Endocrine determinants of breast density and breast cancer risk

Martijn Verheus

Endocrine determinants of breast density and breast cancer risk

Utrecht, Universiteit Utrecht, Faculteit Geneeskunde
Thesis University Utrecht - with a summary in Dutch
Proefschrift Universiteit Utrecht - met een samenvatting in het Nederlands

ISBN	978-90-393-4536-8
Author	Martijn Verheus
Cover	Marius Bauer, "Liggend naakt"
Lay-out	Haaring Automatisering, Scherpenzeel
Print	Gildeprint Drukkerijen B.V., Enschede, the Netherlands

Endocrine determinants of breast density and breast cancer risk

Endocriene determinanten van borstdensiteit en van het risico op borstkanker

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. W.H. Gispen,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 23 mei 2007 des ochtends te 10.30 uur

door

Martijn Verheus

geboren op 8 februari 1975 te Leiderdorp

Promotor	:	Prof. dr. D.E. Grobbee
Co-promotoren	:	Dr. C.H. van Gils
		Dr. P.H.M. Peeters

The studies described in this thesis were supported by the World Cancer Research Fund (WCRF) [grant nr 2002/11 and 2000/30] and by the "Europe Against Cancer" Programme of the European Commission (SANCO) and the institutes and funds supporting the EPIC study

The financial support by the Julius Center for Health Sciences and Primary Care as well as the educational grant from Alpro nv, for the publication of this thesis, are gratefully acknowledged

Manuscripts based on the studies presented in this thesis

Chapter 2.1

Martijn Verheus, Petra H.M. Peeters, Rudolf Kaaks, Paulus A.H. van Noord, Diederick E. Grobbee, Carla H. van Gils
Premenopausal Insulin-like growth factor I serum levels and changes in breast density over menopause. Cancer Epidemiol Biomarkers Prev 2007; 16:451-457.

Chapter 2.2

Martijn Verheus, James D. McKay, Rudolf Kaaks, Carine Biessy, Mattias Johanson, Diederick E. Grobbee, Petra H.M. Peeters, Carla H. van Gils
Common genetic variation in the IGF-1 gene, serum IGF-I levels and breast density. Submitted.

Chapter 3

Martijn Verheus, Petra H.M. Peeters, Paulus A.H. van Noord, Yvonne van der Schouw, Diederick E. Grobbee, Carla H. van Gils
No relationship between circulating levels of sex steroids and breast density; the Prospect-EPIC cohort. Submitted.

Chapter 4

Martijn Verheus, Petra H.M. Peeters, Sabina Rinaldi, Laure Dossus, Carine Biessy, Anja Olsen, Anne Tjønneland, Kim Overvad, Majbritt Jeppesen, Françoise Clavel-Chapelon, Bertrand Téhard, Gabriele Nagel, Jakob Linseisen, Heiner Boeing, Petra H. Lahmann, Athina Arvaniti, Theodora Psaltopoulou, Antonia Trichopoulou, Domenico Palli, Rosario Tumino, Salvatore Panico, Carlotta Sacerdote, Sabina Sieri, Carla H. van Gils, Bas H. Bueno-de-Mesquita, Carlos A. González, Eva Ardanaz, Nera Larranaga, Carmen Martinez Garcia, Carmen Navarro, J.Ramón Quirós, Tim Key, Naomi Allen, Sheila Bingham, Kay-Tee Khaw, Nadia Slimani, Elio Riboli, Rudolf Kaaks
Serum C-peptide levels and breast cancer risk; results from the European Prospective Investigation into Cancer and Nutrition (EPIC). Int J Cancer 2006; 119:659-667.

Chapter 5

Martijn Verheus, Carla H. van Gils, Lital Keinan-Boker, Philip B. Grace, Sheila A. Bingham, Petra H.M. Peeters
Plasma phytoestrogens and subsequent breast cancer risk. J Clin Oncol 2007; 25:648-655.

Contents

Chapter 1	General introduction	1
Chapter 2	Insulin-like growth factor I and breast density	9
Chapter 2.1	Premenopausal insulin-like growth factor I serum levels and changes in breast density over menopause	11
Chapter 2.2	Common genetic variation in the IGF-1 gene, serum IGF-I levels and breast density	33
Chapter 3	No relationship between circulating levels of sex steroids and breast density; the Prospect-EPIC cohort	59
Chapter 4	Serum C-peptide levels and breast cancer risk; results from the European Prospective Investigation into Cancer and Nutrition (EPIC)	79
Chapter 5	Plasma phytoestrogens and subsequent breast cancer risk	105
Chapter 6	General discussion	125
Chapter 7	Summary	139
	Samenvatting	145
	Dankwoord	153
	Curriculum Vitae	161

GENERAL INTRODUCTION

CHAPTER 1

Introduction

With an estimated 1.15 million new cases worldwide in 2002, breast cancer is the most common malignancy among females, accounting for 0.41 million deaths each year. In the last three decennia, breast cancer incidence rates increased in all regions of the world and in 2010 around 1.40 million new cases are expected (1,2).

Most tumors originate in the glandular ducts and the number of glandular cells in the female breast has been hypothesized to predict breast cancer risk (3-5). The amount of glandular tissue can be estimated with mammography. The radiographically dense tissues of the breast (stromal and glandular tissues) appear light on a mammogram. The radiographically lucent fatty tissues appear black (6). Using a semi quantitative computer assisted method, the total breast area and the dense area on a mammogram can be estimated and used to calculate the proportion of the breast that is composed of dense tissue. This percent breast density is the most widely used breast density measure in breast density research. The absolute measure of breast density, the dense area, may, however, better estimate the amount of glandular tissue, which is regarded to be the target tissue for breast cancer (3,4). High percent breast density is one of the strongest known risk factors for developing breast cancer (7). Woman with over 75% of their breasts composed of dense tissues have an estimated 2 to 6 times increased risk of developing breast cancer, compared with women with very low breast density (8,9). Hence breast density has been referred to as a biomarker for breast cancer risk (8). Normally, breast density decreases around menopause, but not to the same extent in all women. The women with persistently high breast density have been hypothesized to have the highest risk of breast cancer. Investigating factors that increase or decrease breast density or affect the process of involution of the breast during the menopausal transition phase, may obtain more insight in the etiology of breast cancer and may help find explanations for the increased risk through common determinants.

Hormones and growth factors that have mitogenic properties or stimulate the growth of epithelial cells can increase breast cancer risk, possibly through an increase in breast density. From in vitro studies and studies using animal models, it has been shown that insulin growth factor I (IGF-I), insulin and sex steroids are all potent mitogens and thus candidates to be studied in relation to breast density and breast cancer risk (10-16).

Aim of the thesis

The aim of this thesis is to investigate the relationships between growth factors (IGF-I) and hormones, both endogenous (insulin, sex steroids) and exogenous (phytoestrogens), on the one hand, and breast density and breast cancer on the other hand.

For this thesis we used data from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort and from the Prospect-EPIC cohort, which is one of two Dutch cohorts participating in the EPIC study. EPIC is a multi center study with 23 participating cohorts from 10 European countries. Between 1992 and 1998, 366,521 women were included into the study (17,18). At baseline, anthropometric measurements were taken and a blood sample was drawn. Detailed questionnaire information was collected about menstrual and reproductive history, current and past use of oral contraceptives and postmenopausal hormone therapy, history of previous illness and surgical operations, lifetime history of tobacco smoking and consumption of alcoholic beverages, habitual diet and physical activity.

The Prospect-EPIC cohort is a breast cancer screening cohort and participating women reside in Utrecht or its vicinity. A total of 17,357 women were included between 1993 and 1997 (19). As Prospect-EPIC is a breast cancer screening cohort, mammograms for most women were available for analyses.

Outline of the thesis

In cross sectional studies, high circulating levels of IGF-I have been associated with high breast density, but only before menopause. As circulating levels of IGF-I are known to decrease with age, postmenopausal breast density may already be determined before menopause, when circulating IGF-I levels are still high. In **Chapter 2** we investigate the relationship between IGF-I and breast density in the Prospect-EPIC cohort. In the study reported in **Chapter 2.1**, the association between premenopausal circulating levels of IGF-I and changes in breast density during the menopausal transition phase, was established. Of 684 women, one mammogram before menopause and one mammogram after menopause were retrieved from the archives and breast density was determined using a semi quantitative computer assisted method. A disadvantage of circulating levels is that they fluctuate over time. As genetic characteristics are stable throughout life, genetic variation in the *IGF-1* gene may reflect lifetime exposure to IGF-I and can therefore be used to verify relations between circulating levels of IGF-I and breast density. Results of analyses of common variation in the *IGF-1* gene and breast density are presented in **Chapter 2.2**. In total, 1928 women were genotyped for 15 single nucleotide polymorphisms, tagging 4 haplotype blocks within the *IGF-1* gene. A mammogram that was made before menopause was available for all women and an additional mammogram made after menopause as well as premenopausal circulating levels of IGF-I were available for a subgroup of 656 women.

Epidemiological studies on circulating levels of endogenous sex steroids and breast density do not show strong evidence for relationships. Most of these studies were small however and only presented results with the relative measure of breast density, the percent breast density. In **Chapter 3** we present results of the second largest study on endogenous androgens, estrogens and sex hormone binding globulin on the one hand, and breast density – measured both as absolute and relative measure – on the other hand among 969 postmenopausal women participating in the Prospect-EPIC cohort.

In **Chapter 4**, we describe the relationship between circulating levels of C-peptide – a marker for pancreatic insulin secretion – and breast cancer risk. Within the total EPIC cohort, prediagnostic C-peptide serum levels were analyzed for 1141 women who were diagnosed with breast cancer during follow-up, and for 2204 matched control subjects.

Phytoestrogens are plant compounds with hormone-like activity. They can compete with endogenous estrogens for the estrogen receptor and this way inhibit binding of the estrogenic more potent endogenous estrogens, potentially decreasing breast cancer risk. So far, only few studies on blood levels of phytoestrogens have been performed, of which most were relatively small. Results of these studies were inconclusive. In **Chapter 5** we present results of the largest prospective study on circulating levels of phytoestrogens and breast cancer risk. Prediagnostic serum levels of seven phytoestrogens – five isoflavones (daidzein, genistein, glycitein, O-desmethylangolensin and equol) and two lignans (enterodiol and enterolactone) – were analyzed in 383 women who were diagnosed with breast cancer during follow-up and 383 control subjects of the Prospect-EPIC cohort.

In **Chapter 6**, we discuss how growth factors and hormones affect the two components of percent breast density separately. We speculate about the consequences of these effects, as not only the dense tissue but also the surrounding fatty tissue may play a role in breast cancer development.

References

1. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
2. Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J.Clin.Oncol. 2006;24:2137-2150.
3. Trichopoulos D, Lipman RD. Mammary gland mass and breast cancer risk. Epidemiology. 1992;3:523-526.
4. Albanes D, Winick M. Are cell number and cell proliferation risk factors for cancer? J.Natl.Cancer Inst. 1988;20;80:772-774.
5. Pike MC, Spicer DV, Dahmoush L, et al. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol.Rev. 1993;15:17-35.
6. Boyd NF, Byng JW, Jong RA, et al. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study. J Natl Cancer Inst 1995;87:670-675.
7. Veronesi U, Boyle P, Goldhirsch A, et al. Breast cancer. Lancet 2005;365:1727-1741.
8. Boyd NF, Rommens JM, Vogt K, et al. Mammographic breast density as an intermediate phenotype for breast cancer. Lancet Oncol. 2005;6:798-808.
9. McCormack VA, dos SS, I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol.Biomarkers Prev. 2006;15: 1159-1169.
10. Kleinberg DL. Role of IGF-I in normal mammary development. Breast Cancer Res Treat 1998;47:201-208.
11. Pollak MN. Endocrine effects of IGF-I on normal and transformed breast epithelial cells: potential relevance to strategies for breast cancer treatment and prevention. Breast Cancer Res Treat 1998;47:209-217.
12. Lee AV, Yee D. Insulin-like growth factors and breast cancer. Biomed Pharmacother. 1995;49:415-421.
13. Chappell J, Leitner JW, Solomon S, et al. Effect of insulin on cell cycle progression in MCF-7 breast cancer cells. Direct and potentiating influence. J Biol Chem 2001;276:38023-38028.
14. Ish-Shalom D, Christoffersen CT, Vorwerk P, et al. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. Diabetologia 1997;40 Suppl 2:S25-S31.
15. Russo IH, Russo J. Role of hormones in mammary cancer initiation and progression. J.Mammary.Gland.Biol.Neoplasia. 1998;3:49-61.
16. Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. J.Steroid Biochem.Mol.Biol. 2006;102:89-96.

17. Riboli E, Kaaks R. The EPIC Project: rationale and study design. European Prospective Investigation into Cancer and Nutrition. Int J Epidemiol 1997;26 Suppl 1:S6-14.
18. Riboli E, Hunt KJ, Slimani N, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002;5:1113-1124.
19. Boker LK, Van Noord PA, van der Schouw YT, et al. Prospect-EPIC Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. Eur J Epidemiol 2001;17:1047-1053.

INSULIN-LIKE GROWTH FACTOR I AND BREAST DENSITY

CHAPTER 2

Premenopausal insulin-like growth factor I serum levels and changes in breast density over menopause

Chapter 2.1

Martijn Verheus
Petra H.M. Peeters
Rudolf Kaaks
Paulus A.H. van Noord
Diederick E. Grobbee
Carla H. van Gils

Cancer Epidemiol Biomarkers Prev 2007; 16:451-457.

Abstract

Background

A high proportion of glandular and stromal tissue in the breast (percent breast density) is a strong risk factor for breast cancer development. Insulin-like growth factor-I (IGF-I) is hypothesized to influence breast cancer risk by increasing breast density.

Objectives

We studied the relation between premenopausal circulating IGF-I levels and pre- and postmenopausal, absolute non-dense and dense area and percent breast density, as well as changes in these measures over menopause.

Design and Methods

Mammograms and blood samples of 684 premenopausal participants of the Prospect-EPIC cohort were collected at baseline. A second mammogram of these women was collected after they became postmenopausal. Premenopausal IGF-I levels were measured in serum. Pre- and postmenopausal breast measures were assessed using a computer-assisted method. Mean values of breast measures were calculated for quartiles of serum IGF-I, using linear regression analysis.

Results

Women with higher premenopausal IGF-I levels showed a slightly smaller decrease in dense area over menopause (-12.2 cm^2 in the highest versus -12.9 cm^2 in the lowest quartile, p-trend=0.58) and, at the same time, a smaller increase in the non-dense (fat) area (p-trend=0.09). Due to the changes over menopause, high premenopausal IGF-I serum levels were associated with lower non-dense area (p-trend=0.05), somewhat higher dense area (p-trend=0.66) and consequently with higher percent breast density (p-trend=0.02) after menopause.

Conclusion and discussion

Women with higher premenopausal IGF-I levels have a smaller increase in non-dense area and also a slightly smaller decrease in absolute dense area during menopause, resulting in higher breast density after menopause.

Introduction

From all types of cancer affecting women in the Western World, breast cancer has the highest incidence and in many countries breast cancer screening programs are implemented using mammography. Connective (stromal) and epithelial tissues of the female breast are radiologically dense and appear light on a mammogram, while the radiologically lucent fat tissue has a dark appearance. High mammographic density has been associated with a 3-6 fold increase in risk, of both pre- and postmenopausal breast cancer and is therefore one of the strongest known risk factors for developing breast cancer (1-4).

Insulin-like growth factor-I (IGF-I) has mitogenic properties and is involved in the development of normal breast tissue (5,6). In vitro studies showed that IGF-I is also a potent mitogen for several breast cancer cell lines, and that it inhibits apoptosis (7,8). Furthermore, in xenograft models, tumor growth was decreased in IGF-I deficient animals (6). Prospective studies on the association between circulating levels of IGF-I and subsequent breast cancer risk showed contradictive results. High levels of IGF-I were associated with increased risk of premenopausal breast cancer in most studies (9-14), but not all (15-17). Most studies with postmenopausal women did not show a clear association between IGF-I levels and breast cancer risk (9-11,13-15,18,19), with few exceptions. Muti et al. reported decreased cancer risk in women with high IGF-I levels (12). Rinaldi et al. found the opposite effect in women over 50 years of age (16).

Several studies have been published on the association between circulating levels of IGF-I and breast density (20-25). IGF-I was positively associated with breast density among premenopausal women in most studies (20,21,23,25), with the exception of two (22,24). Only one of five studies (25), found an association between IGF-I circulating levels and breast density in postmenopausal women (20,22-25), which was however no longer statistically significant, after adjusting for age and waist circumference (25). Guo et al. studied IGF-I staining in paraffin blocks of tissues surrounding benign legions. IGF-I staining was higher in blocks from highly dense breasts, but only among women younger than 50 years (26).

It has been shown that involution of the breast is stronger during the menopausal transition phase compared with involution before menopause (27). It is known that IGF-I levels gradually decrease with age (28), and it is possible that the involution of dense breast tissue during menopause, and consequently also postmenopausal breast density, is largely determined before menopause when IGF-I levels are still high. Results from a recently published study by Rollison et al., reporting higher premenopausal IGF-I levels to be associated with increased postmenopausal breast cancer risk, support this (29). In this longitudinal study we investigated whether premenopausal circulating levels of IGF-I were associated with premenopausal mammographic density and also how these levels affected changes in mammographic density during menopause.

Methods

The study population

This study included women participating in Prospect-EPIC, one of two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition (EPIC). EPIC is a multicentre cohort study with 10 participating European countries. Rationale and design of both EPIC and Prospect-EPIC are described in detail elsewhere (30,31,32). The Prospect-EPIC cohort consists of 17357 women, aged 49-69 years. Women were recruited between 1993 and 1997 through a regional programme for breast cancer screening and reside in Utrecht or its vicinity. The regional programme is part of the national screening programme which covers the entire Dutch population. As part of the population-based screening programme, a mediolateral oblique mammogram is taken every two years, starting at age 50 until age 75, and stored in archives.

At time of study enrolment, pulse rate, blood pressure and anthropometric measurements were taken. Furthermore, a blood sample was drawn and stored at -196 °C under liquid nitrogen, and participants were asked to fill out two questionnaires. A general questionnaire was used to gather information on demographic, reproductive and lifestyle factors and past and current morbidity. To determine regular dietary intake, an extensive self-administered food frequency questionnaire was used containing 178 food items, which was validated and described in more detail by Ocké et al. (33,34).

Approximately 5 years after recruitment, participating women were asked to fill out a follow-up questionnaire, containing questions about demographic, reproductive and lifestyle factors and past and current morbidity.

All participants signed an informed consent and the study was approved by The Institutional Review Board of the University Medical Center Utrecht.

Blood collection and IGF-I measurements in serum

A 30 ml non-fasting blood sample was donated by each participant, using three safety monovettes, one dry monovette for serum and two citrated monovettes for plasma. Within 24 hours, samples of 4 ml serum, 9 ml citrate plasma and 2 ml white blood cells were fractionated into 0.5 ml aliquots and stored in heat-sealed plastic straws under liquid nitrogen at -196 ºC.

IGF-I in serum was measured by Labor Benrath (Dusseldorf, Germany) using immulite 2000 technology with reagents from DPC (Diagnostic Products Corporation, Frankfurt, Germany). The samples were analyzed in 11 batches, each of which contained the same two control samples. The interbatch coefficients of variation for the two control samples were 3.73% and 2.62% respectively.

Study subjects and mammogram selection

From the 16917 subjects that had donated a blood sample, 4055 where premenopausal at baseline. Women were considered premenopausal if they had menses at least once during the last 12 months prior to blood collection. Current users of oral contraceptives (OC) or postmenopausal hormone therapy (HT) were excluded, as were former users who quit using OC or HT less than two years before blood donation. Women having had ovariectomy on both ovaries were also excluded. Three women had had a hysterectomy. However all three still had both their ovaries and met all other selection criteria. Therefore they were not excluded. At the start of mammogram collection, our study group comprised 2325 women. Of these, 264 women, were excluded because their mammograms could not be retrieved from the archives. Another 14 were excluded for having silicone protheses. Twenty two women had breasts too large to fit on a single mammogram. The latter group had therefore more than one mammogram which could not be used because the overlap between these mammograms could not be measured properly. A premenopausal mammogram was available for 2025 women.

A follow-up questionnaire was filled out approximately five years after baseline by 1607 of the 2025 women with a premenopausal mammogram and 1015 women had become postmenopausal at the time that they filled out the follow-up questionnaire. Because no other information about menopausal status of study participants in the time span between baseline and filling out the follow-up questionnaire was available, answers to the follow-up questionnaire were used to determine postmenopausal status. Women were considered postmeno-pausal when they had not had any menses during the 12 months prior to filling out the follow-up questionnaire. The first mammogram after the time women filled out the follow-up questionnaire was collected for these women and could be retrieved from the archives for 695 women. None of these women were current users of OC or HT or had used these compounds less than two years before filling out the follow-up questionnaire. One woman was excluded because she had silicone protheses. None of the participants had breasts too large to fit a single mammogram.

Of the 694 women with both a premenopausal and a postmenopausal mammogram available, 684 had a serum sample available to be used for IGF-I measurements.

Mammographic density analysis

Mammographic density was assessed using the mediolateral oblique mammogram, which is the routine view for breast cancer screening in the Netherlands. It has been observed that the proportions of mammographic density on craniocaudal views and mediolateral oblique views and on left and right views show very strong correlation and that representative information on mammographic density is provided in a single view (35). For each study subject, mammographic density was assessed on the left view.

After digitizing the films using a laser film scanner (Lumiscan 50, Lumisys), mammographic density was quantified using a computer-assisted method based on gray levels of pixels in the digitized mammogram. This computer-assisted method to determine mammographic density has proved to be very reliable and the method is described elsewhere in detail (36). Briefly, for each image, the reader first sets a threshold to determine the outside edge of the breast, to separate the image of the breast from the dark background surrounding it. A second threshold is set to determine the area of dense tissue within the breast, which is the lightest tissue visible on the mammogram. The program then determines the amount of pixels within the total breast area and within the dense area.

To compute percent breast density the dense area of a breast is divided by the total breast area and multiplied by 100. Percent breast density is used in most publications on breast density. However, a small sized breast and a larger sized breast could have the same percent breast density, while the absolute amount of connective and epithelial tissue, which is regarded as the target tissue for breast cancer (37,38), is higher in the larger breast (39). Hence we will present results on both relative and absolute measures of breast density.

All mammograms were assessed by one observer in sets composed of randomly ordered films. Both the pre- and postmenopausal images of the same woman were always read in the same set which contained 36 images. The order in which the mammograms within a set were presented to the reader was also randomized. To assess the reliability of the reader, a library set was created, which consisted of 36 randomly chosen films from our study subjects. This library set was read before the first set and at five time points between sets, which were blinded for the reader. The images in the library set were randomly ordered every time they were read to prevent the observer from recognizing this set. In this study an average intraclass correlation coefficient of 0.99 (range: 0.99-1.00) for total breast area, 0.81 (range: 0.75-0.86) for absolute dense area and 0.90 (range: 0.88-0.92) for percent breast density was reached between repeated readings.

Statistical analyses

Breast measures that were not normally distributed were square root transformed. Breast measures that were transformed included; premenopausal non-dense area and dense area, as well as postmenopausal non-dense area, dense area and percent breast density. These transformed values were used in regression analyses and when calculating correlation coefficients. For ease of interpretation, presented means and 95% confidence intervals (95% CIs) have been transformed to the original scale. Changes in breast measures over menopause were all normally distributed.

Means and 95% CIs of breast measures (total breast area, non-dense breast area, dense breast area and percent breast density) by quartile level of serum IGF-I were estimated with linear regression models using the SAS 'GLM' procedure. The changes of breast measures over menopause were calculated as the absolute difference between post- and premenopausal measures. To test for linear trends over the quartiles, median values within quartiles were calculated and evaluated as a continuous variable using linear regression analysis. Regression coefficients and corresponding p-values were calculated for levels of IGF-I on a continuous scale, in linear models, also using the SAS 'GLM' procedure. Models used to calculate postmenopausal means, 95% CIs and ß-estimates, as well as means, 95% CIs and ß-estimates for menopausal changes in breast measures, also included the premenopausal breast measure of interest as covariate. Potential confounding of various factors was assessed by adding those variables to the crude models. The following characteristics were evaluated for confounding using continuous variables: age, body mass index (BMI), change in BMI over menopause (postmenopausal models only), waist circumference, age at menarche, age at menopause (postmenopausal models only), total physical activity, time span between first and second mammography and dietary intake of total energy, proteins, carbohydrates, fat and alcohol. Dichotomous variables were used to evaluate, ever use of OC, ever use of HT and ever breast feeding. Parity and age at first childbirth were evaluated using a combined variable with a category for nulliparous women and three categories of parous women combined with tertiles of age at birth of the first child. Smoking was evaluated using a variable with three categories, for current smokers, past smokers and never smokers. Analytical batches of IGF-I measurements were added to the regression models using a categorical variable as were the analytical batches of breast measurements. Postmenopausal age, BMI, OC ever use, HT ever use and smoking habits were used in the postmenopausal models. Only variables that changed crude mean breast measures with at least 1% were added to the regression models. Models used to calculate mean values, 95% CIs and ß-estimates of menopausal changes in breast measurements, were adjusting for the same variables included in the postmenopausal models. Breast measurements were measured in a total of 81 batches and adding the analytical batch of measurement, only changed crude mean values of breast measurements marginally. To increase the number of degrees of freedom this variable was not included in the final models.

To evaluate potential confounding of the associations between IGF-I and breast measures by BMI, Pearson's correlation coefficients were calculated between BMI and breast measures on the one hand and serum IGF-I on the other hand.

All p-values are two-sided and when below 0.05, results were considered statistically significant. All analyses were conducted using the Statistical Analysis System (SAS) software package, release 9.1 (SAS Institute, Cary, NC, USA).

Results

All study participants were premenopausal at baseline, when questionnaire data were collected, a blood sample was drawn and the first mammogram was taken. Additional data were collected on average 4.4 years after baseline (inter quartile range; 3.8-5.0 years), when a follow-up questionnaire was filled out. The postmenopausal mammogram was taken on average 1.1 years after this follow-up questionnaire (inter quartile range; 0.6-1.4 years); thus, the average time between the dates of the premenopausal and the postmenopausal mammograms was 5.5 years.

Baseline characteristics of the study population are presented in Table 1. At baseline, mean age was 51.4 years and mean BMI was 25.2 kg/m^2. BMI increased slightly over menopause to an average 25.7 kg/m^2 at time of follow-up, when all women were postmenopausal. Age at baseline was comparable between quartiles of premenopausal serum IGF-I, as were BMI at baseline, BMI at follow-up, age at menarche, age at menopause, being nulliparous, smoking in the past and time span between mammogram measurements. Women with high premenopausal IGF-I levels were somewhat younger at first child birth and the number of women who reported to have breast fed was highest in the upper quartile of IGF-I serum levels. There was no clear association between past smoking and IGF-I level, however most current smokers had low IGF-I levels. Because we excluded current HT users from the study population, only few women reported a history of HT use.

Median values of total breast area, non-dense area, dense area and percent breast density are presented in Table 2. The median of the dense area was 11.8 cm^2 smaller after menopause compared with the median dense area before menopause, likely explained by the involution of glandular tissue during menopause. Both total breast area and percent breast density were also smaller after menopause. The non-dense area, i.e. the amount of adipose tissue, slightly increased over menopause.

Total breast size as well as non-dense breast area was strongly positively correlated with BMI before menopause (Table 3). These correlations were somewhat less strong after menopause. Absolute density was inversely correlated with BMI, both before and after menopause, as was percent breast density. Circulating levels of IGF-I showed no clear linear or non-linear relationship with BMI.

In Table 4, associations between premenopausal IGF-I levels in serum and premenopausal breast measures are presented. There was no clear linear association between premenopausal IGF-I level and total breast size, non-dense area, absolute dense area or percent breast density, although women in the highest quartile of serum IGF-I had a slightly higher total breast area, non-dense area and dense area. As shown in Table 5, the absolute amount of breast density decreased during the menopausal transition phase.

Table 1. General characteristics of the study population

	Quartiles of premenopausal serum IGF-I (cut off points in ng/ml)				
	Total n=684	Q1 (< 94.6)	Q2 (94.6 – 111.3)	Q3 (111.4 – 132.0)	Q4 (≥ 132.0)
Age at baseline (yrs (SD))	51.4 (2.1)	51.4 (2.1)	51.7 (2.2)	51.4 (2.1)	51.1 (2.0)
Age at follow-up (yrs (SD))	55.8 (2.3)	55.9 (2.3)	56.1 (2.4)	55.7 (2.3)	55.4 (2.2)
BMI at baseline (kg/m2 (SD))	25.2 (3.8)	25.2 (4.2)	25.1 (3.9)	24.9 (3.5)	25.4 (3.5)
BMI at follow-up1 (kg/m2 (SD))	25.7 (4.4)	26.0 (5.4)	25.7 (4.7)	25.5 (3.7)	25.6 (3.5)
Time between mammogram measurements2 (yrs (SD))	5.5 (1.2)	5.7 (1.3)	5.4 (1.2)	5.4 (1.3)	5.5 (1.1)
Reproductive factors					
Age at menarche (yrs (SD))	13.2 (1.4)	13.2 (1.4)	13.2 (1.5)	13.2 (1.4)	13.3 (1.5)
Age at menopause (yrs (SD))	52.0 (2.8)	52.0 (2.5)	52.3 (2.7)	51.7 (2.9)	52.0 (2.9)
Nulliparous (nr (%))	70 (11.4)	20 (11.8)	17 (10.7)	25 (14.7)	16 (9.1)
Age at first child birth (yrs (SD))	25.1 (3.9)	25.4 (4.2)	25.4 (3.8)	24.9 (3.8)	24.7 (3.7)
Ever breast feeding3 (nr (%))	458 (75.6)	114 (76.0)	111 (73.0)	109 (75.2)	124 (78.0)
Lifestyle factors					
Ever used OC (nr (%))	490 (71.6)	116 (68.2)	124 (73.4)	116 (68.2)	134 (76.6)
Ever used HT (nr (%))	36 (5.3)	3 (1.8)	13 (7.7)	10 (5.9)	10 (5.8)
Current smoking (nr (%))	152 (22.2)	48 (28.2)	42 (24.9)	37 (21.8)	25 (14.3)
Ever smoked (nr (%))	417 (61.0)	102 (60.0)	108 (63.9)	106 (62.4)	101 (57.7)

[1] calculated using bodyweight at follow-up and body height at baseline
[2] time between the measurement of the pre- and the postmenopausal mammogram
[3] among parous women only

Table 2. Pre- and postmenopausal breast measures and changes in breast measures over menopause

		Pre menopause (baseline)	Post menopause (follow-up)	Absolute change over menopause
Breast area[1]	Median	105.4	95.0	-9.6
	Inter quartile range	(84.4 ; 133.8)	(68.6 ; 120.0)	(-25.5 ; 2.2)
Non-dense area[1]	Median	57.4	63.8	0.5
	Inter quartile range	(36.9 ; 89.2)	(34.5 ; 94.3)	(-13.5 ; 14.7)
Dense area[1]	Median	42.7	26.8	-11.8
	Inter quartile range	(30.1 ; 57.3)	(14.3 ; 37.7)	(-23.5 ; - 3.9)
% Breast density	Median	44.4	34.3	-6.7
	Inter quartile range	(27.4 ; 59.2)	(16.5 ; 52.0)	(-15.0 ; 0.7)

[1] in cm^2

This decrease was slightly smaller in women with high premenopausal IGF-I levels than in those with low IGF-I levels, although the association was not linear (p-trend=0.58). In contrast, the non-dense area increased slightly during menopause, and this increase was strongest in women with low levels of premenopausal IGF-I (p-trend=0.09). This inverse relation between IGF-I and increase in non-dense area remained after corrections for confounding factors. As a consequence of these opposing effects on the absolute size of the dense tissue and the non-dense tissue, the decrease in percent breast density over menopause was lowest in women with high levels of premenopausal IGF-I (p-trend=0.06). Table 6 shows associations between premenopausal IGF-I and postmenopausal breast measures. Higher levels of premenopausal IGF-I were associated with significantly higher percent breast density after menopause (p-trend=0.02), and with lower total breast size and non-dense area (p-trend: 0.21 and 0.05 for breast size and non-dense area respectively). Women with high premenopausal IGF-I levels had slightly higher absolute density (28.6 cm^2 in the highest versus 27.8 cm^2 in the lowest quartile), however there was no evidence for a linear relationship (p-trend=0.66).

Discussion

In this study we did not find serum IGF-I levels to be associated with breast size, non-dense (fat) breast tissue or breast density before menopause. However, high levels of IGF-I *before* menopause were associated with lower total breast size and non-dense breast tissue and with higher breast density *after* menopause. The association with percent breast density after menopause was mostly explained by a smaller increase in non-dense breast tissue over menopause in women with higher levels of premenopausal IGF-I.

Table 3. Pearson's coefficients of correlation and 95% confidence intervals between breast measures, IGF-I serum levels and BMI

	Premenopausal BMI (baseline)[1]	Postmenopausal BMI (follow-up)[2]
Breast area	0.62 (0.57 ; 0.66)	0.46 (0.40 ; 0.52)
Non-dense area	0.61 (0.56 ; 0.66)	0.51 (0.45 ; 0.56)
Dense area	-0.12 (-0.19 ; -0.04)	-0.15 (-0.22 ; -00.7)
% breast density	-0.46 (-0.52 ; -0.40)	-0.40 (-0.46 ; -0.33)
IGF-I	0.02 (-0.06 ; 0.09)	-0.02 (-0.10 ; 0.05)

[1] correlations of premenopausal breast measurements and IGF-I serum levels with premenopausal BMI
[2] correlations of postmenopausal breast measurements and premenopausal IGF-I serum levels with postmenopausal BMI. postmenopausal BMI was calculated with premenopausal body height and postmenopausal body weight

A major strength of our study is its longitudinal design. IGF-I levels in serum as well as premenopausal breast measures were measured on average 5.5 years before the postmenopausal mammogram was taken, which enabled us to look at the effect of premenopausal circulating levels of IGF-I on the menopausal involution process of the breast. To our knowledge, the present study is the first study presenting results of premenopausal IGF-I levels and both pre- and postmenopausal breast measurements of the same women. Another advantage of the present study is its size. With 684 participants, our study is one of the largest studies conducted so far on circulating IGF-I levels and breast density.

All women in the present study had at least one menstrual period in the last 12 months prior to their first mammographic measurement and were therefore classified to be premenopausal. Part of these women were, however, probably already perimenopausal, because all participants were 49 years at age or older at study entry. Furthermore, the exact date of the last menstrual period was unknown. Hence, the time to menopause (for the premenopausal mammogram) and the time from menopause (for the postmenopausal mammogram) could not be determined and may have influenced all of the measures used. Nevertheless, the time between the first and the second mammogram (5.5 years on average) will still have included the main part of the menopausal transition phase, when stronger breast involution occurs compared with breast involution before menopause (27).

Table 4. Crossectional analyses of premenopausal serum IGF-I concentrations and premenopausal breast measures

	Quartiles of premenopausal serum IGF-I (cut off points in ng/ml)						
	Total	Q1 (< 94.6)	Q2 (94.6-111.3)	Q3 (111.4-132.0)	Q4 (≥ 132.0)	p-trend	ß-estimate p-value
Total breast size[1] (cm^2)							
Mean	111.4	111.9	109.7	111.7	112.8		0.013
95% CI	(105.9 ; 117.0)	(105.1 ; 118.7)	(103.2 ; 116.3)	(105.0 ; 118.4)	(106.1 ; 119.5)	0.61	0.74
Non-dense area[2] (cm^2)							
Mean	62.1	62.0	60.1	62.5	63.9		0.001
95% CI	(56.3 ; 68.3)	(54.9 ; 69.6)	(53.2 ; 67.4)	(55.5 ; 69.9)	(56.9 ; 71.4)	0.63	0.73
Dense area[3] (cm^2)							
Mean	37.6	37.7	36.8	37.5	38.6		0.002
95% CI	(33.5 ; 42.0)	(32.7 ; 43.0)	(32.0 ; 41.9)	(32.6 ; 42.7)	(33.6 ; 43.9)	0.68	0.35
% breast density[4]							
Mean	41.8	41.9	42.3	41.4	41.5		0.006
95% CI	(38.1 ; 45.5)	(37.4 ; 46.4)	(37.9 ; 46.7)	(37.1 ; 45.8)	(37.1 ; 45.9)	0.70	0.81

Means and 95% CIs were calculated using linear regression analyses with quartiles of premenopausal serum IGF-I ß-estimates and corresponding p-values were calculated for premenopausal IGF-I levels on a continuous scale, using linear regression analyses. To normalize distributions square root transformed variables of non-dense area and dense area were used in the regression models. For ease of interpretation, means and 95% CIs have been transformed to the original scale.

[1] means, 95% CIs and ß-estimate were adjusted for BMI, former HT use and breast feeding

[2] means and 95% CIs were adjusted for age, BMI, former HT use, breast feeding, age at menarche, age at first child birth, parity, analytical batch of IGF-I measurements

[3] means and 95% CIs were adjusted for former OC use, former HT use, smoking status, age at first child birth, parity, analytical batch of IGF-I measurements

[4] means and 95% CIs were adjusted for age, BMI, former OC use, former HT use, breast feeding, age at first child birth, parity, analytical batch of of IGF-I measurements

95% CI=95% confidence interval; BMI=body mass index; OC=oral contraceptives; HT=postmenopausal hormone therapy

Table 5. Premenopausal serum IGF-I concentrations and changes in breast measures over menopause[1]

	Quartiles of premenopausal serum IGF-I (cut off points in ng/ml)						
	Total	Q1 (< 94.6)	Q2 (94.6-111.3)	Q3 (111.4-132.0)	Q4 (≥ 132.0)	p-trend	ß-estimate p-value
Total breast size[2] (cm^2)							
Mean	-11.5	-8.1	-11.4	-12.7	-12.8		-0.061
95% CI	(-17.0 ; -5.9)	(-15.2 ; -1.1)	(-18.1 ; -4.7)	(-19.5 ; -5.8)	(-19.5 ; -6.0)	0.21	0.14
Non-dense area[3] (cm^2)							
Mean	3.7	7.3	3.6	2.6	2.1		-0.070
95% CI	(-3.3 ; 10.7)	(-0.9 ; 15.4)	(-4.3 ; 11.4)	(-5.3 ; 10.5)	(-5.8 ; 10.0)	0.09	0.06
Dense area[4] (cm^2)							
Mean	-13.0	-12.9	-13.4	-13.2	-12.2		0.017
95% CI	(-16.9 ; -9.1)	(-17.7 ; -8.2)	(-17.9 ; -8.9)	(-17.8 ; -8.7)	(-16.8 ; -7.6)	0.58	0.43
% breast density[5]							
Mean	-8.7	-11.3	-8.6	-8.2	-7.9		0.043
95% CI	(-12.3 ; -5.2)	(-15.4 ; -7.2)	(-12.6 ; -4.7)	(-12.1 ; -4.2)	(-11.9 ; -3.9)	0.06	0.02

Means and 95% CIs were calculated using linear regression analyses with quartiles of premenopausal serum IGF-I ß-estimates and corresponding p-values were calculated for premenopausal IGF-I levels on a continuous scale, using linear regression analyses. To normalize distributions square root transformed variables of premenopausal non-dense area and dense area and postmenopausal non-dense area, dense area and percent breast density were used in the regression models. For ease of interpretation, means and 95% CIs have been transformed to the original scale.

[1] calculated as postmenopausal value – premenopausal value
[2] means and 95% CIs were adjusted for premenopausal total breast size, BMI, former OC use, former HT use, analytical batch of IGF-I measurements
[3] means and 95% CIs were adjusted for premenopausal non-dense area, BMI, change in BMI over menopause, age at menopause, former OC use, former HT use, analytical batch of IGF-I measurements
[4] means and 95% CIs were adjusted for premenopausal dense area, BMI, change in BMI over menopause, smoking status, age at menopause, former HT use, age at first child birth, parity
[5] means and 95% CIs were adjusted for premenopausal percent breast density, BMI, change in BMI over menopause, age at menopause, former OC use, former HT use, analytical batch of IGF-I measurements
95% CI=95% confidence interval; BMI=body mass index; OC=oral contraceptives; HT=postmenopausal hormone therapy

Table 6. Premenopausal serum IGF-I concentrations and postmenopausal breast measures

	Quartiles of premenopausal serum IGF-I (cut off points in ng/ml)						
	Total	Q1 (< 94.6)	Q2 (94.6-111.3)	Q3 (111.4-132.0)	Q4 (≥ 132.0)	p-trend	ß-estimate p-value
Total breast size[1] (cm^2)							
Mean	100.0	103.4	100.1	98.9	98.7		-0.061
95% CI	(94.5 ; 105.6)	(96.3 ; 110.5)	(93.5 ; 106.8)	(92.0 ; 105.7)	(92.0 ; 105.5)	0.21	0.14
Non-dense area[2] (cm^2)							
Mean	65.4	70.5	65.9	63.6	63.6		-0.006
95% CI	(57.5 ; 73.7)	(61.1 ; 80.6)	(57.1 ; 75.2)	(55.0 ; 72.9)	(54.9 ; 72.8)	0.05	0.03
Dense area[3] (cm^2)							
Mean	27.8	27.8	27.8	27.1	28.6		0.001
95% CI	(23.9 ; 31.9)	(23.4 ; 32.6)	(23.6 ; 32.4)	(22.9 ; 31.7)	(24.2 ; 33.4)	0.66	0.52
% breast density[4]							
Mean	30.1	27.8	29.8	30.4	31.4		0.004
95% CI	(26.7 ; 33.6)	(24.2 ; 31.8)	(26.1 ; 33.7)	(26.7 ; 34.4)	(27.6 ; 35.5)	0.02	0.01

Means and 95% CIs were calculated using linear regression analyses with quartiles of premenopausal serum IGF-I ß-estimates and corresponding p-values were calculated for premenopausal IGF-I levels on a continuous scale, using linear regression analyses. To normalize distributions square root transformed variables of premenopausal non-dense area and dense area were used in the regression models.

[1] means and 95% CIs were adjusted for premenopausal total breast size, BMI, former OC use, former HT use, analytical batch of IGF-I measurements
[2] means and 95% CIs were adjusted for premenopausal non-dense area, BMI, change in BMI over menopause, age at menopause, former OC use, former HT use, analytical batch of IGF-I measurements
[3] means and 95% CIs were adjusted for premenopausal dense area, BMI, change in BMI over menopause, smoking status, age at menopause, former HT use, age at first child birth, parity
[4] means and 95% CIs were adjusted for premenopausal percent breast density, BMI, change in BMI over menopause, age at menopause, former OC use, former HT use, analytical batch of IGF-I measurements
95% CI=95% confidence interval; BMI=body mass index; OC=oral contraceptives; HT=postmenopausal hormone therapy

Restricting the analyses to a selection of the women who had had at least 6 menstrual periods in the 12 months prior to their first mammogram measurement, did not change the associations markedly.

A very large proportion of IGF-I in the circulation is bound to IGFBP-3, and blood levels of IGFBP-3 are therefore an important co determinant of the fraction of IGF-I that can diffuse to target tissues. We did not have any measurement of circulating IGFBP-3 levels, and thus could not adjust our analyses. It is hard to speculate if the present results would have been influenced by IGFBP-3 adjustments. IGF-I and IGFBP-3 are positively correlated, which was also shown in the larger EPIC cohort (16). IGFBP-3 may influence breast density in an IGF-I independent manner, by inducing apoptosis and inhibiting cell growth (40). Hence, IGFBP-3 is a potential confounder for the association between IGF-I levels and breast density. The positive relation between IGF-I and IGFBP-3 and the inverse effect of IGFBP-3 on breast density, may have attenuated a positive association between premenopausal IGF-I levels and premenopausal breast density - both as absolute measure and as percentage of the whole breast- in the present study. Indeed, in previous studies on premenopausal women, the direct association of IGF-I with percent breast density generally became stronger after adjustment for IGFBP-3 (20,23,25).

Another explanation for the absence of association between IGF-I levels and premenopausal breast density, may be the relative old age of our study population at baseline (51.4 years) compared to the average age of premenopausal women in studies that did find higher IGF-I levels to be associated with higher breast density (range; 42.9-48.3 years) (20,21,23,25). Maybe a premenopausal association between IGF-I levels and breast density decreases upon menopause. However, two other studies on this association, which were in the same age range (43.3 and 46.5 years), also did not find IGF-I levels and breast density to be associated (22,24).

Our hypothesis was that IGF-I, with its mitogenic and anti-apoptotic properties, increases the amount of dense tissue, which is regarded as the target tissue for breast cancer (37,38). Results of epidemiologic studies showing IGF-I levels to be associated with increased breast density and also with increased breast cancer risk, support this hypothesis, although the associations were only seen in premenopausal women (9-25). Given these effects and the fact that IGF-I gradually decreases with age (28), we have hypothesized that *post*menopausal risk could be mainly determined before menopause, when IGF-I levels are still high. Our finding of the association between high *pre*menopausal IGF-I levels and increased *post*menopausal breast density, supports this hypothesis and is in line with results of a study recently published by Rollinson et al., showing increased *post*menopausal breast cancer risk in women with high *pre*menopausal IGF-I levels (29).

However, we only found IGF-I levels to be associated with the relative measure of breast density and not with the absolute breast density measure, although women in the highest quartile of IGF-I levels had a slightly higher dense area. The association between IGF-I and relative breast density (percent breast density) was mainly driven by the inverse association of IGF-I with the non-dense area (fat tissue), which is part of the denominator of the algorithm to calculate relative breast density. These findings are not in line with the idea that IGF-I increases the number of glandular cells (dense tissue), and in this regard, do not support the hypothesis that IGF-I can increase breast cancer risk through an increase in dense tissue. Most studies on IGF-I and breast density do not report results of the absolute dense area. Only two studies reporting increased percent breast density with high IGF-I levels, also reported associations with the absolute dense area. Maskarinec et al. found comparable associations for the relative and absolute measures, but Boyd et al. showed a somewhat stronger effect for relative breast density compared with absolute density (21,25). A third study did not show any association with either the relative or the absolute measure of breast density (24). Non-dense area was only reported by two studies. The first found a small, non-significant inverse correlation (21), the second reported a borderline significant association, but only among postmenopausal women, which disappeared after correction for confounding factors (24).

The lack of association between premenopausal IGF-I levels and premeno-pausal breast density mirrors the lack of association between IGF-I circulating levels and breast cancer risk in the larger EPIC study, reported by Rinaldi et al. (16). The present study is not a subset of that study, although there is a small overlap (n=21). IGF-I measurements were performed independently, using a different method. Therefore the lack of association could be more than coincidence. Genetic variation in the IGF-I gene was however associated with increased cancer risk within the EPIC cohort, especially among women aged 55 years or younger (42). More research is therefore needed to elucidate the exact role of IGF-I in breast density and breast cancer development.

In the present study, IGF-I levels were borderline significantly associated with smaller increase of the non-dense area during the menopausal transition phase and consequently, with smaller non-dense area after menopause. IGF-I is known to reflect levels of growth hormone (GH), which provides the key stimulus for IGF-I synthesis in the liver and many other tissues. GH has strong lipolytic actions, and will tend to reduce adipose tissue mass (43-47). Furthermore, a study by Mulhall et al. showed two polymorphisms in the GH gene to be associated with increased GH levels and decreased non-dense area (48). This may explain the borderline significant trend for menopausal change in non-dense area (fat), and postmenopausal non-dense area over the quartiles of IGF-I, observed in the present study.

An artifact in measurement of breast density may give another explanation for the lack of association between IGF-I and the absolute measure of breast density we observed. Possibly the absolute measurement of breast density and changes therein are more prone to measurement error than the relative measure caused by the influence of the level of compression of the breast when making a 2-dimensional image. If the number of glandular cells really is associated with breast cancer risk, this implies that the absolute measure of breast density is preferred over the relative measure. Hopefully in the near future, the culprit of the compression of the breast during mammography will be solved using a volumetric method. This method, using digital mammography, adjusted for compression and the amount of radiation used, will enable us to measure absolute density with much more precision (49).

In conclusion, high levels of circulating IGF-I *before* menopause were associated with a slightly smaller decrease of absolute dense tissue over menopause. Consequently, absolute density after menopause was larger in women with higher *pre*menopausal levels of IGF-I, although differences between extreme quartiles of absolute breast density were small and the data did not support a linear relationship. Hence these findings only to some extend support the hypothesis that *pre*menopausal IGF-I levels increase postmenopausal breast density. The significant association between *pre*menopausal IGF-I and *post*menopausal percent breast density was mainly explained by the smaller increase in non-dense area during the menopausal transition phase in women with high levels of IGF-I.

Acknowledgements

This study was supported by grant number 2002/11 of the World Cancer Research Fund (WCRF).

References

1. Boyd NF, Lockwood GA, Martin LJ, et al. Mammographic density as a marker of susceptibility to breast cancer: a hypothesis. IARC Sci Publ 2001;154:163-169.
2. Byrne C, Schairer C, Wolfe J, et al. Mammographic features and breast cancer risk: effects with time, age, and menopause status. J Natl Cancer Inst 1995;87:1622-1629.
3. Boyd NF, Byng JW, Jong RA, et al. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study. J Natl Cancer Inst 1995;87:670-675.
4. Van Gils CH, Hendriks JH, Holland R, et al. Changes in mammographic breast density and concomitant changes in breast cancer risk. Eur J Cancer Prev 1999;8:509-515.
5. Kleinberg DL. Role of IGF-I in normal mammary development. Breast Cancer Res Treat 1998;47:201-208.
6. Pollak MN. Endocrine effects of IGF-I on normal and transformed breast epithelial cells: potential relevance to strategies for breast cancer treatment and prevention. Breast Cancer Res Treat 1998;47:209-217.
7. Lee AV, Yee D. Insulin-like growth factors and breast cancer. Biomed Pharmacother. 1995;49:415-421.
8. Sachdev D, Yee D. The IGF system and breast cancer. Endocr Relat Cancer 2001;8: 197-209.
9. Hankinson SE, Willett WC, Colditz GA, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet 1998;351:1393-1396.
10. Toniolo P, Bruning PF, Akhmedkhanov A, et al. Serum insulin-like growth factor-I and breast cancer. Int J Cancer 2000;88:828-832.
11. Krajcik RA, Borofsky ND, Massardo S, et al. Insulin-like growth factor I (IGF-I), IGF-binding proteins, and breast cancer. Cancer Epidemiol Biomarkers Prev 2002;11:1566-1573.
12. Muti P, Quattrin T, Grant BJ, et al. Fasting glucose is a risk factor for breast cancer: a prospective study. Cancer Epidemiol Biomarkers Prev 2002;11:1361-1368.
13. Allen NE, Roddam AW, Allen DS, et al. A prospective study of serum insulin-like growth factor-I (IGF-I), IGF-II, IGF-binding protein-3 and breast cancer risk. Br.J.Cancer 2005;92:1283-1287.
14. Schernhammer ES, Holly JM, Pollak MN, et al. Circulating levels of insulin-like growth factors, their binding proteins, and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2005;14:699-704.
15. Kaaks R, Lundin E, Rinaldi S, et al. Prospective study of IGF-I, IGF-binding proteins, and breast cancer risk, in northern and southern Sweden. Cancer Causes Control 2002;13:307-316.

16. Rinaldi S, Peeters PH, Berrino F, et al. IGF-I, IGFBP-3 and breast cancer risk in women: The European Prospective Investigation into Cancer and Nutrition (EPIC). Endocr.Relat Cancer 2006;13:593-605.

17. Schernhammer ES, Holly JM, Hunter DJ, et al. Insulin-like growth factor-I, its binding proteins (IGFBP-1 and IGFBP-3), and growth hormone and breast cancer risk in The Nurses Health Study II. Endocr.Relat Cancer 2006;13:583-592.

18. Keinan-Boker L, Bueno De Mesquita HB, Kaaks R, et al. Circulating levels of insulin-like growth factor I, its binding proteins -1,-2, -3, C-peptide and risk of postmenopausal breast cancer. Int J Cancer 2003;106:90-95.

19. Gronbaek H, Flyvbjerg A, Mellemkjaer L, et al. Serum insulin-like growth factors, insulin-like growth factor binding proteins, and breast cancer risk in postmenopausal women. Cancer Epidemiol.Biomarkers Prev 2004;13:1759-1764.

20. Byrne C, Colditz GA, Willett WC, et al. Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. Cancer Res 2000;60:3744-3748.

21. Maskarinec G, Williams AE, Kaaks R. A cross-sectional investigation of breast density and insulin-like growth factor I. Int J Cancer 2003;107:991-996.

22. Lai JH, Vesprini D, Zhang W, et al. A polymorphic locus in the promoter region of the IGFBP3 gene is related to mammographic breast density. Cancer Epidemiol Biomarkers Prev 2004;13:573-582.

23. Diorio C, Pollak M, Byrne C, et al. Insulin-like growth factor-I, IGF-binding protein-3, and mammographic breast density. Cancer Epidemiol Biomarkers Prev 2005; 14:1065-1073.

24. dos Santos Silva I, Johnson N, De Stavola B, et al. The insulin-like growth factor system and mammographic features in premenopausal and postmenopausal women. Cancer Epidemiol.Biomarkers Prev 2006;15:449-455.

25. Boyd NF, Stone J, Martin LJ, et al. The association of breast mitogens with mammographic densities. Br J Cancer 2002;87:876-882.

26. Guo YP, Martin LJ, Hanna W, et al. Growth factors and stromal matrix proteins associated with mammographic densities. Cancer Epidemiol Biomarkers Prev 2001;10:243-248.

27. Boyd N, Martin L, Stone J, et al. A longitudinal study of the effects of menopause on mammographic features. Cancer Epidemiol Biomarkers Prev 2002;11:1048-1053.

28. Goodman-Gruen D, Barrett-Connor E. Epidemiology of insulin-like growth factor-I in elderly men and women. The Rancho Bernardo Study. Am.J.Epidemiol 1997; 145:970-976.

29. Rollison DE, Newschaffer CJ, Tao Y, et al. Premenopausal levels of circulating insulin-like growth factor I and the risk of postmenopausal breast cancer. Int.J.Cancer 2006;118:1279-1284.

30. Riboli E, Kaaks R. The EPIC Project: rationale and study design. European Prospective Investigation into Cancer and Nutrition. Int J Epidemiol 1997;26 Suppl 1:S6-14.

31. Riboli E, Hunt KJ, Slimani N, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002;5:1113-1124.

32. Boker LK, Van Noord PA, van der Schouw YT, et al. Prospect-EPIC Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. Eur J Epidemiol 2001;17:1047-1053.

33. Ocke MC, Bueno-de-Mesquita HB, Pols MA, et al. The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. Int J Epidemiol 1997;26 Suppl 1:S49-S58.

34. Ocke MC, Bueno-de-Mesquita HB, Goddijn HE, et al. The Dutch EPIC food frequency questionnaire. I. Description of the questionnaire, and relative validity and reproducibility for food groups. Int J Epidemiol 1997;26 Suppl 1:S37-S48.

35. Byng JW, Boyd NF, Little L, et al. Symmetry of projection in the quantitative analysis of mammographic images. Eur J Cancer Prev 1996;5:319-327.

36. Byng JW, Boyd NF, Fishell E, et al. The quantitative analysis of mammographic densities. Phys Med Biol 1994;39:1629-1638.

37. Trichopoulos D, Lipman RD. Mammary gland mass and breast cancer risk. Epidemiology 1992;3:523-526.

38. Albanes D, Winick M. Are cell number and cell proliferation risk factors for cancer? J.Natl.Cancer Inst 1988;%20;80:772-774.

39. Haars G, Van Noord PA, Van Gils CH, et al. Measurements of breast density: no ratio for a ratio. Cancer Epidemiol.Biomarkers Prev 2005;14:2634-2640.

40. Ali O, Cohen P, Lee KW. Epidemiology and biology of insulin-like growth factor binding protein-3 (IGFBP-3) as an anti-cancer molecule. Horm.Metab Res 2003;35: 726-733.

41. Kaaks R. Insulin-like growth factor-I and mammographic breast density. Cancer Epidemiol.Biomarkers Prev 2005;14:3019-

42. Canzian F, McKay JD, Cleveland RJ, et al. Polymorphisms of genes coding for insulin-like growth factor 1 and its major binding proteins, circulating levels of IGF-I and IGFBP-3 and breast cancer risk: results from the EPIC study. Br.J.Cancer 2006;94:299-307.

43. Djurhuus CB, Gravholt CH, Nielsen S, et al. Additive effects of cortisol and growth hormone on regional and systemic lipolysis in humans. Am.J.Physiol Endocrinol.Metab 2004;286:E488-E494.

44. Louveau I, Gondret F. Regulation of development and metabolism of adipose tissue by growth hormone and the insulin-like growth factor system. Domest.Anim Endocrinol 2004;27:241-255.

45. Flint DJ, Binart N, Kopchick J, et al. Effects of growth hormone and prolactin on adipose tissue development and function. Pituitary 2003;6:97-102.

46. Hansen TK, Gravholt CH, ORskov H, et al. Dose dependency of the pharmacokinetics and acute lipolytic actions of growth hormone. J.Clin.Endocrinol.Metab 2002;87: 4691-4698.

47. Moller N, Gjedsted J, Gormsen L, et al. Effects of growth hormone on lipid metabolism in humans. Growth Horm.IGF.Res 2003;13 Suppl A:S18-21.:S18-S21.

48. Mulhall C, Hegele RA, Cao H, et al. Pituitary growth hormone and growth hormone-releasing hormone receptor genes and associations with mammographic measures and serum growth hormone. Cancer Epidemiol.Biomarkers Prev 2005;14:2648-2654.

49. van Engeland S, Snoeren PR, Huisman H, et al. Volumetric breast density estimation from full-field digital mammograms. IEEE Trans.Med.Imaging 2006;25:273-282.

Common genetic variation in the *IGF-1* gene, serum IGF-I levels and breast density

Chapter 2.2

Martijn Verheus
James D. McKay
Rudolf Kaaks
Carine Biessy
Mattias Johanson
Diederick E. Grobbee
Petra H.M. Peeters
Carla H. van Gils

Submitted.

Abstract

Introduction

High breast density is one of the strongest known risk factors for developing breast cancer. Insulin-like growth factor I (IGF-I) is a strong mitogen and has been suggested to increase breast cancer risk by increasing the amount of dense tissue in the female breast.

Objectives

We wanted to investigate the effect of common variation in the *IGF-1* gene on serum IGF-I levels and on percent breast density, as well as the absolute dense and non-dense areas of the breast.

Design and Methods

Mammograms and blood samples of 1928 premenopausal participants of the Prospect-EPIC cohort were collected at baseline. Prospect-EPIC is a breast cancer screening cohort participating in the European Prospective Investigation into Cancer and Nutrition (EPIC). Using a haplotype tagging approach, 15 single nucleotide polymorphisms (SNP) from 3 blocks covering the *IGF-1* gene were selected and genotyped in all study participants. Breast measures were assessed using a quantitative computer-assisted method. For a subgroup of women, who went through menopause within 5 years after recruitment (n=656), premenopausal IGF-I levels in serum and additional postmenopausal breast measures were determined. Associations between genetic variation on the one hand, and IGF-I circulating levels and breast measures on the other hand, were assessed using linear regression models.

Results

Five SNPs in block 3 were significantly associated with elevated levels of IGF-I (p-values: 0.01–0.04). The same SNPs were related, although not statistically significantly, with higher percent breast density before menopause and with a smaller decrease of percent density during the menopausal transition phase. The effects were most obvious for two SNPs, of which one has previously been reported to be related to higher IGF-I levels and increased breast cancer risk (rs6220).

Conclusion and discussion

Common genetic variation in the *IGF-I* gene may be related with circulating levels of IGF-I and possibly also with breast density.

Introduction

Insulin-like growth factor I (IGF-I) has mitogenic properties and prohibits apoptosis in several breast cancer cell lines (1,2). In addition, in xenocraft models tumor growth was decreased in IGF-I deficient animals (3). Prospective epidemiological studies on circulating levels of IGF-I and breast cancer risk have shown different results between pre- and postmenopausal women. In premenopausal women, high IGF-I levels were associated with increased breast cancer risk in most studies (4-9), but not all (10-12). Most studies with postmenopausal women did not show a clear association between IGF-I levels and breast cancer risk (4-11,13,14).

IGF-I is also involved in the development of normal breast tissue (3,15) and hence, can influence the amount of stromal and glandular tissue within the breast. This tissue appears light on a mammogram (dense breast tissue), in contrast to the dark appearance of fat tissue (non-dense tissue) (16). High mammographic density has been associated with a 3–6 fold increase in risk, of both pre- and postmenopausal breast cancer and is therefore one of the strongest known risk factors for breast cancer development (17-20). It has been suggested that elevated circulating levels of IGF-I may increase breast cancer risk by increasing breast density. Several studies have been published on the association between circulating levels of IGF-I and breast density (21-27). IGF-I was directly associated with breast density among premenopausal women in most studies (21,22,24,26), but not all (23,25,27). This effect was not seen in postmenopausal women (21,23-26). It is known that IGF-I levels gradually decrease with age (28), and we hypothesized that the involution of breast tissue during menopause, and consequently also postmenopausal breast density and postmenopausal breast cancer risk, are largely determined before menopause when IGF-I levels are still high. Results from a study by Rollison et al., reporting higher premenopausal IGF-I levels to be associated with increased postmenopausal breast cancer risk, support this hypothesis (29). In a previous study, we showed that high premenopausal IGF-I levels were related to larger percent breast density after menopause, although the association was mainly driven by an inverse association between IGF-I levels and the non-dense area instead of a positive association with the dense area (27).

A disadvantage of studying circulating levels of IGF-I is that they fluctuate over time. Genetic polymorphisms, if associated with IGF-I levels, may provide a more accurate indication for inter-individual differences in lifetime exposure to IGF-I. To our knowledge no studies have been published on IGF-I genetic variation and breast density. To date, only a few studies on common genetic variation of the *IGF-1* gene and breast cancer risk have been published (30-32). Setiawan et al. did not find evidence for an association between common genetic variation of the *IGF-1* gene and breast cancer risk, but two other studies found several single nucleotide polymorphisms (SNP) to be related to breast cancer risk (30,31).

In the present study, we set out to study common genetic variation across the *IGF-1* gene in relation to circulating IGF-I levels and in relation to mammographic density, using a haplotype tagging approach.

Methods

The Prospect-EPIC cohort

This study included women participating in Prospect-EPIC, one of two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition (EPIC). The Prospect-EPIC cohort consists of 17,357 women, aged 49–69 years at enrolment. Women were recruited between 1993 and 1997 through a regional programme for breast cancer screening and reside in Utrecht or its vicinity. The regional programme is part of the population based national screening programme in the Netherlands. A mammogram is taken every two years, starting at age 50. At time of study enrolment anthropometric measurements were taken and a 30 ml non-fasting blood sample was drawn and stored at -196 °C under liquid nitrogen. Participants were asked to fill out two questionnaires. A general questionnaire was used to gather information on demographic, reproductive and lifestyle factors and past and current morbidity. To determine regular dietary intake, a validated extensive self-administered food frequency questionnaire was used (33,34). Approximately 3-5 years after study recruitment participating women were asked to fill out a follow-up questionnaire, again containing questions about demographic, reproductive and lifestyle factors and past and current morbidity. Rationale and design of both EPIC and Prospect-EPIC are described in detail elsewhere (35-37).

All participants signed an informed consent and the study was approved by The Institutional Review Board of the University Medical Center Utrecht.

Study population

From the 16,917 subjects that had donated a blood sample, 4055 where premenopausal at baseline. Women were considered pre- or perimenopausal if they had menses at least once during the last 12 months prior to blood collection. Current users of oral contraceptives (OC) or postmenopausal hormone therapy (HT) were excluded, as were former users who quit using these components less than two years prior to blood donation. Women having had ovariectomy on both ovaries were also excluded. Three women had had hysterectomy. All three still had both ovaries however, and met all other selection criteria. Therefore they were not excluded. After these exclusions, our study group comprised 2325 women. Of these, 264 women, were excluded because their mammograms could not be retrieved from the archives. Another 14 were excluded for having silicone protheses. Twenty-two women had breasts too large to fit on a single mammogram. The

latter group had therefore more than one mammogram which could not be used for computation of total breast and dense area because the overlap between these mammograms could not be measured properly. For 2025 women we were able to retrieve a mammogram. Of 1981 of these women, a blood sample could be retrieved from the Prospect-EPIC biobank. The genotype of at least 9 of the 15 SNPs used in the study could be determined for 1928 women.

A follow-up questionnaire was filled in approximately 3 to 5 years after baseline measurements. Part of the women had become postmenopausal at the time that they filled in a follow-up questionnaire. Women were considered postmenopausal when they had not had any menses during the 12 months prior to filling in the follow-up questionnaire. For these women, the first mammogram after filling in the follow-up questionnaire, was retrieved and available for 750 participants. None of these women were current users of OC or HT or had used these compounds less than two years before filling out the follow-up questionnaire. One woman was excluded because she had silicone protheses.

Of the 662 women with genetic data and both a premenopausal and a postmenopausal mammogram available, 656 had a serum sample available to be used for IGF-I measurements. We previously reported the relation between circulating premenopausal IGF-I levels and pre- and postmenopausal breast density as well as the change in breast density over menopause in these women (27). Twenty-two women in the present study were also included in our resent study on genetic variation of the *IGF-1* gene and breast cancer risk in the EPIC cohort (30).

Haplotype block definition and SNP selection

The haplotype blocks within the *IGF-1* gene, as well as the set of haplotype tagging SNPs (htSNPs) that we chose to represent the majority of common genetic variation across the *IGF-1* gene locus, were described by Johansson et al. (38). Briefly, haplotype blocks were identified according to the criteria defined by Gabriel et al. (39). We obtained SNPs genotyped across the *IGF-1* locus in the Caucasian population and used a htSNP approach, tagging haplotypes inside linkage disequilibrium (LD) blocks, along the lines of the criteria suggested by Stram et al. (40). We used the criteria of using the statistic of $R^2_h \geq 0.8$ for haplotypes inside an LD block to capture the maximum amount of genetic variation with the minimum set of SNPs. To these series we added SNPs rs978458, rs5742714 and rs9989002 to increase the average R^2_h described by Johansson et al. from 0.8 to 0.9. We also added rs1520220, which has recently been associated with circulating levels of IGF-I and with breast cancer risk (31), as well as rs7965399, which has recently been associated with prostate cancer risk (41). Finely we tested directly SNP rs6214, which was not in strong linkage disequilibrium with any of the other SNPs. This resulted in 15 SNPs in the *IGF-1* gene, listed in Table 3.

Haplotype frequency estimation and haplotype assigning

Haplotype frequencies of our study population were estimated using the SNP frequencies of the htSNPs of our study population. This was done using the EM-algorithm by Excoffier and Slatkin (42). These frequencies were then used to estimate haplotype dosages, using an individual's genotype, with the method described by Stram et al. (43).

Genotyping

Genotyping was performed by the 5' nuclease assay (TaqMan). Taqman probes were synthesized by either Applied Biosystems (with MGB chemistry) or Proligo (with or without LNA chemistry). Sequences of primers and probes are available on request. The reaction mix included 5 ng genomic DNA, 5 pmol of each primer, 1 pmol of each probe, and 2.5 µl of 2 X master mix (Applied Biosystems) in a final volume of 5 µl. The thermocycling included 50 cycles with 30 seconds at 95 °C followed by 60 seconds at 60 °C. PCR plates were read on an ABI PRISM 7900HT instrument (Applied Biosystems Genotyping call rates were greater than 95% for all assays. The distributions of genotypes of all polymorphisms were in agreement with the Hardy-Weinberg equilibrium. Repeated quality control genotypes (8% of the total) showed greater than 99% concordance for all assays.

IGF-I measurements in serum

IGF-I in serum was measured by Labor Benrath (Düsseldorf, Germany) using immulite 2000 technology with reagents from DPC (Diagnostic Products Corporation, Frankfurt, Germany). The samples were analyzed in 11 batches, each of which contained the same two control samples. The interbatch coefficients of variation for the two control samples were 3.73% and 2.62%.

Mammographic density analysis

The assessment of breast measures in the present study population has been described in detail elsewhere (27,44). In brief, all mammograms were read in batches containing 36 mammograms by one reader. When there were both pre- and postmenopausal images of the same woman, these were always read in the same set which contained 36 images. The order in which the mammograms within a set were presented to the reader was randomized. To assess the reliability of the reader, a library set was created, which consisted of 36 randomly chosen films from our study subjects. This library set was read before the first set and at five time points between sets, which were blinded for the reader. The images in the library set were randomly ordered every time they were read to prevent the observer from recognizing the set. The results of these repeated readings were used to compute

reproducibility. The average intraclass correlation coefficient for total breast area, dense area and percent breast density were 0.99 (range: 0.99-1.00), 0.81 (range: 0.75-0.86), and 0.90 (range: 0.88-0.92) respectively.

Statistical analyses

First, to investigate the association between genotypes and levels of IGF-I we used linear regression models by utilizing the GLM procedure (SAS Base ®). Confounding by age and BMI was evaluated using linear regression models with IGF-I level as the outcome and adding a potential confounder as a covariate to the model in addition to one of the SNPs as the variable of interest. P-values for differences in mean IGF-I levels between genotypes were calculated for three different modes of assumed inheritance of effect; additive, dominant and recessive. Circulating levels of IGF-I were compared between subjects who were heterozygous or homozygous for the rare allele and all others (dominant model) or between subjects who were homozygous for the rare allele and all others (recessive model). In the additive model, serum IGF-I levels were assumed to be linearly related to the number of copies of an allele (0, 1 or 2) carried by the individuals. For SNPs with less than 20 women being homozygous for the rare allele, these women were grouped together with the heterozygous women, to gain power. Hence for these SNPs only the dominant model was assessed.

Secondly, SNPs were analyzed in relation to breast measures (percent breast density, dense breast area and non-dense breast area), and menopausal changes herein, using linear regression models. It is known that during the menopausal transition phase, normally, all breast measures change (decrease in dense area and percent density and increase in non-dense area) (45). Changes were calculated as the absolute difference between post- and premenopausal measurements. Potential confounding factors included age, and BMI at baseline (premenopausal models), age and BMI at follow-up (postmenopausal models and models with menopausal change), age at menarche, parity, age at first child birth, ever use of OC, ever use of HT, smoking habits and physical activity. Models used to analyze postmenopausal breast measures menopausal changes in breast measures, also included the premenopausal breast measure of interest as covariate. P-values for differences in mean values between genotypes were calculated in the same manner as for the differences in mean serum IGF-I.

Thirdly, haplotypes within haplotype blocks were analyzed. We analyzed haplotypes in relation to serum levels of IGF-I and in relation to breast measures by including the dosage variables as covariates in the linear regression model. The dosage value for each haplotype ranged from 0 to 2.0 (haplotypes), and for each individual these dosage values added up to a total value of 2.0 across all possible haplotypes given an individuals genotype. This dosage was used in the additive model. Haplotype dosages for being

Table 1. Baseline characteristics of the study population

	All premenopausal women (n=1928)		Women who became postmenopausal during 5.5 years of follow-up[1] (n=656)	
Age at baseline (years (SD))	51.0	(1.9)	51.4	(2.1)
Age at follow-up (years (SD))	N.A.		55.8	(2.3)
BMI at baseline (kg/m^2 (SD))	25.4	(4.0)	25.1	(3.8)
BMI at follow-up (kg/m^2 (SD))	N.A.		25.7	(4.4)
Time between mammogram measurements (years (SD))			5.5	(1.2)
Nulliparous (nr (%))	242	(12.6)	74	(11.3)
Age at first child birth (years (SD))	24.9	(4.1)	25.1	(3.9)
Age at menopause (years (SD))	N.A.		52.0	(2.8)
IGF-I serum levels	N.A.		114.7	(29.0)

[1] these women are a subgroup of all premenopausal women, and have both pre- and postmenopausal breast measurements as well as premenopausal serum IGF-I levels measured

SD=standard deviation; BMI=body mass index

homozygous for a certain haplotype had values ranging from 0 to 1.0 and were used in the recessive models. The haplotype dosage for having 1 copy of a certain haplotype and the dosage of having 2 copies of this haplotype were added up and used as haplotype dosage in the dominant model. Each haplotype was compared to all other haplotypes as the reference. The same confounding factors used for the analyses with single SNPs were selected for the models with haplotypes.

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

Results

All study participants were premenopausal at baseline, when questionnaire data were collected, a blood sample was drawn and the first mammogram was taken. The subgroup of women that had become postmenopausal during follow-up, which was on average 4.4 years after baseline, also had a postmenopausal mammogram available. The postmenopausal mammogram was collected on average 1.1 years after filling out a follow-up questionnaire. The average time between the dates of the premenopausal and the postmenopausal mammograms was thus 5.5 years.

Baseline characteristics of both the whole study population and the subgroup of women who became menopausal during follow-up, are presented in Table 1. Mean age of all premenopausal women was 51.0 years and mean BMI was 25.4 kg/m^2. In the subgroup of women that became postmenopausal during follow-up, mean age was slightly higher

Table 2. Median pre- and postmenopausal breast values and changes in breast values over menopause

	All premenopausal women (n=1928)	Women who became postmenopausal during 5.5 years of follow-up[1] (n=656)		
	Pre menopause	Pre menopause	Post menopause	Absolute change over menopause
Breast area[2]	109.4	105.3	94.6	-9.6
Dense area[2]	44.4	42.9	26.8	-12.0
Non-dense area[2]	58.7	56.5	62.3	0.5
% breast density	45.1	44.8	34.3	-6.7

[1] these women are a subgroup of all premenopausal women, and have both pre- and postmenopausal breast measurements as well as premenopausal serum IGF-1 levels measured
[2] in cm2

and BMI slightly lower (51.4 years and 25.1 kg/m^2 respectively). Median values of dense area, non-dense area and percent breast density are presented in Table 2. The dense area and percent breast density were slightly higher in the total group of women that were premenopausal at baseline than in the subgroup of women that became postmenopausal during follow-up. The median decrease in dense area during menopause was 12.0 cm^2 in the latter subgroup. This is likely to be explained by the involution of glandular tissue during menopause. Percent breast density was also smaller after menopause. The non-dense area, i.e. the amount of adipose tissue, slightly increased over menopause.

SNPs used for the present study have been numbered from 1 to 15. Rs numbers and other information on the 15 SNPs, are presented in Table 3.

Associations between SNPs and serum IGF-I levels are presented in Table 4. None of the SNPs tagging haplotype block 1 and 2 were significantly associated with IGF-I levels. Among 5 of the 7 SNPs tagging haplotype block 3, IGF-I mean levels differed significantly between women with different genotypes (SNP8, SNP9, SNP10, SNP11 and SNP13). Three SNPs in block 3 showed a significant trend in increasing levels of IGF-I (p-values: 0.04, 0.01 and 0.01 for SNPs 8, 11 and 13 respectively). SNP 9 could only be tested for dominant inheritance and gave a significant difference in IGF-I levels (p-value=0.02). For SNP10 a significant effect was only observed via the recessive mode of inheritance (p-value=0.03). SNP15 was not associated with IGF-I levels. None of the potential confounders tested changed the results substantially and hence the crude results were presented.

In Table 5 mean values of premenopausal breast measures for different genotypes are presented. None of the potential confounders tested changed the results substantially except for BMI, which attenuated effects of genotypes on mean values of percent breast density and non-dense area. Hence BMI was added to the models to calculate these figures.

Table 3. Haplotype tagging SNPs

SNP	rs number	Haplotype block	Minor allele frequency (%)	Polymorphism
SNP1	rs855211	1	16.4	G → A
SNP2	rs7965399	1	4.3	T → C
SNP3	rs35765	1	11.4	C → A
SNP4	rs2162679	1	16.0	A → G
SNP5	rs1019731	2	14.2	C → A
SNP6	rs7956547	2	24.7	A → G
SNP7	rs5742632	2	23.6	A → G
SNP8	rs9989002	3	26.5	C → T
SNP9	rs2033178	3	5.9	C → T
SNP10	rs7136446	3	38.6	T → C
SNP11	rs978458	3	25.7	G → A
SNP12	rs1520220	3	18.5	C → G
SNP13	rs6220	3	27.9	T → C
SNP14	rs5742714	3	10.2	C → G
SNP15	rs6214	-	41.0	C → T

SNP=single nucleotide polymorphism

SNPs tagging block 1 were associated with smaller percent breast density via the recessive mode of inheritance, but none of these associations were statistically significant. The opposite was seen with most SNPs tagging one of the other blocks. For two SNPs this association for the recessive mode of inheritance was borderline significant (p-values: 0.05 and 0.06 for SNPs 6 and 13 respectively). These associations were mainly driven by the significantly lower non-dense area in women being homozygous for the rare allele of these SNPs, although partly also by associations with the dense area. Associations between SNPs and the dense area were in the same direction as seen with percent density, but none of the effects was statistically significant. The rare alleles of all SNPs, with the exception of SNPs tagging haplotype block 1, were associated with the non-dense area in the same way as SNPs 6 and 13, i.e. smaller non-dense area in women being homozygous for the rare type allele, but only the results for SNPs 6 and 13 were statistically significant (p-values: 0.05 and 0.02 for SNPs 6 and 13 respectively).

During menopause, breast density decreases. None of the relations between single SNPs and change in percent breast density was statistically significant, with the exception that women who were homozygous for the rare allele of SNP10 showed a significantly smaller decrease in percent density during menopause (p-value=0.02) (Table 6). As a result, these women showed signifi-cantly higher percent density after menopause (p-value=0.04) (Appendix 1). This effect on percent breast density was explained by both the smaller decrease in the absolute dense area during menopause and by the decrease in non-dense area (as compared with an increase in non-dense area among women not being homozygous for the rare allele of SNP10). Four SNPs were borderline significantly associated with menopausal changes of the dense area (SNPs 2, 3, 8 and 10). None of the

Table 4. Associations between SNPs and serum IGF-I levels, in women who became postmenopausal during follow-up (n=656)

Block	SNP	Nr[1]	Genotype	Mean level	(95% CI)	p-value[2]	p-value[3]	p-value[4]
1	SNP1	445	GG	114.3	(111.6 - 117.0)		0.83	
		203	GA/AA	114.8	(110.9 - 118.8)			
1	SNP2	584	T T	114.9	(112.6 - 117.3)		0.66	
		55	TC/CC	113.1	(105.4 - 120.8)			
1	SNP3	496	CC	114.3	(111.7 - 116.9)		0.41	
		146	CA/AA	116.6	(111.8 - 121.3)			
1	SNP4	458	AA	114.1	(111.4 - 116.8)		0.39	
		195	AG/GG	116.2	(112.2 - 120.3)			
2	SNP5	491	CC	115.4	(112.9 - 118.0)		0.36	
		158	CA/AA	113.0	(108.5 - 117.5)			
2	SNP6	364	AA	113.9	(110.9 - 116.9)	0.36	0.42	0.50
		239	AG	115.4	(111.7 - 119.1)			
		43	GG	117.6	(108.9 - 126.3)			
2	SNP7	369	AA	114.0	(111.0 - 116.9)	0.34	0.64	0.13
		245	AG	114.1	(110.5 - 117.7)			
		33	GG	121.8	(112.0 - 131.5)			
3	SNP8	357	CC	113.0	(110.0 - 116.0)	**0.04**	0.10	0.09
		244	CT	115.8	(112.2 - 119.4)			
		53	T T	121.3	(113.5 - 129.1)			
3	SNP9	563	CC	113.6	(111.2 - 116.0)		**0.02**	
		65	CT/TT	122.5	(115.4 - 129.6)			
3	SNP10	256	T T	114.1	(110.5 - 117.7)	0.13	0.56	**0.03**
		289	TC	113.7	(110.3 - 117.0)			
		99	CC	120.7	(115.0 - 126.4)			
3	SNP11	361	GG	112.9	(109.9 - 115.9)	**0.01**	0.06	**0.02**
		240	GA	116.0	(112.3 - 119.7)			
		40	AA	125.2	(116.2 - 134.3)			
3	SNP12	428	CC	113.6	(110.9 - 116.4)	0.25	0.19	0.91
		197	CG	117.0	(112.9 - 121.0)			
		22	GG	115.4	(103.2 - 127.6)			
3	SNP13	339	T T	112.4	(109.3 - 115.5)	**0.01**	**0.03**	**0.04**
		238	TC	116.5	(112.8 - 120.2)			
		49	CC	122.9	(114.8 - 131.1)			
3	SNP14	493	CC	114.6	(112.0 - 117.2)		0.81	
		129	CG/GG	113.9	(108.9 - 118.9)			
-	SNP15	222	CC	114.1	(110.3 - 117.9)	0.83	0.71	0.92
		330	CT	115.2	(112.1 - 118.3)			
		96	T T	114.4	(108.6 - 120.2)			

Mean levels and CIs were not adjusted for confounding factors.

[1] nr of women with certain genotype

[2] p-value for additive mode of inheritance; IGF-I levels were linearly related to the number of copies of an allele (0, 1 or 2) carried by the individuals

[3] p-value for dominant mode of inheritance; IGF-I levels were compared between subjects who were heterozygous or homozygous for the rare allele and all others

[4] p-value for recessive mode of inheritance; IGF-I levels were compared between subjects who were homozygous for the rare allele and all others

SNP=single nucleotide polymorphism; CI=confidence interval

Block 3	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP8	1029	CC	43.3 (42.2 ; 44.4)	0.74	0.87	0.25	42.9 (41.5 ; 44.3)	0.76	0.45	0.48	63.4 (61.5 ; 65.4)	0.42	0.81	0.13
	744	CT	42.8 (41.5 ; 44.1)				41.8 (40.2 ; 43.5)				63.8 (61.5 ; 66.1)			
	133	TT	45.0 (41.9 ; 48.1)				43.9 (40.1 ; 48.0)				59.4 (54.3 ; 64.7)			
SNP9	1626	CC	43.2 (42.3 ; 44.1)		0.70		42.6 (41.5 ; 43.7)		0.47		63.4 (61.9 ; 65.0)		0.84	
	204	CT/TT	42.7 (40.2 ; 45.1)				41.6 (38.6 ; 44.7)				63.0 (58.8 ; 67.3)			
SNP10	718	TT	43.2 (41.8 ; 44.5)	0.53	0.96	0.19	42.7 (41.1 ; 44.4)	0.99	0.74	0.67	63.6 (61.3 ; 65.9)	0.37	0.82	0.15
	888	TC	42.7 (41.5 ; 43.9)				42.1 (40.7 ; 43.6)				64.0 (61.9 ; 66.1)			
	285	CC	44.4 (42.3 ; 46.6)				43.0 (40.4 ; 45.7)				60.9 (57.4 ; 64.6)			
SNP11	1034	GG	43.3 (42.1 ; 44.4)	0.60	0.84	0.36	42.6 (41.2 ; 44.0)	0.93	0.89	0.94	63.2 (61.3 ; 65.1)	0.32	0.74	0.07
	724	GA	43.2 (41.9 ; 44.5)				42.8 (41.1 ; 44.5)				63.5 (61.2 ; 65.8)			
	122	AA	44.8 (41.6 ; 48.0)				42.5 (38.6 ; 46.7)				58.2 (52.9 ; 63.6)			
SNP12	1255	CC	43.1 (42.1 ; 44.1)	0.65	0.67	0.80	42.5 (41.3 ; 43.8)	0.94	0.99	0.80	63.6 (61.9 ; 65.4)	0.38	0.47	0.45
	587	CG	43.4 (41.9 ; 44.9)				42.6 (40.8 ; 44.5)				62.8 (60.3 ; 65.3)			
	58	GG	43.8 (39.1 ; 48.5)				41.8 (36.2 ; 47.8)				60.3 (52.7 ; 68.3)			
SNP13	964	TT	43.0 (41.9 ; 44.2)	0.28	0.73	0.06	42.3 (40.8 ; 43.7)	0.55	0.76	0.39	63.6 (61.6 ; 65.6)	0.17	0.65	**0.02**
	748	TC	42.8 (41.5 ; 44.1)				42.3 (40.7 ; 44.0)				64.0 (61.8 ; 66.3)			
	143	CC	45.9 (42.9 ; 48.9)				44.0 (40.3 ; 47.9)				57.3 (52.5 ; 62.3)			
SNP14	1473	CC	43.1 (42.2 ; 44.0)		0.42		42.5 (41.3 ; 43.6)		0.63		63.4 (61.8 ; 65.0)		0.43	
	345	CG/GG	44.0 (42.1 ; 45.9)				43.0 (40.7 ; 45.5)				61.9 (58.7 ; 65.2)			

	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP15	644	CC	43.0 (41.6 ; 44.4)	0.62	0.68	0.71	42.2 (40.5 ; 44.0)	0.35	0.63	0.28	64.0 (61.6 ; 66.5)	0.57	0.46	0.92
	962	CT	43.3 (42.1 ; 44.4)				42.4 (41.0 ; 43.9)				62.9 (60.9 ; 64.9)			
	300	TT	43.6 (41.5 ; 45.6)				43.9 (41.3 ; 46.6)				63.1 (59.6 ; 66.7)			

Mean levels and CIs of percent breast density as well as the non-dense area were adjusted for BMI. Mean levels and CIs of the dense area were not adjusted for confounding factors.
[1] p-value for the additive mode of inheritance
[2] p-value for the dominant mode of inheritance
[3] p-value for the recessive mode of inheritance
SNP=single nucleotide polymorphism; CI=confidence interval; BMI=body mass index

Table 5. Associations between SNPs and premenopausal breast measures in the total group of premenopausal women (n=1928)

Block 1			% breast density				Dense area				Non-dense area			
	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP1	1332	GG	43.3 (42.3 ; 44.2)	0.56	0.87	0.16	42.6 (41.4 ; 43.9)	0.44	0.68	0.18	63.4 (61.7 ; 65.1)	0.85	0.69	0.60
	524	GA	43.4 (41.9 ; 45.0)				42.5 (40.6 ; 44.5)				62.5 (59.8 ; 65.2)			
	50	AA	39.6 (34.6 ; 44.7)				38.4 (32.6 ; 44.6)				65.5 (56.9 ; 74.6)			
SNP2	1726	TT	43.2 (42.3 ; 44.0)		0.82		42.5 (41.4 ; 43.6)		0.73		63.3 (61.8 ; 64.8)		0.71	
	155	TC/CC	42.8 (39.9 ; 45.7)				41.8 (38.3 ; 45.5)				64.3 (59.4 ; 69.4)			
SNP3	1478	CC	43.4 (42.4 ; 44.3)	0.47	0.67	0.15	42.8 (41.6 ; 43.9)	0.29	0.48	0.08	63.4 (61.8 ; 65.0)	0.78	0.68	0.65
	390	CA	43.2 (41.4 ; 45.0)				42.3 (40.1 ; 44.6)				62.5 (59.4 ; 65.6)			
	20	AA	37.5 (29.5 ; 45.4)				34.2 (25.8 ; 43.7)				66.4 (53.1 ; 81.3)			
SNP4	1341	AA	43.2 (42.2 ; 44.2)	0.79	0.93	0.24	42.6 (41.4 ; 43.8)	0.65	0.92	0.21	63.5 (61.8 ; 65.2)	0.64	0.57	0.87
	518	AG	43.6 (42.0 ; 45.1)				42.8 (40.9 ; 44.8)				62.4 (59.8 ; 65.2)			
	45	GG	40.1 (34.8 ; 45.4)				38.5 (32.4 ; 45.1)				63.9 (55.0 ; 73.5)			
Block 2	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP5	1406	CC	43.2 (42.2 ; 44.1)	0.70	0.96	0.22	42.6 (41.4 ; 43.8)	0.92	0.91	0.45	63.3 (61.6 ; 64.9)	0.78	0.92	0.18
	469	CA	42.9 (41.3 ; 44.6)				42.2 (40.2 ; 44.3)				64.0 (61.2 ; 66.9)			
	36	AA	46.9 (40.9 ; 52.9)				45.4 (38.0 ; 53.4)				56.5 (47.2 ; 66.7)			
SNP6	1070	AA	43.2 (42.1 ; 44.3)	0.46	0.99	**0.05**	42.7 (41.3 ; 44.0)	0.69	0.91	0.20	63.5 (61.6 ; 65.4)	0.39	0.90	**0.05**
	718	AG	42.7 (41.3 ; 44.0)				42.1 (40.5 ; 43.8)				64.2 (61.9 ; 66.5)			
	110	GG	46.5 (43.1 ; 49.9)				45.3 (41.1 ; 49.8)				57.8 (52.3 ; 63.5)			
SNP7	1095	AA	43.2 (42.1 ; 44.3)	0.81	0.89	0.32	42.7 (41.4 ; 44.1)	0.69	0.61	0.93	63.3 (61.4 ; 65.2)	0.51	0.98	0.06
	709	AG	42.8 (41.5 ; 44.2)				42.1 (40.4 ; 43.8)				64.1 (61.8 ; 66.4)			
	94	GG	45.0 (41.3 ; 48.7)				42.7 (38.2 ; 47.4)				57.6 (51.7 ; 63.8)			

Block 3	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP8	357	CC	-7.44 (-8.81 ; -6.07)	0.63	0.96	0.22	-16.7 (-18.3 ; -15.1)	0.15	0.35	0.09	-0.45 (-3.16 ; 2.27)	0.82	0.41	0.33
	244	CT	-7.95 (-9.61 ; -6.30)				-16.2 (-18.1 ; -14.3)				2.17 (-1.11 ; 5.46)			
	53	TT	-5.34 (-8.88 ; -1.79)				-12.8 (-16.9 ; -8.80)				-3.05 (-10.10 ; 4.02)			
SNP9	563	CC	-7.43 (-8.52 ; -6.34)		0.32		-16.2 (-17.5 ; -15.0)		0.51		0.13 (-2.04 ; 2.29)		0.61	
	65	CT/TT	-5.29 (-8.58 ; -2.00)				-14.9 (-18.6 ; -11.3)				-1.61 (-7.98 ; 4.76)			
SNP10	256	TT	-7.76 (-9.36 ; -6.16)	0.12	0.64	**0.02**	-17.3 (-19.1 ; -15.4)	0.09	0.10	0.26	0.17 (-3.03 ; 3.37)	0.37	0.89	**0.05**
	289	TC	-8.17 (-9.67 ; -6.66)				-15.6 (-17.3 ; -13.8)				2.10 (-0.92 ; 5.11)			
	99	CC	-4.60 (-7.18 ; -2.02)				-14.5 (-17.5 ; -11.6)				-4.35 (-9.51 ; 0.82)			
SNP11	361	GG	-7.70 (-9.07 ; -6.33)	0.45	0.59	0.43	-17.0 (-18.5 ; -15.4)	0.11	0.18	0.20	0.01 (-2.69 ; 2.71)	0.94	0.83	0.81
	240	GA	-7.35 (-9.03 ; -5.67)				-15.7 (-17.6 ; -13.8)				0.66 (-2.65 ; 3.96)			
	40	AA	-5.84 (-9.96 ; -1.72)				-13.3 (-18.0 ; -8.62)				-0.78 (-8.89 ; 7.34)			
SNP12	428	CC	-7.86 (-9.11 ; -6.61)	0.21	0.19	0.74	-16.8 (-18.2 ; -15.3)	0.12	0.12	0.52	0.35 (-2.13 ; 2.83)	0.85	0.82	0.97
	197	CG	-6.41 (-8.25 ; -4.57)				-14.9 (-17.0 ; -12.8)				-0.20 (-3.86 ; 3.46)			
	22	GG	-6.45 (-12.05 ; -0.94)				-14.1 (-20.4 ; -7.80)				0.37 (-10.60 ; 11.30)			
SNP13	339	TT	-7.41 (-8.82 ; -6.01)	0.64	0.81	0.50	-16.9 (-18.5 ; -15.3)	0.18	0.16	0.58	-0.08 (-2.88 ; 2.72)	0.94	0.81	0.52
	238	TC	-7.39 (-9.06 ; -5.71)				-15.2 (-17.1 ; -13.3)				0.97 (-2.36 ; 4.31)			
	49	CC	-6.07 (-9.77 ; -2.38)				-14.9 (-19.2 ; -10.7)				-2.18 (-9.54 ; 5.18)			
SNP14	493	CC	-7.47 (-8.63 ; -6.30)		0.33		-16.5 (-17.8 ; -15.2)		0.28		-0.12 (-2.43 ; 2.20)		0.73	
	129	CG/GG	-6.18 (-8.46 ; -3.91)				-14.9 (-17.5 ; -12.3)				-1.02 (-5.55 ; 3.52)			

	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP15	222	CC	-7.52 (-9.26 ; -5.79)	0.92	0.81	0.89	-16.4 (-18.4 ; -14.4)	0.96	0.80	0.66	0.95 (-2.49 ; 4.40)	0.77	0.54	0.80
	330	CT	-7.17 (-8.60 ; -5.75)				-15.8 (-17.5 ; -14.2)				-0.70 (-3.53 ; 2.12)			
	96	TT	-7.52 (-10.23 ; -4.89)				-16.8 (-19.8 ; -13.8)				0.72 (-4.51 ; 5.95)			

Mean levels and CIs were not adjusted for confounding factors.
[1] p-value for the additive mode of inheritance
[2] p-value for the dominant mode of inheritance
[3] p-value for the recessive mode of inheritance
SNP=single nucleotide polymorphism; CI=confidence interval

Table 6. Associations between SNPs and changes in breast measures during menopause, in women who became postmenopausal during follow-up (n=656)

Block 1			% breast density				Dense area				Non-dense area			
	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP1	445	GG	-7.33 (-8.55 ; -6.10)		0.74		-16.2 (-17.6 ; -14.8)		0.91		-0.51 (-2.95 ; 1.92)		0.25	
	203	GA/AA	-7.70 (-9.52 ; -5.88)				-16.1 (-18.1 ; -14.0)				2.04 (-1.56 ; 5.64)			
SNP2	584	T T	-7.12 (-8.18 ; -6.05)		0.10		-15.8 (-17.1 ; -14.6)		0.06		-0.25 (-2.38 ; 1.88)		0.12	
	55	TC/CC	-10.10 (-13.6 ; -6.63)				-19.8 (-23.8 ; -15.9)				5.56 (-1.37 ; 12.49)			
SNP3	496	CC	-7.72 (-8.88 ; -6.57)		0.38		-16.8 (-18.2 ; -15.5)		0.09		0.01 (-2.31 ; 2.33)		0.66	
	146	CA/AA	-6.63 (-8.77 ; -4.49)				-14.4 (-16.9 ; -12.0)				1.10 (-3.17 ; 5.37)			
SNP4	458	AA	-7.37 (-8.57 ; -6.16)		0.78		-16.4 (-17.7 ; -15.0)		0.55		-0.27 (-2.66 ; 2.13)		0.35	
	195	AG/GG	-7.68 (-9.53 ; -5.83)				-15.6 (-17.7 ; -13.5)				1.82 (-1.85 ; 5.49)			
Block 2	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP5	491	CC	-7.01 (-8.16 ; -5.85)		0.10		-15.8 (-17.1 ; -14.5)		0.14		-0.68 (-2.99 ; 1.63)		0.11	
	158	CA/AA	-5.70 (-8.91 ; -2.49)				-17.8 (-20.2 ; -15.5)				3.11 (-0.97 ; 7.18)			
SNP6	364	AA	-7.28 (-8.64 ; -5.92)	0.58	0.71	0.52	-16.0 (-17.6 ; -14.5)	0.74	0.79	0.75	-0.01 (-2.70 ; 2.69)	0.76	0.74	0.93
	239	AG	-7.48 (-9.16 ; -5.81)				-16.3 (-18.2 ; -14.3)				0.70 (-2.62 ; 4.03)			
	43	GG	-8.70 (-12.71 ; -4.74)				-16.9 (-21.4 ; -12.4)				0.65 (-7.21 ; 8.50)			
SNP7	369	AA	-7.28 (-8.63 ; -5.93)	0.78	0.73	0.98	-16.3 (-17.8 ; -14.7)	0.70	0.96	0.35	-0.08 (-2.76 ; 2.61)	0.60	0.74	0.51
	245	AG	-7.68 (-9.34 ; -6.03)				-16.5 (-18.4 ; -14.6)				0.27 (-3.02 ; 3.56)			
	33	GG	-7.37 (-11.90 ; -2.86)				-13.9 (-19.0 ; -8.73)				3.16 (-5.84 ; 12.15)			

potentially confounding variables had substantial influence on the menopausal changes or postmenopausal breast measures and hence crude figures are presented (Table 6 and Appendix 1).

Results of analyses with haplotypes appeared not to provide additional information and are hence not presented, but are available upon request.

Discussion

The rare alleles of 5 SNPs tagging haplotype block 3 of the *IGF-1* gene, were related to higher circulating levels of premenopausal IGF-I and, to a lesser extent, also with higher percent breast density in premenopausal women. These relationships were statistically significant for SNP13 (rs6220). Menopausal decrease of breast density was smaller in women with two copies of the rare allele of SNPs tagging haplotype block 3. This effect was most obvious for SNP10 (rs7136446).

To our best knowledge, this is the first study on common variation of the *IGF-1* gene and breast density. An advantage of the study is that the relationship between genetic variation in the *IGF-1* gene and serum IGF-I levels as well as breast density could be assessed. Only approximately one third of the study population had become postmenopausal during 5.5 years of follow-up, therefore postmenopausal breast measures were available for only 656 women. It was in this subgroup that premenopausal IGF-I serum levels were measured (27). This limited the power to study effects on circulating IGF-I levels of the SNPs with a low frequency of the minor allele. All women in the present study had at least one menstrual period in the last 12 months prior to their first mammographic measurement and were therefore classified to be premenopausal. Part of these women were, however, probably already perimenopausal, because all participants were 49 years of age or older at study entry.

We hypothesized that premenopausal IGF-I levels may determine postmenopausal breast density. In a previous study we therefore investigated the relation between premenopausal IGF-I serum levels and pre- and postmenopausal breast density (27). Circulating levels however, may fluctuate over time, thus lowering reproducibility and herewith the power to detect associations. Along the lines of Mendelian randomization, genetic information has the advantage that, if related to IGF-I levels, it may give information on the life-long exposure to elevated levels of IGF-I that are not confounded by factors altering IGF-I levels over the short term (46,47). Being able to study genetic variation in relation to breast measures in addition to the relation between circulating levels and breast density, may therefore give us better insight in the relation between IGF-I levels and breast density. We found that the minor allele of 5 SNPs was associated with higher circulating

premenopausal IGF-I levels. We expected that the SNPs that were associated with elevated premenopausal IGF-I levels would also be associated with higher premenopausal breast density. We indeed found this relationship. In our previous study on IGF-I levels and breast density, we did not find premenopausal IGF-I levels to be associated with premenopausal breast measures, although dense area was slightly higher in the highest quantile of serum IGF-I (27). Several other studies, however, did find high IGF-I levels to be associated with higher percent density in premenopausal women (21,22,24,26), but this was not confirmed by others (23,25). The sample size of premenopausal women (n=1928) and thus the power of the current analysis, however, is much higher than in our previous study.

Similarly, we also expected a smaller decrease of breast density during menopause. This was the case for SNPs tagging haplotype block 3, but less so for the other SNPs. With the exception of associations between SNP10 and percent breast density, none of the associations was statistically significant.

Several previous studies have been published on common genetic variation in the *IGF-1* gene in relation to breast cancer risk. In the Multiethnic cohort, rs7965399 (SNP2 in our study), has been reported to be in LD with a CA-repeat polymorphism in the promoter region of the *IGF-1* gene (41). The relation between this polymorphism and both IGF-I levels and breast cancer risk, has been studied intensively with mixed results (48-60) Three previous studies have analyzed genetic variation across the whole *IGF-1* gene in relation to breast cancer risk (30-32), of which two also presented circulating levels of IGF-I (30,31). In the study by Canzian et al., SNPs were selected on the basis of expected functionality while Al-Zahrani et al. and Setiawan et al. used a haplotype tagging approach. Setiawan et al. concluded that there was no evidence in their results for an association between common genetic variation of the *IGF-1* gene and breast cancer risk (32). As in our study, Al Zahrani et al. and Canzian et al. both found that several SNPs were associated with elevated levels of circulating IGF-I. Of particular interest is that SNP13 (rs6220) was associated with elevated levels of IGF-I in both the study from Al Zahrani et al. and in the study from Canzian et al., as well as in the present study. Al Zahrani also found that this SNP is associated with increased breast cancer risk. By contrast Al Zahrani et al. also noted another SNP in the 3' region of *IGF-1*, SNP12, to be associated with IGF-I levels, which we were unable to replicate. We found that women carrying the rare allele of SNP13 had higher premenopausal percent breast density and dense area, a smaller decrease of these measures over menopause and consequently higher percent breast density and dense area after menopause. Although none of these relations with breast measures were statistically significant, our results and those of Al-Zahrani et al. concerning SNP13, support the hypothesis that IGF-I may increase breast cancer risk by increasing the dense area.

As far as we know none of the SNPs has proven functionality. Haplotype block 3 is in the 3'-UTR and is hence suggested to possibly influence IGF-I levels through altering RNA stability (31). SNPs may also be associated with IGF-I levels or breast measures due to being in LD with an unknown functional variant. However, due to the large number of tests, associations may also have risen by chance. To define the possibility of having found false positive results, we used a method described by Wacholder et al., using a Bayesian approach (61). In our study, SNP9, SNP11 and SNP13 had the strongest association with IGF-I levels. These associations were still significant at the 0.5 FPRP-level for priors of 0.1 or higher. The effect of SNP13, when considering the additive mode of inheritance, was still significant at the 0.5 FPRP-level for priors of 0.01 or higher. The statistically most significant association between any of the SNPs and breast density, was the association between the homozygous rare allele genotype of SNP10 and change in percent breast density during menopause. This association was still significant at the 0.5 FPRP-level for priors of 0.1 or higher.

In conclusion, we found some evidence that the number of copies of the rare allele of SNPs tagging haplotype block 3 in our study, was associated with slightly higher percent breast density before menopause and also with a smaller decrease of percent density during the menopausal transition phase. These SNPs were also related with higher circulating levels of IGF-I and effects on menopausal changes in percent density are in line with results of our previous study on IGF-I circulating levels and breast density. While all associations were only marginally statistically significant, meaning we cannot draw strong conclusions from this study, it is worth noting that SNP13 (rs6220) has now been associated with circulating IGF-I levels in three studies, making a strong case against a spurious result. Furthermore, this SNP, and others also in the *IGF-1* gene may affect breast density, possibly increasing breast cancer risk, notions consistent with the principles of Mendelian randomization. Nevertheless, more studies of substantial size need to be done to verify the association of IGF-I genetic variation and breast measures both before and after menopause.

Acknowledgements

This study was supported by grant number 2002/11 of the World Cancer Research Fund (WCRF).

References

1. Sachdev D, Yee D. The IGF system and breast cancer. Endocr Relat Cancer 2001;8: 197-209.
2. Lee AV, Yee D. Insulin-like growth factors and breast cancer. Biomed Pharmacother. 1995;49:415-421.
3. Pollak MN. Endocrine effects of IGF-I on normal and transformed breast epithelial cells: potential relevance to strategies for breast cancer treatment and prevention. Breast Cancer Res Treat 1998;47:209-217.
4. Hankinson SE, Willett WC, Colditz GA, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet 1998;351:1393-1396.
5. Toniolo P, Bruning PF, Akhmedkhanov A, et al. Serum insulin-like growth factor-I and breast cancer. Int J Cancer 2000;88:828-832.
6. Krajcik RA, Borofsky ND, Massardo S, et al. Insulin-like growth factor I (IGF-I), IGF-binding proteins, and breast cancer. Cancer Epidemiol Biomarkers Prev 2002;11:1566-1573.
7. Muti P, Quattrin T, Grant BJ, et al. Fasting glucose is a risk factor for breast cancer: a prospective study. Cancer Epidemiol Biomarkers Prev 2002;11:1361-1368.
8. Allen NE, Roddam AW, Allen DS, et al. A prospective study of serum insulin-like growth factor-I (IGF-I), IGF-II, IGF-binding protein-3 and breast cancer risk. Br.J.Cancer. 2005;92:1283-1287.
9. Schernhammer ES, Holly JM, Pollak MN, et al. Circulating levels of insulin-like growth factors, their binding proteins, and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2005;14:699-704.
10. Kaaks R, Lundin E, Rinaldi S, et al. Prospective study of IGF-I, IGF-binding proteins, and breast cancer risk, in northern and southern Sweden. Cancer Causes Control 2002;13:307-316.
11. Rinaldi S, Peeters PH, Berrino F, et al. IGF-I, IGFBP-3 and breast cancer risk in women: The European Prospective Investigation into Cancer and Nutrition (EPIC). Endocr.Relat Cancer. 2006;13:593-605.
12. Schernhammer ES, Holly JM, Hunter DJ, et al. Insulin-like growth factor-I, its binding proteins (IGFBP-1 and IGFBP-3), and growth hormone and breast cancer risk in The Nurses Health Study II. Endocr.Relat Cancer. 2006;13:583-592.
13. Keinan-Boker L, Bueno De Mesquita HB, Kaaks R, et al. Circulating levels of insulin-like growth factor I, its binding proteins -1,-2, -3, C-peptide and risk of postmenopausal breast cancer. Int J Cancer 2003;106:90-95.
14. Gronbaek H, Flyvbjerg A, Mellemkjaer L, et al. Serum insulin-like growth factors, insulin-like growth factor binding proteins, and breast cancer risk in postmenopausal women. Cancer Epidemiol.Biomarkers Prev. 2004;13:1759-1764.

15. Kleinberg DL. Role of IGF-I in normal mammary development. Breast Cancer Res Treat 1998;47:201-208.

16. Byng JW, Boyd NF, Fishell E, et al. The quantitative analysis of mammographic densities. Phys Med Biol 1994;39:1629-1638.

17. Boyd NF, Lockwood GA, Martin LJ, et al. Mammographic density as a marker of susceptibility to breast cancer: a hypothesis. IARC Sci Publ 2001;154:163-169.

18. Byrne C, Schairer C, Wolfe J, et al. Mammographic features and breast cancer risk: effects with time, age, and menopause status. J Natl Cancer Inst 1995;87:1622-1629.

19. Boyd NF, Byng JW, Jong RA, et al. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study. J Natl Cancer Inst 1995;87:670-675.

20. Van Gils CH, Hendriks JH, Holland R, et al. Changes in mammographic breast density and concomitant changes in breast cancer risk. Eur J Cancer Prev 1999;8:509-515.

21. Byrne C, Colditz GA, Willett WC, et al. Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. Cancer Res 2000;60:3744-3748.

22. Maskarinec G, Williams AE, Kaaks R. A cross-sectional investigation of breast density and insulin-like growth factor I. Int J Cancer 2003;107:991-996.

23. Lai JH, Vesprini D, Zhang W, et al. A polymorphic locus in the promoter region of the IGFBP3 gene is related to mammographic breast density. Cancer Epidemiol Biomarkers Prev 2004;13:573-582.

24. Diorio C, Pollak M, Byrne C, et al. Insulin-like growth factor-I, IGF-binding protein-3, and mammographic breast density. Cancer Epidemiol Biomarkers Prev 2005;14:1065-1073.

25. dos Santos Silva I, Johnson N, De Stavola B, et al. The insulin-like growth factor system and mammographic features in premenopausal and postmenopausal women. Cancer Epidemiol.Biomarkers Prev. 2006;15:449-455.

26. Boyd NF, Stone J, Martin LJ, et al. The association of breast mitogens with mammographic densities. Br J Cancer 2002;87:876-882.

27. Verheus M, Peeters PHM, Kaaks R, et al. Premenopausal IGF-I serum levels and changes in breast density over menopause. Cancer Epidemiol.Biomarkers Prev. 2007;

28. Goodman-Gruen D, Barrett-Connor E. Epidemiology of insulin-like growth factor-I in elderly men and women. The Rancho Bernardo Study. Am.J.Epidemiol. 1997;145:970-976.

29. Rollison DE, Newschaffer CJ, Tao Y, et al. Premenopausal levels of circulating insulin-like growth factor I and the risk of postmenopausal breast cancer. Int.J.Cancer. 2006;118:1279-1284.

30. Canzian F, McKay JD, Cleveland RJ, et al. Polymorphisms of genes coding for insulin-like growth factor 1 and its major binding proteins, circulating levels of IGF-I and IGFBP-3 and breast cancer risk: results from the EPIC study. Br.J.Cancer. 2006;94:299-307.

31. Al Zahrani A, Sandhu MS, Luben RN, et al. IGF1 and IGFBP3 tagging polymorphisms are associated with circulating levels of IGF1, IGFBP3 and risk of breast cancer. Hum.Mol.Genet. 2006;15:1-10.

32. Setiawan VW, Cheng I, Stram DO, et al. Igf-I genetic variation and breast cancer: the multiethnic cohort. Cancer Epidemiol.Biomarkers Prev. 2006;15:172-174.

33. Ocke MC, Bueno-de-Mesquita HB, Pols MA, et al. The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. Int J Epidemiol 1997;26 Suppl 1:S49-S58.

34. Ocke MC, Bueno-de-Mesquita HB, Goddijn HE, et al. The Dutch EPIC food frequency questionnaire. I. Description of the questionnaire, and relative validity and reproducibility for food groups. Int J Epidemiol 1997;26 Suppl 1:S37-S48.

35. Riboli E, Kaaks R. The EPIC Project: rationale and study design. European Prospective Investigation into Cancer and Nutrition. Int J Epidemiol 1997;26 Suppl 1:S6-14.

36. Riboli E, Hunt KJ, Slimani N, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002;5:1113-1124.

37. Boker LK, Van Noord PA, van der Schouw YT, et al. Prospect-EPIC Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. Eur J Epidemiol 2001;17:1047-1053.

38. Johansson M, McKay JD, Stattin P, et al. Comprehensive evaluation of genetic variation in the IGF1 gene and risk of prostate cancer. Int.J.Cancer 2007;120:539-542.

39. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. Science 2002;296:2225-2229.

40. Stram DO, Haiman CA, Hirschhorn JN, et al. Choosing haplotype-tagging SNPS based on unphased genotype data using a preliminary sample of unrelated subjects with an example from the Multiethnic Cohort Study. Hum Hered. 2003;55:27-36.

41. Cheng I, Stram DO, Penney KL, et al. Common genetic variation in IGF1 and prostate cancer risk in the Multiethnic Cohort. J.Natl.Cancer Inst. 2006;98:123-134.

42. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol. 1995;12:921-927.

43. Stram DO, Leigh PC, Bretsky P, et al. Modeling and E-M estimation of haplotype-specific relative risks from genotype data for a case-control study of unrelated individuals. Hum Hered. 2003;55:179-190.

44. van Duijnhoven FJ, Peeters PH, Warren RM, et al. Influence of estrogen receptor alpha and progesterone receptor polymorphisms on the effects of hormone therapy on mammographic density. Cancer Epidemiol.Biomarkers Prev. 2006;15:462-467.

45. Boyd N, Martin L, Stone J, et al. A longitudinal study of the effects of menopause on mammographic features. Cancer Epidemiol Biomarkers Prev 2002;11:1048-1053.

46. Katan MB. Apolipoprotein E isoforms, serum cholesterol, and cancer. 1986. Int.J.Epidemiol. 2004;33:9-

47. Smith GD, Ebrahim S. Mendelian randomization: prospects, potentials, and limitations. Int.J.Epidemiol. 2004;33:30-42.

48. Rosen CJ, Kurland ES, Vereault D, et al. Association between serum insulin growth factor-I (IGF-I) and a simple sequence repeat in IGF-I gene: implications for genetic studies of bone mineral density. J.Clin.Endocrinol.Metab. 1998;83:2286-2290.

49. Yu H, Li BD, Smith M, et al. Polymorphic CA repeats in the IGF-I gene and breast cancer. Breast Cancer Res Treat 2001;70:117-122.

50. Jernstrom H, Chu W, Vesprini D, et al. Genetic factors related to racial variation in plasma levels of insulin-like growth factor-1: implications for premenopausal breast cancer risk. Mol.Genet.Metab. 2001;72:144-154.

51. Jernstrom H, Deal C, Wilkin F, et al. Genetic and nongenetic factors associated with variation of plasma levels of insulin-like growth factor-I and insulin-like growth factor-binding protein-3 in healthy premenopausal women. Cancer Epidemiol.Biomarkers Prev. 2001;10:377-384.

52. Vaessen N, Heutink P, Janssen JA, et al. A polymorphism in the gene for IGF-I: functional properties and risk for type 2 diabetes and myocardial infarction. Diabetes. 2001;50:637-642.

53. Missmer SA, Haiman CA, Hunter DJ, et al. A sequence repeat in the insulin-like growth factor-1 gene and risk of breast cancer. Int.J.Cancer. 2002;%20;100:332-336.

54. Figer A, Karasik YP, Baruch RG, et al. Insulin-like growth factor I polymorphism and breast cancer risk in Jewish women. Isr.Med.Assoc.J. 2002;4:759-762.

55. DeLellis K, Ingles S, Kolonel L, et al. IGF1 genotype, mean plasma level and breast cancer risk in the Hawaii/Los Angeles multiethnic cohort. Br.J.Cancer. 2003;88:277-282.

56. Tran N, Bharaj BS, Diamandis EP, et al. Short tandem repeat polymorphism and cancer risk: influence of laboratory analysis on epidemiologic findings. Cancer Epidemiol.Biomarkers Prev. 2004;13:2133-2140.

57. Wen W, Gao YT, Shu XO, et al. Insulin-like growth factor-I gene polymorphism and breast cancer risk in Chinese women. Int.J.Cancer. 2005;113:307-311.

58. Slattery ML, Baumgartner KB, Byers T, et al. Genetic, anthropometric, and lifestyle factors associated with IGF-1 and IGFBP-3 levels in Hispanic and non-Hispanic white women. Cancer Causes Control. 2005;16:1147-1157.

59. Wagner K, Hemminki K, Israelsson E, et al. Polymorphisms in the IGF-1 and IGFBP 3 promoter and the risk of breast cancer. Breast Cancer Res.Treat. 2005;92:133-140.

60. Jernstrom H, Sandberg T, Bageman E, et al. Insulin-like growth factor-1 (IGF1) genotype predicts breast volume after pregnancy and hormonal contraception and is associated with circulating IGF-1 levels: implications for risk of early-onset breast cancer in young women from hereditary breast cancer families. Br.J.Cancer. 2005;92:857-866.
61. Wacholder S, Chanock S, Garcia-Closas M, et al. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. J.Natl.Cancer Inst. 2004;96:434-442.
62. Hong Y, Pedersen NL, Brismar K, et al. Quantitative genetic analyses of insulin-like growth factor I (IGF-I), IGF-binding protein-1, and insulin levels in middle-aged and elderly twins. J.Clin.Endocrinol.Metab 1996;81:1791-1797.
63. Harrela M, Koistinen H, Kaprio J, et al. Genetic and environmental components of interindividual variation in circulating levels of IGF-I, IGF-II, IGFBP-1, and IGFBP-3. J.Clin.Invest 1996;98:2612-2615.

Block 3	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP8	357	CC	31.7 (30.4 ; 33.1)	0.51	0.90	0.07	24.8 (23.4 ; 26.3)	0.29	0.56	0.15	58.6 (55.7 ; 61.5)	0.83	0.53	0.52
	244	CT	30.9 (29.3 ; 32.5)				24.9 (23.2 ; 26.7)				60.7 (57.1 ; 64.3)			
	53	TT	34.8 (31.3 ; 38.6)				27.8 (24.0 ; 31.9)				56.8 (49.5 ; 64.6)			
SNP9	563	CC	31.6 (30.6 ; 32.7)		0.56		25.0 (23.9 ; 26.2)		0.76		59.4 (57.0 ; 61.7)		0.54	
	65	CT/TT	32.6 (29.5 ; 35.9)				25.6 (22.3 ; 29.2)				57.1 (50.5 ; 64.1)			
SNP10	256	TT	31.5 (29.9 ; 33.1)	0.21	0.79	**0.04**	24.1 (22.5 ; 25.9)	0.13	0.12	0.41	58.7 (55.3 ; 62.2)	0.57	0.59	0.07
	289	TC	30.9 (29.5 ; 32.4)				25.8 (24.2 ; 27.4)				61.7 (58.5 ; 65.1)			
	99	CC	34.2 (31.6 ; 36.9)				26.3 (23.5 ; 29.1)				54.7 (49.4 ; 60.2)			
SNP11	361	GG	31.8 (30.4 ; 33.1)	0.43	0.71	0.22	24.6 (23.2 ; 26.0)	0.17	0.25	0.25	58.3 (55.4 ; 61.3)	0.86	0.77	0.88
	240	GA	31.8 (30.1 ; 33.5)				25.6 (23.8 ; 27.4)				59.2 (55.6 ; 62.8)			
	40	AA	34.4 (30.3 ; 38.8)				27.7 (23.3 ; 32.5)				57.9 (49.5 ; 67.0)			
SNP12	428	CC	31.4 (30.2 ; 32.7)	0.24	0.27	0.49	24.7 (23.4 ; 26.1)	0.22	0.24	0.54	59.2 (56.6 ; 62.0)	0.84	0.79	0.91
	197	CG	32.5 (30.7 ; 34.4)				26.0 (24.0 ; 28.0)				58.5 (54.6 ; 62.5)			
	22	GG	33.7 (28.3 ; 39.6)				27.0 (21.3 ; 33.5)				59.7 (48.3 ; 72.2)			
SNP13	339	TT	31.4 (30.0 ; 32.8)	0.66	0.93	0.38	24.4 (22.9 ; 25.9)	0.26	0.27	0.54	59.1 (56.1 ; 62.1)	0.90	0.63	0.54
	238	TC	31.2 (29.6 ; 32.9)				25.5 (23.7 ; 27.3)				60.8 (57.2 ; 64.5)			
	49	CC	33.0 (29.4 ; 36.9)				26.1 (22.3 ; 30.3)				57.2 (49.6 ; 65.3)			
SNP14	493	CC	31.5 (30.4 ; 32.7)		0.27		24.7 (23.5 ; 25.9)		0.44		58.8 (56.3 ; 61.4)		0.69	
	129	CG/GG	33.0 (30.7 ; 35.3)				25.8 (23.3 ; 28.3)				57.7 (52.9 ; 62.7)			

	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP15	222	CC	31.8 (30.1 ; 33.5)	0.83	0.85	0.52	24.8 (23.0 ; 26.7)	0.97	0.66	0.60	59.4 (55.7 ; 63.2)	0.85	0.74	0.94
	330	CT	32.2 (30.8 ; 33.6)				25.6 (24.1 ; 27.2)				58.4 (55.4 ; 61.5)			
	96	TT	31.1 (28.6 ; 33.7)				24.5 (21.8 ; 27.4)				59.1 (53.5 ; 64.9)			

Mean levels and CIs were not adjusted for confounding factors.
[1] p-value for the additive mode of inheritance
[2] p-value for the dominant mode of inheritance
[3] p-value for the recessive mode of inheritance

Appendix 1. Associations between SNPs and postmenopausal breast measures, in women who became postmenopausal at time of follow-up (n=656)

Block 1			% breast density				Dense area				Non-dense area			
	Nr	Genotype	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3	Mean (95% CI)	P1	P2	P3
SNP1	445	GG	32.0 (30.8 ; 33.2)		0.37		24.9 (23.7 ; 26.3)		0.79		58.2 (55.6 ; 60.9)		0.25	
	203	GA/AA	31.0 (29.2 ; 32.8)				25.3 (23.4 ; 27.2)				61.0 (57.1 ; 65.1)			
SNP2	584	TT	31.9 (30.8 ; 32.9)		**0.04**		25.2 (24.1 ; 26.4)		0.12		58.9 (56.7 ; 61.3)		0.20	
	55	TC/CC	28.3 (25.1 ; 31.6)				22.2 (18.9 ; 25.8)				64.2 (56.6 ; 72.3)			
SNP3	496	CC	31.6 (30.5 ; 32.7)		0.61		24.6 (23.4 ; 25.8)		0.09		58.8 (56.3 ; 61.3)		0.53	
	146	CA/AA	32.2 (30.1 ; 34.4)				26.9 (24.6 ; 29.3)				60.5 (55.9 ; 65.3)			
SNP4	458	AA	31.9 (30.7 ; 33.1)		0.52		24.8 (23.6 ; 26.1)		0.43		58.4 (55.9 ; 61.1)		0.25	
	195	AG/GG	31.2 (29.4 ; 33.0)				25.8 (23.8 ; 27.8)				61.2 (57.2 ; 65.4)			
Block 2	**Nr**	**Genotype**	**Mean (95% CI)**	**P1**	**P2**	**P3**	**Mean (95% CI)**	**P1**	**P2**	**P3**	**Mean (95% CI)**	**P1**	**P2**	**P3**
SNP5	491	CC	32.1 (31.0 ; 33.3)		0.07		25.4 (24.1 ; 26.6)		0.18		58.6 (56.1 ; 61.1)		0.32	
	158	CA/AA	30.0 (28.1 ; 32.0)				23.7 (21.6 ; 25.8)				61.2 (56.8 ; 65.8)			
SNP6	364	AA	31.7 (30.4 ; 33.1)	0.70	0.81	0.63	25.3 (23.9 ; 26.8)	0.62	0.69	0.66	59.3 (56.4 ; 62.3)	0.86	0.84	0.98
	239	AG	31.6 (30.0 ; 33.3)				25.0 (23.3 ; 26.8)				59.8 (56.2 ; 63.5)			
	43	GG	30.7 (26.9 ; 34.6)				24.3 (20.3 ; 28.6)				59.6 (51.4 ; 68.5)			
SNP7	369	AA	31.9 (30.6 ; 33.2)	0.99	0.82	0.65	25.1 (23.7 ; 26.6)	0.81	0.99	0.51	58.7 (55.8 ; 61.6)	0.64	0.75	0.58
	245	AG	31.5 (29.9 ; 33.1)				24.9 (23.2 ; 26.7)				59.1 (55.6 ; 62.7)			
	33	GG	32.8 (28.4 ; 37.5)				26.7 (22.0 ; 31.9)				61.7 (52.1 ; 72.1)			

No relationship between circulating levels of sex steroids and breast density; the Prospect-EPIC cohort

Chapter 3

No relationship between circulating levels of sex steroids and breast density; the Prospect-EPIC cohort

Martijn Verheus
Petra H.M. Peeters
Paulus A.H. van Noord
Yvonne van der Schouw
Diederick E. Grobbee
Carla H. van Gils

Submitted.

Abstract

Background

High breast density is associated with increased breast cancer risk. Epidemiologic studies have shown an increase in breast cancer risk in postmenopausal women with high levels of sex steroids. Hence, sex steroids may increase postmenopausal breast cancer risk via an increase of breast density.

Objectives

To study the relation between circulating estrogens and androgens as well as sex hormone binding globulin (SHBG) in relation to breast density.

Design and Methods

We conducted a cross-sectional study among 969 postmenopausal women, using baseline data of a random sample of the Prospect-EPIC study. Prospect-EPIC is one of two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition (EPIC), and women were recruited via a breast cancer screening programme. At enrolment, a non-fasting blood sample was taken and a mammogram was made. Estrone, estradiol, dehydroepiandrosterone sulfate, androstenedione, testosterone and SHBG levels were measured, using double-antibody radioimmunoassays. Concentrations of free estradiol and free testosterone were calculated from estradiol, testosterone and SHBG. Mammographic dense and non-dense areas were measured using a semi quantitative computerized method and percent breast density was calculated. Mean breast measures for quintiles of hormone or SHBG levels were estimated using linear regression analyses.

Results

None of the estrogens or androgens showed clear relationships with percent breast density or the dense breast area, but all, except free testosterone, were inversely associated with the non-dense area (for free estradiol; 93.5 cm^2 in the highest versus 99.4 cm^2 in the lowest quintile, P-trend=0.09). High levels of SHBG were weakly associated with higher dense area and with higher percent density.

Conclusion and discussion

The results of our study do not support the hypothesis that sex steroids increase postmenopausal breast cancer risk via an increase in breast density.

Introduction

Dense breast tissues (glandular and stromal tissues) appear light on a mammogram, while non-dense tissue (fat tissue) appears black (1). Women with a high percentage of breast density have been shown to have strongly increased risk for breast cancer development (2,3).

In epidemiologic studies, nulliparity, late age at first child birth and late age of menopause have all been related to increased breast density, indicating that sex steroid levels may influence breast density and it has been hypothesized that breast density may reflect lifetime exposure to sex steroids. Moreover, postmenopausal hormone therapy (HT), and in particular combination therapy of estrogens and progesterone, increases breast density in most women (4-7), whereas Tamoxifen, an 'anti-estrogen', decreases breast density (8,9). Reproductive factors as well as HT use have been shown to have the same positive associations with breast cancer risk as described with breast density (10,11).

It has been clearly established that higher circulating levels of estrogens and androgens lead to higher breast cancer risk in postmenopausal women (12,13). In the light of the above described relationships between reproductive factors and breast density and also exogenous hormones and breast density, one would also expect that circulating levels of endogenous sex steroids increase breast cancer risk by altering breast density. Studies on sex steroids and breast density in postmenopausal women, however, failed to show consistent results (14-18). Greendale et al. indeed showed that women with high estradiol levels had significantly increased percent breast density (17). Boyd et al., however, found a statistically significant inverse relation between estradiol levels and percent density (14). Three other studies did not find estrogens and breast density to be associated (15,16,18). Androgens were not related to breast density in any of these studies, but most studies reported higher breast density with higher sex hormone binding globulin (SHBG) levels, which appears to contradict the inverse relation between SHBG levels and postmenopausal breast cancer risk that has been shown in many studies (12,13). Most of the studies on sex steroids and breast density were small however (range; 88-404 subjects), and contained a relatively large percentage of former HT users (range; 30-50%). It is not known what time period is needed for a complete wash out of HT effects on breast tissue, and hence results may be influenced. Furthermore, all (15-18) but one (14) of these studies present results on percent breast density only. It is the dense area measure, however, that may be a more precise marker of breast cancer risk than the percent breast density, as it is the absolute amount of glandular and stromal (dense) tissue that is regarded as the target tissue for breast cancer.

In the present large cross-sectional study we set out to investigate the relation between circulating levels of estrogens, androgens and SHBG on the one hand, and both measures of breast density (dense area and percent density) on the other hand, in a sample of Dutch women, with very few (14%) former HT users.

Methods

The Prospect cohort

This study included women participating in Prospect-EPIC, one of two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition (EPIC). EPIC is a multi center cohort study with 10 participating European countries. Rationale and design of both EPIC and Prospect-EPIC have been described in detail elsewhere (19-21). The Prospect-EPIC cohort consists of 17,357 women, aged 49-69 years at enrolment. Women were recruited between 1993 and 1997 through a regional programme for breast cancer screening and reside in Utrecht or its vicinity. The regional programme is part of the national screening programme which covers the entire Dutch population. As part of the population-based screening programme, mammographic examinations are carried out every two years, starting at age 50 until age 75, and stored in archives.

At time of study enrolment, anthropometric measurements were taken, and a blood sample was drawn and stored at -196 °C under liquid nitrogen. Participants were asked to fill out two questionnaires. A general questionnaire was used to gather information on demographic, reproductive and lifestyle factors and past and current morbidity. To determine regular dietary intake, a validated extensive self-administered food frequency questionnaire was used containing 178 food items (22,23).

All participants signed an informed consent and the study was approved by The Institutional Review Board of the University Medical Center Utrecht.

Study population

A 10% random sample (n=1736) was taken from the total Prospect cohort. Of 1595 women, mammograms could be retrieved from the archives and breast density was successfully measured for 1531 women.

Only women being postmenopausal, either through natural (no menstrual periods for at least twelve months after spontaneous cessation of their menses) or surgical (hysterectomy, ovariectomy or a combination) causes, with available plasma sample were selected. Women using HT or oral contraceptives (OC) at the time of blood donation were excluded, as were women with missing data on alcohol consumption or with a daily energy intake equal to or lower than 500 Kcal or higher than 6000 Kcal (24). Both sex steroid measurements and mammographic measurements were available for 969 postmenopausal women.

Blood collection and measurements of sex steroids and SHBG in plasma

A 30 ml non-fasting blood sample was donated by each participant, using three safety monovettes, one dry monovette for serum and two citrated monovettes for plasma. Within 24 hours, samples of 4 ml serum, 9 ml citrate plasma and 2 ml white blood cells were fractionated into 0.5 ml aliquots and stored in heat-sealed plastic straws under liquid nitrogen at -196 ºC.

Plasma levels of estrone, estradiol, androstenedione, dehydroepiandrosterone (DHEAS), testosterone and SHBG were measured using commercially available double-antibody radioimmunoassay kits (Diagnostics System Laboratories Inc., Webster, TX, USA). The following kits were used: estrone: DSL-8700, estradiol: DSL-39100, androstenedione: DSL-4200, DHEAS: DSL-2700, testosterone: DSL-4100 and SHBG: DSL-6300. The intra-assay coefficients of variation were 5.6%, 3.9%, 4.3%, 5.2%, 7.7% and 3.0% respectively. The inter-assay coefficients of variation were 11.1%, 4.1%, 6.3%, 5.3%, 8.1% and 4.0% respectively (24).
Concentrations of free estradiol and free testosterone were calculated from total plasma concentrations of estradiol, testosterone and SHBG, using theoretical calculations described by Vermeulen et al. (25).

Mammographic density analysis

The mediolateral oblique mammogram, which is the routine view for breast cancer screening in the Netherlands, was used to assess mammographic density. It has been observed that the proportions of mammographic density on craniocaudal views and mediolateral oblique views and on left and right views are very strongly correlated and that representative information on mammographic density is provided in a single view (26). For each study subject, mammographic density was assessed on the left view.

After digitizing the films using a laser film scanner (Lumiscan 50, Lumisys), mammographic density was quantified using a computer-assisted method based on grey levels of pixels in the digitized mammogram. This computer-assisted method to determine mammographic density has proved to be very reliable and the method is described elsewhere in detail (1). Briefly, for each image, the reader first sets a threshold to determine the outside edge of the breast, to separate the image of the breast from the dark background surrounding it. A second threshold is set to determine the area of dense tissue within the breast, which is the lightest tissue visible on the mammogram. The program then determines the amount of pixels within the total breast area and within the dense area.

To compute percent breast density the dense area of a breast is divided by the total breast area and multiplied by 100. Percent breast density is used in most publications on breast density. However, a small sized breast and a larger sized breast could have the same percent breast density, while the absolute amount of glandular and stromal tissue, which is regarded as the target tissue for breast cancer (27,28), is higher in the larger breast (29). Hence we will present results on both relative and absolute measures of breast density.

All mammograms were assessed by one observer in sets composed of 35 randomly ordered films. To assess the reliability of the reader, a library set was created, which consisted of 35 randomly chosen films from our study subjects. This library set was read before the first set, after the last set, and at four time points between sets, which were blinded for the reader. The images in the library set were randomly ordered every time they were read to prevent the observer from recognizing this set. In this study an average intraclass correlation coefficients of 1.00 (range: 0.99–1.00); 0.93 (range: 0.91–0.97) and 0.91 (range: 0.87–0.94) were reached between repeated readings, for total breast area, dense area and percent breast density respectively.

Statistical analyses

Subjects with plasma sex steroid or SHBG values under the detection limit, which were 1.2 pg/ml, 1 pg/ml, 25 ng/ml, 0.02 ng/ml, 0.05 ng/ml and 5 nmol/l for estrone, estradiol, DHEAS, androstenedione, testosterone and SHBG respectively, were given the value of the detection limit. Plasma sex steroid and SHBG levels were log transformed to normalize distributions. These transformed values were then used to compute geometric mean levels.

All three measures of the breast (percent breast density, dense area and non-dense area) were square root transformed to normalize their distributions. These transformed values were used in linear regression analyses. For ease of interpretation, presented means and 95% confidence intervals (95% CIs) have been transformed back to the original scale. Means and 95% CIs of breast measures by quintile level of plasma sex steroids or SHBG were estimated with linear regression models using the 'GLM' procedure. To test for linear trends over the quintiles, median values within quintiles were calculated and evaluated as a continuous variable using linear regression analysis. Potential confounding of various factors with known association with sex steroid levels or with breast measures or with both, was assessed by adding those variables to the crude models. The following characteristics were evaluated for confounding using continuous variables: age, body mass index (BMI), waist circumference, waist-hip ratio (WHR), age at menarche, age at menopause, time since menopause and alcohol consumption. Dichotomous variables were used for ever breast feeding and family history of breast cancer (mother and/or

sister). A variable for ever use of postmenopausal hormone therapy (HT) combined with time since last use of HT was used with one category for never users and two categories for ever users of which the first contained women with a time period since last HT use below the median (5 years), and the second contained women with a time period since last use of median value or greater. Parity and age at first childbirth were evaluated using a combined variable with one category for nulliparous women and three categories of parous women combined with tertiles of age at birth of the first child (tertile cut off points; 23 and 26 years). Smoking was evaluated using a variable with three categories, for current smokers, past smokers and never smokers. In a previous study by Aiello et al. different effects were found between never users and ever users (15). Hence, we stratified the analyses of all three breast measures by HT ever/never use, for comparison.

All p-values are two-sided and when below 0.05, results were considered significant. All analyses were conducted using the Statistical Analysis System (SAS) software package, release 9.1 (SAS Institute, Cary, NC, USA).

Results

Table 1 shows basic characteristics of the study population. Mean age at study recruitment was 59.5 years and mean BMI was 26.1 kg/m2. Age at first childbirth among parous women was on average 25.2 years, and 11.7% (n=113) of the participants was nulliparous. Women were on average 47.2 years at menopause and average time since menopause was 12.0 years. Only 14.0% (n=136) of the participants had previously used HT. Those who did, quit using these compounds on average 6.8 years prior to recruitment. Median values for percent density, dense area and non-dense area were 22.0% (inter quartile range; 14.8–30.9%), 25.5 cm^2 (inter quartile range; 18.4–35.9 cm^2) and 94.9 cm^2 (inter quartile range; 71.2–122.7 cm^2) respectively.

Results of regression analyses for the relation between sex steroids and percent breast density, dense area and the non-dense area are presented in Tables 2, 3 and 4 respectively. Both crude associations and associations adjusted for variables that changed associations are presented. These variables included SHBG levels and BMI in the models to calculate mean percent breast density and mean non-dense area. Adding a second marker for total body fat (WHR) to these models only had additional effect on the models with SHBG. Hence WHR was added to these models only (Tables 2 and 4 for percent density and non-dense area respectively). None of the potential confounders that were tested influenced associations between sex steroids or SHBG and the dense area substantially, and hence only the crude models are presented (Table 3).

The inverse association of levels of both estrogens with percent density was no longer apparent when adjusted for SHBG and BMI (Table 2). There was no clear relation

Table 1. Basic characteristics of the study population

Age (years (SD))		59.5	(5.6)
Height (cm (SD))		163.9	(6.0)
Weight (kg (SD))		70.2	(11.8)
BMI (kg/m² (SD))		26.1	(4.1)
WHR (ratio (SD))		0.79	(0.06)
Family history of breast cancer (nr (%))[3]		130	(13.4)
Reproductive factors			
Age at menarche (years (SD))		13.4	(1.7)
Nulliparous (nr (%))		113	(11.7)
Age at first child birth (years (SD))[1]		25.2	(4.0)
Number of children (average (SD)) [1]		2.6	(0.9)
Ever breast feeding (nr (%))[1]		705	(82.4)
Age at menopause (years (SD))		47.2	(5.8)
Time since menopause (years (SD))		12.0	(6.8)
Lifestyle factors			
Ever used OC (nr (%))		532	(54.9)
Ever used HT (nr (%))		136	(14.0)
Time since stop HT use (years (SD))[2]		6.8	(6.2)
Current smoking (nr (%))		211	(21.8)
Ever smoked (nr (%))		524	(54.1)
Alcohol intake (gram/day)		8.2	(11.4)
Sex steroids and SHBG			
Estrone (pg/ml)[4]		16.05	(4.20 ; 61.31)
Estradiol (pg/ml)		8.98	(2.49 ; 32.33)
Free estradiol (pg/ml)		0.27	(0.07 ; 1.00)
DHEAS (ng/ml)		459.9	(104.7 ; 2012.7)
Androstenedione (ng/ml)		0.47	(0.09 ; 2.30)
Testosterone (ng/ml)		0.26	(0.09 ; 0.74)
Free testosterone (pg/ml)		5.46	(1.51 ; 19.77)
SHBG (nmol/L)		18.89	(4.87 ; 73.21)
Breast measures			
% breast density	(median cm²)	22.0	
	(inter quartile range cm²)	14.8 – 30.9	
Dense area	(median cm²)	25.5	
	(inter quartile range cm²)	18.4 – 35.9	
Non-dense area	(median cm²)	94.9	
	(inter quartile range cm²)	71.2 – 122.7	

[1] among parous women only
[2] among former HT users only
[3] mother and / or sister with history of breast cancer
[4] geometric mean (± 2SD)
SD=standard deviation; BMI=body mass index; WHR=waist-hip ratio; OC=oral contraceptives; HT=postmenopausal hormone therapy

between estrogens and the dense area either (Table 3). Before adjustment, higher levels of both estrogens were significantly associated with higher non-dense area. After corrections, however, these associations reversed to inverse associations (p-trend: 0.04, 0.03 and 0.09 for estrone, estradiol and free estradiol respectively) (Table 4).

Women with high DHEAS levels had high percent breast density (p-trend=0.04). The inverse relation of testosterone with percent density disappeared after adjustments (Table 2). None of the androgens were related to the dense area (Table 3). High levels of both DHEAS and androstenedione were associated with lower non-dense area (Table 4). As was seen with estradiol, the positive relation between testosterone and the non-dense area reversed to a statistically significant inverse relation after adjustments for SHBG and BMI. Circulating levels of free testosterone were, however, not related to the non-dense area after adjustments.

Women with high SHBG levels showed significantly higher percent density than women with low SHBG levels (Table 2). This association was still significant after correction for BMI, but was no longer significant after further adjustment for WHR. Women with high SHBG levels had higher dense area, but this association was statistically non-significant (Table 3). High levels of SHBG were very strongly associated with low non-dense area (Table 4). This relationship became less strong after corrections for BMI and disappeared after additional adjustment for WHR.

When we stratified the analyses according to HT ever use (yes/no), results were very comparable between the groups, except for estrone. Former HT users with high estrone levels had small percent density and low dense area (tertile adjusted mean values and 95% CIs for percent density; 24.9 (21.4–28.7), 22.3 (19.4–25.5), 18.7 (15.9–21.8), and for dense area; 31.9 (27.0–37.2), 27.8 (23.7–32.1), 24.3 (20.4–28.5)). Although the subgroup of former HT users was small (n=136), the trend over the tertiles was statistically significant for both measures of breast density (p-trend: 0.01 and 0.03 for percent density and dense area respectively).

Discussion

No clear relationships were found between sex steroids and both percent breast density and the dense area, except for a positive association between DHEAS levels and percent breast density. Surprisingly, all sex steroids were significantly associated with the non-dense area, although this association was not seen for free testosterone levels. High levels of SHBG were associated with high percent density and a high dense area. The highly significant inverse association between SHBG and the non-dense area disappeared after corrections for BMI and WHR.

An advantage of the present study is its size. With 969 participants, our study is larger than all previous studies (14-17), except one (18). Another advantage is the fact that the blood sample we used for hormone measurements was drawn at the same day as mammography was performed for most of the women (98.5%). In addition to the measurement of total hormone levels, we also calculated free levels of estradiol and

Table 2. Mean percent breast density for quintiles of sex steroids and SHBG

	Q1 Mean (95% CI)	Q2 Mean (95% CI)	Q3 Mean (95% CI)	Q4 Mean (95% CI)	Q5 Mean (95% CI)	p-trend
Estrone[1]						
Crude	24.1 (22.4 - 25.9)	22.5 (20.9 - 24.1)	21.2 (19.6 - 22.8)	22.0 (20.4 - 23.7)	21.9 (20.4 - 23.5)	0.16
+ SHBG and BMI	23.0 (21.0 - 25.0)	21.6 (19.9 - 23.4)	21.5 (19.7 - 23.3)	22.2 (20.4 - 24.2)	23.4 (21.6 - 25.3)	0.28
Estradiol[2]						
Crude	24.2 (22.5 - 26.0)	23.0 (21.3 - 24.6)	21.1 (19.6 - 22.7)	21.9 (20.1 - 23.8)	21.5 (20.1 - 23.0)	0.03
+ SHBG and BMI	22.3 (20.4 - 24.2)	22.1 (20.3 - 23.9)	21.0 (19.3 - 22.8)	22.7 (20.7 - 24.7)	23.3 (21.5 - 25.1)	0.18
Free Estradiol[3]						
Crude	24.3 (22.6 - 26.0)	23.4 (21.7 - 25.1)	21.4 (19.9 - 23.1)	20.7 (19.2 - 22.3)	21.8 (20.2 - 23.5)	0.02
+ BMI	22.3 (20.5 - 24.3)	22.4 (20.6 - 24.3)	21.3 (19.5 - 23.2)	21.8 (20.0 - 23.7)	23.6 (21.7 - 25.6)	0.24
DHEAS[4]						
Crude	21.9 (20.3 - 23.5)	21.8 (20.2 - 23.4)	21.3 (19.7 - 22.9)	23.6 (22.0 - 25.3)	23.0 (21.4 - 24.7)	0.12
+ SHBG and BMI	21.6 (19.8 - 23.5)	21.7 (20.0 - 23.5)	21.2 (19.4 - 23.0)	23.4 (21.5 - 25.3)	23.3 (21.5 - 25.2)	0.04
Androstenedione[5]						
Crude	21.4 (19.8 - 23.1)	22.6 (21.0 - 24.2)	21.8 (20.2 - 23.5)	24.2 (22.6 - 26.0)	21.5 (19.9 - 23.1)	0.87
+ SHBG and BMI	20.8 (19.0 - 22.7)	22.3 (20.6 - 24.1)	21.9 (20.1 - 23.8)	24.2 (22.3 - 26.1)	22.0 (20.2 - 23.8)	0.25
Testosterone[6]						
Crude	22.8 (21.1 - 24.5)	23.4 (21.7 - 25.1)	22.7 (21.0 - 24.3)	22.3 (20.8 - 24.0)	20.6 (19.1 - 22.2)	0.09
+ SHBG and BMI	21.3 (19.5 - 23.2)	22.7 (20.9 - 24.6)	22.4 (20.6 - 24.3)	22.7 (20.9 - 24.6)	22.1 (20.3 - 24.0)	0.46
Free Testosterone[7]						
Crude	25.2 (23.5 - 26.9)	22.8 (21.2 - 24.5)	21.8 (20.2 - 23.4)	21.9 (20.3 - 23.6)	20.0 (18.5 - 21.6)	< 0.0001
+ BMI	23.4 (21.6 - 25.4)	21.9 (20.1 - 23.7)	21.6 (19.8 - 23.5)	22.6 (20.8 - 24.5)	21.8 (20.0 - 23.8)	0.40
SHBG[8]						
Crude	19.0 (17.5 - 20.6)	20.6 (19.1 - 22.1)	22.8 (21.3 - 24.5)	22.9 (21.4 - 24.5)	26.2 (24.5 - 28.0)	< 0.0001
+ BMI	21.2 (19.4 - 23.1)	21.2 (19.4 - 23.1)	22.6 (20.7 - 24.5)	22.2 (20.4 - 24.1)	24.2 (22.3 - 26.2)	0.01
+ BMI and WHR	21.7 (19.9 - 23.6)	21.7 (19.9 - 23.6)	22.3 (20.5 - 24.2)	22.0 (20.3 - 23.9)	23.6 (21.7 - 25.5)	0.11

[1] quintile cut points in pg/ml; 10, 15, 19, 25; [2] quintile cut points in pg/ml; 5.7, 7.6, 9.6, 12.0; [3] quintile cut points in pg/ml; 0.16, 0.22, 0.28, 0.39; [4] quintile cut points in ng/ml; 271, 411, 586, 828; [5] quintile cut points in ng/m; 0.29, 0.45, 0.60, 0.88; [6] quintile cut points in ng/m; 0.17, 0.24, 0.30, 0.40; [7] quintile cut points in pg/ml; 3.22, 4.91, 6.62, 9.35; [8] quintile cut points nmol/l; 11, 18, 24, 34
DHEAS=dehydroepiandrosterone sulfate; SHBG=sex hormone binding globulin; BMI=body mass index; WHR=waist-hip ratio; CI=confidence interval

testosterone, because it is the unbound hormone fraction that is expected to affect the target (dense) tissue most clearly. The free levels used in the present study were not directly measured but calculated from total plasma concentrations of estradiol, testosterone and SHBG, using theoretical calculations (Vermeulen). In a validation study, Rinaldi et al. showed high correlation between measured concentrations and calculated concentrations of both free estradiol and free testosterone in postmenopausal women (r=0.84 and r=0.76 for estradiol and testosterone respectively).

To our best knowledge, seven previous studies on sex steroids and breast density have been published (14-18,30,31), of which five reported results on postmenopausal women (14-18). With the exception of the study by Boyd et al. (14), all reported results with the relative measure of breast density (percent density) only, although Aiello et al. and Tamimi mentioned that analyses with absolute breast density gave similar results (15,16). Although Boyd et al. found slightly stronger effects with percent breast density than with the dense area (14), it is the dense area that is likely to be a more precise marker of breast cancer risk, as it is the absolute amount of glandular and stromal (dense) tissue, that is regarded as the target tissue for breast cancer (27,28). None of the previous studies on sex steroids and breast density showed results of the non-dense area, which is part of the denominator to calculate percent breast density.

In the present study, crude models show both estrone and estradiol to be inversely related with percent density. After correction for confounding variables these relations were no longer apparent. Associations with free estradiol were in the same direction and of similar magnitude. Three other studies also found the crude inverse relation between estradiol and percent density, but only in the study by Boyd et al. this association remained statistically significant after adjustments for confounders, although only with free estradiol (14,18). This inverse relationship appeared to be stronger for percent density than for the absolute dense area. Aiello et al. only showed results of multivariate analyses, which showed estrogens not to be related to percent density (15). Only Greendale et al. found the opposite result with a positive association between estrogen levels and percent density, which even became stronger and statistically significant after correcting for confounding variables (17). We do not know what caused this different finding by Greendale et al. A relatively large percentage of their study population consisted of former HT users and average time since last use of these compounds was smaller compared with other studies. It is not known what time period is needed for a complete wash out of the effect of exogenous hormones on both endogenous sex steroid levels and breast density. Results of ever users should therefore be interpreted cautiously. In our study, only 14% of the study participants were former HT users and median time since last HT use was 5 years (inter quartile range; 2–10 years). When we stratified the analyses to HT ever use, estrogen levels were significantly inverse related to both percent breast density

Table 3. Mean dense breast area for quintiles of sex steroids and SHBG

	Q1 Mean (95% CI)	Q2 Mean (95% CI)	Q3 Mean (95% CI)	Q4 Mean (95% CI)	Q5 Mean (95% CI)	p-trend
Estrone[1]	27.1 (25.2 - 29.1)	27.1 (25.3 - 29.0)	26.3 (24.4 - 28.2)	26.3 (24.5 - 28.3)	27.4 (25.6 - 29.2)	0.84
Estradiol[2]	27.7 (25.8 - 29.6)	27.5 (25.6 - 29.4)	24.5 (22.7 - 26.3)	27.0 (24.9 - 29.2)	27.6 (25.9 - 29.4)	0.78
Free Estradiol[3]	27.4 (25.6 - 29.4)	27.0 (25.2 - 28.9)	25.8 (24.0 - 27.7)	26.0 (24.2 - 27.9)	28.1 (26.2 - 30.0)	0.57
DHEAS[4]	27.3 (25.5 - 29.3)	26.1 (24.3 - 28.0)	26.5 (24.6 - 28.4)	27.3 (25.4 - 29.2)	27.1 (25.3 - 29.0)	0.75
Androstenedione[5]	26.7 (24.8 - 28.6)	26.9 (25.1 - 28.8)	25.8 (24.0 - 27.7)	28.8 (26.9 - 30.8)	26.0 (24.2 - 27.9)	0.87
Testosterone[6]	26.9 (25.0 - 28.9)	27.4 (25.5 - 29.3)	26.4 (24.5 - 28.3)	27.0 (25.2 - 28.9)	26.7 (24.9 - 28.5)	0.86
Free Testosterone[7]	28.4 (26.5 - 30.4)	25.5 (23.7 - 27.3)	26.6 (24.7 - 28.5)	27.4 (25.6 - 29.4)	26.5 (24.6 - 28.4)	0.55
SHBG[8]	25.8 (23.9 - 27.7)	27.0 (25.1 - 28.8)	26.4 (24.6 - 28.3)	27.2 (25.4 - 29.1)	27.9 (26.0 - 29.8)	0.14

[1] quintile cut points in pg/ml; 10, 15, 19, 25; [2] quintile cut points in pg/ml; 5.7, 7.6, 9.6, 12.0; [3] quintile cut points in pg/ml; 0.16, 0.22, 0.28, 0.39; [4] quintile cut points in ng/ml; 271, 411, 586, 828; [5] quintile cut points in ng/m; 0.29, 0.45, 0.60, 0.88; [6] quintile cut points in ng/m; 0.17, 0.24, 0.30, 0.40; [7] quintile cut points in pg/ml; 3.22, 4.91, 6.62, 9.35; [8] quintile cut points nmol/l; 11, 18, 24, 34
DHEAS=dehydroepiandrosterone sulfate; SHBG=sex hormone binding globulin; CI=confidence interval

Table 4. Mean non-dense breast area for quintiles of sex steroids and SHBG

	Q1 Mean (95% CI)	Q2 Mean (95% CI)	Q3 Mean (95% CI)	Q4 Mean (95% CI)	Q5 Mean (95% CI)	p-trend
Estrone[1]						
Crude	87.6 (82.2 - 93.2)	94.1 (88.9 - 99.5)	100.1 (94.6 - 105.8)	95.5 (90.0 - 101.3)	98.7 (93.4 - 104.1)	0.02
+ SHBG and BMI	94.8 (89.4 - 100.4)	100.3 (95.2 - 105.5)	99.4 (94.2 - 104.8)	95.8 (90.5 - 101.2)	91.6 (86.6 - 96.7)	0.04
Estradiol[2]						
Crude	88.6 (83.4 - 94.1)	92.6 (87.4 - 98.1)	94.3 (89.0 - 99.8)	98.1 (91.9 - 104.5)	102.2 (97.1 - 107.3)	< 0.001
+ SHBG and BMI	99.9 (94.5 - 105.5)	98.5 (93.3 - 103.8)	96.2 (91.0 - 101.6)	95.2 (89.6 - 100.9)	93.5 (88.6 - 98.6)	0.03
Free Estradiol[3]						
Crude	87.4 (82.3 - 92.7)	89.7 (84.6 - 95.1)	96.5 (91.1 - 102.1)	101.1 (95.5 - 106.8)	102.5 (96.9 - 108.2)	< 0.0001
+ BMI	99.4 (94.1 - 104.9)	96.2 (90.9 - 101.5)	98.2 (92.9 - 103.7)	95.8 (90.7 - 101.1)	93.5 (88.2 - 98.9)	0.09
DHEAS[4]						
Crude	98.9 (93.4 - 104.5)	97.1 (91.6 - 102.7)	98.7 (93.1 - 104.3)	90.2 (85.0 - 95.6)	92.1 (86.8 - 97.6)	0.03
+ SHBG and BMI	101.3 (95.9 - 106.8)	98.5 (93.3 - 103.8)	100.0 (94.7 - 105.5)	92.5 (87.5 - 97.8)	92.2 (87.2 - 97.3)	< 0.01
Androstenedione[5]						
Crude	99.6 (93.9 - 105.4)	94.6 (89.2 - 100.0)	94.8 (89.4 - 100.3)	91.4 (86.2 - 96.9)	96.7 (91.3 - 102.3)	0.57
+ SHBG and BMI	104.2 (98.7 - 109.9)	96.8 (91.8 - 102.0)	94.9 (89.7 - 100.3)	93.1 (88.0 - 98.3)	94.7 (89.6 - 100.0)	0.01
Testosterone[6]						
Crude	92.7 (87.2 - 98.4)	92.6 (87.3 - 98.2)	92.7 (87.4 - 98.2)	94.6 (89.3 - 100.1)	103.6 (98.1 - 109.2)	0.03
+ SHBG and BMI	102.0 (96.5 - 107.7)	96.8 (91.6 - 102.1)	94.8 (89.8 - 100.1)	93.7 (88.6 - 99.0)	96.1 (90.9 - 101.4)	0.03
Free Testosterone[7]						
Crude	86.6 (81.5 - 91.8)	89.0 (83.9 - 94.3)	96.4 (91.0 - 102.0)	98.4 (93.0 - 104.0)	107.0 (101.3 - 112.8)	< 0.0001
+ BMI	97.2 (91.9 - 102.6)	95.5 (90.3 - 100.8)	98.7 (93.4 - 104.2)	95.2 (90.0 - 100.6)	96.5 (91.1 - 102.0)	0.81
SHBG[8]						
Crude	110.8 (104.9 - 116.8)	106.1 (100.7 - 111.7)	90.9 (85.8 - 96.1)	91.9 (87.0 - 97.0)	80.1 (75.3 - 85.0)	< 0.0001
+ BMI	97.8 (92.4 - 103.3)	103.3 (97.8 - 108.9)	93.7 (88.6 - 99.0)	97.5 (92.3 - 102.8)	91.7 (86.6 - 97.0)	0.02
+ BMI and WHR	95.1 (89.8 - 100.5)	101.0 (95.7 - 106.4)	94.7 (89.6 - 99.9)	98.4 (93.3 - 103.6)	94.4 (89.3 - 99.7)	0.58

[1] quintile cut points in pg/ml; 10, 15, 19, 25; [2] quintile cut points in pg/ml; 5.7, 7.6, 9.6, 12.0; [3] quintile cut points in pg/ml; 0.16, 0.22, 0.28, 0.39; [4] quintile cut points in ng/ml; 271, 411, 586, 828; [5] quintile cut points in ng/m; 0.29, 0.45, 0.60, 0.88; [6] quintile cut points in ng/m; 0.17, 0.24, 0.30, 0.40; [7] quintile cut points in pg/ml; 3.22, 4.91, 6.62, 9.35; [8] quintile cut points nmol/l; 11, 18, 24, 34
DHEAS=dehydroepiandrosterone sulfate; SHBG=sex hormone binding globulin; BMI=body mass index; WHR=waist-hip ratio; CI=confidence interval

and dense area, which was not seen in the subgroup of never HT users. The difference in effect between never HT users and ever HT users was not seen with any of the other sex steroids or SHBG and that the results with estrone levels may be chance findings.

The significantly positive association between DHEAS and percent density we found, was not shown in other studies presenting results of DHEAS (15,16,18). This association was not seen with he dense area.

Our result of a positive association between circulating SHBG levels and percent breast density, are in line with four previous studies (14,16-18). Like in the present study, in most of these studies the crude effect was very explicit, but became weaker after correction for confounding factors, especially BMI or other measures of total body fat. In our study, women with high SHBG levels had slightly, but non significantly higher dense area and smaller non-dense area. The apparently positive association between SHBG levels and percent breast density or dense area seems in contradiction with the inverse relation between these SHBG levels and breast cancer risk that has been described in many epidemiologic studies (12,13). A possible explanation was offered by Greendale et al., suggesting a difference in SHBG-receptor-mediated effects between normal and cancerous breast tissue (17).

Unexpectedly, higher levels of all sex steroids, except free testosterone, were significantly associated with a smaller non-dense area. We do not have a clear explanation for these results. Unfortunately none of the other studies on sex steroids and breast density studied the non-dense area to verify our results.

Conclusions

After studying our results and the results of 5 previous studies on sex steroids and postmenopausal breast density, we conclude that there is not much proof for such associations in postmenopausal women, at least not to the same extent as the associations between sex steroid levels and breast cancer risk. Some of the previous studies were relatively small, but the study by Warren et al., as well as our own study had enough power to detect such effects. The relationship between sex steroids and breast cancer risk as described in the literature, does not seem to be explained by a change in mammographic density.

Acknowledgements

This study was supported the World Cancer Research Fund (grant number 2002/11), and by the Dutch Heart Association (grant number 99.159).

References

1. Byng JW, Boyd NF, Fishell E, et al. The quantitative analysis of mammographic densities. Phys Med Biol 1994;39:1629-1638.
2. Boyd NF, Rommens JM, Vogt K, et al. Mammographic breast density as an intermediate phenotype for breast cancer. Lancet Oncol. 2005;6:798-808.
3. McCormack VA, dos SS, I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol.Biomarkers Prev. 2006;15: 1159-1169.
4. McTiernan A, Martin CF, Peck JD, et al. Estrogen-plus-progestin use and mammographic density in postmenopausal women: women's health initiative randomized trial. J.Natl.Cancer Inst. 2005;97:1366-1376.
5. Greendale GA, Reboussin BA, Slone S, et al. Postmenopausal hormone therapy and change in mammographic density. J.Natl.Cancer Inst. 2003;95:30-37.
6. Chlebowski RT, Hendrix SL, Langer RD, et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. JAMA 2003;289:3243-3253.
7. Boyd NF, Martin LJ, Li Q, et al. Mammographic density as a surrogate marker for the effects of hormone therapy on risk of breast cancer. Cancer Epidemiol.Biomarkers Prev. 2006;15:961-966.
8. Brisson J, Brisson B, Cote G, et al. Tamoxifen and mammographic breast densities. Cancer Epidemiol.Biomarkers Prev. 2000;9:911-915.
9. Cuzick J, Warwick J, Pinney E, et al. Tamoxifen and breast density in women at increased risk of breast cancer. J.Natl.Cancer Inst. 2004;96:621-628.
10. Shah NR, Borenstein J, Dubois RW. Postmenopausal hormone therapy and breast cancer: a systematic review and meta-analysis. Menopause. 2005;12:668-678.
11. Clavel-Chapelon F, Gerber M. Reproductive factors and breast cancer risk. Do they differ according to age at diagnosis? Breast Cancer Res.Treat. 2002;72:107-115.
12. Key T, Appleby P, Barnes I, et al. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606-616.
13. Kaaks R, Rinaldi S, Key TJ, et al. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European prospective investigation into cancer and nutrition. Endocr.Relat Cancer. 2005;12:1071-1082.
14. Boyd NF, Stone J, Martin LJ, et al. The association of breast mitogens with mammographic densities. Br J Cancer 2002;87:876-882.

15. Aiello EJ, Tworoger SS, Yasui Y, et al. Associations among circulating sex hormones, insulin-like growth factor, lipids, and mammographic density in postmenopausal women. Cancer Epidemiol.Biomarkers Prev. 2005;14:1411-1417.

16. Tamimi RM, Hankinson SE, Colditz GA, et al. Endogenous sex hormone levels and mammographic density among postmenopausal women. Cancer Epidemiol.Biomarkers Prev. 2005;14:2641-2647.

17. Greendale GA, Palla SL, Ursin G, et al. The Association of Endogenous Sex Steroids and Sex Steroid Binding Proteins with Mammographic Density: Results from the Postmenopausal Estrogen/Progestin Interventions Mammographic Density Study. Am.J.Epidemiol. 2005;.:

18. Warren R, Skinner J, Sala E, et al. Associations among mammographic density, circulating sex hormones, and polymorphisms in sex hormone metabolism genes in postmenopausal women. Cancer Epidemiol.Biomarkers Prev. 2006;15:1502-1508.

19. Riboli E, Kaaks R. The EPIC Project: rationale and study design. European Prospective Investigation into Cancer and Nutrition. Int J Epidemiol 1997;26 Suppl 1:S6-14.

20. Riboli E, Hunt KJ, Slimani N, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002;5:1113-1124.

21. Boker LK, Van Noord PA, van der Schouw YT, et al. Prospect-EPIC Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. Eur J Epidemiol 2001;17:1047-1053.

22. Ocke MC, Bueno-de-Mesquita HB, Pols MA, et al. The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. Int J Epidemiol 1997;26 Suppl 1:S49-S58.

23. Ocke MC, Bueno-de-Mesquita HB, Goddijn HE, et al. The Dutch EPIC food frequency questionnaire. I. Description of the questionnaire, and relative validity and reproducibility for food groups. Int J Epidemiol 1997;26 Suppl 1:S37-S48.

24. Onland-Moret NC, Peeters PH, van der Schouw YT, et al. Alcohol and endogenous sex steroid levels in postmenopausal women: a cross-sectional study. J Clin Endocrinol Metab 2005;90:1414-1419.

25. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J.Clin.Endocrinol.Metab 1999;84: 3666-3672.

26. Byng JW, Boyd NF, Little L, et al. Symmetry of projection in the quantitative analysis of mammographic images. Eur J Cancer Prev 1996;5:319-327.

27. Trichopoulos D, Lipman RD. Mammary gland mass and breast cancer risk. Epidemiology. 1992;3:523-526.

28. Albanes D, Winick M. Are cell number and cell proliferation risk factors for cancer? J.Natl.Cancer Inst. 1988;20;80:772-774.

29. Haars G, Van Noord PA, Van Gils CH, et al. Measurements of breast density: no ratio for a ratio. Cancer Epidemiol.Biomarkers Prev. 2005;14:2634-2640.

30. Noh JJ, Maskarinec G, Pagano I, et al. Mammographic densities and circulating hormones: a cross-sectional study in premenopausal women. Breast 2006;15:20-28.

31. Meyer F, Brisson J, Morrison AS, et al. Endogenous sex hormones, prolactin, and mammographic features of breast tissue in premenopausal women. J.Natl.Cancer Inst. 1986;77:617-620.

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

Chapter 4

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

Martijn Verheus
Petra H.M. Peeters
Sabina Rinaldi
Laure Dossus
Carine Biessy
Anja Olsen
Anne Tjønneland
Kim Overvad
Majbritt Jeppesen
Françoise Clavel-Chapelon
Bertrand Téhard
Gabriele Nagel
Jakob Linseisen
Heiner Boeing
Petra H. Lahmann
Athina Arvaniti
Theodora Psaltopoulou
Antonia Trichopoulou
Domenico Palli
Rosario Tumino
Salvatore Panico
Carlotta Sacerdote
Sabina Sieri
Carla H. van Gils
Bas H. Bueno-de-Mesquita
Carlos A. González
Eva Ardanaz
Nera Larranaga
Carmen Martinez Garcia
Carmen Navarro
J.Ramón Quirós
Tim Key
Naomi Allen
Sheila Bingham
Kay-Tee Khaw
Nadia Slimani
Elio Riboli
Rudolf Kaaks

Int J Cancer 2006; 119:659-667

Abstract

Background

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.

Design and Methods

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 1141 incident cases of breast cancer and 2204 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.

Results

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 non-significant reduced risk of breast cancer diagnosed up to the age of 50 years (OR=0.70, (95% 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 non-fasting conditions (OR=2.03, (95% CI: 1.20–3.43); p-trend =0.01).

Conclusion and discussion

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.

Introduction

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 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 non-fasting 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 1141 incident breast cancer cases, and 2204 matched control subjects.

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 non-dietary 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 therapy (HT), 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 recruitement 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 hours 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 one dry syringe was used to prepare serum. After centrifugation (1550g for 20 minutes), blood fractions (serum, plasma, red cells, buffy coat) were aliquoted in 28 plastic straws of 0.5 ml each (12 plasma, 8 serum, 4 erythrocytes, 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, non-fasting 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 et al., unpublished data) (21,24). Some of the selection criteria (no use of OC or HT at 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 hormone replacement therapy at the time of blood donation, or any exogenous hormones for contraception or medical purposes, and women who had previous diagnosis of cancer (except non-melanoma skin cancer) were excluded from the study.

At the time this study was started in November 2002, out of a total number of 2271 incident breast cancer cases, 1786 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 1141 cases were available for data-analyses. 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 time of blood donation. From the total 1141 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, two control subjects were chosen at random among appropriate risk sets consisting of all cohort members alive and free of cancer (except non-melanoma 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 hours; 3-6 hours, > 6 hours), 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 time of blood donation), measurements were also made of sex steroids (testosterone, androstenedione, dehydroepiandrosterone sulphate (DHEAS), estrone, 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 due to failed analyses.

Statistical analyses

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. Correlation 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 ($\geq$ 6 hours fasting at blood collection; < 6 hours fasting) and by age at diagnosis ($\leq$ 50 years; 50-60 years; > 60 years).

Heterogeneity of ORs between the study centers, between countries and between fasting and non-fasting subgroups, was assessed on a continuous scale (log2), using chi-square tests. The chi-square statistic was calculated as the deviations of logistic β-

coefficients observed in each of the subgroups, relative to the overall β-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 HT (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 two-sided, and p-values < 0.05 were considered statistically significant. All statistical analyses were done using the Statistical Analysis System (SAS) software package, version 9.1 (SAS Institute, Cary, NC, USA).

Results

The average age at blood donation was 54.5 years, with a 5th to 95th percentile range of 39.9 to 68.8 years (Table 1). Cases had an average age at diagnosis of 56.9 years (5th to 95th percentile range 53.0 to 72.0), and the average time between blood donation and diagnosis was 2.83 years (0.09 – 6.27). Compared to controls, cases were significantly older at first full term pregnancy, and had a slightly, but statistical 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 HT was higher in the control group than among the cases, but these differences were not statistically significant. BMI and waist circumference were slightly higher among control subjects aged 50 years or younger at diagnosis. In women older than 50 however these variables were slightly higher in the case group than among control subjects. Differences were statistically non-significant. 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 to 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

Table 1. Baseline characteristics of the study population

	Cases	Control subjects	p-value [1]
Total number of subjects	1141	2204	
Menopausal status at blood donation			
Premenopausal [2]	400	778	
Postmenopausal [2]	643	1240	
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 HT use (%) [5]	17.3	19.2	0.32
Current smoking (%)	16.9	16.4	0.65
Level of physical activity (MET hours / 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/m²) [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/m²) [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/m²) [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 chi-square 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; HT=postmenopausal hormone therapy

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 2), 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 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 non-fasting 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 3) 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 / non-fasting status at blood donation (Table 4).

When the analyses were stratified by fasting / non-fasting 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 statistically significant in either group separately (Table 4). By contrast, the direct association of C-peptide with the risk of breast cancer diagnosed after age 60 was present only, and statistically significant, in the subgroup of women who had provided a non-fasting 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 HT or women who reported a history of diabetes mellitus or women with breast cancer diagnosed less then two 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 1813 control subjects). For the extreme age groups ($\leq$ 50 years; > 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,

Table 2. 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)	Non fasting (n=621)	Fasting (n=646)	Non fasting (n=1237)	Fasting (n=112)	Non fasting (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 sulfate; SHBG=sex hormone binding globulin

Table 3. 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 (1141;2204)[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 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 due to failed analyses

Non-fasting	Q1	Q2	Q3	Q4	Q5	p-trend[2]
cut off points (ng/ml)	< 2.43	2.43 - 3.21	3.22 - 4.19	4.20 - 5.71	≥ 5.72	
All women (695;1335)						
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 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 due to failed analyses

Table 4. 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	

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 3 and 4. 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 2). 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 (non-fasting) 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 (non-hormonal) 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 statistically significant heterogeneity between fasting and non-fasting 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 statistically 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 non-fasting 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, very much parallel 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 non-fasting C-peptide levels).

A mayor 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 two 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 statistically 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 non-significant 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 two previous prospective studies into the association, higher levels of postmenopausal non-fasting serum C-peptide were associated with small increases in breast cancer risk (ORs and 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 statistically 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 statistically non-significant 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 non-fasting C-peptide levels, although the decrease was not linear and results were not statistically 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 characterised 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 six 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 six 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 population 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 hours) of insulin production) are smaller than those in insulin levels (which is rapidly cleared by the liver), circulating levels of C-peptide are less influenced by food intake (44-48).

In our study population, fasting serum C-peptide levels had higher correlations than non-fasting levels, with anthropometric factors and hormone levels. We expected fasting serum C-peptide to be a better biomarker than non-fasting 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 statistically significant increase in breast cancer risk among women with elevated C-peptide levels, who had provided a non-fasting 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 anti-apoptotic 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 non-fasting blood samples, may be impaired glucose tolerance by delayed insulin production (51).

Besides its possible direct anti-apoptotic 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 effects of elevated insulin independently 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 parallel 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.

Acknowledgements

The EPIC study was funded by "Europe Against Cancer" Programme of the European Commission (SANCO); Ligue contre le Cancer (France); Société 3M (France); Mutuelle Générale de l'Education Nationale; Institut National de la Santé et de la Recherche Médicale (INSERM); German Cancer Aid; German Cancer Research Center; German Federal Ministry of Education and Research; Danish Cancer Society; Health Research Fund (FIS) of the Spanish Ministry of Health; the participating regional governments and institutions of Spain; USCIII, Red de Centros RCESP, C03/09; Cancer Research UK; Medical Research Council, UK; the Stroke Association, UK; British Heart Foundation; Department of Health, UK; Food Standards Agency, UK; the Wellcome Trust, UK; Greek Ministry of Health; Greek Ministry of Education; Italian Association for Research on Cancer; Italian National Research Council; Dutch Ministry of Public Health, Welfare and Sports; Dutch Ministry of Health; Dutch Prevention Funds; LK Research Funds; Dutch ZON (Zorg Onderzoek Nederland); World Cancer Research Fund (WCRF); Swedish Cancer Society; Swedish Scientific Council; Regional Government of Skane, Sweden; Norwegian Cancer Society.

References

1. van den Brandt PA, Spiegelman D, Yaun SS, et al. Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. Am J Epidemiol 2000;152:514-527.
2. Lahmann PH, Hoffmann K, Allen N, et al. Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer And Nutrition (EPIC). Int J Cancer 2004;111:762-771.
3. Friedenreich CM, Orenstein MR. Physical activity and cancer prevention: etiologic evidence and biological mechanisms. J Nutr 2002;132:3456S-3464S.
4. Henriksen EJ. Invited review: Effects of acute exercise and exercise training on insulin resistance. J Appl Physiol 2002;93:788-796.
5. Kahn BB, Flier JS. Obesity and insulin resistance. J Clin Invest 2000;106:473-481.
6. Chlebowski RT, Pettinger M, Stefanick ML, et al. Insulin, physical activity, and caloric intake in postmenopausal women: breast cancer implications. J Clin Oncol 2004;22: 4507-4513.
7. Laban C, Bustin SA, Jenkins PJ. The GH-IGF-I axis and breast cancer. Trends Endocrinol Metab 2003;14:28-34.
8. Sachdev D, Yee D. The IGF system and breast cancer. Endocr Relat Cancer 2001;8:197-209.
9. Shi R, Yu H, McLarty J, et al. IGF-I and breast cancer: a meta-analysis. Int J Cancer 2004;111:418-423.
10. Chappell J, Leitner JW, Solomon S, et al. Effect of insulin on cell cycle progression in MCF-7 breast cancer cells. Direct and potentiating influence. J Biol Chem 2001;276:38023-38028.
11. Ish-Shalom D, Christoffersen CT, Vorwerk P, et al. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. Diabetologia 1997;40 Suppl 2:S25-S31.
12. Shafie SM, Grantham FH. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 1981;67:51-56.
13. Shafie SM, Hilf R. Insulin receptor levels and magnitude of insulin-induced responses in 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats. Cancer Res 1981;41: 826-829.
14. Heuson JC, Legros N. Influence of insulin deprivation on growth of the 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction. Cancer Res 1972;32:226-232.
15. Heuson JC, Legros N. Effect of insulin and of alloxan diabetes on growth of the rat mammary carcinoma in vivo. Eur J Cancer 1970;6:349-351.
16. Cocca C, Gutierrez A, Nunez M, et al. Suppression of mammary gland tumorigenesis in diabetic rats. Cancer Detect Prev 2003;27:37-46.

17. Cocca C, Martin G, Rivera E, et al. An experimental model of diabetes and cancer in rats. Eur J Cancer 1998;34:889-894.

18. Byrne C, Colditz GA, Willett WC, et al. Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. Cancer Res 2000;60:3744-3748.

19. Nestler JE. Insulin regulation of human ovarian androgens. Hum Reprod 1997;12 Suppl 1:53-62.

20. Awartani KA, Cheung AP. Metformin and polycystic ovary syndrome: a literature review. J Obstet Gynaecol Can 2002;24:393-401.

21. Kaaks R, Rinaldi S, Key TJ, et al. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European prospective investigation into cancer and nutrition. Endocr.Relat Cancer. 2005;12:1071-1082.

22. Key T, Appleby P, Barnes I, et al. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606-616.

23. Kaaks R. Nutrition, hormones, and breast cancer: is insulin the missing link? Cancer Causes Control 1996;7:605-625.

24. Kaaks R, Berrino F, Key T, et al. Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). J Natl Cancer Inst 2005;97:755-765.

25. Travis RC, Key TJ. Oestrogen exposure and breast cancer risk. Breast Cancer Res 2003;5: 239-247.

26. Toniolo P, Bruning PF, Akhmedkhanov A, et al. Serum insulin-like growth factor-I and breast cancer. Int J Cancer 2000;88:828-832.

27. Kaaks R, Lundin E, Rinaldi S, et al. Prospective study of IGF-I, IGF-binding proteins, and breast cancer risk, in northern and southern Sweden. Cancer Causes Control 2002;13:307-316.

28. Keinan-Boker L, Bueno De Mesquita HB, Kaaks R, et al. Circulating levels of insulin-like growth factor I, its binding proteins -1,-2, -3, C-peptide and risk of postmenopausal breast cancer. Int J Cancer 2003;106:90-95.

29. Mink PJ, Shahar E, Rosamond WD, et al. Serum insulin and glucose levels and breast cancer incidence: the atherosclerosis risk in communities study. Am J Epidemiol 2002;156:349-352.

30. Muti P, Quattrin T, Grant BJ, et al. Fasting glucose is a risk factor for breast cancer: a prospective study. Cancer Epidemiol Biomarkers Prev 2002;11:1361-1368.

31. Riboli E, Hunt KJ, Slimani N, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002;5: 1113-1124.

32. Bingham S, Riboli E. Diet and cancer—the European Prospective Investigation into Cancer and Nutrition. Nat Rev Cancer 2004;4:206-215.

33. Ainsworth BE, Haskell WL, Whitt MC, et al. Compendium of physical activities: an update of activity codes and MET intensities. Med Sci Sports.Exerc. 2000;32:S498-S504.

34. Rinaldi S, Dechaud H, Biessy C, et al. Reliability and validity of commercially available, direct radioimmunoassays for measurement of blood androgens and estrogens in postmenopausal women. Cancer Epidemiol Biomarkers Prev 2001;10:757-765.

35. Rinaldi S, Geay A, Dechaud H, et al. Validity of free testosterone and free estradiol determinations in serum samples from postmenopausal women by theoretical calculations. Cancer Epidemiol Biomarkers Prev 2002;11:1065-1071.

36. Bruning PF, Bonfrer JM, Van Noord PA, et al. Insulin resistance and breast-cancer risk. Int J Cancer 1992;52:511-516.

37. Schairer C, Hill D, Sturgeon SR, et al. Serum concentrations of IGF-I, IGFBP-3 and c-peptide and risk of hyperplasia and cancer of the breast in postmenopausal women. Int J Cancer 2004;108:773-779.

38. Yang G, Lu G, Jin F, et al. Population-based, case-control study of blood C-peptide level and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2001;10:1207-1211.

39. Hirose K, Toyama T, Iwata H, et al. Insulin, insulin-like growth factor-I and breast cancer risk in Japanese women. Asian Pac J Cancer Prev 2003;4:239-246.

40. Del Giudice ME, Fantus IG, Ezzat S, et al. Insulin and related factors in premenopausal breast cancer risk. Breast Cancer Res Treat 1998;47:111-120.

41. de Waard F, de LAIVE J, BAANDERS-VANHALEWIJN EA. On the bimodal age distribution of mammary carcinoma. Br J Cancer 1960;14:437-448.

42. Barcelo A. Monograph series on aging-related diseases: VIII. Non-insulin-dependent diabetes mellitus (NIDDM). Chronic Dis Can 1996;17:1-20.

43. Wolf I, Sadetzki S, Catane R, et al. Diabetes mellitus and breast cancer. Lancet Oncol 2005;6:103-111.

44. Waldhausl W, Bratusch-Marrain P, Gasic S, et al. Insulin production rate following glucose ingestion estimated by splanchnic C-peptide output in normal man. Diabetologia 1979;17:221-227.

45. Polonsky K, Jaspan J, Pugh W, et al. Metabolism of C-peptide in the dog. In vivo demonstration of the absence of hepatic extraction. J Clin Invest 1983;72:1114-1123.

46. Polonsky KS, Rubenstein AH. C-peptide as a measure of the secretion and hepatic extraction of insulin. Pitfalls and limitations. Diabetes 1984;33:486-494.

47. Hovorka R, Jones RH. How to measure insulin secretion. Diabetes Metab Rev 1994;10: 91-117.

48. Tura A, Ludvik B, Nolan JJ, et al. Insulin and C-peptide secretion and kinetics in humans: direct and model-based measurements during OGTT. Am J Physiol Endocrinol Metab 2001;281:E966-E974.

49. Palmqvist R, Stattin P, Rinaldi S, et al. Plasma insulin, IGF-binding proteins-1 and -2 and risk of colorectal cancer: a prospective study in northern Sweden. Int J Cancer 2003;107:89-93.

50. Giovannucci E. Insulin and colon cancer. 1995;6:164-179.

51. Ruige JB, Dekker JM, Nijpels G, et al. Hyperproinsulinaemia in impaired glucose tolerance is associated with a delayed insulin response to glucose. Diabetologia 1999;42:177-180.

52. Key TJ, Allen NE, Verkasalo PK, et al. Energy balance and cancer: the role of sex hormones. Proc Nutr Soc 2001;60:81-89.

53. De L, V, la Marca A, Petraglia F. Insulin-lowering agents in the management of polycystic ovary syndrome. Endocr Rev 2003;24:633-667.

54. Potischman N, Swanson CA, Siiteri P, et al. Reversal of relation between body mass and endogenous estrogen concentrations with menopausal status. J Natl Cancer Inst 1996;88: 756-758.

Plasma phytoestrogens and subsequent breast cancer risk

Chapter 5

Plasma phytoestrogens and subsequent breast cancer risk

M. Verheus
C.H. van Gils
L. Keinan-Boker
P.B. Grace
S.A. Bingham
P.H.M. Peeters

J Clin Oncol 2007; 25:648-655.

Abstract

Introduction

Phytoestrogens are plant compounds that are structurally and functionally similar to mammalian estrogens. By competing for estrogen receptors, phytoestrogens possibly inhibit binding of the more potent endogenous estrogens and decrease their potential effects on breast cancer risk. We investigated the association between plasma phytoestrogen levels and breast cancer risk in a prospective manner.

Methods

We performed a nested case-control study within the Prospect-EPIC cohort, one of the two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition. A total of 383 women (87 pre/perimenopausal (mean age 52) and 296 postmenopausal (mean age 59)) who developed breast cancer, were selected as cases and matched to 383 controls, on date of blood sampling. Plasma levels of isoflavones (daidzein, genistein, glycitein, O-desmethylangolensin and equol) and lignans (enterodiol and enterolactone) were measured. The isotope dilution liquid chromatography / tandem mass spectrometry method incorporating triply ^{13}C-labelled standards was used for all analyses. Breast cancer odds ratios were calculated for tertiles of phytoestrogen plasma levels using conditional logistic regression analysis.

Results

For genistein, the risk estimate for the highest versus the lowest tertile was 0.68 (95% confidence interval=0.47-0.98). Similar protective effects, although not statistically significant, were seen for the other isoflavones. Lignan levels did not appear to be related to breast cancer risk. Results were the same in pre/peri- and postmenopausal women.

Conclusion

High genistein circulation levels are associated with reduced breast cancer risk in the Dutch population. No effects of lignans on breast cancer risk were observed.

Introduction

Phytoestrogens are plant compounds that are structurally and functionally similar to mammalian estrogens. The two main classes found in the human diet are; isoflavones (daidzein, genistein and glycitein) and lignans (enterodiol and enterolactone). The isoflavone daidzein can be metabolized by intestinal bacteria into O-desmethylangolensin (O-DMA) and, in approximately 30-50% of individuals, into equol. The primary food sources of isoflavones are soy and soy products and for lignans these are cereals, flaxseed and berries (1-4).

In Asian countries, the incidence of breast cancer is much lower compared to Western countries (5). Studies with Asian women who migrated to Western countries show that breast cancer incidence rates change in only a few generations (6,7). Hence the role of genetics is probably relatively small compared to lifestyle habits, such as dietary intake. The intake of phytoestrogens is much higher among Asian women living in Asia, compared with intake among women living in the Western world. It has therefore been hypothesized that high phytoestrogen intake could protect against breast cancer (8).

Estrogens, estradiol in particular, are known to have strong mitogenic properties (9) and epidemiologic studies on circulating estrogen levels and breast cancer showed increased risk with higher levels of both estrone and estradiol, although only in postmenopausal women (10-12). Phytoestrogens are capable of binding to the estrogen receptor (ER) and have weak estrogenic potential (13-16), possibly increasing breast cancer risk. They can however also compete with endogenous estrogens for ERs and this way inhibit binding of the estrogenic more potent endogenous estrogens (13,15,17). It has been postulated that in an environment with low circulating levels of endogenous estrogens, phytoestrogens may act as weak estrogens, but have an anti-estrogenic effect in an environment with high circulating levels of endogenous estrogens. Hence they could protect against premenopausal breast cancer and increase breast cancer risk after menopause (18). Other properties of phytoestrogens, like inhibition of aromatase and tyrosine kinase activity, have also been described (1-4,18).

Prospective studies on soy intake and breast cancer risk found no significant protective effect for higher intake (19,20). Studies on phytoestrogen levels in blood or urine samples are scarce and have only been performed in Western study populations. Higher levels of isoflavones in blood and urine were found to be associated with (non-significantly) increased breast cancer risk in a study with *pre*menopausal German women and also in a study in the UK with predominantly postmenopausal women (21,22). This direct association was not found by den Tonkelaar et al. studying urinary isoflavone levels in a Dutch population of *post*menopausal women (23). Prospective epidemiologic studies with circulating levels or urinary excretion levels of lignans show conflicting results. In

*pre*menopausal women, both increased and decreased breast cancer risk were found (22,24,25). Most studies with *post*menopausal women, or studies showing results of pre- and postmenopausal women combined, did not report a relationship between lignans and breast cancer risk (21,23-26), although decreased risk, especially for ER-breast tumors, was found in a Danish study (27).

In the present large prospective study we investigated the effect of phytoestrogens measured in plasma on subsequent breast cancer occurrence, in pre/peri- and postmenopausal women, in the, Prospect-EPIC cohort.

Methods

The Prospect-EPIC cohort and subject selection

We designed a nested case-control study within Prospect-EPIC, one of two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition (EPIC). Rationale and design of both EPIC and Prospect-EPIC are described in detail elsewhere (28,29,30). All women with an incident breast cancer diagnosis until January 2003 were selected for the present study. In a mean follow-up period of 6.5 years, 395 breast cancer cases were diagnosed. One control subject was matched to every case on recruitment date (plus or minus 6 months) making storing time of biological samples comparable. The majority of women participating in the Prospect-EPIC cohort is postmenopausal. To increase power in the premenopausal sub group, pre- and perimenopausal women were grouped together. A plasma sample was available for 87 pre/perimenopausal and 296 postmenopausal case/control sets.

All participants signed an informed consent and the study was approved by The Institutional Review Board of the University Medical Center Utrecht.

Menopausal status

Women were categorized as premenopausal when they had had at least 6 menstrual periods in the 12 months prior to inclusion and were not currently using oral contraceptives (OC) or postmenopausal hormone therapy (HT). Women without any menstrual periods were categorized as being postmenopausal. Women with menstrual cycles in the 12 months prior to inclusion were classified to be perimenopausal when they had had less than 6 menstrual periods or when they were using OC or HT at the time of inclusion or when they had equivocal data.

Laboratory analyses

Plasma samples were analyzed for three isoflavones (daidzein, genistein and glycitein), two metabolites of daidzein (O-DMA and equol) and two mammalian lignans (enterodiol and enterolactone). Laboratory personnel were blinded for case-control status when performing the analyses. Information on the methodology of the analyses and quality assurance are described in detail elsewhere (31). Empirically calculated limits of detection were < 10 pg/ml for all phytoestrogens except equol. The detection limit for equol was calculated to be 100 pg/ml. All analyses were performed at the MRC Dunn Human Nutrition Unit in Cambridge, The UK.

Statistical analyses

Subjects with plasma phytoestrogen values under the detection limit were given the value of the detection limit. This was done for all phytoestrogens except equol, as only an estimated 30-50% of the population is able to produce equol in the intestinal tract (3). Plasma phytoestrogen levels were log transformed to normalize distributions. These transformed values were then used to compute geometric means.

Odds ratios (ORs) and 95% confidence intervals (95% CIs) for cancer risk by tertile level of the plasma phytoestrogens were estimated with conditional logistic regression models using the SAS 'PHREG' procedure. Tertile cut-off points were based on the frequency distribution of controls. Due to small numbers of women with detectable equol levels (24%), these women were compared with women without detectable levels. Potential confounding was evaluated using conditional logistic regression models with breast cancer as the outcome and adding one potential confounder as a covariate to the model in addition to one of the phytoestrogens as the variable of interest. Variables were judged to be a confounder when addition to the model changed the ORs for the phytoestrogen of interest with at least 10%. The following characteristics were evaluated for confounding: age at baseline, body mass index (BMI), family history of breast cancer, age at menarche, parity, age at first childbirth, age at menopause (postmenopausal women only), current and ever use of OC and HT, current and ever cigarette smoking physical activity. Analyses were done separately for pre/peri- and postmenopausal women. To test for linear trends in ORs over the tertiles, median values within tertiles were calculated and evaluated as a continuous variable using conditional logistic regression analysis.

All p-values are two-sided and when below 0.05, results were considered significant. All analyses were conducted using the Statistical Analysis System (SAS) software package, release 8.02 (SAS Institute, Cary, NC, USA).

Table 1. Baseline characteristics of the study population

		Pre- and perimenopausal (n=174)				Postmenopausal (n=592)			
		Cases (n=87)		Control subjects (n=87)		Cases (n=296)		Control subjects (n=296)	
Age	(year)[1]	52.2	(3.5)	51.6	(2.7)	59.4	(5.8)	58.6	(5.8)
BMI	(kg/m²)[1]	25.6	(4.0)	26.0	(4.7)	26.4	(3.5)	26.3	(3.9)
Family history [3]	yes[2]	14	(16%)	8	(9%)	58	(20%)	40	(14%)
Age at menarche	(year)[1]	13.1	(1.4)	13.2	(1.5)	13.4	1.7	13.5	1.70
Parity	nulliparous[2]	25	(29%)	13	(15%)	38	(13%)	49	(17%)
Age at first child birth[4]	(year)[1]	25.4	(4.3)	25.0	(4.6)	25.5	(3.9)	25.3	(3.7)
Age at menopause[5]	(year)[1]					47.9	(5.6)	47.2	(5.9)
Current OC use	yes[2]	10	(12%)	12	(14%)	0	(0%)	0	(0%)
OC use ever	yes[2]	69	(80%)	74	(85%)	161	(54%)	170	(57%)
Current HT use	yes[2]	19	(22%)	18	(21%)	0	(0%)	0	(0%)
HT use ever	yes[2]	30	(34%)	28	(32%)	75	(25%)	73	(25%)
Current smoker	yes[2]	17	(20%)	23	(26%)	64	(22%)	76	(26%)
Ever smoking	yes[2]	54	(62%)	58	(67%)	164	(55%)	178	(60%)
Physical activity	inactive[2]	8	(9%)	9	(10%)	13	(4%)	13	(4%)
	moderately inactive	20	(23%)	20	(23%)	51	(17%)	55	(19%)
	moderately active	36	(41%)	28	(32%)	168	(57%)	176	(59%)
	active	23	(26%)	30	(34%)	64	(22%)	52	(18%)
Total energy intake	(Mj/day)[1]	7.86	(1.84)	7.51	(2.09)	7.39	(1.86)	7.49	(1.86)
Protein intake	(gram/day)[1]	74.3	(16.8)	70.1	(19.4)	70.1	(17.4)	72.1	(18.5)
Alcohol intake	(gram/day)[1]	11.6	(14.5)	11.5	(15.1)	9.6	(13.4)	8.3	(11.5)
Dietary fiber intake	(gram/day)[1]	23.3	(5.8)	21.7	(5.1)	23.0	(6.4)	22.3	(5.6)

[1] mean (standard deviation)
[2] number (%)
[3] mother and / or sister with breast cancer
[4] parous women only
[5] postmenopausal women only
BMI=body mass index; OC=oral contraceptives; HT=postmenopausal hormone therapy

RESULTS

Basic characteristics at baseline are given in Table 1. Among isoflavones, genistein had the highest geometric mean concentration (Table 2). Enterolactone was the main lignan.

Higher levels of all isoflavones were associated with lower breast cancer risk (table 3). For genistein, the strongest decrease was seen and the 32% reduction in the upper tertile compared with the lowest tertile was statistically significant (OR=0.68, 95% CI: (0.47-0.98)). A test for trend showed a borderline significant decrease in risk for this particular isoflavone (p=0.07). Women with detectable equol levels were shown to have decreased breast cancer risk, compared to women with non-detectable levels (OR=0.87, 95% CI: (0.63-1.21)), and when women with detectable levels above median were compared with women with undetectable levels, the protection was somewhat stronger (OR=0.77, 95% CI: (0.49-1.21)) (data not shown). Adjustment for various possible confounders did not change the direction or magnitude of the associations of circulating levels of any of the isoflavones with breast cancer risk by more than 10% and therefore none of these variables were added to the crude models.

Relations between circulating levels of isoflavones and breast cancer risk in pre/peri- and in postmenopausal women were all in the same direction compared to analyses in the total group: higher levels of all isoflavones were associated with decreased breast cancer risk (Table 4). In pre/perimenopausal women, results were less stable, probably due to smaller numbers. Several confounding factors did change the magnitude of the associations between some of the phytoestrogens and breast cancer risk in this subgroup by more than 10% and were added to the crude models to calculate adjusted risk estimates. No potential counfounders substantially changed postmenopausal ORs.

Results for lignans showed no relations with breast cancer risk in the total study population (Table 3) and adjustment for various possible confounders again did not change the direction or magnitude of the observed associations. In pre/perimenopausal women, circulating levels of both enterodiol and enterolactone were associated with an increase in breast cancer risk (Table 4). Especially for enterolactone, the increase in risk was quite substantial (OR=1.72), however confidence intervals were very wide and there was no significant trend over the tertiles. In postmenopausal women circulating levels of these compounds were not associated with breast cancer occurrence (Table 5).

Isoflavone levels were mutually adjusted by analyzing genistein and one of the other isoflavones in the same model. The beneficial effect of genistein became slightly stronger, whereas the effects of the other isoflavones (except equol) changed toward the null relation (data not shown).

		Pre- and perimenopausal (n=174)		Postmenopausal (n=592)	
		Cases (n=87)	Control subjects (n=87)	Cases (n=296)	Control subjects (n=296)
Equol	Women with detectable levels	16 (18%)	19 (22%)	72 (24%)	77 (26%)
	Geometric mean (± 2SD)[2]	0.25 (0.05 - 1.17)	0.30 (0.02 - 3.98)	0.31 (0.03 - 3.24)	0.37 (0.03 - 5.37)
	Median[2]	0.22	0.17	0.18	0.20
	Inter quartile range [2]	0.12 - 0.52	0.13 - 0.37	0.13 - 0.60	0.14 - 0.73
	Range[2]	0.10 - 0.77	0.10 - 13.30	0.10 - 5.43	0.10 - 19.20
	Women with undetectable levels	71	68	224	219
Enterodiol	Geometric mean (± 2SD)	0.27 (0.02 - 4.17)	0.19 (0.01 - 2.95)	0.18 (0.01 - 2.69)	0.18 (0.01 - 2.34)
	Median	0.29	0.19	0.20	0.19
	Inter quartile range	0.11 - 0.63	0.09 - 0.49	0.08 - 0.41	0.08 - 0.44
	Range	0.00 - 6.08	0.00 - 13.99	0.00 - 20.25	0.00 - 10.11
	Nr under detection limit	18	22	87	85
Enterolactone	Geometric mean (± 2SD)	2.98 (0.29 - 30.90)	2.66 (0.39 - 18.20)	2.71 (0.25 - 29.51)	2.65 (0.26 - 26.30)
	Median	2.98	3.07	2.95	2.99
	Inter quartile range	1.66 - 6.24	1.66 - 5.01	1.58 - 5.12	1.48 - 5.49
	Range	0.15 - 36.77	0.09 - 37.57	0.00 - 57.77	0.00 - 52.00
	Nr under detection limit	0	1	5	5

[1] number of subjects with a value under the detection limit that has been given the detection limit as value
[2] figures for equol are based on women with detectable equol levels only
SD=standard deviation; O-DMA=O-desmethylangolensin

Table 2. Plasma levels of phytoestrogens in ng/ml

		Pre- and perimenopausal (n=174)		Postmenopausal (n=592)	
		Cases (n=87)	Control subjects (n=87)	Cases (n=296)	Control subjects (n=296)
Daidzein	Geometric mean (± 2SD)	2.27 (0.19 - 26.92)	2.57 (0.23 - 28.20)	2.76 (0.29 - 26.30)	2.91 (0.28 - 30.20)
	Median	2.34	2.62	2.86	3.27
	Inter quartile range	1.13 - 5.49	1.22 - 6.40	1.30 - 6.30	1.61 - 6.23
	Range	0.01 - 21.69	0.11 - 20.25	0.07 - 36.77	0.00 - 77.98
	Nr under detection limit[1]	1	0	1	4
Genistein	Geometric mean (± 2SD)	3.58 (0.33 - 38.90)	3.74 (0.28 - 50.12)	3.88 (0.35 - 43.65)	4.58 (0.36 57.54)
	Median	3.08	3.75	3.78	4.89
	Inter quartile range	1.64 - 8.91	1.85 - 8.66	1.89 - 8.71	2.08 - 9.71
	Range	0.25 - 55.13	0.00 - 45.41	0.00 - 57.50	0.00 - 92.62
	Nr under detection limit	0	1	2	4
Glycitein	Geometric mean (± 2SD)	0.06 (0.00 - 1.02)	0.07 (0.00 - 1.23)	0.07 (0.00 - 1.38)	0.08 (0.00 - 1.35)
	Median	0.06	0.08	0.09	0.10
	Inter quartile range	0.02 - 0.22	0.00 - 0.19	0.02 - 0.21	0.02 - 0.23
	Range	0.00 - 1.32	0.00 - 1.86	0.00 - 3.17	0.00 - 8.62
	Nr under detection limit	54	46	157	152
O-DMA	Geometric mean (± 2SD)	0.05 (0.00 - 1.41)	0.06 (0.00 - 1.58)	0.08 (0.00 - 1.95)	0.09 (0.00 - 2.29)
	Median	0.04	0.08	0.08	0.08
	Inter quartile range	0.01 - 0.15	0.01 - 0.19	0.02 - 0.32	0.02 - 0.27
	Range	0.00 - 4.71	0.00 - 7.77	0.00 - 8.59	0.00 - 10.62
	Nr under detection limit	58	48	154	160

Table 3. Associations of plasma phytoestrogens in tertiles with breast cancer

	T 1	T 2	T 3	p-trend
Daidzein				
Case / control	140 / 129	129 / 129	119 / 130	
OR (95% CI) crude [1]	1.00	0.92 (0.66 - 1.29)	0.83 (0.58 - 1.19)	0.33
Genistein				
Case / control	158 / 129	118 / 129	112 / 130	
OR (95% CI) crude	1.00	0.73 (0.51 - 1.04)	0.68 (0.47 - 0.98)	0.07
Glycitein				
Case / control	132 / 123	136 / 133	120 / 132	
OR (95% CI) crude	1.00	0.95 (0.67 - 1.33)	0.83 (0.59 - 1.18)	0.32
O-DMA				
Case / control	140 / 125	127 / 133	121 / 130	
OR (95% CI) crude	1.00	0.87 (0.62 - 1.20)	0.83 (0.59 - 1.18)	0.39
Equol [2]				
Case / control	300 / 290	88 / 98	-	
OR (95% CI) crude	1.00	0.87 (0.63 - 1.21)	-	
Enterodiol				
Case / control	128 / 129	122 / 128	138 / 131	
OR (95% CI) crude	1.00	0.97 (0.68 - 1.37)	1.07 (0.75 - 1.53)	0.63
Enterolactone				
Case / control	121 / 129	133 / 128	134 / 131	
OR (95% CI) crude	1.00	1.11 (0.78 - 1.56)	1.10 (0.76 - 1.57)	0.67

[1] ORs were calculated using conditional logistic regression analysis; cut off points for tertiles were based on the distribution of the control subjects

[2] OR for women with an equol level above the detection limit versus women with an equol level under the detection limit

None of the potential confounding factors changed crude ORs by more than 10%. Hence only the crude figures are presented.

OR=odds ratio; CI=confidence interval; O-DMA=O-desmethylangolensin

Discussion

In this study, high levels of plasma genistein were associated with significantly lower risk of breast cancer development. Similar trends, although not statistically significant were shown for daidzein, glycitein, O-DMA and equol. Lignans were not related to breast cancer occurrence. Results were the same in pre/peri- and postmenopausal women.

The main advantage of the present study is its prospective design. Information about lifestyle factors and dietary habits as well as blood samples were collected before diagnosis. Therefore our results cannot have been biased by behavioral or metabolic changes after breast cancer diagnosis or by cancer treatment. Because of the prospective design we were also able to select controls from the same source population from which

the cases arose, avoiding selection bias. A second major strength of the study is the use of plasma levels of phytoestrogens instead of dietary intake. Blood levels may reflect more accurately relevant biological dose levels, since phytoestrogen metabolism by bacteria in the gastrointestinal tract is highly variable. This is not reflected by dietary intake. Another important advantage is the size of our study, which exceeds the sample size of three other prospective studies on circulating levels or urinary excretion levels of isoflavones (21-23). Furthermore our study is one of the largest prospective studies to investigate the association between lignans and breast cancer risk (21-27).

We have measured seven different phytoestrogens. It should be noted however, that their relationships with breast cancer development are unlikely to be independent. Levels of these phytoestrogens are highly correlated, especially among isoflavones. Relations of isoflavones with breast cancer risk must therefore be interpreted with caution, because they may have been caused by the relation between breast cancer risk and genistein, as it is this compound that has the highest affinity for the estrogen receptor (15,32-34) and is the most abundant isoflavone in the blood stream. Moreover, when daidzein, glycitein or O-DMA were added to a regression model with genistein, the association between genistein and breast cancer risk became slightly stronger, while the protective effect of the other isoflavones disappeared.

Formally, the capability of producing equol in the intestinal tract from its precursor daidzein, can only be determined after challenging persons for several days with a high dose of dietary daidzein (3). In our population, without preceding such dietary challenge, detectable/undetectable levels may reflect the capability of producing equol. However, as detectable levels also depend on daidzein levels, some women capable of metabolizing equol may have had undetectable levels. Hence the association between metabolizing capability and breast cancer risk may be stronger when we would have been able to compare metabolizers with non-metabolizers.

We did not have information on race. However, only 5.5% of the women in our study filled out not to have been born in the Netherlands. Furthermore, women had to speak proper Dutch to fill out the questionnaires. In the Netherlands, we can then assume that most women of the study population are Caucasian.

Our results are opposite to results of a recently published study with *pre*menopausal women in Germany, showing increased breast cancer risk in women with higher levels of genistein in plasma (22), which was however non-significant. Grace et al. also found increased breast cancer risk with elevated levels of five different types of isoflavones, measured in both serum and urine samples in women living in the UK (21). Results were based on analyses with *pre*- and *post*menopausal women combined, but the majority of women was *post*menopausal. This direct association was not found by den Tonkelaar et al. in a study with urinary genistein levels in a Dutch population of *post*menopausal women (23).

Table 4. Associations of plasma phytoestrogens in tertiles with breast cancer in pre/peri menopausal women

	T 1	T 2	T 3	p-trend
Daidzein				
Case / control	30 / 28	34 / 30	23 / 29	
OR (95% CI) crude [1]	1.00	1.03 (0.48 - 2.22)	0.70 (0.30 - 1.61)	0.31
OR (95% CI) adjusted [2]	1.00	1.21 (0.54 - 2.70)	0.80 (0.34 - 1.88)	0.44
Genistein				
Case / control	34 / 28	27 / 30	26 / 29	
OR (95% CI) crude	1.00	0.70 (0.31 - 1.57)	0.73 (0.35 - 1.52)	0.51
OR (95% CI) adjusted [2]	1.00	0.78 (0.33 - 1.83)	0.80 (0.38 - 1.69)	0.65
Glycitein				
Case / control	27 / 26	32 / 32	28 / 29	
OR (95% CI) crude [3]	1.00	0.96 (0.42 - 2.18)	0.92 (0.42 - 2.03)	0.85
O-DMA				
Case / control	29 / 29	36 / 29	22 / 29	
OR (95% CI) crude	1.00	1.33 (0.62 - 2.85)	0.67 (0.29 - 1.56)	0.30
OR (95% CI) adjusted [4]	1.00	1.36 (0.60 - 3.08)	0.66 (0.26 - 1.65)	0.32
Equol 5				
Case / control	71 / 58	16 / 19	-	
OR (95% CI) crude [4]	1.00	0.81 (0.39 - 1.69)	-	
Enterodiol				
Case / control	20 / 26	34 / 32	33 / 29	
OR (95% CI) crude [3]	1.00	1.40 (0.64 - 3.04)	1.45 (0.69 - 3.05)	0.45
Enterolactone				
Case / control	26 / 28	23 / 30	38 / 29	
OR (95% CI) crude	1.00	0.80 (0.36 - 1.80)	1.35 (0.66 - 2.75)	0.29
OR (95% CI) adjusted [6]	1.00	1.13 (0.47 - 2.71)	1.72 (0.80 - 3.71)	0.13

[1] ORs were calculated using conditional logistic regression analysis; cut off points for tertiles were based on the distribution of the control subjects
[2] ORs and 95% CIs adjusted for; age at menarche
[3] None of the potential confounding factors changed crude ORs by more than 10%. Hence only the crude figures are presented
[4] ORs and 95% CIs adjusted for; age at menarche, ever OC use, parity and age at first child birth
[5] OR for women with an equol level above the detection limit versus women with an equol level under the detection limit
[6] ORs and 95% CIs adjusted for; age at menarche, having a family history of breast cancer
OR=odds ratio; CI=confidence interval; O-DMA=O-desmethylangolensin; OC=oral contraceptives

In the present study we found no relations between enterodiol or enterolactone levels and breast cancer occurrence, although in *pre/peri*menopausal women, there was a tendency of increased risk, but confidence intervals were wide and results were non-significant. Only two other prospective studies with circulating levels of enterolactone, present results for *pre*- and *post*menopausal women separately. Results of the present study are in line with results of a large case control study, nested within the New York

Table 5. Associations of plasma phytoestrogens in tertiles with breast cancer in postmenopausal women

	T 1	T 2	T 3	p-trend
Daidzein				
Case / control	111 / 98	86 / 99	99 / 99	
OR (95% CI) crude	1.00	0.78 (0.53 - 1.15)	0.88 (0.59 - 1.32)	0.59
Genistein				
Case / control	118 / 98	93 / 99	85 / 99	
OR (95% CI) crude	1.00	0.76 (0.51 - 1.14)	0.69 (0.45 - 1.04)	0.09
Glycitein				
Case / control	99 / 90	103 / 103	94 / 103	
OR (95% CI) crude	1.00	0.90 (0.61 - 1.33)	0.81 (0.53 - 1.24)	0.34
O-DMA				
Case / control	104 / 97	104 / 100	88 / 99	
OR (95% CI) crude	1.00	0.98 (0.67 - 1.44)	0.82 (0.55 - 1.23)	0.64
Equol[6]				
Case / control	224 / 219	72 / 77		
OR (95% CI) crude	1.00	0.91 (0.63 - 1.33)		
Enterodiol				
Case / control	103 / 97	95 / 99	98 / 100	
OR (95% CI) crude	1.00	0.89 (0.59 - 1.35)	0.91 (0.60 - 1.39)	0.76
Enterolactone				
Case / control	93 / 98	112 / 99	91 / 99	
OR (95% CI) crude	1.00	1.18 (0.80 - 1.76)	0.97 (0.63 - 1.48)	0.77

None of the potential confounding factors changed crude ORs by more than 10%. Hence only the crude figures are presented.
OR=odds ratio; CI=confidence interval; O-DMA=O-desmethylangolensin; OC=oral contraceptives

University Women's Health Study cohort, showing increased breast cancer risk with high circulating levels of enterolactone in *pre*menopausal women, but not in *post*menopausal women. As in the present study, results were statistically non-significant (24). A second study reported weak non-significant effects in the opposite direction: high levels of enterolactone were associated with decreased breast cancer risk in *pre*menopausal women, but with increased risk in *post*menopausal women in Finland (25). Piller et al. also found a protective effect in women with high levels of circulating enterolactone in a German study that consisted of *pre*menopausal women only (22). A Dutch study with exclusively *post*menopausal women, found the opposite effects with non-significantly increased breast cancer risk in women with high urinary excretion levels of enterolactone (23). Other prospective studies with enterolactone report combined results of *pre*- and *post*menopausal women. Two such studies from England and Sweden did not find breast cancer occurrence to be associated with enterodiol and enterolactone in blood and urine (21,26). In Denmark, however, plasma enterolactone levels significantly

decreased breast cancer risk, especially for ER-tumors (27). Median plasma levels in the latter study were higher compared to the present study (28.1 versus 10.0 nmol/L), suggesting that the lack of association between enterolactone levels in the present study may have been caused because absolute levels of circulating enterolactone were to low. Piller et al. however, were able to show significantly decreased breast cancer risk with comparable plasma levels to the present study (9.7 nmol/L) (22).

We have no clear explanation for the opposing effects of isoflavones between our study and the studies by Grace et al. and Piller et al. (21,22). An explanation may be that serum levels of isoflavones, besides their own effects, are also markers for other dietary compounds. Only small part of the dietary intake of isoflavones in Western women is via soy or soy products. If other sources of isoflavones differ between countries and isoflavones therefore reflect different dietary components in each country, this may also cause different effects. In addition, variants of genes involved in the metabolism of sex steroid hormones may be important for the effects of phytoestrogens on breast cancer risk (35). Stronger decreased breast cancer risk in women with high dietary intake and in women with high plasma concentrations of lignans was shown for a certain variant of the cyp17 gene (36,37).

In our opinion, the hypothesis that phytoestrogens protect against breast cancer in an environment of high circulating levels of endogenous estrogens (i.e. in premenopausal women), but increase risk in environments of relatively low levels of endogenous estrogens (i.e. after menopause), is not supported in general by the results of prospective studies published so far, including our own. Furthermore, because endogenous estrogen levels severely decrease after menopause, the excess of circulating levels of phytoestrogens over circulating estradiol levels is much larger in postmenopausal women compared to premenopausal women. Hence, competition between phytoestrogens and estradiol may be more effective after menopause.

Circulating levels of isoflavones in women living in Western countries are much lower compared with circulating levels in Asian women. However, comparing endogenous estrogen levels with phytoestrogen levels from Western women, shows that phytoestrogen levels are 50-1000 times higher than endogenous estrogen levels (10-12,21,22,24-27). Hence, an effect can be expected even at low circulating levels of isoflavones, as are common in European and American populations.

Other proposed mechanisms of action of phytoestrogens, like scavenging of free radicals and inducement of apoptosis and of tyrosine kinase activity, may also result in decreased breast cancer risk. Some of these effects were however only shown in in vitro experiments with much higher phytoestrogens levels, compared to in vivo levels and it is therefore questionable whether these mechanisms explain our results (1-4,18). Inhibition of aromatic enzymes, however, was described with levels that can be reached with a phyto-estrogen-

enriched diet (2). This effect is particularly interesting for relations after menopause, when there is no estrogen production from the ovaries and aromatic conversion of androgens is the main pathway of estrogens production.

In conclusion, high circulating levels of genistein were associated with decreased breast cancer risk in Dutch women with relatively low overall circulating levels of isoflavones which are characteristic for Western populations. These results suggest that this compound may be protective against breast cancer development. Other isoflavones may have similar properties, but evidence is less strong. Circulating levels of lignans did not seem to be associated with breast cancer occurrence.

Acknowledgements

This study was supported by grant number 21000027 of the Dutch 'Zorg Onderzoek Nederland' Fund (ZON), grant number 2000/30 of the World Cancer Research Fund (WCRF) and the European Committee 'Europe against Cancer'.

References

1. Duncan AM, Phipps WR, Kurzer MS. Phyto-oestrogens. Best Pract Res Clin Endocrinol Metab 2003;17:253-271.
2. Magee PJ, Rowland IR. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. Br J Nutr 2004;91:513-531.
3. Setchell KD, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. J Nutr 2002;132:3577-3584.
4. Ososki AL, Kennelly EJ. Phytoestrogens: a review of the present state of research. Phytother.Res 2003;17:845-869.
5. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
6. Stanford JL, Herrinton LJ, Schwartz SM, et al. Breast cancer incidence in Asian migrants to the United States and their descendants. Epidemiology 1995;6:181-183.
7. Ziegler RG, Hoover RN, Pike MC, et al. Migration patterns and breast cancer risk in Asian-American women. J Natl Cancer Inst 1993;85:1819-1827.
8. Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest Suppl 1990;201:3-23.
9. Preston-Martin S, Pike MC, Ross RK, et al. Increased cell division as a cause of human cancer. Cancer Res 1990;50:7415-7421.
10. Kaaks R, Berrino F, Key T, et al. Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). J Natl Cancer Inst 2005;97:755-765.
11. Kaaks R, Rinaldi S, Key TJ, et al. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European prospective investigation into cancer and nutrition. Endocr.Relat Cancer. 2005;12:1071-1082.
12. Key T, Appleby P, Barnes I, et al. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606-616.
13. Kuiper GG, Lemmen JG, Carlsson B, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139:4252-4263.
14. Markiewicz L, Garey J, Adlercreutz H, et al. In vitro bioassays of non-steroidal phytoestrogens. J Steroid Biochem Mol Biol 1993;45:399-405.
15. Miksicek RJ. Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. J Steroid Biochem Mol Biol 1994;49:153-160.

16. Welshons WV, Murphy CS, Koch R, et al. Stimulation of breast cancer cells in vitro by the environmental estrogen enterolactone and the phytoestrogen equol. Breast Cancer Res Treat 1987;10:169-175.
17. Adlercreutz CH, Goldin BR, Gorbach SL, et al. Soybean phytoestrogen intake and cancer risk. J Nutr 1995;125:757S-770S.
18. Glazier MG, Bowman MA. A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. Arch Intern Med 2001;161: 1161-1172.
19. Keinan-Boker L, Der Schouw YT, Grobbee DE, et al. Dietary phytoestrogens and breast cancer risk. Am J Clin Nutr 2004;79:282-288.
20. Peeters PH, Keinan-Boker L, van der Schouw YT, et al. Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. Breast Cancer Res Treat 2003;77:171-183.
21. Grace PB, Taylor JI, Low YL, et al. Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. Cancer Epidemiol Biomarkers Prev 2004;13:698-708.
22. Piller R, Chang-Claude J, Linseisen J. Plasma enterolactone and genistein and the risk of premenopausal breast cancer. Eur.J.Cancer Prev. 2006;15:225-232.
23. den Tonkelaar I, Keinan-Boker L, Veer PV, et al. Urinary phytoestrogens and postmenopausal breast cancer risk. Cancer Epidemiol Biomarkers Prev 2001;10:223-228.
24. Zeleniuch-Jacquotte A, Adlercreutz H, Shore RE, et al. Circulating enterolactone and risk of breast cancer: a prospective study in New York. Br J Cancer 2004;91:99-105.
25. Kilkkinen A, Virtamo J, Vartiainen E, et al. Serum enterolactone concentration is not associated with breast cancer risk in a nested case-control study. Int J Cancer 2004;108:277-280.
26. Hulten K, Winkvist A, Lenner P, et al. An incident case-referent study on plasma enterolactone and breast cancer risk. Eur J Nutr 2002;41:168-176.
27. Olsen A, Knudsen KE, Thomsen BL, et al. Plasma enterolactone and breast cancer incidence by estrogen receptor status. Cancer Epidemiol Biomarkers Prev 2004;13:2084-2089.
28. Riboli E, Kaaks R. The EPIC Project: rationale and study design. European Prospective Investigation into Cancer and Nutrition. Int J Epidemiol 1997;26 Suppl 1:S6-14.
29. Riboli E, Hunt KJ, Slimani N, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002;5: 1113-1124.
30. Boker LK, Van Noord PA, van der Schouw YT, et al. Prospect-EPIC Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. Eur J Epidemiol 2001;17:1047-1053.

31. Grace PB, Taylor JI, Botting NP, et al. Quantification of isoflavones and lignans in serum using isotope dilution liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2003;17:1350-1357.

32. Song TT, Hendrich S, Murphy PA. Estrogenic activity of glycitein, a soy isoflavone. J.Agric.Food Chem. 1999;47:1607-1610.

33. Kinjo J, Tsuchihashi R, Morito K, et al. Interactions of phytoestrogens with estrogen receptors alpha and beta (III). Estrogenic activities of soy isoflavone aglycones and their metabolites isolated from human urine. Biol.Pharm.Bull. 2004;27:185-188.

34. Matsumura A, Ghosh A, Pope GS, et al. Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. J.Steroid Biochem.Mol.Biol. 2005;94:431-443.

35. Low YL, Taylor JI, Grace PB, et al. Phytoestrogen exposure correlation with plasma estradiol in postmenopausal women in European Prospective Investigation of Cancer and Nutrition-Norfolk may involve diet-gene interactions. Cancer Epidemiol.Biomarkers Prev. 2005;14: 213-220.

36. Piller R, Verla-Tebit E, Wang-Gohrke S, et al. CYP17 genotype modifies the association between lignan supply and premenopausal breast cancer risk in humans. J.Nutr. 2006;136:1596-1603.

37. McCann SE, Moysich KB, Freudenheim JL, et al. The risk of breast cancer associated with dietary lignans differs by CYP17 genotype in women. J.Nutr. 2002;132:3036-3041.

GENERAL DISCUSSION

CHAPTER 6

Absolute or percent breast density

Glandular and stromal tissues of the female breast are radiologically dense and appear light on a mammogram, while the radiologically lucent fat tissue has a dark appearance (1). High breast density is a risk factor for breast cancer development. Women with a large part of their breasts composed of dense tissue, have an estimated 2 to 6 times higher risk of developing breast cancer compared with women with very low breast density (2,3). Most of the studies on breast density used the relative measure of breast density, the percent breast density, which is defined as the dense area on the mammogram divided by the total breast area, multiplied by 100%. However, it is the amount of glandular cells within the breast that is believed to determine breast cancer risk (4,5), because most tumors originate in the glandular ducts (6). The amount of glandular cells is better estimated by the absolute dense area than by percent breast density. To illustrate: a small sized breast and a larger sized breast could have the same percent density, whereas the absolute amount of glandular and stromal tissue is higher in the larger breast. Although the absolute dense area has been regarded as a more relevant measure, only few studies have been published on the effect of absolute breast density on breast cancer risk, compared to over 40 studies on relative measures of breast density and breast cancer (3). Most of the studies presenting both measures of breast density, found similar effects for the dense area and percent density (7-10), but Byrne et al. found the associations of percent density with breast cancer risk to be stronger than the association between the dense area and risk of breast cancer development (11). Overall, the results of these studies thus do not yet give an indication that the relationship is stronger for the absolute measure of breast density. As the relative measure of breast density is strongly affected by the size of the non-dense area, these unexpected results suggest that the non-dense area also relates to breast cancer risk. It is as yet unclear whether and how the amount of fat tissue (non-dense area) surrounding the glandular and stromal tissue has a role in breast cancer development.

Percent density, effects of different components. Is the non-dense area just an innocent bystander?

Because of its strong relationship with breast cancer risk, breast density has often been regarded as an intermediate marker for breast cancer. Many researchers have tried to identify factors that increase or decrease breast density to obtain more insight in the etiology of breast cancer and find explanations for the increased risk through common determinants. These particularly concentrate on factors related to excessive exposure to sex hormones at critical time periods. Parity, and a young age at first child birth have been found to be associated with lower breast density (12-15). BMI has also been shown to be inversely related to percent breast density (12-14), but not to the dense area (16,17).

Breast density decreases gradually with age, but this decrease is stronger during the menopausal transition phase (18,19). Most women using postmenopausal hormone therapy (HT) have higher breast density, but these effects seem to be restricted to women using combined estrogen and progesterone HT, instead of estrogen only treatment (20-24). In the research described in this thesis, we focused on endogenous hormones and growth factors with known mitogenic properties, as determinants of breast density. We hypothesized that these determinants would affect the dense area, and thus percent density, by increasing proliferation of the glandular tissue. We indeed found women with the highest IGF-I levels to show the highest percent density. This effect, however, was largely driven by a smaller non-dense area, whereas the dense area was mostly unaffected. Sex steroids and SHBG did not appear to have a large effect on the dense area or percent density. However, again a number of hormones was significantly related to the non-dense area. Other investigators have reported similar findings. Genes and growth factors, that on the basis of their proliferative and anti apoptotic capacities had been hypothesized to increase mammographic density, appeared to be related to a smaller non-dense area, instead of a larger dense area (25,26). Furthermore, Maskarinec et al. found that the lower percent breast density in women with high levels of insulin-like growth factor-1 binding protein-3 (IGFBP-3), was mainly caused by a larger non-dense area, rather than a smaller dense area. IGFBP-3 decreases the fraction of bioavailable IGF-I and has also been shown to enhance apoptosis in an IGF-I independent way (27).

Explaining the contradiction

These observations lead to the question how high circulating levels of sex steroids and IGF-I, that are known to increase breast cancer risk, at the same time decrease the non-dense area and hardly affect the dense area? To elucidate this apparent contradiction, we will discuss explanations for these findings on the basis of four theorems. These are;

1. *The relationship between IGF-I and sex steroids on the one hand and the non-dense area on the other hand, can biologically be explained and is not likely to be a chance finding.*
2. *Not only the size of the dense area but also the size of the non-dense area directly affects breast cancer risk.*
3. *Effects of IGF-I and sex steroids on the dense area are not detected because of measurement error.*
4. *The effect of IGF-I and sex steroids on breast cancer risk is not mediated by breast density.*

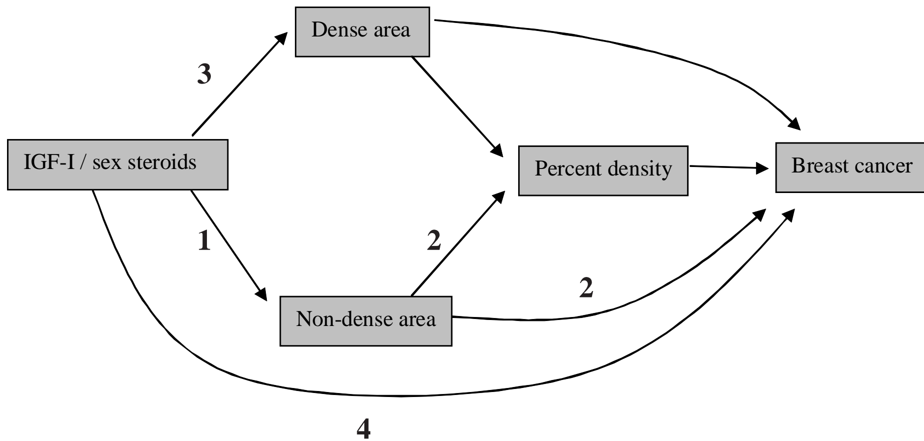

Figure 1. Schematic view of relations between IGF-I, sex steroids, breast measures and breast cancer. Numbers indicate potential pathways, as described above

1. The relationship between IGF-I and sex steroids on the one hand and the non-dense area on the other hand, can biologically be explained and is not likely to be a chance finding.

We did not expect the mitogens IGF-I and sex steroids to affect the non-dense area. A possible explanation may therefore be that IGF-I and sex steroids do not truly influence the non-dense area and that the relations we found are chance findings. Many other studies on the relationship between IGF-I and sex steroids and breast density have been performed, but unfortunately only very few of these presented results on the non-dense area. Maskarinec et al. also found an inverse correlation between IGF-I levels and the non-dense area in a study with premenopausal women (r=-0.10; p=0.13) (23). IGF-I and non-dense area were, however, not related in another study among premenopausal women (28). Postmenopausal women with high IGF-I levels in this study, had an even higher non-dense area, although this relation was not linear (28). To our best knowledge, no studies have been published presenting relations between sex steroids and the non-dense area.

An important question with respect to the likelihood of a chance rather than a true finding is whether or not it is biologically plausible that IGF-I and hormones would decrease the non-dense area or the amount of fat tissue? On the one hand, IGF-I has been described to increase proliferation of adipocytes, possibly increasing the non-dense area (29). On the other hand, IGF-I strongly correlates with growth hormone (GH), which

provides the key stimulus for IGF-I synthesis in the liver and many other tissues. GH has strong lipolytic actions, and will tend to reduce adipose tissue mass (30-34). Furthermore, a study by Mulhall et al. showed two polymorphisms in the GH gene to be associated with increased GH levels and decreased non-dense area (25). Therefore, the lower non-dense area in women with high IGF-I levels may only be a reflection of the lipolytic actions of GH.

The relationship between estrogens and fat tissue is complex. An important indication that estrogens are involved in fat metabolism lies in the fact that women gain weight after menopause when the ovaries stop producing estrogens. Moreover, one of the effects of postmenopausal hormone therapy (HT) is a decrease in body weight (35,36). In vitro studies have shown that estrogens inhibit the activity of lipoprotein lipase (LPL) and hence promote lipolysis, resulting in lower fat storage in the adipocyte, ultimately decreasing total fat mass (35). This finding seems to contradict with the fact that after menopause, obese women have higher circulating estrogen levels than non-obese women. In postmenopausal women, however, estrogens are no longer produced in the ovaries and circulating estrogen levels after menopause, reflect the conversion rate of androgens to estrogens by aromatase, that takes place in the adipocytes (37). Estrogens thus exert a negative feed back on their own production. Estradiol has also been shown to decrease fat tissue in an indirect way, by regulating energy intake via effects on satiety and nutritional intake (hypothalamus) and energy metabolism (liver) (35).

The effect of androgens on non-dense tissue could be indirect and explained by the fact that androgen levels and estrogen levels are strongly correlated. Testosterone has also been shown to have an estrogen independent effect on adipocytes by lowering catecholamine-induced lipolysis and thus increasing fat storage by the adipocyte (38). This effect differs, however, between different sites of adipose depots.
Taken together, it is not likely that associations with the non-dense area resulted from chance, since associations with the non-dense area have been observed before, and both IGF-I and sex steroids are involved in several biological mechanisms concerning adipocyte metabolism.

2. Not only the size of the dense area but also the size of the non-dense area directly affects breast cancer risk.

Percent density is an established breast cancer risk factor. It is calculated by dividing the dense area by the sum of the dense and the non-dense area. As mentioned earlier, little is known about the separate effects of its components. The dense area is often considered most relevant, but in theory factors that decrease the non-dense area and by that, increase the percent density could also increase breast cancer risk. There is, however, no biologically plausible explanation for why a smaller non-dense area would increase breast

cancer risk. The non-dense area consists of radiologically lucent fat tissue (39). Fat tissue is an important source of estrogens, particularly in postmenopausal women, because of the conversion of androgens to estrogens in the adipocytes (37). Circulating levels of estrogens have been shown to increase breast cancer risk (40-42), and locally produced estrogens have been shown to act predominantly at the local tissue level in a paracrine way, affecting glandular tissue (37). According to this reasoning, a smaller non-dense area is much more likely to decrease than to increase the risk of breast cancer development. This is also supported by a study of our research group that focuses on the separate as well as the combined effects of the dense and the non-dense tissue in relation to breast cancer risk. In this case-cohort study with 309 breast cancer cases and a subcohort of 599 women for comparison, we found that a large non-dense area was associated with significantly increased breast cancer risk. Breast cancer odds ratios and 95% confidence intervals for tertiles of non-dense area were 1.23 (0.84–1.81) and 2.23 (1.41–3.53) for the middle and highest tertile respectively, taking the lowest tertile as reference (Haars et al., submitted).

In conclusion, a decrease in non-dense area increases the percent density which is directly related to breast cancer risk. However, we do not have a biologically plausible explanation why a smaller non-dense area would directly increase breast cancer risk.

3. Effects of IGF-I and sex steroids on the dense area are not detected because of measurement error.

An explanation for not finding relationships between IGF-I and sex steroids on the one hand and the dense breast area on the other hand, may relate to the techniques used for measuring breast tissues. Using a two dimensional projection to measure three dimensional tissues gives a limitation. Both the total breast area and the dense breast area on a mammogram are influenced by the level of compression of the breast and the amount of radiation used during mammography. We made use of mammograms from a breast cancer screening cohort and neither compression nor the amount of radiation used was registered during routine mammography. Inability to adjust for these variables may lower the precision of measurements and hence decreases power to detect associations with the breast measures. Although previous studies did show effects of other risk factors on the dense area when measured with two dimensional mammography (22,43-46), it is possible that small effects on the dense area cannot be detected using this technique. Future research may make use of digitally recorded mammograms with which compression and radiation intensity are recorded automatically. Currently, techniques are being developed for estimating the three dimensional volume of the dense tissue on the basis of these features (47). This will increase the precision of the breast tissue measurements.

4. The effect of IGF-I and sex steroids on breast cancer risk is not mediated by breast density.

A last explanation for our results would be that both IGF-I and sex steroids increase breast cancer risk, but not through an increase in breast density. We selected growth factors and hormones with known proliferative properties, but did not find the expected increase in glandular tissue, which in turn is associated with increased breast cancer risk. Possibly IGF-I and sex steroids do not materially affect the normal proliferation of dense tissue, but rather affect development of cancerous cells in a later stage of tumor genesis. Another explanation lies in the composition of dense tissue. Only an estimated 20% of total dense tissue is composed of glandular tissue, the rest is stromal tissue. If high levels of growth factors and hormones only affect the glandular tissue, effects on the total dense area may be small and hard to detect, while at the same time the risk of breast cancer development is increased. Stromal tissue has also been shown to affect breast cancer development (48-51) and it has been hypothesized that breast density might relate to risk through an interplay between the stromal and epithelial structures (52,53). Hence, future research should not only focus on factors that affect glandular tissues, but also focus on factors that affect stromal tissues.

In conclusion, as percent density is the ratio between the dense area and the non-dense area (as part of the denominator), any association between percent density and breast cancer risk may thus reflect associations of either the dense area or the non-dense area or both, with breast cancer risk. There are important biological pathways via which IGF-I and sex steroids may decrease adipose tissues and hence the association between growth factors and hormones and percent breast density can, at least partly, be explained by the influence of the non-dense area on percent breast density. The association between an increase in dense area and increased breast cancer risk can be explained by the higher absolute amount of glandular cells, which is suggested to predict breast cancer risk. The association between a decrease in non-dense area and increased breast cancer risk is hard to explain, and results of the only study on non-dense area and breast cancer risk so far, indeed contradict such association (Haars et al. in preparation).

Future research will benefit from techniques that estimate dense area and non-dense area with more precision, making it possible to detect smaller effects and possibly disclose separate risk relations with greater precision. Application of these techniques may help to elucidate the apparent contradiction between the effect of estrogens and IGF-I on the breast tissue and their effect on breast cancer risk. Furthermore, factors that affect stromal tissues may better explain the relation between breast density and the risk of breast cancer than factors that are known to stimulate proliferation of the glandular tissue, such as estrogens and IGF-I.

References

1. Boyd NF, Byng JW, Jong RA, et al. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study. J Natl Cancer Inst 1995;87:670-675.
2. Boyd NF, Rommens JM, Vogt K, et al. Mammographic breast density as an intermediate phenotype for breast cancer. Lancet Oncol. 2005;6:798-808.
3. McCormack VA, dos SS, I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol.Biomarkers Prev. 2006;15: 1159-1169.
4. Trichopoulos D, Lipman RD. Mammary gland mass and breast cancer risk. Epidemiology. 1992;3:523-526.
5. Albanes D, Winick M. Are cell number and cell proliferation risk factors for cancer? J.Natl.Cancer Inst. 1988;20;80:772-774.
6. Pike MC, Spicer DV, Dahmoush L, et al. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol.Rev. 1993;15:17-35.
7. Maskarinec G, Meng L. A case-control study of mammographic densities in Hawaii. Breast Cancer Res Treat 2000;63:153-161.
8. Ursin G, Ma H, Wu AH, et al. Mammographic density and breast cancer in three ethnic groups. Cancer Epidemiol.Biomarkers Prev. 2003;12:332-338.
9. Maskarinec G, Pagano I, Lurie G, et al. Mammographic density and breast cancer risk: the multiethnic cohort study. Am.J.Epidemiol. 2005;162:743-752.
10. Maskarinec G, Pagano I, Chen Z, et al. Ethnic and geographic differences in mammographic density and their association with breast cancer incidence. Breast Cancer Res.Treat. 2006;
11. Byrne C, Schairer C, Wolfe J, et al. Mammographic features and breast cancer risk: effects with time, age, and menopause status. J Natl Cancer Inst 1995;87:1622-1629.
12. Vachon CM, Kuni CC, Anderson K, et al. Association of mammographically defined percent breast density with epidemiologic risk factors for breast cancer (United States). Cancer Causes Control. 2000;11:653-662.
13. Jeffreys M, Warren R, Gunnell D, et al. Life course breast cancer risk factors and adult breast density (United Kingdom). Cancer Causes Control 2004;15:947-955.
14. Titus-Ernstoff L, Tosteson AN, Kasales C, et al. Breast cancer risk factors in relation to breast density (United States). Cancer Causes Control 2006;17:1281-1290.
15. de WF, Rombach JJ, Collette HJ, et al. Breast cancer risk associated with reproductive factors and breast parenchymal patterns. J.Natl.Cancer Inst. 1984;72:1277-1282.
16. Haars G, Van Noord PA, Van Gils CH, et al. Measurements of breast density: no ratio for a ratio. Cancer Epidemiol.Biomarkers Prev. 2005;14:2634-2640.

17. Verheus M, Peeters PHM, Kaaks R, et al. Premenopausal IGF-I serum levels and changes in breast density over menopause. Cancer Epidemiol.Biomarkers Prev. 2007;

18. Goodman-Gruen D, Barrett-Connor E. Epidemiology of insulin-like growth factor-I in elderly men and women. The Rancho Bernardo Study. Am.J.Epidemiol. 1997;145:970-976.

19. Boyd N, Martin L, Stone J, et al. A longitudinal study of the effects of menopause on mammographic features. Cancer Epidemiol Biomarkers Prev 2002;11:1048-1053.

20. McTiernan A, Martin CF, Peck JD, et al. Estrogen-plus-progestin use and mammographic density in postmenopausal women: women's health initiative randomized trial. J.Natl.Cancer Inst. 2005;97:1366-1376.

21. Greendale GA, Reboussin BA, Slone S, et al. Postmenopausal hormone therapy and change in mammographic density. J.Natl.Cancer Inst. 2003;95:30-37.

22. van Duijnhoven FJ, Peeters PH, Warren RM, et al. Influence of estrogen receptor alpha and progesterone receptor polymorphisms on the effects of hormone therapy on mammographic density. Cancer Epidemiol.Biomarkers Prev. 2006;15:462-467.

23. Chlebowski RT, Hendrix SL, Langer RD, et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. JAMA 2003;289:3243-3253.

24. Boyd NF, Martin LJ, Li Q, et al. Mammographic density as a surrogate marker for the effects of hormone therapy on risk of breast cancer. Cancer Epidemiol.Biomarkers Prev. 2006;15:961-966.

25. Mulhall C, Hegele RA, Cao H, et al. Pituitary growth hormone and growth hormone-releasing hormone receptor genes and associations with mammographic measures and serum growth hormone. Cancer Epidemiol.Biomarkers Prev. 2005;14:2648-2654.

26. Takata Y, Maskarinec G, Le Marchand L. Breast Density and Polymorphisms in Genes Coding for CYP1A2 and COMT: The Multiethnic Cohort. 2007;

27. Maskarinec G, Williams AE, Kaaks R. A cross-sectional investigation of breast density and insulin-like growth factor I. Int J Cancer 2003;107:991-996.

28. dos Santos Silva I, Johnson N, De Stavola B, et al. The insulin-like growth factor system and mammographic features in premenopausal and postmenopausal women. Cancer Epidemiol.Biomarkers Prev. 2006;15:449-455.

29. Siddals KW, Westwood M, Gibson JM, et al. IGF-binding protein-1 inhibits IGF effects on adipocyte function: implications for insulin-like actions at the adipocyte. J.Endocrinol. 2002;174:289-297.

30. Djurhuus CB, Gravholt CH, Nielsen S, et al. Additive effects of cortisol and growth hormone on regional and systemic lipolysis in humans. Am.J.Physiol Endocrinol.Metab. 2004;286:E488-E494.

31. Louveau I, Gondret F. Regulation of development and metabolism of adipose tissue by growth hormone and the insulin-like growth factor system. Domest.Anim Endocrinol. 2004;27:241-255.

32. Flint DJ, Binart N, Kopchick J, et al. Effects of growth hormone and prolactin on adipose tissue development and function. Pituitary. 2003;6:97-102.

33. Hansen TK, Gravholt CH, ORskov H, et al. Dose dependency of the pharmacokinetics and acute lipolytic actions of growth hormone. J.Clin.Endocrinol.Metab. 2002;87:4691-4698.

34. Moller N, Gjedsted J, Gormsen L, et al. Effects of growth hormone on lipid metabolism in humans. Growth Horm.IGF.Res. 2003;13 Suppl A:S18-21.:S18-S21.

35. Cooke PS, Naaz A. Role of estrogens in adipocyte development and function. Exp.Biol.Med.(Maywood.) 2004;229:1127-1135.

36. Chmouliovsky L, Habicht F, James RW, et al. Beneficial effect of hormone replacement therapy on weight loss in obese menopausal women. Maturitas 1999;32:147-153.

37. Simpson ER. Sources of estrogen and their importance. J.Steroid Biochem.Mol.Biol. 2003;86:225-230.

38. Dicker A, Ryden M, Naslund E, et al. Effect of testosterone on lipolysis in human pre-adipocytes from different fat depots. Diabetologia 2004;47:420-428.

39. Byng JW, Boyd NF, Fishell E, et al. The quantitative analysis of mammographic densities. Phys Med Biol 1994;39:1629-1638.

40. Kaaks R, Berrino F, Key T, et al. Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). J Natl Cancer Inst 2005;97:755-765.

41. Kaaks R, Rinaldi S, Key TJ, et al. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European prospective investigation into cancer and nutrition. Endocr.Relat Cancer. 2005;12:1071-1082.

42. Key T, Appleby P, Barnes I, et al. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606-616.

43. Maskarinec G, Meng L. An investigation of soy intake and mammographic characteristics in Hawaii. Breast Cancer Res. 2001;3:134-141.

44. Heng D, Gao F, Jong R, et al. Risk factors for breast cancer associated with mammographic features in Singaporean chinese women. Cancer Epidemiol.Biomarkers Prev. 2004;13: 1751-1758.

45. Boyd NF, Stone J, Martin LJ, et al. The association of breast mitogens with mammographic densities. Br J Cancer 2002;87:876-882.

46. van Duijnhoven FJ, Bezemer ID, Peeters PH, et al. Polymorphisms in the estrogen receptor alpha gene and mammographic density. Cancer Epidemiol.Biomarkers Prev. 2005;14: 2655-2660.

47. van Engeland S, Snoeren PR, Huisman H, et al. Volumetric breast density estimation from full-field digital mammograms. IEEE Trans.Med.Imaging. 2006;25:273-282.

48. Kim JB, Stein R, O'Hare MJ. Tumour-stromal interactions in breast cancer: the role of stroma in tumourigenesis. Tumour.Biol. 2005;26:173-185.

49. Haslam SZ, Woodward TL. Host microenvironment in breast cancer development: epithelial-cell-stromal-cell interactions and steroid hormone action in normal and cancerous mammary gland. Breast Cancer Res. 2003;5:208-215.

50. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. Science 2002;296:1046-1049.

51. Ellis MJ, Singer C, Hornby A, et al. Insulin-like growth factor mediated stromal-epithelial interactions in human breast cancer. Breast Cancer Res.Treat. 1994;31:249-261.

52. Warren R, Lakhani SR. Can the stroma provide the clue to the cellular basis for mammographic density? Breast Cancer Res. 2003;5:225-227.

53. Haslam SZ, Woodward TL. Host microenvironment in breast cancer development: epithelial-cell-stromal-cell interactions and steroid hormone action in normal and cancerous mammary gland. Breast Cancer Res. 2003;5:208-215.

Summary

Samenvatting

Dankwoord

Curriculum Vitae

Chapter 7

Summary

Worldwide, breast cancer is the most common malignancy among females. Glandular tissue as well as stromal tissue of the breast are radiologically dense and appear light on a mammogram, whereas the radiologically lucent fatty tissue appears black. Mammographic density, calculated as the area of dense tissue on a mammogram or as the proportion of the total breast area that is composed of dense tissue (percent density), is a strong breast cancer risk factor. Exposure to high levels of growth factors and hormones with known mitogenic properties could increase breast cancer risk, through an increase in the amount of dense tissue of the breast. In this thesis we aimed to investigate the relationships between growth factors (insulin-like growth factor I (IGF-I)) and hormones, both endogenous (insulin and sex steroids) and exogenous (phytoestrogens), on the one hand, and breast density and breast cancer on the other hand.

In cross sectional studies, high circulating levels of IGF-I have been associated with high breast density, but only before menopause. During menopause, breast density normally decreases, but not to the same extent in all women. The women with persistently high breast density have been hypothesized to have the highest risk of breast cancer. As circulating levels of IGF-I are known to decrease with age, their effect on the involution of the breast during menopause and consequently on postmenopausal breast density, may have already been determined before menopause, when circulating IGF-I levels are still high. We investigated the effects of premenopausal circulating levels of IGF-I (**Chapter 2.1**) and genetic variation in the *IGF-1* gene (**Chapter 2.2**) on menopausal changes in breast density. Women with high premenopausal IGF-I levels showed a slightly smaller decrease in dense area over menopause (-12.2 cm^2 in the highest versus -12.9 cm^2 in the lowest quartile, p-trend=0.58) and, at the same time, a smaller increase in the non-dense (fat) area (p-trend=0.09). Due to these changes over menopause, high premenopausal IGF-I serum levels were associated with somewhat higher dense area (p-trend=0.66), with lower non-dense area (p-trend=0.05) and consequently with higher percent breast density (p-trend=0.02) after menopause. We concluded that higher premenopausal IGF-I levels affect percent breast density after menopause and that this effect is largely explained by the smaller increase of the non-dense area during the menopausal transition phase, in women with high premenopausal circulating levels of IGF-I. For the genetic analyses, women were genotyped for 15 single nucleotide polymorphisms (SNP), tagging 3 haplotype blocks within the *IGF-1* gene. Several SNPs tagging haplotype block 3 were significantly associated with higher IGF-I levels. These same SNPs were related, but to a lesser extent and statistically non-significantly, with a smaller decrease of percent density during the menopausal transition phase. The effects were most obvious for two SNPs, of which one was previously reported to be related to

higher IGF-I levels and increased breast cancer risk (rs6220). We concluded that common genetic variation in the *IGF-I* gene is related with IGF-I circulating levels and possibly also with breast density.

Epidemiologic studies have shown an increase in breast cancer risk in postmenopausal women with high circulating levels of sex steroids. Using data of 969 women, participating in the Prospect-EPIC cohort, we investigated whether this increased risk, is mediated by an increase in breast density (**Chapter 3**). Estradiol had a U-shaped relation with both percent breast density and the dense breast area, but was inversely associated with quartiles of non-dense area (p-trend=0.01). Androgens were not related to absolute or percent breast density, but did show inverse relationships with the non-dense area (p-trend for DHEAS, androstenedione and testosterone was 0.03, < 0.01 and 0.23 respectively). We concluded that the results of our study do not support the hypothesis that sex steroids increase postmenopausal breast cancer risk via an increase in breast density. The unexpected finding that circulating levels of sex steroids were more strongly associated with the non-dense area than with the dense area is intriguing.

In **Chapter 4**, we focus on hyperinsulinemia and describe the relationship between circulating levels of C-peptide - a marker for pancreatic insulin secretion - and breast cancer risk. Within the large EPIC cohort, prediagnostic C-peptide serum levels were analyzed for 1141 women who were diagnosed with breast cancer on average 2.8 years after baseline, and for 2204 matched control subjects. Elevated serum C-peptide levels were associated with a non significantly reduced risk of breast cancer diagnosed up to the age of 50 years (OR=0.70, (95% CI: 0.39-1.24), p-trend=0.05). By contrast, higher levels of C-peptide were associated with an increased risk of breast cancer, among women above 60 years of age, however only among those women who had provided their blood sample under non-fasting conditions (OR=2.03, (95% CI: 1.20-3.43), p-trend=0.01). We concluded that 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 higher breast cancer risk. Phytoestrogens are plant compounds with hormone-like activity. They can compete with endogenous estrogens for the estrogen receptor and in this way inhibit binding of the estrogenic more potent endogenous estrogens, potentially decreasing breast cancer risk. So far, only few studies on blood levels of phytoestrogens have been performed, most of which were relatively small. Results of these studies were inconclusive. In **Chapter 5** we present results of the largest prospective study on circulating levels of phytoestrogens and breast cancer risk. Plasma levels of seven phytoestrogens - five isoflavones (daidzein, genistein, glycitein, O-desmethylangolensin and equol) and two lignans (enterodiol and enterolactone) - were analyzed in 383 women who were diagnosed with breast cancer during an average follow-up time of 6.5 years and 383 control subjects

of the Prospect-EPIC cohort. For genistein, the risk estimate for the highest versus the lowest tertile was 0.68 (95% CI: 0.47-0.98). Similar protective effects, although not statistically significant, were seen for the other isoflavones. The results suggest that high circulating levels of isoflavones may protect against breast cancer, at least in the Dutch population with low overall circulating isoflavone levels, as is common in Western countries. Lignan levels did not appear to be related to breast cancer risk.

In this thesis we investigated growth factors and hormones with known mitogenic properties and hypothesized that these determinants would affect the dense area, and thus percent density, by increasing proliferation of the glandular tissue. To our surprise most associations between these determinants and percent breast density mainly existed because of an association with the non-dense area, whereas the dense area was largely unaffected. In **Chapter 6**, we discuss possible explanations for these findings. First, biological pathways are described that explain the relationship between IGF-I and sex steroids on the one hand, and the non-dense area on the other hand. These pathways make it less likely that the relationships between IGF-I and sex steroids and the non-dense area that we found are in fact change findings. Secondly, as percent density is the ratio between the dense area and the non-dense area (as part of the denominator), any association between percent density and breast cancer risk may thus reflect associations of either dense area or non-dense area or both, with breast cancer risk. The association between a decrease in non-dense area and increased breast cancer risk is, however, hard to explain, and results of the only study on non-dense area and breast cancer risk so far, indeed contradict such association. Thirdly, effects of IGF-I and sex steroids on the dense area may be too subtle to be detected. The advantage of using new techniques that are able to measure breast density with more precision are discussed. Finely, the lack in associations between IGF-I and sex steroids on the one hand, and the dense area on the other hand, may be explained by the fact that the effect of these mitogens on breast cancer risk is not mediated by breast density.

Summary

Samenvatting

Dankwoord

Curriculum Vitae

Chapter 7

Samenvatting

Borstkanker is wereldwijd de meest voorkomende vorm van kanker bij vrouwen. Klier- en bindweefsel in de borst zijn radiologisch ‘dens’ ofwel: deze weefsels hebben een hoge radiologische dichtheidsgraad. Deze dense weefsels zijn wit op een mammogram (röntgenfoto van de borst) in tegenstelling tot het radiologisch niet-dense vetweefsel, dat zwart is op een mammogram. Mammografische densiteit, wordt berekend als de absolute oppervlakte van het dense weefsel op een mammogram (hoeveelheid dens weefsel) of als de proportie van de totale oppervlakte van de borst (percentage densiteit) en is een sterke risicofactor voor borstkanker. Blootstelling aan hoge bloedspiegels van groeifactoren en hormonen die de celdeling verhogen, zou het risico op borstkanker kunnen verhogen via een verhoging van de mammografische densiteit.

In dit proefschrift hebben we de relatie onderzocht tussen groeifactoren (insulin-like growth factor I (IGF-I)) en hormonen (insuline, geslachtshormonen (lichaamseigen) en fyto-oestrogenen (uit de voeding)) enerzijds, en mammografische densiteit en borstkanker anderzijds.

Dwarsdoorsnede studies hebben aangetoond dat vrouwen met hoge IGF-I concentraties in hun bloed een hoge mammografische densiteit hebben. Deze associatie is echter alleen gevonden bij vrouwen vóór de menopauze (overgang). Tijdens de overgang neemt de hoeveelheid klier- en bindweefsel in de borst normaal gesproken af. De mate van afname verschilt echter per vrouw. Er wordt gedacht dat vrouwen die ná de overgang nog steeds een hoge mammografische densiteit hebben, ook het hoogste risico op borstkanker hebben. De IGF-I concentratie in het bloed neemt af met het ouder worden. Mogelijk wordt de afname van de borstdensiteit tijdens de menopauze -en daardoor de postmenopauzale borstdensiteit – beïnvloed door IGF-I spiegels vóór de overgang, wanneer de IGF-I concentratie nog hoog is. We hebben het effect onderzocht van de hoogte van de premenopauzale IGF-I bloedspiegel (**Hoofdstuk 2.1**) en van genetische variatie in het IGF-1 gen (**Hoofdstuk 2.2**) op de veranderingen in de borstdensiteit tijdens de overgang. De afname van de absolute hoeveelheid dens weefsel tijdens de overgang was iets kleiner bij vrouwen met hoge premenopauzale IGF-I spiegels (-12,2 cm^2 in het hoogste versus -12,9 cm^2 in het laagste kwartiel van IGF-I spiegels, p-trend=0,58). Tegelijkertijd was de toename van de absolute hoeveelheid niet-dens (vet)weefsel minder groot bij de vrouwen met hoge IGF-I spiegels (p-trend=0,09). Als gevolg van deze veranderingen tijdens de overgang, waren hoge IGF-I bloedspiegels vóór de overgang geassocieerd met een iets hogere absolute hoeveelheid dens weefsel (p-trend=0,66), met een lagere hoeveelheid niet-dens weefsel (p-trend=0,05) en daardoor met een hoger percentage borstdensiteit (p-trend=0,02) ná de overgang. We concludeerden dat hogere

premenopauzale IGF-I spiegels, het percentage borstdensiteit ná de overgang beïnvloeden en dat dit effect voornamelijk verklaard wordt doordat bij deze vrouwen een minder grote toename in het vetweefsel is te zien tijdens de overgang.

Alle erfelijke informatie van de mens ligt opgeslagen in genen. Het IGF-I gen bevat de genetische code om de groeifactor IGF-I te maken. Zoals de meeste genen, is het IGF-I gen polymorf, ofwel: er bestaan verschillende varianten van het gen waardoor de eigenschappen (hoeveelheid en precieze werking) van het IGF-I kunnen verschillen van mens tot mens. Het IGF-I gen is op meerdere punten polymorf. Die plekken worden polymorfismen genoemd en kunnen worden bepaald met behulp van een genetische analyse. Voor de genetische analyse van het IGF-I gen werden vrouwen onderzocht op 15 zogenaamde "single nucleotide polymorphisms" (SNP). Een aantal van deze SNPs was statistisch significant geassocieerd met hogere IGF-I spiegels. Dezelfde SNPs waren geassocieerd - zij het minder sterk en niet statistisch significant - met een kleinere afname van het percentage borstdensiteit tijdens de overgang. De associaties waren het meest uitgesproken voor twee SNPs, waarvan er één in eerder onderzoek ook aan hogere IGF-I spiegels was gerelateerd, alsmede aan een hoger risico op borstkanker. We concludeerden dat genetische variatie in het IGF-I gen gerelateerd is aan IGF-I bloedspiegels en mogelijk ook aan borstdensiteit.

Epidemiologische studies hebben aangetoond dat bij postmenopauzale vrouwen, hoge concentraties van geslachtshormonen in het bloed een verhoogde kans op borstkanker geven. Gebruikmakend van gegevens van 969 vrouwen uit het Prospect-EPIC cohort, hebben we onderzocht of dit verhoogde risico via een toename in borstdensiteit loopt (**Hoofdstuk 3**). Oestradiol had zowel met het percentage borstdensiteit als met de absolute densiteit een U-vormige relatie. Tegelijkertijd hadden vrouwen met hoge oestradiolspiegels een kleinere hoeveelheid niet-dens (vet)weefsel (p-trend=0,01). Androgeenspiegels hingen niet samen met de absolute borstdensiteit of het percentage borstdensiteit. Wel hadden vrouwen met hoge androgeenspiegels en kleinere hoeveelheid niet-dens weefsel (p-trend voor DHEAS, androstenedione en testosteron was respectievelijk 0,03, <0,01 en 0,23). We concludeerden dat de hypothese dat geslachtshormonen het risico op borstkanker na de overgang vergroten door een verhoging van de borstdensiteit niet ondersteund wordt door onze resultaten. De bevinding dat de bloedspiegels van geslachtshormonen sterker geassocieerd waren met de hoeveelheid niet-dens weefsel dan met de hoeveelheid dens weefsel, is onverwacht en niet duidelijk verklaarbaar.

In **Hoofdstuk 4** focussen we op hyperinsulinemia en beschrijven we de relatie tussen bloedspiegels van C-peptide - een marker voor insulinesecretie door de alvleesklier - en het risico op borstkanker. Binnen het grootschalige EPIC cohort werden van 1141 vrouwen,

bij wie gemiddeld 2,8 jaar na de start van de studie borstkanker gediagnosticeerd werd, en 2204 gematchte controles prediagnostische C-peptide serumconcentraties bepaald. Verhoogde C-peptide spiegels waren geassocieerd met een lichte afname van het risico op borstkanker voor het 51ste levensjaar (OR=0,70, (95% CI 0,39-1,24), p-trend=0,05). Bij vrouwen met een leeftijd boven de 60 jaar daarentegen, waren hogere C-peptide spiegels geassocieerd met een hoger risico op borstkanker, echter alleen bij vrouwen die niet-nuchter waren tijdens de bloedafname. We concludeerden dat onze resultaten de hypothese dat chronische hyperinsulinemia, het risico op borstkanker vergroot, niet bevestigen. Desalniettemin zou hyperinsulinemia bij oudere vrouwen na de overgang, het risico op borstkanker kunnen verhogen.

Fyto-oestrogenen zijn plantaardige bestanddelen met hormoonachtige werking. Tijdens het binden aan de oestrogeenreceptor, zijn ze in competitie met de endogene oestrogenen en remmen op deze wijze de binding van het qua oestrogene werking meer potente endogene oestrogeen. Hierdoor zouden ze het risico op borstkanker mogelijk verminderen. Tot op heden zijn er slechts enkele studies naar de concentraties van fyto-oestrogenen in het bloed gepubliceerd, waarvan de meeste relatief klein waren. De resultaten van deze studies waren niet eenduidig. In **Hoofdstuk 5** presenteren we de resultaten van de grootste prospectieve studie naar concentraties van fyto-oestrogenen in het bloed in relatie tot het risico op borstkanker. Van 383 vrouwen die gedurende een gemiddelde follow-uptijd van 6,5 jaar met borstkanker gediagnosticeerd waren en van 383 controlepersonen van het Prospect-EPIC cohort, werden plasmaniveaus van zeven fyto-oestrogenen - vijf isoflavonen (daidzeïne, genisteïne, glyciteïne, O-desmethylangolensin en equol) en twee lignanen (enterodiol en enterolactone) - geanalyseerd. Het borstkankerrisico voor vrouwen uit het hoogste versus vrouwen uit het laagste tertiel van genisteïne plasmaniveaus was 0,68 (95% CI 0,47-0,98). Vergelijkbare beschermende effecten, hoewel statistisch niet significant, werden gevonden voor de andere isoflavonen. De resultaten suggereren dat hoge concentraties van isoflavonen in het bloed tegen borstkanker zouden kunnen beschermen in Nederlandse vrouwen die in het algemeen lage bloedspiegels van isoflavonen hebben, net als vrouwen in andere Westerse landen. Lignanen-spiegels leken de kans op borstkanker niet te beïnvloeden.

In dit proefschrift onderzochten we groeifactoren en hormonen die de celdeling bevorderen met als hypothese dat deze determinanten de hoeveelheid dens weefsel, en dus het percentage densiteit van de borst zouden beïnvloeden door verhoogde proliferatie van het klierweefsel. Tot onze verbazing berustten de meeste associaties tussen deze determinanten en het percentage borstdensiteit voornamelijk op een associatie met de absolute hoeveelheid niet-dens weefsel, terwijl de absolute hoeveelheid dens weefsel

slechts in zeer kleine mate bijdroeg aan de associatie met het percentage densiteit. In **Hoofdstuk 6** bespreken we mogelijke verklaringen voor deze bevindingen. Allereerst worden biologische mechanismen beschreven die de relatie tussen IGF-I en geslachtshormonen enerzijds en de hoeveelheid niet-dens weefsel anderzijds verklaren. Het bestaan van dit soort mechanismen maakt het minder aannemelijk dat de gevonden relaties tussen IGF-I en geslachtshormonen en de hoeveelheid niet-dens weefsel, kansbevindingen zijn. Ten tweede, aangezien het percentage densiteit wordt bepaald door de verhouding tussen de absolute hoeveelheid dens weefsel en de absolute hoeveelheid niet-dens weefsel, kan elke associatie tussen percentage densiteit en het risico op borstkanker worden verklaard door ofwel de hoeveelheid dens weefsel ofwel de hoeveelheid niet-dens weefsel of beide. Dat een afname van de hoeveelheid niet-dens (vet)weefsel de kans op borstkanker zou vergroten is echter moeilijk te verklaren. De resultaten van de enige studie naar de hoeveelheid niet-dens weefsel en het risico op borstkanker tot dusver, wijzen er juist op dat een grotere hoeveelheid vetweefsel de kans op borstkanker verhoogt. Ten derde, effecten van IGF-I en geslachtshormonen op de hoeveelheid dens weefsel zouden te subtiel kunnen zijn om gedetecteerd te worden. De voordelen van het gebruik van nieuwe technieken die de borstdensiteit met meer precisie meten worden besproken. Tot slot zou de afwezigheid van het verband tussen IGF-I en geslachtshormonen enerzijds en de hoeveelheid dens weefsel anderzijds, verklaard kunnen worden door het feit dat de effecten van deze mitogenen op het risico op borstkanker niet via een effect op de borstdensiteit lopen, maar via een ander mechanisme.

SUMMARY

SAMENVATTING

DANKWOORD

CURRICULUM VITAE

CHAPTER 7

Dankwoord

Het lijkt het intrappen van een open deur, maar zonder data zou het onderzoek dat in dit proefschrift beschreven staat nooit gedaan kunnen zijn. Een eerste woord van dank gaat dan ook uit naar al de vrouwen die bereid zijn geweest om in het kader van het Prospect-EPIC onderzoek vragenlijsten in te vullen, een bloedmonster af te staan en hun mammogrammen beschikbaar te stellen ten behoeve van onderzoek naar het ontstaan van kanker.

I would like to thank all women participating in the EPIC study for their willingness to fill out questionnaires and to donate blood samples. Without their affords we would not have been able to perform the research described in this thesis.

Een aantal mensen wil ik graag persoonlijk bedanken.

Beste Carla, ik geloof dat je één week lang geen tijd voor me had, toen je een aanvraag moest indienen. Verder kon ik altijd een beroep op je doen. Je hebt een erg prettige manier van begeleiden. Je op- en aanmerkingen in mijn manuscripten dreven me soms tot wanhoop, maar zijn altijd het bewijs geweest dat je al mijn werk zeer zorgvuldig bekeek en de stukken werden er altijd beter van. Ik heb ontzettend veel van je geleerd de afgelopen drie en een half jaar en ik heb veel waardering voor je intelligente benadering van problemen en je analyserend vermogen. Ik hoop dan ook dat onze laatste paper samen nog niet geschreven is.

Beste Petra, ik heb letterlijk en figuurlijk een gat in de lucht gesprongen toen je mij op een vrijdagavond belde met het goede nieuws dat ik op het Julius mocht komen werken. Bedankt voor je kritische commentaar en voor het vertrouwen, ook als mijn stukken wat langer op zich lieten wachten. Onze eerste coproductie is uiteindelijk toch aardig geslaagd! Je enthousiasme voor ons vak is aanstekelijk. Tot slot, dank voor de gezellige etentjes in Utrecht en Lyon.

Beste Rick, ik vind het als globetrotter een mooi idee dat jij mijn discussie van commentaar hebt voorzien op 10 km boven de aarde op weg van Kuala Lumpur naar New York (althans zo hadden Giene en ik dat berekend). Ik vond het inspirerend om te zien hoe je naast het runnen van onze afdeling ook altijd wist waar ik mee bezig was. Dank voor je sturing en je scherpe aanwijzingen.

Beste Rudolf, ik zou je willen bedanken voor de mogelijkheid die je me bood om een jaar onder jouw supervisie op het IARC in Lyon te komen werken. Zeker gezien het onderwerp van mijn proefschrift was het zeer leerzaam om een jaartje met een wandelende encyclopedie over hormonen en kanker te kunnen werken. Je gedrevenheid als onderzoeker heb ik altijd inspirerend gevonden.

Ik ben veel dank verschuldigd aan alle mensen die geholpen hebben bij het verzamelen, scannen en lezen van de mammogrammen. Als eerste zou ik Joke Metselaar willen bedanken. Joke, ik ken niemand die zo assertief en kordaat is als jij. Het was erg prettig zoals je het verzamelen en terugzetten coördineerde. Je hebt me veel werk uit handen genomen. Annemieke Bartels en Christien Grasmayer, bedankt voor de medewerking van de kant van Preventicon bij de dataverzameling. David Beijerinck, bedankt voor de uitleg bij het beoordelen van mammogrammen.

Het Almereteam, Jens en Steven, dank voor alle hulp bij het ophalen en terugzetten van de mammogrammen bij Hulshof en natuurlijk ook voor de gezellige treinritten van en naar Almere (ik met het oma-boodschappentasje op wieltjes voor de mammogrammen, jullie met de lunch van de Boterham express). Thomas, bedankt voor je hulp bij het ophalen van de mammogrammen en natuurlijk voor alle inside information over mijn copromotor die je aan me doorspeelde. Jelmer en Bert, jullie waren bereid dagen in het beruchte labje te zitten, ingebouwd tussen dozen en kasten, in een hokje waar het daglicht nimmer doorbreekt. Samen hebben jullie een paar duizend mammogrammen ingescand, waarvoor veel dank.

De collega's van het VIC. Karin Nijssen, Truus Meijers en Francis Verheij, bedankt voor jullie hulp voor de coördinatie van het lezen van de mammogrammen. Esther en Arthur, jullie hebben beiden heel wat mammogrammen voor mij gelezen. Ik vond het leuk dat jullie beiden zo betrokken waren bij het onderwerp. Frank Leus en Peter Huistra, bedankt voor het datamanagement van deze klus.

Paul van Noord, mijn dank voor je adviezen en suggesties tijdens het schrijven van dit proefschrift.

Bernard Slotboom, bedankt voor de talloze datasets die je me hebt aangeleverd.

Cees Haaring, dank voor je hulp bij de lay-out. Ik ben blij dat ik met jouw hulp de ergernissen van verspringende teksten en tabellen heb kunnen voorkomen.

Albert Zwart en Jettie van der Walle-Kelderman, dank voor de coördinatie van de IGF-I bepalingen.

Ron Voorbij en Mark Roest, ik ben jullie veel dank verschuldigd voor de mogelijkheid die jullie mij geboden hebben om de DNA-isolaties bij jullie op het lab te doen. Het was erg gezellig, maar de volgende keer doen we gewoon kits! Mirjam, dank voor je grote inzet bij het isoleren, je hebt mij veel werk uit handen genomen. Ik vind het leuk dat je nu zelf ook aan een promotieonderzoek begonnen bent. Arjan, Bas, Sven, Thomas en Nicki, bedankt voor de hulp en de gezelligheid op het lab.

I would like to thank the EPIC Group in Lyon for a fantastic time. Dear Elio, thank you for having me on the team for a year. Carine, Laure, Bertrand and Christophe thanks for all your work. Especially Carine, for your patience on the phone. Dear Kofi, thanks for the peace keeping. I wouldn't have survived my stay without you. Thanks also for helping me with submitting! Thanks Becki for the good times we had as room mates. Dear Sabina, thanks for all the fun we had in and outside the office. You always did your best to make me feel at home in Lyon, which I appreciate very much. Dear James, thanks for helping me with the genotyping, for your expertise on the field of genetics and for the solid teamwork in the defense of the IARC football team.

Al mijn kamergenoten op het Julius, Marja, Sjoerd, Charlotte, Daphne, Joke, Fränzel, Audrey, Hamid, Laura, Irene, Fleur, Jettie en Peter. Bedankt voor de leuke tijd op het Julius. Ik heb veel kamergenoten gehad, maar slechts 2 roomies. Marjolein en Nadine, ik heb een hele leuke tijd gehad met jullie, eerst op de zesde en later op de vierde. Nadine, ik vind het jammer dat je ons verlaten hebt en benijd je nieuwe collega's. Gelukkig zit je niet ver weg, dus kunnen we onze excursies naar Huffels voor snoep, koek en chocola voortzetten. Marjolein, met een beetje geluk zijn we nog jarenlang roomies zodat we verder kunnen discussiëren over p-waarden en of ze überhaupt wel relevant zijn. Ik heb bewondering voor het feit dat je na een mooi proefschrift te hebben geschreven, er nog even een studie geneeskunde achteraan doet. Meteen na de verdediging verder en nog geen tentamen gemist.

Lieve Fränzel, het eerste wat je deed in mijn eerste vergadering op het Julius was mij met een tevreden grijns op je gezicht bevorderen tot notulist van het poly-overleg. Volgens jou viel het allemaal best mee en zou ik zelf ook snel weer afgelost worden door de volgende nieuweling. Het kwam er echter gewoon op neer dat ik een jaar lang elke week braaf heb zitten opschrijven hoe het met jouw project ging. Ik had overigens geen klagen, want ik beschreef voornamelijk jouw pionierswerk met het lezen van de mammogrammen met het Cumulus-programma, waar ik later veel van heb geprofiteerd. Je incasseerde daarbij alle klappen van de opstartmoeilijkheden als een volleerd bokser. Ik heb dan ook groot respect voor je volharding en ben je dankbaar dat ik altijd een beroep kon en kan

doen op je kennis en ervaring met het lezen en beoordelen van mammogrammen. Het enige waar ik eigenlijk jaloers op was is dat tripje naar Canada... Na een korte onderbreking zijn we inmiddels weer collega's en moet die eerste Duinheus et al. er nu eindelijk maar eens van komen.

Tjer, Fred, Jaap, Wal, Lutser en Knip van het Panadolteam, dank dat jullie me fit hebben gehouden de afgelopen drie jaar met jullie kansloze zondagochtend-exercities op het voetbalveldje aan de Burggravenlaan. Nog meer dank voor de inleidende tactiekbesprekingen de avonden daarvoor in de kroeg. Misschien dat er met behulp van de beroemde Tjer-spreadsheet versie 2, toch nog een trainingskamp gepland kan worden voor het einde van 2007, opdat we het BOB-team ooit nog eens mogen verslaan.

Willem en Jeantine dank voor het sollicitatieadvies een kleine vier jaar geleden. Ari, voor alle keren dat je naar Leiden kwam in plaats van dat ik naar Amsterdam ging. Voor het geval je je afvraagt of er überhaupt nog iets over vezels in dit boekje staat, zie pagina 112. Tony, dank voor je pogingen om mij aan het wandelen te krijgen ter ontspanning na een dag noeste arbeid. Ik heb het nooit echt onder de knie gekregen, maar het is zo ook wel gelukt. Nog twee jaar in het onderzoek en dat vaatje is van mij! Pieter, veerkracht en doorzettingsvermogen zijn onontbeerlijke eigenschappen voor het schrijven van een proefschrift. Je bent op dat vlak altijd een voorbeeld voor mij. Dank je wel Bar, natuurlijk voor je vriendschap maar in het bijzonder ook voor je diepgaande interesse in het onderwerp van dit proefschrift. Maarten, dank voor de bijtankweekenden in het zuiden van Frankrijk en de huislijke sfeer op de Lijsterbes. Bas, dank je voor je hulp met de kaft.

Beste paranimfen, jullie waren mijn slaapadressen in Utrecht waar ik altijd terecht kon als ik geen puf had om voor de tienmiljoenste keer in de boemel naar Leiden te stappen. Lieve Es, je bent in korte tijd een ontzettend goeie vriendin geworden. Dank voor je steun en je positivisme de afgelopen jaren en voor alle bakkies tussen de vissen en de schildpadden. Ik vind het wel sneaky van je dat je er tussenuit geknepen bent, net nu het tijd werd voor paranimftaken. Tegen jouw wens in zal er geen promotiediner bij Mr. Jacks komen, maar misschien kunnen we nog eens langs om de 50 keer vol te maken.

Hup, relatief gezien ben jij langer mijn vriend dan ik de jouwe omdat jij nou eenmaal 33 dagen ouder bent. Ondertussen weten we dus wel wat we aan elkaar hebben. Ik ben blij met je back-up straks tijdens de verdediging. Ik hoop dat je voorraad oude port maar nooit mag uitdrogen, zodat we ook in de toekomst nog van een glaasje kunnen genieten voor het slapen gaan op de Snip 6.

Vader en moeder, dit boek is nu af, ik hoop dat het volgende ook jullie goedkeuring weg kan dragen! Bedankt voor jullie steun, ook in de mindere tijden.

Pa en Ma, mijn dank voor jullie steun op allerlei vlakken. Zonder jullie vertrouwen en hulp al zoveel jaren was dit natuurlijk nooit gelukt. Geen van ons had dit verwacht ten tijden van “Koen koopt een kans”, dus we kunnen best trots zijn. Het is prettig te weten dat ik altijd op jullie terug kan vallen.

Lieve Sas, dank je wel dat je zo af en toe even de maatschappelijke relevantie van mijn vraagstellingen en stukken onder de loep nam en mij daarmee van de verdieping weer terugbracht naar de basis van het onderzoek. Dank voor je steun en voor je begrip voor de promovendus in de eindfase.

Summary
Samenvatting
Dankwoord
Curriculum Vitae

Chapter 7

Curriculum Vitae

Martijn Verheus was born on February 8th, 1975 in Leiderdorp, the Netherlands. In 1994, after graduating from secondary school at the Rijnlands Lyceum in Oegstgeest, he studied Nutrition & Dietetics at the Hogeschool van Amsterdam, the Netherlands. In 1998, he undertook an internship at the Instituto Politecnico de Viana do Castelo, Portugal, where he designed a Portuguese food frequency questionnaire. He also received a paramedical training at the Medical Center Alkmaar, the Netherlands. After obtaining his Bachelor of Science degree in 1999, he studied Nutrition & Health at the Wageningen University, the Netherlands. As part of this traineeship he investigated calcium intake, polymorphisms in the vitamin D receptor gene and colorectal adenomas (supervised by Dr. E. Kampman). In 2001 he obtained a Masters in Science with a major in molecular epidemiology. In 2003 he started his thesis work at the Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, the Netherlands (supervised by Prof. dr. D.E. Grobbee, Dr. C.H. van Gils and Dr. P.H.M. Peeters). He performed part of the work at the International Agency for the Research on Cancer in Lyon, France, where he worked for one year (supervised by Dr. R. Kaaks). In January 2007 he obtained a postdoc position at the Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, the Netherlands to perform research on soy intake, intestinal metabolism of equol and breast density (supervised by Dr. Y.T. van der Schouw). On June 19th 2007 he will obtain a Master of Science degree in Genetic Epidemiology at the Netherlands Institute for Health Sciences, Erasmus Medical Center Rotterdam, the Netherlands.