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Phytohemagglutinin-Induced Mitotic Index in Blood Lymphocytes: A Potential Biomarker for Breast Cancer Risk

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Abstract

Background: Cell proliferation is associated with the pathogenesis of cancer because it provides opportunities for accumulating genetic mutations. However, biomarkers of cell proliferation in response to environmental stimuli have not been adequately explored for breast cancer risk.

Methods: In a case-control study of 200 breast cancer patients and 360 healthy controls, we investigated the association between phytohemagglutinin (PHA)-induced mitotic index in blood lymphocyte and breast cancer risk.

Results: Having high mitotic index ($>3.19\%$) was associated with an increased risk of breast cancer, with adjusted odds ratios (95% confidence interval) of 1.54 (1.03–2.30) and 2.03 (1.18–3.57) for all women and post-menopausal women, respectively. Mitotic index was correlated with some reproductive factors and body mass index in controls.

Conclusions: Our data suggest increased PHA-induced mitotic index in blood lymphocytes is associated with an increased breast cancer risk and that this association may be modulated by reproductive and other hormones.

Keywords: breast cancer, mitogens, mitotic index, biomarker, cell proliferation, lymphocytes

Introduction

Breast cancer is the most common malignancy[1] and the second leading cause of cancer-related death in women.[2,3] In the United States, over the last two decades the annual incidence of breast cancer has been increasing[4] while survival rates of patients with early breast cancer have improved steadily due to early diagnosis and more effective therapy regimens.[5] Additional gains would come by the development of methods that can predict which individual woman is most likely to develop the disease in the general population, allowing focused prevention on women at the greatest risk. Of the nearly 241,000 women diagnosed each year, about 90% are sporadic cases among women without a significant family history of breast cancer and no other strong identifiable risk factors other than age and reproductive or hormonal risk factors.[6]

Cell proliferation has long been associated with the pathogenesis of cancer because it provides opportunities for the accumulation of genetic mutations. Cells that do not replicate in adults, such as cardiomyocytes, never develop tumors.[7] In contrast, malignant tumors occur frequently in tissues that are characterized by active renewal, such as the epithelium of the mammary gland that undergoes dramatic morphogenetic changes, especially during the reproductive years of a woman.[8] Moreover, hyperproliferative lesions of the breast such as hyperplasia of the ductal epithelium is associated with a 1.5–2 fold increased risk of breast cancer.[9] In the breast, higher cellular proliferative activity within the mammary gland confers a higher susceptibility for transformation by chemical carcinogens.[10] From studying breast biopsy tissues, estrogens appear to trigger breast cell proliferation and estrogens with progesterone together induce more mitoses than estrogens alone.[11] The normal physiology of the female breast is dependent on the proliferative effects of the ovarian hormones; estrogens are primarily responsible for elongation and branching of the breast ducts, whereas progesterone is necessary for the lobular development and maturation.[12]

Proliferation markers, such as Ki-67, mitotic index of tumor cells and cell nuclear antigen (PCNA) expression in breast tumor tissues have been extensively studied among breast cancer survivors and published data were recently reviewed in a meta-analysis.[13]

These markers of cancer tissue proliferation predicted clinical outcomes such as response to medical therapy,[14] risk of relapse,[15] incidence of lymph node metastasis[16] and overall survival of the breast cancer patient.[17] However, these cancer tissue markers are not applicable to assessing cancer risk in the general healthy population. Given the crucial role of cell proliferation in breast carcinogenesis, biomarkers of cell proliferation are potentially valuable tools for breast cancer risk assessment.

Circulating lymphocytes are commonly used surrogate cells to measure the cellular function/events of other healthy somatic cells. Blood lymphocytes are typically resting in G_0 of the cell cycle and can be stimulated by mitogens to divide in vitro. Among the mitogens, phytohemagglutinin (PHA), an extract of red kidney beans, stimulates T-cell (thymus dependent) fraction of lymphocytes while it has little or no affect on the B-cell (bone-marrow-dependent) lymphocyte fraction.[18] Lymphocyte growth rate in response to PHA stimulation varies among healthy individuals.[19] In the context of cancer, there are few studies that demonstrated a reduced mitogenic stimulation of peripheral blood lymphocytes in cancer cases compared to cancer free controls.[20–23] The observed lower mitotic index in blood lymphocytes in response to PHA stimulation in cancer patients has been interpreted as the result of compromised immunocompetence in cancer patients. However, the direct evidence to support this interpretation is weak and PHA-induced mitotic index in blood lymphocytes is likely a complex phenotype partly representing immune function and cell proliferation potentials. In breast cancer, one previous study reported that PHA-induced lymphocyte proliferation was correlated with breast cancer stage and recurrence and that the rate of PHA-induced lymphocyte proliferation was lower in cases than in healthy controls. This study was small (90 cases and 60 controls) and other host or environmental factors were not considered in the analysis.[20] We conducted a case-control study of 200 cases and 360 controls to further understand the relationship between PHA-induced mitotic index in blood lymphocytes and breast cancer risk. To shed some light on potential etiology, we also examined the association between PHA-induced mitotic index in blood lymphocytes and other known breast cancer risk factors.

Materials and Methods

Study population

The study population was described previously.[24] Breast cancer cases (n = 200) were recruited at the Georgetown University Hospital clinics (Lombardi Comprehensive Cancer Center's [LCCC] Division of Medical Oncology, Department of Surgery and the Betty Lou Ourisman Breast Health Clinic). The inclusion criteria for cases included a diagnosis of breast cancer within the prior 6 months, in women who have not yet received chemotherapy and/or radiotherapy and were able to provide informed consent in English. Exclusion criteria included having a prior history of cancer, prior chemotherapy and/or radiation therapy, or an active infection or immunological disorder that required treatment with antibiotics or immunosuppressive medication within one month prior to enrollment. From 2006 through 2008, a total of 254 newly diagnosed breast cancer patients were identified as eligible and 214 (70%) participated in our study. Common reasons for non-participation were: too busy or not interested (21%), overwhelmed by cancer diagnosis (5%), and not responsive to phone call or e-mail contact (4%). Four cases (2%) did not provide a blood sample, 6 blood cultures failed (3%), and blood culture was not performed on 4 blood samples due to holiday schedule when the laboratory was closed (2%). Therefore, the final number of cases with mitotic index data was 200.

Between 2006 and 2008, a total of 380 women who were recruited by random selection from healthy women who visited the mammography screening clinic at Georgetown University Hospital and each donated a blood sample for the lymphocyte culture. The exclusion criteria for healthy women were the same as for cases. Additionally, women who had a breast biopsy within the past 6 months or were currently pregnant or breast feeding were not eligible. The overall participation rate among the eligible women was 60%. The major reasons for non-participation were: being too busy (19%) or not interested (20%). Blood cultures failed in 10 samples (3%), and 10 samples (3%) yielded poor quality slides for mitotic index ascertainment, thus the final number of subjects with mitotic index data was 360.

After providing informed consent, subjects completed a structured, in-person interview assessing prior medical history, tobacco smoke exposures, current medications, family medical history, reproductive history, and socioeconomic characteristics. Trained phlebotomists obtained venous blood using heparinized tubes. The study was approved by the MedStar Research Institute-Georgetown University Oncology Institutional Review Board.

Blood lymphocyte culture and mitotic index ascertainment

Blood cultures were established within 48 hours of blood collection as described previously.[23] Briefly, 1 ml of fresh blood was added to 9 ml of RPMI-1640 medium supplemented with 15% bovine serum, 1.5% phytohemagglutinin (Invitrogen, Rockville, MD), 2 mM L-glutamine and 100 U/ml each of penicillin and streptomycin. To arrest the cells at metaphase, 0.2 μg/ml colcemid was added to the culture 1 hour before harvest. The cells were treated in hypotonic solution (0.06M KCL) and fixed in fixative (methanol:acetic acid = 3:1). The cells were then dropped onto clean microscopic slides, air dried, and stained with 4% Gurr's Giemsa solution (BDH Laboratory Supplies, Poole, Dorset, UK). One thousand cells were examined to visually score the percentage of mitotic cells. The slides were coded and scored without the knowledge of case-control status. In order to assess the reproducibility of the mitotic index measurements, blood samples from ~10% n = 50 randomly selected subjects were assayed in duplicates. The results indicated that mitotic index score in assay 1 was very similar to that in assay 2 and significantly correlated [Pearson correlation coefficiency (r) = 0.80, $P = 0.01$] and the average coefficient of variation for the 50 pairs of duplicates was 15%. The overall blood culture success rate was 97%.

Statistical Analysis

Descriptive analyses were conducted to characterize the population using the Student t test for continuous variables and chi-square test for categorical variables. We used general Linear Models (GLM) to examine the association of selected host characteristics and mitotic index in the control group, adjusting for age and race where appropriate. We categorized the mitotic index using informative cutoff points based on the distributions among the controls and the P-for-trend across

categories was examined. Smoking status was stratified into two categories: never smokers—individuals who had never smoked more than 100 cigarettes in their life and ever (former/current) smokers—individuals who had smoked more than 100 cigarettes in their life. Family history of female cancers was defined as having breast or ovarian cancer in first or second degree biological relatives. Physical activity was defined as any physical activity on a regular basis (at least once a week on average) for at least 20 minutes at a time that was reported to either have made the subjects sweat or increased their heart rate. Pearson Correlation was used to estimate the correlations between mitotic index and the variable of interest. Multivariate logistic regression was used to estimate the risk association between mitotic index and breast cancer, adjusting for age, race, state as well as other known breast cancer risk factors and other potential confounders. An individual was considered to have high mitotic index if the mitotic index score was equal to or greater than the 50th percentile value in controls (3.19%). To assess for a dose-response trend between breast cancer risk and mitotic index, women were categorized to three groups: lowest quartile, 2 middle quartiles combined and highest quartile based on control distributions. All P-values were two-sided and considered significant if $P < 0.05$. Analyses were performed using SAS software, version 9 (SAS Institute Inc., Cary, NC).

Results
Study population
The demographic characteristics of case-control subjects (200 cases and 360 controls) are presented in Table 1. The mean age was 52.7 for cases and 54.2 for controls and the population was predominantly white (73% of cases versus 62% of controls). There were no significant case-control differences in the distribution of tobacco smoking status, menopausal status, family history of female cancers, hormonal replacement therapy (HRT) use and mean body mass index (BMI). Eighty one percent of cases and 80% of controls reported to have been pregnant at some point in their lifetime and the average number of full term pregnancies was significantly higher in controls than in cases (Table 1). Sixty-six percent of cases and 69% of controls ever breastfed and the mean lifetime duration of breastfeeding was 57 weeks for cases and 70 weeks

for controls ($P = 0.13$, Table 1). Seventy-nine percent of controls and 65% of cases had regular physical activities in their teens. Forty percent of cases and 43% of controls had completed college or higher education, and 56% of cases and 52% of controls had median family income $\geq$\$100k, reflecting the high socioeconomic characteristics of patients seen at the Georgetown University Medical Center.

The mean percent of mitotic cells in blood lymphocytes was significantly higher in cases (3.59%) than in controls (3.26%, $P < 0.01$, Table 2). When the case-control comparison was stratified by age, race, menopausal status, BMI, tobacco smoking and physical activity during the teenage years, cases consistently showed a higher mitotic index across all subgroups of women than controls (Table 2).

Mitotic index in blood lymphocytes and breast cancer risk
We examined the association between mitotic index and breast cancer risk using multivariate logistic regression (Table 3). Using the median (3.19%) in controls as a cut point, subjects were dichotomized into high (equal or above median) or low (below median) mitotic index groups. Women who had high mitotic index had significantly increased breast cancer risk compared with women with low mitotic index (adjusted odds ratio [OR] = 1.54, 95% Confidence Interval [CI] = 1.03–2.30) in the overall study population (Table 3). ORs were adjusted for age, race, menopausal status, number of full-term pregnancies and lifetime duration of breast-feeding. When stratified by menopausal status, the ORs were 1.14 (95% CI = 0.62–2.09) and 2.06 (95% CI = 1.18–3.57) for pre- and post-menopausal women, respectively. We also found that the significant associations between mitotic index and breast cancer risk were restricted to women who were overweight or obese (BMI > 25), (OR = 2.02, 95% CI = 1.19–3.43, Table 3). To assess for the presence of a dose-response trend between breast cancer risk and mitotic index, women were categorized into three groups: low mitotic index (lowest quartile), intermediate mitotic index (2 middle quartile categories) and high mitotic index (highest quartile). Women with low mitotic index (the lowest quartile) were used as the reference. A significant dose-response relationship was observed ($P_{trend} < 0.01$) in all women, and the lowest-versus-highest quartile

Table 1. Characteristics of study population by case-control status.

Characteristic	Cases (n = 200)	Controls (n = 360)	P-value*
Age (years), mean (SD)	52.7 (10.9)	54.2 (10.2)	0.11
Race, N (%)			
White	147 (73)	224 (62)	
Black	42 (21)	126 (35)	
Other	11 (6)	10 (3)	<0.01
BMI, mean (SD)	27.1 (6.4)	27.7 (7.2)	0.26
Active smoking, N (%)			
Ever	71 (36)	148 (41)	0.2
Age at menarche (years), mean (SD)	12.6 (1.5)	12.5 (1.5)	0.58
Ever pregnant, N (%)			
Yes	161 (81)	288 (80)	0.67
FTP (N), mean (SD)	1.61 (1.2)	2.00 (1.2)	<0.01
Age at 1st FTP (years), mean (SD)	27.3 (6.3)	27.4 (6.6)	0.97
Age at last FTP (years), mean (SD)	31.7 (6.1)	32.0 (5.8)	0.63
Years since last FTP (years), mean (SD)	22.7 (13.3)	23.1 (12.1)	0.75
Ever breastfed (%)			
Yes	82 (66)	178 (69)	0.43
Weeks breastfed (N), mean (SD)	56.6 (66.7)	70.0 (70.1)	0.13
Menopausal Status, N (%)			
Post-	108 (56)	218 (61)	0.23
Use of HRT (%)			
Ever	53 (51)	120 (55)	0.54
Physical activity at age 13–19, N (%)			
Yes	130 (65)	277 (79)	<0.01
Family history of female cancers, N (%)			
0 relatives affected	180 (90)	329 (93.7)	
1 relative affected	9 (4.5)	21 (6)	
2 relatives affected	1 (0.5)	1 (0.3)	0.76
Educational level (%)			
Equal/above college	80 (40)	153 (43)	0.63
Household income (%)			
Equal/above $100K	78 (56)	134 (52)	0.38

Notes: *P-values were computed using t-tests for continuous characteristics and chi-square tests for categorical characteristics. Missing values were excluded from the P-value computation. Physical activity was defined as any weekly physical activity, longer than 20 minutes at a time that would make the subject sweat or increase their heart rate. Family history of female cancers was defined as any breast or ovarian cases among 1st and 2nd degree blood relatives.
Abbreviations: BMI, body mass index; FTP, full term pregnancy, analyses among parous women only; HRT, hormonal replacement therapy, analysis among post-menopausal women, weeks breastfeeding computed only among women that breastfed.

OR was 2.00 (95% CI = 1.16–3.47). A significant dose-response relationship was also observed among whites, post-menopausal women and women who were overweight or obese (Table 3). No significant dose response relationship was observed for black women, premenopausal women, and women who had BMI ≤ 25. Further adjustment of the models for BMI resulted in comparable results.

Correlation between mitotic index and host factors

We evaluated the relationship between mitotic index and selected host factors among 360 healthy woman controls (Table 4). Two hundred and twenty four women were white (62%), 126 were African American (35%) and 10 were of 'other' race/ethnicity (3%). The overall mean mitotic index among all healthy women subjects was 3.25%. White women had a significantly higher mitotic index (3.35%) compared with African American women (3.06%, $P < 0.01$). We observed a trend of decreasing mitotic index with increasing age (Table 4). However, there was no noticeable difference in mitotic index between pre- and post-menopausal women (Table 2). Using Pearson correlation, we observed a modest inverse association between mitotic index and BMI (r = −0.19, $P = 0.0003$, Table 4),

Table 2. Case-control comparison of mean mitotic index.

Host factors	Cases		Controls		*P*-value
	N	Mean (SD)	N	Mean (SD)	
All subjects	200	3.59 (1.2)	360	3.26 (1.0)	<0.01
Age					
<55	124	3.65 (1.3)	196	3.31 (1.0)	0.05
≥55	76	3.51 (1.0)	164	3.10 (1.0)	0.04
Race					
White	147	3.59 (1.2)	224	3.35 (1.0)	0.03
African American	42	3.47 (1.2)	126	3.06 (1.0)	0.02
Active smoking					
Ever	71	3.59 (1.0)	148	3.21 (0.9)	0.01
Never	126	3.61 (1.3)	208	3.30 (0.8)	0.02
Menopausal status					
Pre-	85	3.53 (1.3)	138	3.28 (1.0)	0.08
Post-	108	3.69 (1.2)	218	3.26 (1.0)	<0.01
BMI categories					
<20	14	4.09 (1.8)	19	3.64 (1.0)	0.37
20–25	65	3.75 (1.4)	135	3.52 (1.0)	0.13
26–30	54	3.49 (1.0)	88	3.07 (1.0)	0.01
>30	49	3.29 (1.0)	105	2.99 (0.9)	0.07
Physical activity at age 13–19					
Yes	130	3.53 (1.3)	277	3.28 (1.0)	0.03
No	68	3.70 (1.0)	74	3.22 (0.9)	<0.01

Note: *P*-values were computed using t-tests.
Abbreviation: BMI, body mass index.

and this correlation was only significant among postmenopausal women (r = −0.24, *P* = 0.0004) and white women (r = −0.26, *P* < 0.0001, Table 4). We also found that mitotic index was modestly correlated with household income (r = 0.13, *P* = 0.04) and educational level (r = 0.17, *P* < 0.01). There were no associations between mitotic index and family history of female cancers, smoking status or physical activity during teenage years.

Correlation between mitotic index and reproductive factors

We examined the correlations between mitotic index and reproductive factors known to contribute to breast cancer risk in 360 healthy women controls. Because mitotic index differed significantly by race and reproductive characteristics also differ between white women and African American women, this part of the analysis was stratified by race and adjusted for age. Among white women, mitotic index was significantly correlated with age at menarche (*P* < 0.01) and number of full-term pregnancies (*P* < 0.01, Table 4). Among African American women, mitotic index was

significantly correlated with age of last full-term pregnancy (*P* = 0.03), and borderline significantly correlated with age of first full-term pregnancy (*P* = 0.08). Consistent with previous reports,[22–25] the distributions of reproductive characteristics differed between Whites and African Americans in our study population. For example, we observed that a higher percentage of African American women had an early age of menarche (<11 years of age) compared to white women (31% versus 20%). Twenty-eight percent of African American women had their first child at age ≤20 years while only 2% of white women did. African American women were less likely to give birth at late age (>36 years) compared with white women (26% versus 41%). Breast feeding seemed to only be associated with the mitotic index among white women where increasing lifetime duration of breastfeeding was associated with a non-statistically significant increase in mitotic index (Table 4).

Discussion

In this report, we demonstrated that, after adjusting for known breast cancer risk factors, high mitotic index in cultured blood lymphocytes was significantly

Table 3. Logistic regression examining the association between mitotic index and breast cancer risk.

	Cases/controls	OR* (95% CI)	Cases/controls	OR* (95% CI)
Mitotic index (%)	**All subjects**			
By median				
Low (≤3.19)	74/178	1		
High (>3.19)	126/182	1.54 (1.03–2.30)		
By quartiles				
Q1 (≤2.59)	40/96	1		
Q2 + Q3 (2.60–3.98)	83/173	1.20 (0.69–1.99)		
Q4 (≥3.99)	77/91	2.00 (1.16–3.47)		
P trend		<0.01		
	White women		**African American women**	
By median				
Low (≤3.19)	56/104	1	16/71	1
High (>3.19)	91/120	1.46 (0.90–2.35)	26/55	1.44 (0.64–3.22)
By quartiles				
Q1 (≤2.59)	30/48	1	8/45	1
Q2 + Q3 (2.60–3.98)	60/115	0.93 (0.49–1.77)	20/56	1.77(0.65–4.80)
Q4 (≥3.99)	57/61	1.76 (0.91–3.41)	14/25	2.10 (0.67–6.51)
P trend		0.04		0.19
	Premenopausal women		**Postmenopausal women**	
By median				
Low (≤3.19)	35/66	1	35/109	1
High (>3.19)	50/72	1.14 (0.62–2.09)	73/109	2.06 (1.18–3.57)
By quartiles				
Q1 (≤2.59)	19/36	1	18/58	1
Q2 + Q3 (2.60–3.98)	35/69	0.96 (0.44–2.09)	46/102	1.42 (0.69–2.81)
Q4 (≥3.99)	31/33	1.68 (0.73–3.88)	44/58	2.41 (1.15–5.07)
P trend		0.17		<0.01
	BMI ≤ 25		**BMI > 25**	
By median				
Low (≤3.19)	26/54	1	43/117	1
High (>3.19)	53/100	1.03 (0.51–2.05)	60/76	2.02 (1.19–3.43)
By quartiles				
Q1 (≤2.59)	18/29	1	20/64	1
Q2 + Q3 (2.60–3.98)	22/71	0.43 (0.16–1.16)	51/95	1.69 (0.88–3.25)
Q4 (≥3.99)	39/54	1.50 (0.62–3.61)	32/34	2.49 (1.17–5.32)
P trend		0.07		0.02

Notes: *ORs are adjusted for age, race, menopausal status (where appropriate), number of full term pregnancies and weeks of breastfeeding. Further adjustment for BMI resulted in comparable results.
Abbreviations: HRT, hormonal replacement therapy, analysis among post–menopausal women only; BMI, body mass index.

associated with an increased risk of breast cancer. This result seems support the concept that cellular hyper-proliferation in response to mitogens is associated with an increased risk of breast cancer. Although the endogenous factors that contribute to PHA-induced mitotic index phenotype are likely complex and remain to be elucidated by future studies, our data provided some clues that reproductive or other hormonal exposures may partly contribute to the mitotic index phenotype.

Our data is not in agreement with a previous report by Wiltschke et al showing that PHA-induced lymphocyte proliferation was lower in breast cancer patients compared with that in healthy women controls.[20] There are several limitations in this previous small study (90 cases and 60 controls). First, recruitment of the study subjects was not described and it is unknown what type of control subjects were included in the analysis. Second, the characteristics of the study population were not described, thus it is hard to evaluate the comparability of the case-control population. Third, other known reproductive and host factors were not considered in the analysis. It is also

Table 4. Correlation between mitotic index, host factors and reproductive factors in healthy controls (N = 360).

Host factors	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
	All (N = 360)		White (N = 224)		African American (N = 126)	
Race	360	3.25 (1.0)	224	3.35 (1.0)	126	3.06 (0.9)
Age categories (y)						
≤45	78 (22)	3.39 (1.0)	45	3.40 (0.9)	31 (25)	3.22 (0.9)
46–55	118 (33)	3.26 (0.9)	71	3.39 (1.0)	42 (33)	3.00 (0.8)
56–65	102 (28)	3.30 (1.0)	63	3.46 (1.0)	36 (28)	3.08 (0.9)
≥66	62 (17)	3.01 (1.0)	45	3.07 (0.9)	17 (14)	2.84 (1.1)
P trend		0.06		0.19		0.28
BMI						
<20	19 (5)	3.63 (0.9)	16 (7)	3.78 (1.0)	2 (2)	2.34 (0.1)
20–25	135 (38)	3.49 (1.0)	107 (48)	3.53 (0.9)	23 (18)	3.27 (1.0)
26–30	88 (24)	3.10 (0.9)	48 (21)	3.11 (1.0)	36 (29)	3.09 (1.0)
>30	105 (29)	3.00 (0.9)	46 (21)	3.08 (1.0)	59 (47)	2.92 (0.8)
P trend		<0.01		<0.01		0.32
Reproductive factors						
Age at menarche (y)						
9–11	86 (24)	3.12 (1.0)	45 (20)	3.23 (1.0)	39 (31)	3.02 (1.0)
12	110 (30)	3.35 (0.9)	70 (31)	3.35 (1.0)	35 (28)	3.19 (1.0)
13	85 (24)	3.22 (1.0)	54 (24)	3.38 (0.9)	28 (22)	2.97 (1.0)
≥14	76 (21)	3.34 (0.9)	53 (24)	3.47 (1.0)	23 (18)	3.05 (0.9)
P trend		0.04		<0.01		0.65
FTP						
Never pregnant	69 (19)	3.29 (1.0)	47 (21)	3.30 (1.0)	19 (15)	3.23 (1.0)
No FTP	34 (9)	3.42 (0.9)	17 (8)	3.70 (0.9)	17 (13)	3.11 (0.8)
Yes FTP	254 (71)	3.23 (1.0)	158 (70)	3.34 (1.0)	89 (71)	3.01 (0.8)
P value		0.06		0.34		0.48
Number of FTP						
1	57 (22)	3.01 (1.0)	33 (21)	3.11 (1.0)	23 (26)	2.93 (0.9)
2	112 (44)	3.31 (0.9)	74 (47)	3.35 (1.0)	35 (39)	3.16 (0.9)
≥3	85 (34)	3.26 (1.0)	51 (32)	3.45 (1.1)	31 (35)	2.89 (0.9)
P trend		0.13		<0.01		0.94
Age of first FTP (y)						
≤20	30 (12)	2.92 (1.1)	3 (2)	2.84 (0.9)	25 (28)	2.85 (0.9)
21–24	59 (24)	2.99 (1.0)	31 (19)	3.14 (1.1)	27 (31)	2.77 (0.9)
25–29	64 (26)	3.50 (1.0)	44 (28)	3.63 (1.0)	17 (19)	3.21 (1.0)
≥30	94 (38)	3.30 (1.0)	74 (47)	3.28 (1.0)	19 (21)	3.37 (1.0)
P trend		0.04		0.6		0.08
Age of last FTP						
<30	78 (31)	3.14 (1.1)	33 (21)	3.44 (1.1)	41 (46)	2.73 (0.9)
30–35	83 (33)	3.27 (1.0)	56 (36)	3.38 (1.0)	25 (28)	3.18 (0.8)
36–40	67 (26)	3.35 (1.1)	48 (30)	3.38 (0.9)	18 (20)	3.28 (0.9)
≥40	23 (9)	3.02 (1.0)	18 (11)	2.94 (1.3)	5 (6)	3.45 (1.3)
P trend		0.23		0.27		0.03
Years since last FTP						
≤10	51 (20)	3.29 (1.5)	36 (23)	3.10 (1.0)	14 (16)	3.35 (0.7)
11–20	56 (22)	3.24 (1.1)	36 (23)	3.23 (1.3)	18 (20)	3.26 (0.9)
21–30	68 (27)	3.29 (1.0)	36 (23)	3.53 (0.9)	32 (36)	3.00 (1.0)
≥30	76 (30)	3.12 (1.6)	47 (29)	3.48 (1.0)	25 (28)	2.63 (0.9)
P trend		0.23		0.37		0.3
Ever breastfed						
Yes	178 (70)	3.28 (1.0)	130 (82)	3.40 (1.0)	44 (49)	3.13 (0.9)
No	76 (30)	3.13 (1.0)	28 (18)	3.32 (1.0)	45 (51)	2.94 (0.9)
P value		0.23		0.7		0.36

(*Continued*)

Table 4. (*Continued*)

Host factors	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
	All (N = 360)		White (N = 224)		African American (N = 126)	
Weeks breast feeding						
≤20	43 (24)	3.07 (1.0)	28 (22)	3.20 (1.0)	14 (32)	2.93 (0.9)
21–56	41 (23)	3.24 (1.0)	29 (22)	3.09 (1.0)	12 (27)	3.64 (0.8)
57–108	43 (24)	3.48 (1.0)	36 (28)	3.49 (1.1)	6 (14)	2.89 (1.2)
≥109	47 (27)	3.39 (1.0)	35 (27)	3.48 (1.1)	10 (23)	2.92 (1.0)
P trend		0.11		0.12		0.91

Notes: **P*-trend and *P*-values were computed using general linear models adjusting for age and race apart from when age, race and menopausal status were examined. For analysis stratified by race, age adjusted values are presented.
Abrrevaitons: FTP, full term pregnancy, analyses among parous women only, weeks breast feeding analysis was performed among women who breast fed; BMI, body mass index.

worth to note that the study by Wiltschke et al used purified mononuclear cells by Ficoll gradient centrifugation for lymphocyte culture, while the present study used whole blood for lymphocyte cultures. It is possible that lymphocyte growth as measured by mitotic index in our culture system is affected by circulating hormonal and other factors in the blood serum.

We found that mitotic index was positively associated with number of full-term pregnancies and age at first full-term pregnancy, suggesting exposure to very high level of reproductive hormones during pregnancy may increase PHA-induced mitotic index in blood lymphocytes. The ability of the ovarian hormones estrogen and progesterone to promote cell proliferation in the normal breast epithelium can explain key epidemiologic observations regarding reproductive history and breast cancer risk.[25] However, whether the reproductive hormones influence the proliferative potential of blood lymphocytes is unknown, although estrogen receptors are present on blood lymphocytes.[26] Pregnancy is associated with very high levels of estrogen and progesterone that induce both cell proliferation and differentiation and therefore pregnancy is related to a dual effect on breast cancer; a short term increase in risk for up to 10 years after full term pregnancy and long term decrease.[27] The high levels of circulating reproductive hormones during pregnancy result in the differentiation of the terminal duct-lobular unit and confers a protective effect.[28] Early age at pregnancy,[29] higher parity[30] and prolonged lactation are found to be protective against breast cancer.[31] Additionally, it is well known that during pregnancy the maternal immune system and cytokine profile are modified in order to achieve immune tolerance towards paternal antigen expressed on fetal cells.[32] It is therefore possible that the observed associations between reproductive factors and the PHA-induced mitotic index in blood lymphocytes are the result of pregnancy-related endocrine and immune-function changes.

We are surprised to find a positive correlation between PHA-induced mitotic index and age at menarche and an inverse correlation between PHA-induced mitotic index and BMI. Both early age at menarche and obesity at post-menopausal result in prolonged exposure to physiological level estrogens and progesterone. Prolonged exposure to estrogens and progesterone are well documented risk factors for breast cancer. Early age at menarche (less than 12 years of age) has been associated with a 10%–20% increase in breast cancer risk compared to late age (>14 years of age) at menarche.[33] Obesity has a complex relationship with breast cancer that was modulated by menopausal status. In general, BMI has been found to be positively associated with breast cancer risk among postmenopausal women, whereas it is inversely associated with breast cancer risk among premenopausal women.[34] Since adipose tissue is an important source of estrogens[35] in postmenopausal women, it is expected that obese postmenopausal women have higher levels of endogenous estrogen than lean women. Our data seem to indicate that prolonged exposure to physiological levels of estrogens decreases PHA-induced mitotic index in blood lymphocytes, suggesting that the mitotic index phenotype might be a breast cancer risk factor independent of low level reproductive hormonal exposures.

We also found that the mitotic index was significantly lower in African American women than in white

women, which is consistent with African American women having lower overall breast cancer incidence compared with white women.[3] Lower breast cancer incidence in African American women can be partially explained by the differences in distributions of the two key reproductive risk factors, younger age at first full term pregnancy and parity.[25] Approximately 50% of black women have their first child before age 20, whereas only 20% of white women have their first child at age <20.[36,37] Also, a higher proportion of white women have one or two children and black women more often have three or more children.[37] African American women are also less likely to breastfeed their children.[38] In agreement with current literature we observed racial differences in reproductive factors and these could explain the differential effect of mitotic index as a marker of breast cancer risk. Given the fact that African American women tend to have their first and last child at an earlier age than white women and are less likely to breastfeed, the cumulative effect of reproductive hormones on lymphocyte proliferation during pregnancy would be shifted to an earlier age, thus may not be captured in our study population in which greater than 90% of the women were older than 40 years of age. The small number of African American cases in our study precluded a detailed analysis to understand racial differences in the association between mitotic index and breast cancer risk. Thus future larger studies are needed to characterize the relationship between PHA-induced mitotic index and breast cancer risk in African American women.

Given that this is a case-control study, a theoretical concern is that mitotic index in blood lymphocytes is affected by case status (reverse causality). To evaluate whether having breast cancer increases blood lymphocyte mitotic index, we examined if surgical removal of breast tumor affect the mitotic index among cases. We found that the mean mitotic index in blood samples collected before surgery (3.6%, n = 23) was similar to blood samples collected after surgery (3.6%, n = 89). We further examined the effect of time since surgery on the mitotic index in post-surgical cases. We observed that there were no significant changes in mitotic index between cases who's blood was drawn within 30 days of post-surgery (3.5%, n = 32) and cases who's blood was drawn between 30–60 days of post-surgery (3.8%, n = 34) and cases who's blood was drawn between 60–180 days of post-surgery (3.2%, n = 23).

There were no significant differences in mitotic index by stage of the disease (DCIS 3.9%, stage I–II 3.7% and stage III–IV 3.4%, $P = 0.22$) among cases. Our study is limited by its moderate sample size and do not have sufficient power for subgroup analyses. Thus, our results need to be replicated by large independent studies for more precise risk estimation. Other more relevant mitogens such as estrogens and interleukins should also be tested in future studies.

In summary, our study revealed that high PHA-induced mitotic index in blood lymphocytes was significantly associated with an increased risk of breast cancer. If confirmed by future studies, mitotic index of cultured blood lymphocytes may serve as an independent biomarker for breast cancer risk. Our data also suggested that this association may be modulated by menopausal status and BMI and its interactive effects with lifetime exposures to reproductive and other hormones on breast cancer risk warrants further investigation.

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Disclosure

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