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Association of TGF- β 2 levels in breast milk with severity of breast biopsy diagnosis

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Abstract

Purpose—TGF- β plays a dual role in breast carcinogenesis, acting at early stages as tumor-suppressors and later as tumor-promoters. TGF- β isoforms are expressed in breast tissues and secreted in milk, suggesting that analysis of levels in milk might be informative for breast cancer risk. Accordingly, we assessed TGF- β 2 levels in milk from women who had undergone a breast biopsy and related the concentrations to diagnosis.

Methods—Milk donated by women who had undergone or were scheduled for a breast biopsy was shipped on ice for processing and testing. Breast cancer risk factors were obtained through a self-administered questionnaire and biopsy diagnoses were extracted from pathology reports. TGF- β 2 levels in milk, assessed as absolute levels and in relation to total protein, were analyzed in bilateral samples donated by 182 women. Linear regression was used to estimate relationships of log transformed TGF- β 2 levels and TGF- β 2:total protein ratios to biopsy category.

Results—Milk TGF- β 2 levels from biopsied and non-biopsied breasts within women were highly correlated ($r^2=0.77$). Higher mean TGF- β 2 milk levels (based on average of bilateral samples) were marginally associated with more severe breast pathological diagnosis, after adjusting for duration of nursing current child (adjusted p-trend=0.07).

Conclusions—Our exploratory analysis suggests a borderline significant association between higher mean TGF- β 2 levels in breast milk and more severe pathologic diagnoses. Further analysis

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

of TGF- β signaling in milk may increase understanding of post-partum re-modeling and advance efforts to analyze milk as a means of assessing risk of breast pathology.

Keywords

TGF- β 2; breast milk; breast cancer; epidemiology

INTRODUCTION

Nearly 25% of breast cancers occur prior to age 50 years [1]. These cancers are often hormone receptor negative, disproportionately affect African American women, are missed by mammography, grow rapidly, and are fatal [2,3]. Parity, breastfeeding and postpartum breast re-modeling are emerging as factors that may affect the development of early onset breast cancers, but mechanisms and biomarkers related to risk following childbirth are largely unknown [4].

The transforming growth factor (TGF)- β pathway is implicated in postpartum breast re-modeling and carcinogenesis [5,6] through its influences on cell division differentiation, proliferation, and apoptosis [7,8]. TGF- β is proposed to have a dualistic role in breast carcinogenesis, acting to suppress tumor formation in benign cells, but promoting tumor aggressiveness once cancer is established [8-10]. Deregulation of the TGF- β pathways in part explains this paradox, which triggers a cascade of downstream events that drive multiple oncogenic pathways, such as uncontrolled proliferation, loss of apoptosis, and sustained angiogenesis [11]. TGF- β signaling patterns have also been found to vary with age and pathologic features of prognostic significance [12], further providing evidence for the complex role of the TGF- β pathway in breast carcinogenesis. Further complicating the role of TGF- β in breast cancer progression is the finding that TGF- β and its receptors can be epigenetically silenced in early breast cancer by histone modifications [13].

Given that the blood-milk barrier isolates the breast from the circulation, breast milk is thought to represent a unique biospecimen for analyzing the breast epithelium and its microenvironment [14]. Of the three TGF- β isoforms, TGF- β 2 is the predominant isoform detected in human milk compared with TGF- β 1 or TGF- β 3 [15-17]. Furthermore, TGF- β 2 levels in milk and serum do not seem to be correlated [18], suggesting that levels in milk may better reflect the breast microenvironment. TGF- β 2 levels in milk have been shown to vary greatly among women and these variations have been associated with psychosocial factors [19], maternal immunity [20], infant birth or mother's weight [21], and phase of nursing [22,23]. Less is known about the association with breast biopsy diagnosis. In a previous study of five breast cancer cases, TGF- β 2 levels were significantly higher in the affected breast compared with the normal breast [24], suggesting that breast-specific TGF- β 2 levels measured in the milk might discriminate breast-specific cancer risks.

To further our knowledge on the association between TGF- β 2 levels in breast milk with breast biopsy diagnosis, we examined TGF- β 2 levels in milk from 182 women who had undergone or were scheduled for a breast biopsy and donated milk from both breasts. Given the inter-individual variability of TGF- β 2 levels in breast milk and its numerous roles in

normal mammary gland development as well as breast cancer, we also examined whether TGF- β 2 levels in breast milk were correlated with several breast cancer risk factors.

METHODS

Study population

Subject recruitment and milk collection procedures are described in detail elsewhere [25]. Briefly, we recruited lactating women aged 18 years or older, who either had a breast biopsy or were scheduled to have a breast biopsy across the U.S. from 2006-2011. Consented women completed a questionnaire, provided a copy of their breast biopsy pathology report, and donated milk from both breasts. The study was approved by the Institutional Review Boards of the University of Massachusetts, the Congressionally Directed Medical Research Program, and the National Cancer Institute. Here we present data from 182 women with sufficient frozen milk from both their biopsied and non-biopsied breasts, including six women who had bilateral biopsies.

Breast milk collection

Enrolled women were asked to separately collect up to 100 mL of milk from left and right breasts by pumping or hand expression. Women returned fresh milk with an ice pack. Milk was processed as previously described to collect cell populations, and the cellular-depleted milk was frozen at -20°C [25]. One woman provided two sets of milk samples in one shipment and both sets were tested for TGF- β 2 and total protein levels. Analyses based on values from either sample set or the average of the two sets did not appreciably change the overall results, thus we present the analysis based on values from the first set for this woman.

Breast cancer risk and other related factors

At time of breast milk collection, women returned a self-administered questionnaire that assessed basic breast cancer risk factors including demographic factors (age at donation, race), reproductive factors (age at first/last birth, number of live births), lifestyle factors (body mass index, smoking), and breastfeeding factors (total number of children breastfed, duration of nursing current child captured by baby's age).

Biopsy diagnosis

Breast biopsy pathology reports were abstracted and each woman was categorized into one of the 4 following biopsy category: (1) non-proliferative benign breast disease, (2) benign/proliferative lesions without atypia, (3) atypical ductal or lobular hyperplasia, and (4) ductal carcinoma *in situ* or invasive carcinomas. One woman had an indeterminate biopsy result. Six women underwent bilateral biopsies concurrently and all of these women had concordant diagnoses between breasts. Nine women provided biopsy reports from two different biopsy dates, with no change in diagnoses for all but four women, who advanced from non-proliferative benign breast disease to invasive carcinoma in about a five year span between biopsies. Analysis based on the biopsy-related factors obtained from the most recent biopsy reports are presented as the main result. As a sensitivity analysis, we examined

associations between TGF- β 2 levels with biopsy-related factors from the earlier biopsy date for women with more than two biopsies.

TGF- β 2 levels in breast milk

Epithelial depleted and diluted milk samples (1.5 PBS: 1 milk) were prepared as 500 μ L aliquots and frozen at -20°C for batch testing, along with a pooled specimen from four women. Four TGF- β 2 standard curves (8-point) were run in duplicate over the course of testing. Samples were plated irrespective of biopsy status or other known characteristics of the women. Activated protein levels of TGF- β 2 were determined using the *Quantikine Human TGF- β 2 Immunoassay* kit (R&D Systems DB250) as per the manufacturer's instructions. Two optical density readings were taken at 450 and 570 nm (VERSAmix plate reader, Molecular Devices) and absorbance at 570 nm was subtracted from the absorbance reading at 450 nm correcting for optical imperfections in the plate. The optical density of a zero control was subtracted from each value of the standard curve and each sample. Sample concentrations were calculated from a four parameter logistic curve-fit generated in Excel using the Solver Add-In (Microsoft Office 2013). Every 96-well plate included technical duplicates of the activated milk samples along with technical duplicates of at least four controls: two independently activated aliquots of pooled milk and two independently activated aliquots of pooled TGF- β 2-spiked milk. The immunoassay kit was considered appropriate for reliably measuring TGF- β 2 in human breast milk as analysis of control samples from nine plates (a total of 78 wells) demonstrated good reproducibility for both the low level and TGF- β 2-spiked control samples (coefficients of variation = 14.4% and 18.3% respectively). The detection limit of the assay is reported by the manufacturer as 7.0 pg/mL. Samples from two women with TGF- β 2 values out of range were retested after diluting the samples another 5 fold. Reports are based on the rerun for these samples.

Total protein in each milk sample was quantified in duplicate using the *Coomassie Plus (Bradford) Assay Kit* (Thermo Scientific) following the manufacturer's instructions. Samples were diluted with PBS to be within the assay working range of 100-1500 μ g/mL. The lab performing the assay was masked to sample annotation, such as age, race, or if the sample had been taken from a biopsied or non-biopsied breast.

Statistical analysis

TGF- β 2 levels were evaluated individually and as a ratio to total proteins (TGF- β 2 divided by total protein). TGF- β 2 and TGF- β 2:total protein ratios were log transformed to approximate a normal distribution. We determined the correlation between the duplicate measures of TGF- β 2 using Pearson's correlation and compared the levels between biopsied vs. non-biopsied breasts within women using paired t-tests.

TGF- β 2 duplicate measures were highly correlated (Supplementary Figure 1; $r^2=0.97$). Thus, analyses were conducted at the breast level (biopsied breast: n=182 breasts; non-biopsied breast: 176 breasts), by taking an average of the duplicate TGF- β 2 measurements in the six women with both breasts biopsied, and at the woman level (n=182 women) by taking an average of the duplicate TGF- β 2 measurements from both breasts (i.e. four data points).

Linear regression was used to compute geometric TGF- β 2 levels or the ratio of TGF- β 2/total protein across risk factor categories and biopsy-related factors. In multivariable analyses, any risk factor statistically significantly associated with TGF- β 2 or the ratio of TGF- β 2/total protein identified in univariate analyses at $P \leq 0.05$ were included. Tests for linear trends across risk factors were calculated by entering the ordinal values representing categories of risk factors as a continuous variable in the models and based on Spearman correlation. All P -values were two-sided and considered statistically significant if $P \leq 0.05$. Analyses were performed using STATA 13.1.

RESULTS

Selected characteristics and milk sample

Key characteristics of the women included in our analysis are shown in Table 1. Median age of participant was 34 years with 89% self-identified as White and 35% self-reported as having a first degree relative with breast cancer. Majority (95%) of women was biopsied prior to milk donation. The difference in breast milk volume between the two breasts ranged from 0 - 89 mL (mean: 15.4; standard deviation: 16.2). TGF- β 2 and total protein levels varied greatly among the 364 samples, ranging from 245.25 to 85,687.5 pg/mL for TGF- β 2 and from 2,764.5 to 33,572.5 μ g/mL for total protein. Furthermore, TGF- β 2 levels in milk from biopsied versus non-biopsied breasts were not significantly different (Figure 1A). Similarly, ratios of TGF- β 2/total protein between biopsied and non-biopsied breasts did not differ (Figure 1B).

TGF- β 2 and breast cancer risk factors

In woman-level analyses, older age of current baby breast fed was positively associated with higher TGF- β 2 levels and a higher ratio of TGF- β 2 to total protein (Table 2). None of the other demographics, reproductive factors, lifestyle factors, or breastfeeding factors we examined were statistically significantly associated with either absolute TGF- β 2 levels or as a ratio to total proteins (p -trend >0.05).

TGF- β 2 and biopsy diagnosis

We examined the relation between TGF- β 2 levels and three factors related to a woman's biopsy: age at biopsy, duration between biopsy and milk donation, and biopsy diagnosis. In unadjusted models and models adjusted for baby's age which was the only significant factor associated with TGF- β 2 in univariate analysis, we found that a higher TGF- β 2 level and higher ratio of TGF- β 2 to total protein was marginally associated with more severe pathologic diagnosis (Table 3). Analyses based exclusively on TGF- β 2 levels and TGF- β 2:total protein ratios in milk from the biopsied breast versus the non-biopsied breast were similarly related to biopsy diagnoses (Table 3). In a sensitivity analysis, we re-examined the biopsy-related factors using the earlier biopsy from nine women whom we had acquired biopsy information from more than one biopsy. We observed similar trends, albeit the association with pathologic severity was slightly diminished (woman-level analysis: adjusted p -trend=0.16 for TGF- β 2 and adjusted p -trend=0.10 for ratio of TGF- β 2 to total protein). Furthermore, sensitivity analyses omitting the atypia cases ($n=2$) as well as combining the atypia with malignant cases ($n=12$) were run and the results did not

appreciably change, albeit the association with pathologic severity was diminished (woman-level analysis: adjusted p-trend=0.16 and 0.11 for TGF- β 2 for atypia cases omitted and atypia cases combined with malignant cases, respectively).

DISCUSSION

Our analysis of bilateral milk samples collected from 182 women with available breast biopsy results show that TGF- β 2 levels varied greatly among women. Our exploratory analysis suggests a borderline significant association between higher TGF- β 2 levels and increasing lesion severity. We also observed that levels were highly correlated between biopsied and non-biopsied breasts within women, suggesting that systemic factors may influence levels more strongly than local tissue factors.

Our results showing high correlation between biopsied and non-biopsied breasts within women contradicts with a prior study based on a subset of five cancer cases included in our current analysis, which found that TGF- β 2 levels were higher in the breast with cancer versus the normal breast [24]. To explore possible reasons for these discordant findings, we carefully reviewed TGF- β 2 results of the women included in the prior analysis, which was conducted at a different laboratory using the same assay as our present analysis (Supplementary Table 1). We observed that in four of the five cases in the previous report, the TGF- β 2 levels were higher in the biopsied breast compared with the non-biopsied breasts; however, we observed the opposite in the remaining case from the previous report plus three additional cases that were included in our current report. The discrepancy between our observation and the previous study for the cancer cases might be related to methodological issues, including differential storage protocols of the breast milk samples. However, the laboratory that conducted the assays previously found higher levels of TGF- β 2 at time of weaning compared with the start of lactation [23], a finding which is consistent with the results we present here. Thus we cannot rule out the possibility that the previous study observed the difference between biopsied and non-biopsied breasts by chance alone because of the small number of cancer cases previously evaluated.

Our data suggest that increased TGF- β 2 levels are marginally associated with more severe pathologic diagnosis among biopsied women. While this supports previous findings of increased expression of TGF- β 2 associated with malignant conversion and progression in breast cancer [26,27], our finding needs to be cautiously interpreted given that it was based on small numbers of atypia cases (n=2). Mammary gland development, differentiation, and neoplasia are influenced by actions of sex steroid hormones, but it has been postulated that TGF- β 2 may mediate some of these effects. [28]. Confirmation that higher cytokine levels in breast milk might be informative for breast cancer risk is of interest, given that the process of post-partum remodeling shares commonalities with wound healing and may potentiate tumor aggressiveness [5]. Although biological roles of TGF- β isoforms overlap, each isoform is structurally and biologically possibly unique [29], warranting further expansion of this analysis to include TGF- β 1 and TGF- β 3. Gaining knowledge about the pathogenesis of early onset and pregnancy-associated breast cancer is important because these tumors are aggressive, exact a high societal cost and currently are not readily detected or prevented.

A major strength of the current analysis is the large number of women with available biopsy results and breast milk donation from both breasts. A limitation of this study was that the method of milk collection was not identical among subjects (i.e. the time of day milk was expressed, the type of pump used, the elapsed time between milk donation and processing); thus, milk volumes and composition may have varied in accordance with these considerations, but this was probably non-differential by biopsy diagnosis and therefore should not affect our results [30]. By analyzing TGF- β 2 as a ratio to total protein levels, we tried to minimize the impact of variation in collection timing and methods, and our results showed that conclusions derived from interpretation of these ratios were similar to those for absolute TGF- β 2 levels. Although our data suggest that assay performance was reliable, efforts to optimize methods of collection, transport and fractionation of milk for analysis of cytokines are ongoing. In addition, we cannot exclude that our analysis is limited by incomplete adjustment for potential confounders, including other psychosocial factors, microbial exposure, and phase of nursing (transitional, lactation, weaning), which may affect milk composition [19-23]. If these factors are related to severity of pathology, they could confound our analysis. However, we did assess important breast cancer risk factors and other exposures as possible confounders of the relationships between milk TGF- β 2 levels and breast biopsy diagnosis, and baby's age was accounted for in the analyses. We were unable to evaluate the cellular source of TGF- β 2 and could not evaluate TGF- β 2 expression and activity in biopsies, particularly tumor tissue or surrounding benign tissues. Thus, we were unable to determine whether elevated TGF- β 2 levels among those with more severe pathologic diagnoses may reflect involution secondary to breast tissue re-modeling, over-expression by benign tissues retained following surgery for neoplastic lesions, or perhaps other tissue changes related to biopsy and therapy.

In conclusion, we found suggestion that detecting higher levels of TGF- β 2 in milk among women may be associated with pathologic abnormalities including neoplastic lesions. We did not find that higher levels were restricted to the affected breast, but rather that TGF- β 2 levels were similar for left and right breasts within a woman, irrespective of unilateral biopsy. Confirmation that higher cytokine levels in breast milk are related to breast cancer risk in additional large studies of milk with annotation for potential confounders could advance the use of milk as a valuable biospecimen for assessing health. Given that the milk-blood barrier may isolate the breast environment from the systemic circulation, studies of milk may provide opportunities to better understand breast physiology and carcinogenesis and help identify tools for aggressive, early onset tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Log TGF- β 2 (n=176 women)Pearson $r^2 = 0.75$ (p-value<0.001)

Paired t-test: p-value=0.77

B. Log Ratio TGF- β 2/Total Protein (n=176 women) Pearson $r^2 = 0.80$ (p-value<0.001)

Paired t-test: p-value=0.62

Figure 1. TGF- β 2 level by biopsied breast compared to non-biopsied breast

Numbers do not add up to total number in study because 6 women had both breasts biopsied.

Log transformed values are presented in the figures.

Table 1Study population characteristics and TGF- β 2 levels in breast milk among 182 nursing mothers

Characteristics	Median	(10th, 90th percentile)
Age at donation (years)	34	(29,40)
Age at first birth (years)	30	(25,36)
Age at last birth (years)	33	(28,39)
Current BMI (kg/m ²)	23.2	(19.6,30.0)
	N	%
Race		
White	162	89%
Black, African-American	3	2%
Asian/Pacific Islander	4	2%
Hispanic	12	7%
Other	1	1%
Age at menarche (years)		
<13	95	52%
13	49	27%
14+	38	21%
Ever smoke		
No	80	63%
Yes	47	37%
Live births (number)		
1	57	35%
2	85	56%
3+	40	8%
Children breastfed (number)		
1	56	35%
2	89	56%
3+	37	8%
Biopsy		
Biopsy in 1 breast	176	97%
Biopsy in 2 breasts	6	3%
Time from biopsy to milk donation		
Biopsied 3+ years before donation	91	50%
Biopsied 1-2 years before donation	44	24%
Biopsied <1 year before donation	37	20%
Biopsied after milk donation	10	5%
1st degree family history of cancer		
Breast cancer (yes)	58	35%
	N	Median* (10th, 90th percentile) Geometric mean**

Characteristics		Median	(10th, 90th percentile)	
Individual woman level analysis				
TGF- β 2 (pg/mL)	182	3,760.0	(1330, 16877.8)	3,604.72
Total protein (μ g/mL)	182	14,712.3	(5446, 19406.5)	14,617.87
Ratio TGF- β 2/total protein (pg/ μ g)	182			0.31
Breast level analysis**				
<i>Biopsied Breast (n=182 breasts)</i>				
TGF- β 2 (pg/mL)	182	3,795.0	(1121.5,16453)	3,789.54
Total protein (μ g/mL)	182	14,558.5	(5888,19157)	14,617.87
Ratio TGF- β 2/total protein (pg/ μ g)	182			0.28
<i>Non-biopsied Breast (n=176 breasts)</i>				
TGF- β 2 (pg/mL)	176	3,418.0	(1087.5,16845.5)	3,428.92
Total protein (μ g/mL)	176	14,365.5	(5360,19141)	14,328.42
Ratio TGF- β 2/total protein (pg/ μ g)	176			0.31

* Average of multiple measurements presented for a milk sample set for each woman/breast.

** Values were log transformed to approximate a normal distribution.

Table 2

Unadjusted and adjusted TGF- β 2 levels and ratio of TGF- β 2/total protein in breast milk in relation to breast cancer risk factors and breastfeeding factors

DEMOGRAPHICS	N*	Geometric Mean TGF-β2 (Pg/mL)		Geometric mean TGF-β2/Total Protein (pg/μg)	
		Unadjusted	Adjusted**	Unadjusted	Adjusted**
Age at donation (years)					
<32	57	3,569	2,322	0.29	0.17
32-35	74	4,537	2,322	0.36	0.20
>35	51	4,024	1,287	0.31	0.13
	<i>Spearman r (p)</i>	<i>0.05 (0.53)</i>		<i>0.03 (0.72)</i>	
	<i>P-trend***</i>	0.53	0.96	0.70	0.93
Race					
White	162	4,146	2,039	0.34	0.17
Non-white	20	3,569	1,652	0.24	0.14
	<i>Spearman r (p)</i>	<i>−0.02 (0.77)</i>		<i>−0.08 (0.30)</i>	
REPRODUCTIVE FACTORS					
Age at first birth (years)					
<=28	58	4,024	1,669	0.31	0.12
29-32	70	4,230	2,039	0.33	0.19
>32	54	3,944	2,253	0.32	0.20
	<i>Spearman r (p)</i>	<i>−0.02 (0.74)</i>		<i>−0.01 (0.91)</i>	
	<i>P-trend***</i>	0.91	0.98	0.89	0.75
Age at last birth (years)					
<=30	46	3,641	2,080	0.30	0.15
31-35	81	4,537	2,368	0.36	0.20
>35	55	3,866	1,495	0.30	0.14
	<i>Spearman r (p)</i>	<i>0.02 (0.79)</i>		<i>0.001 (0.98)</i>	
	<i>P-trend***</i>	0.83	0.97	0.99	0.88
Live births (number)					
1	57	3,361	2,253	0.27	0.19
2	85	4,583	2,122	0.36	0.20
3+	40	4,230	1,097	0.34	0.07
	<i>Spearman r (p)</i>	<i>0.01 (0.18)</i>		<i>0.0.8 (0.28)</i>	
	<i>P-trend***</i>	0.20	0.51	0.29	0.61
LIFESTYLE FACTORS					
Current BMI (kg/m²)					
<25	114	4,188	2,059	0.32	0.17
25-<30	44	4,230	2,322	0.37	0.15
30+	18	3,395	1,572	0.28	0.15
	<i>Spearman r (p)</i>	<i>176</i>	<i>−0.03 (0.70)</i>	<i>0.01 (0.86)</i>	

DEMOGRAPHICS	N*	Geometric Mean TGF- β 2 (Pg/mL)		Geometric mean TGF- β 2/Total Protein (pg/ μ g)	
		Unadjusted	Adjusted**	Unadjusted	Adjusted**
<i>P-trend***</i>		0.53	0.67	0.95	0.92
Ever smoke					
No	80	3,866	1,620	0.30	0.12
Yes	47	4,024	1,959	0.31	0.17
<i>Spearman r (p)</i>	127	0.06 (0.49)		0.05 (0.56)	
<i>P-trend***</i>		0.86	0.84	0.79	0.80
BREASTFEEDING FACTORS					
<i>Children breastfed (number)</i>					
1	56	3,165	2,276	0.25	0.17
2	89	4,629	2,039	0.36	0.21
3+	37	3,294	1,153	0.35	0.07
<i>Spearman r (p)</i>		0.14 (0.06)		0.12 (0.11)	
<i>P-trend***</i>		0.09	0.28	0.12	0.32
Age of current baby breastfed (days)					
<160	62	2,836		0.23	
160-275	59	4,188		0.35	
>275	60	5,884		0.44	
<i>Spearman r (p)</i>	181	0.30 (0.0001)		0.26 (0.005)	
<i>P-trend***</i>		<0.001		0.001	

* Ns are based on number of women. Numbers do not add to total due to missingness.

** Adjusted for risk factor with p-trend<0.05: baby's age (<160, 160-275, >275 days).

*** We calculated p-trend from linear regression model using a continuous variable for each risk factor.

Table 3
Unadjusted and adjusted TGF-β2 levels and ratio of TGF-β2/total protein in breast milk in relation to biopsy related factors

	Woman Level						Biopsied breast						Nonbiopsied breast					
	Geometric mean TGF-β2 (pg/mL)			Geometric mean TGF-β2/Total Protein			Geometric mean TGF-β2 (pg/mL)			Geometric mean TGF-β2/Total Protein			Geometric mean TGF-β2 (pg/mL)			Geometric mean TGF-β2/Total Protein		
	N*	Unadjusted	Adjusted**	Unadjusted	Adjusted**	N	Unadjusted	Adjusted**	Unadjusted	Adjusted**	N*	Unadjusted	Adjusted**	Unadjusted	Adjusted**	N*	Unadjusted	Adjusted**
Age at biopsy (years)																		
<25	29	3,463	821	0.29	0.06	29	3,866	953	0.33	0.06	28	3,134	706	0.26	0.06			
25-29	57	4,273	3,072	0.33	0.24	57	4,105	3,605	0.31	0.24	53	4,492	2,864	0.35	0.24			
30-34	51	3,984	2,080	0.31	0.18	51	3,944	2,368	0.31	0.18	51	3,984	1,826	0.32	0.18			
35+	45	4,359	1,772	0.35	0.20	45	4,230	1,510	0.34	0.20	44	4,583	2,039	0.37	0.20			
<i>Spearman r (p)</i>		0.04 (0.61)		0.03 (0.67)			0.004 (0.96)		-0.002 (0.98)			0.06 (0.46)		0.05 (0.52)				
<i>P-trend</i> ***		0.50	0.84	0.54	0.81		0.79	0.89	0.80	0.81		0.32	0.55	0.39	0.73			
Time from biopsy to milk donation																		
Biopsied 3+ years before donation	91	4,024	1,510	0.31	0.11	91	3,790	1,556	0.30	0.11	87	4,230	1,495	0.34	0.11			
Biopsied 1-2 years before donation	44	3,569	2,298	0.27	0.24	44	3,569	2,752	0.28	0.24	44	3,605	1,920	0.27	0.24			
Biopsied <1 year before donation	37	4,964	2,951	0.43	0.26	37	5,324	3,072	0.45	0.26	36	4,675	2,893	0.42	0.26			
Biopsied after milk donation	10	3,984	2,465	0.32	0.47	10	4,675	3,752	0.35	0.47	9	3,429	1,556	0.30	0.45			
<i>Spearman r (p)</i>		0.03 (0.65)		0.06 (0.41)			0.08 (0.29)		0.09 (0.23)			-0.01 (0.85)		0.03 (0.66)				
<i>P-trend</i> ***		0.52	0.59	0.35	0.39		0.17	0.19	0.15	0.39		0.94	0.88	0.72	0.31			
Biopsy diagnosis																		
Non-proliferative lesions	141	3,866	1,920	0.31	0.18	141	3,828	2,080	0.30	0.18	137	3,944	1,755	0.32	0.18			
Prolif lesions without atypia	28	4,188	1,249	0.30	0.08	28	4,359	1,495	0.32	0.08	27	3,828	1,097	0.28	0.08			
Prolif lesions with atypia	2	11,499	11,499	0.87	0.87	2	9,997	9,997	0.81	0.87	2	13,095	13,095	0.94	0.87			
Malignant lesions	10	6,836	22,471	0.61	0.85	10	6,568	7,480	0.59	0.85	9	7,785	12,836	0.73	0.74			
<i>Spearman r (p)</i>		0.10 (0.20)		0.08 (0.29)			0.12 (0.11)		0.09 (0.25)			0.07 (0.34)		0.06 (0.45)				
<i>P-trend</i> ***		0.06	0.07	0.07	0.09		0.07	0.09	0.09	0.09		0.08	0.07	0.08	0.05			

* Based on most recent biopsy information acquired. Numbers do not add to total due to missingness.
** Adjusted for risk factor with p-trend<0.05: baby's age (<160, 160-275, >275 days).
*** We calculated p-trend from linear regression model using a continuous variable for each risk factor.