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Circulating concentrations of insulin-like growth factor-I, insulin-like growth factor binding protein-3, genetic polymorphisms and mammographic density in premenopausal Mexican women: results from the ESMaestras cohort

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Abstract

The insulin-like growth factor (IGF) axis plays an essential role in the development of the mammary gland. High circulating levels of IGF-I and of its major binding protein IGFBP3 have been related with increased mammographic density in Caucasian premenopausal women. Some common single nucleotide polymorphisms (SNPs) in genes of the IGF pathway have also been suggested to play a role in mammographic density. We conducted a cross-sectional study nested within the large Mexican ESMaestras cohort, to investigate the relation between circulating levels of IGF-I, IGFBP-3, the IGF-I/IGFBP-3 ratio, five common SNPs in the *IGF-1, IGFBP-3 and IGF-1R* genes, and mammographic density in 593 premenopausal Mexican women. Mean age at mammogram was 43.1 (standard deviation–SD=3.7) years, and average body mass index (BMI) at recruitment was 28.5 kg/m^2. Mean percent mammographic density was 36.5% (SD: 17.1), with mean dense tissue area of 48.3 (SD: 33.3) cm^2. Mean IGF-I and IGFBP-3 concentrations were 15.33 (SD: 5.52) nmol/l and 114.96 (SD: 21.34) nmol/l, respectively. No significant associations were seen between percent density and biomarker concentrations but women with higher IGF-I and IGF-I/IGFBP-3 concentrations had lower absolute dense (p_{trend} =0.03 and 0.09, respectively)

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and non-dense tissue areas (p_{trend} <0.001 for both parameters). However, these associations were null after adjustment by BMI. SNPs in specific genes were associated with circulating levels of growth factors, but not with mammographic density features. These results do not support the hypothesis of a strong association between circulating levels of growth hormones and mammographic density in Mexican premenopausal women.

Keywords

IGF; mammographic density; genetic polymorphisms; Mexican premenopausal women

Introduction

Mammographic density is among the strongest predictors of breast cancer, with women having more than 75% of dense tissue showing between 4 to 6 times the risk of breast cancer compared to women with little dense tissue (1). Mammographic density is influenced by age, body mass index (BMI) and by several reproductive and lifestyle factors (such as parity, menopause, hormone use), which also are associated with breast cancer risk (2;3). Overall, however, these factors explain only between 20 and 30% of inter-women variability in breast density, while a larger proportion (up to 60% in homozygous twins) appears to be associated with genetic factors (3–5). Identifying genetic variants associated with mammographic density may help better understand the strong association between mammographic density and breast cancer risk.

Endogenous hormones play an essential role in the development of the mammary gland. Insulin-like growth factor-I (IGF-I) is a polypeptide hormone with mitogenic and anti-apoptotic properties, and co-regulates the proliferation of many different types of cells, including breast epithelium (6;7). Most of circulating IGF-I present is synthesized by the liver, and about 90% of it is bound to IGF-binding protein 3 (IGFBP-3), the most abundant IGF-binding protein present in blood. In addition to regulating IGF-I bio-availability, IGFBP-3 has independent anti-mitogenic and anti-proliferative properties on several cell types, including breast epithelium (8). To exert their biological functions, both IGF-I and IGFBP-3 bind to receptors, and although several receptors have been identified for IGFs, it appears that most of the effects are mediated through the IGF-I receptor (IGF-IR) (6).

High circulating levels of IGF-I have been associated with increased breast cancer risk (9), but results on the associations of this hormone with mammographic density are inconsistent (10–15). Results from large international studies indicate that some common genetic variations in *IGF-1* and *IGFBP-3* in Caucasian women are associated with circulating levels of the IGF-I and IGFBP-3 (16;17), but not with breast cancer risk (17–19). Conversely, in women from other ethnic groups, some *IGF-1* polymorphisms were significantly associated with breast cancer risk (20), especially in young women (21). Results from large cohort studies, including mainly Caucasian women, suggested that some common genetic variations in growth hormone pathways (especially for the minor allele for rs6220 in the IGF-I pathway) are associated with higher mammographic density (10;22–24), although

sometimes modestly (23;24). It has also been suggested that associations may differ among different racial/ethnic groups (24).

The purpose of this study was to evaluate the relation between five candidate IGF-I pathway single nucleotide polymorphisms (SNPs), circulating concentrations of IGF-I and IGFBP-3 and mammographic density a sample of Mexican pre-menopausal women who are part of the large ESMaestras cohort (25).

Material and Methods

Study population and blood collection

The ESMaestras cohort is a cohort of 115,345 female teachers from 12 Mexican states that was established in 2006–08 to identify risk factors related to cancer and other chronic diseases among Mexican women (25). Participants responded to questionnaires on demographics, socio-economic status, reproductive history and use of oral contraceptives, menopausal hormone therapy, medical conditions, anthropometry, lifestyle (including a food frequency questionnaire), physical activity, smoking habits and early-life risk factors. In 2007, a subsample of 2,084 ESMaestras teachers from two Mexican states (Jalisco and Veracruz) participated in a clinical evaluation that included an interview, anthropometric measurements, a mammogram, and the collection of biological specimens. Fasting blood samples (approximately 25 ml) were obtained by venepuncture by trained nurses and in 5 different tubes, two of them containing disodium ethylene diamine tetraacetic acid (EDTA). In no more than 30 minutes after blood draw, plasma, serum, erythrocytes and buffy coat were separated by centrifugation at 2500 rpm during 10 minutes in a refrigerated centrifuge (4°C), and aliquoted into several cryotubes at the field work site. Samples were frozen and kept in ultra low freezers at −70°C at the National Institute of Public Health (INSP) until shipment to IARC where they were stored at −80 °C in ultra-low freezer until hormone analyses were performed.

All participants gave informed consent for future use of biological specimens and questionnaire data. The study was approved by the Institutional Review Board at INSP and by the IARC Ethics Committee.

Selection of subjects

Among the 2,084 ESMaestras teachers who participated in the clinical sub-cohort, we excluded 230 women who had insufficient information on metabolic syndrome components (because of a parallel study on metabolic syndrome in the same population (26)), 67 who had an unknown menopausal status and 624 who were postmenopausal at the time of their mammogram (women were considered as pre-menopausal if they had menstruated at least once over the 12 months prior to the visit, and were considered as postmenopausal if they had no menstruation over the last 12 months prior to the visit, and those with surgical menopause who reported bilateral oophorectomy or those who did not know the type of surgery but who were over 48 years, given that mean age at menopause in Mexican women is 48 years (27)). We then stratified women by 4 breast density categories: <10%, 10 to <25%, 25 to <50% and >=50% (28). Women were randomly selected from each group

proportionally to its size. Thirty-five were selected for the first group, 158 for the second, 247 for the third and 160 for the fourth group. Out of these 600, 7 declared to be older than 55 and be premenopausal, and were therefore excluded from the analyses. Our final study population was composed of 593 women.

Mammographic density

Measurement of mammographic density was performed and validated as previously described (26). Briefly, a radiology technician performed mammography using the Giotto Image M (Internazionale Medico Scientifica, Bologna, Italy) in Jalisco and the Hologic Lorad M-III (Hologic, Bedford, MA) in Veracruz. Mammograms were developed using the Agfa CP1000 (Agfa-Gevaert Group, Belgium) developer. Cranio-caudal (CC) views were taken on each breast. An Astra 2400S scanner (Umax, Fremont, CA) was used to digitize the analogue films. A single observer measured mammographic density on the left CC view using Mamgr, a computer-assisted program developed at the Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine (29). This is a quantitative interactive-threshold method based on Byng et al. (30) and Ursin et al. (31), in which an observer selects one threshold grey level to identify pixels within the breast area on the digitized image and another to distinguish dense pixels from lucent ones within the breast gland. Percent mammographic density is automatically calculated as the percent of "dense" pixels within the breast area. Non-dense area was calculated by subtracting the dense area from the total breast area. Absolute dense and non-dense area values are converted to cm^2 according to the pixel size used in the digitisation. The intra-observer intra-class correlation in 108 duplicate mammograms was 0.84.

Laboratory assays

Hormone analyses—All laboratory analyses were performed on never-thawed serum samples continuously stored at −80C. Serum IGF-I and IGFBP3 concentrations were measured by immunoradiometric assays by Beckmann Coulter (Marseille, France) at the laboratory of hormone analyses, Biomarkers Group, International Agency for Research on Cancer (Lyon, France). Samples were batched by and randomly ordered within states of recruitment (Jalisco/Veracruz). The intra-assay and inter-assay coefficients of variations were 0.8% and 4.2%, respectively, for a concentration of 19.5 nmol/l for IGF-I, and 1.3% and 3.0%, respectively, for a concentration of 125 nmol/l for IGFBP-3.

DNA extraction—Genomic DNA from participants was extracted from a 0.5 ml aliquot of buffy coat, which had been kept frozen since blood collection and processing (32). All DNAs were extracted at IARC by use of the Gentra Autopure LS DNA preparation platform (Gentra Systems, Minneapolis, USA).

SNPs selection and genotyping—Five candidate SNPs which were observed in previous studies to be associated with IGF-I or IGFBP-3 levels, mammographic density, or breast cancer risk, especially in premenopausal women, were selected for genotyping: rs1549593 and rs1520220, in intron 3 of *IGF-1*, rs6220 in the 3′UTR of *IGF-1*, rs2854744 upstream of *IGFBP-3* (promoter region), and rs2229765, a synonymous SNP (p.Glu1043Glu) in *IGFR*.

To assess these five candidate SNPs, genotyping was performed on 10 ng of genomic DNA using TaqMan pre-designed SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). The fluorescence reading and allelic discrimination analyses were performed with the Applied Biosystems ABI PRISM 7900HT Sequence detection system.

DNA from study participants was randomized on plates and all samples were analysed simultaneously. For quality control purposes, duplicates of 10% of the samples were interspersed throughout the plates.

Statistical analysis

The age of the subjects was calculated based on the date of birth and the date of the clinical visit. BMI was defined as measured weight (kg) divided by measured height squared (m^2). Means and standard deviations, or percentages (where appropriate), of selected baseline characteristics and biomarkers were estimated. Correlations between hormone concentrations, mammographic density measures, age at recruitment and anthropometry were calculated as Spearman's partial correlation coefficients, adjusting for age, batch of laboratory analyses, and region, as appropriate. Multivariate regression analyses were performed to compare means of mammographic characteristics by quartiles of IGF-I, IGFBP-3 and the ratio IGF-I/IGFBP3. Covariates included in the analyses included BMI (continuous), age, batch of analyses, state (Jalisco and Veracruz), family history of breast cancer (yes/no), benign breast disease (yes/no), age at menarche (<12, 12, 13, 14+, unknown), oral contraceptive use (never used, used for less than 5 years, used for more than 5 years, ever unknown duration, unknown), number of full term pregnancies (nulliparous, 1, 2, 3, 4+, missing), age at first full term pregnancy (nulliparous, <20 years, 20–25 years, 25–30 years, >30 years, missing), alcohol intake (0, <0.1 drinks/day, 0.1–0.2 drinks/day, >0.2 drinks/day, missing), smoking status (never, past, current, missing), physical activity (as a continuous variable expressed as MetS/h) and social economic status (low, medium, high, missing. These categories were based on questionnaire data about having a telephone, a mobile telephone, a car, a computer, a vacuum cleaner, a microwave oven and internet access, and were classified as follows: low social economic status: ≤3 items, medium: SES 4–5 items, high SES: 6+ items). We tested for trends across categories of variables by assigning equally spaced scores to the categories (p-trend). As BMI may be a mediator of the IGF-mammographic density associations, all analyses were conducted with and without further adjustment for BMI. Women with missing BMI values (n=19) were excluded from the BMI-specific analyses. To further evaluate possible effect modification of body size on the association between hormones and measures of mammographic density, we explored these associations separately in obese women (BMI ≥30 kg/m^2, n=198), using multivariate regression analyses through tertiles of exposure.

SNPs analysis—For each of the genotyped SNPs, the average call rate was 90.2 % (range 87.5- 93.9) and quality control analysis showed a concordance rate >99% between duplicate samples. Allele and genotype frequencies were calculated and Hardy-Weinberg equilibrium (HWE) was tested in the studied sample set for each SNP. The five SNPs were in HWE among the subjects analysed.

Genotype-trend regression models were used to assess whether genotypes were associated with mammographic features, and with hormone levels. For each SNP, we estimated the effects of heterozygote and rare-allele homozygote genotype relative to the common homozygote genotype as this approach does not imply any assumptions regarding the structure of associations across genotypes. Covariates included in the analyses were age, batch (where applicable), BMI and state. All analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC).

Results

Selected characteristics of the study population are presented in Table 1. Mean age at mammogram was 43.1 (standard deviation–SD=3.7) years, and average BMI at the clinical assessment was 28.5 kg/m^2. Mean percentage mammographic density was 36.5 (SD: 17.1), with mean dense tissue area of 48.3 (SD: 33.3) cm^2. Mean IGF-I and IGFBP-3 concentrations were, respectively, 15.33 (SD: 5.52) nmol/l, and 114.96 (SD: 21.34) nmol/l. Smoking status was unknown for 65 subjects, while 378 women were never smokers, 59 were current smokers and 91 previous smokers (results not shown). Habits for alcohol drinking were unknown for 46 subjects, while 170 women were teetotallers, 267 women drank less than 0.1 drink/day, 77 between 0.1 and 0.2 drinks/day, and 33 equal or more than 0.2 drinks/day (data not shown). Only 15 women declared to take hormone replacement therapy at the time of the clinical assessment.

In our study population, IGF-I concentrations were positively correlated with IGFBP-3 concentrations and with the ratio IGF-I/IGFBP-3 (Spearman's r = 0.64 and 0.86, respectively, p<0.01; data not shown). Concentrations of IGF-I, IGFBP-3 and the ratio of IGF-I/IGFBP-3 were negatively correlated with age at the clinical visit (Spearman's r = −0.22, p<0.001, r = −0.13, p=0.002 and r = −0.20, p<0.001, respectively), while only IGF-I and IGF-I/IGFBP-3 (but not IGFBP-3) were significantly, and negatively, correlated with BMI (Spearman's r = −0.32, p<0.001 and r= −0.34, p<0.001, respectively). BMI was negatively correlated with percentage of breast density (Spearman's: −0.17, p<0.001), and positively correlated with absolute dense area (Spearman's r = 0.18, p<0.001) and, more strongly so, with absolute non-dense area (Spearman's r = 0.61, p<0.001).

Means of mammographic characteristics by quartiles of biomarkers are shown in Table 2. No statistically significant relations were seen between increasing biomarker concentrations and percentage mammographic density. Women with higher IGF-I and IGF-I/IGFBP-3 concentrations had lower absolute dense and non-dense tissue areas (for dense tissue area: 40.08 (29.5–52.2) *vs* 50.0 (38.9–61.0) p_{trend}=0.03; and 42.8 (31.4–54.2) *vs* 48.3 (37.4–59.3) p_{trend} = 0.09 (non-significant) highest *vs* lowest IGF-I and IGF-I/IGFBP-3 ratio quartile, respectively. For non-dense tissue area: 72.2 (59.0–85.4) *vs* 90.4 (77.5–103.2) p_{trend}<0.0001; and 68.8 (55.7–82.0) *vs* 89.8 (77.1–102.4) p_{trend} < 0.0001 highest vs lowest IGF-I and IGF-I/IGFBP-3 ratio quartile, respectively). However, associations were attenuated and no longer statistically significant after adjustment by BMI (Table 2). When exploring the associations between mammographic characteristics and biomarker concentrations separately in obese women (BMI≥30, n=198), absolute non-dense tissue area was significantly lower among women with higher IGF-I/IGFBP-3 ratio levels (highest *vs*

lowest tertile of absolute non-dense tissue area= 103.7 (75.9–131.5) vs 126.9 (98.7–155.0), p_{trend}: 0.01; data not shown). No significant relations were observed between other mammographic characteristics and hormone concentrations in obese women (data not shown).

Allele and genotype frequencies in the *IGF-1, IGFBP-3 and IGFR* genes are shown in Table 3, together with single SNP associations with hormone levels and mammographic density characteristics. In *IGF-1,* the number of copies of the minor allele (T) of rs1549593 was inversely associated with IGF-I and IGF-I/IGFBP-3 ratio (p_{trend}= 0.01 and 0.02, respectively). In contrast, there were no associations between the rs1520220 or the rs6220 and circulating hormone levels. In *IGFBP-3*, the number of copies of the minor allele (T) of rs2854744 was associated with increased levels of IGF-I and IGFBP-3 concentrations, and decreased levels of the IGF-I/IGFBP-3 ratio (p_{trend}: 0.03, <0.0001, and 0.02, respectively). The examined SNP in *IGFR* (rs2229765) was not related to hormone concentrations. No significant associations were observed between the studied SNPs and mammographic density measures.

Mean IGF-I concentrations and mean percent breast density values (with 95% confidence intervals) by deciles of BMI are shown in Figure 1. The lowest IGF-I concentrations (mean: 10.7 nmol) were observed in women with the highest BMI (>36.3 kg/m^2), who, in turn, had also the smallest percent of breast density (mean: 23%).

Discussion

In the present study, we observed that premenopausal Mexican women with higher IGF-I levels had lower absolute dense and non-dense tissue areas. However, these associations were attenuated after adjustment for BMI. In the same population, one SNP in *IGF-1* and one SNP in *IGFBP-3*, were significantly associated with circulating hormone levels, but not with mammographic density. When exploring the association between IGF-I concentrations and mammographic density by adiposity, the lowest IGF-I concentrations, as well as the lowest percent breast density, were observed in women in the highest decile of BMI.

Previously published cross-sectional studies reporting on the associations between measures of mammographic density and circulating growth factors in pre-menopausal women, mainly focussed on percent mammographic density. They have mostly shown positive associations between percent mammographic density, IGF-I concentrations and the IGF-I/IGFBP-3 ratio (10;11;14;33), mainly independently of BMI, while reported no significant associations with IGFBP-3 concentrations. However, a study within the Guernsey cohort (15), and a recent study undertaken within the Nurses' Health Study (12), showed no associations at all between circulating growth factors and percent density. The results from our study are consistent with this lack of association. Nevertheless, our population is of Mexican origin, whereas the populations from the previously mentioned studies are composed mainly of Caucasian women, therefore comparison of results should be taken cautiously.

Only a few cross-sectional studies presented data specifically on the associations between blood levels of IGF-I, IGFBP-3 and their ratio, with absolute dense and absolute non-dense

areas in pre-menopausal women (11;14;15;23): two studies (11;15) showed a direct association between increasing absolute dense area and increasing IGF-I concentrations, one study (14) reported a positive association with the IGF-I/IGFBP-3 ratio, one study (15) showed positive associations with both IGF-I and IGFBP-3 concentrations, which however lost statistical significance after adjustment for BMI, and two studies reported no significant associations (12;23). In our study, we observed an inverse association between absolute dense and non-dense areas with increasing concentrations of IGF-I and the IGF-I/IGFBP-3 ratio, as women who had lower absolute tissue areas had higher hormone concentrations. However, these associations were attenuated and null after adjustment for BMI, suggesting that overall adiposity is an important confounder (or mediator) in these associations.

Indeed, it is extremely important to consider adiposity when exploring the associations between growth factors and mammographic density (2;34). In Caucasian populations, increased BMI is generally associated with a moderate decrease in IGF-I concentrations, although this association appears to be non-linear (35;36). Women with very high BMI have higher free IGF-I concentrations, which suppress growth hormone secretion, and therefore IGF-I production in the liver, through a negative feedback control (37). As high BMI inversely correlates with percent mammographic density, a positive correlation between IGF-I and mammographic density may be observed, which is however mainly obesity-driven. The importance of adiposity on the IGF-breast density association is even more evident for absolute non-dense area, which primarily represents the amount of adipose tissue that is present in the breast (2). Although absolute dense area is less associated with adiposity than the non-dense area, it is interesting to notice that in our study the association between endogenous hormones and absolute dense area was also substantially attenuated after adjustment for overall adiposity.

In our study, IGF-I concentrations were linearly inversely related to BMI. These results contrast somewhat with those observed in Caucasian women, where results from cross-sectional studies indicate a non-linear, inverse U-shape correlation between adiposity and growth factor concentrations, with the lowest IGF-I concentrations observed in women with BMI<20 as well as in women with BMI > 30 (9;35;36). This difference in results confirms previous observations from the Multi Ethnic Cohort (38) where discrepancies in the strength and in the shape of associations between circulating IGF-I concentrations and BMI have been observed in women from different ethnicities. Nevertheless, it should also be noted that, in our population, mean BMI was about 28, while mean BMI in most of the studies reported on Caucasian women was about 25 or lower (35).

Results from the large Breast and Prostate Cancer Cohort Consortium (BPC3) (16;19), reporting associations between 18 genes and more than 300 SNPs in the IGF signalling pathway from more than 5,000 Caucasian women (mainly post-menopausal), showed that the rs1520220 polymorphism in the *IGF-1* and the rs2854744 polymorphism in the *IGFBP-3* were associated with an increase in circulating hormone levels, while no significant relation was observed for rs1549693 polymorphisms. Similar to the BPC3 study, we observed a strong association between rs2854744 and circulating levels of IGFBP-3. But, in contrast to the BCP3 study, *IGF-1* rs1520220 was not associated with circulating IGF-I levels; however we found levels to be associated with rs1549693 polymorphism.

Overall, these observations may suggest that associations between common genetic variants in *IGFBP-3* pathway and IGFBP-3 circulating levels seem to be independent from ethnicity, while variants in *IGF1* seem to differ in different ethnicities, supporting previous results (although on different SNPs) comparing Caucasian and African American pre-menopausal women (39). Inconsistency in the associations among specific SNPs in the *IGF-1* and circulating hormone levels may also suggest that the SNPs that have been genotyped are not tagging the sequence variant(s) having a direct/functional impact on the circulating level of the studied hormones.

Few studies have explored the association between circulating IGF-I concentrations and polymorphisms in *IGF-1* rs6220, with some studies undertaken in Caucasian premenopausal women, indicating a strong association (40), while others (including pre and post-menopausal women) showing no association. The results from our study are not supportive of an association between this SNP and biomarker levels in our Mexican population.

Despite the significant associations observed between some genetic polymorphisms in *IGF-1* and *IGFBP3*, and circulating IGF-I and IGFBP-3 levels, no associations could be seen between these polymorphisms and any of the mammographic density measures. This confirms observations in previous studies (5;24). The influence of these variants, when tested individually, on circulating levels might be small, and have therefore very little influence on the association with mammographic density measures.

Our study has several strengths: to our knowledge, this is the first study to examine the association between IGF-I and IGFBP-3 concentrations, SNPs and mammographic density in a large sample of premenopausal Mexican women. Because of detailed questionnaire information and anthropometric measurements, we were able to adjust for several potential confounders. The large sample size allowed the stratification of analyses by BMI. Biological samples were collected according to standard operating procedures and have been stored at −70C. Hormone levels were measured on never-thawed aliquots. Our study has also some weaknesses: we only had one biological sample collected per subject, and no information was collected on phase of the menstrual cycle at blood donation/mammography. However, previous studies have shown high intra-class correlations between IGF-I and IGFBP-3 concentrations overtime in premenopausal women (35), and indicate that variations of mammographic density (as well as IGF-I concentrations) through the menstrual cycle are modest (41).

In conclusion, the results of our study do not support the hypothesis of a strong association between circulating levels of IGF-I and IGFBP-3 and mammographic density in a sample of Mexican premenopausal women. The relatively strong associations observed between IGF-I levels and absolute dense and non-dense areas seemed to be mainly driven by body fatness. Polymorphisms in specific genes were found to be associated with circulating levels of insulin-like growth factors, but not with mammographic density measures. Further research is needed to better understand the potential interaction between body fatness, genetic factors and mammographic density, especially in non-Caucasian populations.

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Novelty and impact of the work

This study is the first to evaluate the association between *IGF-1* pathway SNPs, circulating concentrations of IGF-I and IGFBP-3 and mammographic density in a large sub-sample of Mexican premenopausal women. Although our results are not supportive of a strong association between concentrations of growth factors and mammographic density in this population, they are important to better understand the relations among breast cancer risk factors in a population with high breast cancer rates in young women.

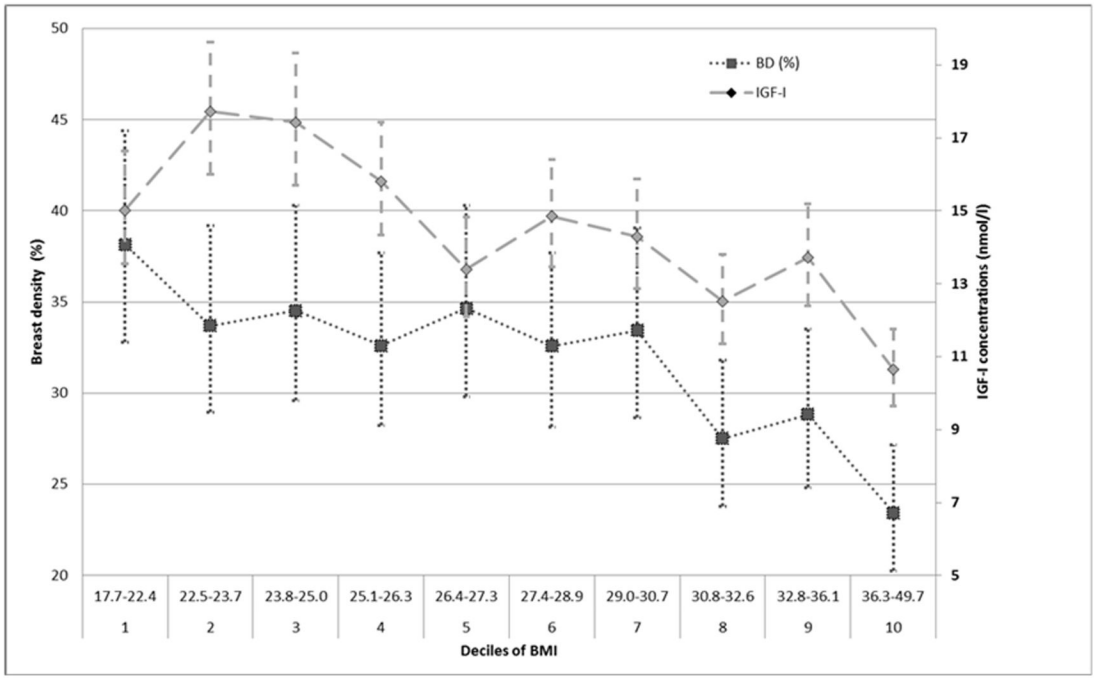

Fig 1.

Adjusted[a] mean IGF-I circulating levels and mean percent breast density (with 95% confidence intervals) in 593 premenopausal Mexican women by deciles of BMI

[a]Adjusted by age, batch (for IGF-I) and state

Table 1

Selected characteristics of the 593 premenopausal women included in the study.

***Mean* (± standard deviation)**	**All**
Age at screening (years)	43.1 (3.7)
Age at menarche (years)	12.6 (1.5)
Age at first birth* (years)	24.8 (4.5)
Number of full term pregnancies*	2.4 (1.0)
Duration of breast feeding* (months)	15.8 (14.3)
BMI# (kg/m^2)	28.5 (5.3)
Frequency (%)	
Parous	88.6
Family history of breast cancer	4.7
History of benign breast disease	14.7
Ever hormone use	51.8
***Mean* (± standard deviation)**	
Density (%)	36.52 (17.05)
Dense tissue area (cm^2)	48.34 (33.25)
Non-dense tissue area (cm^2)	82.33 (38.80)
IGF-I (nmol/l)	15.33 (5.52)
IGFBP-3 (nmol/l)	114.96 (21.34)
IGF-I/IGFBP-3	0.13 (0.04)

* among parous women

N=19 missing

Table 2

Adjusted means (95% confidence intervals) of mammographic characteristics by quartiles of IGF-I, IGFBP-3 and their ratio[1]

	IGF-I (nmol/l)				
	< 11.5	**11.5–15.4**	**15.4–18.8**	**> 18.8**	
Number of subjects	**148**	**149**	**147**	**149**	**p trend**
Percent mammographic density	37.3 (31.7–42.8)	35.3 (30.0–40.6)	37.5 (32.0–42.9)	36.9 (31.2–42.6)	0.85
Further adjusted[2]	37.9 (32.1–43.6)	36.3 (30.8–41.7)	36.3 (30.6–42.0)	35.4 (29.5–41.3)	0.29
Absolute dense tissue area (cm^2)	50.0 (38.9–61.0)	43.6 (33.0–54.1)	42.3 (31.4–53.2)	40.8 (29.5–52.2)	0.03
Further adjusted[2]	46.5 (35.1–57.9)	42.2 (31.4–53.1)	43.6 (32.3–54.9)	43.4 (31.6–55.1)	0.56
Absolute non dense tissue area (cm^2)	90.4 (77.5–103.2)	85.6 (73.3–97.9)	76.6 (63.9–97.9)	72.2 (59.0–85.4)	<.0001
Further adjusted[2]	82.3 (71.4–93.2)	79.6 (69.2–89.9)	83.3 (72.5–94.1)	82.6 (71.4–93.8)	0.73
	IGFBP-3 (nmol/l)				
	< 101.9	101.9–116.8	116.8–129.4	> 129.4	
Number of subjects	148	148	149	148	
Percent mammographic density	36.1 (30.5–41.6)	37.5 (32.0–43.0)	37.0 (31.6–42.4)	35.7 (30.3–41.1)	0.81
Further adjusted[2]	36.6 (30.9–42.3)	37.3 (31.7–42.9)	37.1 (31.5–42.6)	35.3 (29.7–40.9)	0.52
Absolute dense tissue area (cm^2)	46.4 (35.3–57.5)	44.7 (33.7–55.7)	43.7 (32.8–54.6)	43.3 (32.5–54.1)	0.42
Further adjusted[2]	44.5 (33.1–55.8)	43.9 (32.7–55.1)	43.1 (31.9–54.2)	43.5 (32.3–54.7)	0.77
Absolute non dense tissue area (cm^2)	87.8 (74.7–100.8)	81.1 (68.2–94.1)	82.3 (69.5–95.0)	80.7 (68.0–93.4)	0.18
Further adjusted[2]	82.2 (71.3–93.0)	80.6 (69.9–91.4)	80.8 (70.2–91.4)	82.5 (71.9–93.2)	0.91
	Ratio IGF-I/BP-3				
	< 0.11	0.11–0.13	0.13–0.16	> 0.16	
Number of subjects	148	148	149	148	
Percent mammographic density	36.7 (31.3–42.2)	35.8 (30.4–41.1)	36.6 (31.1–42.1)	38.6 (32.9–44.3)	0.31
Further adjusted[2]	37.4 (31.7–43.1)	36.2 (30.7–41.8)	35.8 (30.2–41.4)	37.1 (31.2–43.0)	0.87
Absolute dense tissue area (cm^2)	48.3 (37.4–59.3)	45.3 (34.7–56.0)	39.6 (28.7–50.6)	42.8 (31.4–54.2)	0.09
Further adjusted[2]	45.1 (33.8–56.4)	44.3 (33.2–55.3)	40.7 (29.6–51.9)	45.8 (34.1–57.5)	0.94
Absolute non dense tissue area (cm^2)	89.8 (77.1–102.4)	85.7 (73.4–98.0)	77.0 (64.4–89.6)	68.8 (55.7–82.0)	<.0001

	IGF-I (nmol/l)				
	< 11.5	**11.5–15.4**	**15.4–18.8**	**> 18.8**	
Number of subjects	**148**	**149**	**147**	**149**	**p trend**
Further adjusted[2]	82.0 (71.2–92.8)	81.5 (70.9–92.0)	81.8 (71.1–92.5)	79.8 (68.6–91.0)	0.62

[1] Adjusted for age, batch, state, family history of breast cancer (yes/no), benign breast disease (yes/no), age at menarche (<12, 12, 13, 14+, unknown), oral contraceptive use (never, ever <5 years, ever 5+ years, ever unknown duration, unknown), number of full term pregnancies (0, 1, 2, 3, 4+, missing), age at first full term pregnancy (nulliparous, <20, [20–25[, [25–30[, 30+, missing), alcohol intake (0, <0.1, [0.1–0.2[, 0.2+, missing), smoking status (never, past, current, missing) and social economic status (low, medium, high, missing)

[2] Model 2 is Model 1 plus additional adjustment for BMI

Table 3

Common genetic variations in *IGF-1*, *IGFBP-3* and *IGFR* and concentrations of IGF-I, IGFBP-3 and their ratio, and mammographic density

			IGF-I[a] (nmol/l)	IGFBP-3[a] (nmol/l)	Ratio[a]	%density[b]	Absolute dense area[b]	Non-dense area[b]
IGF-1								
rs1549593	Minor frequency allele Major frequency allele	A1/A1 A1/A2 A2/A1						
0.GG	G = 0.80 T = 0.20	333	15.91 (15.35–16.48)	116.7 (114.4–119.0)	0.13 (0.13–0.14)	36.60 (34.87–38.33)	48.67 (45.196–52.15)	83.29 (79.99–86.59)
1.GT		169	14.35 (13.74–15.32)	113.5 (110.3–116.8)	0.12 (0.12–0.13)	35.68 (33.25–38.10)	46.07 (41.18–50.96)	82.27 (77.64–86.91)
2.TT		18	15.17 (12.78–17.56)	112.4 (102.6–122.3)	0.13 (0.12–0.15)	40.76 (33.31–48.20)	59.16 (44.19–74.14)	80.15 (65.95–94.35)
p_{trend}[#]			*0.01*	*0.09*	*0.02*	*0.90*	*0.93*	*0.62*
rs1520220	Frequency allele 1 Frequency allele 2	A1/A1 A1/A2 A2/A1						
0.CC	C = 0.76 G = 0.24	287	15.06 (14.44–15.68)	114.7 (112.2–117.3)	0.13 (0.13–0.13)	36.31 (34.43–38.19)	47.27 (43.50–51.04)	82.76 (79.18–86.33)
1.CG		179	15.55 (14.78–16.32)	115.5 (112.3–118.6)	0.13 (0.13–0.1)	36.46 (34.10–38.81)	50.30 (45.59–55.02)	81.96 (77.48–86.43)
2.GG		37	16.50 (14.80–18.20)	122.5 (115.6–129.5)	0.13 (0.12–0.15)	34.70 (29.47–39.93)	40.22 (29.73–50.71)	81.67 (71.72–91.62)
p_{trend}[#]			*0.10*	*0.10*	*0.28*	*0.74*	*0.81*	*0.76*
rs6220	Frequency allele 1 Frequency allele 2	A1/A1 A1/A2 A2/A1						
0.AA	A = 0.71 G = 0.29	253	15.11 (14.46–15.76)	115.5 (112.8–118.1)	0.13 (0.13–0.13)	36.23 (34.25–38.22)	47.12 (43.12–51.12)	83.02 (79.23–86.81)
1.AG		197	15.37 (14.63–16.11)	114.4 (111.4–117.4)	0.13 (0.13–0.14)	36.59 (34.34–38.83)	50.05 (45.53–54.56)	80.88 (76.60–85.16)
2.GG		50	16.16 (14.69–17.62)	121.1 (115.2–127.1)	0.13 (0.12–0.14)	35.77 (31.28–40.26)	42.74 (33.70–51.78)	84.37 (75.80–92.94)
p_{trend}[#]			*0.22*	*0.33*	*0.39*	*0.99*	*0.55*	*0.86*
IGFBP–3								
rs2854744	Frequency allele 1 Frequency allele 2	A1/A1 A1/A2 A2/A1						
0.GG	G = 0.72 T = 0.28	280	15.04 (14.42–15.66)	109.6 (107.3–112.0)	0.14 (0.13–0.14)	37.08 (35.20–38.97)	48.88 (45.13–52.62)	80.91 (77.33–84.49)

			IGF-I[a] (nmol/l)	IGFBP-3[a] (nmol/l)	Ratio[a]	%density[b]	Absolute dense area[b]	Non-dense area[b]
1.GT		214	15.65 (14.94–16.36)	120.9 (118.2–123.6)	0.13 (0.12–0.13)	35.49 (33.32–37.65)	47.57 (43.26–51.88)	84.47 (80.35–88.58)
2.TT		43	16.84 (15.29–18.40)	131.4 (125.4–137.4)	0.13 (0.12–0.14)	36.16 (31.31–41.01)	46.28 (36.65–55.91)	83.82 (74.62–93.02)
p_{trend}[#]			*0.03*	*<.0001*	*0.02*	*0.38*	*0.55*	*0.26*
IGFR								
rs2229765	Frequency allele 1 Frequency allele 2	A1/A1 A1/A2 A2/A1						
0.GG	G = 0.67 A = 0.33	231	15.54 (14.86–16.23)	116.0 (113.1–118.8)	0.13 (0.13–0.14)	35.83 (33.74–37.91)	47.16 (43.01–51.31)	82.28 (78.32–86.24)
1.GA		238	14.87 (14.19–15.55)	114.6 (111.9–117.4)	0.13 (0.12–0.13)	36.66 (34.60–38.71)	47.73 (43.64–51.83)	82.36 (78.45–86.27)
2.AA		53	16.48 (15.07–17.88)	120.7 (115.0–126.5)	0.13 (0.12–0.14)	34.20 (29.82–38.58)	45.17 (36.44–53.90)	82.39 (74.06–90.72)
p_{trend}[#]			*0.86*	*0.47*	*0.73*	*0.85*	*0.85*	*0.60*

[a]Means and (95% confidence intervals), adjusted by age, state, batch of laboratory analyses and BMI

[b]Means and (95% confidence intervals), adjusted by age, state and BMI

[#]p-value for a linear trend in hormone/density levels across genotype categories estimated by assigning scores equal to the number of minor alleles to the categories (0, 1 or 2 alleles).