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EPIDEMIOLOGY

Pro-inflammatory cytokines and growth factors in human milk: an exploratory analysis of racial differences to inform breast cancer etiology

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Abstract

Background Analysis of cytokines and growth factors in human milk offers a noninvasive approach for studying the microenvironment of the postpartum breast, which may better reflect tissue levels than testing blood samples. Given that Black women have a higher incidence of early-onset breast cancers than White women, we hypothesized that milk of the former contains higher levels of pro-inflammatory cytokines, adipokines, and growth factors.

Methods Participants included 130 Black and 162 White women without a history of a breast biopsy who completed a health assessment questionnaire and donated milk for research. Concentrations of 15 analytes in milk were examined using two multiplex and 4 single-analyte electrochemiluminescent sandwich assays to measure pro-inflammatory cytokines, angiogenesis factors, and adipokines. Mixed-effects ordinal logistic regression was used to identify determinants of analyte levels and to compare results by race, with adjustment for confounders. Factor analysis was used to examine covariation among analytes.

Results Thirteen of 15 analytes were detected in $\geq 25\%$ of the human milk specimens. In multivariable models, elevated BMI was significantly associated with increased concentrations of 5 cytokines: IL-1β, bFGF, FASL, EGF, and leptin (all p-trend < 0.05). Black women had significantly higher levels of leptin and IL-1β, controlling for BMI. Factor analysis of analyte levels identified two factors related to inflammation and growth factor pathways.

Conclusion This exploratory study demonstrated the feasibility of measuring pro-inflammatory cytokines, adipokines, and angiogenesis factors in human milk, and revealed higher levels of some pro-inflammatory factors, as well as increased leptin levels, among Black as compared with White women.

Keywords Human milk · Breast cancer risk · Prevention · Race

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Introduction

Increased lifetime duration of lactation in developed countries has been associated with a modest lowering of breast cancer risk overall, with a potentially stronger protective effect on risk for aggressive, early-onset estrogen receptor (ER)-negative or triple-negative breast cancers [1–4]. Differences in exposures that might increase risk of ER-negative tumors, such as increased parity, less breastfeeding and greater body mass index (BMI) may contribute to the higher age-adjusted incidence of ER-negative breast cancers among Black compared with White women [5]. Findings from the AMBER consortium show that an early age at first birth, particularly without breastfeeding, may contribute to an elevated risk of triple-negative breast cancer among Black women [3, 4]. Further, a meta-analysis found that premenopausal obesity is associated with increased risk of triple-negative breast cancer [6]. Understanding the interplay of parity, lactation, BMI, and early-onset breast cancer is potentially important for risk assessment and prevention, but developing approaches to pursue this research is challenging.

We hypothesized that factors associated with early-onset breast cancer, such as obesity and race, might be related to increased levels of cytokines, angiogenesis factors, and hormones in the postpartum period and that such factors are measurable in milk. Given that previous studies have shown that levels of specific cytokines in human milk and serum differ [7–9], measurement of these components in milk may provide a better reflection of the breast microenvironment than analysis of blood. Further, limited data suggest that inflammatory protein levels differ between affected and unaffected breasts of women with unilateral breast cancer [10], supporting the idea that milk may provide information about cancer risk that pertains specifically to the sampled breast. Several studies have measured cytokines in milk [11, 12], including sample sizes ranging from 5 to 182 specimens, but none has focused on comparing analyte concentrations in Black and White women. In the current exploratory study, we demonstrate the feasibility of comparing cytokine and growth factor levels in human milk by participant characteristics to develop hypotheses that may account for racial differences in risk of early-onset breast cancers.

Methods

Study population

Participant recruitment, milk collection, and processing are described in detail elsewhere [11–13]. Briefly, from 2007 to 2013 lactating women aged 18 years or older were recruited to donate human milk samples, which were frozen and archived at the University of Massachusetts-Amherst Breastmilk Laboratory. Women were recruited for milk donation through websites, lactation consultants, or, for those living within 20 miles of the laboratory, local advertisement or fliers. For the purposes of this analysis, we selected a total of 292 samples, 130 donated by Black women and 162 by White women, all without a history of a breast biopsy or breast cancer. Women provided informed consent and a self-completed questionnaire related to medical history, lactation, and breast cancer risk. The study was approved by the Institutional Review Boards of the University of Massachusetts Amherst and the National Cancer Institute.

Enrolled women were provided with pre-labeled glass bottles or BPA-free plastic milk bags and asked to express the full breast contents of each breast upon waking into separate containers by either hand expressing or using their own breast milk pump. For participants living with 30 miles of the University of Massachusetts-Amherst, milk was collected at their home and transported to the laboratory in a cooler. All other participants were provided with coolers, ice packs, and prepaid labels for home pick-up and express (overnight) shipment of their milk to the laboratory.

Laboratory methods

Milk samples were processed immediately upon arriving at the laboratory. Cells were separated from the milk after centrifugation resulting in cell-depleted milk diluted 1.5x with phosphate buffered saline (PBS). Diluted milk samples were stored at − 20 °C.

Cytokines and growth factors were measured using two multiplex and four single-analyte assays from Mesoscale Discovery (MSD, Gaithersburg, MD). A custom-ordered Human V-PLEX Proinflammatory Panel 1 included assays for five cytokines: interleukin-6 (IL-6, lower limit of detection (LLOD): 0.14 pg/mL), interleukin-8 (IL-8, LLOD: 0.12 pg/mL), tumor necrosis factor-alpha (TNF-α, LLOD: 0.09 pg/mL), interleukin-1 beta (IL-1β, LLOD; 0.07 pg/mL), and interferon-gamma (IFN-γ, LLOD: 0.74 pg/mL). A custom-ordered Human V-PLEX Angiogenesis Panel 1 included assays for six growth factors: vascular endothelial growth factor C (VEGFC, LLOD: 13.81 pg/mL), vascular endothelial growth factor D (VEGFD, LLOD: 3.26 pg/mL), tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE-2, LLOD: 21.69 pg/mL), fms-related tyrosine kinase 1 (FLT1, LLOD: 1.53 pg/mL), placental growth factor (PLGF, LLOD: 0.42 pg/mL), and basic fibroblast growth factor (bFGF, LLOD: 0.15 pg/mL). Single-analyte assays were used for leptin (LLOD: 93.96 pg/mL), adiponectin (LLOD: 0.008 ng/mL), Fas ligand (FASL,

LLOD: 0.415 pg/mL), and epidermal growth factor (EGF, LLOD: 0.075 pg/mL). Assays were performed according to the manufacturer's instructions. Each 96-well plate included 38 human milk samples, eight standards in duplicate, and 4 control wells. Eight-point standard curves generated with a 4-parameter logistic regression model were used to provide calculated concentrations for each analyte. The standard curves demonstrated adequate consistency across plates (data not shown). One sample was repeated in duplicate on all 8 plates, and one of the two other samples was included in duplicate on each of the four plates. Plates ($n = 8$) were read on the SECTOR® Imager 2400A (MSD). MSD uses electrochemiluminescence technology with a tagged double-antibody sandwich assay to detect each analyte of interest.

Statistical analysis

All 15 analytes were measured in technical replicates and analyzed as both continuous and categorical variables based on the proportion of samples within the detectable range. Assay precision was assessed using generalized linear models with correction for analyte levels within plates to calculate coefficients of variation (CVs) and intraclass correlation coefficients (ICCs). Final calculated concentrations of analytes were further categorized based on the proportion of individuals with measurements less than the LLOD as follows: analytes for which ≥ 75% of samples yielded measurement above the LLOD were categorized into quartiles (adiponectin, IL-8, EGF, FLT1, PLGF, VEGFD, FASL); analytes for which 50–75% of samples measured above the LLOD were analyzed in four categories, defined as less than LLOD and tertiles of detectable measurements (bFGF, IL-6, IL-1-beta, Leptin, TNFα); and analytes for which 25–50% of sample values were above the LLOD were analyzed as three categories: less than LLOD and below and above the median (IFNγ). Most (121 of 130; 93%) milk samples donated by Black women were mailed to the laboratory, whereas 129 of 162 (80%) White women residing within a 1-h drive from the laboratory had their samples collected at home and brought to the laboratory. The primary data analysis proceeded with a pooled sample of 292 women, based on sensitivity analyses that demonstrated that associations were independent of method of donation, irrespective of race (data not shown).

Ordinal logistic regression models with a random effect for plate were used to test for associations between maternal demographic characteristics (predictor variables) and analytes in human milk, which were categorized after taking into account detectability as described in the previous paragraph. Consistent with an exploratory analysis, we considered $p < 0.05$ statistically significant. We present unadjusted and adjusted model results. Adjustment variables were selected by forward stepwise selection, including race plus all variables with p-values less than 0.4 retained in final models. We used factor analysis, with varimax rotation, to explore the relationships of cytokines, angiogenesis factors, and adipokines in human milk. We computed analyte factor scores for each participant, categorized them into quartiles, and evaluated their relationship with maternal and breastfeeding characteristics using ordinal logistic regression, controlling for plate. Adjustment variables were selected by forward stepwise selection as described above.

Descriptive statistics and ordinal logistic regression analyses were performed using STATA 13 (College Station, TX). CVs, ICCs, and factor analyses were computed using SAS version 9.3 (Cary, NC).

Results

Population

Demographic and health characteristics of the 292 women are summarized in Table 1, stratified by self-reported race for 130 Black, and 162 White women. Black women tended to have a higher BMI, and greater parity. White women more frequently reported a history of a first-degree relative with breast cancer and ever smoking. Black and White women did not differ in age distribution, the age of their nursing baby, whether their menses had returned since delivery, their age at first birth, or their total lifetime days of lactation. Black women were more likely than White women to have shipped their milk samples (vs. local collection—see Table 1). Demographic characteristics stratified by collection method are shown in Table S1. Women who shipped samples had higher median lactation duration (59% vs. 43% over 180 days), and lower rates of ever smoking (13% vs. 42%).

Assay performance

Coefficients of variation (CVs) and intraclass correlation coefficient (ICCs) for 32 repeated samples run across all plates are shown in Table S2. CVs for 8 of the 15 analytes were below 15%, ranging from 2.97 to 14.31% (FASL, EGF, IL-8, FLT1, PLGF, VEGFD, leptin, and adiponectin). CVs and ICCs were similar in measurement of technical replicates across all 292 women in the study (Table S3). TIE-2 and VEGFC were dropped from further analysis due to low detectability (< 25%), high CVs and lower ICCs.

For most analytes, the median and range of concentrations, as well as the percent of samples with detectable concentrations, were similar for both locally collected and shipped milk samples (see Table 2). There was only one statistically significant difference: compared with locally collected milk, shipped milk had a higher median value for

Table 1 Demographic and health characteristics of human milk donors

	Black $n=130$		White $n=162$		p-value*
	n	%	n	%	
Age (year)					
<25	17	13	24	15	0.4
25–<30	34	26	32	20	
30–<35	47	36	53	33	
35–<40	26	20	40	25	
40+	6	5	13	8	
Body mass index (BMI) (kg/m^2)					
<25	59	45	89	55	0.05
25–<30	35	27	47	29	
≥30	36	28	26	16	
Baby's age (days)					
21 –<187	56	43	86	53	0.09
187–1500	74	57	76	47	
Menses have returned since giving birth					
No	65	50	93	57	0.13
Yes	64	49	64	40	
Missing	1	1	5	3	
First-degree relative with breast cancer					
No	122	94	139	86	0.03
Yes	8	6	23	14	
Age at menarche (year)					
<12	43	33	42	26	0.38
12–<14	68	52	84	52	
14+	15	12	30	19	
Unknown/missing	4	3	6	3	
Number of pregnancies					
1	39	30	65	40	0.02
2	33	25	46	28	
3	28	22	35	22	
4+	30	23	16	10	
Ever smoke cigarettes					
No	114	87	100	62	0.0001
Yes	16	12	62	38	
Age at first birth (year)					
15–<25	46	35	41	25	0.17
25–43	84	65	121	75	
Type of milk collection					
Local	9	7	129	80	0.0001
Shipped	121	93	33	20	
Total lifetime days of breastfeeding					
Q1 21–<165	26	20	47	29	0.30
Q2 165 –<390	35	27	38	24	
Q3 390–<753	38	29	38	24	
Q4 753–3900	31	24	39	23	

*χ^2 p-value

leptin ($p=0.03$), but similar levels of detectability (70 and 80%, respectively, $p=0.07$). Because results from shipped and locally collected samples were largely similar, we included results from all 292 women in this exploratory analysis.

Associations of participant characteristics with analytes in human milk

Associations between analyte levels and select demographic and health status predictors adjusted for plate effects are shown in Table 3. Compared with White women, Black women had significantly higher levels of IL-1β (per category OR 1.82, 95% CI 1.19–2.79, $p<0.001$) and leptin (OR 2.56, 95% CI 1.65–3.96, $p<0.001$). Obesity (BMI > 30 Kg/m^2) was associated with significantly increased levels of INFγ, IL-1β, bFGF, EGF, FASL, and leptin (all p-trend < 0.05). Obesity was also associated with statistically significantly lower levels of adiponectin (OR 0.56, 95% CI 0.32–0.97, $p<0.001$); however, the trend across all BMI categories did not reach statistical significance (p-trend = 0.06). Predictors including parity, baby's age at donation, total lifetime days lactation and return of menses were related to several analytes as shown in Table S4, although values did not always show consistent trends across categories within variables.

Multivariable models are shown in Table 4. All multivariable models included race; given the exploratory nature of this analysis, we additionally included covariates whose univariate association with the analyte of interest was $p<0.4$. As compared with White women, Black women had elevated levels of IL-1β and leptin, which persisted in multivariate models adjusted for BMI and other factors such as baby's age in days. Overall, in multivariable models adjusted for race, elevated BMI was significantly associated with increased concentrations of IL-1β, bFGF, EGF, leptin, and FASL (all p-trend < 0.05). Adiponectin was inversely associated with BMI, but this was not statistically significant. Return of menses since delivery was associated with increased levels of FLT1, PLGF, and EGF. A history of smoking cigarettes was significantly positively associated with adiponectin concentrations, after controlling for race, BMI, and total lifetime days of lactation. Baby's age was significantly associated with increased levels of IL-1β and IL-8 after controlling for race. Total lifetime days of lactation was generally associated with lower concentrations of bFGF and adiponectin, but the trends were not statistically significant (p-trends 0.56 and 0.70, respectively).

Exploratory factor analysis of analytes identified two factors with significant contributions, with positive loading of VEGFD, FLT1, FASL, EGF, and PLGF to Factor 1, and of IL-6, IL-1β, and TNFα to Factor 2 (Table 5). Factor 1 was predominantly composed of angiogenesis factors, but also FASL, which functions in apoptosis, whereas significant

Table 2 Concentrations of select analytes in human milk

Analytes	Locally collected ($n=138$)			Shipped to laboratory ($n=154$)		
	Median	IQR	Detectable (%)	Median	IQR	Detectable (%)
Pro-inflammatory panel						
Interferon gamma (IFNγ)	0	0–6.22	37	0	0–7.00	35
Interleukin 1 beta (IL-1β)	0.36	0–1.02	73	0.67	0.23–1.78	74
Interleukin-6 (IL-6)	0.89	0–3.02	59	1.05	0–3.48	57
Interleukin-8 (IL-8)	210.25	102.50–401.72	97	255.48	127.97–620.37	96
Tumor necrosis factor-alpha (TNFα)	1.06	0.49–2.26	59	0.70	0–2.02	58
Angiogenesis panel						
Basic fibroblast growth factor (bFGF)	0.65	0.0–1.70	61	0.76	0–1.58	60
Fms-Related Tyrosine Kinase 1 (FLT1)	1758.01	1299.81–2353.27	99	1762.68	1318.11–2496.71	99
Placenta growth factor (PLGF)	67.29	37.04–118.33	99	72.96	39.13–144.45	99
Vascular endothelial growth factor D (VEGF-D)	293.81	229.89–449.74	99	274.81	188.84–411.39	98
Singleplex assays						
Adiponectin	21.15	15.15–28.46	100	20.26	14.43–26.27	100
Epidermal growth factor (EGF)	4373.99	2991.79–6053.52	100	4480.19	2881.58–5987.60	100
Fas ligand (FASL)	44.31	32.62–59.88	100	43.63	30.88–56.99	100
Leptin	605.21	0–1433.18	70	1047.06	334.97–2208.45*	80

pg/mL for all analytes except adiponectin, which is μg/mL; analytes with <25% detectability excluded from table. If both duplicate levels were above the lower limit of detection (LLOD), the final calculated concentration was the mean of the two levels. If one technical replicate level was above the LLOD and one was below, then the final calculated concentration was the mean of the analyte above the LLOD and ½ the LLOD. If both analytes were below the LLOD then the result was recorded as zero

*$\chi^2=4.96$, $p=0.03$

loading on Factor 2 primarily included pro-inflammatory cytokines. In univariate analysis (Table 6), higher levels of Factor 1 scores were associated with increased age, BMI and with resumption of menses since delivery (all $p<0.05$). Higher levels of Factor 2 scores were inversely associated with baby's age (p-trend <0.05) and positively with mother's age (p-trend <0.01). Multivariable analyses for factor scores, which included race in all of the models, demonstrated significant positive relationship of Factor 1 scores with BMI and menses since delivery ($p<0.05$), controlling for maternal age and baby's age. The multivariable model for Factor 2 showed that higher scores were associated with Black race ($p<0.05$), but had an inverse association with baby's age in days (p-trend <0.01), controlling for maternal age and race (Table 7).

Discussion

Our exploratory analysis demonstrates the feasibility of measuring many analytes in human milk in epidemiologic studies seeking to identify differences in breast biology between Black and White women. Preliminarily, we find that certain analytes in human milk may vary by BMI, race, and other characteristics of the lactating mother and nursing baby. Thus, we suggest that analysis of human milk may inform hypotheses about early events in breast carcinogenesis for women with different levels of early-onset breast cancer risk.

We successfully analyzed 13 out of 15 candidate analytes tested but excluded TIE2 and VEGFC because of poor detectability. The other angiogenic and adipokines markers achieved reasonable CVs and ICCs; for the cytokines, the CVs were adequate given the relatively high ICCs (for the most part exceeding 90%). Agarwal's 2011 review [20] of 25 pro-inflammatory cytokine studies of human milk included some of the analytes measured in our study with some differences and similarities in results. Among reviewed reports, levels of IFNγ, IL-6, and TNF-α were higher than in our study, though their ranges fell within the wide range of median quantities reported for each analyte, whereas levels of IL1-β and of IL-8 were similar across studies [20]. Most summarized studies used singleplex enzyme-linked immunosorbent assays (ELISA), while our study used electrochemiluminescent, multiplex technology. In addition, another systematic review of 26 studies of leptin concentrations in milk, primarily measured with ELISA, found levels similar to our data [16].

Our results suggest that BMI and race represent potential correlates of several analytes in human milk. Although highly preliminary, our work demonstrates that levels of leptin in human milk are positively associated with BMI, as

Table 3 Univariate odds ratios and 95% confidence intervals for associations between select maternal demographic predictors and analytes in human milk

Predictors	Race	Age (years: quartiles)				BMI (kg/m^2: normal weight, overweight, obese)		
Analytes	Ref: whit	Q1 19–28	Q2 29–31	Q3 32–35	Q4 36–45	≤24	25–<30	≥30
		Ref	OR [95% CI]	OR [95% CI]	OR [95% CI]	Ref	OR [95% CI]	OR [95% CI]
Pro-inflammatory cytokines panel								
IFNγ	1.10 [0.69, 1.68]	–	0.86 [0.44, 1.67]	1.22 [0.64. 2.31]	1.04 [0.54, 1.99]	–	1.68 [0.96, 2.93]	2.03 [1.10, 3.74]**
IL-1β	1.82 [1.19, 2.79]*	–	1.02 [0.57, 1.84]	1.15 [0.65, 2.04]	0.67 [0.37, 1.23]	–	1.53 [0.94, 2.50]	1.79 [1.05, 3.00]**
IL-6	1.19 [0.77, 1.84]	–	0.47 [0.25, 0.87]	0.73 [0.40, 1.32]	0.53 [0.29, 0.97]	–	1.27 [0.76, 2.14]	1.48 [0.85, 2.58]
IL-8	1.39 [0.91, 2.15]	–	0.82 [0.45, 1.46]	1.14 [0.63, 2.04]	0.76 [0.42, 1.40]	–	1.25 [0.76, 2.06]	0.87 [0.50, 1.50]
TNFα	0.66 [0.43, 1.01]	–	0.69 [0.37, 1.26]	1.20 [0.66, 2.17]	0.18 [0.60, 1.95]	–	1.75 [1.05, 2.91]	1.46 [0.84, 2.53]
Angiogenesis panel								
bFGF	1.18 [0.78, 1.79]	–	0.70 [0.40, 1.26]	1.14 [0.64, 1.96]	1.05 [0.59, 1.89]	–	1.53 [0.94, 2.50]	1.79 [1.06, 3.04]**
FLT1	1.03 [0.68, 1.56]	–	0.74 [0.41, 1.34]	1.10 [0.63, 1.91]	1.09 [0.61, 1.96]	–	1.75 [1.08, 2.85]	1.19 [0.70, 2.03]
PLGF	1.31 [0.86, 1.98]	–	0.64 [0.36, 1.42]	1.12 [0.64, 1.94]	1.02 [0.57, 1.83]	–	1.31 [0.81, 2.12]	1.35 [0.79, 2.33]
VEGF-D	0.72 [0.48, 1.10]	–	0.99 [0.55, 1.79]	0.85 [0.49, 1.50]	0.86 [0.49, 1.51]	–	1.54 [0.94, 2.52]	0.75 [0.44, 1.29]
Singleplex								
Adiponectin	0.70 [0.46, 1.07]	–	1.05 [0.59, 1.88]	0.89 [0.50, 2.58]	1.16 [0.64, 2.11]	–	0.95 [0.58, 1.56]	0.56 [0.32, 0.97]
EGF	0.92 [0.61, 1.41]	–	1.48 [0.80, 2.71]	1.41 [0.80, 2.49]	1.26 [0.69, 2.30]	–	2.34 [1.41, 3.87]	1.51 [0.87, 2.61]**
FASL	1.06 [0.70, 1.61]	–	0.81 [0.45, 1.47]	1.20 [0.69, 2.12]	0.97 [0.53, 1.76]	–	1.92 [1.17, 3.15]	2.05 [1.18, 3.57]**
Leptin	2.56 [1.65–3.96] *	–	0.17 [0.70, 2.31]	1.68 [0.94, 2.99]	1.47 [0.81, 2.63]	–	5.83 [3.37, 10.18]	19.88 [10.03, 39.41]**

Univariate analyses conducted using mixed effects ordinal logistic regression with random effect for plate, $n = 292$ women, with odds ratios (ORs) presented. Other predictors such as age at first birth, age at menarche, first-degree relative with breast cancer, and use of over the counter pain medicine were also tested, with null results

*$p < 0.05$

**p-trend < 0.05

Table 4 Multivariable odds ratios and 95% confidence intervals for associations of select participant characteristics with analytes in human milk

Predictor	IL-8	IL-1β	bFGF	FLT1	PLGF	EGF	Adiponectin	Leptin	FASL
	OR [95% CI]	OR [95% CI]	OR [95% CI]	OR [95% CI]	OR [95% CI]	OR [95% CI]	OR [95% CI]	OR [95% CI]	OR [95% CI]
Race (ref.: white)	1.20 [0.77–1.87]	1.59 [1.03–2.45]*	1.16 [0.75–1.79]	0.93 [0.60–1.43]	1.18 [0.77–1.79]	0.85 [0.55–1.32]	0.91 [0.58–1.44]	2.16 [1.37–3.42]*	0.99 [0.65–1.52]
Body mass index (BMI)									
< 25	N/A	Ref	Ref	Ref	N/A	Ref	Ref	Ref	Ref
25–< 30		1.57 [0.94–2.60]	1.51 [0.91–2.49]	1.76 [1.06–2.90]		2.31 [1.38–3.86]	0.88 [0.53–1.41]	6.01 [3.46–10.43]	1.92 [1.17–3.15]
≥ 30		1.82 [1.05–3.16]**	1.77 [1.02–3.08]**	1.12 [0.65–1.95]		1.49 [0.85–2.62]**	0.57 [0.32–1.01]	18.36 [9.23–36.53] **	2.05 [1.17–3.58]**
Menses since delivery	N/A	N/A	N/A	2.37 [1.49–3.77]*	2.84 [1.84–4.38]*	1.75 [1.13–2.77]*	N/A	N/A	N/A
Pregnancies									
1	N/A	N/A	N/A	N/A	N/A	N/A	Ref	N/A	N/A
2							0.47 [0.26–0.85]		
3+							0.63 [0.34–1.12]		
Baby's age in days									
Q1 21–98	Ref	Ref	Ref	Ref	N/A	N/A	N/A	N/A	N/A
Q2 100–189	2.21 [1.18–4.11]	0.93 [0.51–1.72]	0.36 [0.20–0.67]	0.42 [0.23–0.77]					
Q3 194–315	4.15 [2.18–7.90]	1.42 [0.77–2.62]	0.47 [0.22–0.97]	0.48 [0.26–0.89]					
Q4 316–1500	8.74 [4.42–17.2]**	2.44 [1.32–4.50]**	0.84 [0.43–1.68]	0.77 [0.39–1.49]					
Ever smoked cigarettes	N/A	N/A	N/A	N/A	N/A	N/A	1.82 [1.09–3.05]*	N/A	N/A
Total lifetime days of breastfeeding									
Q1 21–120	N/A	N/A	ref	N/A	N/A	N/A	Ref	N/A	N/A
Q2 127–255			0.47 [0.22–1.02]				0.35 [0.19–0.64]		
Q3 270–735			0.56 [0.29–1.08]				0.73 [0.38–1.38]		
Q4 750–3900			0.61 [0.31–1.21]				0.87 [0.42–1.40]		

Associations tested in ordinal logistic regression with random effect for plate. Multivariable models were built in forward stepwise selection, with race and all predictors $p < 0.4$ retained in final models. Factors with cells labeled N/A (Not Applicable) were not included in the model for the analyte listed because they failed to meet criteria for selection

*$p < 0.05$

**p-trend < 0.05

Table 5 Factor patterns-varimax rotation of analyte factor correlations of residuals after removal of plate effects

	Factor 1	Factor 2
VEGFD	84*	0
FLT1	84*	3
FASL	73*	3
EGF	70*	9
PLGF	69*	9
Leptin	47	11
Adiponectin	47	31
BFGF	46	37
IL-8	38	10
IL-6	10	93*
IL-1β	7	79*
TNFα	13	74*
IFNγ	8	26

Values are multiplied by 100 and rounded to the nearest integer. Starred (*) values represent greater than 49% of the variance associated with a factor

Table 6 Univariate ordinal logistic regression of demographic predictors on quartiles of factor scores

Predictor	Factor 1-growth		Factor 2-inflammation	
	OR	95% CI	OR	95% CI
Race	1.28	[0.84–1.93]	1.43	[0.94–2.16]
Age				
Q1 19–28	Ref		Ref	
Q2 29–31	1.09	[0.61–1.92]	0.75	[0.42–1.33]
Q3 32–35	1.61	[0.92–2.80]	1.12	[0.63–1.97]
Q4 36–45	1.71	[0.95–3.10]**	1.01	[0.56–1.79]**
Body mass index (BMI)				
< 25	Ref		Ref	
25–< 30	2.13	[1.31–3.46]	1.24	[0.76–2.01]
≥ 30	1.92	[1.11–3.20]*	1.25	[0.73–2.14]
Baby's age in days				
Q1 21–98	Ref		Ref	
Q2 100 – 89	0.72	[0.41–1.26]	0.50	[0.28–0.90]
Q3 194–315	0.93	[0.52–1.65]	0.39	[0.22–0.71]
Q4 316–1500	1.48	[0.82–2.67]	0.45	[0.25–0.82]**
Menses since delivery	2.07	[1.35–3.17]*	0.89	[0.59–1.36]
Ever smoked cigarettes	1.15	[0.72–1.83]	1.04	[0.66–1.65]
Pregnancies				
1	Ref		Ref	
2	1.13	[0.68, 1.90]	0.61	[0.36, 1.04]
3+	1.12	[0.69, 1.81]	0.77	[0.48, 1.25]
Lifetime days of breastfeeding	1.12	[0.93, 1.35]	0.86	[0.72, 1.04]

Associations tested in mixed effects multiple level ordinal logistic regression on quartiles of factor scores with a random effect for plate. Factors with cells labeled N/A (Not Applicable) were not included in the model for the analyte listed because they failed to meet criteria for selection

*$p < 0.05$

**p-trend < 0.05

expected and previously reported (reviewed in [16–19]), and that levels are higher among Black as compared with White women, even after controlling for BMI, findings which are consistent with prior studies [21, 22]. Many findings implicate leptin in breast carcinogenesis, including studies of obese women showing higher ratios of circulating leptin to BMI in breast cancer patients vs. age-matched controls, and data suggesting that leptin is increased in breast cancer tissues compared with benign specimens [22, 23]. Epidemiologic studies of circulating leptin and breast cancer risk are conflicting, but inverse associations with premenopausal breast cancer have been reported [24, 25]. In our analysis, obesity was also associated with higher milk levels of IL-1β, bFGF, EGF, leptin, and FASL. A prior analysis of serial milk samples from normal-weight and heavy women found associations between higher BMI and elevated leptin and insulin levels, with substantially higher concentrations of insulin in breastmilk vs. plasma [26]. Similar to our findings, BMI was not significantly related to levels of IL-6, IL-8, and TNFα [26].

Our data suggest that milk from Black women may contain higher levels of IL-1β than in White women, independent of BMI and lactation duration. IL-1β is an important "alarm" inflammatory mediator produced by monocytes and macrophages in response to cellular necrosis that has important effects in multiple aspects of tumorigenesis [27]. This finding contrasts with that of Burch et al. [28], who measured associations between demographic characteristics, allergy history and immune factors in human milk in a group of 115 women of different racial groups: 77 White, 20 Black, and 18 "Other." They noted that breast milk of Black women had higher levels of IL-6 (6.60 vs. 1.55 pg/mL) and IFN-γ (6.56 vs. 2.58 pg/mL) than that of White women, controlling for covariates such as history of atopy, smoking, and BMI. There was no statistically significant difference in IL1-β levels in Black and White women in the study, perhaps due to low statistical power, lack of samples from late in the breastfeeding trajectory, or different control variables.

We and others have hypothesized that increased inflammation within the breast of Black women compared with White women might be linked to the higher rates of early-onset breast cancer in Black women [29, 30]. The transformation of the lactating breast back to an inactive gland (involution) has long-lasting effects on the health of the breast, as evidenced by a study in mice that found evidence that involution is similar to the tissue remodeling that occurs during wound healing [31]. Thus, our preliminary data showing that Black race and obesity are linked to

Table 7 Multivariable ordinal logistic regression of demographic predictors on quartiles of factor scores

Predictor	Factor 1-growth		Factor 2-inflammation	
	OR	95% CI	OR	95% CI
Race	1.11	[0.74–1.74]	1.59	[1.03–2.39]*
Age				
Q1 19–28	Ref		Ref	
Q2 29–31	1.09	[0.61–1.96]	0.84	[0.47–1.50]
Q3 32–35	1.42	[0.80–2.51]	1.25	[0.70–2.23]
Q4 36–45	1.62	[0.88–2.95]	1.14	[0.64, 2.06]
Body mass index (BMI)				
< 25	Ref		N/A	
25–< 30	2.04	[1.24–3.34]		
≥ 30	1.61	[0.93–2.81]*		
Baby's age in days				
Q1 21–98	Ref		Ref	
Q2 100–189	0.72	[0.41–1.26]	0.50	[0.28–0.90]
Q3 194–315	0.93	[0.52–1.65]	0.38	[0.21–0.69]
Q4 316–1500	1.48	[0.82–2.67]	0.45	[0.22–0.75]**
Menses since delivery	1.98	[1.29–3.05]*	N/A	

Associations tested in ordinal logistic regression on quartiles of factor scores with a random effect for plate. Multivariable models were built in forward stepwise selection with race and all predictors $p < 0.4$ retained in final models. Factors with cells labeled N/A (Not Applicable) were not included in the model for the analyte listed because they failed to meet criteria for selection

*$p < 0.05$

**p-trend < 0.05

higher levels of inflammatory cytokines and growth factors in human milk, if confirmed, suggest potential strategies to reduce racial disparities in breast cancer risk through interventions such as weight control and perhaps short courses of anti-inflammatory agents. This would support the preclinical results reported by Lyons et al. showing that a postpartum pro-inflammatory mechanism may promote development of aggressive breast cancer and that this process can be inhibited by NSAIDs [31].

Along with the poor detectability and reliability of some analytes in human milk, challenges in this study included the heterogenous nature of milk collection (local vs. shipped samples). Specifically, nearly all specimens donated by Black women were shipped, whereas specimens from White women were collected near the laboratory and more rapidly processed, raising concerns that mode of collection could confound associations between participant characteristics and analytes levels. Although collection of milk samples by mail offers reduced control over sample handling, it offers advantages with respect to enrolling large numbers of diverse participants. Still, the difference in collection methods represents a limitation that we attempted to minimize through statistical adjustment. Our findings are supported by a classic review by Lawrence [32] that concluded that milk proteins and immune components are relatively stable over a range of collection and storage conditions, and another study showed that leptin levels did not vary between fresh, frozen, and pasteurized milk samples [33]. In our study, all women donated specimens using their own breast pump, following specific instructions for standardized collection. Table 2 demonstrates that shipped samples have higher levels of leptin, while all other analytes had similar levels, suggesting that location of donation did not have major influences on most cytokines. When we stratified analyses by shipping, leptin was positively associated with BMI in both shipped and locally collected samples (data not shown); therefore, we concluded that differences in leptin levels by collection method were likely due to race, not method of collection, although this interpretation is tentative.

Strengths of this analysis include collection of a large number of samples from a racially diverse group of women with comprehensive annotation, and the use of standardized collection methods. The innovative use of local recruitment along with the Internet to facilitate an off-site collection protocol, allowed for representation of lactating women throughout the lactation trajectory. In our cross-sectional study, we treated the length of lactation largely as a control variable in analysis. Other longitudinal studies demonstrated changes in cytokine expression related to length of lactation [11, 34, 35], though with different cytokine panels and different durations of lactation, limiting our ability to integrate these findings with ours. For example, Qin et al. [11] recruited women who provided "transitional," "mature," and "wean" milk at varying timepoints, using breastfeeding pattern to differentiate the samples instead of number of days since birth of the baby. Chollet-Hinton et al. [34] and Collado et al. [35] analyzed milk from women at varying timepoints up to 12 weeks in the breastfeeding trajectory, with no indication that any sample was a wean sample. Future research in this area with collection of repeated samples over time from larger numbers of women, with comprehensive annotation of feeding patterns, medical history, and medication use throughout the postpartum period, would represent an important next step. Additional specimens could include both serum and tissue samples from matched time points in the breastfeeding trajectory.

The ease of collection of human milk, along with the potential to assess biomarkers such as adipokines, cytokines, and growth factors, as well as genetic and epigenetic changes in luminal epithelial cells [13], suggest that human milk analysis may inform our understanding of breast carcinogenesis. If high levels of inflammation in human milk are linked to breast cancer risk, women with such forms of "inflammatory mastopathy" might be candidates for trials assessing

screening at early ages, and possibly short-term chemoprevention with anti-inflammatory agents. While this hypothesis is supported by data from elegant preclinical models [31], testing this hypothesis among women will require both methods development and large population-based studies.

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Compliance with ethical standards

Conflict of interest The authors state they have no conflict of interest.

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