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Insulin-Like Growth Factor-I (IGF-1), IGF-Binding Protein-3 (IGFBP-3) and mammographic features

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SUMMARY: **Insulin-Like Growth Factor-I (IGF-1), IGF-Binding Protein-3 (IGFBP-3) and mammographic features.**

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Introduction. *The IGF system has recently been shown to play an important role in the regulation of breast tumor cell proliferation. However, also breast density is currently considered as the strongest breast cancer risk factor. It is not yet clear whether these factors are interrelated and if and how they are influenced by menopausal status. The purpose of this study was to examine the possible effects of IGF-1 and IGFBP-3 and IGF-1/IGFBP-3 molar ratio on mammographic density stratified by menopausal status.*

Patients and methods. *A group of 341 Italian women were interviewed to collect the following data: family history of breast cancer, reproductive and menstrual factors, breast biopsies, previous administration of hormonal contraceptive therapy, hormone replacement therapy (HRT) in menopause and lifestyle information. A blood sample was drawn for determination of IGF-1, IGFBP-3 levels. IGF-1/IGFBP-3 molar ratio was then calculated. On the basis of recent mammograms the women were divided into two groups: dense breast (DB) and non-dense breast (NDB). Student's t-test was employed to assess the association between breast density and plasma level of IGF-1, IGFBP-3 and molar ratio. To assess if this relationship was similar in subgroups of pre- and postmenopausal women, the study population was stratified by menopausal status and Student's t-test was performed. Finally, multivariate analysis was employed to evaluate if there were confounding factors that might influence the relationship between growth factors and breast density.*

Results. *The analysis of the relationship between mammographic density and plasma level of IGF-1, IGFBP-3 and IGF-1/ IGFBP-3 molar ratio showed that IGF-1 levels and molar ratio varied in the two groups resulting in higher mean values in the DB group (IGF-1: 109.6 versus 96.6 ng/ml; p= 0.001 and molar ratio 29.4 versus 25.5 ng/ml; p= 0.001) whereas IGFBP-3 showed similar values in both groups (DB and NDB). Analysis of plasma level of IGF-1, IGFBP-3 and IGF-1/IGFBP-3 molar ratio compared to breast density after stratification of the study population by menopausal status (premenopausal*

RIASSUNTO: **Fattore di crescita insulino-simile I (IGF-1), IGF-proteina legante 3 (IGFBP-3) e caratteristiche mammografiche.**

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Introduzione. *Scoperte recenti hanno dimostrato l'importante ruolo del sistema IGF nella regolazione della proliferazione cellulare del tumore della mammella. Inoltre, la densità della mammella è attualmente considerata come il fattore di rischio maggiore in questa patologia. Non è ancora chiaro se questi fattori siano intercorrelati e se e come siano influenzati dallo stato menopausale. Lo scopo dello studio è stato quello di esaminare i possibili effetti dell'IGF-1 e dell'IGFBP-3 e della loro concentrazione molare sulla densità mammografica nella menopausa.*

Pazienti e metodi. *Una coorte di 341 donne italiane sono state contattate e intervistate per raccogliere i seguenti dati: storia familiare di cancro della mammella, caratteristiche riproduttive e del ciclo mestruale, biopsie del seno, terapie ormonali contraccettive precedenti, terapia ormonale sostitutiva (HRT) in menopausa e informazioni sullo stile di vita. Un campione di sangue è stato prelevato per determinare i valori di IGF-1 e IGFBP-3. Sono state calcolate quindi le loro concentrazioni molari. Sulla base dei risultati dell'esame mammografico, le pazienti sono state divise in due gruppi: seno denso (dense breast, DB) e seno non-denso (NDB). Lo Student's t-test è stato usato per valutare l'associazione tra la densità del seno e i valori plasmatici di IGF-1, IGFBP-3 e le loro concentrazioni molari. Per valutare se questa correlazione fosse simile nei sottogruppi di donne in pre- e post-menopausa, la coorte è stata stratificata secondo lo stato menopausale. È stata utilizzata l'analisi multivariata per valutare la presenza di fattori concomitanti che potessero influenzare la relazione tra i fattori di crescita presi in esame e la densità del seno alla mammografia.*

Risultati. *L'analisi della relazione tra densità mammografica e livelli plasmatici di IGF-1, IGFBP-3 e concentrazione molare IGF-1/IGFBP-3 ha messo in evidenza che i valori di IGF-1 e le concentrazioni molari variavano nei due gruppi risultando maggiori nel gruppo DB (IGF-1: 109.6 ng/ml versus 96.6 ng/ml, p=0.001; concentrazione molare 29.4 ng/ml versus 25.5 ng/ml, p=0.001) mentre l'IGFPB-3 ha mostrato valori simili nei due gruppi. L'analisi dei livelli plasmatici di IGF-1, IGFBP-3 e IGF-1/IGFBP-3 correlati alla densità del seno nella popolazione in studio stratificata per lo stato menopausale (pre- e post-menopausa) non ha evidenziato alcuna correlazione tra i fattori plasmatici e la densità del seno. L'analisi multivariata ha mostrato che solo la nulliparità, lo stato premenopausale e il Body Mass Index (BMI) sono determinanti della densità del seno.*

Conclusioni. *Il presente studio suggerisce una forte evidenza di associazione tra la densità del seno e i valori plasmatici di IGF-1 e le sue*

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and postmenopausal) showed that there was no association between the plasma of growth factors and breast density, neither in premenopausal nor in postmenopausal patients. Multivariate analysis showed that only nulliparity, premenopausal status and body mass index (BMI) are determinants of breast density.

Conclusions. *Our study provides a strong evidence of a crude association between breast density and plasma levels of IGF-1 and molar ratio. On the basis of our results, it is reasonable to assume that the role of IGF-1 and molar ratio in the pathogenesis of breast cancer might be mediated through mammographic density. IGF-1 and molar ratio might thus increase the risk of cancer by increasing mammographic density.*

concentrazioni molari. Sulla base dei nostri risultati, è plausibile ipotizzare il valore di IGF-1 come fattore patogenetico nel cancro della mammella tramite incremento della densità mammografica. Il rischio di cancro cresce con l'aumento della densità mammografica.

KEY WORDS: Breast cancer - Mammographic density - IGF-1 - IGFBP-3.
Cancro della mammella - Densità mammografica - IGF-1 - IGFBP-3.

Introduction

Insulin-like growth factor-1 (IGF-1) is an essential growth factor for the regulation of proliferation and apoptosis in normal mammary cells (1-4). There is also increasing evidence of the role of IGF-1 in the growth of tumors in a number of different cancers (prostate, colon and lung cancer) including breast cancer (2,5,6). The IGF-1 mechanism of action in the carcinogenesis and development of breast cancer is complex and multifactorial and has led to numerous studies reported in the literature. IGF-1 is a growth factor that circulates in the blood and it is known to have a very short lifetime in free form, approximately 12 minutes (4). Its action is strongly influenced by the association with one of six existing different insulin-like growth factor binding proteins (IGFBP) which increases its average lifetime to about 12 hours (2). Over 90% of IGF-1 in circulation is bound to form-3 of IGFBP (IGFBP-3) (7). This complex remains stable in the blood due to the presence of a binding protein, specific protease inhibitor.

In the extravascular space, the lack of this inhibitor allows specific metalloproteases to break the link between IGF and IGFBP-3 thus favoring the association between IGF and its specific cellular receptor expression in the breast tissue where IGF-1 fulfills its regulatory role (8).

In addition to the regulatory effect on the action of IGF-1 through modulation of the association with IGF receptors (IGFR), the IGFBP-3 also seems to directly promote cell apoptosis independently of IGF-1 (8-10). Several studies have demonstrated an association between the IGF system and breast cancer risk, but only in premenopausal women (6, 11-14), suggesting that IGF-1 might interact with the estrogen signal to increase cell proliferation (15-17). Other more recent studies have reported an association between the IGF system and carcinogenesis also in postmenopausal women (18).

Schernhammer et al. demonstrated a weak association between IGF-1 and breast cancer in premenopausal women but did not find a significant association between IGFBP-3, IGFBP-1, free IGF and breast cancer risk neither in pre- nor in postmenopausal women (19). Also in a later study of premenopausal women, the same authors found no significant association between IGF-1, IGFBP-1, IGFBP-3, growth hormone levels and breast cancer risk (20). The debate concerning the association between menopausal status, IGFBP, IGF-1 and breast cancer risk is therefore still open. On the other hand, breast density is currently considered as the strongest breast cancer risk factor (21). Women with mammographic density ≥75% have a five-fold increased risk of developing breast cancer compared to women with fatty breast tissue and density <5% (22-25).

Given the regulatory function of IGF-1on the proliferation of normal breast tissue, the question has been raised whether there is a possible association between IGF and breast density. Some authors have shown a significant association between IGF and breast density in premenopausal women (8, 26-29). However, the results reported in the literature related to postmenopausal women are still discordant, as most authors found no correlation between IGF and breast density (8, 26, 27, 30), whereas some authors reported a weak relationship also in postmenopausal women (31) whether they were receiving hormonal therapy (32) or not (31).

The present study had three objectives. The main objective was to analyze whether there was a relationship between plasma levels of IGF-1, IGFBP-3, IGF-1/IGFBP-3 molar ratio and mammographic density in a study population of Italian Caucasian women. Secondary objective was to assess whether this relationship

was similar in subgroups of pre-and postmenopausal women. Tertiary objective was to assess whether there were confounding factors that might influence this relationship after dividing the groups according to reproductive factors and lifestyle, today considered among the factors that influence IGF-1 and IGFBP levels along with breast density.

Patients and methods

Ethical approval for this single-center, prospective, observational study was granted by the Medical Research Ethics Committee of our institution and written informed consent was obtained from all patients.

The sample was built up continuously in the order of presentation and 7.000 women were selected among those who spontaneously turned to the Breast Care section of the Department of Gynecology, Perinatology and Childcare of the University of Rome "Sapienza" for a breast examination between March 2005 and March 2007.

According to the protocol we selected only Italian women of childbearing age (regular menstrual cycles during the past year) or naturally postmenopausal women (absence of menstrual cycles for at least 12 months) who had performed mammographic examination, negative for breast cancer pathology, at the section of radiology of our department maximum 3 months prior to recruitment. Premenopausal women were enrolled in the study only if they had undergone mammographic examination within the first ten days of the menstrual cycle.

After recruitment the women were interviewed by a medical doctor (trained in medical research). Collected information included: age at mammography, family history of breast cancer (those with at least two first degree relatives with breast cancer were considered positive), reproductive and menstrual factors such as age at menarche, menopause, parity (nulliparous or with at least one full-term pregnancy), age at first pregnancy, lactation and infertility, previous breast biopsies (yes/no), previous administration of hormonal contraceptive therapy (yes/no), HRT in menopause (yes/no). Accurate lifestyle information was also collected: smoking status (never, past or current; only current smokers were considered positive), alcohol consumption (yes/no; intake of ≥15 g per day during the past year was considered positive), chest X-ray examinations before the age of 20, physical activity before the age of 20 and current physical activity (yes/no; ≥ 3 hours of physical activity per week for at least one year was considered positive) and previous slimming diets. Height without shoes (cm) and weight in light clothes (kg) were registered by a trained nurse for the calculation of body mass index (BMI).

Women with a clinical history positive for breast cancer and/or for colon and lung cancer, administration of hormone therapy for up to 12 months before recruitment such as menopause hormone replacement therapy (HRT) and hormonal contraceptives, pre-menopausal status (irregular menstrual cycles with or without menopausal symptoms), surgical menopause, participation in assisted fertilization programs and previous breast reduction or augmentation surgery were excluded from the study.

The objective of the study was explained to all the selected subjects. The first phase of the study included signing of an informed consent form, collection of recent mammograms as well as drawing of blood samples for the evaluation of serum IGF-1 and IGFBP-3 and calculation of IGF-1/IGFBP-3 molar ratio. Patients were divided into two groups: dense breast (DB) or non-dense breast (NDB) according to the mammographic parenchymal category assigned at the evaluation of the presented mammograms. Subsequently patients were stratified by menopausal status.

Mammographic classification

To determine the mammographic parenchymal category all mammograms were examined by three physicians (two radiologists and a gynecologist and breast specialist) all blinded to the clinical data and to the classification already assigned. Particular attention was paid to the craniocaudal projections of both breasts, and the distribution of glandular parenchyma was qualitatively evaluated in percentage of the total area of the breast. The patients were then assigned to one of the four categories of breast parenchymal density distribution established by the Breast Imaging Reporting and Data System (BI-RADS): type 1, the breast is almost entirely fat (glandular parenchyma <25% of the total area of both breasts); type 2, scattered fibroglandular densities (25%-50%); type 3, heterogeneously dense breast tissue (51%-75%); type 4 extremely dense (> 75% glandular).

It is well-known that the sensitivity of mammography is decreased in type 3 and 4 (33-34), and the patients participating in our study were therefore divided into two groups: DB which included BI-RADS type 3 and 4, and NDB which included BI-RADS type 1 and 2. In case of contradictory judgments, the classification assigned by at least two readers out of three was considered correct.

Peptide assays

At recruitment, a peripheral venous blood sample was drawn for determination of IGF-1 and IGFBP-3 levels. One sample was considered enough as previous studies have shown that a single assessment accurately reflects long-term concentration of IGF-1 (35).

All blood samples were drawn between 8am and 11am after an overnight fast; in women of childbearing age samples were drawn between the 6th and 10th day of the menstrual cycle. Serum obtained by centrifuging the blood samples was immediately frozen at -25°C until analysis which was performed in a single block. Blood samples were analyzed by a laboratory technician who was blinded to the parenchymal group (DB or NDB) assigned to the patients. A serum sample of each patient was stored for possible later tests. Determination of IGF-1 and IGFBP-3 levels was performed using Immulite 2000 (Siemens Medical Solutions Diagnostics) based on automated sandwich chemiluminescence immunoassay. Values were determined and calibration was performed on a laboratory instrument according to the producer's instructions.

Statistical analysis

In order to assess whether classification of DB and NDB was consistent, agreement between the three readers was evaluated using Cohen's kappa before further statistical analysis.

Univariate analysis, involving examination of each of the considered variables was carried out; particularly percentages, mean values and standard deviations of quantitative and qualitative variables in the two subgroups were calculated. All data were also graphically represented in a scatter diagram to provide an overview of the series and also to identify any outliers or incorrect data. To assess the association or dependence relation between categorical variables, Pearson's chi-square test was employed.

Group mean values were compared using the Student's t-test. Significance level was set at 0.05. Multivariate analysis was performed by building logistic regression models using the Enter method and subsequently the Stepwise procedure on the variables selected using univariate analysis. When a dichotomous dependent variable had been identified, logistic regression selected, from a pool of independent variables, those variables that had relevantly and significantly influenced the outcome. The odd ratio (OR) which expresses how much greater the probability of an outcome is among those that are exposed to a factor compared to those that are not exposed, as well as interval estimate 95%. The goodness of fit of the logistic regression model was assessed using the Hosmer-Lemeshow test.

Results

A total of 7000 women were assessed for eligibility; 3099 were excluded because they did not meet the inclusion criteria and 3560 were excluded according to exclusion criteria. This selection produced a final sample of 341 women.

Evaluation of mammographic features showed the presence in the sample of 196 (57.5%) patients with DB (BI-RADS 3 and 4) and 145 (42.5%) patients with NDB (BI-RADS 1 and 2*).* Assessment of inter-operator variability did not show statistically significant differences; Cohen's kappa values ranged from 0.85 to 0.89 (p= 0.001) thus indicating a high level of agreement.

Table 1 lists the data collected at the anamnestic interview: demographic information, reproductive history, family medical history, anthropometric measurements and life style related to the two groups DB and NDB.

Women with DB were generally younger than those with NDB (mean age about 49 and 59 years, respectively; p= 0.001) and the patients in the DB group were less frequently postmenopausal (36.7% versus 81.4%; p= 0.001).

Women with DB were more frequently nulliparous (32.7% versus 11.3%; p=0.001), among the women who had had at least one full-term pregnancy, pregnancy had occurred at a later age (mean age 27.1 versus 24.8; p= 0.001) and they had lactated less (49.5% versus 68.3%; p= 0.001); women with DB had a lower BMI (22.5 versus 26.6; p= 0.001).

As regards lifestyle factors a positive association was found between breast density and current smoking status (19.9% versus 11.7%; p= 0.041) and sports activities both at a young age (37.8% versus 24.8%; p=0.01) and at the time of recruitment (27.0% versus 15.2%; p= 0.008). An inverse association was furthermore found between DB and previous slimming diets (18.9% versus 35.2%; p= 0.001).

Analysis of the relationship between mammographic density and plasma levels of IGF-1, IGFBP-3 and IGF-1/ IGFBP-3 molar ratio showed that IGF-1 levels and molar ratio varied in the two groups resulting in higher mean values in the DB group (IGF-1: 109.6 versus 96.6 ng/ml; p= 0.001 and molar ratio 29.4 versus 25.5 ng/ml; p= 0.001) whereas IGFBP-3 showed similar values in the two groups (Tab.1).

After division of IGF-1 values into tertiles (I: <85 ng/ml, II: 85.1-110 ng/ml, III: > 110 ng/ml) the DB group showed the highest percentage distribution in tertile III (41.8%) compared to tertile II (35.7%) and tertile I (22.5%), whereas the women with NDB showed the highest percentage distribution in tertile I (42.8%) (p=0.001).

Similar results were obtained for molar ratio. In particular after division in tertiles (I: <24, II: 24-30, III: ≥ 30) the DB group showed the highest percentage distribution in tertile III (42.9%) compared to tertile II (30.6%) and tertile I (26.5%) whereas the women with NDB showed the highest percentage distribution in tertile I (43.2%) (p=0.001).

After division of IGFBP-3 values into tertiles (I: <3.1 ng/ml, II: 3.2-3.7 ng/ml, III: >3.7 ng/ml) the NDB group showed a higher percentage distribution than the DB group in tertile I (37.2% versus 31.6%) and in tertile III (37.3% versus 27.1%) whereas the DB group showed a higher percentage distribution than the NDB group in tertile II (41.3% versus 25.5%) (p=0.01). These results are shown in Table 2.

As regard menopausal status, 151(44.3%) women were premenopausal and 190 (55.7%) were postmenopausal at the time of mammography. Mean age was 43.9 years for premenopausal women and 61 years for postmenopausal women. BMI was higher in postmenopausal than in premenopausal women (25.7 versus 22.5; p= 0.001).

Comparing the association between plasma level of IGF-1, IGFBP-3 and IGF-1/IGFBP-3 molar ratio and breast density after stratification of the study population by menopausal status (premenopausal and postmenopausal), it was observed that there was no association neither in premenopausal nor in postmenopausal patients (Table 3).

After division into tertiles of plasma level of IGF-1, IGFBP-3 and IGF-1/IGFBP-3 molar ratio it was observed that the premenopausal women with DB showed the highest percentage distribution in tertile III of molar ratio (50.8%) compared to tertile II (29.8%) and tertile I (19.4%) whereas the premenopausal women with NDB showed the highest percentage distribution in tertile I (42.3%) and in tertile III (46.2%) compared to tertile II (11.5%) (p=0.025). Among postmenopausal women, the NDB group showed a higher percentage distribution than the DB group in tertile I (38.7% versus 33.3%) and in tertile III (39.5% versus 27.8%) whereas the DB group showed a higher percentage distribution than the NDB group in tertile II (38.9% versus 21.8%) (p=0.035) (Table 3).

Table 4 shows the results of logistic regression analysis related to the main variables. The binary logistic regression model built up using the enter method and subsequently the stepwise procedure, selected three variables among those found significantly associated with mammographic density when submitted to univariate analysis: BMI being inversely related and menopausal status and parity being directly related to mammographic density.

Each of these factors, BMI, menopausal status and parity affected breast density with an odd ratio of 0.76 (95% CI: 0.69 - 082), 3.88 (95% CI: 2.20 - 6.86) and 2.38 (95% CI: 1.16 - 4.89), respectively. Particularly me-

TABLE 1 - MAIN CHARACTERISTICS OF THE PATIENTS VERSUS MAMMOGRAPHIC FEATURES: PERCENTAGE OF QUALITATIVE VARIABLES, MEAN VALUE AND STANDARD DEVIATION FOR THE QUANTITATIVE VARIABLES IN THE TWO GROUPS: NON-DENSE BREAST (NDB) AND DENSE BREAST (DB). To assess the association between mammographic density and qualitative variables, Pearson's chi-square was employed, whereas for quantitative variables Student's t-test was used. Significant level was α=0.05. If p-value was > 0.05 the result was reported as not significant (NS).

VARIABLES	Non-dense breast (n = 145; 57.5%)	Dense breast (n = 196; 42.5%)	p-value
DEMOGRAPHIC DATA			
Age at mammography (years)	58.8 ± 10.2	49.5 ± 10.2	0.001
REPRODUCTIVE DATA			
Parity (at least one full-term pregnancy; %)	130 (897%)	132 (67.3%)	0.001
Nulliparity (%)	15 (11.3%)	64 (32.7%)	0.001
Age at menarche (years)	12.3 ± 1.6	12.6 ± 1.5	NS
Age at first pregnancy (years)	24.8 ± 4.1	27.1 ± 5.7	0.001
Breast feeding (yes)	100 (68.5%)	97 (49.5%)	0.001
Menopausal status (menopause)	119 (81.5%)	72 (36.7%)	0.001
FAMILY RISK			
Family history of breast cancer (yes)	28 (19.3%)	36 (18.4%)	NS
ANTHROPOMETRIC DATA			
BMI (kg/m^2)	26.6 ± 4.2	22.5 ± 2.7	0,001
LIFESTYLE			
Alcohol (yes)	46 (31.7%)	49 (25%)	NS
Smoking status (current)	17 (11.6%)	39 (19.9%)	0.041
Past physical activity (yes)	36 (24.8%)	74 (37.8%)	0.01
Current physical activity (yes)	22 (15.2%)	53 (27.0%)	0.008
Previous slimming diet (yes)	51 (35.2%)	37 (18.9%)	0.001
Ever on oral contraception (yes)	19 (13.1%)	16 (8.2%)	NS
Ever on HRT (yes)	16 (11.0%)	10 (5.1%)	NS
X-ray exposure in childhood (<18 yrs)	38 (26.2%)	69 (35.2%)	NS
Past biopsies (yes)	18 (12.4%)	20 (10.2%)	NS
SERUM PEPTIDE ASSAYS			
IGF-1 (ng/ml; mean)	96.6 ± 35.0	109.6 ± 36.1	0.001
IGFBP-3 (ng/ml; mean)	3.8 ± 1.0	3.8 ± 0.8	NS
Molar ratio (mean)	25.5 ± 7.6	29.4 ± 8.6	0.001

nopausal status and parity ≥1 showed a direct association with mammographic density; thus menopausal women or women with parity ≥1 should have less dense breasts and women with low BMI should have denser breasts. Logistic regression analysis did not reveal IGF-1 and molar ratio as determinants of breast density. The goodness of fit of the logistic regression model was assessed using the Hosmer-Lemeshow test (p 0.38).

TABLE 2 - CHARACTERISTICS OF BLOOD SAMPLES VERSUS MAMMOGRAPHIC FEATURES.

VARIABLES	Non-dense breast	Dense breast	p-value
IGF-1			
Tertile I (<85)	62 (42.8%)	44 (22.5%)	0.001
Tertile II (85.1-110)	41 (28.3%)	70 (35.7%)	
Tertile III (>110)	42 (29%)	82 (41.8%)	
IGFBP-3			
Tertile I (<3.1)	54 (37.2%)	62 (31.6%)	0.01
Tertile II (3.2-3.7)	37 (25.5%)	81 (41.3%)	
Tertile III (>3.7)	54 (37.2%)	53 (27%)	
Molar ratio			
Tertile I (< 24)	63 (43.2%)	52 (26.5%)	0.001
Tertile II (24-29)	46 (31.5%)	60 (30.6%)	
Tertile III (≥ 30)	37 (25.3%)	84 (42.9%)	

Discussion

There is an increasing interest in early detection of risk factors for developing breast cancer. Mammographic density is one factor (21, 22, 25), but the IGF system has recently been shown to have a role in the development of breast cancer (2, 5, 6). However, it is not yet clear whether these factors are interrelated and if and how they are influenced by menopausal status (8, 26-30).

The purpose of this cross-sectional study was to examine the possible effects of IGF-1, IGFBP-3 and IGF-1/ IGFBP-3 molar ratio on mammographic density and assess whether this relationship was similar in subgroups of pre-and postmenopausal women.

The study sample was fairly homogeneous as only Italian Caucasian women were enrolled, while women of different ethnic origins were excluded due to the possibility that plasma levels of IGF-1 and IGFBG-3 and parenchymal density might vary among different ethnicities. This choice was dictated by the need to build a homogeneous study sample, as previous studies of IGF-1 and IGFBG-3 reported in the literature seem not to have paid attention to ethnic differences but only to geographic location thereby suggesting environmental rather than genetic influence, whereas parenchymal density is thought to differ according to ethnicity rather than geographical location (28,36-37). Also women who had received HRT for up to 12 months before recruitment were excluded from this study because the use of postmenopausal hormones has been reported to lower circulating IGF-1 levels and increase breast density (38-40).

Particular attention was paid to the uniformity of blood sampling for determining IGF-1 and IGFBP-3 levels. Analysis of a single sample was considered sufficient, as most authors claim that one evaluation can predict long-term levels of these peptides (41,42) although not all authors are of the same opinion (43). In premenopausal women, the blood sample was drawn between 8am and 11am after an overnight fast between the 6th and 10th day of the menstrual cycle as these values may vary according to the menstrual cycle; however also in this case not all authors are of the same opinion (44-46). Blood analysis was carried out in a single block by one single laboratory technician who was blinded to the parenchymal classification. Using this strategy, peptide levels measured in our group of Italian women were generally lower than those reported by other authors (14).

This study has some limitations. One concerns the reliability of mammographic classification which was performed qualitatively and not by a computer-assisted method. However, BI-RADS mammographic classification is the most common technique used in the USA for the assessment of mammographic density (33). In order to further reduce the risk of measurement error, both premenopausal and postmenopausal women were enrolled in the study only if they had undergone mammographic examination at our center not more than three months before recruitment and blood sampling. Rigid criteria were furthermore used for assessing breast density (47). Using this method, inter-operator variances were not statistically significant as there was a high level of concordance in the evaluation carried out by the three blinded readers. Furthermore, BI-RADS was developed to alert the referring clinician that the ability to detect small cancers in the dense breast is reduced and it not related to the risk *per se* (34).

A second limitation is that blood sampling and mammographic examination were not carried out at the same time. A third limitation is that the temporality of the relation between growth factors and breast density cannot be determined due to the cross-sectional design. A fourth limitation is that the study population was inhomogeneous when stratified by menopausal status. Among the premenopausal women 82.7% had DB and only 17.3% had NDB, and among the postmenopausal women 63.4% had NDB and only 36.6% had DB.

TABLE 3 - ASSOCIATION BETWEEN PLASMA LEVEL OF IGF-1, IGFBP-3 AND IGF-1/IGFBP-3 MOLAR RATIO WITH BREAST DENSITY IN PREMENOPAUSAL AND POSTMENOPAUSAL WOMEN.

VARIABLES	PREMENOPAUSAL (n= 150; 43.9%)			POSTMENOPAUSAL (n= 191; 56.1%)		
	NDB (n=26; 7.3%)	DB (n=124; 2.7%)	p-value	NDB (n=119; 63.4%)	DB (n=72; 6.6%)	p-value
IGF-1 (ng/ml)	107.9± 39.3	115,7± 36.2	NS	96.6 ± 44.3	98.9± 33.5	NS
IGFBP-3 (ng/ml)	3.8 ± 0.9	3.7 ± 0.7	NS	3.8 ± 1	3.7 ± 0.9	NS
IGF-1/IGFBP-3 molar ratio	28.7± 8.7	31.0 ± 9	NS	29.9 ± 9	26.5 ± 7.1	NS
IGF-1						
Tertile I (<85)	7 (26.9%)	16 (12.9%)	NS	54 (45.4%)	28 (38.9%)	NS
Tertile II(85.1-110)	10 (38.5%)	46 (37.1%)		31 (26.1%)	24 (33.3%)	
Tertile III (>110)	9 (34.6%)	62 (50%)		34 (28.6%)	20 (27.8%)	
IGFBP-3						
Tertile I (<3.1)	87 (26.9%)	38 (30.6%)	NS	46 (38.7%)	24 (33.3%)	0.035
Tertile II(3.2-3.7)	11 (42.3%)	53 (42.7%)		26 (21.8%)	28 (38.9%)	
Tertile III (>3.7)	8 (30.8%)	33 (26.6%)		47 (39.5%)	20 (27.8%)	
Molar ratio						
Tertile I (< 24)	11 (42.3%)	24 (19.4%)	0.025	52 (43.7%)	28 (38.9%)	NS
Tertile II(24-29)	3 (11.5%)	37 (29.8%)		43 (36.1%)	23 (31.9%)	
Tertile III (30)	12 (46.2%)	63 (50.8%)		24 (20.2%)	21 (29.2%)	

TABLE 4 - STEPWISE LOGISTIC REGRESSION: VARIABLES IN THE MODEL.

VARIABLES	B	Wald test	Odd ratio	95% CI odd ratio
BMI	- 0.283	p= 0.001	0.76	0.69 – 082
Menopause	1.358	p = 0.001	3.88	2.20 - 6.86
Parity	0.866	p = 0.02	2.38	1.16 - 4.89

Hosmer-Lemeshow test: p = 0.38.

Finally the analysis of the potential confounders of the relationship between mammographic density and plasma level of IGF-1, IGFBP-3 and molar ratio was carried out using logistic regression instead of linear regression because mammographic density was not expressed as a continuous variable.

Univariate analysis showed that IGF-1 values and molar ratio were higher in the DB group compared to the NDB group. The same results were obtained after division into tertiles. IGFBP-3 values were similar in the two groups.

The distribution of IGFBP-3 tertiles in the two groups (DB and NDB) is not easy to interpret as the NDB showed a higher percentage distribution in tertile I than in tertile III, whereas the DB women showed the highest percentage distribution in tertile II. Furthermore, the association between IGFBP-3 expressed in tertiles and breast density is less strong than the association between IGF-1 and molar ratio (also expressed in tertiles) as demonstrated by the p-values (0.01 versus 0.001).

When the levels of IGF-1, IGFBP and molar ratio were compared to breast density stratifying by menopausal status, no association was found.

However after division in tertiles we found that among the premenopausal women, the highest percentage of DB was in tertiles III and II whereas the highest percentage of NDB was in tertile I (p= 0.025).

However, also in this case the results are not unam-

biguous, since 48.1% of the women with NDB were located in tertile III. Although this percentage is lower than the percentage found in the DB group, this result is not sufficient for asserting that among premenopausal women molar ratio levels were related to breast density, also in view of the fact that an association was not found when the molar ratio was expressed as a continuous variable. This also counts for IGFBP-3 levels.

These results might be due to the lack of homogeneity in the sample related to breast density after stratification by menopausal status. Among the premenopausal women 82.7% had DB and only 17.3% had NDB, and among the postmenopausal women 63.4% had NDB and only 36.6% had DB.

Multivariate logistic regression showed that nulliparity and premenopausal status are positively associated with mammographic density, whereas BMI is inversely associated. It is particularly interesting to note that the analysis did not reveal IGF-1, IGFBP-3 and molar ratio plasma level as determinant of breast density.

This might explain the lack of association between mammographic density and growth factors when the analysis was stratified by menopausal status.

Previous studies showed that breast cancer risk rose steadily with increased percentage of the breast area with a dense appearance on a prediagnostic mammogram, and this association was not explained by other breast cancer risk factors such as age, weight, age at first child birth, family history of breast cancer, alcohol intake, prior benign breast disease, age at menarche, and age at menopause (22,25). It is still not known through what mechanism breast density is related to cancer risk.

On the other hand current breast density reflecting the proportion of stromal and epithelial proliferation, may simply indicate the area of susceptible tissue (number of epithelial cells) or may represent the interaction between stromal and epithelial proliferation influenced by local growth factors, including epidermal growth factor, transforming growth factors, IGF-1, and IGF-2 (53). Growing evidence indicates that breast development and involution are influenced by IGFs (which increase proliferation) and IGFBPs (which reduce proliferation) (54). Thus, greater breast density may be a consequence of higher IGF and molar ratio levels and an associated increase in proliferation and/or of decreased IGFBP levels with a resulting reduction in the involution process.

Our study provides a strong evidence of a crude association between breast density and plasma levels of IGF-1 and molar ratio, but unlike previous studies by other authors, they do not confirm that IGF-1 can be considered determinant in breast density neither in premenopausal (8, 26-29) nor in postmenopausal women (30).

Conclusions

On the basis of our results it is reasonable to assume that the role of IGF-1 and molar ratio in the pathogenesis of breast cancer is mediated through mammographic density. Thus IGF-1 and molar ratio might increase the risk of cancer by increasing the mammographic density.

Further studies are required to clarify these issues, particularly the mechanisms regulating the IGF bioavailability in the biological systems which may explain the development of not only breast cancer, but also prostate, colon and lung cancer in which growth factors have been implicated.

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