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New Title: Changes in the *total effective xenoestrogen burden* (TEXB) of breast cancer patients during an 18-month post-surgical follow-up

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Short Title: Total effective xenoestrogen burden in breast cancer follow-up

Research Highlights

1. Total effective estrogenic burden (TEXB) reflects the combined effect of xenoestrogens
2. TEXB was measured in breast cancer women at surgery and during 18-month follow-up
3. Breast adipose tissue was collected at surgery and abdominal tissue during follow-up
4. Median TEXB concentrations significantly increased during follow-up
5. Tumor characteristics (ER positivity) and age were related to TEXB values

Abstract

We aimed to assess changes in the *total effective xenoestrogen burden* (TEXB) -a biomarker of combined effect of mixtures of xenoestrogens- in breast cancer patients at surgery (breast adipose tissue) and at different time points during an 18-month follow-up (abdominal adipose tissue), and to analyze the potential influence of socio-demographic, reproductive, tumor, and treatment characteristics on TEXB levels. TEXB-alpha (due to persistent organohalogenated chemicals) and TEXB-beta (due mainly to endogenous estrogens) were quantified in 44 women. TEXB values significantly increased over follow-up (p <0.001); the largest difference was observed at 6-12 months post-surgery (p<0.001); then decreased over time. TEXB-alpha at 6-18 months was significantly higher in younger patients with estrogen receptor positivity (p = 0.034) and in those receiving anti-neoplasm chemotherapy. Cancer treatment may be responsible for the increase in TEXB-alpha observed in patients with hormone-dependent tumors, which may confer to xenoestrogens a role in the progression of the disease.

List of abbreviations

BMI: body mass index

DDT: dichlorodiphenyltrichloroethane

EDCs: endocrine-disrupting chemicals

Eeq: estradiol equivalents units

ER: estrogen receptor

GLM: general linear models

HPLC: high-pressure liquid chromatography

LOD: limit of detection

PCBs: polychlorinated biphenyls

pM: picomolar concentration

PR: progesterone receptor

TEXB: the total effective xenoestrogen burden

TNM: patients were classified according to pathology reports based on histological study and tumor size (T), lymph node involvement (N), and metastasis (M).

Keywords: breast cancer; follow up; total effective xenoestrogen burden; TEXB; estrogen receptor tumors; cancer treatment; chemotherapy.

1. Introduction

The burden of breast cancer remains huge worldwide. Incidence rates are partly explained by the distribution of risk factors associated with a greater lifetime cumulative exposure to estrogens; notably, age, early menarche, late menopause, nulliparity, late first full-term pregnancy, lack of breast feeding, and hormone replacement therapy [1-4]. Family history also plays a role. However, these factors alone do not explain the magnitude of changes in incidence rates. Considerable attention has been paid to the role of exposure to endocrine-disrupting chemicals (EDCs); nevertheless, studies that analyzed the association between the body burden of individual EDCs and cancer risk have yielded inconsistent results [5-11].

Research on these issues faces difficult challenges, which may explain some lack of consistency in results. The association between body burden of xenoestrogens and breast cancer is likely to vary among population or ethnic groups or among subgroups exposed to different mixtures of environmental chemicals [12, 13], limiting the replication of results. Studies that do not measure exposures in periods of life critical for cancer development may also underestimate risks [9, 11, 14, 15]. Moreover, individual xenoestrogens can interact with each other and with other environmental, dietary, lifestyle, and reproductive factors, which are not systematically measured across studies [13, 16]. Crucially, a hypothetical association between xenoestrogens and breast cancer risk cannot be tested solely through measures of the concentrations of individual compounds; account must be taken of possible synergetic, additive, or antagonistic interactions among them [10, 17-19].

Humans are exposed to multi-component chemical mixtures present in the environment [12, 13]. Over the past decades, a wide variety of synthetic chemicals have been demonstrated to have estrogenic effects [9, 17]. Xenoestrogen assessment based exclusively on chemical analysis appears to be unrealistic given the multitude of residues to be measured and the structural diversity of these compounds [20]. It has therefore been proposed to evaluate the potential contribution of the total effective estrogenic burden (TEXB) in biological samples, using a specific bioassay for estrogenicity, as a reliable marker of the combined effect of mixtures of xenoestrogens in the organism. TEXB comprises two fractions; TEXB-alpha, which indicates the estrogenicity of the lipophilic fraction (including most persistent pollutants), and TEXB-beta, which is largely influenced by endogenous estrogens (but also containing more polar xenoestrogens) [17]. Using this approach, two breast cancer case-control studies have provided evidence of a significant relationship between cancer risk and the combined effect of environmental estrogens in human adipose

tissue [21], and the estrogenic potential of EDC mixtures present in blood samples [22]. TEXB-alpha at the time of surgery was associated with environmental, dietary, lifestyle, genetic, and reproductive factors [23, 24].

All previous studies assessed TEXB in a single sample taken at breast surgery [21, 22, 24], and no data are available on TEXB during the follow-up of breast cancer patients to evaluate the influence of treatment (chemotherapy and/or radiotherapy) or tumor progression. It is critical to know whether cancer treatment modifies xenoestrogen content in the organism, whether TEXB is influenced by this modification, and how TEXB evolves during the clinical course. These issues are also relevant for methodological research on biomarkers [25].

Our objective was to assess changes in TEXB levels in newly-diagnosed breast cancer patients between the time of surgery and at four time post-surgical points during follow-up (<6 months, 6-12 months, 12-18 months, and >18 months), and to analyze the potential influence of socio-demographic, reproductive, tumor, and treatment characteristics on TEXB values.

2. Material and Methods

2.1. Study design and population

The available study population was all women with newly diagnosed breast cancer scheduled for surgery at San Cecilio University Hospital, Granada (Southern Spain) during 2002-2003. Exclusion criteria were the presence of gynecological or endocrinal disease, history of cancer, and refusal to sign informed consent. After application of these criteria, a final study sample of 56 women was obtained. All women were Caucasian, resided in Granada Province, and diagnosed with infiltrating ductal carcinoma. The study had a prospective longitudinal design and participants were followed up until June 2004. Breast adipose samples were obtained during the surgery and abdominal adipose samples (fat pad aspiration) during the follow up. Surgical treatments were: tumorectomy and axillary lymphadenectomy (12%), quandrantectomy and homolateral lymphadenectomy (40%), and the Madden technique (48%), a modified radical mastectomy [26]. In our study most patients (83.6%) were treated post-surgery with antineoplastic drugs, with only a small variation in the schedule and type. Most of them received Adriamycin and Cyclophosphamide, for four cycles, followed by either no further therapy or four cycles of Taxol. Three out of four (75%) also received radiotherapy. Patients completed antineoplastic treatment

during the first year post-surgery. None of the subjects received neoadjuvant (before surgery) chemotherapy treatment, neither hormonal treatment during the follow up period.

Structured face-to-face interviews were conducted at the hospital by a trained interviewer to gather data on socio-demographic characteristics, reproductive history and fertility, menopausal status, use of exogenous hormones, diet, tobacco and alcohol consumption, and family history of breast cancer. Clinical-pathological information was also collected from medical records, including details on diagnostic procedures and laboratory results. Patients were classified according to pathology reports based on histological study and tumor size, lymph node involvement, and metastasis (TNM). Tumors were also classified as estrogen receptor (ER) and/or progesterone receptor (PR) positive depending on the immunostaining (intensity of reactivity and number of cells observed under light microscopy, and following a semi-quantitative scale (considering 0 as negative, and 1 +, 2 + and 3 + categories as light, moderate, and intense positive, respectively). Hospital discharge data were also recorded. The study was approved by the ethics committee of the hospital.

2.2 Sample collection and follow-up

Fifty five patients agreed to participate in the study. All women had at least one adequate adipose tissue sample, interview reports, and estrogenicity bioassay results. Only 52 breast adipose samples were obtained at the time of surgery. Adequate abdominal adipose samples after surgery were available for: 37 women, during first 6 months (median 95 days), 25 women between 6-12 months (median 192 days), 29 women at 12-18 months (median 371 days), and 26 women at >18 months (median 542 days). Women were followed for a median time of 16.8 months after surgery. Of the 52 patients with sample at the time of surgery, 44 had at least an additional sample during the follow-up, 33 had two more, and 21 had three samples. For n=14, adipose tissue were available at surgery, <6 months, 6-12 months or 12-18 months, and >18 months. At each follow-up session, height and weight were measured, and body mass index (BMI-Kg/m^2) calculated.

2.3 Chromatographic conditions and quality control analysis

An adipose tissue sample (~5 g) was obtained from each patient at each time point, immediately coded and sent to the laboratory for chemical and biological analysis. Xenoestrogens were extracted from 200 mg of adipose tissue with hexane using a previously described method (Rivas et al., 2001; Fernandez et al., 2004). Dried adipose tissue extracts were reconstituted in 200 μL hexane, halved, and eluted in duplicate (100+100 μL) using high-performance liquid chromatography (HPLC).The HPLC technique collects two fractions, the alpha- (first 11 min) and beta-fraction (from 13-32 min), and allows the

separation of natural estrogens (beta-fraction) from more lipophilic xenoestrogens (alpha-fraction) without their destruction. The normal-phase column separates xenoestrogens according to their polarity, with the most lipophilic compounds eluting in the shortest time. Thus, dichlorodiphenyltrichloroethane (DDT) and metabolites, dieldrin, aldrin, and lindane, among other organochlorines, as well as other chlorinated and/or brominated organohalogenated chemicals elute in the alpha-fraction [27]. The beta-fraction contains endogenous sex steroids and more polar xenoestrogens (pharmaceutical estrogens, nonylphenol, octylphenol, and bisphenol-A), distinct from those eluted in the alpha-fraction [17, 27].

2.4 TEXB assessment

After HPLC fractionation, duplicated of each fraction were combined, dry and resuspended in 2.5 ml of experimental steroid-free medium (phenol red–free medium supplemented with charcoal-dextran fetal bovine serum), and tested for estrogenic activity with the E-Screen bioassay [28]. Each fraction was assayed at three different dilutions (1:1, 1:5, and 1:10), along with the negative control (experimental steroid-free medium) and the positive control (treated with 100 pmol of estradiol), in triplicate (three wells), in each culture plate. Proliferative effect of fractions was referred to the maximal effect obtained with estradiol and transformed into estradiol equivalent units (Eeq) by reading from a dose–response curve (estradiol concentration range from 0.1 pM to 10 nM) [17] and expressed as TEXB-alpha and TEXB-beta values in pM Eeq per milliliter (pM Eeq/ml) and per gram of lipid (pM Eeq/g lipid) [21, 24]. Lipid content was quantified gravimetrically [27].

The limit of detection (LOD) for TEXB-alpha and TEXB-beta was 0.1 Eeq pM/mL, which corresponded to the minimum concentration of estradiol needed to produce a significantly different proliferative effect from that observed in steroid-free control cells (negative control). When a sample had a concentration below the detection threshold, it was assigned the mid-value of this limit.

For quality control, 10 adipose samples were analyzed in triplicate through independent extraction, HPLC fractionation, and E-Screen bioassay. The inter-assay coefficients of variation for TEXB-alpha and TEXB-beta were 17.1% and 12.2%, respectively. In addition, researchers responsible for the extraction, fractionation, and cell culture (E-Screen) analysis were blinded to the characteristics of the study population

2.5 Statistical analysis

Univariate statistics were computed as customary [29, 30]. The Kruskal–Wallis test and Mann–Whitney U test for independent samples, and Wilcoxon Signed Ranks test and Friedman test for related samples were used for comparisons of continuous variables. Spearman's rank correlation coefficient (ρ) was used to evaluate the linear correlations between TEXB alpha and beta results in the samples obtained at surgery and during the follow up.

For women with TEXB values at 6-12 months or at 12-18 months of the follow-up, we created a new variable named "second follow-up measurement". The concentration of TEXB-alpha or -beta at 6-12 months was preferably considered; if missing, the concentration at 12-18 months was used instead. The number of patients with information on this new variable was 34, with a median follow-up time from surgery of 203 days; 32 of these women also had TEXB values at surgery.

General linear models (GLMs) were applied to analyze the relative influence of socio-demographic and clinical factors on the TEXB concentrations at the "second follow-up measurement". TEXB values were normalized by natural logarithmic transformation. The main effects of all predictors were independently explored in the base models, and age was included in final models as a potential confounder. In the statistical models, account was taken of weight changes, time interval between surgery and the collection of the fat samples at the "second follow-up measurement" (measured in months), and number of reproductive cycles, categorized in tertiles. BMI, weight changes and time interval between surgery and the collection of additional fat samples were also explored in the models as possible confounding variables.

We did not consider performing multivariate analysis of repeated measures for several reasons. First, the limited number of repeated adipose tissue samples from the same women, both at surgery and at the follow-up measurements. Second, the limited available data for some variables in the different follow-up points, such as for example BMI. Third, for many women the time intervals of different follow up points overlapped. And finally, the very low TEXB values at surgery compared to the "second follow-up measurement" allow interpret results using linear regression models in an approximate way to those obtained in the analysis for repeated measures, without losing participants. The statistical significance level was set at 0.05, and all tests were two-tailed. SPSS version 18 (SPSS, Armonk, NY, USA, 2009) was used for analyses.

3. Results

Table 1 exhibits the main characteristics of the study population. The age of the 55 newly-diagnosed breast cancer patients ranged from 34 to 80 years (mean, 61.1; SD, 11.4) years. Mean BMI at surgery was 29.4 Kg/m^2. The high BMI of these patients (>71% overweight or obese) is consistent with previous findings in our area [21, 31]. Around 80% of subjects were diagnosed in stages I or II of the disease. Tumors were classified as ER-positive in 74.5% of women and PR-positive in 49.1%.

Patients treated with antineoplastic drugs after surgery (83.6%) completed their treatment during the first year post-surgery. Women not receiving chemotherapy had only a slightly higher mean BMI at surgery than those receiving chemotherapy (median 30.6 *vs*. 28.1 Kg/m^2, respectively, $p = 0.341$). During the first year post-surgery, median weight increased by 0.9 Kg in non-chemotherapy treated patients and by 2.0 Kg in patients with chemotherapy ($p = 0.230$).

Figure 1 (and Table 1S) shows TEXB-alpha and -beta levels at surgery and during follow-up. Median TEXB-alpha and -beta values significantly increased during follow-up ($p < 0.001$, Kruskal Wallis test), were maximal at 6-12 months, and then decreased lightly with time. Statistical significant differences ($p < 0.001$, Mann-Whitney's *U* test) were also found between TEXB-alpha and beta median values estimated at surgery and those at 6-12 months, between surgery and 6-18 months, and between surgery and >18 months. Interestingly, TEXB-alpha or -beta levels in breast adipose tissue collected at surgery (2.5 and 16.1 pM Eeq/g lipid, for TEXB-alpha and -beta, respectively) and the earliest abdominal fat samples (first follow-up measurement) (<6 months, 5.3 and 17.0 pM Eeq/g lipid, for TEXB-alpha and -beta, respectively) were similar and not statistically different. These samples were obtained before the onset of chemotherapy and/or radiotherapy treatment. Maximal significant differences ($p < 0.001$, Mann-Whitney's *U* test) were found between median values at 6-12 months (174 and 252 pM Eeq/g lipid for TEXB-alpha and -beta, respectively) and levels at surgery (Table 1S). In addition, a slight and statistically non-significant decrease in mean and median TEXB-alpha and -beta values was observed between the top values (6-12 months) and final follow-up measurements (12-18 months, and > 18 months).

When we analyzed individual data for the patients (n=14) in whom TEXB was obtained at four time points (surgery, <6 months, 6-12 months or 12-18 months, and >18 months), we again found a significant increase in median TEXB values over the follow-up period ($p < 0.001$, Friedman test).

The greatest increase in TEXB values was observed for those patients (n=32) with TEXB-alpha and -beta values at surgery (median 1.7 and 13.8 pM Eeq/g lipid for TEXB-alpha and -beta) and at 6-18 months-"second follow-up measurement" (median 169 and 320 pM Eeq/g lipid for TEXB-alpha and -beta, respectively) (Table 2). Among those women a significant correlation was also observed between TEXB-alpha and -beta values; and it became weaker over time: the coefficient ρ was 0.619 (p <0.01) at surgery and 0.372 ($p \leq 0.030$) at the "second follow-up measurement". The median crude increase in TEXB-alpha values (surgery vs. second follow-up measurement) was 9 pM Eeq/g lipid in non-chemotherapy treated patients and 165 pM Eeq/g lipid in chemotherapy patients, being statistically significant only in the latter ($p < 0.001$).

The potential influence of socio-demographic, reproductive, tumor, and treatment characteristics on the TEXB-alpha values at the "second follow-up measurement" were assessed by GLM models. First, main effects of all predictors were independently explored. Second, the model was adjusted by age as a potential confounder (Table 3), TEXB-alpha (pM Eeq/ml) levels were significantly associated with high-to-moderate positive *versus* low or negative ER tumor phenotype ($\beta = 2.121$, $p = 0.034$). The association was weaker when TEXB-alpha values were adjusted by lipid content (pM Eeq/g lipid) ($\beta = 1.964$, $p = 0.065$). When the models were further adjusted by the time from surgery to the follow-up measurement, TEXB-alpha values remained statistically significant for ER-positive tumor phenotype [$\beta = 2.073$; $p = 0.041$for TEXB-alpha (pM Eeq/ml) and $\beta = 1.899$; $p = 0.078$ for TEXB-alpha (pM Eeq/g lipid)] (data not shown in Tables). Linear regression analysis of TEXB values at 6-18 months follow up, excluding one case with values below detection limit (n = 33), revealed a negative beta coefficient for age ($\beta = -0.093$, $p = 0.008$) (data not shown in Tables), indicating a reduction in post-surgical TEXB-alpha concentrations with higher patient age.

4. Discussion

During post-surgical follow-up, women with breast cancer experienced an increase in the combined estrogenicity of extracted chemicals bioaccumulated in fat. The size of this increase peaked at 6-12 months post-surgery and slightly decreased thereafter. The TEXB-alpha peak median value (252 pM Eeq/g lipid) was similar to values reported in women at highest risk of breast cancer in a previous case-control study in the same area [21]. TEXB-alpha concentrations at 6-18 months after surgery were larger in younger women with high-to-moderate positive ER tumors.

The observation of an increase in the xenoestrogenicity of adipose tissue during follow-up poses the question of the role of xenoestrogens in the progression of the disease, particularly in the case of estrogen-dependent tumors. It has been hypothesized that xenoestrogen compounds stored in breast adipose tissue adjacent to the breast carcinoma may affect the tumor microenvironment favoring onset or progression among hormone receptor-positive neoplasms [32]. Muñoz de Toro and coworkers found PR expression to be positively associated with higher xenoestrogen concentrations in tissues adjacent to ER-alpha positive tumors, suggesting an up regulation of PR expression *via* an ER-alpha dependent mechanism. They proposed an interaction between epithelium and stromal compartments through a network of diffusible factors, which might modulate cell growth and differentiation and facilitate tumor development/progression in a permissive, xenoestrogen-rich microenvironment [32]. However, whether bioaccumulated xenoestrogens affect both ER positive and ER negative breast cancer subtypes needs further investigation.

It has also shown that some xenoestrogens interfere with hormonally responsive tissue functions via dysregulation in hormone signaling and cell function; and in addition to their tumorigenic properties, some of them may also possess proinvasive and prometastatic abilities [33, 34]. The mechanisms of this invasive and metastatic potential are not clearly understood. However, it is now widely accepted that the development and progression of a tumor toward the malignant phenotype is highly dependent on interactions between tumor cells and different components of the tumor microenvironment [35, 36]. Nevertheless, an estrogenic stimulus could be reached by multiple combinations of low-dose chronic xenoestrogens exposure, synergism and timing of exposure, as well as complementation of mechanistic effects of these agents, reflected in the complex nature of carcinogenesis [10].

It is now well documented that the surrounding stromal cells, including adipocytes, the majority of cells in breast tissue, have a significant effect on breast cancer progression [37]. A major function of adipose tissue is to store and release fatty acids, which are incorporated in adipocyte triglycerides to meet whole body energy demands [38]. Lipophilic xenoestrogens bind to adipocyte lipids, where they are stored and then distributed to all parts of the organism according to need [39]. The turnover of adipocyte lipids may also favor depot or mobilization of these chemicals, given that triglycerides are replaced around six times during the lifespan of the adipocyte (mean age of 9.5 years), enabling a dynamic regulation of lipid storage and mobilization over time [38, 40]. The bioavailability of xenoestrogens in adipose tissue is regulated by the integrity of fat stores, and individual compounds may be affected differently during

weight enhances or loss, diet, or therapeutically-induced lipolysis [41]. The precise equilibrium of xenoestrogens between fat and blood circulation may be altered by weight changes and/or metabolic disruption of adipose tissue during cancer treatment, perhaps accounting for the observed changes in TEXB.

A recent revision suggests a causal link between environmental pollutants exposure through diet and their bioaccumulation in adipose promotes the development of obesity and ultimately influences breast cancer development and/or progression [42]. Commonly cancer patients lose weight in the course of the disease and/or treatment, but most breast cancer patients experience weight gain. This gain is more evident in premenopausal women in Western countries, especially in those receiving multi-agent *versus* single chemotherapy regimens and in those with early-stage disease [43-45]. These changes in body weight were related to changes in body composition, with a significant increase in body fat and decrease in the percentage of lean soft tissue and skeletal mass, similar to observations in gradual sarcopenic obesity with aging and menopause [46]. Weight changes occurred in our patients during the year following the diagnosis; especially in women receiving chemotherapy, whom showed a weight gain of 2.0 Kg *versus* a gain of 0.9 Kg in women without chemotherapy, however differences were not significant ($p = 0.230$).

A relationship between weight variation during treatment and poorer prognosis has frequently been reported [47, 48]; there is also an increased risk of breast cancer recurrence and mortality in patients overweight at the time of the diagnosis [48, 49]. Again, possible mechanisms underlying the adverse effects of weight gain on risk of cancer recurrence and mortality include the possibility of adipose tissue disruption, i.e., a greater aromatase activity in the excess adipose tissue and an inhibition of the synthesis of sex hormone-binding globulin that result in a higher bio-availability of estrogens, which could stimulate neoplastic cells [47]. This possible explanation is enriched by the major increase in the bio-availability of xenoestrogens extracted from fat tissues shown in our estimations of TEXB-alpha; however, bioavailability was not assessed in our study. Further research is needed to understand the role of xenoestrogens in the progression of breast and other cancers.

A potential study limitation is first that adipose tissue was obtained from mammary gland at the time of surgery and from parietal adipose tissue during follow-up, because these tissues may differ in fat composition and/or rates of accumulation of lipophilic xenoestrogens. Nevertheless, we found no differences in TEXB values between mammary tissue at surgery and parietal fat obtained at the first

follow up (<6 months), confirming a previous report [17, 21] that TEXB values of mammary and parietal adipose tissue are equivalent when lipid-adjusted (Figure 1, Table S1). In addition, according to Petreas et al., [50] measurements in breast and abdominal adipose tissues were correlated and that concentrations of environmental pollutants in one tissue could be derived from measurements in the other tissue. Moreover, the distribution of xenoestrogens in fatty tissues appears to be more dependent on the nature rather than site of tissue, and differences in xenoestrogen accumulation between organs largely depend on the lipid content of tissues [50, 51]. In this regard, breast, liver, omental fat, and subcutaneous fat are known to contain similar concentrations of chlorinated pesticides and their metabolites and of PCBs [52]. Therefore, regardless of the specific xenoestrogens responsible for the TEXB-alpha, it is plausible that they are similarly distributed in mammary fat and subcutaneous adipose tissue. Secondly, sample size in the present study is somewhat limited. Nevertheless, it was sufficient to perform this exploratory study and to yield statistically significant associations. In addition, it should be borne in mind that studies with repeated measures from abdominal fat are extremely uncommon in this and other research areas.

Our goal was also to respond to the often stated but seldom practiced methodological requirement to collect multiple samples of biomarkers over time from individual study participants [25]; the results illustrate how challenging this requirement remains. It is necessary to emphasize the exploratory character of the present study, which gave interesting and potentially relevant results (a profound variation of TEXB values during the follow-up of patients) and set the basis for future confirmatory studies.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the submitted work.

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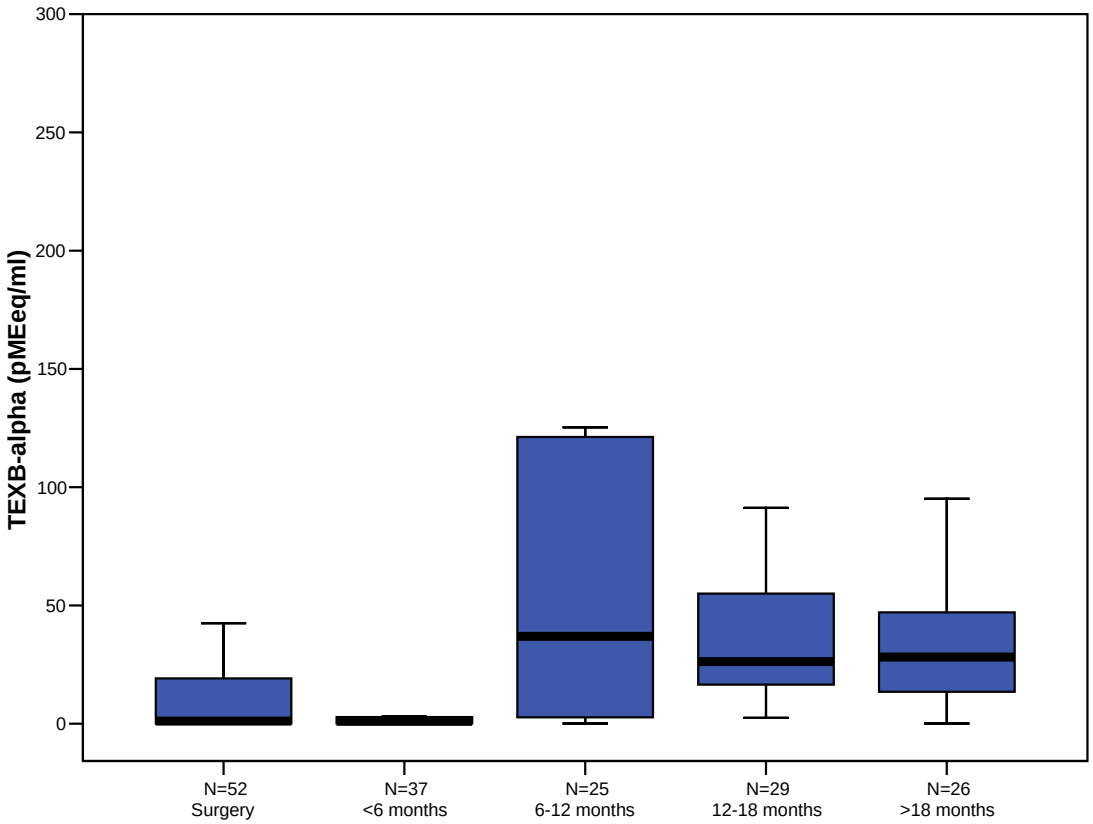
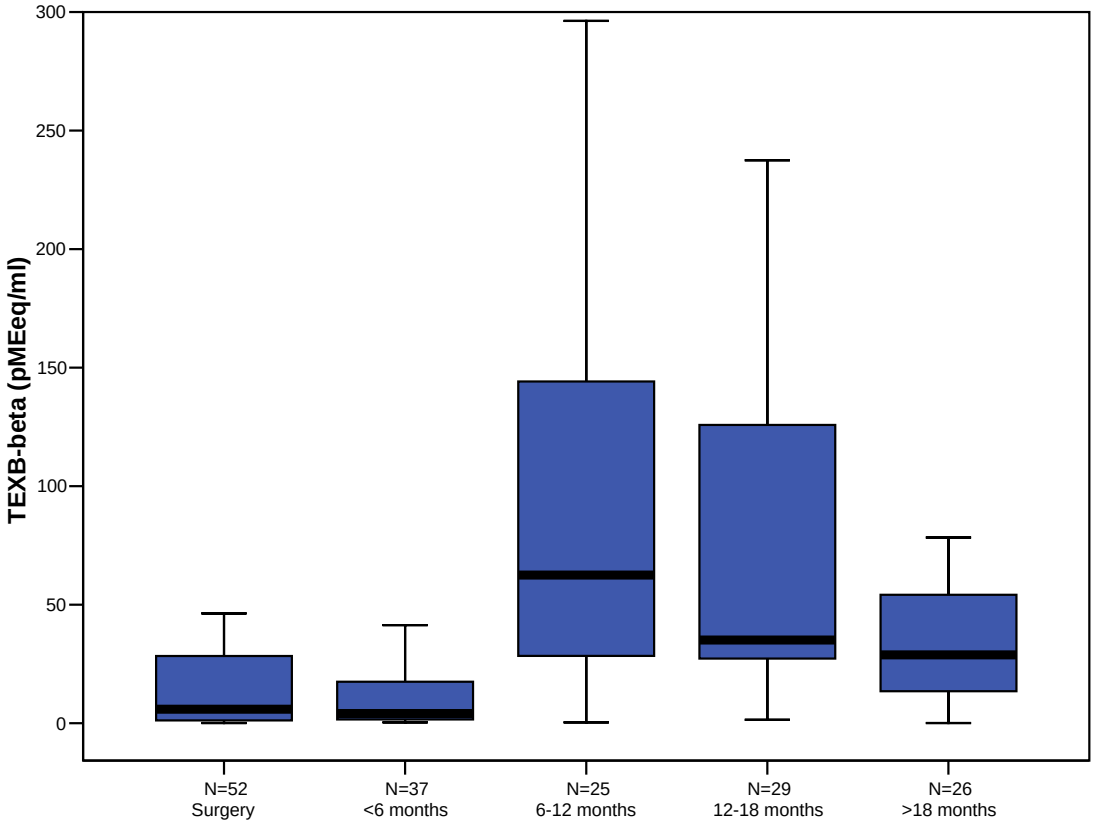
Figure 1a. Total effective xenoestrogen burden [TEXB-alpha (pM Eeq/ml)] at surgery and during the follow-up



* Samples were collected from breast adipose tissue at surgery and from abdominal fat depot during follow-up.
First 6 months (median 95 days); 6-12 months (median 192 days), 12-18 months (median 371 days), and >18 months (median 542 days)

Figure 1b. Total effective xenoestrogen burden [TEXB-beta (pM Eeq/ml)] at surgery and during the follow-up



* Samples were collected from breast adipose tissue at surgery and from abdominal fat depot during follow-up.
First 6 months (median 95 days); 6-12 months (median 192 days), 12-18 months (median 371 days), and >18 months (median 542 days)

Table 1. Characteristics of the study participants

Characteristics	Number (%)*
Total	55
Age (years)	61.1 ± 11.4
Body mass index at surgery (Kg/m^2)	29.4 ± 6.2
Normal weight	14 (25.5)
Overweight	17 (30.9)
Obese	22 (40.1)
Educational level	
Without formal education	32 (55.8)
Formal education	19 (36.5)
Past exposure to pesticides	
Yes	4 (7.3)
No	47 (85.5)
Parity	
Yes	44 (80.0)
Nulliparous	7 (12.7)
Number of children	2.3 ± 1.3
Lactation	
Yes	36 (65.5)
No	15 (27.3)
Months of lactation	13.1 ± 15.6
Number of reproductive cycles	392.0 ± 67.4
≤366	17 (30.9)
367-414	17 (30.9)
>414	17 (30.9)
Family history of breast cancer	
Yes	15 (27.3)
No	36 (65.5)
Tumor stage at diagnosis	
I	9 (16.4)
II	35 (63.6)
III	11 (20.0)
Postoperative chemotherapy	
Yes	44 (80.0)
No	10 (18.2)
Radiotherapy & chemotherapy	
Yes	41 (74.5)
No	12 (21.8)
Estrogen receptor (ER)	
Intense & moderate positivity	41 (74.5)
Low & negative positivity	13 (23.6)
Progesterone receptor (PR)	
Intense & moderate positivity	27 (49.1)
Low & negative positivity	27 (49.1)

*Continuous data are presented as mean ± standard deviation of mean.
Number of children, Lactation and Months of lactation calculated in parous women
End-points don´t add up to 100% in some categories because of missing data

Table 2. Total effective xenoestrogen burden (TEXB) at surgery and at "*second follow-up measurement*" (6-18 months) in the same individual patients*

	Sample		
TEXB concentrations	**Surgery**	**"Second follow-up"**	***p* value[a]**
TEXB-alpha (pM Eeq/ml)			
Mean (SD)	89.7 (463)	124 (220)	
Median	0.8	32.4	0.001
Minimum	0.1	0.1	
Maximum	2625	750	
TEXB-alpha (pM Eeq/g lipid)			
Mean (SD)	188 (938)	572 (1121)	
Median	1.7	169	<0.001
Minimum	0.1	0.1	
Maximum	5320	4212	
TEXB-beta (pM Eeq/ml)			
Mean (SD)	13.4 (21.5)	103 (90.3)	
Median	3.1	66.9	<0.001
Minimum	0.1	0.4	
Maximum	111	296	
TEXB-beta (pM Eeq/g lipid)			
Mean (SD)	32.1 (54.4)	483 (510)	
Median	13.8	320	<0.001
Minimum	0.1	0.8	
Maximum	235	1780	

SD standard deviation of mean
* n = 32
[a] Wilcoxon Signed Ranks test (two-tail)
Breast adipose samples were collected at surgery and from abdominal fat depot during follow-up

Table 3. Variables influencing total effective xenoestrogen burden (TEXB) values at "*second follow-up measurement*" (6-18 months)

Fraction	Independent variables	β[a]	(95% CI)	*p* value[b]
TEXB-alpha (pM Eeq/ml)	Age (years)	-0.064	(-0.147, 0.018)	0.122
	BMI (Kg/m^2)	-0.001	(-0.150, 0.147)	0.988
	Parity	0.741	(-1.861, 3.343)	0.566
	Months of lactation	-0.024	(-0.081, 0.033)	0.393
	Number of children	-0.415	(-1.082, 0.253)	0.215
	Number of reproductive cycles			
	367-414 cycles vs. ≤366 cycles	-0.725	(-3.174, 1.724)	0.550
	>414 cycles vs. ≤366 cycles	0.602	(-2.898, 1.694)	0.596
	Educational level			
	Formal education vs. WFE	0.630	(-1.759, 3.019)	0.595
	Exposure of pesticides	-0.030	(-2.837, 2.778)	0.983
	Familiar history of breast cancer	-0.136	(-2.185, 1.914)	0.894
	Tumor stage			
	Stage II vs. stage I	-1.592	(-3.991, 0.807)	0.185
	Stage III vs. stage I	-2.211	(-4.990, 0.568)	0.115
	Postoperative chemotherapy	0.116	(-2.856, 3.087)	0.937
	Radiotherapy & chemotherapy	0.945	(-1.587, 3.478)	0.452
	Estrogen receptor (RE)			
	High & moderate vs. low & negative positivity	2.121	(0.176, 4.067)	**0.034**
	Progesterone receptor (PR)			
	High & moderate vs. low & negative positivity	0.268	(-1.551, 2.087)	0.766
TEXB-alpha (pM Eeq/g lipid)	Age (years)	-0.064	(-0.152, 0.023)	0.143
	BMI (Kg/m^2)	-0.042	(-0.201, 0.118)	0.597
	Parity	-0.977	(-3.720, 1.766)	0.473
	Months of lactation	-0.026	(-0.086, 0.034)	0.387
	Number of children	-0.392	(-1.102, 0.317)	0.268
	Number of reproductive cycles			
	367-414 cycles vs. ≤366 cycles	-0.953	(-3.535, 1.628)	0.457
	>414 cycles vs. ≤366 cycles	-0.716	(-3.137, 1.704)	0.550
	Educational level			
	Formal education vs. WFE	0.630	(-1.898, 3.158)	0.615
	Exposure of pesticides	0.012	(-2.957, 2.981)	0.993
	Familiar history of breast cancer	-0.060	(-2.228, 2.108)	0.955
	Tumor stage			
	Stage II vs. stage I	-1.424	(-3.998, 1.151)	0.268
	Stage III vs. stage I	-1.904	(-4.886, 1.079)	0.202
	Postoperative chemotherapy	0.317	(-2.824, 3.457)	0.838
	Radiotherapy & chemotherapy	1.004	(-1.659, 3.667)	0.447
	Estrogen receptor (RE)			
	High & moderate vs. low & negative positivity	1.964	(-0.131, 4.059)	**0.065**
	Progesterone receptor (PR)			
	High & moderate vs. low & negative positivity	0.189	(-1.736, 2.114)	0.843

[Continued next page]

Fraction	Independent variables	β[a]	(95% CI)	*p* value[b]
TEXB-beta (pM Eeq/ml)	Age (years)	-0.028	(-0.081, 0.025)	0.290
	BMI (Kg/m^2)	-0.043	(-0.154, 0.068)	0.433
	Parity	-1.128	(-2.755, 0.499)	0.167
	Months of lactation	-0.028	(-0.063, 0.008)	0.118
	Number of children	-0.257	(-0.686, 0.172)	0.232
	Number of reproductive cycles			
	367-414 cycles vs. ≤366 cycles	-0.256	(-1.780, 0.167)	0.733
	>414 cycles vs. ≤366 cycles	0.709	(-0.719, 2.137)	0.319
	Educational level			
	Formal education vs. WFE	0.320	(-1.215, 1.856)	0.673
	Exposure of pesticides	-0.594	(-2.383, 1.194)	0.503
	Familiar history of breast cancer	-0.273	(-1.585, 1.039)	0.675
	Tumor stage			
	Stage II vs. stage I	-0.630	(-2.187, 0.926)	0.415
	Stage III vs. stage I	-1.260	(-3.063, 0.543)	0.164
	Estrogen receptor (RE)			
	High & moderate vs. low & negative positivity	-0.142	(-1.486, 1.201)	0.830
	Progesterone receptor (PR)			
	High & moderate vs. low & negative positivity	-0.441	(-1.599, 0.717)	0.443
TEXB-beta (pM Eeq/g lipid)	Age (years)	-0.028	(-0.086, 0.029)	0.322
	BMI (Kg/m^2)	-0.083	(-0.202, 0.036)	0.163
	Parity	-1.354	(-3.112, 0.403)	0.126
	Months of lactation	-0.030	(-0.068, 0.009)	0.127
	Number of children	-0.227	(-0.698, 0.244)	0.333
	Number of reproductive cycles			
	367-414 cycles vs. ≤366 cycles	-0.472	(-2.130, 1.185)	0.565
	>414 cycles vs. ≤366 cycles	0.614	(-0.940, 2.169)	0.426
	Educational level			
	Formal education vs. WFE	0.285	(-1.387, 1.957)	0.731
	Exposure of pesticides	-0.543	(-2.493, 1.407)	0.574
	Familiar history of breast cancer	-0.211	(-1.640, 1.218)	0.765
	Tumor stage			
	Stage II vs. stage I	-0.447	(-2.170, 1.276)	0.600
	Stage III vs. stage I	-0.950	(-2.946, 1.046)	0.339
	Estrogen receptor (RE)			
	High & moderate vs. low & negative positivity	-0.326	(-1.784, 1.132)	0.651
	Progesterone receptor (PR)			
	High & moderate vs. low & negative positivity	-0.535	(-1.792, 0.721)	0.392

All variables are adjusted for age. *WFE* Without formal education
* n = 34
[a] β: Regression coefficient (all concentrations of TEXB were log-transformed)
[b] *p* value of each category of the variable when comparing with the reference group
* The variable named "*second follow-up measurement*" was created for those patients with TEXB-alpha and TEXB–beta data values at the time of surgery as well as at 6-12 months or 12-18 months of the

follow-up; if available, the concentration of TEXB at 6-12 months was preferably considered and, if it was missing, the concentration at 12-18 months

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