Insulin-like Growth Factor I (IGF-I), IGF-binding Proteins, and Breast Cancer

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Abstract

Epidemiological evidence supports a role for the insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) in the induction and progression of various cancers. Estrogen, which plays a role in the etiology of breast cancer, both regulates and is influenced by the IGF family. Risk of breast cancer associated with serum levels of IGF-I and/or IGFBPs may therefore depend upon menopausal status. A nested, case-control study was conducted on 66 women who were premenopausal and 60 who were postmenopausal at the time of diagnosis of primary breast cancer; they were selected from a cohort of 95,000 women who underwent multiphasic health check-ups > 30 years ago when enrolled in the Kaiser Permanente Medical Care Program. For each case, one control who matched by age, date of examination, and length of follow-up was chosen. Concentrations of IGF-I, insulin, glucose, and IGFBP-1, IGFBP-2, and IGFBP-3 in serum drawn at least 2 years before diagnosis (mean times of 10.5 and 15.8 years for pre- and postmenopausal cases, respectively) were compared using conditional logistic regression analysis. All statistical tests were two-sided. Serum IGF-I, adjusted for insulin, glucose, and body mass index, was weakly associated with breast cancer risk across quartiles for premenopausal women only (P for trend = 0.05). Serum IGFBP-3 was higher in premenopausal cases *versus* controls (P = 0.04) and showed a positive trend in risk for increasing quartiles (P for trend = 0.033). After adjusting for insulin, glucose, body mass index, and IGF-I, premenopausal women in the highest quartile of IGFBP-3 had an elevated risk of breast cancer [odds ratio (OR) = 5.28, 95% confidence interval (CI) = 1.13–24.7]. Conversely, IGFBP-3 was lower in postmenopausal cases *versus* controls (P = 0.04) but showed no significant trend in risk. Postmenopausal women with glucose levels in the diabetic range were at increased risk for developing breast cancer (OR = 2.06, 95% CI = 0.87–4.91), whereas those in the highest quartile of IGFBP-2 had a substantial reduction (71%) in risk relative to those in the lowest quartile (OR = 0.29, 95% CI = 0.09–0.92). Serum IGFBP-1 was not associated with breast cancer risk in either pre- or postmenopausal women. In premenopausal women, elevated serum IGF-I and IGFBP-3 are associated with increased breast cancer risk, whereas elevated serum IGFBP-2 is inversely associated with risk of postmenopausal breast cancer.

Introduction

A number of epidemiological studies implicate the IGF[2] family in the development and progression of cancer (recently reviewed in Refs. 1, 2). These studies cite as possible mechanisms the influence of IGF-I on the rate of epithelial cell proliferation and its role as a survival factor for transformed cells. Because nutritional intake is a strong determinant of IGF-I serum concentration (3), a potential programming effect of early nutrition on future cancer risk may be mediated by circulating levels of IGF-I.

Liver is the primary source of serum IGF-I, which peaks at puberty and declines with age. IGF-I is regulated by a family of six IGFBPs, which can inhibit the action of IGF-I by blocking its binding to the IGF-I receptor. Under some circumstances, however, the IGFBPs may increase IGF-Is bioavailability by protecting it from proteolysis and clearance or by delivering IGF-I to target tissues. IGFBPs can also exhibit IGF-independent activities (4).

IGFBP-3 is the major carrier of IGFs in serum and, of the six IGFBPs, is present in the highest concentration. Serum concentrations of IGFBP-3 are fairly constant throughout the day with a half-life of the IGF-I/IGFBP-3/acid-labile subunit ternary complex of 16 h (5). Although *in vitro* studies show both inhibition and potentiation of IGF activity, *in vivo* studies largely support the concept that IGFBP-3 provides a stable serum reservoir of bioactive IGF-I, thereby enhancing its growth-inducing effects (5, 6). Serum levels of IGFBP-2 are stable and unaffected by acute changes in metabolism but rise after a fast of many days (5, 7) and are age-dependent, with higher levels in infancy and old age and lower levels in young adults (8, 9). IGFBP-2 generally inhibits the activity of IGFs *in vitro* and *in vivo* (10), particularly of IGF-II, which it binds with 4-fold higher affinity than it does IGF-I (4, 5). IGFBP-1 levels are very dependent on metabolic status and on insulin that suppresses it; this dynamic pattern of regulation suggests a glucoregulatory role (6).

Several studies suggest important ways that IGFs and

Received 4/8/02; revised 8/6/02; accepted 9/23/02.
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[2] The abbreviations used are: IGF, insulin-like growth factor; BMI, body mass index; CV, coefficient of variability; E2, estradiol; IGFBP, IGF-binding protein; MHC, multiphasic health check-up; OGTT, oral glucose tolerance test; OR, odds ratio; CI, confidence interval.

estrogen, specifically 17β-E2, could interact in the development of breast cancer (reviewed in Refs. 11, 12). IGF-I and E2 synergistically increase cell proliferation *in vitro*: IGF-I is necessary for maximum estrogen receptor activation in breast cancer cell lines (11), whereas E2 up-regulates not only the genes for IGF-I and IGF-II (13) but also for IGF-I receptor and insulin-receptor substrate-1 (13, 14), which are required for IGF-I activity (13). E2 also significantly increases IGF-I and IGF-II mRNA *in vivo* in ovariectomized rhesus monkey mammary glands while decreasing IGFBP-2 mRNA levels (15). Therefore, the combined effect of the two tightly linked growth-regulatory pathways may stimulate proliferation in normal mammary epithelium, thereby increasing breast cancer risk.

Some disagreement exists regarding risk of breast cancer associated with the IGF family according to menopausal status. Several clinical case-control studies (16–18), but not all (19), and prospective studies (20, 21) generally support an increased risk of breast cancer in association with elevated serum levels of IGF-I in pre- but not postmenopausal women. However, one case-control study found higher levels of IGF-I in cases *versus* controls in women both under and over the age of 50 (22). The evidence regarding risk associated with IGFBP-3 is much less clear. A nonsignificant inverse association between IGFBP-3 and breast cancer risk in premenopausal women was noted by Hankinson *et al.* (20) and Bohlke *et al.* (18) but not by Toniolo *et al.* (21) who found no association, whereas Del Giudice *et al.* (19) and Vadgama *et al.* (17) found a positive association of IGFBP-3 with premenopausal risk. IGFBP-1 was found not to be associated with premenopausal breast cancer risk in one study (19) but has not been widely examined. Hyperinsulinemia was also positively associated with breast cancer in two case-control studies: one involving premenopausal (19) and the other both pre- and postmenopausal women (23). Some evidence suggests that diabetes is a risk factor as well particularly for older women (24, 25).

This report describes the results of a nested, case-control study in a cohort of women who underwent MHC $>$ 30 years ago when enrolled in the Kaiser Permanente Medical Care Program, which was undertaken to assess the association of circulating levels of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 with pre- and postmenopausal breast cancer risk.

Subjects and Methods

Subjects. This investigation used residual serum from a previous study conducted to determine whether high levels of high density lipoprotein cholesterol increase the risk of breast cancer (26). Briefly, the source population was a cohort of 95,000 women enrolled in the Kaiser Permanente Medical Care Program who underwent a routine MHC between 1964 and 1971, which comprised a battery of tests (described in Refs. 27, 28), including a 1-h OGTT. Two hundred cases, 100 diagnosed premenopausally and 100 diagnosed postmenopausally, were randomly selected from the $>$2000 breast cancer cases that subsequently developed in this cohort while still in the health plan through 1991. For each case, one control who matched on age, date of examination (within 1 year), and duration of follow-up was chosen. All subjects were white women with no history of previous breast cancer and not pregnant at the time of serum collection. The cases were diagnosed at least 2 years after blood draw, and menopausal status at the time of diagnosis was confirmed by medical record review. Postmenopausal status in this study refers to women without a natural menstrual cycle in the previous 12 months. Of this original study, 66 premenopausal and 60 postmenopausal case-control pairs had enough residual serum for the present investigation. In these groups, recognized risk factors (29, 30) were generally not statistically associated with breast cancer, but most available risk factors exhibited apparent associations of the expected magnitude and in the expected direction (data not shown). The source population and medical chart review are discussed in detail by Moorman *et al.* (26). Separate aliquots of serum from the postmenopausal group were previously analyzed for a study on vitamin D and breast cancer risk as well (31).

Materials and Methods

All analytes were measured in serum and assayed in duplicate using commercially available immunoassay methods. The intra- and interassay CVs were determined from two levels of quality control specimens provided by the manufacturer of the assay kits that were measured in duplicate in each assay along with the serum specimens. IGF-I was measured by radioimmunoassay after acid-ethanol extraction using kits from Nichol's Institute Diagnostics (San Capistrano, CA) (intra- and interassay CVs of 7.0 and 6.3%). Insulin and IGFBP-1, IGFBP-2, and IGFBP-3 were measured using kits from Diagnostics Systems Laboratories, Inc. (Webster, TX). The methods for IGFBP-1 and IGFBP-3 were immunoradiometric assays with intra-assay CVs of 7.8 and 5.3% and interassay CVs of 6.7 and 2.1%, respectively. Insulin was measured by ELISA with intra- and interassay CVs of 3.4 and 7.3%. IGFBP-2 was measured by radioimmunoassay with intra- and interassay CVs of 9.6 and 8.2%. The glucose values are the original values that were obtained on the date of the MHC. To verify this historical data, our laboratory measured glucose in the premenopausal samples using Sigma Diagnostics (Saint Louis, MO) Glucose (Trinder) reagent. The Pearson correlation coefficient (r) between the original MHC data and ours was 0.79.

The mean insulin and glucose values represent the 1-h time point post-75gm glucose challenge given at a minimum of 4 h after the last meal (mean time, 5.24 $\pm$ 1.5 h). Individuals with elevated 1-h glucose values were also drawn at the 2-h time point for glucose determination. OGTTs are no longer recommended for routine screening for diabetes and, if given, need to be verified by repeat testing on another day. Furthermore, the present day diagnostic glucose level for diabetes ($\geq$200 mg/dl) refers to the 2-h time point, not 1-h, post-75gm glucose challenge after an 8–12-h fast (32). Any interpretation of the data must be assessed with these historical limitations in mind. Serum IGFBP-1 levels, although responsive to insulin, remain unchanged at 1-h post-75gm oral glucose challenge and do not drop significantly until the 2.5-h time point (33). Therefore, the IGFBP-1 levels approximate reported fasting levels, which correlate very strongly ($r = 0.93$) with 24-h mean values (34).

Since samples were collected from 1964 through 1971 and stored at -23°C until 1980 then at -40°C until present, sodium concentration, which serves as an indicator of evaporative loss in the samples, was performed using a Beckman Electrolyte Model E2A instrument. Samples with evidence of desiccation were adjusted by multiplying the measured value of each analyte by the average sodium level in the study population (145 mM) divided by the individual sodium level; 7.5% of the samples (14 cases and 5 controls) were normalized by this method. Analyses conducted either including or excluding pairs where either case or control required adjustment yielded similar results, thus all pairs were retained.

Although the specimens were thawed once and then refrozen before this study, a report (9) shows that IGF-I and

Table 1 Baseline characteristics of breast cancer cases and controls by menopausal status

	Premenopausal			Postmenopausal		
	Cases $n = 66$	Controls $n = 66$	P	Cases $n = 60$	Controls $n = 60$	P
Age at MHC (range) yr	33.7 (19–46)	34.3 (19–52)		49.5 (29–73)	49.2 (28–73)	
Age at diagnosis (range) yr	44.2 (31–53)			65.3 (52–82)		
BMI (range)	24.0 (17–58)	23.1 (18–42)	0.22	24.5 (19–35)	24.2 (17–37)	0.96
IGF-I ng/ml	258 ± 86	244 ± 90	0.15	227 ± 71	243 ± 76	0.11
Insulin μIU/ml	66 ± 41	67 ± 55	0.58	51 ± 29	60 ± 41	0.33
Glucose mg/dl	170 ± 39	175 ± 45	0.60	191 ± 45	174 ± 50	0.10
IGFBP-1 ng/ml	46 ± 36	42 ± 33	0.70	44 ± 35	43 ± 29	0.98
IGFBP-2 ng/ml	376 ± 208	393 ± 226	0.78	470 ± 247	550 ± 253	0.10
IGFBP-3 ng/ml	2510 ± 700	2310 ± 670	0.04	2220 ± 530	2420 ± 660	0.04

IGFBP-3 withstand multiple (five) freeze-thaw cycles, which was also verified in our laboratory. Additionally, we measured IGF-I levels in 16 specimens after 29 years in frozen storage and the same specimens again 6 years later using the Nichol's IGF-I kit. The Pearson correlation coefficient (r) between the two measurements was 0.98 (unpublished data). Furthermore, our mean values and ranges for IGF-I, IGFBP-1, and IGFBP-2 compare favorably with those obtained for white women of similar ages in studies using serum that was in frozen storage for much shorter periods of time and using either the same or different methods of analysis (9, 16, 20, 21, 35), indicating minimal degradation during prolonged storage at −40°C. IGFBP-3 levels for both cases and controls, however, are marginally lower than previously reported (9, 18, 19, 21, 35), probably indicating some instability of this protein during prolonged storage because, in at least one instance (18), the same assay method as ours was used. However, as cases and controls were matched on date (within 1 year) of blood draw, any degradation that occurred would be expected to affect cases and controls equally.

Statistical Analysis. Because controls were matched to cases on age, baseline comparisons for BMI (weight in kilograms/height in meters^2), IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3, insulin, and glucose between the two groups were conducted using the Wilcoxon sign-rank test. (The parametric *t* test was also run on the log-transformation of the data; both approaches produced similar levels of significance.) Age-adjusted Pearson coefficients of correlation, which were obtained by combining measurements from all cases and controls because the blood samples were drawn before cancer diagnosis, were used to examine the cross-sectional relationships between natural log-transformed values for serum IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3, insulin, glucose, and untransformed BMI values. To examine changes and trends in risk, the distributions of IGF-I and IGFBP-1, IGFBP-2, and IGFBP-3 were split into quartiles. Quartiles were defined for pre- and postmenopausal women separately using case and control values. ORs were generated using the lowest quartile as the reference group. Trends in the crude ORs were determined using the score test, and CIs for crude ORs were computed using SEs derived as the square root of the variance of the score statistic (36).

For multivariate analysis, conditional logistic regression was used to maintain the case-control matching. These models were developed using approaches described by Hosmer and Lemeshow (37). All models were developed separately for IGF-I and the three IGFBP measures and included insulin, glucose, BMI, IGF-I (in the IGFBP analyses), and IGFBP-3 (in the IGF-I analysis). IGF-I and IGFBP measurements were entered into the models in three different ways depending on the question being addressed. To estimate the adjusted OR and associated CIs for each quartile relative to the lowest quartile, three design variables representing the four quartiles of IGF measures were modeled. To determine whether a trend in the ORs existed after adjusting for other covariates, a single IGF variable was entered along with the covariates. In this model, the IGF variable was ordinal with four levels, where each level was coded as the median of the quartile range. Finally, in the postmenopausal group, missing data for BMI precluded an estimate of the adjusted OR for the highest quartile of IGFBP-2. Therefore, BMI was not included as a covariate in the logistic model estimating the ORs for quartiles of IGFBP-2 but was included in the analysis of IGFBP-2 as a continuous variable by dropping pairs with missing data. For all models significance testing was conducted using the Wald statistic at a critical level of $P = 0.05$. All statistical tests were two-sided. Analyses were completed using Stata v7.0 (College Station, TX).

Results

Baseline characteristics are shown in Table 1. The mean time to diagnosis was 10.5 years for pre- and 15.8 years for postmenopausal cases. No statistically significant differences occurred between cases and controls for either group for BMI, IGF-I, insulin, glucose, IGFBP-1, or IGFBP-2. However, IGF-I tended to be nonsignificantly higher in premenopausal cases *versus* controls ($P = 0.15$), with the opposite trend occurring in the postmenopausal women ($P = 0.11$), which mirrored the differences in IGFBP-3. IGFBP-3 was significantly higher in premenopausal cases ($P = 0.04$) and lower in postmenopausal cases ($P = 0.04$) *versus* controls. This is not unexpected because levels of IGF-I and IGFBP-3 are both growth hormone dependent and interrelated (38). IGFBP-2 was also nonsignificantly lower in the postmenopausal cases ($P = 0.10$).

Table 2 shows the cross-sectional age-adjusted Pearson correlation coefficients for IGF-I, IGFBPs, insulin, glucose, and BMI. As expected, there was a positive correlation between IGF-I and IGFBP-3 ($r = 0.37$) but no strong correlation between IGF-I and IGFBP-1, IGFBP-2, insulin, glucose, or BMI. BMI correlated negatively with both IGFBP-1 ($r = -0.43$) and IGFBP-2 ($r = -0.31$) and positively with insulin ($r = 0.29$). In addition to the negative correlation with BMI, IGFBP-2 was also inversely correlated with both insulin ($r = -0.22$) and glucose ($r = -0.17$).

The mean insulin and glucose values (Table 1), representing 1-h post-75gm glucose challenge, indicate that both groups included women who would be considered glucose intolerant or diabetic based on comparisons to 1-h glucose values reported by Reaven *et al.* (39). In addition, postmenopausal cases tended

Table 2 Cross-sectional correlations between IGF-I, IGFBPs, insulin, glucose, and BMI

	Correlation[a]					
	IGF-I	IGFBP-1	IGFBP-2	IGFBP-3	Insulin	Glucose
IGFBP-1	−0.13 (<.05)					
IGFBP-2	0.05 (.42)	0.09 (.19)				
IGFBP-3	0.37 (<.001)	0.05 (.43)	0.13 (.05)			
Insulin	−0.06 (.33)	−0.07 (.30)	−0.22 (.001)	0.03 (.60)		
Glucose	−0.06 (.38)	0.07 (.30)	−0.17 (<.01)	0.03 (.65)	0.23 (<.001)	
BMI	−0.18 (<.01)	−0.43 (<.001)	−0.31 (<0.001)	−0.03 (.67)	0.29 (<.001)	0.10 (.15)

[a] Partial Pearson correlation coefficients for the natural log-transformed variables, adjusted for age (BMI and age were not transformed) for case and control subjects combined. Values in parentheses = *P*s for statistical tests.

Table 3 ORs for breast cancer by quartiles of serum protein measurements for premenopausal women

	Quartile				Quartile 4 95% CI	*P* for trend
	1	2	3	4		
IGF-I (ng/ml)						
Median	148	220	278	351		
Range	80–181	185–240	243–307	308–483		
Cases/controls	13/20	16/17	19/14	18/15		
OR, crude	1.0	1.45	2.09	1.85	0.68–5.01	0.18
OR, adjusted[a]	1.0	2.0	3.63	3.49	0.65–18.7	0.051
OR, adjusted[b]	1.0	1.82	2.65	2.01	0.33–12.4	0.24
IGFBP-1 (ng/ml)						
Median	9.7	24.9	42.2	94.9		
Range	1.2–18.8	19.2–34.7	35.8–63.3	63.8–147.2		
Cases/controls	18/15	12/21	18/15	18/15		
OR, crude	1.0	0.48	1.0	1.0	0.38–2.66	0.56
OR, adjusted[c]	1.0	0.53	2.06	2.40	0.61–9.51	0.18
IGFBP-2 (ng/ml)						
Median	175	269	406	630		
Range	67–217	218–346	349–485	494–1236		
Cases/controls	14/19	21/12	17/16	14/19		
OR, crude	1.0	2.38	1.44	1.00	0.37–2.67	0.54
OR, adjusted[c]	1.0	2.37	2.06	1.10	0.30–4.07	0.69
IGFBP-3 (ng/ml)						
Median	1650	2120	2690	3190		
Range	970–1868	1872–2410	2440–2880	2890–4670		
Cases/controls	13/20	16/18	17/15	20/13		
OR, crude	1.0	1.37	1.74	2.37	0.85–6.55	0.075
OR, adjusted[c]	1.0	0.41	2.26	5.28	1.13–24.7	0.033

[a] Adjusted for insulin, glucose, and BMI.
[b] Adjusted for insulin, glucose, BMI, and IGFBP-3.
[c] Adjusted for insulin, glucose, BMI, and IGF-I.

to be more glucose intolerant or diabetic with a higher mean glucose/insulin ratio than controls (191/51 = 3.8 *versus* 174/60 = 2.9, respectively). Using Reaven's (39) 1-h glucose ranges for normal (<165 mg/dl), borderline tolerant (165–185 mg/dl), and diabetic (>185 mg/dl), 28% of postmenopausal cases fell in the normal range compared with 38% of controls. Among premenopausal women, 38% of cases and 45% of controls were normal. A χ^2 test based on the distribution of postmenopausal cases and controls in the normal, borderline tolerant, and diabetic ranges (medians of 126, 177, and 216 mg/dl glucose, respectively) produced an elevated OR of 2.06 (95% CI = 0.87–4.91, $P = 0.095$; P for trend = 0.08) for the highest glucose level only. There was no evidence of a trend in risk with increasing glucose levels in the premenopausal group.

Fasting glucose and fasting insulin values are needed to use the homeostasis model to estimate insulin resistance (40). Moreover, there is considerable variability in the insulin response of any given individual on successive OGTTs (39) limiting the usefulness of a single 1-h value. C-peptide, used as an indicator of chronic insulin levels and a potential substitute for insulin in this analysis, was not measurable in these archived samples. Because of these limitations, whereas there was no apparent risk associated with increasing levels of insulin in either group (data not shown), we do not consider that the data allow conclusions to be drawn regarding risk associated with hyperinsulinemia or insulin resistance.

Unadjusted and adjusted ORs for breast cancer by quartiles of serum protein measurements derived from conditional logistic regression analyses of cases and controls matched on age for women who were premenopausal at the time of diagnosis are shown in Table 3. The trend for risk of breast cancer with increasing quartiles of IGF-I approached significance (P for trend = 0.051) only after adjusting for insulin, glucose, and BMI. The risk for the highest quartile of IGF-I relative to the lowest was 3.49 (95% CI = 0.65–18.7) after adjusting for insulin, glucose, and BMI, but the CI overlaps substantially with the unadjusted (0.68–5.01). Adjustment for IGFBP-3 levels, which were positively related to both IGF-I (Table 2) and

Table 4 ORs for breast cancer by quartiles of serum protein measurements for postmenopausal women

	Quartile				Quartile 4 95% CI	*P* for trend
	1	2	3	4		
IGF-I (ng/ml)						
Median	153	203	250	330		
Range	95–173	176–227	230–284	288–450		
Cases/controls	18/13	15/15	13/16	14/16		
OR, crude	1.0	0.72	0.59	0.63	0.23–1.76	0.37
OR, adjusted[a]	1.0	1.04	0.56	0.77	0.23–2.56	0.067
OR, adjusted[b]	1.0	1.27	0.38	1.22	0.21–6.78	0.74
IGFBP-1 (ng/ml)						
Median	9.4	30.8	49.2	76.4		
Range	0.8–20.9	21.3–35.7	36.6–56.2	58.3–158.2		
Cases/controls	17/13	15/15	11/19	17/13		
OR, crude	1.0	0.76	0.44	1.0	0.36–2.80	0.85
OR, adjusted[c]	1.0	1.07	0.54	1.96	0.35–10.9	0.75
IGFBP-2 (ng/ml)						
Median	245	402	539	810		
Range	18–324	339–473	477–631	651–1394		
Cases/controls	17/13	20/10	15/16	8/21		
OR, crude	1.0	1.53	0.72	0.29	0.09–0.92	0.007
OR, adjusted[d]	1.0	3.23	1.36	0.11	0.02–0.66	0.002
IGFBP-3 (ng/ml)						
Median	1660	2070	2410	3040		
Range	980–1870	1880–2230	2240–2620	2630–4250		
Cases/controls	16/14	17/13	17/13	10/20		
OR, crude	1.0	1.14	1.14	0.44	0.15–1.28	0.10
OR, adjusted[c]	1.0	1.22	0.97	0.32	0.07–1.41	0.09

[a] Adjusted for insulin, glucose, and BMI.
[b] Adjusted for insulin, glucose, BMI, and IGFBP-3.
[c] Adjusted for insulin, glucose, BMI, and IGF-I.
[d] Adjusted for insulin, glucose, and IGF-I.

breast cancer risk, reduced the risk associated with IGF-I (OR = 2.01, 95% CI = 0.33–12.4). Breast cancer risk also increased significantly with increasing quartiles of IGFBP-3 (*P* for trend = 0.033). Women in the highest IGFBP-3 quartile had a 5-fold risk of breast cancer relative to those in the lowest quartile (OR = 5.28, 95% CI = 1.13–24.7, *P* = 0.034) after adjusting for IGF-I, insulin, glucose, and BMI. It should be noted, however, that the wide CI is an indication of instability in the risk estimate. IGFBP-1 was not associated with risk whether adjusted for insulin or not.

The unadjusted and adjusted ORs for breast cancer by quartiles of serum proteins for women who were postmenopausal at the time of diagnosis are shown in Table 4. The only significant findings in this group involve IGFBP-2. Those in the highest quartile of IGFBP-2 had a 71% reduction in risk of breast cancer compared with the lowest quartile (OR = 0.29, 95% CI = 0.09–0.92, *P* = 0.025). The reduction in risk increased to 89% after adjustment for IGF-I, insulin, and glucose (OR = 0.11, 95% CI = 0.02–0.66, *P* = 0.016). Although the sample size is too small for statistical analysis, when we stratify the 60 postmenopausal pairs according to age at time of blood draw, the data suggest that the reduction in risk of postmenopausal breast cancer associated with IGFBP-2 was greatest for women who were ≤50 years of age (31 pairs) at the time of MHC [case/control IGFBP-2 quartile distributions: (*a*) 12/4; (*b*) 8/7; (*c*) 7/8; and (*d*) 4/12]; there was no corresponding relationship between IGFBP-2 and risk of breast cancer among women diagnosed premenopausally. Also, unlike the women who developed premenopausal breast cancer and had higher IGFBP-3 *versus* controls (Table 1), the women who were ≤50 years of age at blood draw and who developed postmenopausal breast cancer had lower IGFBP-3 levels *versus* controls (2134 *versus* 2523 ng/ml). The case/control IGFBP-3 quartile distributions for this younger subset were (*a*) 11/5; (*b*) 9/6; (*c*) 5/10; and (*d*) 6/10. IGF-I was not associated with risk in this subset (31 pairs) or the entire group combined (60 pairs). Not unexpectedly, merging the 31 younger pairs from the group that developed postmenopausal breast cancer with the 66 premenopausal case/control pairs resulted in no significant risk associated with either IGF-I or IGFBP-3. IGFPB-1 was not associated with risk in any analysis.

Table 5 shows ORs for breast cancer risk by serum protein levels treated as continuous variables according to menopausal status. The risk associated with each 100 ng/ml increase in IGF-I in premenopausal women was 1.33 (95% CI = 0.82–2.18, *P* = 0.24) and was not significant even after adjusting for covariates (OR = 1.70, 95% CI = 0.90–3.20, *P* = 0.10). The OR associated with each 1000 ng/ml rise in IGFBP-3 in premenopausal women was 1.12 (95% CI = 1.02–1.23, *P* = 0.02) after including insulin, glucose, IGF-I, and BMI in the analysis. The OR associated with each 100 ng/ml rise in IGFBP-2 in postmenopausal women was 0.76 (95% CI = 0.61–0.96, *P* = 0.02), which decreased to 0.67 (95% CI = 0.50–0.90, *P* = 0.008) after adjusting for insulin, glucose, IGF-I, and BMI.

Finally, because previous studies reported results for the IGF-I/IGFBP-3 ratio, we also considered this variable and found no evidence of an association of this ratio with breast cancer risk in either the pre- or postmenopausal women (data not shown).

Discussion

Our prospective data indicate that breast cancer risk related to IGF-I and its binding proteins differs according to menopausal status at the time of diagnosis. In agreement with most previous

Table 5 ORs for breast cancer by serum protein levels according to menopausal status

	Unit	Premenopausal		Postmenopausal	
		OR	95% CI	OR	95% CI
IGF-I					
Crude	100 ng/ml	1.33	0.82–2.18	0.67	0.38–1.20
Adjusted[a]	100 ng/ml	1.34	0.82–2.19	0.74	0.41–1.34
Adjusted[b]	100 ng/ml	1.70	0.90–3.20	0.77	0.38–1.57
IGFBP-2					
Crude	100 ng/ml	0.94	0.79–1.13	0.76	0.61–0.96
Adjusted[c]	100 ng/ml	0.97	0.82–1.14	0.85	0.70–1.03
Adjusted[d]	100 ng/ml	0.98	0.82–1.17	0.67	0.50–0.90
IGFBP-3					
Crude	1000 ng/ml	1.07	0.99–1.15	0.96	0.89–1.03
Adjusted[c]	1000 ng/ml	1.06	0.99–1.14	0.95	0.88–1.02
Adjusted[d]	1000 ng/ml	1.12	1.02–1.23	0.95	0.87–1.03

[a] Adjusted for insulin and glucose ($n = 66$ pairs premenopausal; $n = 55$ pairs postmenopausal).
[b] Adjusted for insulin, glucose, and BMI ($n = 53$ pairs premenopausal; $n = 44$ pairs postmenopausal).
[c] Adjusted for insulin, glucose, and IGF-I ($n = 66$ pairs premenopausal; $n = 55$ pairs postmenopausal).
[d] Adjusted for insulin, glucose, BMI, and IGF-I ($n = 53$ pairs premenopausal; $n = 44$ pairs postmenopausal).

reports (16–18, 20, 21), although not as robustly, high serum levels of IGF-I increased the risk of pre- but not postmenopausal breast cancer. This corresponded with a moderate elevation in risk with increased levels of IGFBP-3 again in premenopausal women only. In contrast, IGFBP-2 was significantly associated with reduced risk of postmenopausal breast cancer. To our knowledge, this is the first report to look for and find a protective effect of IGFBP-2 against breast cancer. Also, women with the highest glucose levels, and who were likely to be diabetic (41), were at increased risk of developing postmenopausal breast cancer in agreement with several other reports (24, 25). IGFBP-1 was not associated with risk in premenopausal women supporting a previous finding (19), and our data indicate no association in postmenopausal women as well.

Strengths of this study include the prospective design, unbiased selection of control subjects, and collection of blood samples at least 2 years before cancer diagnosis, which makes it unlikely that the results were affected by metabolic changes resulting from the disease. In fact, the long mean time between sample collection and diagnosis (10.5 and 15.8 years for pre- and postmenopausal cases, respectively) exceeds that of most prospective studies. Therefore, the value of the data rests to some extent on the degree to which a single determination of protein levels for an individual represents their long-term or chronic levels. Blood levels of IGF-I and IGFBP-3 have a relatively low intrasubject variation over time (42). Our laboratory measured IGF-I longitudinally in women over a 5-year period and reported a good intraclass coefficient of reliability of 0.69 (43). Long-term longitudinal data are not available for IGFBP-1 or IGFBP-2, but 1-year correlations have been reported for women ($r = 0.76$ and 0.62, respectively; Ref. 35).

A drawback is the relatively small size of the study; a larger sampling would have allowed us to differentiate the effects of pre- from postmenopausal levels of the IGF family on postmenopausal breast cancer risk. The age range of 29 to 73 years at the time of blood collection for those diagnosed with primary incident breast cancer after menopause clearly indicates that the group included both pre- and postmenopausal women. Also, the long interval between sampling and diagnosis increases the likelihood of unrecorded confounding events such as changes in BMI or the development of diabetes.

Most clinical (16–18) and prospective (20, 21) case-control studies support a positive relationship between plasma IGF-I and breast cancer risk primarily in premenopausal women. The risk associated with IGFBP-3 is less straightforward. Two clinical studies found that premenopausal women with early-stage operable breast cancer (16) or ductal carcinoma *in situ* (18) had lower levels of IGFBP-3 than controls and that the ratio of IGF-I to IGFBP-3 was most closely related to breast cancer risk, a finding also reported in a large prospective study nested within the Nurses' Health Study cohort (20). However, in the clinical studies, blood collection occurred after diagnosis, and disease effects on concentrations cannot be ruled out. In the prospective study, the mean time between blood collection and diagnosis was only 28 months (range, 1–57 months), suggesting that some women had undiagnosed breast cancer at the time of blood draw; the authors do note, however, that excluding women diagnosed in the first year after blood collection gave similar results (20). On the other hand, a clinical case-control study involving premenopausal women with node-negative invasive carcinoma of the breast found a positive association of breast cancer with IGFBP-3, which approached significance (OR = 2.05, 95% CI = 0.93–4.53; Ref. 19) and approximated our unadjusted risk estimate (OR = 2.37, 95% CI = 0.85–6.55). Another study examining subjects diagnosed a mean of 57 months (range, 7–102 months) after blood collection found no association between premenopausal breast cancer and IGFBP-3 or the IGF-I/IGFBP-3 ratio. However, when the analysis was confined to women who were diagnosed before age 50, comparable with our premenopausal group, the unadjusted ORs and 95% CIs for both IGF-I and IGFBP-3 agreed fairly closely with ours (1.91, 0.96–3.83 *versus* 1.85, 0.68–5.01, and 2.25, 1.08–4.69 *versus* 2.37, 0.85–6.55 respectively; Ref. 21). Our adjustment of IGFBP-3 for covariates increased the OR to 5.28 but produced a wide CI (1.13–24.7) and substantial imprecision. Therefore, the source population and timing of blood collection in relation to diagnosis are more critical for the analysis of IGFBP-3 than IGF-I. Confounding factors not yet recognized or considered may further explain some of the discrepancies that are also evident regarding IGFBP-3 and prostate cancer risk (44–47), ruling out a strictly gender effect.

The regulation of the ternary complex (IGF-I/IGFBP-3/acid-labile subunit) is complicated, and the role it plays in determining the level of bioavailable or free IGF-I is still unclear. The ternary complex does not pass through blood vessel walls and probably serves as a storage pool for IGF-I, delivering it to target tissues while protecting the IGF-I receptor from down-regulation (4). Uncomplexed IGFBP-3, on the other hand, is present in mammary tissue (48) where it may interact with its own membrane receptor to inhibit growth (49), induce apoptosis, or mediate the cell growth arrest induced by other molecules (50). However, as with serum levels, the weak and inconsistent relationship of tumor tissue levels of IGFBP-3 with breast cancer prognosis underscores the complexity of IGFBP-3 effects on cell proliferation (48). Speculating, at some point the reservoir function of the ternary complex may become less important than the local effects of free IGFBP-3. Nonetheless, our data indicate a moderate increase in risk for premenopausal breast cancer associated with elevated levels of IGFBP-3, supporting the hypothesis of a chaperone function for this binding protein.

The association of IGF-I and IGFBP-3, both growth hormone and nutrition dependent (3), with breast cancer risk in younger women is biologically plausible and conceptually supports the data that show elevated risk associated with leanness and greater height in premenopausal women (30). Maximal growth in early life,

driven largely by the growth hormone/IGF-I axis, may predispose women to breast cancer while E2 production is high.

Postmenopausal breast cancer, which is associated with obesity (30) not leanness, appears to be influenced by factors other than IGF-I and IGFBP-3. In postmenopausal women, we found no association between IGF-I and breast cancer risk in agreement with other prospective studies (20, 21), which may emphasize the importance of the interaction of E2 and IGF-I on breast epithelial cell proliferation in younger women. Interestingly, the postmenopausal cases even had nonsignificantly lower IGF-I, as reported by others (20, 21), and significantly lower IGFBP-3 as well; but reductions in risk associated with increasing quartiles of either IGF-I or IGFBP-3, even after mutual adjustment, were not significant. This may not be the case for IGFBP-3 levels in younger women ($\leq$50 years of age) who develop breast cancer in their menopausal years. Elevated IGFBP-3, which is a risk factor for premenopausal breast cancer, may reduce risk of postmenopausal breast cancer, perhaps through a long-term negative relationship with obesity or some other unrecognized association.

IGFBP-2, although only modestly lower in postmenopausal cases, proved to be significantly inversely associated with postmenopausal cancer in determining post-menopausal breast caner risk. Even though IGFBP-2 correlated negatively with BMI, ($r = -0.31$) risk reduction associated with elevated IGFBP-2 remained after adjustment for BMI, indicating a mechanism independent of reduced peripheral conversion of testosterone to E2 by aromatase in adipose tissue. When data for the women who developed postmenopausal breast cancer were separated according to age at time of blood collection, the difference between mean IGFBP-2 levels for cases and controls was greater for women who were age 50 years or under (438 *versus* 557 ng/ml) than over 50 years (507 *versus* 517 ng/ml; data not shown). This finding needs to be verified by a larger study but suggests that it is long-term or premenopausal levels of IGFBP-2, that may be an important factor in determining postmenopausal breast cancer risk.

IGFBP-2 was shown to be the only IGFBP, of those examined, responsive to sex-steroid regulation in monkey mammary glands; its expression was significantly decreased by *in vivo* E2 administration (15). Oral E2 supplementation in postmenopausal women, however, does not affect the levels of serum IGFBP-2 (51), which is derived primarily from the liver (5), indicating tissue-specific regulation of IGFBP-2 by E2. However, higher bioavailable E2 concentrations increase risk of breast cancer in older as well as in younger women (52), which may reflect, in part, the suppression of breast tissue IGFBP-2 expression by E2. IGFBP-2 tissue levels correlate positively with mammary epithelial apoptosis and negatively with proliferation (15).

IGFBP-2 binds IGF-II with ~4-fold higher affinity than IGF-I, and its level increases with age, inversely reflecting growth hormone status. Of potential importance, growth hormone administration suppresses serum levels of IGFBP-2 (7). The apparent protective effect of IGFBP-2 against breast cancer in older women, therefore, raises a question about the role, if any, that either circulating or locally produced IGF-II plays in postmenopausal breast cancer etiology. Unlike serum IGF-I, which declines gradually with age, serum levels of IGF-II remain fairly stable after puberty, at least according to cross-sectional data (9), are higher than IGF-I throughout life, and may become more important as the ratio of IGF-II to IGF-I increases with age. IGF-II could be relatively more important, as well, in younger individuals with low IGF-I, although the role IGF-II plays in postnatal life remains an enigma (53).

In monkey mammary glands, IGF-II is expressed in normal epithelium as well as in normal stroma and correlates more closely with proliferation than does IGF-I, whereas IGF-I correlates more closely with reduced apoptosis (15). Ellis *et al.* (54) hypothesize that the evolution of breast cancer involves the loss of IGF-I-expressing stroma and the induction of IGF-II-expressing stroma with IGF-II then acting in a paracrine manner. Yee *et al.* (55) showed that changes in the level of IGF-II mRNA correlated directly with E2-mediated changes in xenotransplant breast tumor growth, whereas Yu *et al.* (56) found levels of IGF-II in breast tumor tissue cytosol to correlate with unfavorable prognostic indicators.

No studies to date have found any significant association between serum levels of IGF-II and breast cancer risk. Although breast tissue levels of IGF-II, rather than circulating levels, may be an important factor in postmenopausal breast cancer risk, as well as during early breast cancer while the tumor is still growth factor-responsive (57), it might be worthwhile to reexamine serum levels of IGF-II relative to IGFBP levels in postmenopausal women. The (IGF-I + IGF-II)/IGFBP-3 molar ratio is remarkably constant. Therefore, although not growth hormone dependent, the level of total IGF-II is determined by the IGF binding capacity, principally that of IGFBP-3 (5). IGFBP-6, which has a strong preferential affinity for IGF-II (58), should probably be measured as well. We are currently investigating these and other areas.

In conclusion, our prospective study supports the hypothesis that breast cancer risk associated with IGF-I and its binding proteins varies according to menopausal status, perhaps signaling a shift in the relative importance of IGF-I/IGFBP-3 *versus* IGF-II/IGFBP-2 with age. Our data show that elevated IGF-I and IGFBP-3 are associated with risk of premenopausal breast cancer, whereas elevated IGFBP-2 is markedly associated with decreased risk of postmenopausal breast cancer. Higher circulating levels of an inhibitory binding protein such as IGFBP-2 over a lifetime may be biologically relevant. Additional work is needed to confirm these results, but if verified, potential lifestyle changes or pharmacological approaches to increase IGFBP-2 could be an area for future research.

Acknowledgments

We thank Dr. Paul Visintainer for the statistical analysis, Leslie Mauchline for technical assistance, Sylvia Duffy for manuscript preparation, and Nancy P. Durr for editing.

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