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Serum C-peptide levels and breast cancer risk: Results from the European Prospective Investigation into Cancer and Nutrition (EPIC)

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It has been hypothesized that chronic hyperinsulinemia, a major metabolic consequence of physical inactivity and excess weight, might increase breast cancer risk by direct effects on breast tissue or indirectly by increasing bioavailable levels of testosterone and estradiol. Within the European Prospective Investigation into Cancer and Nutrition (EPIC), we measured serum levels of C-peptide—a marker for pancreatic insulin secretion—in a total of 1,141 incident cases of breast cancer and 2,204 matched control subjects. Additional measurements were made of serum sex hormone binding globulin (SHBG) and sex steroids. Conditional logistic regression models were used to estimate breast cancer risk for different levels of C-peptide. C-peptide was inversely correlated with SHBG and hence directly correlated with free testosterone among both pre and postmenopausal women. C-peptide and free estradiol also correlated positively, but only among postmenopausal women. Elevated serum C-peptide levels were associated with a nonsignificant reduced risk of breast cancer diagnosed up to the age of 50 years [odds ratio (OR) = 0.70, (95% confidence interval (CI), 0.39–1.24); $p_{trend} = 0.05$]. By contrast, higher levels of C-peptide were associated with an increase of breast cancer risk among women above 60 years of age, however only among those women who had provided a blood sample under nonfasting conditions [OR = 2.03, (95% CI, 1.20–3.43); $p_{trend} = 0.01$]. Our results do not support the hypothesis that chronic hyperinsulinemia generally increases breast cancer risk, independently of age. Nevertheless, among older, postmenopausal women, hyperinsulinemia might contribute to increasing breast cancer risk.

Key words: C-peptide; breast cancer; prospective; cohort; EPIC

Excess body weight is a well-established risk factor for breast cancer among postmenopausal women,[1,2] whereas among both pre and postmenopausal women, regular physical activity has been generally associated with a reduced risk.[3] One major metabolic consequence of physical inactivity and excess body weight is the

Abbreviations: BMI, body mass index; CI, confidence interval; DHEAS, dehydroepiandrosterone sulphate; EPIC, European Prospective Investigation into Cancer and Nutrition; GH, growth hormone; HRT, hormone replacement therapy; IARC, International Agency for Research on Cancer; IGF-1, insulin-like growth factor 1; MET, metabolic equivalent values; OC, oral contraceptives; OR, odds ratio; SHBG, serum sex hormone binding globulin; WHR, waist-to-hip ratio.

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development of insulin resistance, accompanied by chronic hyperinsulinemia.[4–6]

Recently several studies have been published, on the growth hormone (GH) and insulin-like growth factor 1 (IGF-I) axis, and the adverse effects of IGF-I on breast tissue.[7–9] Similar, but independent of IGF-I and the GH/IGF-I axis, insulin also has mitogenic effects on normal breast tissue and on breast cancer cell-lines.[10,11] Experiments with insulin-deficient (diabetic) animals have shown that insulin promotes tumor growth and development in xenograft models and in chemical models of carcinogenesis.[12–17]

In addition, elevated insulin levels lead to a reduction in serum SHBG levels, and hence to increases in levels of bioavailable testosterone and estradiol[18–20]—factors that have all been associated with an increased risk of breast cancer among postmenopausal women.[21,22] It has thus been hypothesized that, especially among postmenopausal women, the increase of breast cancer risk related to physically inactivity and excess body weight might at least in part be due to elevated insulin levels.[23] In premenopausal women, associations of SHBG and bioavailable testosterone and estradiol with breast cancer are less clear.[24,25]

So far, only few prospective studies have addressed the possible relationship of breast cancer risk with prediagnostic circulating insulin or C-peptide levels.[26–30] Most of these studies had relatively small numbers of incident breast cancers and results have been inconsistent, suggesting an increase in risk,[30] a decrease in risk[27] or no clear association at all[26,29] in premenopausal women. In postmenopausal women, one study showed decreased breast cancer risk in association with fasting insulin samples,[27] whereas other studies, examining relationships with elevated nonfasting C-peptide levels showed increased breast cancer risk.[26,28] In none of these previous studies did associations reach statistical significance.[26–30]

We here present findings from a case–control study, nested within the European Prospective Investigation into Cancer and Nutrition (EPIC), a prospective cohort that is conducted in 10 Western European countries, on the relationship of breast cancer risk with serum C-peptide—a marker for pancreatic insulin secretion. In total, our study included 1,141 incident breast cancer cases and 2,204 matched control subjects.

Material and methods

Study population

EPIC recruitment procedures, and collection of questionnaire data, anthropometric measurements and blood samples have been described in detail previously.[31,32] In brief, extensive standardized questionnaire data on diet and nondietary variables, anthropometric measurements, and blood samples were collected between 1992 and 1998, from 366,521 women and 153,457 men living around 23 research centers spread over 10 Western European countries. Detailed questionnaire information was also collected about menstrual and reproductive history, current and past use of oral contraceptives (OC), postmenopausal hormone replacement therapy (HRT), history of previous illness and surgical operations, lifetime history of tobacco smoking and consumption of alcoholic beverages, habitual diet and physical activity.

The present study includes breast cancer cases and control subjects from 19 recruitment centers in 8 of the participating countries: France, the Netherlands, the United Kingdom, Germany, Spain, Italy, Denmark and Greece. Norway was not included in the present study because blood samples have been collected only recently on a sub-sample of cohort participants, and when the project was started, only very few cases of breast cancer had accumulated after blood collection; Sweden was not included because the association between plasma insulin levels and breast cancer occurrence has been examined within an independent study.[27] The present study includes some breast cancer cases ($N = 55$) and controls ($N = 6$) that were previously also part of a Dutch study on IGF-I, C-peptide and breast cancer in postmenopausal women.[28]

Follow up of cancer incidence and vital status

Incident cancer cases were identified through record linkage with regional cancer registries in Denmark, the Netherlands, the United Kingdom, Spain and Italy. In Germany, France, Greece and Naples, follow-up was based on a combination of methods, including checking of health insurance records, cancer and pathology registries, and active follow-up through study subjects and their next-of-kin. Data on vital status in most EPIC study centers were collected from mortality registries at the regional or national level, in combination with data collected by active follow-up (Greece). For each EPIC study center, closure dates of the study period were defined as the latest dates of complete follow-up for both cancer incidence and vital status. Closure dates varied between the EPIC recruitment centers, and ranged from June 1988 to December 2000.

Anthropometric measurements, menopausal status, fasting status, and level of physical activity

Anthropometric measurements (height, weight, and waist and hip circumferences) were measured according to standardized protocols, in light dressing. In part of the Oxford cohort, height, weight and body circumferences were self-reported. All measurements were reported to the nearest centimeter (height, body circumferences) and to the nearest kilogram (weight). Body mass index (BMI) was calculated as kilograms divided by the square of the height expressed in meters. Waist-to-hip ratio (WHR) was calculated as waist circumference divided by hip circumference.

Women were considered premenopausal when they reported having had regular menses over the past 12 months, or when they were less than 42 years of age. Women were considered postmenopausal when they reported not having had any menses over the past 12 months, or when they reported bilateral ovariectomy. Women who had incomplete or missing questionnaire, or who reported having had a hysterectomy, were considered postmenopausal when they were older than 55 years. Women who were between 42 and 55 years of age, with equivocal data for menopausal status, or who reported a hysterectomy, were classified as ''perimenopausal/unknown''.

Women were considered to be fasting, when they had not consumed any food or drinks for at least 6 hr prior to blood collection.

To determine levels of physical activity, women were asked questions on frequency and duration of recreational and household activity for a typical week during the summer and the winter of the past year. The various recreational and household activities were applied intensity codes using metabolic equivalent values (MET) where a MET is defined as the ratio of work metabolic rate to a standard resting metabolic rate, as described in detail by Ainsworth *et al.*[33] Using the data on frequency and duration, MET hours per week for both the summer and winter period were estimated, and a variable for household activity as well as for recreational activity was then created taking the average MET hours per week during the summer and the winter for household activity and recreational activity, respectively. The sum of household and recreational activity was then estimated and used as the variable representing physical activity in the statistical analyses.

Blood collection and storage

In France, the Netherlands, the United Kingdom, Germany, Spain, Italy and Greece, blood samples were collected according to a standardized protocol. From each subject, 30 ml of blood was drawn using 10 ml Safety Monovettes (Sartstedt, Nümbrecht, Germany). Filled syringes were kept at 5–10°C, protected from light, and transferred to a local laboratory for further processing and aliquoting. Two of the 3 syringes contained trisodium citrate as anticoagulant for the preparation of blood plasma, buffy coat and red cells, and 1 dry syringe was used to prepare serum. After centrifugation (1550 $\times$ g for 20 min), blood fractions (serum, plasma, red cells and buffy coat) were aliquoted in 28 plastic straws of 0.5 ml each (12 plasma, 8 serum, 4 erythrocytes and 4 buffy coat for

DNA), which were heat-sealed and stored under liquid nitrogen (−196°C). Mirror half of the 28 aliquots were stored locally and the other half centrally at the International Agency for Research on Cancer (IARC). In Denmark, nonfasting blood samples were drawn, and serum, plasma, red cells or buffy coat were aliquotted into 1-ml tubes stored in the vapor phase in liquid nitrogen containers (−150°C).

Selection of case and control subjects

For the present study, the same case and control subjects were used as for studies recently performed on sex steroids, growth factors and breast cancer (Rinaldi and coworkers).[21,24] Some of the selection criteria (no use of OC or HRT at the time of blood donation) and matching criteria (phase of the menstrual cycle) are therefore not directly relevant for the present study, but use of this study population gave us the opportunity to adjust for the effects of SHBG and sex steroids on breast cancer risk in our analyses on C-peptide and breast cancer. Case subjects were selected among women who developed breast cancer after their recruitment into the EPIC study, and before the end of the study period, for each study center defined by the latest end-date of follow-up. Women who used any HRT at the time of blood donation, or any exogenous hormones for contraception or medical purposes, and women who had previous diagnosis of cancer (except nonmelanoma skin cancer) were excluded from the study.

At the time this study was started in November 2002, out of a total number of 2,271 incident breast cancer cases, 1,786 had donated a blood sample. Of these, 549 cases were excluded because of previously mentioned selection criteria (previous history of cancer ($N = 20$) and use of exogenous hormones ($N = 529$)). Another 92 cases were excluded because of missing serum samples ($N = 30$) or missing data on fasting status ($N = 47$) or serum C-peptide ($N = 15$). After matching to controls, another 4 cases were excluded because they were poorly matched on fasting status. Thus, data on a total 1,141 cases were available for dataanalyses. A total of 400 incident cases of breast cancer were identified among women classified as premenopausal at the time of blood donation. Of these, 44 had a carcinoma *in situ* and all others ($N = 356$) had an invasive tumor. The number of incident cases among women classified as postmenopausal at the time of blood donation was 643 (58 with *in situ* tumors, and 585 with invasive tumors). A total of 98 women were diagnosed with breast cancer (5 with carcinoma *in situ* and 93 with an invasive tumor) among those women classified as being perimenopausal or having unknown menopausal status at the time of blood donation. From the total 1,141 case subjects included in our analyses, 75 were from France, 249 from the Netherlands, 202 from the United Kingdom, 62 from Germany, 191 from Spain, 295 from Italy, 32 from Denmark and 35 from Greece.

For each case subject with breast cancer, 2 control subjects were chosen at random among appropriate risk sets consisting of all cohort members alive and free of cancer (except nonmelanoma skin cancer) at the time of diagnosis of the index case.[24] An incidence density sampling protocol for control selection was used, such that controls could include subjects who became a case later in time, while each control subject could also be sampled more than once. Matching characteristics were the study center where the subjects were enrolled in the cohort, menopausal status (premenopausal, postmenopausal, perimenopausal/unknown), age (±6 months) at enrolment, time of the day at blood collection, fasting status (<3, 3–6, >6 hr), and phase of menstrual cycle for premenopausal women (“early follicular” (days 0–7 of the cycle), “late follicular” (days 8–11), “peri-ovulatory” (days 12–16), “midluteal” (days 20–24) and “other luteal” (days 17–19 or 25–40)).

All participants had given their written consent for future analyses of their blood samples and the Internal Review Board (IRB) of IARC had approved the hormone/C-peptide analyses as part of the previously described nested case–control study on endogenous hormone metabolism and breast cancer risk.

Laboratory assays

Hormone assays were performed at the laboratory of the Hormones and Cancer Group, IARC, using serum aliquots that had never been thawed before. C-peptide was measured by radioimmunoassay from Diagnostic Systems Laboratories (DSL, Webster, Texas). On the same samples (except for the samples of women who were perimenopausal or had undetermined menopausal status at the time of blood donation), measurements were also made on sex steroids (testosterone, androstenedione, dehydroepiandrosterone sulphate [DHEAS], estrone, and estradiol) and SHBG, using direct radioimmunoassays that were all previously validated against a reference method.[34] Free testosterone and free estradiol concentrations were calculated from the absolute concentrations of each of the steroids and SHBG using mass action equations, and assuming a constant serum albumin concentration of 43 g/l.[35]

The laboratory personnel performing the assays were blinded as to the case–control status of the study subjects. Cases and matched control subjects were always analyzed in the same analytical batch. For C-peptide, the mean intra-batch and inter-batch coefficients of variation were 6.7 and 9.8%, respectively. For the other hormonal parameters, measured in premenopausal women, details about assays used and accuracy of the assays have been reported elsewhere.[24] For postmenopausal women, the same assays were used accept for estradiol, which was measured using a radioimmunoassay from Diagnostic Systems Laboratories (DSL, Webster, Texas). Intra-batch coefficients of variation for sex steroids and SHBG, measured in postmenopausal women were 7.0% for DHEAS, 10.8% for testosterone, 4.8% for androstenedione, 10.2% for estrone, 5.8% for estradiol and 8.0% for SHBG.[21] Sex steroids and SHBG were not measured in women who where perimenopausal or had an unknown menopausal status and were also not measured in premenopausal cases that were not matched to control subjects on phase of the menstrual cycle (94 cases and 186 control subjects). Data of sex steroids and SHBG for 13 postmenopausal cases and 23 matched control subjects could not be used because of failed analyses.

Statistical analysis

Levels of C-peptide and other hormones were transformed using the natural logarithm to normalize their distributions. An analysis of variance was used to examine age, study center, analytical batches (clustered by single assay kit), BMI, combined household and recreational activity and menopausal status as determinants of measured C-peptide levels. A pairwise *t*-test was used to test for mean case–control differences in age at blood donation, age at first full term pregnancy, number of full term pregnancies, age at menarche, anthropometric measures and combined household and recreational activity, and a chi-square test was used to test for differences in percentage of parous women, percentage of past hormone users and percentage of current smokers.

Partial Pearson’s correlation coefficients were calculated between C-peptide and anthropometric factors and combined household and recreational activity, and between C-peptide and SHBG and sex steroids (for pre and postmenopausal women only), adjusting for age, case–control status and analytical batch. Correlations coefficients were calculated separately for women who were premeopausal, postmenopausal or who were perimenopausal or had unknown menopausal status at baseline of the study.

Odds ratios (ORs) for disease by quintile level of serum C-peptide were estimated by conditional logistic regression models using the SAS “PHREG” procedure. Quintile cut-off points were based on the serum C-peptide distribution of the control subjects. Likelihood ratio tests were used to assess linear trends in ORs over the quintiles, using the quintile medians for the quintile categories. Analyses were stratified by fasting status (≥6 hr fasting at blood collection, <6 hr fasting) and by age at diagnosis (≤50, 50–60, >60 years).

Heterogeneity of ORs between the study centers, between countries and between fasting and nonfasting subgroups, was assessed

TABLE I – BASELINE CHARACTERISTICS OF THE STUDY POPULATION

	Cases	Control subjects	*p*-Value[1]
Total number of subjects	1,141	2,204	
Menopausal status at blood donation			
Premenopausal[2]	400	778	
Postmenopausal[2]	643	1,240	
Perimenopausal or unknown[2]	98	186	
Age at blood donation (years)[3]	54.6 [39.9–68.7]	54.5 [39.9–68.8]	0.11
Age at diagnosis (years)[3]	56.9 [53.0–72.0]	–	
Years between blood donation and diagnosis[3]	2.83 [0.09–6.27]	–	
Age at menarche (years)[3]	13.0 [11.0–16.0]	13.1 [11.0–16.0]	0.15
Parous (%)	84.6	85.7	0.57
Age at first full term pregnancy (years)[3,4]	25.8 [20.0–34.0]	25.3 [19.0–33.0]	<0.01
Number of full term pregnancies[3,4]	2.3 [1.0–4.0]	2.4 [1.0–5.0]	<0.01
Previous OC use (%)	43.4	45.7	0.25
Previous HRT use (%)[5]	17.3	19.2	0.32
Current smoking (%)	16.9	16.4	0.65
Level of physical activity (MET hr/week)[3,6]	108.1 [28.7–204.3]	111.2 [33.8–202.6]	0.10
Age at diagnosis; (≤50 years)			
Body mass index (kg/m^2)[3,7]	24.9 [19.6–32.9]	25.3 [19.8–34.7]	0.26
Waist circumference (cm)[3]	78.1 [64.0–99.6]	78.8 [65.0–100.5]	0.37
Waist-hip ratio[3]	0.78 [0.69–0.88]	0.78 [0.69–0.88]	0.33
C-peptide (ng/ml)[8]	2.70 [2.57–2.85]	2.86 [2.76–2.97]	0.04
Age at diagnosis; (50–60 years)			
Body mass index (kg/m^2)[3]	26.4 [20.3–34.6]	26.1 [20.2–34.9]	0.23
Waist circumference (cm)[3]	83.2 [66.8–105.0]	82.2 [67.0–103.4]	0.11
Waist-hip ratio[3]	0.80 [0.70–0.91]	0.80 [0.70–0.91]	0.32
C-peptide (ng/ml)[8]	3.18 [3.04–3.32]	3.15 [3.05–3.26]	0.80
Age at diagnosis; (>60 years)			
Body mass index (kg/m^2)[3]	27.2 [21.1–35.4]	27.1 [20.7–35.7]	0.60
Waist circumference (cm)[3]	86.0 [69.3–104.2]	85.2 [70.0–106.0]	0.25
Waist-hip ratio[3]	0.81 [0.72–0.92]	0.82 [0.72–0.92]	0.76
C-peptide (ng/ml)[8]	3.83 [3.64–4.03]	3.59 [3.46–3.73]	0.02

[1]*p*-Value for the difference between cases and control subjects, tested with a paired *t*-test or a χ^2 test.–[2]Number of subjects.–[3]Mean [5th–95th percentile range].–[4]Among parous women only.–[5]Among postmenopausal women only.–[6]Combined household and recreational activity.–[7]Anthropometric measures were collected at baseline.–[8]Geometric mean [95%CI]. OC, oral contraceptives; HRT, hormone replacement therapy.

on a continuous scale (log2), using chi-square tests. The chi-square statistics was calculated as the deviations of logistic beta-coefficients observed in each of the subgroups, relative to the overall beta-coefficient. Multivariate logistic regression was used to estimate ORs adjusted for possible confounders other than those controlled for by matching, including age at first full-term pregnancy, number of full-term pregnancies, age at menarche, parity, past use of HRT (for perimenopausal and postmenopausal women) or OC, age at menopause (for postmenopausal women). The influences of obesity, combined household and recreational activity and serum levels of sex steroids on the association between serum C-peptide and breast cancer occurrence were evaluated using multivariate logistic regression.

All statistical tests and corresponding *p*-values were 2-sided, and *p*-values <0.05 were considered significant. All statistical analyses were done using the Statistical Analysis System (SAS) software package, version 9.1 (SAS Institute, Cary, NC).

Results

The average age at blood donation was 54.5 years, with a 5th to 95th percentile range of 39.9–68.8 years (Table I). Cases had an average age at diagnosis of 56.9 years (5th to 95th percentile range of 53.0–72.0 years), and the average time between blood donation and diagnosis was 2.83 years (0.09–6.27 years). Compared to controls, cases were significantly older at first full term pregnancy, and had a slightly, but significantly lower number of full term pregnancies. Age at menarche, parity (having had any children, or not), the percentage of women who smoked at time of recruitment and combined household and recreational activity did not differ significantly between cases and controls. Reported previous use of both OC and HRT was higher in the control group than among the cases, but these differences were not significant. BMI and waist circumference were slightly higher among control subjects aged 50 years or younger at diagnosis. In women older than 50 years, however these variables were slightly higher in the case group than among control subjects. Differences were nonsignificant. WHR was comparable between cases and control subjects. Serum levels of C-peptide were higher in older women. In women aged 50 years or younger at diagnosis, serum C-peptide levels were significantly higher among control subjects than among the cancer cases. C-peptide levels were comparable between cases and controls in the intermediate age group of women aged 50–60 years. In women over 60 years at diagnosis, cases had significantly higher circulating levels of C-peptide.

We used an analysis of variance to examine the effects of age, fasting status, different study center, BMI, combined household and recreational activity and menopausal status on C-peptide levels. BMI and fasting status explained 9.2 and 7.4 % of the variation in serum C-peptide levels, respectively. By contrast, differences between study centers, menopausal status, age at blood donation and combined household and recreational activity accounted for only very small percentages of between-subject variation in serum C-peptide levels (2.8, 0.2, 0.1 and 0.1 %, respectively).

Adjusting for age, case–control status and analytical batch, serum C-peptide concentrations correlated with BMI (Table II), as well as with WHR and waist circumference measurements. These correlations were relatively similar for women who were classified as being premenopausal, postmenopausal or perimenopausal or having unknown menopausal status, at the time of blood donation. By contrast, there was no correlation between C-peptide and combined household and recreational activity in either of the subgroups. In addition, C-peptide was inversely correlated with SHBG, and directly correlated with levels of free testosterone (unbound to SHBG), in both pre and postmenopausal women. Among postmenopausal women only, C-peptide levels correlated with serum estrogen levels and especially with calculated values

TABLE II – PEARSON'S PARTIAL CORRELATION COEFFICIENTS BETWEEN SERUM C-PEPTIDE AND ANTHROPOMETRIC FACTORS, COMBINED HOUSEHOLD AND RECREATIONAL ACTIVITY, SHBG AND SEX STEROIDS[1]

	Premenopausal at baseline		Postmenopausal at baseline		Perimenopausal or unknown menopausal status at baseline[2]	
	Fasting (n = 557)	Nonfasting (n = 621)	Fasting (n = 646)	Nonfasting (n = 1237)	Fasting (n = 112)	Nonfasting (n = 172)
BMI	0.50 (0.44; 0.56)	0.23 (0.16; 0.31)	0.45 (0.39; 0.51)	0.29 (0.24; 0.34)	0.64 (0.51; 0.74)	0.30 (0.16; 0.43)
WHR	0.33 (0.26; 0.41)	0.27 (0.20; 0.34)	0.39 (0.32; 0.46)	0.23 (0.18; 0.29)	0.36 (0.19; 0.51)	0.25 (0.10; 0.38)
Waist circumference	0.50 (0.49; 0.60)	0.28 (0.20; 0.35)	0.48 (0.42; 0.54)	0.31 (0.26; 0.36)	0.62 (0.49; 0.72)	0.33 (0.19; 0.47)
Physical activity	−0.01 (−0.09; 0.08)	0.02 (−0.06; 0.10)	−0.02 (−0.09; 0.06)	−0.02 (−0.08; 0.04)	−0.07 (−0.25; 0.11)	−0.03 (−0.18; 0.12)
Testosterone	0.01 (−0.08; 0.09)	0.01 (−0.07; 0.09)	0.06 (−0.02; 0.13)	0.03 (−0.03; 0.09)		
Androstenedione	0.01 (−0.07; 0.09)	−0.04 (−0.12; 0.04)	0.07 (−0.01; 0.14)	0.02 (−0.04; 0.07)		
DHEAS	0.11 (0.03; 0.19)	0.05 (−0.03; 0.13)	0.01 (−0.07; 0.08)	0.04 (−0.01; 0.10)		
Estrone	−0.03 (−0.12; 0.05)	−0.01(−0.11; 0.08)	0.11 (0.03; 0.18)	0.08 (0.03; 0.14)		
Estradiol	−0.08 (−0.16; 0.01)	−0.08 (−0.18; 0.02)	0.16 (0.09; 0.24)	0.17 (0.12; 0.22)		
SHBG	−0.40 (−0.47; −0.33)	−0.29 (−0.36; −0.21)	−0.46 (−0.52; −0.39)	−0.30 (−0.35; −0.25)		
Free testosterone	0.23 (0.15; 0.31)	0.15 (0.08; 0.23)	0.28 (0.20; 0.35)	0.18 (0.13; 0.24)		
Free estradiol	0.00 (−0.08; 0.09)	−0.03 (−0.13; 0.07)	0.31 (0.24; 0.38)	0.25 (0.20; 0.3)		

[1]Analyses were adjusted for age, laboratory batch, and case-control status.–[2]For women with a perimenopausal status or with unknown menopausal status at time of blood donation, SHBG and sex steroids were not measured. BMI, body mass index; WHR, waist–hip ratio; DHEAS, dehydroepiandrosterone sulphate; SHBG, sex hormone binding globulin.

of free estradiol. Androstenedione and DHEAS concentrations were either very weakly or not at all correlated with serum C-peptide, in any of the subgroups. As expected, the observed correlations were generally higher with fasting than with nonfasting levels of serum C-peptide.

All study subjects combined, conditional logistic regression analyses showed no association of breast cancer risk with circulating C-peptide levels, either in quintile categories (Table III) or as a continuous variable (results not shown). An inverse association with C-peptide levels was observed, however, when restricting the analysis to breast cancer diagnosis at, or before the age of 50 years. By contrast, higher levels of C-peptide were associated with higher cancer risk when breast cancer was diagnosed after age 60. There was no clear association between C-peptide levels and breast cancer risk at the intermediate ages of diagnosis (age 51–60), either in overall analyses, or in analyses stratified by fasting/nonfasting status at blood donation (Table IV).

When the analyses were stratified by fasting/nonfasting status at blood donation, the inverse association of C-peptide with risk of breast cancer up to age 50 was present in both subgroups, although not significant in either group separately (Table IV). By contrast, the direct association of C-peptide with the risk of breast cancer diagnosed after age 60 was present only, and significant in the subgroup of women who had provided a nonfasting blood sample [OR = 2.03 (95% CI = 1.20–3.43) between top and bottom quintiles; $p_{trend} = 0.01$].

The exclusion of cases with *in situ* tumors (and their matched controls) from the analysis did not materially alter any of the relative risk estimates, and neither was there any such change when past users of HRT or women who reported a history of diabetes mellitus or women with breast cancer diagnosed less than 2 years after intake, were excluded from the analysis.

Adjustments for BMI only changed the association between C-peptide and breast cancer occurrence substantially in the subgroup of women who had provided a fasting blood sample and were aged 50 or younger [OR, 0.96 (95% CI, 0.67–1.39), between extreme quintiles]. Serum levels of sex steroids and SHBG were available for most women in our study (935 cases and 1,813 control subjects). For the extreme age groups ($\leq$50, >60 years), crude associations between serum C-peptide levels and breast cancer risk, calculated for those women who had available data on serum sex steroids and SHBG, changed only marginally, compared with crude associations calculated for the whole study population (data not shown). In the intermediate age group, associations changed moderately and became more similar to the associations in the highest age group, because most women without data on sex steroids and SHBG were premenopausal or perimenopausal [OR, 1.48 (95% CI, 0.89–2.48), between extreme quintiles]. Adjusted associations between serum C-peptide level and breast cancer risk are presented in Tables III and IV. The negative association between serum C-peptide level and breast cancer occurrence in women of the lowest age group became stronger after adjustment for serum free testosterone levels [OR, 0.51 (95% CI, 0.29–0.90), between extreme quintiles], a factor that was directly related to breast cancer risk up to age 50,[24] and that was positively correlated with C-peptide levels (Table II). Adjustment for estradiol and free estradiol, however, slightly weakened the association [OR, 0.78 (95% CI, 0.40–1.53) and 0.76 (95% CI, 0.42–1.37), respectively, between extreme quintiles]. The direct association of (nonfasting) C-peptide levels with risk of breast cancer after age 60 was less strong after introducing either free testosterone or free estradiol to the model. Adjustments for combined household and recreational activity or any (nonhormonal) potential confounding factor did not show any major effect on relative risk estimates with respect to C-peptide levels.

When center and country-specific cut points were used, relative risk estimates were close to those from analyses with EPIC-wide cut points. Estimated relationships of breast cancer with serum C-peptide level, expressed on a continuous scale, showed no sig-

TABLE III – ASSOCIATIONS BETWEEN SERUM C-PEPTIDE AND BREAST CANCER RISK, STRATIFIED BY AGE AT DIAGNOSIS[1]

	Q1	Q2	Q3	Q4	Q5	p_{trend}[2]
Cut off points (ng/ml)	<2.17	2.17–2.73	2.74–3.47	3.48–4.80	≥4.81	
All women (1,141; 2,204)[3]						
Crude	ref.	0.85 (0.68–1.07)	0.93 (0.74–1.18)	0.96 (0.75–1.22)	1.02 (0.79–1.32)	0.66
Number of subjects	241/441	209/447	222/438	226/437	243/441	
Adjusted[4,5]	ref.	0.94 (0.72–1.22)	0.94 (0.72–1.23)	0.92 (0.70–1.21)	1.07 (0.80–1.44)	0.67
Number of subjects	187/367	172/346	181/355	182/362	213/358	
≤50 years (288; 563)						
Crude	ref.	0.80 (0.54–1.20)	0.48 (0.30–0.76)	0.58 (0.36–0.93)	0.70 (0.39–1.24)	0.05
Number of subjects	97/147	68/124	43/122	45/105	35/65	
Adjusted	ref.	0.84 (0.53–1.34)	0.42 (0.25–0.73)	0.55 (0.32–0.96)	0.64 (0.32–1.29)	0.04
Number of subjects	78/120	55/95	34/99	38/83	25/45	
50–60 years (445; 853)						
Crude	ref.	0.71 (0.49–1.02)	0.98 (0.68–1.42)	1.12 (0.78–1.61)	0.97 (0.64–1.45)	0.61
Number of subjects	97/173	75/187	90/169	103/170	80/154	
Adjusted	ref.	0.83 (0.53–1.31)	1.05 (0.67–1.64)	1.16 (0.73–1.83)	1.40 (0.83–2.37)	0.12
Number of subjects	64/127	53/123	61/120	67/122	62/95	
>60 years (408; 788)						
Crude	ref.	1.23 (0.78–1.92)	1.52 (1.00–2.33)	1.28 (0.81–2.01)	1.53 (0.98–2.38)	0.09
Number of subjects	47/121	66/136	89/147	78/162	128/222	
Adjusted	ref.	1.20 (0.76–1.91)	1.43 (0.92–2.23)	1.11 (0.70–1.78)	1.26 (0.79–2.00)	0.56
Number of subjects	45/120	64/128	86/136	77/157	126/218	

[1]Odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated by conditional logistic regression, for quintiles of serum C-peptide (quintile cut points based on the distribution of the control subjects).–[2]Likelihood ratio tests were used to assess linear trends in ORs over the quintiles, using the quintile medians for the quintile categories.–[3]Total number of cases and control subjects per stratum.–[4]ORs and 95% CIs, adjusted for free testosterone and free estradiol.–[5]The number of cases and control subjects used to calculate the adjusted ORs was smaller than the number of cases and control subjects used to calculate the crude ORs, because circulating levels of testosterone and estradiol were not measured in women who were perimenopausal or had an unknown menopausal status and were also not measured in premenopausal cases that were not matched to control subjects on phase of the menstrual cycle (94 cases and 186 control subjects). Data of sex steroids and SHBG for 13 postmenopausal cases, and 23 matched control subjects could not be used because of failed analyses.

nificant heterogeneity between fasting and nonfasting sub groups. *p*-values for heterogeneity among all women, among women aged 50 or less, among women in the age group between 51 and 60 years and among women over 60 years of age were 0.39, 0.66, 0.60 and 0.37, respectively. Tests for heterogeneity did not show significant differences among neither study centers nor countries (results not shown).

Discussion

Within the large, prospective EPIC study, we examined the relationships of breast cancer risk with prediagnostic serum concentrations of C-peptide—a marker for pancreatic insulin secretion. Our major findings were a moderate reduction in the risk of breast cancer diagnosed before or at age 50, among women who had elevated serum C-peptide levels. By contrast, after age 60, breast cancer risk was found to be increased among women with elevated C-peptide levels, but only when measured in nonfasting serum samples. No clear association was observed between circulating C-peptide levels and breast cancer risk at the intermediate ages of diagnosis (age 51–60).

Our observed relationships of breast cancer risk with C-peptide, by different age groups, were very much parallel to the relationships generally observed for breast cancer risk with BMI, or other measures of excess weight, i.e., a reduction of risk among obese, premenopausal women, and an increase in risk among postmenopausal women.[1,2] These parallel observations are not surprising, since obesity is a cause of insulin resistance and hyperinsulinemia (also in our data, there was a moderately strong correlation between BMI and serum C-peptide levels). Nevertheless, our observed relationships of risk with C-peptide levels remained relatively unaffected by adjustments for BMI or waist circumference, suggesting that the effects of insulin on breast cancer risk could be relatively independent from those of excess weight or adiposity. Only for women who had a diagnosis of breast cancer before age 50 and whose C-peptide levels were measured in fasting blood, did the adjustment for BMI abolish the moderate, inverse relationship of C-peptide with breast cancer risk (the inverse relationship remained, however, in combined statistical analyses of fasting and nonfasting C-peptide levels).

A major strength of our study is its prospective design. Compared to the classical case–control design, prospective cohort studies have the advantage of avoiding bias in the selection of appropriate control subjects and of having prediagnostic blood samples, collected and processed under the same conditions for women who eventually develop the cancer (cases) and those who do not (control subjects). The prospective design also avoids "reverse causation" biases that may occur if the presence of a tumor, or especially its diagnosis and treatment of disease, leads to changes in the metabolic risk factor examined. When cancer is diagnosed only shortly after intake, the tumor may already have been present at the time of intake, and hence may have influenced baseline measurements. In our study, associations between C-peptide levels and breast cancer risk did not change when we excluded cases with a breast cancer diagnosis within 2 years after baseline. A limitation of our study was the lack of data on menopausal status at the time of diagnosis. Hence, we chose to use age at diagnosis as an estimation of menopausal status at the time of diagnosis when stratifying the analyses of the association of C-peptide levels and breast cancer risk.

In 1992, Bruning *et al*. published results from a first case–control study,[36] showing significant increase in breast cancer risk with elevated serum C-peptide levels [OR, 2.9 (95% CI, 1.7–5.1), between extreme quintiles]. Other case–control studies on circulating insulin or C-peptide levels and breast cancer risk showed similar results for postmenopausal women [ORs between extreme tertiles/quartiles ranging from 1.5 to 2.9].[37–39] Increased risk of the same magnitude was shown among premenopausal women by some studies,[38,40] but not all.[39] Hirose *et al*. even showed a small but nonsignificant decrease in cancer risk in women with elevated insulin levels.[39]

Most prospective studies published so far did not show strong relationships of breast cancer with circulating insulin or C-peptide levels.[26–30] In 2 previous prospective studies into the association, higher levels of postmenopausal nonfasting serum C-peptide were associated with small increases in breast cancer risk [ORs and

TABLE IV – ASSOCIATIONS BETWEEN SERUM C-PEPTIDE AND BREAST CANCER RISK, STRATIFIED BY AGE AT DIAGNOSIS AND FASTING STATUS[1]

Fasting	Q1	Q2	Q3	Q4	Q5	p_{trend}[2]
Cut off points (ng/ml)	<1.98	1.98–2.33	2.34–2.75	2.76–3.37	≥3.38	
All women (446; 869)[3]						
Crude	ref.	0.80 (0.56–1.15)	0.86 (0.60–1.22)	0.76 (0.52–1.09)	0.96 (0.67–1.39)	0.76
Number of subjects	100/172	85/176	85/170	82/183	94/168	
Adjusted[4,5]	ref.	0.85 (0.57–1.26)	0.99 (0.67–1.46	0.76 (0.50–1.13)	0.96 (0.63–1.47)	0.66
Number of subjects	82/149	75/154	82/145	68/156	82/146	
≤50 years (150; 295)						
Crude	ref.	0.91 (0.51–1.62)	0.78 (0.44–1.38)	0.58 (0.30–1.11)	0.80 (0.41–1.55)	0.25
Number of subjects	42/69	31/55	31/65	22/59	24/47	
Adjusted	ref.	0.94 (0.50–1.77)	0.92 (0.50–1.71)	0.49 (0.23–1.03)	0.77 (0.36–1.64)	0.21
Number of subjects	35/60	28/49	29/53	17/51	20/38	
50–60 years (170; 330)						
Crude	ref.	0.56 (0.31–1.02)	0.72 (0.40–1.30)	0.80 (0.44–1.46)	1.13 (0.63–2.00)	0.45
Number of subjects	41/66	27/75	29/64	32/65	41/60	
Adjusted	ref.	0.61 (0.31–1.19)	0.91 (0.47–1.79)	0.84 (0.43–1.64)	1.27 (0.64–2.52)	0.39
Number of subjects	31/53	21/60	27/51	24/50	33/48	
>60 years (126; 244)						
Crude	ref.	1.25 (0.58–2.72)	1.33 (0.61–2.88)	1.01 (0.48–2.13)	1.04 (0.49–2.21)	0.78
Number of subjects	17/37	27/46	25/41	28/59	29/61	
Adjusted	ref.	1.29 (0.56–2.94)	1.36 (0.61–3.03)	1.06 (0.47–2.36)	1.01 (0.44–2.36)	0.74
Number of subjects	16/36	26/45	26/41	27/55	29/60	
Nonfasting						
Cut off points (ng/ml)	<2.43	2.43–3.21	3.22–4.19	4.20–5.71	≥5.72	
All women (695; 1,335)						
Crude	ref.	1.06 (0.79–1.43)	1.08 (0.79–1.46)	1.05 (0.76–1.43)	1.26 (0.92–1.72)	0.18
Number of subjects	130/269	136/266	136/266	133/267	160/267	
Adjusted	ref.	1.07 (0.76–1.52)	1.03 (0.71–1.47)	1.11 (0.77–1.60)	1.20 (0.83–1.73)	0.33
Number of subjects	96/208	106/208	100/206	112/208	132/208	
≤50 years (138; 268)						
Crude	ref.	1.06 (0.57–1.96)	0.45 (0.23–0.86)	0.56 (0.27–1.15)	1.09 (0.53–2.22)	0.56
Number of subjects	45/73	31/45	20/69	18/48	24/33	
Adjusted	ref.	0.73 (0.34–1.57)	0.43 (0.20–0.94)	0.68 (0.30–1.53)	0.74 (0.30–1.82)	0.35
Number of subjects	34/51	20/38	16/47	17/33	14/22	
50–60 years (275; 523)						
Crude	ref.	0.88 (0.55–1.39)	1.30 (0.81–2.07)	1.16 (0.73–1.85)	0.90 (0.54–1.49)	1.00
Number of subjects	55/108	53/119	61/93	62/107	44/96	
Adjusted	ref.	0.83 (0.45–1.51)	1.00 (0.54–1.87)	1.24 (0.67–2.29)	1.08 (0.56–2.08)	0.51
Number of subjects	33/65	32/74	32/64	41/66	33/56	
>60 years (282; 544)						
Crude	ref.	1.46 (0.87–2.47)	1.59 (0.92–2.75)	1.43 (0.82–2.48)	2.03 (1.20–3.43)	0.01
Number of subjects	30/88	52/102	55/104	53/112	92/138	
Adjusted	ref.	1.70 (0.98–2.95)	1.66 (0.93–2.95)	1.41 (0.80–2.50)	1.69 (0.97–2.95)	0.22
Number of subjects	29/92	54/96	52/95	54/109	85/130	

[1]Odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated by conditional logistic regression, for quintiles of serum C-peptide (quintile cut points based on the distribution of the control subjects).–[2]Likelihood ratio tests were used to assess linear trends in ORs over the quintiles, using the quintile medians for the quintile categories.–[3]Total number of cases and control subjects per stratum.–[4]ORs and 95% CIs, adjusted for free testosterone and free estradiol.–[5]The number of cases and control subjects used to calculate the adjusted ORs was smaller than the number of cases and control subjects used to calculate the crude ORs, because circulating levels of testosterone and estradiol were not measured in women who were perimenopausal or had an unknown menopausal status and were also not measured in premenopausal cases that were not matched to control subjects on phase of the menstrual cycle (94 cases and 186 control subjects). Data of sex steroids and SHBG for 13 postmenopausal cases, and 23 matched control subjects could not be used because of failed analyses.

95% CIs between extreme quartiles; 1.2 (0.7–2.3) and 1.3 (0.7–2.7)].[26,28] In one of these studies, a separate analysis with premenopausal women showed a small inverse association of C-peptide with breast cancer [OR and 95% CI between extreme quartiles; 0.8 (0.4–1.3)].[26] In both studies however, numbers of cases were relatively small and linear trends in ORs over quartiles were not significant. Other prospective studies, all based on fasting blood samples, did not show any increase in breast cancer risk in the older age groups,[27,29,30] and one study even found a nonsignificant decrease in risk among women older than 55 years [OR = 0.5, between extreme quartiles], although this age-stratified analysis was not published.[27] Studies with fasting levels of insulin in premenopausal women were inconclusive.[27,30] Only one of the previous prospective studies restricted their analysis to women aged 50 years or younger at diagnosis. As in our study, that analysis also showed a decrease in risk with increasing nonfasting C-peptide levels, although the decrease was not linear and results were not significant [OR and 95% CI between extreme quartiles; 0.6 (0.3–1.3)].[26] None of the previous prospective studies examined relationships of breast cancer risk with serum insulin or C-peptide in women after 60 years of age.

Already in 1960, de Waard *et al*. hypothesized that obesity, essential hypertension, decreased glucose tolerance, or a combination of these, could increase the risk of breast cancer development.[41] Type 2 diabetes is generally characterized by increased levels of insulin for many years, both before and after its clinical onset.[42] Literature on the association between type 2 diabetes and breast cancer has recently been reviewed.[43] A pooled analysis of 6 prospective studies showed a small, but significant increase in breast cancer risk among women with type 2 diabetes [OR and 95% CI; 1.25 (1.19–1.31)]. However, the authors noted that most of the 6 studies had not properly adjusted for confounding factors and thus they concluded that type 2 diabetes might increase breast cancer risk, but that more research on this topic is still needed.[43] In our own study popula-

tion, the baseline prevalence of diabetes was too small to allow separate analyses of the relationship of C-peptide with breast cancer risk in this subgroup ($N = 33$ and $N = 62$ for cases and control subjects, respectively). Exclusion of diabetic subjects did not attenuate associations between circulation C-peptide levels and breast cancer risk.

Although fluctuations in circulating levels of C-peptide (which is a short-term indicator (2–3 hr) of insulin production) are smaller than those in insulin levels (which is rapidly cleared by the liver), circulating levels of C-peptide are influenced by food intake.[44–48] In our study population, fasting serum C-peptide levels had higher correlations than nonfasting levels, with anthropometric factors and hormone levels. We expected fasting serum C-peptide to be a better biomarker than nonfasting C-peptide for average circulating insulin concentrations and therefore expected to see stronger associations with breast cancer in the fasting subgroup. However, after age 60, we did not observe a clear relationship of breast cancer risk with fasting levels of C-peptide, but we did observe a significant increase in breast cancer risk among women with elevated C-peptide levels, who had provided a nonfasting blood sample. We have no clear explanation for this difference, although it has been suggested that tumor development could be enhanced especially by high postprandial insulin peaks, possibly because of direct antiapoptotic or mitogenic effects of insulin itself.[49,50] An alternative explanation for the observation that the increased breast cancer risk for high C-peptide levels in women aged over 60 years is stronger in nonfasting blood samples may be impaired glucose tolerance by delayed insulin production.[51]

Besides its possible direct antiapoptotic or mitogenic effects, elevated insulin levels could influence breast cancer risk by regulating sex steroid synthesis and/or bioavailability. Elevated insulin strongly reduces the hepatic synthesis and blood levels of SHBG, and thus increases blood levels of bioavailable testosterone and estradiol, unbound to SHBG.[19,20] In our study population[21] and others,[22] postmenopausal women who had elevated serum concentrations of bioavailable testosterone and estradiol were shown to be at increased risk of breast cancer. In our study, however, adjustment for the effects of sex steroids on breast cancer risk did not lead to any substantial attenuation of the association between circulating levels of C-peptide and breast cancer risk. The latter suggests that the increase in breast cancer risk observed in our study could be due to the effects of elevated insulin independent of any changes in bioavailable sex steroid levels. Among premenopausal women, contrary to postmenopausal women, reductions in circulating SHBG levels have not generally been found to increase bioavailable estradiol, probably because of negative feedback regulations of ovarian estradiol synthesis, through the hypothalamo-pituitary axis.[52] The lack of increase in circulating bioavailable estradiol might at least partially explain the lack of increase in breast cancer risk among hyperinsulinemic, premenopausal women. The possible reduction in risk among premenopausal women with elevated insulin, as among obese premenopausal women, could be due to insulin's stimulatory effects on ovarian androgen synthesis and, in a susceptible subgroup of women, the development of ovarian hyperandrogenism.[19,23,53] It has been hypothesized that ovarian hyperandrogenism could reduce breast cancer risk among premenopausal women because of chronic anovulation and reduced ovarian progesterone production.[54]

In conclusion, we found that the risk of breast cancer development before age 50 was decreased among women who had elevated C-peptide levels, whereas risk of breast cancer after age 60 was increased. These results are parallel to the observations of decreased and increased breast cancer risk depending on menopausal status, by obesity. Our results do not support the hypothesis that insulin is a major risk factor for breast cancer in general, irrespective of age at diagnosis, although at more advanced age, after menopause, it cannot be ruled out that hyperinsulinemia contributes to increased risk, e.g., by direct effects on breast tissue or by lowering circulating SHBG levels, thus increasing levels of bioavailable estradiol and testosterone.

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