Influence of Insulin-like Growth Factors on the Strength of the Relation of Vitamin D and Calcium Intakes to Mammographic Breast Density

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Abstract

Diets with higher vitamin D and calcium contents were found associated with lower mammographic breast density and breast cancer risk in premenopausal women. Because laboratory studies suggest that the actions of vitamin D, calcium, insulin-like growth factor (IGF)-I, and IGF-binding protein-3 (IGFBP-3) on human breast cancer cells are interrelated, we examined whether IGF-I and IGFBP-3 levels could affect the strength of the association of vitamin D and calcium intakes with breast density. Among 771 premenopausal women, breast density was measured by a computer-assisted method, vitamin D and calcium intakes by a food frequency questionnaire, and levels of plasma IGF-I and IGFBP-3 by ELISA methods. Multivariate linear regression models were used to examine the associations and the interactions. The negative associations of vitamin D or calcium intakes with breast density were stronger among women with IGF-I levels above the median ($\beta = -2.8$, $P = 0.002$ and $\beta = -2.5$, $P = 0.002$, respectively) compared with those with IGF-I levels below or equal to the median ($\beta = -0.8$, $P = 0.38$ and $\beta = -1.1$, $P = 0.21$; $P_{interaction} = 0.09$ and 0.16, respectively). Similar results were observed within levels of IGFBP-3 ($P_{interaction} = 0.06$ and 0.03, respectively). This is the first study to report that the negative relation of vitamin D and calcium intakes with breast density may be seen primarily among women with high IGF-I or high IGFBP-3 levels. Our findings suggest that the IGF axis should be taken into account when the effects of vitamin D and calcium on breast density (and perhaps breast cancer risk) are examined at least among premenopausal women.

Introduction

Emerging evidence supports the idea that vitamin D and calcium as well as the insulin-like growth factor (IGF) pathway may be involved in the etiology of breast cancer. Increased intakes of vitamin D and calcium seem to be associated with a reduction of the risk of cancer, including breast cancer (reviewed in refs. 1, 2). Thus far, prospective epidemiologic studies have consistently shown high intakes of vitamin D (3, 4) and calcium (4, 5) to be associated with decreased breast cancer risk. Moreover, mammographic breast density, which reflects breast epithelial and/or stromal proliferation and is one of the strongest and well-recognized breast cancer risk indicators (reviewed in ref. 6), was found to decrease as vitamin D and calcium intakes from food increased (7–9). These negative associations with breast cancer risk or breast density might be limited to premenopausal women (4, 7–9). In addition, IGF-I is a well-established mitogen to various cell types and seems to be implicated in the development of a number of human cancers, including breast cancer (10). In contrast, its principal carrier protein, IGF-binding protein-3 (IGFBP-3), seems to be able to promote an IGF-I-independent action on apoptosis (11). Among premenopausal women, high levels of IGF-I and/or low levels of IGFBP-3 may be associated with increased breast cancer risk (12, 13) and breast density (14, 15).

Laboratory studies suggest that the antiproliferative and proapoptotic effects of vitamin D and calcium in the breast may vary according to levels of IGF-I and IGFBP-3. The effects of vitamin D (including its analogues) and calcium on breast cancer cells are enhanced in the presence of high IGF-I and/or high IGFBP-3 levels. Calcium, vitamin D, and IGFBP-3 seem to potentiate each other's antiproliferative and proapoptotic effects. IGFBP-3 alone or bound to IGF-I increases intracellular calcium concentrations (16) which promote the inhibition of cell proliferation and/or induction of apoptosis. The expression of IGFBP-3, a growth factor known to inhibit breast cell growth, is induced by vitamin D (17). This effect seems to be mediated via the vitamin D receptor interaction with a vitamin D response element recently identified on the human IGFBP-3 promoter (18). In contrast, vitamin D seems to counter the proliferative and antiapoptotic IGF-I activities. Vitamin D is known to inhibit the mitogenic effects of IGF-I (17, 19–23), attenuate the antiapoptotic effects of IGF-I (19–23), and down-regulate the expression of IGF-I receptors (19, 20, 22, 23). The inhibitory effect of vitamin D on cell proliferation was seen to be greater in the presence (19–21, 23) or with increasing levels (19) of IGF-I. Finally, the vitamin D receptor mediates, at least in part, the antiproliferative and proapoptotic activities of vitamin D, and its own activity has been found to be promoted by IGF-I (24, 25). Up to now, one epidemiologic study found that ff *Fok*I polymorphism in the vitamin D receptor gene was associated with a reduction of prostate cancer risk but only among men with high circulating levels of IGFBP-3 (26).

Taken together, epidemiologic and laboratory findings suggest that the IGF pathway may influence the effect of vitamin D and calcium on breast cancer risk and breast density. In the perspective that vitamin D and calcium are emerging as promising preventive agents, this idea is important as it proposes that the beneficial effect of vitamin D and calcium may be experienced only by a subgroup of individuals defined by their IGF levels. Thus, the present study was carried out to examine the hypothesis that the negative associations of vitamin D and calcium intakes with mammographic breast density are stronger among women with high circulating levels of IGF-I or IGFBP-3.

Materials and Methods

Study population and recruitment procedures. Details of the study design and methods have been published elsewhere (15). Briefly, the study subjects for the present analysis were premenopausal women who received a screening mammogram between February and December 2001 at the Clinique radiologique Audet (Quebec, Canada). Women were classified as premenopausal if they had at least one natural menstrual cycle within 12 months or after hysterectomy without bilateral oophorectomy and were younger than 48 years (if a nonsmoker) or 46 years (if a smoker; ref. 27). The eligibility was restricted to women not taking hormonal derivatives within 3 months of the mammography, not pregnant, never having used tamoxifen or raloxifene, without a history of cancer at any site, without breast reduction or implants, and without diabetes mellitus, dwarfism/acromegaly, thyroid, adrenal, or hepatic disease.

Of the 9,559 women who received a screening mammogram and were approached, 1,021 refused to participate in our study. In the remaining 8,538 women, 787 premenopausal and 804 postmenopausal women were found eligible. Among these premenopausal women, eight declined to be interviewed or to return the food frequency questionnaire, six had incomplete answers for some breast cancer risk factors, and the mammograms of two women could not be retrieved for review. Thus, 771 premenopausal women were included in the present analysis.

This study was reviewed and approved by the Research Ethics Committee of the Centre hospitalier affilié universitaire de Québec, Québec, Canada. All study participants provided written informed consent.

Data collection. At the time of mammography, a trained nurse measured the women's weight (kg) and height (cm) and collected 20 mL of blood. Information on potential breast cancer risk factors was collected by trained interviewers and included menstrual and reproductive histories, family history of breast cancer, personal history of breast biopsies, past use of hormonal derivatives, smoking status, alcohol intake, education, and physical activity (28).

Diet was assessed with a validated (29) and self-administered semiquantitative food frequency questionnaire (97GP copyrighted at Harvard University). Women provided, on average over the past year, the frequency of consumption for specific foods, ranging from "never" to "six or more times per day." Intake of foods was then translated into daily nutrient intake at Harvard University using the composition of foods derived from U.S. Department of Agriculture tables supplemented with other data. Women also provided the duration, brand, type, and weekly dose of multivitamins, the daily dose of calcium supplements (<400, 400-900, 901-1,300, or >1,300 mg), and the use of vitamin D if taken on a regular basis (assumed to be 400 IU/d). A generic code was used for brands of multivitamins available in Canada but not in United States. The daily nutrient intake from food and supplements, total intake, was obtained by adding the amounts from multivitamins and individual supplements to the intake from food.

All mammograms were scanned at 260 μm per pixel with a Kodak Lumiscan85 digitizer. Mammographic breast density, expressed as the percentage of the breast showing epithelial and/or stromal densities, was assessed by one trained author (C.D.) without any information on women using a computer-assisted method developed by one of us (M.Y.) and described elsewhere (30). For each woman, mammogram showing the cranio-caudal view of a randomly chosen breast was assessed. Random images were repeated; the within-batch intraclass correlation coefficient (n = 210 duplicate images) was 0.98 and the between-batch coefficient of variation (n = 10 images repeated 21 times) was 4%. In addition, the intraclass correlation coefficient between the right and left breasts (n = 100) was 0.95 in our preliminary validation study (15).

At the time of mammography, blood constituents were rapidly aliquoted and stored at −80°C until analysis. Under the supervision of one of us (M.P.), levels of IGF-I and IGFBP-3 (ng/mL) in plasma were blindly assayed by ELISA with reagents from Diagnostic Systems Laboratory (Webster, TX). The intrabatch coefficients of variation were <13% and the between-batch coefficients of variation were <11% for both growth factors.

Statistical methods. All statistical analyses were carried out using the SAS (SAS Institute, Inc., Cary, NC) software system. Statistical significance was based on two-sided P values. The analysis focused on premenopausal women because in previous analyses of these data, the negative associations of vitamin D and calcium with breast density were observed only among these women (7). The molar ratio IGF-I/IGFBP-3 was calculated as (0.130 × level of IGF-I in ng/mL) / (0.036 × level of IGFBP-3 in ng/mL) to better reflect availability of IGF-I in tissue (31).

Multivariate-adjusted means of breast density by category of vitamin D and/or calcium intakes or levels of growth factors were estimated using generalized linear models. The same model was used to obtain multivariate-adjusted mean levels of the natural logarithms of growth factors by category of these nutrient intakes, presented as back-transformed values. A log transformation of levels of growth factors was used to normalize the distribution of residuals.

Associations between continuous measurements (Table 1) were evaluated using generalized linear models and expressed as changes in the dependent variable for increases of 100 IU/d and 250 mg/d for vitamin D and calcium, respectively. To calculate the P values for trend in the analysis of combined intakes of both nutrients with continuous measure of breast density (Table 2), women were first cross-classified according to both tertiles of vitamin D and tertiles of calcium. Then, two variables were created. For the first variable, women were assigned a score of 1 if they were classified in tertile 1 of vitamin D intake and tertile 1 of calcium intake; a score of 2 if they were classified in tertile 2 of vitamin D intake and tertile 2 of calcium intake; a score of 3 if they were classified in tertile 3 of vitamin D intake and tertile 3 of calcium intake; and a value of 0 if they were classified in any of the other categories. For the second variable, a value of 0 was assigned to women who, for the first variable, had a score from 1 to 3, and a value of 1 if otherwise. Thus, when both of these two variables are in a model, the P values for the first variable provide a test for trend of the simultaneous increase in intakes of both nutrients.

The strength of associations of vitamin D and/or calcium to breast density among women with growth factors levels above the median was compared with that among women with levels at or below the median (Tables 3 and 4) by using the above models to which an interaction term between levels of growth factors (≤median/>median) and intakes of vitamin D and/or calcium was added. The P values of the interaction term between levels of growth factors (≤median/>median) and the continuous intakes of vitamin D and/or calcium were used to assess the effect modification of growth factors. For the analysis of combined nutrients (Table 4), some cells comprise very few women due to the high correlation between intakes of vitamin D and calcium from food (Pearson correlation coefficient = 0.73, $P < 0.0001$) or from food and supplements (Pearson correlation coefficient = 0.61, $P < 0.0001$). A similar approach was used to compare the strength of associations between levels of growth factors and breast density among women reaching adequate intakes of both vitamin D (≥200 IU/d) and calcium (≥1,000 mg/d) to that among women not reaching adequate intakes of vitamin D (<200 IU/d) and/or calcium (<1,000 mg/d; Table 5). These adequate daily vitamin D and calcium intakes are those recommended for women ages ≤50 years (32).

In the present analysis, factors included as potential confounders in all multivariate models are the same as in our previous study (7) and included several factors potentially related to breast density, intakes of vitamin D or calcium, as well as growth factors (see legend of tables). However, conclusions of this analysis remain essentially unchanged when minimum adjustment (such as for age, BMI, and energy intake) is made. Models using

Table 1. Relations of vitamin D and calcium intakes with breast density and IGFs in premenopausal women

	n	Mean of breast density (%)	Mean of IGF-I (ng/mL)*	Mean of IGFBP-3 (ng/mL)*	Mean of IGF-I/IGFBP-3 molar ratio*
Vitamin D and calcium from food only					
Vitamin D (IU/d)[†]					
<100.0	191	43.4	211.2	4,726	0.161
100.0-199.9	303	43.3	215.0	4,717	0.165
200.0-299.9	163	41.3	225.6	4,795	0.170
300.0-399.9	65	39.3	208.9	4,682	0.161
≥400.0	49	36.8	208.1	4,645	0.162
β (P) for 100 IU/d	771	−1.8 (0.008)	0.001 (0.92)	−0.002 (0.77)	0.003 (0.71)
β (P) for 100 IU/d[‡]	610	−2.1 (0.007)	−0.002 (0.84)	−0.003 (0.66)	0.001 (0.91)
Calcium (mg/d)[§]					
<500.0	116	44.2	211.0	4,643	0.164
500.0-749.9	236	44.0	215.8	4,704	0.166
750.0-999.9	178	42.0	213.6	4,746	0.163
1,000.0-1,249.9	121	42.7	217.2	4,768	0.164
≥1,250.0	120	36.5	219.0	4,793	0.165
β (P) for 250 mg/d	771	−1.8 (0.005)	0.013 (0.14)	0.007 (0.26)	0.007 (0.36)
β (P) for 250 mg/d[‡]	610	−2.4 (0.002)	0.010 (0.34)	0.010 (0.15)	−0.0003 (0.97)
Vitamin D and calcium from food and supplements					
Vitamin D (IU/d)[‖]					
<100.0	139	45.2	212.5	4,732	0.162
100.0-199.9	239	43.7	216.0	4,727	0.165
200.0-299.9	132	41.5	229.1	4,866	0.170
300.0-399.9	63	41.4	211.4	4,630	0.165
400.0-499.9	60	36.0	202.4	4,683	0.156
500.0-599.9	53	45.6	218.2	4,759	0.166
600.0-699.9	35	40.5	227.9	4,735	0.174
≥700.0	50	33.9	194.5	4,511	0.156
β (P) for 100 IU/d	771	−1.4 (0.004)	−0.008 (0.27)	−0.005 (0.28)	−0.003 (0.63)
β (P) for 100 IU/d[‡]	610	−1.9 (0.01)	−0.007 (0.53)	−0.005 (0.52)	−0.002 (0.81)
Calcium (mg/d)[¶]					
<500.0	78	46.6	207.9	4,614	0.163
500.0-749.9	209	44.1	214.6	4,681	0.166
750.0-999.9	165	42.4	217.6	4,843	0.162
1,000.0-1,249.9	126	43.5	216.0	4,747	0.164
1,250.0-1,499.9	90	39.8	217.6	4,772	0.165
1,500.0-1,749.9	59	35.1	212.9	4,557	0.169
≥1,750.0	44	34.8	219.4	4,824	0.164
β (P) for 250 mg/d	771	−1.9 (0.0004)	0.005 (0.53)	0.001 (0.79)	0.003 (0.56)
β (P) for 250 mg/d[‡]	610	−2.1 (0.002)	0.013 (0.19)	0.009 (0.14)	0.003 (0.68)

NOTE: β represents absolute mean decrease in breast density (%) or in growth factors (log ng/mL, if applicable). Means and β's are adjusted for alcohol (drinks/wk) and total energy (kcal/d) intakes in the past year, age (y), body mass index (BMI; kg/m^2), age at menarche (y), age at first full-term pregnancy (y), number of full-term pregnancies, number of personal breast biopsies, duration of past use of oral contraceptive and of hormone-replacement therapy (y), family history of breast cancer in first-degree relative (yes/no), physical activity (metabolic equivalents-h/wk), education (primary or less, secondary, college, or university diploma), and smoking status (never, former, or current smoker).

*Means of the natural logarithms of IGF-I, IGFBP-3, and IGF-I/IGFBP-3 molar ratio are presented as back-transformed values.

[†]Adjusted also for vitamin D intake from supplements (IU/d).

[‡]Among women not using multivitamins.

[§]Adjusted also for calcium intake from supplements (mg/d).

[‖]Adjusted also for vitamin D supplement use (yes/no).

[¶]Adjusted also for calcium supplement use (yes/no).

vitamin D or calcium intakes from food as independent variables were adjusted for intakes of vitamin D or calcium from supplements, respectively. Supplement use is recognized as being a behavioral marker for other factors related to cancer risk (33–35). To adjust for such factors, an indicator variable for current use of vitamin D or calcium supplements (yes/no) was included in multivariate models using vitamin D or calcium intakes from food and supplements as independent variables.

Results

Characteristics of the study population are described elsewhere (7, 15). Briefly, the 771 premenopausal women in the present study reported mean (±SD) daily dietary and total vitamin D intakes of 187.2 (±121.6) and 289.8 (±239.7) IU, respectively. Mean daily dietary and total calcium intakes were 856.8 (±361.9) and 981.2

(±445.3) mg, respectively. The proportions of vitamin D and calcium supplement users were 26.1% (n = 201) and 33.3% (n = 257), respectively. The proportion of women reaching adequate intakes of both vitamin D and calcium based on those recommended for women ages ≤50 years was 33.6% (n = 259) in the present study.

Table 1 shows that daily dietary or total intakes of vitamin D or calcium were not associated with levels of IGF-I, IGFBP-3, or the molar ratio in these premenopausal women.

Individual (Table 1) or combined (Table 2) vitamin D and calcium daily intakes from food only or from food and supplements were associated with lower breast density. These negative associations were similar or stronger after exclusion of women taking multivitamins, except the one for combined tertiles of vitamin D and calcium intakes from food and supplements.

The strength of the negative associations of vitamin D or calcium intakes from food with breast density varies according to levels of IGF-I and IGFBP-3 (Table 3). The negative associations of vitamin D or calcium from food with breast density were strong among women whose IGF-I levels were above median value ($\beta = -2.8$, $P = 0.002$ and $\beta = -2.5$, $P = 0.002$, respectively) but not among those whose IGF-I levels were below or equal to median value ($\beta = -0.8$, $P = 0.38$ and $\beta = -1.1$, $P = 0.21$, respectively) with P values for the interaction of 0.09 and 0.16, respectively. Similarly, the negative associations of vitamin D or calcium intakes from food with breast density were strong among women with levels of IGFBP-3 above the median value ($\beta = -2.7$, $P = 0.001$ and $\beta = -2.7$, $P = 0.0007$, respectively) but not among those with levels of IGFBP-3 below or equal to the median value ($\beta = -0.5$, $P = 0.61$ and $\beta = -0.6$, $P = 0.45$, respectively). The P values for the interaction were 0.06 and 0.03 for intakes of vitamin D and calcium from food, respectively. The effect modifications were similar after exclusion of women using multivitamins although were no longer statistically significant for intake of calcium from food (P value changes from 0.03 to 0.09).

Although not statistically significant, there were suggestions that IGF-I and IGFBP-3 levels modified the negative associations of calcium intake from food and supplements with breast density ($P = 0.14$ and $P = 0.24$, respectively) but not those of total intake of vitamin D with breast density ($P = 0.76$ and $P = 0.79$, respectively; Table 3). However, after exclusion of women using multivitamins, all of these multivariate-adjusted negative associations were stronger among women with levels of IGF-I or IGFBP-3 above median values than among those with levels of IGF-I or IGFBP-3 below or equal to median value (borderline significant for modification of calcium intake by IGF-I, $P = 0.06$).

The strength of the negative associations of dietary or total vitamin D and calcium intakes with breast density was reduced after further mutual adjustment in both levels of IGF-I and IGFBP-3. However, all modifying effects were largely unaffected by such adjustment (data not shown).

Table 2. Relations of the combined intakes of vitamin D and calcium with breast density in premenopausal women

Vitamin D and calcium from food only*

		Tertile of vitamin D (IU/d)			
		≤120.50	120.51-210.68	>210.68	
Tertile of calcium (mg/d)	≤655.08	45.6 (172)	43.1 (77)	47.2 (8)	
	655.09-979.48	41.5 (69)	42.0 (124)	41.5 (64)	
	>979.48	48.9 (16)	40.5 (56)	39.0 (185)	
					$P_{trend} = 0.01$
					$P_{trend} = 0.002$†

Vitamin D and calcium from food and supplements‡

		Tertile of vitamin D (IU/d)			
		≤144.33	144.34-302.93	>302.93	
Tertile of calcium (mg/d)	≤725.19	45.3 (164)	45.6 (71)	45.9 (22)	
	725.20-1,105.98	41.9 (76)	42.6 (114)	44.0 (67)	
	>1,105.98	47.3 (17)	36.3 (72)	38.3 (168)	
					$P_{trend} = 0.02$
					$P_{trend} = 0.11$†

NOTE: Values in cells are means of breast density (%) and the numbers in parentheses are the number of women in the category of the combined intakes of vitamin D and calcium. Means and linear models for trend are adjusted for alcohol (drinks/wk) and total energy (kcal/d) intakes in the past year, age (y), BMI (kg/m^2), age at menarche (y), age at first full-term pregnancy (y), number of full-term pregnancies, number of personal breast biopsies, duration of past use of oral contraceptive and of hormone replacement therapy (y), family history of breast cancer in first-degree relative (yes/no), physical activity (metabolic equivalents-h/wk), education (primary or less, secondary, college, or university diploma), and smoking status (never, former, or current smoker).

*Means and linear models for trend also adjusted for vitamin D (IU/d) and calcium (mg/d) intakes from supplements.

†Among women not using multivitamins.

‡Means and linear models for trend also adjusted for vitamin D and calcium supplement use (yes/no, respectively).

Table 3. Modifying effect of IGFs on the relation of vitamin D and calcium intakes with mammographic breast density in premenopausal women

	IGF-I (ng/mL)					IGFBP-3 (ng/mL)					IGF-I/IGFBP-3 molar ratio				
	≤ median = 218.4		> median = 218.4		$P_{interaction}$	≤ median = 4,694.9		> median = 4,694.9		$P_{interaction}$	≤ median = 0.1652		> median = 0.1652		$P_{interaction}$
	n	Mean of breast density (%)	*n*	Mean of breast density (%)		*n*	Mean of breast density (%)	*n*	Mean of breast density (%)		*n*	Mean of breast density (%)	*n*	Mean of breast density (%)	
Vitamin D and calcium from food only															
Vitamin D (IU/d)*															
<100.0	95	43.1	96	43.7		98	43.6	93	43.3		100	42.2	91	44.9	
100.0-199.9	158	42.7	145	44.1		157	45.8	146	40.7		154	41.6	149	45.1	
200.0-299.9	70	43.9	93	39.4		74	45.3	89	37.9		72	43.6	91	39.5	
300.0-399.9	36	36.6	29	42.5		34	41.1	31	37.1		34	32.8	31	46.2	
≥400.0	27	42.1	22	30.1		22	45.3	27	29.8		26	36.2	23	37.3	
β (P) for 100 IU/d	386	−0.8 (0.38)	385	−2.8 (0.002)	0.09	385	−0.5 (0.61)	386	−2.7 (0.001)	0.06	386	−1.7 (0.05)	385	−1.8 (0.04)	0.91
β (P) for 100 IU/d†	308	−0.9 (0.38)	302	−3.6 (0.0008)	0.04	313	−0.7 (0.46)	297	−3.3 (0.001)	0.06	302	−1.8 (0.07)	308	−2.4 (0.03)	0.66
Calcium (mg/d)‡															
<500.0	63	42.6	53	46.1		63	43.9	53	44.5		61	41.9	55	46.6	
500.0-749.9	117	44.0	119	44.0		122	46.1	114	41.9		113	44.2	123	43.7	
750.0-999.9	94	42.9	84	40.9		92	44.2	86	39.6		95	41.2	83	42.8	
1,000.0-1,249.9	56	42.8	65	42.6		55	45.2	66	40.5		59	40.3	62	45.1	
≥1,250.0	56	37.9	64	35.2		53	43.3	67	31.0		58	34.0	62	39.0	
β (P) for 250 mg/d	386	−1.1 (0.21)	385	−2.5 (0.002)	0.16	385	−0.6 (0.45)	386	−2.7 (0.0007)	0.03	386	−2.0 (0.02)	385	−1.7 (0.03)	0.76
β (P) for 250 mg/d†	308	−1.4 (0.15)	302	−3.3 (0.0006)	0.10	313	−1.2 (0.19)	297	−3.2 (0.001)	0.09	302	−2.5 (0.01)	308	−2.2 (0.02)	0.85
Vitamin D and calcium from food and supplements															
Vitamin D (IU/d)§															
<100.0	71	45.6	68	44.8		78	46.7	61	43.4		77	43.6	62	47.3	
100.0-199.9	127	43.6	112	43.9		124	46.4	115	41.0		117	41.2	122	46.1	
200.0-299.9	51	45.7	81	38.9		57	45.0	75	38.9		54	46.4	78	38.1	
300.0-399.9	35	37.4	28	46.5		34	44.1	29	38.1		31	35.0	32	47.5	
400.0-499.9	32	36.3	28	35.4		26	35.5	34	36.2		30	35.5	30	36.5	
500.0-599.9	24	46.8	29	44.4		25	50.4	28	40.8		28	45.7	25	45.8	
600.0-699.9	16	37.0	19	43.4		15	43.7	20	37.8		16	36.6	19	43.8	
≥700.0	30	34.7	20	32.5		26	35.6	24	31.9		33	33.0	17	35.7	
β (P) for 100 IU/d	386	−1.5 (0.008)	385	−1.3 (0.02)	0.76	385	−1.5 (0.01)	386	−1.3 (0.02)	0.79	386	−1.4 (0.01)	385	−1.4 (0.02)	0.97
β (P) for 100 IU/d†	308	−1.5 (0.07)	302	−2.6 (0.006)	0.28	313	−1.6 (0.06)	297	−2.2 (0.01)	0.49	302	−1.6 (0.05)	308	−2.3 (0.02)	0.49
Calcium (mg/d)‖															
<500.0	44	43.7	34	50.4		43	48.0	35	45.0		42	43.4	36	50.2	
500.0-749.9	105	44.4	104	43.7		113	46.4	96	41.6		97	43.6	112	44.5	
750.0-999.9	84	43.6	81	41.2		79	43.4	86	41.5		86	42.0	79	42.9	
1,000.0-1,249.9	56	43.2	70	43.8		60	45.7	66	41.6		62	42.3	64	44.9	
1,250.0-1,499.9	43	38.0	47	41.4		37	44.0	53	36.7		46	36.3	44	43.3	
1,500.0-1,749.9	33	37.7	26	31.9		35	38.9	24	29.0		30	32.2	29	38.1	
≥1,750.0	21	39.0	23	30.7		18	42.6	26	29.2		23	37.0	21	32.3	
β (P) for 250 mg/d	386	−1.3 (0.06)	385	−2.5 (0.0002)	0.14	385	−1.3 (0.05)	386	−2.3 (0.0005)	0.24	386	−1.7 (0.01)	385	−2.1 (0.002)	0.57
β (P) for 250 mg/d‡	308	−1.1 (0.21)	302	−3.0 (0.0004)	0.06	313	−1.4 (0.09)	297	−2.4 (0.005)	0.35	302	−1.8 (0.04)	308	−2.4 (0.004)	0.59

NOTE: β represents absolute mean decrease in breast density (%). Means and β's are adjusted for alcohol (drinks/wk) and total energy (kcal/d) intakes in the past year, age (y), BMI (kg/m^2), age at menarche (y), age at first full-term pregnancy (y), number of full-term pregnancies, number of personal breast biopsies, duration of past use of oral contraceptive and of hormone-replacement therapy (y), family history of breast cancer in first-degree relative (yes/no), physical activity (metabolic equivalents-h/wk), education (primary or less, secondary, college, or university diploma) and smoking status (never, former, or current smoker).

*Adjusted also for vitamin D intake from supplements (IU/d).

†Among women not using multivitamins.

‡Adjusted also for calcium intake from supplements (mg/d).

§Adjusted also for vitamin D supplement use (yes/no).

‖Adjusted also for calcium supplement use (yes/no).

Table 4. Modifying effect of IGFs on the relation of the combined intakes of vitamin D and calcium with breast density in premenopausal women

Vitamin D and calcium from food only*

		IGF-I ≤ median = 218.4 ng/mL				IGF-I > median = 218.4 ng/mL				$P_{interaction}$
		Tertile of vitamin D (IU/d)				Tertile of vitamin D (IU/d)				
		≤120.50	120.51-210.68	>210.68		≤120.50	120.51-210.68	>210.68		
Tertile of calcium (mg/d)	≤655.08	45.8 (89)	40.0 (39)	49.0 (4)		45.3 (83)	46.3 (38)	45.6 (4)		
	655.09-979.48	39.9 (38)	43.4 (68)	40.3 (30)		43.6 (31)	40.4 (56)	42.6 (34)		
	>979.48	56.6 (6)	36.9 (23)	41.5 (89)		44.0 (10)	42.8 (33)	36.5 (96)		
					P_{trend} = 0.24				P_{trend} = 0.009	0.28
					P_{trend} = 0.27†				P_{trend} = 0.0005†	0.06†
		IGFBP-3 ≤ median = 4,694.9 ng/mL				IGFBP-3 > median = 4,694.9 ng/mL				
		Tertile of vitamin D (IU/d)				Tertile of vitamin D (IU/d)				
		≤120.50	120.51-210.68	>210.68		≤120.50	120.51-210.68	>210.68		
Tertile of calcium (mg/d)	≤655.08	47.0 (96)	45.1 (41)	50.2 (3)		43.6 (76)	40.6 (36)	46.1 (5)		
	655.09-979.48	42.5 (31)	43.3 (70)	43.0 (31)		40.8 (38)	40.5 (54)	40.1 (33)		
	>979.48	45.9 (4)	43.3 (21)	44.9 (88)		49.8 (12)	38.7 (35)	33.6 (97)		
					P_{trend} = 0.50				P_{trend} = 0.003	0.06
					P_{trend} = 0.30†				P_{trend} = 0.0008†	0.05†

Vitamin D and calcium from food and supplements‡

		IGF-I ≤ median = 218.4 ng/mL				IGF-I > median = 218.4 ng/mL				$P_{interaction}$
		Tertile of vitamin D (IU/d)				Tertile of vitamin D (IU/d)				
		≤144.33	144.34-302.93	>302.93		≤144.33	144.34-302.93	>302.93		
Tertile of calcium (mg/d)	≤725.19	44.5 (89)	44.6 (31)	42.5 (13)		46.3 (75)	46.5 (40)	50.4 (9)		
	725.20-1,105.98	44.3 (37)	45.3 (57)	37.9 (34)		39.7 (39)	40.0 (57)	50.0 (33)		
	>1,105.98	46.5 (8)	39.1 (30)	39.1 (87)		48.5 (9)	34.4 (42)	37.3 (81)		
					P_{trend} = 0.14				P_{trend} = 0.02	0.42
					P_{trend} = 0.77†				P_{trend} = 0.02†	0.09†
		IGFBP-3 ≤ median = 4,694.9 ng/mL				IGFBP-3 > median = 4,694.9 ng/mL				
		Tertile of vitamin D (IU/d)				Tertile of vitamin D (IU/d)				
		≤144.33	144.34-302.93	>302.93		≤144.33	144.34-302.93	>302.93		
Tertile of calcium (mg/d)	≤725.19	47.8 (97)	46.2 (33)	46.3 (13)		41.8 (67)	45.2 (38)	45.1 (9)		
	725.20-1,105.98	43.5 (33)	45.1 (57)	42.5 (30)		40.8 (43)	40.1 (57)	45.3 (37)		
	>1,105.98	49.8 (9)	40.8 (33)	42.6 (80)		45.0 (8)	32.8 (39)	34.0 (88)		
					P_{trend} = 0.14				P_{trend} = 0.04	0.54
					P_{trend} = 0.48†				P_{trend} = 0.13†	0.47†

NOTE: Values in cells are means of breast density (%) and the numbers in parentheses are the number of women in the category of the combined intakes of vitamin D and calcium. Means and linear models for trend are adjusted for alcohol (drinks/wk) and total energy (kcal/d) intakes in the past year, age (y), BMI (kg/m^2), age at menarche (y), age at first full-term pregnancy (y), number of full-term pregnancies, number of personal breast biopsies, duration of past use of oral contraceptive and of hormone-replacement therapy (y), family history of breast cancer in first-degree relative (yes/no), physical activity (metabolic equivalents-h/wk), education (primary or less, secondary, college, or university diploma) and smoking status (never, former, or current smoker).

*Means and linear models for trend also adjusted for vitamin D (IU/d) and calcium (mg/d) intakes from supplements.

†Among women not using multivitamins.

‡Means and linear models for trend also adjusted for vitamin D and calcium supplement use (yes/no, respectively).

Table 5. Modifying effect of adequate intakes of both vitamin D and calcium on the relation of IGFs with mammographic breast density in premenopausal women

	<200 IU/d of vitamin D and/or <1,000 mg/d of calcium		≥200 IU/d of vitamin D and ≥1,000 mg/d of calcium		$P_{interaction}$
	n	Mean of breast density (%)	*n*	Mean of breast density (%)	
IGF-I (ng/mL)*					
Quartile 1: ≤179.5	134	41.3	59	37.9	
Quartile 2: 179.6-218.2	126	42.2	66	40.6	
Quartile 3: 218.3-260.9	127	43.9	66	43.0	
Quartile 4: >260.9	125	45.4	68	39.1	
P_{trend} for quartile	512	0.12	259	0.67	0.51
P_{trend} for quartile †	445	0.10	165	0.69	0.18
IGFBP-3 (ng/mL) ‡					
Quartile 1: ≤4,181.5	133	44.7	60	44.6	
Quartile 2: 4,181.6-4,694.9	128	46.0	64	44.5	
Quartile 3: 4,695.0-5,296.7	117	41.2	76	37.8	
Quartile 4: >5,296.7	134	40.6	59	34.4	
P_{trend} for quartile	512	0.05	259	0.002	0.13
P_{trend} for quartile †	445	0.02	165	0.005	0.23

NOTE: Means and linear models for trend of quartiles are adjusted for alcohol (drinks/wk) and total energy (kcal/d) intakes in the past year, age (y), BMI (kg/m^2), age at menarche (y), age at first full-term pregnancy (y), number of full-term pregnancies, number of personal breast biopsies, duration of past use of oral contraceptive and of hormone-replacement therapy (y), family history of breast cancer in first-degree relative (yes/no), physical activity (metabolic equivalents-h/wk), education (primary or less, secondary, college, or university diploma), smoking status (never, former, or current smoker), and vitamin D and calcium supplement use (yes/no, respectively).

*Adjusted also for IGFBP-3 (ng/mL).

†Among women not using multivitamins.

‡Adjusted also for IGF-I (ng/mL).

Table 4 shows the negative associations of combined vitamin D and calcium daily intakes with breast density according to circulating levels of growth factors. Among women with levels of IGF-I or IGFBP-3 above median values, the multivariate-adjusted means of breast density were progressively lower as vitamin D and calcium intakes from food (P_{trend} = 0.009 and P_{trend} = 0.003, respectively) or from food and supplements (P_{trend} = 0.02 and P_{trend} = 0.04, respectively) simultaneously increased. These trends were weaker among women who had below or equal to median values of IGF-I or IGFBP-3 (all $P_{trend} \geq 0.14$). The effect modification was similar or stronger after exclusion of women using multivitamins.

No effect modification of the levels of IGF-I/IGFBP-3 molar ratio on the relation of individual (Table 3) or combined (data not shown) vitamin D and calcium daily intakes from food or from food and supplements with mammographic breast density was observed.

Table 5 shows the associations of IGF-I or IGFBP-3 levels with breast density according to intakes of both vitamin D and calcium. Multivariate-adjusted mean breast density was higher with increasing quartiles of IGF-I levels among women not taking adequate intakes of vitamin D and/or calcium (41.3%, 42.2%, 43.9%, and 45.4%) and, with the exception of the last quartile, among those taking adequate intakes of both nutrients (37.9%, 40.6%, 43.0%, and 39.1%). However, none of these trends was statistically significant (P_{trend} = 0.12 and P_{trend} = 0.67, respectively). In contrast, the negative association of IGFBP-3 levels with breast density was stronger among women taking adequate intakes of both nutrients (44.6%, 44.5%, 37.8%, and 34.4%; P_{trend} = 0.002) compared with those not taking adequate intakes of vitamin D and/or calcium (44.7%, 46.0%, 41.2%, and 40.6%; P_{trend} = 0.05). However, statistical significance of the interaction term was not reached (P = 0.13). An effect modification of IGF-I was suggested after exclusion of women using multivitamins (P value changes from 0.51 to 0.18).

Among postmenopausal women, there was little or no association of vitamin D or calcium to breast density irrespective of the levels of IGF-I and IGFBP-3 (data not shown).

Discussion

These data support our a priori hypothesis that, among premenopausal women, higher intakes of vitamin D and calcium are associated with lower breast density but that this association is much stronger among those with high levels of IGF-I or IGFBP-3. Because mammographic breast density is strongly related to breast cancer risk (reviewed in ref. 36), our results suggest that the association of vitamin D and calcium with breast cancer risk may vary according to growth factor levels.

Vitamin D and calcium regulate expression of some members of the IGF family at the cellular or tissue level (17, 19–23), but whether these nutrients have little or no effect on circulating levels of IGF still needs to be clarified. In our study, we found no association of intakes of vitamin D or calcium with circulating levels of growth factors. These results are consistent with randomized placebo-controlled trials showing that increases in calcium supplementation through diet and/or supplements had little or no effect on

circulating IGF-I (37–39) and IGFBP-3 (37) levels among postmenopausal women. Moreover, DeLellis et al. (40) have found among postmenopausal women no association of vitamin D and calcium intakes from food with circulating IGF-I, IGFBP-3, or the molar ratio levels. However, in a study conducted among premenopausal and postmenopausal women of the Nurses' Health Study, Holmes et al. (41) observed that levels of IGF-I were positively associated with vitamin D and calcium intakes from food, but not from food and supplements. These authors also observed a positive association of IGFBP-3 levels with vitamin D intake from food and supplements. Further studies conducted among premenopausal women would help to clarify these associations.

Whenever the association of vitamin D intake with breast density (and perhaps breast cancer risk) is examined, calcium intake should be taken into consideration because the intakes of these nutrients are strongly correlated and share similar effects on breast tissue. For instance, both nutrients have been shown to inhibit cell proliferation and to induce differentiation and apoptosis in mammary cells (24, 25, 42–45). On one hand, the signaling effect of vitamin D is mediated by vitamin D receptor present in both normal mammary gland and breast cancer cells (22, 24, 46). In mice lacking vitamin D receptor, the lobuloalveolar development is accelerated and the postlactational involution is delayed (47). Moreover, laboratory and animal studies suggest that vitamin D (including its analogues) may suppress tumorigenesis of normal mammary epithelial cells (reviewed in ref. 22). On the other hand, the calcium-sensing receptor responds to changes in blood calcium levels and functional calcium-sensing receptors are expressed in normal and abnormal breast tissues (48). Moreover, disruption of calcium-sensing receptor function may contribute to the progression of neoplastic disease (48, 49). There is growing evidence that vitamin D and calcium activities are also interrelated. For instance, vitamin D regulates systemic and intracellular calcium homeostasis (50) and calcium has been shown to induce apoptosis in vitamin D–treated breast cancer cells (51). In addition, both promoters of the calcium-sensing receptor gene contain a vitamin D response element (52). Recently, results of a study conducted by Chakrabarty et al. (53) suggest that both calcium and vitamin D may stimulate calcium-sensing receptor expression in human colon carcinoma cells.

To our knowledge, this is the first epidemiologic study to examine the modifying effect of levels of IGF-I and IGFBP-3 on the relation of vitamin D and calcium intakes with mammographic breast density. Among cancer risk studies, we identified only one study showing that ff *Fok*I polymorphism in the vitamin D receptor gene was associated with a reduction of prostate cancer risk among men with high levels of IGFBP-3 but not among those with high IGF-I levels (26). In our study, levels of IGF-I and IGFBP-3 are highly correlated ($r = 0.56$, $P < 0.0001$); thus, it is difficult to establish if only one or both growth factors modify the strength of the relation of vitamin D and calcium intakes with breast density. In the present study, total IGF-I (free and bound) levels were measured and the molar ratio IGF-I/IGFBP-3 was estimated and used to reflect availability of IGF-I in tissue (31). Because >95% of circulating IGF-I is bound to IGFBP-3 (54), the lack of modifying effect of the molar ratio suggests that high levels of the IGFBP-3-IGF-I complex may confer the modifying effect instead of high levels of IGF-I alone. For instance, Ricort et al. (16) observed that IGFBP-3-IGF-I complex induces a larger calcium increase in breast cancer cells than does IGFBP-3 alone.

Given that mammographic breast density is strongly associated with breast cancer risk, our findings suggest that levels of growth factors should be taken into account when the effects of vitamin D and calcium on breast cancer risk are examined at least among premenopausal women. From a public health perspective, the identification of women who would benefit the most from increases in vitamin D and calcium intakes could be of great interest. Our results support the idea that, for breast cancer, premenopausal women with high levels of IGF-I or IGFBP-3 might be those who would benefit the most from increases of these promising breast cancer prevention agents.

A stronger positive association of IGF-I levels with breast density among women with lower daily intakes of vitamin D and calcium was expected on the basis of laboratory studies. However, this interaction did not reach statistical significance in our data. More research will be needed to determine if one of the roles of these nutrients on epithelial and/or stromal breast tissue could be, at least in part, to attenuate the proliferative effects of IGF-I. On the other hand, the stronger negative association between levels of IGFBP-3 and breast density among women taking adequate daily intakes of both nutrients suggests another role: to promote the antiproliferative effects of IGFBP-3 on breast tissue. Overall, vitamin D and calcium, directly or indirectly, may inhibit IGF-I and enhance IGFBP-3 effects in breast tissue, two activities suspected to reduce breast density and breast cancer risk (reviewed in ref. 10).

In the present study, misclassification of mammographic breast density and levels of IGF-I and IGFBP-3 are possible. However, all of these measurements were done without knowledge of the women's status on other variables and their reliability was shown to be high (15). Thus, the misclassification of these measures should be relatively small, most likely be random, and therefore should not have substantially biased our results.

Misclassification of vitamin D and calcium intakes is also likely. Although the reliability and validity of the food frequency questionnaire used here have been established (55), the translation of food intake into nutrient intake was based on U.S. rather than Canadian nutrient tables. Intake of nutrients from supplements is particularly vulnerable to misclassification. For intake from multivitamins, a generic code had to be used for supplements available in Canada but not in the United States. Moreover, women who answered that they were using supplements of vitamin D alone on a regular basis (at least once a week) were assumed to take such supplements every day and at a dose of 400 IU. In contrast, the daily dose of supplements of calcium alone was queried. This nondifferential misclassification of vitamin D and calcium intakes likely attenuated observed associations of intake from food only and, even more, those from food and supplements with breast density.

Whereas such random misclassification could not create the observed effect modifications, it may have attenuated some of them. The modifying effect of IGF-I or IGFBP-3 on the relation of calcium intake with breast density was observed when calcium derived from food only and calcium derived from food and supplements were considered. However, the modifying effect of IGF-I or IGFBP-3 on the relation of vitamin D intake with breast density was observed only when vitamin D derived from food only was considered. We believe that this discrepancy may be explained, at least in part, by the above-mentioned random misclassification of nutrients intakes from supplements, which was more pronounced for vitamin D than for calcium. Supplements accounted for 74% of the variance in vitamin D intake from food and supplements, compared with 34% for calcium.

When women taking multivitamins were excluded from the analyses, the reduction in breast density associated with elevated intakes of vitamin D or calcium was more pronounced and the modifying effect of growth factors was more evident. The decision to exclude multivitamin users was made to avoid two potential problems. First, exclusion of these women avoided some misclassification of vitamin D and calcium intakes from supplements as mentioned above. Second, residual confounding by nutrients contained in multivitamins and by behavioral factors associated with multivitamins use (33–35) could be reduced. Therefore, results among women who do not take multivitamins give, in our view, the most valid estimates of the modifying effects although this group of women is smaller and thus suffers from a reduction of statistical power.

In summary, our results are consistent with our a priori hypothesis. In premenopausal women, higher vitamin D and calcium intakes are associated with lower mammographic breast density but these associations were primarily observed in women with high circulating levels of IGF-I or IGFBP-3. These interrelations are plausible biologically but they need to be confirmed by other studies.

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