

Serum Insulin-Like Growth Factors, Insulin-Like Growth Factor Binding Proteins, and Breast Cancer Risk in Postmenopausal Women

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Abstract

Background: Studies have shown a positive association between serum insulin-like growth factor (IGF)-I and breast cancer risk in premenopausal but not postmenopausal women. IGF-II and estrogen receptor (ER) status has never been investigated. We examined the association between IGF-I, IGF-II, IGF binding protein (IGFBP)-2, IGFBP-3, and IGFBP-3 protease activity and breast cancer risk in postmenopausal women, taking ER status of the breast cancer into consideration.

Methods: We conducted this case-cohort study within a Danish follow-up study based on 24,697 postmenopausal women. We identified 411 cases with breast cancer and a matched control group including 397 cohort members. We estimated breast cancer risk using Cox regression analysis with adjustment for known breast cancer risk factors.

Results: We observed no association for IGF-I but a positive association between levels of IGFBP-3 and

breast cancer risk. Per 500 units higher levels of IGFBP-3, an incidence rate ratio [IRR; 95% confidence interval (95% CI)] of 1.14 (1.00-1.30) was estimated. For ER-positive breast cancer, the IRR (95% CI) was 1.18 (1.05-1.33). IGFBP-3 protease activity was not associated with breast cancer risk. Per 275 units higher levels of IGF-II, an IRR (95% CI) of 1.35 (1.10-1.66) was observed for ER-positive tumors, whereas IGFBP-2 was not associated with breast cancer risk. Adjustment for potential confounders did not change the risk estimate. There was no association between IGF-I, IGF-II, IGFBP-2, or IGFBP-3 and risk of ER-negative breast cancer.

Conclusion: Serum IGFBP-3 and IGF-II levels were positively associated with ER-positive breast cancer risk. This may suggest an important relationship among IGFs, IGFBPs, the ER system, and breast cancer development in postmenopausal women. (Cancer Epidemiol Biomarkers Prev 2004;13(11):1759-64)

Introduction

Insulin-like growth factor (IGF) and IGF binding protein (IGFBP) may play a role in the development and progression of various cancers including breast cancer (1).

The IGF system comprises low molecular weight peptides involved in physiologic cell proliferation and differentiation. Furthermore, they are mitotic, antiapoptotic, and proangiogenic, which are important qualities for tumor genesis in various organs including the breast. In the circulation and tissues, the IGFs are bound to specific IGFBPs (2). In the circulation, these IGFBPs are believed to prolong the half-life of the IGFs and to regulate the endocrine effects of the IGFs. At the cellular level, they are believed to modulate local actions of IGFs in both an inhibitory and a stimulatory fashion as well as having IGF-independent actions (3, 4). Accordingly,

addition of IGFBP-2 and IGFBP-3 *in vitro* increased cell proliferation in the MCF-7 breast cancer cell line (5). IGF-II is mitogenic in various breast cancer cell lines (6, 7). Further, IGFBP-2 binds IGF-II with high affinity and thus may modulate IGF-II actions. Proteases for the IGFBPs may change the affinity for IGF-I and IGF-II by cleavage of IGFBPs, thereby releasing IGFs to the receptors located on breast tissue cells (8).

In previous follow-up studies, serum IGF-I was generally positively associated with breast cancer risk in premenopausal (9-12) but not postmenopausal (9-13) women. The association between IGFBP-3 and breast cancer risk is less clear in both premenopausal and postmenopausal women (9-13). IGFBP-3 protease activity seems to be increased in breast cancer patients, with reduction following Tamoxifen treatment (14, 15).

To our knowledge, however, IGFBP-3 protease has not been examined in a prospective study, nor has IGF-II. In addition, none of the previous prospective studies concerning the IGF system and breast cancer risk included information on estrogen receptor (ER) status of the tumors. We therefore examined whether IGF-I, IGF-II, IGFBP-2, IGFBP-3, and IGFBP-3 protease activity were associated with breast cancer risk, taking into account the ER status of the breast cancer tumors.

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Materials and Methods

Study Population. We conducted this case-cohort study within the Danish follow-up study "Diet, Cancer and Health." From December 1993 to May 1997, all individuals who lived in Copenhagen or Aarhus areas and who fulfilled the inclusion criteria [ages 50-64 years; born in Denmark; and not having had no previous diagnosis of cancer registered in the Danish Cancer Registry (which collects information on all cancer diagnoses in Denmark)] at the time of invitation were invited to participate.

All participants completed a detailed food frequency questionnaire and a questionnaire regarding social status, health, and family history of cancer, reproductive factors, and lifestyle. Professional staff members did height and weight measurements.

At inclusion, blood (30 mL) was drawn from each participant. The samples were collected, nonfasting, in citrated and plain Venoject tubes, centrifuged, and divided into tubes of plasma, serum, erythrocytes, and buffy coat. All samples were processed and within 2 hours were frozen at -20°C . At the end of the day of collection, all samples were transferred into liquid nitrogen vapor-cooled containers at a maximum allowed temperature of -150°C .

Of the 79,729 women invited to participate in the follow-up study, a total of 29,875 women responded. Of these, 326 women were excluded who—although not identified in the Danish Cancer Registry with a cancer diagnosis at the preinvitation record linkage—did have an established cancer diagnosis prior to visiting one of the study clinics. Also excluded were eight women who had not completed the lifestyle questionnaire and 46 women for whom information regarding menopausal status or use of hormone replacement therapy (HRT) was missing.

Because the study aimed at evaluating only postmenopausal women, we excluded the 4,798 women who reported at least one menstruation within the last 12 months prior to entry into the study and no use of HRT and thus were classified as premenopausal at baseline. The remaining 24,697 women were classified as either "known postmenopausal" or "probably postmenopausal." The criteria for being assigned to the "known postmenopausal" group were oophorectomy, a reported age for menopause, or no natural menstruation during the last 12 months.

Cohort members were linked to the Central Population Registry for information on vital status and emigration by use of the unique personal identification number, a 10-digit civil registry number assigned to all Danish citizens shortly after birth.

Follow-up for breast cancer was initiated on the date of visit to the study clinic and continued until the date of diagnosis of any cancer (except nonmelanoma skin cancer), date of death, date of emigration, or until December 31, 2000, whichever came first. Cancers diagnosed during follow-up were identified by linkage to the Danish Cancer Registry. In addition to the general Danish Cancer Registry, a registry exclusively concerning breast cancer has been established. The Danish Breast Cancer Cooperative Group holds records on a range of details for $\sim 90\%$ of breast cancers diagnosed in Denmark, including ER status (16). The

cases were classified as "ER positive" if immunohistochemical staining showed $>10\%$ positive staining for ERs. This implies in the current context that "ER negative" should be interpreted more as ER poor than as complete absence of ERs. Linkage to the Danish Breast Cancer Cooperative Group registry was done by use of the personal identification number as well. During follow-up, a total of 434 cases were identified.

Cases and Matched Cohort Members. One control was selected for each of the cases matched on age at interview (half-year intervals), certainty of the postmenopausal status (known or probably postmenopausal), and use of HRT (current, former, or never) using a design corresponding to a nested case-cohort design within the cohort. The selected controls were then used as subcohort members in the case-cohort design with the modification that the controls did not enter the subcohort before the age at which they were included as study controls (i.e., at the age at breast cancer diagnosis for the corresponding cases). Each of the controls was included in the analyses from this delayed entry and until the end of follow-up for breast cancer.

Of the 866 women (434 cases and 434 controls including two cases), 9 cases and 37 controls were excluded because of missing data regarding potential confounders. A further 8 cases for whom information on IGFs were missing were excluded from the analyses, and for 6 cases, none of the controls in the match group were considered at risk at the age at diagnosis of the cases. This study therefore comprised 411 cases and a control group of 397 cohort members.

The "Diet, Cancer and Health" study and the substudy reported here were approved by the regional ethical committees on human studies and by the Danish Data Protection Agency.

IGF Data. Serum total IGF-I and IGF-II were determined using a noncompetitive time-resolved monoclonal immunofluorometric assay (DELFLIA) following acid ethanol extraction as described previously (17). Intra-assay and interassay coefficients of variation were $<5\%$ and $<10\%$, respectively. Serum IGFBP-3 was measured by an immunoradiometric two-site noncompetitive "sandwich" assay (Diagnostic System Laboratories, Inc., Webster, TX), with intraassay and interassay coefficients of variation at $<5\%$ and $<10\%$, respectively. Serum IGFBP-2 was determined by RIA (Diagnostic System Laboratories), with intraassay and interassay coefficients of variation both at $<10\%$. SDS-PAGE and Western ligand blot analysis of IGFBPs were done according to the method of Hossenlopp et al. (18) as described previously (19). Autoradiographs of ligand blots were scanned using a laser densitometer (Shimadzu, CS-9001PC, Shimadzu Corp., Kyoto, Japan) and the relative densities of the bands were measured as arbitrary absorbency units. Intraassay and interassay coefficients of variation were both $\sim 10\%$.

IGFBP-3 protease assay was done as described previously using human recombinant ^{125}I -IGFBP-3 obtained from Diagnostic System Laboratories. ^{125}I -IGFBP-3 ($\sim 30,000$ cpm) was incubated for 18 hours at 37°C with $5\ \mu\text{L}$ serum samples from controls and patients and subjected to SDS-PAGE (20). On each gel, internal control sera from normal controls and term-pregnant women were included. Electrophoresed gels were fixed in a

Table 1. Characteristics of cases and controls (subcohort) in the Danish "Diet, Cancer and Health" study, 1993-2000

	Cases		Subcohort	
	n = 411 (%)	Median (5th-95th percentiles)	n = 397* (%)	Median (5th-95th percentiles)
Nulliparous	59 (14.4)		50 (12.6)	
Benign tumor of the breast	83 (20.2)		54 (13.6)	
Use of HRT				
Never	138 (33.6)		135 (34.0)	
Past	52 (12.7)		52 (13.1)	
Current	221 (53.8)		210 (52.9)	
Duration of HRT (y) [†]		6 (0.5-20)		5 (0.5-21)
Years of school education				
≥7	118 (28.7)		137 (34.5)	
8-10	198 (48.2)		191 (48.1)	
>10	95 (23.1)		69 (17.4)	
Age at first birth (y) [‡]		23 (18-32)		23 (18-30)
BMI		24.84 (19.77-33.6)		24.66 (19.8-33.7)
Alcohol (g/d) [§]		11.20 (1.12-44.35)		10.46 (0.71-42.53)
IGF-I (μg/L)		126 (78-203)		124 (76-187)
IGF-II (μg/L)		1,039 (743-1,409)		1,008 (733-1352)
IGFBP-2 (μg/L)		412 (141-877)		406 (136-948)
IGFBP-3 (μg/L)		4,157 (2,996-5,564)		4,063 (2,865-5,470)

*Including two cases.

[†]Among current and past HRT users.

[‡]Among women who have had one or more children.

[§]Among current drinkers.

solution of 7% acetic acid, dried, and autoradiographed. The amount of proteolysis for each sample was given as the percentage of proteolytic cleavage products for each lane (*in vitro* proteolysis). Intraassay and interassay coefficients of variation were both ~10%.

Confounders. Data regarding potential confounders [parity, age of first birth, benign breast tumor, years of school education, body mass index (BMI), alcohol, and duration of HRT use] were investigated as described above and included in the statistical analysis.

Statistics. The possible associations between levels of IGFs and breast cancer risk were evaluated using Cox proportional hazards models. The estimation procedure followed the principles described by Barlow et al. (21), with the modification that the controls were only included in the risk sets from the time when they were individually matched. The analyses were done unweighed and stratified according to the sampling strata. Age was used as the time scale in the analyses. 95% Confidence intervals (95% CI) were based on Wald's tests using the robust estimate of the variance-covariance matrix for the regression variables in the Cox regression models.

The associations between levels of the IGFs and breast cancer incidence rates are presented crude, mutually adjusted, and adjusted for known risk factors for breast cancer such as parity (entered as two variables): the categorical variable (parous or nulliparous) and the linear variable (number of births), age at first birth (linear), history of benign breast disease (yes or no), school education (short, medium, or long), duration of HRT (linear in years), alcohol intake (linear), and BMI (linear).

The adequacy of the assumed linearity of the effect of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 in the Cox regression model was evaluated using linear splines with three boundaries placed at the 25th, 50th, and 75th percentiles among cases (22).

Results

Baseline characteristics for the cases and the controls (subcohort members) are presented in Table 1. Slightly larger proportions of cases than subcohort members had more education, were nulliparous, and had had a benign tumor of the breast. Only minor differences in the levels of serum IGFs and levels of IGFBPs were observed. Serum levels of IGF-I, IGF-II, IGFBP-3, and IGFBP-2 were within normal levels for this age group.

In Table 2, the associations among IGFs, IGFBPs, and breast cancer are presented as crude and mutually adjusted incidence rate ratio (IRR). No substantial associations were observed between levels of IGF-I or IGF-II and increased breast cancer risk. In contrast, there was a positive association between levels of IGFBP-3 and breast cancer risk. Adjustment for potential confounders did not change the risk estimates. IGFBP-2 levels were not associated with increased breast cancer risk.

Of the 411 cases in the present study, 285 women had an ER-positive tumor, 80 tumors were ER negative, and ER status was not established for 46 women. In Table 3, ER-positive and ER-negative tumors were considered separately, and regarding ER-positive breast cancer, we found positive associations between IGF-I and IGFBP-3 and breast cancer risk and no association with ER-negative breast cancer risk. However, when IGF-I and IGFBP-3 were mutually adjusted, only IGFBP-3 were positively associated with ER-positive breast cancer risk. Further, IGF-II levels were positively associated with ER-positive breast cancer risk, whereas IGFBP-2 levels were slightly negatively associated. Mutual adjustment for IGF-II and IGFBP-2 did not change the results. In relation to ER-negative breast cancer, a weak statistically insignificant negative association with IGF-II was observed, whereas a stronger positive association with IGFBP-2 was seen.

Table 2. IRR and 95% CI for postmenopausal breast cancer in relation to IGF-I, IGF-II, IGFBP-2, and IGFBP-3 in the Danish "Diet, Cancer and Health" study, 1993-2000

	Units	Crude IRR (95% CI)	Mutually adjusted* IRR (95% CI)	Mutually adjusted† IRR (95% CI)
IGF-I	25	1.04 (0.96-1.14)	0.98 (0.88-1.08)	0.97 (0.87-1.08)
IGFBP-3	500	1.11 (1.02-1.20)	1.12 (1.02-1.24)	1.13 (1.02-1.26)
IGF-II	275	1.20 (1.00-1.44)	1.20 (1.00-1.44)	1.19 (0.98-1.44)
IGFBP-2	340	0.97 (0.81-1.15)	0.99 (0.83-1.18)	1.03 (0.84-1.25)

*IGF-I and IGFBP-3 or IGF-II and IGFBP-2 are adjusted for each other, respectively.

†Adjusted for parity, age of first birth, benign breast tumor, BMI, years of school education, alcohol, and duration of use of HRT.

Increased IGFBP-3 protease activity may interfere with the IGFBP-3 measured by immunoradiometric assay; we therefore examined IGFBP-3 levels by Western ligand blotting and an IGFBP-3 protease assay was done in a total of 154 cases and respective controls. There was no difference in IGFBP-3 protease activity between cases [20.5 (2.2-32.4)] and controls [21.7 (2.5-30.2)] and no association with breast cancer risk [0.78 (0.52-1.15)].

Discussion

We found a positive association between serum IGFBP-3 and IGF-II levels and breast cancer risk in postmenopausal women. No associations were observed for IGF-I and IGFBP-2. When considering ER-positive and ER-negative tumors separately, IGFBP-3 and IGF-II levels were positively associated with ER-positive breast cancer risk, whereas IGF-II were weakly negatively and IGFBP-2 levels were weakly positively associated with ER-negative breast cancer risk. There was no evidence for an association between IGFBP-3 protease activity and breast cancer risk.

The strengths of the present study include the complete follow-up of study participants and a thorough collection of questionnaire data and blood samples obtained prior to development of cancer. The latter was ensured by linkage to the Danish Cancer Registry, and the social security number was also used for identifying ER status from the Danish Breast Cancer Cooperative Group, which holds data on ~90% of breast cancers in Denmark.

In our analysis, we have adjusted data for known risk factors for breast cancer and also mutually corrected data, as IGF-I and IGFBP-3 as well as IGF-II and IGFBP-2 may be associated. Although BMI may seem both as a confounder and as an intermediate variable in the causal pathway between IGFs and breast cancer, exclusion of BMI from the multivariate model did not change the risk estimates.

In the present study, serum IGFBP-3 was measured by immunoradiometric assay; however, this is only a correct estimate of IGFBP-3 levels in the absence of IGFBP-3 proteolysis, as the immunoradiometric assay measures both intact and fragmented IGFBP-3. Increased IGFBP-3 protease activity has been described in incident breast cancer patients (15, 23) and IGFBP-3 fragmentation was reduced in patients responding to Tamoxifen treatment, whereas nonresponders exhibited increased IGFBP-3 fragmentation (15, 23). We therefore examined IGFBP-3 proteolysis by Western ligand blot and a specific IGFBP-3 protease assay, and there was no evidence for increased IGFBP-3 proteolysis (data not shown).

Evidence for a role for IGFs and IGFBPs and breast cancer risk has emerged from *in vitro*, animal, and human studies (1). In general, human studies may be divided into follow-up studies and case-control studies. In the latter, blood samples are collected from cases at the time of diagnosis of breast cancer with the possibility that the breast cancer alters the IGF-system per se.

Four follow-up studies comprising premenopausal women (9-12) and five studies with postmenopausal women (9-13) who subsequently developed breast cancer have been published. In *premenopausal* women, IGF-I was

Table 3. IRR and 95% CI for postmenopausal breast cancer by ER status in relation to IGF-I and IGFBP-3 as well as IGF-II and IGFBP-2 in the Danish "Diet, Cancer and Health" study, 1993-2000

	Units	Crude IRR (95% CI)	Mutually adjusted* IRR (95% CI)	Adjusted† IRR (95% CI)	Mutually adjusted‡ IRR (95% CI)
ER positive					
IGF-I	25	1.11 (1.01-1.22)	1.02 (0.91-1.14)	1.12 (1.01-1.23)	1.01 (0.90-1.14)
IGFBP-3	500	1.17 (1.06-1.29)	1.16 (1.04-1.30)	1.19 (1.08-1.31)	1.18 (1.05-1.33)
IGF-II	275	1.35 (1.10-1.66)	1.32 (1.07-1.62)	1.36 (1.09-1.70)	1.34 (1.07-1.67)
IGFBP-2	340	0.81 (0.66-0.99)	0.94 (0.69-1.03)	0.79 (0.63-0.99)	0.82 (0.65-1.02)
ER negative					
IGF-I	25	0.90 (0.75-1.07)	0.87 (0.70-1.07)	0.87 (0.69-1.09)	0.85 (0.65-1.12)
IGFBP-3	500	0.99 (0.83-1.17)	1.07 (0.88-1.32)	0.96 (0.80-1.14)	1.05 (0.84-1.32)
IGF-II	275	0.90 (0.62-1.32)	0.95 (0.65-1.40)	0.87 (0.69-1.09)	0.92 (0.62-1.38)
IGFBP-2	340	1.30 (0.93-1.81)	1.29 (0.92-1.80)	1.34 (0.91-1.96)	1.32 (0.90-1.94)

*IGF-I and IGFBP-3 or IGF-II and IGFBP-2 are adjusted for each other, respectively.

†Adjusted for parity, age of first birth, benign breast tumor, years of school education, BMI, alcohol, and duration of use of HRT.

‡IGF-I and IGFBP-3 or IGF-II and IGFBP-2 are adjusted for each other, respectively, and also for parity, age of first birth, benign breast tumor, years of school education, BMI, alcohol, and duration of HRT use.

positively associated with breast cancer in three studies (9-11); no association was found in the study by Kaaks et al. (12). Serum IGFBP-3 was negatively associated with breast cancer in one study (10), positively associated in another (11), and not associated in the study by Kaaks et al. (12). Data for serum IGFBP-3 were not provided by Hankinson et al.; however, when correcting IGF-I for IGFBP-3, IGF-I was still positively associated with cancer risk (9). This may suggest that IGFBP-3 inhibits IGF actions possibly through reduced bioactive IGF-I. Data on ER status were not provided in any of the studies.

In *postmenopausal* women, serum IGF-I and IGFBP-3 levels were not associated with breast cancer risk in any of the published studies (9-13). In our study, when crude data were used, both IGF-I and IGFBP-3 were associated with increased risk of ER-positive breast cancer. The risk for IGF-I disappeared after adjusting for IGFBP-3, whereas IGFBP-3 remained a risk factor for breast cancer. This may suggest an important link between the IGF system, especially concerning IGFBP-3, and ER-positive breast cancer risk in postmenopausal women.

IGFBP-3 may have dual effects on IGF-I actions along with IGF-I-independent actions. From *in vitro* studies, IGFBP-3 may inhibit IGF-I-stimulated proliferation and apoptosis, but enhancement of IGF-I has also been described. Local proteases and not detectable in the circulation may attack IGFBP-3 and reduce the affinity for IGF-I with release of IGF-I in the extracellular environment of the tumor (24). Another mechanism may be IGFBP-3 cotransport of IGF-I into the cell nucleus where IGF-I may induce mitogenic signaling (25). For a comprehensive review of IGFBP-3 and breast cancer, see ref. 26.

This is the first follow-up study we are aware of where IGF-II was examined and no overall association with breast cancer risk was observed. However, considering ER-positive tumors, IGF-II was associated with increased breast cancer risk, whereas IGFBP-2 was not associated with breast cancer risk. This is in opposition to the study of Krajcik et al. (11) who observed an inverse relation between IGFBP-2 and breast cancer risk in postmenopausal women. Kaaks et al. (12) have assessed insulin, IGFBP-1, and IGFBP-2 in a subgroup from a prospective follow-up study composed of three different substudies, two from northern Sweden and one from southern Sweden with partly self-reported and retrospective data collection. They found no association with breast cancer risk; this has recently been confirmed for IGFBP-1 (13). However, fasting conditions were not secured by overnight fasting but only roughly registered as being 4 to 8 hours postprandially. This may reduce the validity of that study, especially for data on insulin and IGFBP-1, which are strictly dependent on nutritional status. IGFBP-2 is less dependent on fasting and was therefore used in the present study where we found no substantial association to breast cancer risk. Due to the nonfasting condition of the women in the present study, we did not measure IGFBP-1, insulin, or C-peptide.

One may speculate that the difference between premenopausal and postmenopausal women concerning IGF-I and IGFBP-3 and breast cancer risk is dependent on different effects of IGF-I and IGFBP-3 on breast tissues along with differences in menstrual status and estrogen levels. Interactions between IGF-I and estrogen have been shown in some studies (for review, see ref. 27). The

emerging model suggests that estrogen via its receptor may induce IGF-I expression. On the other hand, IGF-I may activate, via its specific receptor, a cascade of phosphorylations that subsequently activates nuclear transcription factors including the ER (28, 29). The balance between higher IGF-I and estrogen levels in premenopausal women may be absent or changed in postmenopausal women with very low estrogen levels and thus partly explain the observed difference in breast cancer risk and IGF-I levels in premenopausal and postmenopausal women. The increased IGFBP-3 levels associated with ER-positive breast cancer risk in postmenopausal women may be explained by either an increased availability of IGF-I to the IGF receptor delivered by IGFBP-3 or an IGF-independent action of IGFBP-3 per se.

In conclusion, we have shown a significant association between serum IGFBP-3 and ER-positive breast cancer risk in postmenopausal women without evidence of association between IGFBP-3 protease activity and breast cancer risk. As observed previously, serum IGF-I was not associated with breast cancer risk; however, as a novel finding, IGF-II was associated with increased breast cancer risk. This may suggest an important relationship among IGFs, IGFBPs, the ER system, and breast cancer development in postmenopausal women.

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