

# Circulating Insulin and C-Peptide Levels and Risk of Breast Cancer among Predominately Premenopausal Women

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## Abstract

Insulin and insulin resistance have been hypothesized to increase the risk of breast cancer as insulin increases breast cell proliferation and inhibits sex hormone binding globulin. Although insulin is directly related to body weight, adiposity is inversely associated with breast cancer risk in premenopausal women but directly related to risk in postmenopausal women. To explore the association between insulin and c-peptide levels and breast cancer risk, we conducted a nested case-control study of predominantly premenopausal women within the Nurses' Health Study II cohort. From 1996 to 1999, blood samples were collected from 29,611 participants. A total of 317 cases were diagnosed after blood collection and before June 2003 and matched to 634 controls; 75% of these women were premenopausal at

blood collection. Logistic regression models, controlling for breast cancer risk factors, were used to calculate relative risks (RR) and 95% confidence intervals (95% CI). Among women with fasting blood samples ( $n = 211$  cases), insulin was suggestively inversely associated with breast cancer risk (highest versus lowest quartile: RR, 0.5; 95% CI, 0.3-1.0;  $P_{\text{trend}} = 0.06$ ). Among all women, c-peptide was not associated with breast cancer risk (highest versus lowest quartile: RR, 1.1; 95% CI, 0.7-1.7;  $P_{\text{trend}} = 0.79$ ); results were similar among fasting samples. These associations did not differ by age, body mass index, or waist-to-hip ratio. Overall, higher levels of insulin and c-peptide were not associated with a higher risk of breast cancer among predominantly premenopausal women.

## Introduction

Experimental evidence suggests that insulin may be important in breast carcinogenesis, as insulin increases proliferation of breast cancer cells (1, 2). It also inhibits sex hormone binding globulin (SHBG) production (3), leading to an increase in bioavailable hormones. Higher free estradiol and testosterone levels have been associated with an increased breast cancer risk among premenopausal (4, 5) and postmenopausal women (6, 7).

During insulin secretion, proinsulin is cleaved into equimolar amounts of c-peptide and insulin. Although insulin levels fluctuate acutely with meals, c-peptide has a longer half-life and is therefore a useful marker of insulin secretion (8). In previous prospective studies of c-peptide or insulin levels, nonsignificant positive (9-11), null (12, 13), and inverse (14) associations have been observed, with conflicting findings by menopausal status (10, 11, 13).

We conducted a nested case-control study within the Nurses' Health Study II to examine the associations of insulin and c-peptide with breast cancer risk among 317 cases and 634

matched controls, the majority of whom were premenopausal at blood collection.

## Materials and Methods

**Study Population.** The Nurses' Health Study II was established in 1989 when 116,609 female registered nurses, ages 25 to 42 years, completed and returned a mailed questionnaire. The cohort has been followed biennially to update exposures and ascertain newly diagnosed disease. Between 1996 and 1999, 29,611 cohort members who were cancer-free and between the ages of 32 and 54 years provided blood samples (described in ref. 15). Briefly, participants were sent a short questionnaire and a blood collection kit containing necessary supplies to have blood samples drawn by a local laboratory or a colleague. Premenopausal women who had not taken oral contraceptives, been pregnant, or breast-fed within 6 months ( $n = 18,521$ ) provided blood samples drawn on the 3rd to 5th day of their menstrual cycle (follicular draw) and 7 to 9 days before the anticipated start of their next cycle (luteal draw). All other women ( $n = 11,090$ ) provided a single 30-mL, untimed blood sample. In the current analysis, only luteal and untimed samples were used; these samples were collected in a similar manner, shipped via overnight courier with an ice pack to our laboratory, and separated into plasma, RBC, and WBC components. Samples have been stored in liquid nitrogen freezers since collection. Menopausal status determination for women providing untimed samples has been described previously (15). Follow-up of the blood cohort was 98% in 2003. The study was approved by the Committee on the Use

of Human Subjects in Research at Harvard School of Public Health and Brigham and Women's Hospital.

Breast cancer cases were identified on biennial questionnaires; the National Death Index was searched for non-responders. Cases had no previously reported cancer diagnosis and were diagnosed with breast cancer after blood collection but before June 1, 2003. Overall, 317 cases of breast cancer were reported and confirmed by medical record review ( $n = 298$ ) or by the nurse herself ( $n = 19$ ). Given the 99% confirmation rate upon medical record review, these latter cases were included. Mean time from blood draw to diagnosis was 31 months (range = 1-87). Each case was matched to two controls (total  $n = 634$ ) on age ( $\pm 2$  years), menopausal status at blood collection and diagnosis (premenopausal, postmenopausal, unknown), month/year of blood draw ( $\pm 2$  months), race/ethnicity (African American, Asian, Hispanic, Caucasian, Other), luteal day (timed samples only, date of next period - date of luteal draw,  $\pm 1$  day), and for each blood collection, time of day ( $\pm 2$  hours), and fasting status ( $< 2$  h, 2-4, 5-7, 8-11,  $\geq 12$ ). For each matching variable,  $> 90\%$  of matches were exact.

**Laboratory Assays.** Insulin and c-peptide were assayed in luteal and untimed samples. Insulin was measured in fasting samples only ( $n = 211$  cases and 414 controls) by RIA (Linco Research, Inc., St. Charles, MO) in the laboratory of Dr. Christos Mantzoros. c-Peptide was assayed using ELISA (Diagnostic Systems Laboratory, Webster, TX) in the laboratory of Dr. Michael Pollak. Estradiol and testosterone were assayed at Quest Diagnostics (San Juan Capistrano, CA) by RIA following extraction and celite column chromatography. Free estradiol and testosterone were calculated per Sodergard et al. (16). SHBG was measured at the Royal Marsden Hospital by chemiluminescent immunoassay with the Immulite auto-analyzer (Diagnostic Products, Gwynedd, United Kingdom).

Case-control sets were assayed together and were ordered randomly and labeled to mask case-control status. Samples were assayed in two batches; the inter-assay coefficients of variation from masked replicate samples were  $< 6\%$  for insulin and c-peptide and  $< 14\%$  for estradiol, SHBG, and testosterone.

**Statistical Analysis.** We identified and excluded statistical outliers [ref. 17; insulin  $> 40$   $\mu\text{U}/\text{mL}$  ( $n = 8$ ), luteal free estradiol  $> 10.6$   $\text{pg}/\text{mL}$  ( $n = 1$ )]. Several samples had missing hormone values related to technical difficulties or low sample volume; the final case/control sample sizes for insulin and c-peptide analyses were 208/409 and 316/629, respectively. Quartile cut points and Spearman correlation coefficients were based on control distributions. We used mixed effects regression, by case-control set to account for matching, to test the paired differences in log-transformed hormone levels between cases and controls.

We used conditional logistic regression to estimate relative risks (RR) and 95% confidence intervals (95% CI). Multivariate models adjusted for common breast cancer risk factors, including body mass index (BMI) at age 18, BMI at blood collection, ages at menarche and first birth, parity, family history of breast cancer, and history of benign breast disease. We used unconditional logistic regression, adjusting for matching factors, in stratified analyses; results from multivariate unconditional and conditional logistic regression models were essentially identical. Tests for trend were conducted by modeling continuous quartile median concentrations and calculating the Wald statistic. Tests for interaction compared the slope of the quartile medians between groups (Wald test). All  $P$ s were based on two-sided tests and were considered statistically significant if  $< 0.05$ .

## Results

Characteristics of cases and controls have been published previously (15, 18). Briefly, mean age at blood collection was 45 years. Cases had slightly lower BMI than controls at blood

collection (25.3 versus 25.7  $\text{kg}/\text{m}^2$ ). Cases had a higher prevalence of family history of breast cancer (16% versus 10%) and history of benign breast disease (24% versus 16%). Characteristics among women who were fasting at the time of blood collection were similar. Among all women, 75% were premenopausal at blood collection; 71% of the fasting women were premenopausal.

Insulin and c-peptide levels were similar between cases and controls (Table 1). c-Peptide levels were lower among women who gave fasting blood samples and, again, similar between cases and controls. Insulin and c-peptide levels were both moderately correlated with BMI ( $r = 0.41$  and  $0.40$ , respectively) and fairly strongly correlated with one another ( $r = 0.68$ ; Table 2). Both insulin and c-peptide were modestly, but significantly, inversely associated with follicular total estradiol and total testosterone (insulin:  $r = -0.24$  and  $-0.12$ , respectively; c-peptide:  $r = -0.19$  and  $-0.20$ , respectively). Insulin and c-peptide were significantly positively associated with free testosterone ( $r = 0.24$  and  $0.20$ , respectively) and inversely associated with SHBG ( $r = -0.36$  and  $-0.39$ , respectively).

In the simple conditional model, insulin levels were nonsignificantly inversely associated with breast cancer risk (top versus bottom quartile: RR, 0.7; 95% CI, 0.4-1.1;  $P_{\text{trend}} = 0.20$ ; Table 3). Multivariate adjustment resulted in a lower point estimate in the fourth quartile (largely due to adjustment for BMI) and a nearly significant trend (RR, 0.5; 95% CI, 0.3-1.0;  $P_{\text{trend}} = 0.06$ ). Results were nearly identical among only premenopausal women. When restricting to invasive ( $n = 148$ ) or estrogen/progesterone receptor-positive ( $n = 99$ ) cases, results again were similar, although the trends were less clear ( $P_{\text{trend}} = 0.25$  and  $0.11$ , respectively). Adjustment for total or free estradiol among premenopausal women with timed samples attenuated the association (e.g., RR, 0.7; 95% CI, 0.3-1.5;  $P_{\text{trend}} = 0.56$  with follicular estradiol). Adjustment for androgens or SHBG did not alter the results.

c-Peptide levels were not significantly associated with breast cancer risk, either in the simple (top versus bottom quartile: RR, 1.0; 95% CI, 0.7-1.6;  $P_{\text{trend}} = 0.73$ ) or multivariate-adjusted (RR, 1.1; 95% CI, 0.7-1.7;  $P_{\text{trend}} = 0.79$ ) model (Table 3). Results were similar among premenopausal women and when cases were restricted to invasive ( $n = 218$ ) tumors. There was a significant increased risk in the top quartile among estrogen/progesterone receptor-positive tumors (RR, 2.0; 95% CI, 1.0-3.9), but the trend was not significant ( $P = 0.15$ ). When we restricted the analysis to fasting samples, the overall results were not altered when using either overall quartile cut points (top quartile  $\geq 2.29$   $\text{ng}/\text{mL}$ : RR, 1.1; 95% CI, 0.6-2.2;  $P_{\text{trend}} = 0.87$ ) or fasting-specific cut points (top quartile  $\geq 1.82$   $\text{ng}/\text{mL}$ : RR, 1.2; 95% CI, 0.6-2.1;  $P_{\text{trend}} = 0.62$ ). When we adjusted for sex steroid hormones and SHBG, the results were unchanged with the exception of adjusting for luteal estradiol (RR, 1.8; 95% CI, 0.9-3.8;  $P_{\text{trend}} = 0.24$ ) and luteal free estradiol (RR, 1.7; 95% CI, 0.8-3.6;  $P_{\text{trend}} = 0.31$ ).

**Table 1. Plasma insulin and c-peptide levels according to case/control status**

Hormone	Cases		Controls		$P^*$
	$n$	Median (range <sup>†</sup> )	$n$	Median (range <sup>†</sup> )	
Insulin ( $\mu\text{U}/\text{mL}$ )					
Fasting only	208	7.22 (5.23-11.89)	409	7.50 (5.48-12.48)	0.57
c-Peptide ( $\text{ng}/\text{mL}$ )					
All	316	1.46 (0.84-3.02)	629	1.49 (0.84-3.19)	0.75
Fasting only	210	1.36 (0.77-2.42)	410	1.35 (0.81-2.35)	0.88

\* $P$  from mixed-effects regression models comparing cases with controls, controlling for matching factors (two sided).

<sup>†</sup>From the median of the bottom quartile (12.5%) to the median of the top quartile (87.5%).

**Table 2. Spearman correlations for body mass index and sex steroid hormones with insulin and c-peptide levels among controls\***

	Insulin	c-Peptide
Insulin	1.0	<b>0.68</b>
c-Peptide	<b>0.68</b>	1.0
BMI	<b>0.41</b>	<b>0.40</b>
Estradiol, follicular	-0.24	-0.19
Estradiol, luteal	-0.10	-0.16
Free estradiol, follicular	-0.11	-0.06
Free estradiol, luteal	0.12	0.07
Testosterone	-0.12	-0.20
Free testosterone	<b>0.24</b>	<b>0.20</b>
SHBG	-0.36	-0.39

NOTE: Numbers in boldface denote  $P$ s < 0.05.

\* All variables measured continuously.

The associations among insulin, c-peptide, and breast cancer risk also were similar among cases with ductal tumors and by tumor size ( $\leq 2$  versus  $> 2$  cm; data not shown). No substantial differences were observed when stratifying by age, BMI, waist-to-hip ratio, or time since diagnosis. Additionally, exclusion of cases diagnosed in the first 2 years following blood draw did not change the results. When we included both insulin and c-peptide in the same model, insulin results were unchanged, and the RR for the top quartile of c-peptide increased to 1.4 (95% CI, 0.6-3.2), although the trend was still nonsignificant ( $P = 0.44$ ).

## Discussion

In this nested case-control study among predominantly premenopausal women, we did not observe increased breast cancer risks with higher levels of insulin or c-peptide. Insulin levels were suggestively inversely associated with risk, but a similar finding was not observed with c-peptide, which is a marker of insulin secretion.

Insulin and insulin resistance have been hypothesized to increase breast cancer risk and may factor into the association between weight and breast cancer risk. Insulin may affect risk independently because it increases proliferation in breast cancer cells, and because the insulin receptor is expressed in normal and malignant breast tissue (1, 2). Alternatively, insulin may affect risk indirectly by inhibiting SHBG produc-

tion (3) and increasing circulating ovarian androgens (19). Finally, insulin increases the bioavailability of insulin-like growth factor I, which may be associated with breast cancer (20), by inhibiting the synthesis of the insulin-like growth factor binding protein 1 (21). Although these hypotheses indicate a role for insulin in breast carcinogenesis, the inverse association between adiposity and breast cancer among premenopausal women (22) suggests that the association between insulin and breast cancer may be complex in premenopausal women.

Results from observational studies have been mixed. Positive associations with diabetes have been reported in prospective studies among postmenopausal women or all women combined (23), but no associations were observed among premenopausal women in two studies (24, 25). In the Women's Health Study, higher levels of hemoglobin A1c were significantly inversely associated with risk among postmenopausal, but not premenopausal, women (26). Nonsignificant positive (9-11), null (12), and inverse (13, 14) associations have been observed with c-peptide (9, 10, 13) or insulin (11, 12, 14) in prospective studies. In the most recent study, within the EPIC cohort, c-peptide was inversely associated with risk among younger women ( $\leq 50$  at diagnosis; top versus bottom quintile: RR, 0.7; 95% CI, 0.4-1.2;  $P_{\text{trend}} = 0.05$ ; ref. 13), similar to the estimate for insulin in a Swedish study (top versus bottom quartile: RR, 0.6; 95% CI, 0.3-1.2;  $P_{\text{trend}} = 0.31$ ; ref. 14). Although we observed an inverse association between insulin levels and risk, the lack of trend and the inconsistency with the c-peptide results suggests this may be a chance finding. We would expect similar results for the two biomarkers given that proinsulin is cleaved into equimolar amounts of insulin and c-peptide, and we used fasting insulin levels to avoid the acute fluctuations with meals (8). An inverse association among premenopausal women is possible if insulin is merely a marker of adiposity, and adjustment for BMI in the EPIC analyses did attenuate the estimate to the null (13). However, adjustment for BMI in our analyses resulted in a slightly stronger association. Given the inverse correlations of insulin and c-peptide with estradiol, and the positive association we observed between estradiol and breast cancer risk (5), it is logical that adjustment for estradiol increased the RRs for both insulin and c-peptide, but neither trend was significant.

**Table 3. RR and 95% CI of breast cancer by quartiles of circulating insulin and c-peptide levels**

Plasma hormone	Cases/controls	Q1	Q2	Q3	Q4	$P_{\text{trend}}$
<b>Insulin</b>						
Cut points ( $\mu\text{U}/\text{mL}$ )		$\leq 6.15$	6.16-7.50	7.51-9.91	$\geq 9.92$	
All (simple*)	208/409	1.0	0.7 (0.4-1.1)	0.8 (0.5-1.3)	0.7 (0.4-1.1)	0.20
All (multivariate <sup>†</sup> )	208/409	1.0	0.7 (0.4-1.2)	0.7 (0.4-1.3)	0.5 (0.3-1.0)	0.06
Premenopausal <sup>‡</sup>	148/289	1.0	0.6 (0.3-1.1)	0.6 (0.3-1.0)	0.5 (0.3-1.0)	0.08
Invasive <sup>‡</sup>	148/409	1.0	0.7 (0.4-1.2)	0.8 (0.4-1.4)	0.6 (0.3-1.2)	0.25
ER <sup>+</sup> /PR <sup>+</sup> <sup>‡</sup>	99/409	1.0	0.7 (0.4-1.3)	0.8 (0.4-1.5)	0.5 (0.2-1.1)	0.11
<b>c-Peptide</b>						
Cut points (ng/mL)		$\leq 1.03$	1.04-1.49	1.50-2.28	$\geq 2.29$	
All (simple*)	316/629	1.0	1.3 (0.9-2.0)	1.1 (0.7-1.6)	1.0 (0.7-1.6)	0.73
All (multivariate <sup>†</sup> )	316/629	1.0	1.4 (1.0-2.2)	1.1 (0.7-1.7)	1.1 (0.7-1.7)	0.79
Premenopausal <sup>‡</sup>	238/472	1.0	1.4 (0.9-2.2)	1.2 (0.7-2.0)	1.2 (0.7-2.1)	0.86
Invasive <sup>‡</sup>	218/629	1.0	1.4 (0.9-2.2)	1.0 (0.6-1.7)	1.4 (0.8-2.4)	0.45
ER <sup>+</sup> /PR <sup>+</sup> <sup>‡</sup>	144/629	1.0	2.0 (1.1-3.4)	1.3 (0.7-2.4)	2.0 (1.0-3.9)	0.15

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

\*Conditional logistic regression.

<sup>†</sup>Conditional logistic regression, adjusting for BMI at age 18 (<21, 21 to <23,  $\geq 23$  kg/m<sup>2</sup>), BMI at blood (continuous), age at menarche (<12, 12, 13,  $\geq 14$  years), parity/age at first birth (nulliparous, age at first birth <25 years/1-4 children, age at first birth 25-29 years/1-4 children, age at first birth  $\geq 30$  years/1-4 children, age at first birth <25 years/ $\geq 5$  children, age at first birth  $\geq 25$  years/ $\geq 5$  children), family history of breast cancer (yes, no), and history of benign breast disease (yes, no).

<sup>‡</sup>Unconditional logistic regression, adjusting for matching factors [days from luteal draw to next menstrual cycle (0-5, 6-7, 8-9, 10-28 days), age at blood collection (continuous), date of blood collection (continuous), fasting at blood collection (yes, no), time of blood collection (1-4 a.m., 5-6 a.m., 7 a.m. to 12 p.m., continuous), race/ethnicity (Caucasian, other), and menopausal status at blood collection (premenopausal, postmenopausal, unknown)] in addition to covariates above.

Our study has several strengths, including the prospective nature, with blood samples collected before diagnosis. Although c-peptide levels are less susceptible than insulin to meal-related fluctuations, we were able to analyze both hormones and perform secondary analyses of c-peptide among women with fasting blood samples. In addition, we were able to analyze estrogen/progesterone receptor-positive and invasive cases separately. However, we were somewhat limited in that we could not examine estrogen/progesterone receptor-negative tumors or an interaction by menopausal status. Although we used a single blood sample, insulin levels have been shown to be fairly consistent over time (ICC = 0.70 for samples collected a year apart; ref. 11). Finally, our case numbers limited our ability to detect meaningful differences among subgroups.

Higher insulin levels were inversely associated with risk, but we did not observe a similar association with c-peptide. Thus, high levels of insulin and c-peptide do not seem to be substantial risk factors for breast cancer among predominantly premenopausal women.

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