0.1)	1.7(0.1)
Body Mass Index (kg/m^2)	24.9(5.5)	24.5(5.0)
Body Mass Index at age 18 (kg/m^2)	21.4(3.4)	20.9(3.2)
Parity among parous	2.2(0.9)	1.9(0.8)
	%	
Never reported a successful pregnancy, %	25.3	38.3
Family history of breast cancer, %	4.8	5.3
Current oral contraceptive use		
- Never, %	16.2	10.0
- Past, %	71.9	80.6
- Current, %	11.8	9.4
Age at first menstrual period		
- <12 years, %	24.0	27.9
- 12 years, %	30.0	30.3
- 13 years, %	27.7	26.3
- 14 years, %	10.5	9.2
- >14 years, %	7.8	6.4
Mammogram in 1991		
- No, %	64.4	60.9
- Yes Symptoms, %	33.5	36.4
- Yes Screening, %	2.1	2.7
Breast Exam		
- No, %	16.8	12.5
- Yes Symptoms, %	81.4	85.0
- Yes Screening, %	1.8	2.5
Hysterectomy, %	4.1	21.0
Oophorectomy		
- No procedure, %	98.3	82.8
- Unilateral, %	0.6	3.1
- Bilateral, %	1.2	14.1
Postmenopausal hormone use		
- Never User, %	87.5	61.7
- Past User, %	9.6	21.4
- Current User, %	2.9	16.8

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

[a] health seeking behavior defined by whether participant sought recent medical exam for screening

Table 2.2: Laparoscopically confirmed endometriosis in relation to BBD risk in the Nurses' Health Study II (1991-2003)

Endometriosis	Cases/ Person-years	Model 1	Model 2	Model 3
		HR (95% CI)		
		Nonproliferative BBD[1]		
No	627/ 859,322	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	48/ 55,715	1.31 (0.97-1.75)	1.25 (0.93-1.69)	1.15 (0.84-1.58)
		Proliferative BBD[1]		
No	1,234/ 858,710	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	102/ 55,662	1.35 (1.11-1.66)	1.23 (1.01-1.51)	1.22 (0.98-1.52)
		BBD[1] (Combined Nonproliferative + Proliferative)		
No	1,861/ 858,147	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)
Yes	150/ 55,618	1.34 (1.13-1.58)	1.24 (1.05-1.47)	1.20 (1.01-1.44)

Model 1: age (months) and calendar time (years) adjusted
Model 2: adjusted for Model 1 + potential confounders: family history of breast cancer in a mother or sister, age at menarche(<12, 12, 13, 14, >14), BMI (kg/m^2) (<18.5, 18.5-22.4, 22.5-24.9, 25-29.9, ≥ 30), BMI at 18 (<18.5, 18.5-22.4, 22.5-24.9, 25-29.9, ≥ 30), smoking history (never, past, current), alcohol intake (no alcohol, <5 gms/day, 5-10 gms/day, >10 gms/day), recent health seeking behavior, height, oral contraceptive use history (never, past, current), parity and age at first birth (nulliparous,1-2 children and <25 years at first birth, 1-2 children and ≥ 25 at first birth, ≥ 3 children and <25 years at first birth, ≥ 3 children and ≥ 25 at first birth), total breast feeding (<1 month, 1-3 months, 3-12 months, >12 months) , adolescent alcohol intake (no alcohol, <5gms alcohol per day, 5-10 gms/day, >10 gms/day)
Model 3: Model 2+ potential mediators: post menopausal hormone use (never, past, current), hysterectomy, oophorectomy, any analgesic greater than 2+ times per week, menopause status
[1] Based on centralized pathology review of H&E slides

Table 2.3: Laparoscopically confirmed endometriosis in relation to BBD risk in the Nurses' Health Study II (1991-2003) stratified by infertility history

Endometriosis	Cases/ Person-years	Model 1	Model 2	Model 3	Test for heterogeneity P-value
		HR (95% CI)			
Nonproliferative BBD					
Never Infertility					
No	508/ 898,457	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)	0.78
Yes	20/ 31,053	1.32 (0.84-2.07)	1.23 (0.78-1.94)	1.05 (0.65-1.70)	
Ever Infertility					
No	119/ 229,902	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)	
Yes	27/ 41,553	1.36 (0.89-2.07)	1.40 (0.90-2.17)	1.29 (0.82-2.03)	
Proliferative BBD					
Never Infertility					
No	992/ 897,973	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)	0.05
Yes	34/ 31,039	1.08 (0.77-1.52)	0.97 (0.69-1.37)	0.95 (0.66-1.36)	
Ever Infertility					
No	243/ 229,773	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)	
Yes	63/ 41,517	1.48 (1.11-1.96)	1.37 (1.03-1.83)	1.37 (1.01-1.85)	
BBD (Combined Nonproliferative + Proliferative)					
Never Infertility					
No	1,500/ 897,551	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)	0.92
Yes	54/ 31,020	1.16 (0.88-1.52)	1.05 (0.80-1.39)	1.01 (0.75-1.34)	
Ever Infertility					
No	362/ 229,672	1.0 (Referent)	1.0 (Referent)	1.0 (Referent)	
Yes	90/ 41,494	1.44 (1.14-1.82)	1.37 (1.08-1.75)	1.33 (1.04-1.72)	

Model 1: age and calendar time adjusted; Model 2: adjusted for Model 1 + potential confounders: family history of breast cancer in a mother or sister, age at menarche(<12, 12, 13, 14, >14), BMI (kg/m^2) (<18.5, 18.5-22.4, 22.5-24.9, 25-29.9, $\geq$ 30), BMI at 18 (<18.5, 18.5-22.4, 22.5-24.9, 25-29.9, $\geq$ 30), smoking history (never, past, current), alcohol intake (no alcohol, <5 gms/day, 5-10 gms/day, >10 gms/day), recent health seeking behavior, height, oral contraceptive use history (never, past, current), parity and age at first birth (nulliparous,1-2 children and <25 years at first birth, 1-2 children and $\geq$ 25 at first birth, $\geq$ 3 children and <25 years at first birth, $\geq$ 3 children and $\geq$ 25 at first birth), total breast feeding (<1 month, 1-3 months, 3-12 months, >12 months) , adolescent alcohol intake (no alcohol, <5gms alcohol per day, 5-10 gms/day, >10 gms/day) Model 3: Model 2+ potential mediators: post menopausal hormone use (never, past, current), hysterectomy, oophorectomy, any analgesic greater than 2+ times per week, menopause status

In sensitivity analyses stratified by report of mammogram or breast exam for screening purposes, we found no significant difference in the relationship between endometriosis and proliferative BBD (P-value: 0.95) or nonproliferative BBD (P-value:0.67) between those who did and who did not report screening behaviors. Additional sensitivity analyses which 1) excluded prevalent endometriosis cases, 2) predated endometriosis diagnosis, and 3) expanded endometriosis cases to include those diagnosed without laparoscopic confirmation were not materially different results than those reported in the main analysis (data not shown).

Discussion

Benign breast disease is an important risk factor for breast cancer. In our analysis, we observed a modest risk of biopsy confirmed BBD, of both proliferative and nonproliferative lesions among women with endometriosis. The strongest increased risk of proliferative BBD in women with endometriosis was observed among women with a history of infertility.

Contrary to our a priori hypothesis, we found that endometriosis conferred increased risk of both proliferative and nonproliferative lesions. In their seminal study, Dupont and Page found no increased breast cancer risk among women with nonproliferative BBD [6]. However, more recent research has found modest increased risk of breast cancer among nonproliferative lesions, however the definition of this category across study populations has varied[22-24]. Wang et al. in a companion study to the National Surgical Adjuvant Breast and Bowel Project (NSABP) Breast Cancer Prevention Trail found that women in the "lower category" of BBD had 1.6 times the risk of breast cancer compared to healthy women[22]. Hartman et. al. found that women with nonproliferative BBD had a 1.27 (95% CI: 1.15-1.41) fold risk of breast cancer among a cohort of women from the Mayo Clinic[23]. And most recently, Castells et. al. found nonproliferative

lesions conferred a 2.23 fold (95% CI: 1.86- 2.68) increased risk of breast cancer in a population based screening cohort[24]. Few studies have investigated risk factors for both proliferative and nonproliferative lesions given the mixed findings related to breast cancer risk.

The relationship between endometriosis and proliferative BBD, the histologic type of BBD which confers the highest risk of breast cancer, was strongest among women who had reported previous history of infertility. While endometriosis is often thought to be highly correlated with infertility, these women may have varying levels of disease severity. Women with endometriosis and infertility may represent more severe cases of endometriosis since the endometriosis lesions were severe enough to have caused impaired fecundity, or these women may represent asymptomatic endometriosis cases who were diagnosed during a routine fertility evaluation because of pre-existing infertility caused by another condition (Prescott, Submitted 2015). Unfortunately information on reason for endometriosis diagnosis (pain or infertility presentation) was not collected in this study. Future research in this area should investigate if mechanism of endometriosis diagnosis modifies the relationship with BBD.

Women who received a diagnosis of endometriosis may be more connected with the medical system than women who are not diagnosed. Since BBD is often diagnosed through screening procedures such as a mammogram or breast exam, it is possible that any relationship found between endometriosis and BBD may represent a spurious association being driven by connection to medical system. While the effect of this bias is likely minimal given that this is a cohort of medical professionals, all analyses were adjusted for recent health seeking behavior which could be a proxy for connection with the medical system (including mammography, clinical breast exam, and other screening behaviors including Pap smear, colonoscopy and hypertension screening). Additionally, sensitivity analyses were conducted stratifying by use of

mammography or clinical breast exam for screening, and no significant differences in the relationship between endometriosis and BBD were observed between women who did and did not undergo breast cancer screening behavior. However, we cannot fully rule out the possibility of residual confounding by connection with the medical system, which our self-reported proxy measures may not fully capture.

Women with endometriosis may have an altered hormonal and inflammatory milieu[8, 25-27]. It is well established that sex steroid hormones play an important role in endometriosis[25], with endometrial lesions depending on circulating estrogen for growth and maintenance[8]. Recent research has found increased levels of steroid hormones, particularly endogenous estrogens among women with BBD compared to healthy controls[28]. Thus, the pathogenesis of both diseases could be regulated through a shared hormonal etiology.

While our study has many strengths, we must also recognize its limitations. Due to the delay in endometriosis diagnosis, some members of our cohort may have asymptomatic disease or disease that has not yet been diagnosed. Since the prevalence of endometriosis is ~10%, the inclusion of undiagnosed endometriosis cases in the unexposed group would likely have a limited effect among the large number of truly unexposed women in this cohort[29], and while this misclassification still may bias our estimates, the bias would most likely attenuate our findings. A bias by diagnostic delay was investigated more thoroughly through the sensitivity analyses which predated endometriosis diagnoses. We found that predating endometriosis cases did not materially alter results. While the participants in our study were not a random sample of all US women, it is unlikely that the biologic relationships that we analyzed would differ between this cohort of female nurses and women in general. The level of health education in this cohort

allows for high quality information to be collected by self-report and reduces confounding by education and socio-economic status.

This study has many strengths. To reduce the likelihood of misclassification of our outcomes, our BBD case definition was centrally reviewed by a team of trained pathologists. Additionally, our validated exposure definition was restricted to endometriosis cases confirmed by laparoscopy to decrease misclassification. In the first study to investigate the association between endometriosis and risk of BBD, we were able to adjust for time-varying covariates and assess possible mediating factors.

Women with endometriosis may be at a modest increased risk of benign breast disease, which may put them at a heightened risk for breast cancer later in life. Future research should focus on replicating this novel finding.

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Aim 3: Endometriosis and Mammographic Density in the Nurses' Health Study II

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Abstract:

Introduction: The association between endometriosis and breast cancer risk has been inconsistent in epidemiologic studies, with some suggesting a modest increased risk. We investigated the association between endometriosis and mammographic density, a consistent and independent risk factor for breast cancer.

Methods: We conducted a cross-sectional analysis among 1,581 pre- and post-menopausal women not previously diagnosed with breast cancer in the Nurses' Health Study II cohort. We measured average percent mammographic density and absolute dense and non-dense breast area using a validated computer-assisted method. Multivariable linear regression was used to estimate the association between endometriosis and mammographic density among pre- and postmenopausal women separately.

Results: Among premenopausal women, average percent mammographic density was 43.1% among women with endometriosis (n=91) and 40.5% among women without endometriosis (n=1,150). Endometriosis was not associated significantly with mammographic density among premenopausal (% difference: 2.00 percentage points, 95% CI:-1.33,5.33) or among postmenopausal women (% difference: -0.89 percentage points, 95% CI:-5.10,3.33). Among premenopausal women, there was heterogeneity by BMI at age 18 (P-value: 0.003), with a suggested association among those who were lean at age 18 (BMI< 20.6 kg/m^2) (% difference: 3.74 percentage points, 95% CI:-0.29,7.78).

Conclusion: Endometriosis was not associated with average percent mammographic density. If endometriosis increases breast cancer risk, it may be mediated through a pathway independent of breast density.

Introduction

One of the strongest and most consistent risk factors for breast cancer is mammographic density, a measure of the amount of fibroglandular tissue in the breast comprised of epithelial and stromal cells. Mammographic density can be assessed on a mammogram--dense breast tissue appears light on a mammogram, whereas non-dense tissue appears dark. Women with $\geq$ 75% mammographic density have a four- to six-fold increased risk of developing breast cancer compared to women with almost entirely fatty breasts, i.e. mammographic density <5% [1-3]. Nationally, mammographic density has been brought to the forefront, with legislation in many states mandating patients be notified of their mammographic density levels after receiving a mammogram[4]. Higher mammographic density has been found to be associated with exogenous hormone use[5-12], with body mass index (BMI)(kg/m^2)[13], and with menopausal status[7-9, 11, 12, 14-16].

Women with endometriosis, a chronic gynecologic disorder that affects approximately 10% of women[17-19], may have an altered hormonal and inflammatory milieu[5, 18, 20, 21]. It is well established that sex steroid hormones play an important role in endometriosis[5] with endometriosis lesions depending on circulating estrogen for growth and maintenance[18]. Recent research has indicated that women with endometriosis may be at increased risk for several chronic diseases including cardiovascular disease and certain types of cancer, including ovarian and possibly breast[22]. Evidence for a relationship between endometriosis and breast cancer risk has been mixed[22, 23] with several studies suggesting modest positive associations, although many lacked statistical significance[24-29]. Despite the possibility that women with endometriosis may be at increased risk of breast cancer and that women with endometriosis may have an altered hormonal environment, no prior study has investigated the relationship between endometriosis and mammographic density.

We investigated the relationship between endometriosis and mammographic density within the Nurses' Health Study II (NHSII) cohort. We hypothesized that women with endometriosis would have higher mammographic density compared to women without the disease.

Methods

The NHSII is a prospective cohort study that began in 1989 when 116,430 registered nurses, 25-42 years old, returned a mailed questionnaire on their health and lifestyle patterns. Follow-up questionnaires were sent biennially to collect information on environmental, dietary, and lifestyle risk factors with cumulative response rates over 90%. This study was approved by the Institutional Review Board of Brigham and Women's Hospital.

Study Population

Within the NHSII, mammogram collection was originally conducted within a breast cancer case-control study, which was nested within the sub-cohort of women who provided a blood sample. Controls were randomly selected from the sub-cohort of women who returned a blood sample and had never reported a diagnosis of cancer. One or two controls were matched to breast cancer cases on year of birth, menopausal status, hormonal therapy use, race/ethnicity, and time of day, month, and fasting status at time of blood draw. Mammograms were received from approximately 80% of eligible women in the nested case-control study and were collected for years close to blood collection (1996-1999). We additionally collected mammograms (conducted from around 1997) from eligible women (breast cancer cases and non-cases) who provided cheek cell samples or completed an adolescent diet questionnaire in NHSII. Women for whom mammograms could not be obtained did not differ from those with available mammograms with

regard to breast cancer risk factors, including BMI, parity and family history of breast cancer[30, 31].

Given that high mammographic density is associated positively with increased risk of breast cancer, to prevent a spurious association between endometriosis and mammographic density, we restricted the study population to controls for this analysis (n=1,581, after exclusions). If women reported hormone therapy use, they were removed from the analysis if they were a smoker and between the ages of 46-54 or a non-smoker and between the ages of 48-56 due to inability to determine menopausal status. Women missing information on endometriosis were excluded from the analysis.

Exposure Definition

On the 1993 questionnaire, women were first asked if they had ever had "physician diagnosed endometriosis." If they answered "yes," they were asked to report when the diagnosis had occurred and if their disease had been confirmed by laparoscopy. Endometriosis diagnosis has been assessed on every biennial subsequent questionnaire. Self-reported endometriosis diagnosis was previously validated using the medical records of 200 randomly selected participants [19]. Among women who reported laparoscopic confirmation, a laparoscopic (surgical) diagnosis of endometriosis was confirmed in 96% of women. Conversely, among women without laparoscopic confirmation, evidence of clinical diagnosis was found in only 54% of medical records. Thus, to reduce the magnitude of misclassification, we restricted our definition of endometriosis to those women with a laparoscopic confirmation. Women who only reported clinical, not surgical, diagnosis were excluded from both the endometriosis and non-endometriosis groups.

Outcome Definition

From the mammograms collected, the cranio-caudal views of both breasts were digitized 261μm/pixel with a Lumysis 85 laser film scanner (Lumysis, Sunnyvale, CA) or a VIDAR CAD PRO Advantage scanner (VIDAR Systems Corporation; Herndon, VA) (using comparable resolution of 150 dots per inch and 12 bit depth inch and 12 bit depth). Average percent mammographic density has been the primary measure of breast density given its consistent relationship to breast cancer risk[31, 32], however recent literature has suggested that both absolute dense and non-dense area are independent predictors of breast cancer risk[32-35]. To estimate absolute dense area, absolute non-dense area (total breast area minus dense area), and percent mammographic density (dense area divided by total breast area), we used Cumulus software (University of Toronto, Toronto, Canada) for computer-assisted thresholding[36, 37]. The reader selects two thresholds according to the intensity of the pixels: one to delineate the breast edge and the other to distinguish dense tissue from non-dense tissue. The software then calculates the number of pixels of the entire breast and those of the area identified as dense. Our trained reader is blinded to case and control status and consistently achieves a within-person intraclass correlation coefficient of 0.91 and an intraclass correlation coefficient of 0.90 for comparison with an external expert[31]. As we found evidence of batch-to-batch variability in mammogram readings, all mammographic density measurements were corrected to produce the measurement that would have been obtained had the mammogram been included in the first batch, using a statistical technique described in detail previously[30, 38].

Statistical Methods

Given the consistent relationship between menopausal status and breast density[1], with breast density being lower among postmenopausal women, all analyses were stratified by

menopausal status *a priori*. Women were considered postmenopausal if they reported 1) no menstrual periods for 12 months, 2) having had a bilateral oophorectomy, or 3) being 54 years old or older if a smoker or 56 years or older if a non-smoker.

We fit multivariable linear regression models to quantify the cross-sectional association between endometriosis (independent variable) and measures of mammographic density (dependent variable). Generalized estimating equations were used to account for correlation among matched controls using an unstructured correlation matrix. Robust (sandwich) standard errors were used to minimize potential violations in the assumptions of normality of residuals and homoscedasticity for linear regression. Model 1 adjusted for current age and BMI, known important predictors of both endometriosis and mammographic density. Model 2 additionally adjusted for potential confounding by other *a priori* risk factors for breast density: alcohol intake (<4 gms/day vs. $\geq$ 4 gms/day), family history of breast cancer, smoking history (current, never, smoker), history of benign breast disease, BMI at age 18 (<18.5, 18.5-22.4, 22.5-24.9, 25-29.9, $\geq$ 30), parity (1, 2, 3, $\geq$ 4) , age at menarche (<12, 12, 13, 14, >14), oral contraceptive use history (never, past, current), and breastfeeding history (never, 1-5 months, 6-11 months, $\geq$ 12 months). Since some treatments for endometriosis may alter mammographic density, model 3 additionally adjusted for those covariates that may be potential mediators of the relationship between endometriosis and mammographic density: hysterectomy, hormone therapy use (never, past, current), and oophorectomy (none, unilateral, bilateral) (postmenopausal women only). Endometriosis and covariate status was defined at the closest questionnaire cycle to the time of the mammogram. Effect modification was investigated for menopausal status, BMI at age 18, and current BMI, and likelihood ratio tests were used to test for significance of interaction terms. To account for possible diagnostic delay of endometriosis, we conducted sensitivity analyses that

used endometriosis diagnostic status of 2, 4, and 6 years before the nurses' reported date of surgical diagnosis.

Results

Around the time of mammogram, pre- and post-menopausal women with endometriosis reported lower current BMI and lower BMI at age 18, were more likely to be nulliparous or to have low parity, to have undergone oophorectomy (unilateral or bilateral) or hysterectomy, and to be past or current users of hormone therapy, compared to women without endometriosis (**Table 3.1**). Premenopausal women with endometriosis were more likely to have had earlier age at menarche compared to premenopausal women without endometriosis, whereas postmenopausal women with endometriosis were more likely to report later age at menarche compared to women without endometriosis.

Among premenopausal women, women with endometriosis appeared to have modestly higher average percent mammographic density of 43.1% (SD:17.5) compared to healthy women (40.5% (17.9)) (**Table 3.2**). However, in models adjusting for potential confounding and mediating factors, endometriosis was not associated with percent mammographic density (% density: 2.00 percentage points difference, 95% CI: -1.33,5.33), nor was endometriosis associated with average dense (0.10 cm^2 difference, 95% CI: -9.74,9.94) or non-dense area (-8.45 cm^2 difference, 95% CI: -22.34,5.44) (Table 2; Model 3). Among postmenopausal women, there was also no significant difference in average percent mammographic density (% density:-0.89 percentage points difference, 95% CI: -5.10, 3.33), average dense area (-2.71 cm^2 difference, 95% CI: -15.34, 9.93), or average non-dense area (3.38 cm^2 difference, 95% CI: -15.82, 22.57) for women with and without endometriosis (Table 2; Model 3). Given the opposing

Table 3.1 Characteristics of women in the Nurses' Health Study II at time of mammogram by endometriosis status (n=1,581)

	Premenopausal		Postmenopausal	
	Endometriosis			
	No (n=1,150)	Yes (n=91)	No (n=263)	Yes (n=77)
	Mean(SD)			
Age*	44.4(4.1)	44.4(3.1)	50.5(4.0)	48.2(4.8)
Body Mass Index (BMI) (kg/m2)	26.0(5.6)	25.3(4.5)	26.3(5.6)	25.2(5.5)
BMI at age 18	21.1(2.9)	20.9(2.4)	21.3(3.1)	20.4(2.5)
Alcohol (gms/day)	4.3(7.4)	5.0(8.5)	2.9(5.9)	2.7(4.0)
Percent Mammographic Density	40.5(17.9)	43.1(17.5)	32.2(17.5)	32.3(16.3)
	%			
Family history of breast cancer, %	9.3	5.3	10.4	14.5
Biopsy confirmed BBD, %	18.1	16.4	17.9	22.7
Parity				
- Nulliparous, %	16.4	31.8	22.7	19.8
- 1 pregnancy, %	12.9	16.6	14.7	36.5
- 2 pregnancies, %	39.6	31.2	35.5	34.3
- 3+ pregnancies, %	31.0	20.4	27.2	9.4
Age at menarche				
- <12 years, %	23.4	34.7	25.1	19.6
- 12-13 years, %	29.5	27.7	31.2	18.3
- >=14 years, %	47.1	37.5	43.8	62.1
Oral contraceptive use				
- Never, %	14.6	8.0	9.0	12.4
- Past, %	78.6	86.7	90.7	87.6
- Current, %	6.7	5.3	0.3	0.0
Smoking status				
- Never, %	69.8	59.6	69.9	60.1
- Past, %	23.9	36.2	17.4	26.5
- current, %	6.3	4.2	12.7	13.3
Oophorectomy				
- No procedure, %	96.3	83.1	46.8	4.0
- Unilateral, %	3.7	16.9	0.6	1.0
- Bilateral, %	0.0	0.0	52.6	95.0
Hysterectomy, %	4.5	17.6	51.9	95.4
HT use				
- Never, %	79.3	66.5	5.1	0.0
- Past, %	17.5	29.6	14.5	7.8
- Current, %	3.2	3.9	80.3	92.2

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

Table 3.2 The association between endometriosis and average mammographic density measurements (linear regression estimates) in the Nurses' Health Study II

		Model 1	Model 2	Model 3[a]
	Mean +/- SD		Difference (95% CI)	
Premenopausal Women (n=1,241) (No endometriosis n=1,150, Endometriosis n=91)				
Average Percent Mammographic Density				
No Endometriosis	40.54 +/- 17.87	0 (ref)	0 (ref)	0 (ref)
Endometriosis	43.07 +/- 17.54	1.77 (-1.71, 5.24)	2.03 (-1.28, 5.34)	2.00 (-1.33, 5.33)
Average Dense Area (cm^2)				
No Endometriosis	94.86 +/- 52.52	0 (ref)	0 (ref)	0 (ref)
Endometriosis	96.61 +/-49.58	1.17 (-9.49, 11.83)	1.32 (-8.82, 11.47)	0.10 (-9.74, 9.94)
Average Non-Dense Area (cm^2)				
No Endometriosis	147.52 +/-76.15	0 (ref)	0 (ref)	0 (ref)
Endometriosis	137.75 +/- 75.95	-5.70 (-19.00, 7.61)	-8.80 (-22.39, 4.80)	-8.45 (-22.34, 5.44)
Postmenopausal Women(n=340) (No endometriosis n=263, Endometriosis n=77)				
Average Percent Mammographic Density				
No Endometriosis	32.24 +/- 17.47	0 (ref)	0 (ref)	0 (ref)
Endometriosis	32.33 +/- 16.26	-1.38 (-5.02,2.26)	-2.04 (-5.74,1.67)	-0.89 (-5.10,3.33)
Average Dense Area (cm^2)				
No Endometriosis	75.12 +/- 48.91	0 (ref)	0 (ref)	0 (ref)
Endometriosis	73.86 +/-39.77	-4.02 (-14.84,6.80)	-3.60 (-14.27,7.07)	-2.71(-15.34,9.93)
Average Non-Dense Area (cm^2)				
No Endometriosis	167.97+/-84.81	0 (ref)	0 (ref)	0 (ref)
Endometriosis	167.34 +/- 79.31	4.28 (-12.93, 21.50)	4.41 (-13.88, 22.70)	3.38 (-15.82, 22.57)

Model 1 Adjusted for age and BMI at mammogram
Model 2 Additionally adjusted for alcohol consumption, family history of breast cancer, smoking history, history of benign breast disease, bmi at age 18, parity, age at menarche, oral contraceptive use history, and breastfeeding history
Model 3 a:Pre-menopausal additionally adjusted for hysterectomy, postmenopausal hormone use
a:Post-menopausal additionally adjusted for hysterectomy, oophorectomy, and postmenopausal hormone use

directions of percent mammographic density in pre- and post-menopausal women the effect to endometriosis on percent mammographic density varied by menopausal status (P-value test for interaction: 0.02). Sensitivity analyses predating onset of endometriosis diagnosis did not substantially alter the results (results not shown).

When assessing effect modification of the relationship between endometriosis and mammographic density among premenopausal women, we found significant heterogeneity by BMI at age 18 (P-value test for interaction: 0.03), but not by current BMI (i.e., near time of mammogram) (P-value test for interaction: 0.31) (**Table 3.3**). Among women who were lean at age 18 (BMI < 20.6, the median value in the population), those with endometriosis had significantly higher percent mammographic density in models adjusted for age and current BMI (4.44 percentage points difference, 95% CI: 0.12, 8.76) compared to those without endometriosis, which was attenuated slightly in fully adjusted models (3.74 percentage points difference, 95% CI: -0.29, 7.78).

Discussion

In this study nested within the NHSII, endometriosis was not found to be associated with average percent mammographic density, dense area, or non-dense area in premenopausal or postmenopausal women overall. However, the relationship between endometriosis and percent mammographic density in premenopausal women was modified by BMI at age 18. Among women who were lean at age 18, those with endometriosis had moderately higher percent mammographic density later in life, compared to those without endometriosis.

Table 3.3: The association between endometriosis and average percent mammographic density among pre-menopausal women at time of mammogram in the Nurses' Health Study II stratified by covariates of interest

	N (%)	Model 1	Model 2	Model 3	P-value[1]
		Difference (95% CI)			
BMI 18<20.6[2]	614	4.44(0.12, 8.76)	3.68(-0.35, 7.71)	3.74(-0.29, 7.78)	0.03
BMI 1 ≥ 20.6	616	-0.8 (-5.50, 3.77)	-0.07(-4.85,4.71)	-0.21(-5.09, 4.67)	
BMI < 25	670	2.04 (-1.82, 5.91)	1.69(-2.07, 5.46)	1.79 (-1.98, 5.57)	0.31
BMI ≥ 25	523	2.89 (-2.66, 8.44)	3.46(-1.86, 8.77)	2.59(-3.05, 8.23)	

Model 1 Adjusted for age and BMI at mammogram
Model 2 Additionally adjusted for alcohol consumption, family history of breast cancer, smoking history, history of benign breast disease, bmi at age 18, parity, age at menarche, oral contraceptive use history, breast feeding history,
Model 3 Additionally adjusted for hysterectomy, postmenopausal hormone use
1 Test for heterogeneity
[2] Median value

To our knowledge, this study is the first investigation of endometriosis and mammographic density to date. Women with endometriosis were not found to have altered mammographic density compared to women without endometriosis. Thus, if endometriosis is associated with increased risk of breast cancer, our data suggest that the relationship may not be mediated through mammographic density. In this cohort, we previously found that while women with endometriosis were not at increased risk for overall breast cancer, they were at an increased risk for estrogen receptor positive, progesterone receptor negative (ER+/PR-) tumors. A recent pooled analysis has found that the effect of mammographic density on breast cancer risk did not vary by tumor PR status, but did appear stronger among ER- compared to ER+ tumors in women <55 years old (0.04)[39].

Early life body size has been inversely associated with endometriosis[40-42], mammographic density [13, 43, 44], and breast cancer [44, 45]. In *a priori* sensitivity analyses, we found that among premenopausal women who were lean at age 18, those with endometriosis had higher mammographic density than those without endometriosis in models adjusted for age and BMI (4.4% difference), although our findings were attenuated slightly in fully adjusted models (3.7% difference). This finding is striking given the large magnitude of effect compared to other reproductive risk factors. For example, it is estimated that each pregnancy decreases average percent mammographic density by 2% and exogenous hormone therapy usage increases mammographic density by 3.1-4.8%[6, 46]. Additionally, women who are lean at age 18 already have higher mammographic density than overweight women, putting them at higher risk of breast cancer. Samimi et al. reported that higher mammographic density in those with lean body size at age 18, independent of adult body fatness, was estimated to correspond to a 5-15% increased risk of breast cancer[13].

While the prospective cohort design of our study allowed for detailed, updated information on exposure and covariate status, the mammographic density measurements are from one cross-sectional point in time and thus may not accurately capture density across the life course. As breast density is known to change as women age, all analyses were adjusted for age and stratified by menopausal status. Due to a potential delay between symptom onset and endometriosis diagnosis, some members of our cohort may have asymptomatic endometriosis or endometriosis that has not yet been diagnosed at time of mammogram. However, since the prevalence of endometriosis is ~10%, the inclusion of undiagnosed endometriosis cases in the unexposed group would have a small effect, given the number of truly unexposed women[47] and while this misclassification still may bias our estimates, the bias would most likely be non-differential in relation to mammographic density and thus attenuate our findings toward the null. In sensitivity analyses, we investigated this potential diagnostic delay by predating endometriosis exposure, which did not substantially alter our results. As discussed previously, hormonal treatments for endometriosis may influence mammographic density, and thus were conceptualized not as traditional confounders but rather as potential mediators. While our participants contributed information on hormone therapy, we did not have sufficiently detailed information on other hormonal treatments which are sometimes used for endometriosis such as Danazol and Lupron. Lupron has been shown to reduce mammographic density and has also been used as a breast cancer treatment [48, 49]. Thus, there may be unmeasured confounding or mediation by these hormonal treatments, which would lead to an underestimation of the association between endometriosis and mammographic density, if a true relationship exists. However, these treatments were more commonly used early in cohort follow-up and sensitivity analyses restricting incident endometriosis after 1989 did not alter results. Furthermore, due to

the limited number of endometriosis cases, we may not have been adequately powered to detect modest differences in mammographic density.

This investigation into endometriosis and mammographic density is the first study to investigate this relationship. It has many strengths including its validated exposure definition of laparoscopically confirmed endometriosis and validated quantitative outcome assessment of percent and absolute mammographic density from screening mammograms with high intra-reader reliability. We were also able to adjust for potentially important confounding and mediating factors of the endometriosis and mammographic density relationship.

Mammographic density is an important risk factor for breast cancer and public awareness is growing as more patients are informed of their mammographic density after receiving a mammogram. In this study, endometriosis was not significantly associated with mammographic density, suggesting that if endometriosis increases breast cancer risk, it may not be mediated through breast density. Future research could focus on replicating our finding among women lean women at age 18, among whom a consistent association with high mammographic density, endometriosis, and risk of breast cancer has been found [42, 44, 50]. However, overall women with endometriosis do not appear to have greater mammographic density.

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Aim 4: Breastfeeding History and Risk of Endometriosis in the Nurses' Health Study II

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Abstract:

Background: Endometriosis is a chronic gynecologic condition with few known modifiable risk factors and a suspected hormonal etiology. Breastfeeding has been shown to mitigate risk of other chronic diseases that are hypothesized to be influenced by circulating hormones. We investigated the association between breastfeeding and incidence of endometriosis in the Nurses' Health Study II.

Methods: From 1989 until 2011, 67,610 parous women were followed, among whom 3,741 laparoscopically confirmed endometriosis cases were diagnosed. Women reported duration of total breastfeeding, exclusive breastfeeding, and amenorrhea for each pregnancy. Cox proportional hazard models, adjusted *a priori* for potential confounding factors were used to calculate hazard ratios (HR) and 95% confidence intervals (CI) of endometriosis.

Results: History of total and exclusive breastfeeding duration were significantly associated with decreased risk of endometriosis. For every three additional months of total breastfeeding per pregnancy, women experienced an 8% lower risk of endometriosis (HR:0.92, CI:0.90-0.94; P-trend<0.0001) and a 14% lower risk for every three additional months of exclusive breastfeeding (HR: 0.86, CI: 0.82-0.90; P-trend<0.0001). Parous women who never breastfed were at 1.6-fold higher risk of endometriosis compared to women who breastfed for $\geq$ 36 months (HR:1.64, CI: 1.38-1.97).The magnitude of the effect of breastfeeding appeared strongest among women who gave birth within the last 5 years (P-value, interaction: 0.04), however the protective association was consistently significant across all groups of women.

Conclusion: We found that among parous women, breastfeeding was inversely associated with risk of endometriosis. Given the chronic and incurable nature of endometriosis, breastfeeding should be further investigated as an important modifiable behavior to mitigate risk.

Introduction

Endometriosis is a chronic gynecologic disorder affecting approximately 10% of women in the United States[1, 2]. Its symptoms include chronic fatigue, chronic pelvic pain, dysmenorrhea (painful periods), dyspareunia (painful intercourse), dysuria (painful urination) and dyschezia (painful defecation)[3, 4] and it has no known cure. Endometriosis lesions depend on circulating estrogen for growth and maintenance and it is hypothesized that endometriosis etiology involves retrograde menstruation[3, 5]. While scientists are beginning to understand the risk profile for endometriosis incidence, there are very few known modifiable risk factors for the disease. Breastfeeding may have important implications as a modifiable risk factor for endometriosis.

The nutritional benefits of breastfeeding for infants and the metabolic benefits of breastfeeding for the mother are well known[6, 7]. The World Health Organization (WHO) and the American Academy of Pediatrics recommend that women breastfeed each child for 12 months with 6 months of exclusive breastfeeding[7]. Emerging research has found lasting benefits of breastfeeding for long-term maternal health including aiding in weight loss and reducing risk of chronic disease, including breast cancer and ovarian cancer[6, 8, 9]. Breastfeeding may alter maternal disease risk by prolonging amenorrhea, promoting circulating levels of oxytocin and prolactin, and inhibiting circulating gonadotropins[10].

Despite the plausible mechanism for an association between breastfeeding and endometriosis, research on this topic is restricted to two studies, one previously conducted by our team in this cohort. Previous research has been limited with crude, cross-sectional measures of breastfeeding duration, small sample size, and short follow-up[11, 12]. Our current analysis followed women for over twenty years, fourteen additional years from the previous study, and leverages

detailed lifetime breastfeeding history applied to each pregnancy to investigate more thoroughly the relationship between the duration of total breastfeeding, as well exclusive breastfeeding and postpartum amenorrhea, and risk of endometriosis. We hypothesized that breastfeeding duration would be protective against risk for endometriosis, with exclusive breastfeeding and postpartum amenorrhea conferring the strongest protective effect on endometriosis risk.

Methods

The Nurses' Health Study II (NHSII) is a prospective cohort study that began in 1989 when 116,430 registered nurses, 25-42 years old, returned a mailed questionnaire on their health and lifestyle. Follow-up questionnaires that collect information on environmental, dietary, and lifestyle risk factors, have been sent biennially with cumulative follow-up rates $\geq$ 90%. The study was approved by the Institutional Review Board of Brigham and Women's Hospital.

Exposure Definition

Since 1989, participants have reported all pregnancies (lasting $\geq$ 6 months) every two years. In 1993, women were asked about lifetime breastfeeding history. In 1997, women were asked detailed information on their breastfeeding history and duration for each of their first four children, as described in detail previously[13]. Women with more than four children were asked to report combined breastfeeding information for each additional child. A supplementary questionnaire was sent to women reporting pregnancies subsequent to 1997 so that breastfeeding duration information could be collected through 2003 (at which point the youngest participant was 43). Combining detailed child-specific information on breastfeeding duration with the annual information on pregnancy history allows us to update breastfeeding history over time.

To estimate total breastfeeding duration, women were asked, "If you breastfed, at what month did you stop breastfeeding altogether?" and were given the following categories: "1-2 months, 3-5 months, 6-8 months, 9-11 months, 12-18 months, 19+ months". To approximate exclusive breastfeeding duration, women were asked "At what month did you start giving formula or purchased milk at least once daily?" and "At what month did you start giving solid food at least once daily (baby food, cereal, table food, etc)?" and could respond "0-2 months, 3 months, 4-5 months, 6-7 months, 8-11 months, 12+ months". We defined exclusive breastfeeding duration as the earlier of these two time points. Lastly, to quantify postpartum amenorrhea, women were asked "At what month after delivery did your menstrual periods return?" and were given the following categories: "1-2 months, 3-5 months, 6-9 months, 10+ months, pregnant again, or never." Our derived breastfeeding and amenorrhea exposures were updated every 2 years and were summed across reproductive history.

Outcome definition

We defined our outcome as laparoscopically confirmed endometriosis. On the 1993 questionnaire, women were first asked if they had ever had "physician diagnosed endometriosis." If they answered "yes," they were asked to report when the diagnosis had occurred and if their disease had been confirmed by laparoscopy. Endometriosis diagnosis was assessed on every subsequent questionnaire since 1993.

Self-reported endometriosis was validated previously among 200 participants using medical records[11]. Among women who reported laparoscopic confirmation, a laparoscopic diagnosis of endometriosis was confirmed in 96% of medical records. Conversely, among those women without laparoscopic confirmation, evidence of clinical diagnosis was found in only 54%

of medical records. Thus, to reduce the magnitude of misclassification, endometriosis cases were restricted to those women with laparoscopic confirmation. Women who reported endometriosis diagnosis without laparoscopic confirmation who then later had a confirmation by laparoscopy were assigned to the endometriosis case group at the time of the initial clinical diagnosis. Those who reported endometriosis diagnosis but never received laparoscopic confirmation were censored at the report of clinical diagnosis.

Study Population

Cohort participants were included in this analysis population from first report of pregnancy (lasting 6 months or greater) through the 2011 questionnaire cycle. We excluded women who reported endometriosis diagnosis or cancer (other than non-melanoma skin cancer) prior to June 1989. Person-months at risk were calculated from entry into the cohort until confirmed i) death, ii) cancer (other than non-melanoma skin cancer), iii) postmenopausal status, or iv) laparoscopically confirmed endometriosis. Because breastfeeding history was last collected in 2003, we did not include women who reported first pregnancy after 2003 (n = 108).

Statistical Analyses

Cox proportional hazard models, stratified by calendar time with age (months) as the time metameter, were used to calculate the Hazard Ratios (HR) and 95% confidence intervals (CI) of endometriosis. The proportional hazard assumption was assessed using a likelihood ratio test for the interaction between exposure and age (months); the assumption was not violated ($p>0.05$). *A priori* hypothesized risk factors for endometriosis were adjusted for and time-varying covariates were updated biennially at every questionnaire cycle: current BMI (<18.5, 1.5-22.4, 22.5-24.9, 25-29.9, $\geq$ 30 kg/m^2), BMI at age 18 (<18.5, 1.5-22.4, 22.5-24.9, 25-29.9, $\geq$ 30 kg/m^2), smoking

history (never, former, current), oral contraceptive use (never, former, current), parity (1, 2, 3+ pregnancies), age at menarche (<12, 12, 13, 14, > 14 years), history of infertility (unable to get pregnant for ≥ 12 months), and time since last birth (<5, 5-10, >10 years).

We examined the possibly non-linear relation between total breastfeeding and exclusive breastfeeding and the relative risk of endometriosis non-parametrically with restricted cubic splines[14] .Tests for non-linearity used the likelihood ratio test, comparing the model with only the linear term to the model with the linear and the cubic spline terms.

Sensitivity Analyses

Previous analyses in this cohort reported a stronger magnitude of association of breastfeeding on endometriosis within five years of pregnancy[11], thus we stratified our analysis by time since last reported birth (≤5, >5 years). Additionally, we assessed whether the association of breastfeeding on endometriosis risk differed by age at first birth (<30, 30-35, >35 years), history of infertility (ever attempting to conceive for ≥ 12 months without success), parity (1, 2, 3+ pregnancies), and BMI (<18.5, 1.5-22.4, 22.5-24.9, 25-29.9, ≥ 30 kg/m^2). In studies of endometriosis, there is concern about a lengthy diagnostic delay between symptom onset and surgical diagnosis. In the NHSII, the average diagnostic delay from symptom onset to surgical diagnosis was approximately 4 years[11], while international multicenter studies have observed an average delay of 7 years[15]. To investigate the effect of this temporal misclassification of exposure, we set the diagnostic date of endometriosis earlier by 2, 4, and 6 years.

Amenorrhea is inversely associated with risk of endometriosis[11, 16] and amenorrhea postpartum can be influenced by duration and intensity of breastfeeding[6, 17]. To further elucidate how the components of breastfeeding influence endometriosis risk, we considered postpartum

amenorrhea a potential mediator of breastfeeding and endometriosis risk. We calculated the percentage and 95% confidence interval of the total breastfeeding and exclusive breastfeeding effect estimates that were mediated by postpartum amenorrhea[18, 19].

Results

At baseline in 1989, women who reported longer duration of total breastfeeding tended to be older, multiparous, and to have never smoked or used oral contraceptives (**Table 4.1**). Women who reported longer duration of total breastfeeding were less likely to be currently overweight or obese, overweight or obese at age 18, and to report earlier age at menarche. From 1989 until return of the 2011 questionnaire, 67,610 parous women were followed among whom 3,741 laparoscopically confirmed endometriosis cases were diagnosed.

Compared to women who breastfed $\geq$ 36 months, parous women who breastfed for <1 month were at a 1.6-fold higher risk of endometriosis (HR:1.64, 95% CI: 1.38-1.97). We observed a statistically significant inverse relationship between total breastfeeding duration and risk of endometriosis (p-value, test for linear trend <0.0001). For every additional 3-months of total breastfeeding among women, we observed a 3% reduction in endometriosis risk (3-month increase HR: 0.97, 95% CI: 0.96-0.98) (**Table 4.2, Figure 4.1**).

Compared to women who breastfed exclusively for eighteen months or more, women who did not exclusively breastfeed had nearly a 40% higher risk for endometriosis (HR:1.37, 95% CI:1.09-1.71). In models using restricted cubic splines, the relationship between exclusive breastfeeding and endometriosis appeared non-linear (P-value, test for non-linearity: 0.01) but

Table 4.1 Characteristics of parous women in the Nurses' Health Study II at baseline by selected categories of breastfeeding status[1]

	Total months of reported breastfeeding			
	<1 (n=11,994)	6-<12 (n=12,434)	18-<24 (n=5,838)	>=36 (n=4,085)
	Mean (SD)			
Age*	35.7(4.6)	34.3(4.4)	35.3(3.9)	36.2(3.5)
Parity	1.8(0.8)	1.8(0.7)	2.4(0.8)	3.3(0.9)
	%			
Parity group				
- One, %	38	33	12	0.1
- Two, %	47	53	48	17
- ≥ Three, %	15	14	40	83
Smoking Status				
- Never, %	62	65	68	75
- Past, %	21	24	24	20
- Current, %	17	12	8	5
Oral Contraceptive Use				
- Never, %	12	13	17	25
- Past, %	75	77	77	72
- Current, %	13	10	6	3
Body Mass Index (BMI) (kg/m2)				
- <18.5, %	3	3	3	3
- 18.5-<22.5, %	39	44	47	47
- 22.5-<25.0, %	23	23	23	23
- 25.0-<30.0, %	21	19	18	18
- ≥30.0, %	14	10	9	8
Body Mass Index at age 18				
- <18.5, %	15	14	15	13
- 18.5-<22.5, %	59	64	67	67
- 22.5-<25.0, %	15	14	13	14
- 25.0-<30.0, %	9	6	5	5
- ≥30.0, %	3	2	1	1
Age at Menarche				
- <12 years, %	26	23	23	22
- 12-13 years, %	31	31	30	32
- ≥14 years, %	44	46	47	46

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

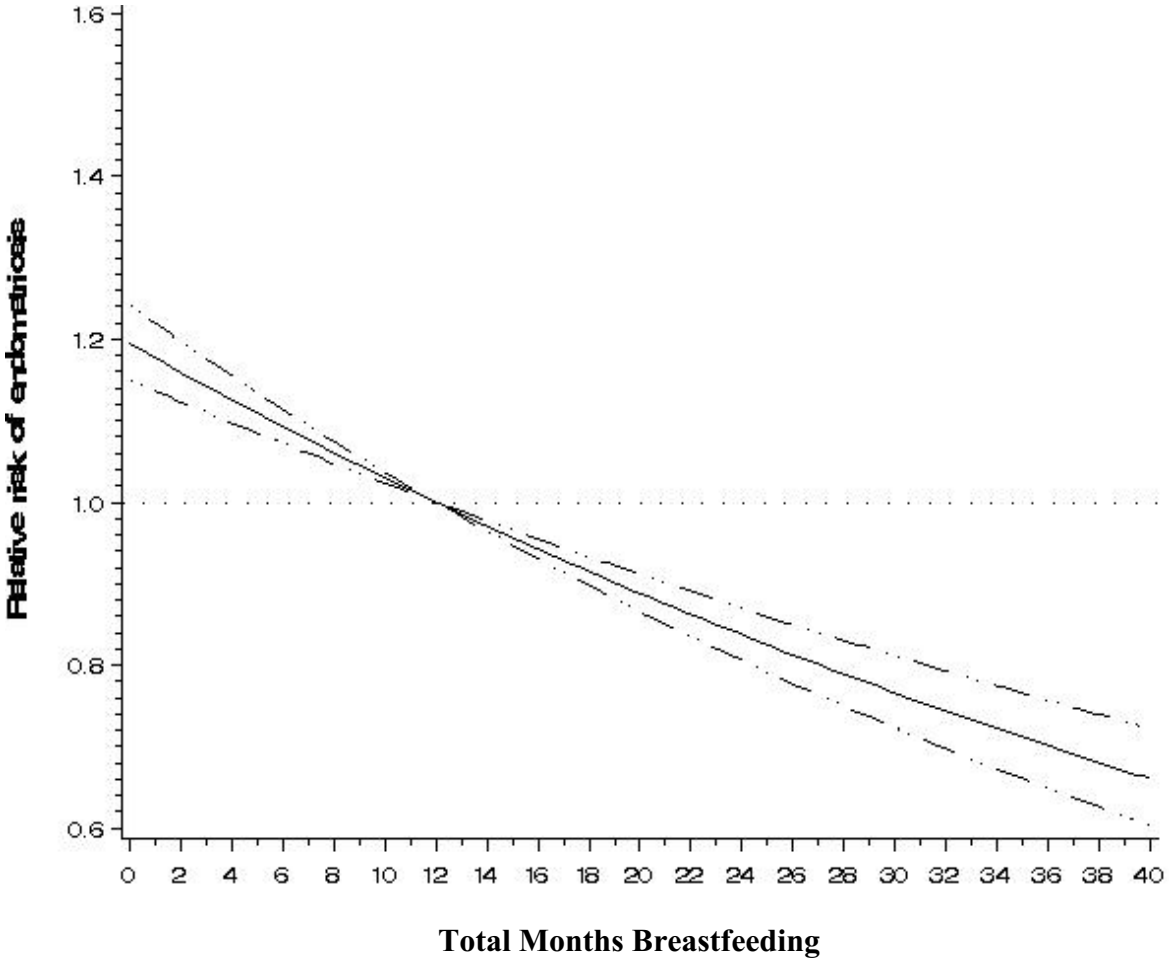
[1] This distribution represents women who reported parity in 1989, which is approximately 89% of women in our study across follow-up

Table 4.2. The relative risk of laparoscopically confirmed endometriosis among parous women by breastfeeding history in the Nurses' Health Study II

Breastfeeding Classification	Cases/Person year	Age and calendar time adjusted[1]	Multivariable adjusted[2]
		HR 95% CI	
Total Breastfeeding[3]			
<1 month	740 /163,381	1.40 (1.24-1.57)	1.25 (1.11-1.41)
1-<3 months	278 /58,655	1.44 (1.24-1.68)	1.30 (1.11-1.51)
3-<6 months	364/92,570	1.21 (1.05-1.39)	1.08 (0.94-1.24)
6-<12 months	644/187,910	1.07 (0.95-1.20)	1.00 (0.89-1.13)
12-<18 months (ref)	456/ 142,668	1.00 (ref)	1.00 (ref)
18-<24 months	291/106,221	0.87 (0.75-1.00)	0.93(0.80-1.08)
24 -<36 months	314/ 130,855	0.76 (0.66-0.88)	0.85 (0.73-0.98)
≥ 36 months	167/90,929	0.60 (0.50-0.71)	0.76 (0.63-0.93)
3 month increase			
P-value, test for linear trend		0.94 (0.93-0.95) <0.0001	0.97 (0.96-0.98) <0.0001
Exclusive Breastfeeding[4]			
<1 month	1,437/348,287	1.40 (1.27-1.54)	1.25 (1.13-1.38)
1-<3 months	358/98,291	1.23 (1.07-1.40)	1.11 (0.97-1.26)
3-<6 months	364/120,233	1.03 0.91-1.18)	0.96 (0.84-1.10)
6-<12 months (ref)	594/205,202	1.00 (ref)	1.00 (ref)
12-<18 months	221/96,770	0.81 (0.69-0.94)	0.94 (0.80-1.10)
≥ 18 months	87/43,790	0.71 (0.57-0.89)	0.91(0.73-1.15)
P-value, test for overall significance of the curve[5]		<0.0001	<0.0001

1: Stratified jointly by age and calendar time (months)
2: Model 1 and additionally adjusted for current BMI, BMI at age 18, smoking history, oral contraceptive use history, parity, age at menarche, infertility history, and time since last birth
3: Total breastfeeding derived from question "If you breastfed, at what month did you stop breastfeeding altogether?"
4: Exclusive breastfeeding derived from the earlier time of questions "At what month did you start giving formula or purchased milk at least once daily?" and "At what month did you start giving solid food at least once daily (baby food, cereal, table food, etc)?"
5:Test for non-linear trend: 0.01

Figure 4.1: Relative risk of endometriosis by total breastfeeding history



Test for non-linearity: 0.07
Test for linear trend: < 0.0001

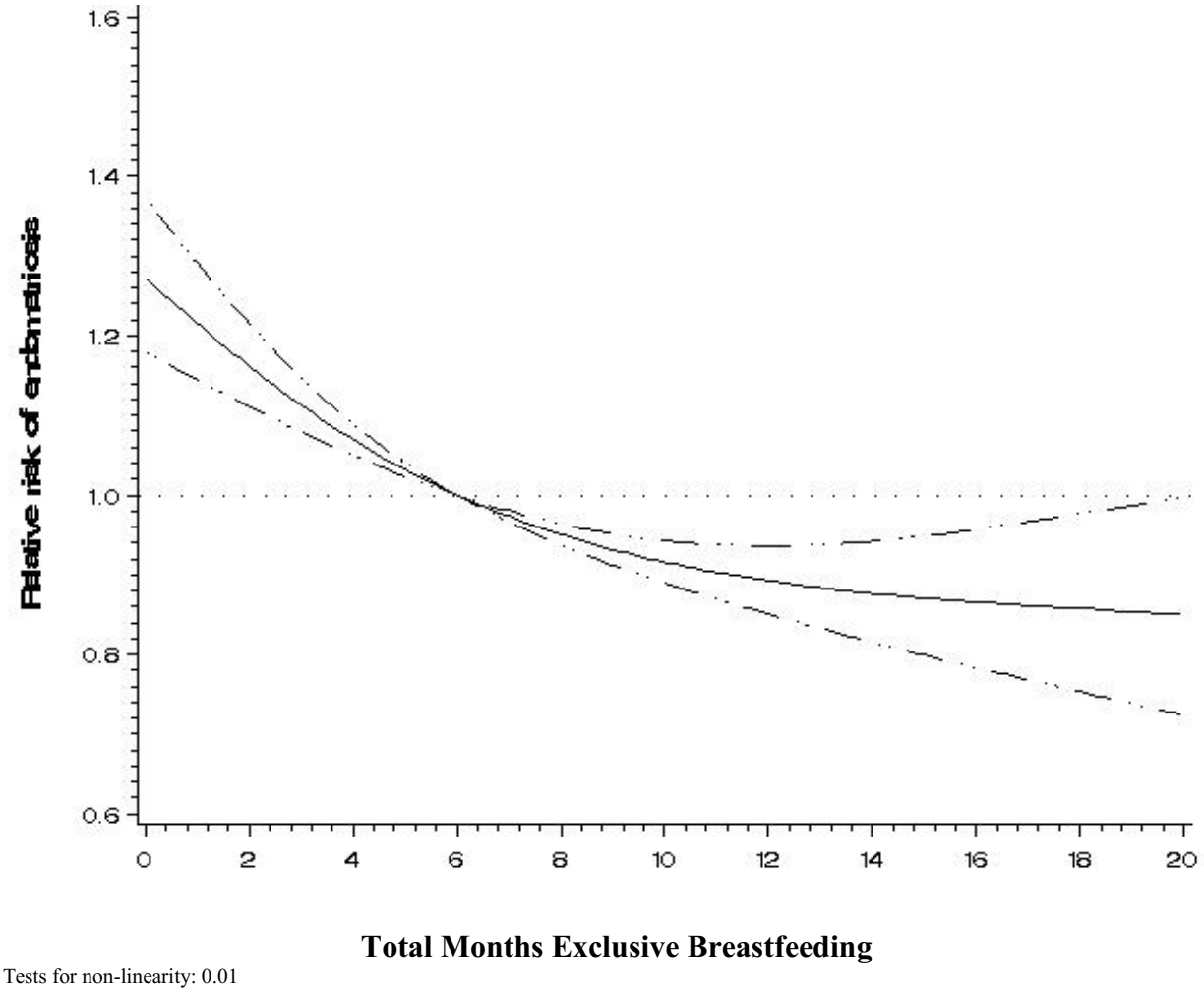
remained statistically significant: (P-value, test for overall significance of the curve:0.001) (**Figure 4.2**).

We then evaluated the pregnancy-specific impact of breastfeeding. For each pregnancy, a 3-month increase in average total breastfeeding duration was associated with an 8% reduction in endometriosis risk (HR: 0.92; 95% CI: 0.90-0.95) (P-value, test for linear trend <0.0001) (**Table 4.3**). For each pregnancy, a 3-month increase in average exclusive breastfeeding duration per pregnancy was associated with a 14% decreased risk of endometriosis (HR: 0.86, 95% CI: 0.82-0.90) (P-value, test for trend: <0.0001).

Postpartum amenorrhea was also inversely associated with endometriosis risk. Compared to women who had experienced amenorrhea postpartum for 6-12 months, parous women who experienced <1 month of amenorrhea had an increased risk of endometriosis (HR:1.22, 95% CI: 1.09-1.35) (P-value, test for linear trend <0.0001) (**Table 4.4**). Postpartum amenorrhea was a significant mediator of the effect of total breastfeeding on endometriosis (% mediated: 31%, 95% CI: 12-58%) and of the effect of exclusive breastfeeding on endometriosis risk, assuming a linear relationship between exclusive breastfeeding and endometriosis (% mediated: 50%, 95% CI: 23-76%). However, even after accounting for postpartum amenorrhea, both total breastfeeding (RR per 3-month increase: 0.98, 95% CI: 0.96-0.99) and exclusive breastfeeding (RR per 3-month increase: 0.97, 95%CI: 0.94-1.00) remained associated with a decreased risk for endometriosis.

While the inverse association of breastfeeding on endometriosis risk was stronger among women who gave birth within the last 5 years (P-value, test for interaction: 0.04) (HR per 3-month increase: 0.95, 95% CI: 0.93-0.97), the association was attenuated but remained significant even after five years since last birth (HR per 3-month increase: 0.98, 95% CI: 0.96-

Figure 4.2: Relative risk of endometriosis by exclusive breastfeeding history



Tests for non-linearity: 0.01
Test for non-linear trend: < 0.0001

Table 4.3: The relative risk of laparoscopically confirmed endometriosis among parous women by breastfeeding history per pregnancy in the Nurses' Health Study II

Breastfeeding Classification	Cases/Person year	Age and calendar time adjusted[1]	Multivariable adjusted[2]
		HR (95% CI)	
Average total breastfeeding duration per pregnancy			
<1 month	801 / 181,739	1.69 (1.50-1.91)	1.47 (1.30-1.66)
1-<3 months	421/ 112,388	1.42 (1.24-1.63)	1.34 (1.17-1.54)
3-<6 months	646 /198,410	1.27 (1.12-1.43)	1.21 (1.07-1.37)
6-<12 months	817/ 293,141	1.09 (0.97-1.23)	1.09 (0.96-1.22)
≥ 12 months (ref)	412/162,328	1.00 (ref)	1.00 (ref)
3 month increase		0.89 (0.87-0.91)	0.92 (0.90-0.94)
P-value, test for trend		<0.0001	<0.0001
Average exclusive breastfeeding duration per pregnancy			
<1 month	1409 /346,874	1.43 (1.24-1.63)	1.33 (1.16-1.53)
1-<3 months	674 /232,930	1.03 (0.89-1.19)	1.07 (0.93-1.24)
3-<6 months	609/ 226,048	0.97 (0.83-1.12)	1.02 (0.88-1.19)
≥ 6 months (ref)	246 /88,452	1.00 (ref)	1.00 (ref)
3 month increase		0.89 (0.85-0.93)	0.86 (0.82-0.90)
P-value, test for trend		<0.0001	<0.0001

1: Stratified jointly by age and calendar time (months)
2: Model 1 and additionally adjusted for current BMI, BMI at age 18, smoking history, oral contraceptive use history, parity, age at menarche, infertility history, and time since last birth

Table 4.4: The relative risk of laparoscopically confirmed endometriosis among parous women by Postpartum amenorrhea history in the Nurses' Health Study II

Postpartum Amenorrhea[1]	Cases/Person year	Age and calendar time adjusted[2] HR (95% CI)	Multivariable adjusted[3]
<1 month	707/158,078	1.30 (1.17-1.44)	1.22 (1.09-1.35)
1-3 months	200/37378	1.60 (1.35-1.85)	1.31 (1.11-1.56)
3-6 months	709/191199	1.09 (0.98-1.20)	1.06 (0.96-1.18)
6 -12 months (ref)	748/220236	1.00 (ref)	1.00 (ref)
12-18 months	361 /153091	0.70 (0.62-0.80)	0.80 (0.71-0.92)
≥ 18 months	262/137267	0.60 (0.50-0.67)	0.72 (0.62-0.84)
3 months increase		0.90 (0.88-0.92)	0.95 (0.93-0.96)
P-value, test for trend		<0.0001	<0.0001

1: Derived from question "About what month after delivery did your menstrual periods start returning?"
2: Stratified jointly by age and calendar time (months)
3: Model 1 and additionally adjusted for current BMI, BMI at age 18, smoking history, oral contraceptive use history, parity, age at menarche, secondary infertility, and time since last birth

0.99). In multivariate adjusted models, we found no effect modification by BMI, age at first birth, parity, or infertility status. In sensitivity analyses predating endometriosis diagnosis by 2, 4, and 6 years, the overall patterns between breastfeeding and endometriosis did not vary (results not shown).

Discussion

We observed that breastfeeding overall, as well as exclusive breastfeeding, were associated with lower risk of endometriosis. Approximately 30% of the inverse association of total breastfeeding duration on endometriosis could be attributed to postpartum amenorrhea. For each pregnancy, women who did not breastfeed were at a significant increased risk of endometriosis compare to women who followed WHO and American Academy of Pediatrics breastfeeding guidelines of breastfeeding for at least 12 months. While we observed heterogeneity in the strength of the association by time since last birth, breastfeeding consistently was protective in all groups of women.

Our findings confirm previous reports of an inverse relationship between breastfeeding and endometriosis risk[11, 12]. In previous research within our cohort, with six-years of follow-up and with 448 incident cases, we reported a significant relationship between a one-time measure of lifetime duration of breastfeeding and endometriosis (P-value linear trend: 0.008)[11]. Women who reported breastfeeding for > 23 months were at significantly decreased risk of endometriosis compared to women who reported never breastfeeding (RR: 0.7, 95% CI: 0.5-1.0). However we found that the inverse relationship was only evident within 5 years of last birth (P-value test for

heterogeneity: <0.001). Heilier et. al. also found a protective association of "ever" vs. "never" breastfeeding in a case-control setting, however their sample size was limited (88 endometriosis cases) and they did not adjust for parity or other confounding factors[12]. While these earlier investigations used crude, cross-sectional measures of lifetime breastfeeding and had limited endometriosis cases, our current analysis incorporated detailed time-varying information on breastfeeding for each individual pregnancy and had over twenty years of follow-up for this large cohort of women (with 3,741 incident endometriosis cases). We also expanded the definition of breastfeeding to investigate total and exclusive breastfeeding, as well as adding an investigation of the impact of postpartum amenorrhea.

We found that total breastfeeding and exclusive breastfeeding duration were protective for risk of endometriosis among parous women, with a statistically significant proportion of the reduction in risk mediated by postpartum amenorrhea (30% and 50%, respectively). The most common and supported etiologic theory for endometriosis onset, "Sampson's theory," posits that endometriosis is caused or at least initiated by retrograde menstruation[5]. Thus, the more menstrual periods a woman experiences, the greater her exposure to retrograde menstruation, and thus greater the risk for developing endometriosis. Our analysis lends support to this theory and found that longer duration of postpartum amenorrhea significantly decreased risk of endometriosis and was an important mediator of total and exclusive breastfeeding.

However, it is estimated that 90% of women experience retrograde menstruation[20], which suggests that the true differences between women with and without endometriosis may be caused by factors that influence adherence, proliferation, and maintenance of the cells and lesions. Alternatively, there may be a component of the pathways that initiate and support the

return of menses postpartum that are also implicated in the pathophysiology of endometriosis. These pathways may or may not overlap with the well established relationship between earlier age at menarche and increased risk of endometriosis[11, 21].

The majority of the inverse relationship of total and exclusive breastfeeding on risk of endometriosis could not be explained by amenorrhea. Breastfeeding is known to increase circulating levels of oxytocin and inhibit circulating levels of gonadotropin-releasing hormone, luteinizing hormone, and follicle stimulating hormone[10, 17]. Oxytocin receptors are expressed in endometriosis lesions[22], and rat models have found oxytocin injections can lead to decreased endometriosis lesion volume and VEGF expression[23], which has been hypothesized to contribute to the angiogenesis of endometriosis lesions. Gonadotropin agonists are also commonly utilized treatments for endometriosis[24]. However, little is known regarding the duration of hormonal and inflammatory changes once breastfeeding ceases. Nevertheless, breastfeeding conferring protection against endometriosis risk has biologic plausibility. Moreover, breastfeeding is hypothesized to influence risk of other chronic diseases including, breast cancer[6, 8, 9], ovarian cancer[6], and type-two diabetes[6, 13] through similar mechanisms of altering circulating hormones and prolonging amenorrhea[10].

Despite the strengths of our investigation, our findings also have some limitations. Misclassification of breastfeeding history is possible, as women were asked retrospectively to report their breastfeeding experience. Previous research indicates that women can accurately report their breastfeeding for up to twenty years after index birth ($r=0.82$)[25], which implies that the likelihood for misclassification from recall is minimal. Additionally, if women reported more than four pregnancies in our questionnaire, they were asked to sum lactation duration across

pregnancies ≥5 (2.8% of pregnancies in 2003), which may lead to misclassification. However, we would expect the effect of any misclassification of breastfeeding to be non-differential with respect to the outcome, endometriosis, and thus the bias would cause an underestimate of any true association.

Due to a potential diagnostic delay in endometriosis diagnosis, some members of our cohort may have asymptomatic disease or disease that has not yet been diagnosed. Since the prevalence of endometriosis is believed to be ~10%, the inclusion of undiagnosed endometriosis cases in the non-case group (~62,000) would likely have a limited effect[26] among the large truly non-case women in this cohort. While this misclassification still may bias our estimates, the bias is likely non-differential with respect to breastfeeding duration and would most likely attenuate our findings. In addition to an erroneous lack of diagnosis, a diagnostic delay in endometriosis was explored more thoroughly through the sensitivity analyses that predated endometriosis diagnosis by 2, 4, and 6 years. This did not significantly alter our results.

To our knowledge, this is the largest study to prospectively estimate the association of breastfeeding and the first study to estimate the components of breastfeeding on endometriosis risk. With over twenty-years of follow-up and validated measurement of endometriosis, our study is well powered to estimate risk. Additionally, with its time-varying and detailed estimates of breastfeeding duration across the entire reproductive history, as well as detailed information on postpartum amenorrhea, our study allowed for a clearer understanding of the public health importance of breastfeeding as a modifiable risk factor for endometriosis risk.

Endometriosis is a chronic disease with no known cure and debilitating symptomatology. At present, very few modifiable risk factors are known to prevent its occurrence. Future research

should investigate breastfeeding as mechanism for symptom mitigation among women diagnosed with endometriosis. The strong inverse relationship we found between breastfeeding duration and endometriosis risk may have important clinical implications for advising women to modify their endometriosis risk profile. Our findings lend support to the body of public health and policy literature that advocates for the promotion of breastfeeding. All pregnant women should be counseled regarding the health benefits of breastfeeding for both the mother and child.

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