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Breast cancer risk and the combined effect of environmental estrogens

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Abstract

Objective: The present study aimed to determine whether the combined effects of environmental estrogens measured as the total effective xenoestrogen burden (TEXB-alpha) are a risk factor for breast cancer over and above the risk potentially linked to specific pesticides.
Methods: We measured the levels of 16 organochlorine pesticides as well as TEXB in adipose tissue of 198 women at the time of breast cancer diagnosis. These were compared with findings in 260 age and hospital matched control women without breast cancer.
Results: The median levels of *p,p′*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene), aldrin, endosulfan ether and lindane (the pesticides detected in $>40\%$ of the study population) were higher in cases than controls, although the differences did not reach statistical significance. After adjusting for potential confounders, the odds ratio (OR) for breast cancer in women with detectable levels of aldrin was 1.55 (95% confidence interval (CI) 1.00–2.40). Among the postmenopausal women, the OR for aldrin and lindane was 1.84 (95% CI 1.06–3.18) and 1.76 (95% CI 1.04–2.98), respectively. Among cases with body mass index (BMI) below the median (28.6 kg/m^2), the OR was 3.42 (95% CI 1.22–9.58) for women in the highest quartile of TEXB-alpha *versus* those in the lowest. The subgroup of leaner postmenopausal women showed an increased risk (OR: 5.67; 95% CI 1.59–20.21) for those in the highest tertile *versus* those in the lowest.
Conclusions: We found an increased risk for breast cancer in the leaner women, especially in the leaner postmenopausal subgroup, related to the TEXB-alpha. The pesticides aldrin and lindane are also individually associated with risk.

Introduction

The disturbing possibility that the bioaccumulation of environmental estrogens (xenoestrogens) may cause breast cancer was raised by some past epidemiological studies on environmental and occupational exposure [1–3]. Certain organochlorine compounds such as 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) and related metabolites, dieldrin, hexachlorocyclohexane isomers (HCH), and some polychlorinated biphenyls (PCBs) have been described as candidates for this effect [4, 5]. Associations have been reported between breast cancer risk and serum or fat tissue levels of 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) or DDT [4, 6–10], sometimes linked to women with estrogen positive tumors [8]. Serum levels of dieldrin have been associated with a significance increase in breast cancer risk and mortality [11–13]. PCBs have also

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been related to breast cancer risk, either individually or collectively, mainly in subgroups of study populations [13–15]. Some authors have also associated exposure to PCBs with the aggressiveness of the tumor [16]. Some weakly positive results have been described for PBB [17], OCDD [18] and hexachlorobenzene [15]. However, the evidence is contradictory and many other studies found no association between these chemicals and breast cancer risk [19–33]. More recent studies have focused on the relationship between exposure to xenoestrogens and polymorphism in the genes encoding biotransformation enzymes [34], pointing to the need for a better definition of susceptible population groups.

Investigation of this issue faces difficult challenges, which may explain the lack of consistency in the results. The association may vary among population or ethnic groups [23], as well as among subgroups defined by genetic predisposition, thereby limiting the replication of the results. Xenobiotics may also interact with other environmental, dietary, lifestyle and reproductive factors, which are not systematically measured across studies [35]. More importantly, a hypothetical association between organochlorines and breast cancer risk cannot be tested on the basis of individual compound levels, and account must also be taken of possible synergetic, additive, or antagonistic interactions between the chemicals.

There has been scant research on interactions between xenoestrogens and natural estrogens or between chemicals with hormonal activity, and only a few compounds have been studied [36–39]. Different methods have been proposed to overcome the unpredictability of xenoestrogen interactions, which derives from possible additive, synergistic, or antagonistic effects. According to Payne *et al.* [39], mixture effects can be predicted from the potency of individual agents if the effects of individual agents and mixtures are analyzed within the same system in relation to identical endpoints, regardless of the complexity of the system. The major drawback of this approach is that enormous resources would be required to test all the compounds known to have anti-estrogenic or estrogenic activity. Moreover, an unknown number of such compounds have yet to be identified.

In order to facilitate the rigorous testing of this putative link between exposure to xenoestrogens and disease, we developed and standardized a method to assess the total effective xenoestrogen burden (TEXB) in human adipose tissue and serum [40–43]. High performance liquid chromatography (HPLC) is used to separate environmental estrogens (alpha-fraction) from sex-steroids (beta-fraction), and the combined estrogenic effect of the extracts is then determined from its proliferative effect on MCF-7 human breast cancer cells [36]. Extensive testing [41–43] demonstrated that the pesticides DDT and metabolites, dieldrin, aldrin and lindane, among other organochlorines, as well as other chlorinated and/or brominated organohalogenated chemicals, all elute in the HPLC alpha-fraction. The beta-fraction eluted by HPLC contains endogenous sex-steroids and more polar xenoestrogens, distinct from those eluted in the alpha-fraction, such as sex-steroids, nonylphenol, octylphenol, and bisphenol-A. The estrogenicity of the alpha-fraction, which contains no endogenous sex-hormones, can be considered a marker of the TEXB of environmental organohalogenated estrogens [43].

The present study aimed to determine whether the combined estrogenic effects of environmental estrogens are a risk factor for breast cancer and to establish the potential role of specific pesticides. Our measurement was performed on adipose tissue samples collected in a hospital-based, case–control study on breast cancer. The combined effect of chemical residues was assessed in a biological assay for estrogenicity, and patients were classified according to their TEXB.

Materials and methods

Participants

A hospital-based case–control study was conducted from April 1996 through June 1998 in the three largest public hospitals serving Granada and Almeria provinces in Southern Spain. Cases were recruited from women aged between 35 and 70 years undergoing surgery for newly diagnosed malignant breast carcinoma (77.2% infiltrating ductal carcinoma, 9.8% lobular carcinoma and 13% others), either invasive (95.5%) or *in situ* (4.5%), and without previous history of cancer. Controls were matched for age (± 3 yrs) and hospital. Because adipose tissue was needed for the study purpose, controls were recruited from women undergoing non-cancer-related surgery (65% gall bladder surgery; 20% inguinal hernia or abdominal surgery; 5% varicose vein surgery; and 10% other surgery). Exclusion criteria for controls were the presence of gynecological or endocrine disease, including diabetes, and history of cancer. All the women participating in the study were of Caucasian origin.

We identified 260 cases and 352 controls; 10 (4%) cases and 12 (3%) controls declined to participate. All participants signed informed consent. Adequate adipose tissue samples and interview reports were obtained for

219 (84%) cases and 307 (87%) controls. Breast or abdominal adipose tissue from cases and controls, respectively, were obtained from participants in the course of surgery and always before the initiation of chemotherapy or radiotherapy. Structured face-to-face interviews before surgery were conducted at the hospitals by trained interviewers to gather data on sociodemographic characteristics, reproductive history and fertility, menopausal status, use of exogenous hormones, diet, tobacco and alcohol consumption, and family history of breast cancer. The questionnaire, chemical analysis, and estrogenicity assay were carried out in 198 (76%) cases and 260 (73%) controls.

Laboratory analyses

The laboratory methods have been described in detail elsewhere [43]. Briefly, 200 mg of adipose tissue was extracted in hexane, and pooled fractions were separated by HPLC. Fractions alpha and beta (eluted from 0 to 11, and 13 to 30 min, respectively) underwent parallel chemical and biological analyses.

The presence of aldrin, dieldrin, endrin, lindane, methoxychlor, endosulfan I and II, mirex, *p*-*p*′-DDT, *o*-*p*′-DDT, *o*,*p*′-DDD, *p*,*p*′-DDE, endosulfan 1-1 diol, sulfate, lactone and ether was analyzed by gas chromatography with electron-capture detection, using *p*,*p*′-dichlorobenzofenone as internal standard. The identity of all chemicals was confirmed by gas chromatography and mass spectrometry (GC/MS), as described elsewhere [44, 45]. The reproducibility of the process was established by running 10 fat samples 10 times. Spiked fat samples were run in parallel to assess the recovery of pesticides from adipose tissue. Recoveries of the organohalogenated compounds ranged from 83.45% for lindane to 102.12% for dieldrin. Coefficients of variation ranged from 3.63 (p,p′DDT) to 10.95 (endosulfan-ether) [43]. Operational quality control procedures also included daily calibrations. We previously reported the limits of detection (LD), which ranged from 0.1 ng/ml for endosulfan ether to 3 ng/ml for endrin [43]. Lipid content was quantified gravimetrically and xenoestrogen concentrations were expressed in nanograms per gram of lipid [43].

To assess the biological effect of the tissue extracts, we used a slightly modified version [46] of the MCF7 cell-estrogenicity test [36]. Each sample was assayed in triplicate with a negative (vehicle) and positive (estradiol) control in each plate. The proliferative effect of the fractions was referred to the maximal effect obtained with estradiol and expressed as TEXB (TEXB-alpha and TEXB-beta) in estradiol equivalent units (Eeq) per gram of lipid [43].

Statistical analysis

The organochlorine content and TEXB-alpha and -beta values were converted to natural logarithms. A concentration equal to half the LD was assumed for samples with organochlorine levels below the LD. Descriptive statistics are reported as geometric mean and standard deviation. The Student's *t* test was used to compare log-transformed adipose concentrations of target chemicals between cases and controls. Associations among continuous variables were assessed with Spearman correlation coefficient. A two-sided p less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS statistical software [47].

Odds ratios (ORs) for breast cancer and 95% confidence interval (CI) were computed by unconditional logistic regression. The OR was only calculated for DDE, aldrin, lindane, and endosulfan-ether, i.e., the chemicals detected above the assay LD in at least 40% of the samples. For aldrin, lindane and endosulfan-ether, the analysis compared the proportion of women above and below the LD. For DDE, TEXB-alpha and TEXB-beta, quartiles based on the frequency distribution of the controls were used in the statistical analysis.

Adjustment was made for potential confounders and the matching variables, age and hospital. Potential confounders included marital status, education level, social class, occupation, number of full-term pregnancies, age at first full-term pregnancy, months of lactation, natural logarithm of the body mass index (BMI), first-degree family history of breast cancer, use of oral contraceptives, use of hormone replacement therapy, menopausal status, age at menarche, age at menopause, and tobacco and alcohol consumption. We investigated the modifying effect of these variables and the association with organochlorine levels and TEXB values. We also conducted stratified analyses for menopausal status, BMI (above/below median value) and parity (nulliparous/parous).

Results

Mean age of the cases was slightly lower than controls (54.8 *versus*. 56.8; $p = 0.06$), despite the matching on age. BMI was also lower in cases (27.3 kg/m^2 *versus*. 29.6 kg/m^2; $p < 0.01$). Differences in other risk factors for breast cancer between case and control subjects were statistically significant for marital status, education level, occupation, number of full-term pregnancies, age at first full-term pregnancy, months of lactation, BMI, family history of breast cancer, menopausal status, and smoking and alcohol consumption (Table 1).

Table 1. Characteristics of the study population

	Cases (n = 198)	Controls (n = 260)	OR[a] (95% CI)	p for trend
Marital status				
Single	17	7	1	
Married	152	205	0.31 (0.11–0.81)	
Separated	29	48	0.25 (0.08–0.74)	
Education level				
Illiterate	30	68	1	
Write and read	80	107	1.69 (0.98–2.94)	
Secondary	68	78	1.98 (1.11–3.51)	
University	20	7	6.48 (2.28–19.07)	< 0.01
Occupation[b]				
Homemaker	50	91	1	
NCO groups 4–9	120	153	1.43 (0.92–2.22)	
NCO groups 1–3	28	16	3.19 (1.49–6.85)	
No. of full-term pregnancies				
0–1	35	24	1	
2–3	110	130	0.58 (0.31–1.07)	
4–5	40	68	0.40 (0.20–0.81)	
≥6	13	38	0.23 (0.10–0.57)	< 0.01
Age at 1st full-term pregnancy				
≤19	17	32	1	
20–25	80	138	1.09 (0.54–2.20)	
≥26	76	73	1.96 (0.95–4.05)	< 0.01
Lactation (months)				
0	53	59	1	
1–10	59	55	1.19 (0.69–2.08)	
11–33	54	63	0.95 (0.55–1.66)	
≥34	32	83	0.43 (0.24–0.77)	< 0.01
BMI (kg/m^2)				
≤25.2	70	44	1	
25.3–28.5	49	63	0.49 (0.28–0.86)	
28.6–32.0	42	75	0.35 (0.20–0.62)	
≥32.1	37	78	0.30 (0.17–0.53)	< 0.01
Family history of breast cancer				
Yes	21	6	5.02 (1.99–12.70)	
No	177	254	1	
Contraceptives				
Yes	60	64	1.33 (0.88–2.02)	
No	138	196	1	
Menopausal status				
Premenopausal	80	77	1	
Postmenopausal	118	183	0.62 (0.41–0.93)	
Age at menopause				
≤44	20	30	1	
45–49	37	62	0.90 (0.42–1.91)	
≥50	61	91	1.01 (0.50–2.03)	0.87
Age at menarche				
≤11	32	62	1	
12–13	103	106	1.88 (1.10–3.22)	
≥14	63	92	1.33 (0.75–2.34)	0.54
Tobacco				
Never	150	223	1	
Former	15	15	1.49 (0.67–3.32)	
Current	33	22	2.23 (1.21–4.14)	< 0.01
Alcohol				
Never	144	217	1	
Former	13	12	1.63 (0.68–3.95)	
Current	41	31	1.99 (1.16–3.43)	< 0.01

[a] Unadjusted. An OR of 1 denotes the reference category.

[b] The occupations of the women were codified according to the Spanish adaptation of the International Standard Classification of Occupations (NCO), International Labour Office: groups 1–3, directors, managers, technicians and professionals; groups 4–9, administrative and general workers.

All the women, both cases and controls, had measurable concentrations of at least one of the 16 pesticides. There were no statistically significant differences between cases and controls in the mean level of any of the pesticides, although the levels of chemicals were higher in cases than in controls for *p,p*'-DDE, aldrin, lindane and endosulfan-ether (Table 2). There was no association or only a very weak association among the different pesticide residues ($-0.07 \geq r_s \geq 0.26$). Interestingly, age ($r_s = 0.16$, $p < 0.01$) and BMI ($r_s = 0.14$, $p < 0.01$) showed a positive correlation with *p,p'*-DDE but not with any other pesticide, with both older and heavier women having higher levels. The TEXB-alpha or -beta fractions showed no association with any of the 16 pesticides quantified, except for endosulfan-ether, which was negatively correlated with TEXB-beta ($r_s = -0.10$, $p = 0.04$). TEXB-alpha and TEXB-beta were positively correlated ($r_s = 0.60$, $p < 0.01$).

In the whole study population, the ORs of the pesticides DDE, aldrin, lindane and endosulfan-ether were greater than unity but were only statistically significant for aldrin (Table 3). Interestingly, among women with a BMI below median, TEXB-alpha showed an OR of 2.44 for the highest quartile with a statistically significant linear trend ($p = 0.03$). After including TEXB-beta in the logistic regression model of the latter subgroup of women, the ORs for the second, third, and fourth quartiles of TEXB-alpha were 1.25 (95% CI 0.54–2.90), 2.00 (95% CI 0.81–4.95), and 3.42 (95% CI 1.22–9.58), with a statistically significant trend ($p = 0.01$). The premenopausal women showed no significant association with the pesticides or with TEXB-alpha or -beta (Table 3). In the postmenopausal group, however, the ORs were statistically significant for aldrin (1.84; 95% CI 1.06–3.18), lindane (1.76; 95% CI 1.04–2.98), and the third quartile of TEXB-alpha (2.18; 95% CI 1.09–4.36), although the trend across all quartiles of TEXB-alpha was not statistically significant (Table 3). Finally, when the postmenopausal women were stratified by BMI, the leaner group showed a statistically significant association with TEXB-alpha; the ORs for the second and third tertile were 2.78 (95% CI 1.00–7.72) and 5.67 (95% CI 1.59–20.21), with a significant trend ($p < 0.01$) (data not shown).

TEXB was found to be dependent on age. TEXB-alpha and -beta decreased with age and, therefore, with the shift from premenopausal to postmenopausal status (data not shown). Although this decrease was statistically significant for both TEXB-alpha and -beta, the reduction in TEXB-beta was greater than that in TEXB-alpha. Interestingly, the BMI did not show statistically significant association with the estrogenicity of the alpha or beta fractions.

Table 2. Concentration of xenoestrogens and TEXB in adipose tissue samples from cases and controls

	Cases (n = 198)		Controls (n = 260)		
	GM	GSD[a]	GM	GSD	P[b]
DDE[c]	326.86	2.78	307.34	3.62	0.57
Aldrin[c]	2.84	4.12	2.37	4.21	0.33
Endosulfan-ether[c]	0.79	1.95	0.75	1.81	0.66
Lindane[c]	6.12	2.84	5.82	3.02	0.67
TEXB-alpha[d]	44.60	14.73	31.79	14.30	0.20
TEXB-beta[d]	76.48	13.74	72.70	14.44	0.86

[a] GM, geometric mean; GSD, geometric standard deviation.
[b] Student's t test.
[c] ng/g of lipid.
[d] Picomolar of Estradiol equivalent (Eeq)/g of lipid.

Discussion

A novel method was used to measure the exposure of women to xenoestrogens by estimating the TEXB from tissue extracts. Estrogenicity from organohalogenated chemicals (alpha-fraction) could be distinguished from that due to endogenous estrogens and the most polar xenoestrogens (beta-fraction). We were unable to detect a statistically significant relationship between cancer risk and TEXB-alpha in the study population as a whole. However, when different groups of women were considered according to epidemiologically relevant factors, relationships of considerable interest emerged. Indeed, separate analysis of the results for the leaner women (BMI < median) allows us to report the first demonstration of a significant relationship between breast cancer risk and the estrogenicity of the alpha-fraction, i.e. the estrogenicity due to bioaccumulated organohalogenated xenoestrogens. Among this leaner group, the women with highest levels of estradiol equivalent in the alpha-fraction (> 197.51 pM Eeq/g lipid; fourth quartile) had a 2.4-fold significantly greater risk of breast cancer than those with the lowest levels (≤0.25 pM Eeq/g lipid; first quartile). When the estrogenicity of the beta-fraction was included in the model, the leaner women with highest levels in the alpha-fraction showed an even greater risk (OR: 3.42; 95% CI 1.22–9.59). Among the leaner postmenopausal women, the risk for those in the highest tertile of TEXB-alpha increased to 5.67 (95% CI 1.59–20.21). No association with breast cancer risk was found for TEXB-beta or for TEXB-alpha and -beta combined, in either the whole study population or any subgroup.

Both the cases and controls came from the same geographical area, in which the Regional Health Service

Table 3. Concentration of xenoestrogens and total effective xenoestrogen burden (TEXB) in adipose tissue and the risk of breast cancer

	All[a]	BMI < Median			BMI > Median			Premenopausal			Postmenopausal		
	OR[b] (95% CI)	Cases n = 121	Controls n = 109	OR[c] (95% CI)	Cases n = 77	Controls n = 151	OR[c] (95% CI)	Cases n = 80	Controls n = 76	OR[c] (95% CI)	Cases n = 118	Controls n = 184	OR[c] (95% CI)
DDE[d]													
≤201.72	1	33	32	1	17	33	1	30	23	1	20	42	1
201.73–397.67	1.04 (0.59–1.84)	40	29	1.29 (0.59–2.83)	14	36	0.74 (0.30–1.82)	22	17	1.07 (0.40–2.87)	32	48	1.14 (0.54–2.43)
397.68–675.97	1.23 (0.69–2.17)	29	19	1.94 (0.83–4.52)	21	46	0.80 (0.35–1.87)	17	20	0.85 (0.30–2.40)	33	45	1.41 (0.66–3.00)
≥675.98	1.22 (0.68–2.21)	19	29	0.91 (0.38–2.14)	25	36	1.46 (0.62–3.43)	11	16	0.64 (0.20–1.99)	33	49	1.58 (0.74–3.37)
	p-trend = 0.40			*p*-trend = 0.85			*p*-trend = 0.33			*p*-trend = 0.48			*p*-trend = 0.18
Aldrin[d]													
< LD	1	80	78	1	51	110	1	53	50	1	78	138	1
> LD	1.55 (1.00–2.40)	41	31	1.74 (0.92–3.30)	26	41	1.50 (0.79–2.84)	27	26	1.07 (0.47–2.42)	40	46	1.84 (1.06–3.18)
Endosulfan-ether[d]													
< LD	1	59	51	1	34	83	1	40	39	1	53	95	1
> LD	1.35 (0.90–2.02)	62	58	1.09 (0.61–1.94)	43	68	1.63 (0.91–2.93)	40	37	0.83 (0.37–1.84)	65	89	1.47 (0.88–2.45)
Lindane[d]													
< LD	1	67	67	1	45	99	1	47	46	1	65	120	1
> LD	1.40 (0.92–2.13)	54	42	1.51 (0.84–2.74)	32	52	1.39 (0.76–2.56)	33	30	1.10 (0.50–2.37)	53	64	1.76 (1.04–2.98)
TEXB-alpha[e]													
≤0.25	1	25	34	1	18	37	1	15	12	1	28	59	1
0.26–41.00	1.15 (0.64–2.05)	25	30	1.12 (0.50–2.52)	17	29	1.24 (0.52–2.95)	16	23	0.57 (0.17–1.87)	26	36	1.76 (0.85–3.67)
41.01–197.50	1.33 (0.76–2.33)	35	25	1.58 (0.70–3.58)	22	40	1.08 (0.48–2.42)	22	21	0.63 (0.20–2.02)	35	44	2.18 (1.09–4.36)
≥197.51	1.31 (0.74–2.31)	36	20	2.44 (1.03–5.78)	20	45	0.80 (0.35–1.84)	27	20	1.27 (0.39–4.14)	29	45	1.39 (0.68–2.84)
	p-trend = 0.30			*p*-trend = 0.03			*p*-trend = 0.55			*p*-trend = 0.53			*p*-trend = 0.22
TEXB-beta[e]													
≤9.95	1	23	26	1	22	37	1	14	11	1	31	52	1
9.96–100.00	1.08 (0.61–1.90)	37	35	1.19 (0.53–2.66)	15	31	1.01 (0.42–2.45)	16	19	0.34 (0.09–1.23)	36	47	1.52 (0.77–3.00)
100.01–550.00	1.05 (0.59–1.86)	35	24	1.48 (0.63–3.48)	18	41	0.80 (0.35–1.83)	25	21	0.69 (0.19–2.49)	28	44	1.18 (0.58–2.38)
≥550.01	0.99 (0.55–1.79)	26	24	0.96 (0.39–2.38)	22	42	0.91(0.42–2.02)	25	25	0.69 (0.20–2.39)	23	41	0.99 (0.47–2.10)
	p-trend = 0.99			*p*-trend = 0.91			*p*-trend = 0.72			*p*-trend = 0.82			*p*-trend = 0.88

[a] All women.
[b] OR adjusted for age, reference hospital, ln BMI, number of children, age at first full-term pregnancy, family history of breast cancer, and alcohol and tobacco consumption.
[c] OR adjusted for age, reference hospital, number of children, age at first full-term pregnancy, family history of breast cancer, and alcohol and tobacco consumption.
[d] ng/g of lipid.
[e] picomolar of Estradiol equivalent (Eeq)/g of lipid.

provides universal medical cover. There are no private hospitals with Oncology Departments in the area under study. One possible shortcoming of the present work is that most of the controls underwent surgery for diseases of the gall bladder and hernia, which are associated with obesity as a risk factor. The high mean BMI of the controls in our study is consistent with a recent report by the European Prospective Investigation into Cancer and Nutrition (EPIC), which stated that 92% of Granada women in the same age range were overweight (BMI > 25) [48]. Wolff and Anderson [49] proposed a pharmacokinetic model by which heavier women show a lower concentration of organochlorine compounds during the uptake period and subsequently for around 15 years, after which time they show higher concentrations of these compounds in comparison with leaner women. According to their model, our study might underestimate the risk, because the greater weight of our controls would imply higher xenoestrogen levels than if they had been leaner. To control for this variable, the whole study population was stratified by BMI. It is not clear why the significant effect of TEXB-alpha in the leaner women was not found in the more obese group. One possible explanation may be a greater relative impact of exogenous estrogens in leaner women, in whom there is no counterbalance due to endogenous hormones accumulated in fat.

A further potential limitation is that breast fat was obtained for cases and abdominal fat for controls. However, several studies have shown a good correlation between measurements at these two sites [50]. Studies that used fat from sites other than the breast as controls, such as those by Van't Veer *et al.* [25] and Stellman *et al.* [26] did not report that the different origin of the tissue modified the results. In fact, Stellman *et al.* [26] found that levels of DDE and other organochlorines did not differ between adipose breast tissue samples from controls with benign breast disease and samples derived from surgery for gall bladder disease or abdominal hernia, very similar to the present study.

When the results were studied according to menopausal status, a further significant relationship was disclosed between breast cancer risk and the estrogenicity of the alpha-fraction. The third quartile of the postmenopausal women had a 2.18-fold increased risk in comparison to those with lowest estrogenic levels in the alpha-fraction. Moreover, when postmenopausal status was stratified by BMI, the leaner postmenopausal women in the highest tertile of TEXB-alpha showed a 5-fold increased risk for breast cancer. Interestingly, this relationship was not found among the premenopausal women, and may be related to the decline in ovarian estrogen production with menopause, and to the increased relative importance of the xenoestrogens bioaccumulated in their adipose tissue. Indeed, the TEXB of both alpha and beta fractions varied according to menopausal status, with a 2.6-fold decline in the total burden of the beta-fraction and a lower fall (1.6-fold) in that of the alpha-fraction after menopause. The age had a similar effect on TEXB values, with a 3.5- and 5-fold decline in the estrogenicity of the alpha and beta fractions, respectively, between the first and last quartiles.

Endogenous estrogens, responsible for the hormonal activity of the beta-fraction, may arise from the local production and depot of circulating precursors. Adipose tissue is known to be the primary source of endogenous estrogens after menopause. These estrogens are produced in the fat of both pre- and postmenopausal women through the conversion of precursors by aromatase cytochrome P450, product of the CYP19 gene. Interestingly, a similar mechanism has been suggested for the transcriptional regulation and expression of the CYP19 gene in adipose tissue, regardless of its localization (breast or abdomen) [51]. This expression is strongly dependent on the age of the subject, with an increasing expression of aromatase activity in adipose tissue and, consequently, a higher estrone production. This appears to be inconsistent with the observed decline in the estrogenicity of the beta-fraction with the onset of menopause. However, adipose tissue also plays an important role in the storage and regulation of estrogen in premenopausal women. For example, concentrations of estradiol esters were reported to be 3-fold higher in the adipose tissue of premenopausal *versus* postmenopausal women [52], in agreement with the decrease in estrogenicity observed in our patients with the onset of menopause.

Older women could be expected to have longer exposure to xenoestrogens, especially to bioaccumulative, fat-soluble xenobiotics, and to show higher levels of xenoestrogen-derived estrogenicity (alpha-fraction). However, the TEXB of the alpha-fraction significantly decreased with the age of the patients and at the onset of menopause, as occurred with the estrogenicity of the beta-fraction. The fall in estrogenic activity with age was not accompanied by a decline in organohalogenated chemical levels, which would account for this finding. In fact, DDE levels increased with age, confirming previous observations of the age-dependent bioaccumulation of lipophilic compounds in adipose tissue [53]. The other organochlorine residues showed no variation with age.

All women had measurable concentrations of at least one of the sixteen organochlorines quantified, clearly reflecting the ubiquity of exposure to pesticides in the population, which hampers the demonstration of an

etiologic role. No single chemical could be positively and statistically significantly associated with the biological effect measured by TEXB-alpha. There may be several reasons for this lack of concordance: (i) the estrogenic effects depicted in the E-Screen bioassay are a consequence of the combined effect of several organohalogens; and (ii) the proliferative effect is due to other chemicals not measured, either other organochlorine pesticides or other lipophilic compounds. However, we found that aldrin and lindane may increase the risk of breast cancer. This relationship is biologically supported by the estrogenic properties of both pesticides [54, 55]. Our finding for aldrin may corroborate previous results for dieldrin [11], because aldrin can readily degrade to dieldrin, as reported in soils and multiple species. Both aldrin and dieldrin tend to accumulate in adipose tissue. The use of lindane has been prohibited or restricted in many countries; in Spain and several other European countries, it is allowed for certain specific agricultural purposes and as a medication for head and body lice.

We found no differences in DDE levels between cases and their matched controls. DDE levels were lower in our series compared with other studies, which may be because this pesticide has not been in use since 1980. It has been recommended [33] to explore the association between DDE and breast cancer in populations with more recent exposure, such as Colombia or Mexico City, where epidemiological studies have found a moderately high risk of breast cancer in women with higher levels of DDE [8, 9]. However, negative results were also observed in these areas [20, 22, 56].

The estrogenicity of adipose tissue extracts due to bioaccumulated xenoestrogens, measured as the TEXB-alpha, was associated with a higher risk of breast cancer in the leaner women, especially in the postmenopausal leaner group. Complex interactions between chemicals, endogenous or exogenous hormones and their natural ligands and receptors may alter the internal homeostasis of the estrogenic environment of mammary tissue, leading to malignant transformation and cancer. Thus, future studies of the association between environmental estrogens and breast cancer or other adverse human health effects should analyze the combined effect of these compounds and the interactions with endogenous hormones and other substances that affect endocrine function.

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References

1. Wassermann M, Nogueira DP, Tomatis L, *et al.* (1976) Organochlorine compounds in neoplastic and adjacent apparently normal breast tissue. *Bull Environ Contam Toxicol* **15**: 478–484.
2. Unger M, Olsen J (1980) Organochlorine compounds in the adipose tissue of deceased people with and without cancer. *Environ Res* **23**: 257–263.
3. Unger M, Kiaer H, Blichert-Toft M, Olsen J, Clausen J (1984) Organochlorine compounds in human breast fat from deceased with and without breast cancer and in a biopsy material from newly diagnosed patients undergoing breast surgery. *Environ Res* **34**: 24–28.
4. Djordjevic MV, Hoffmann D, Fan J, Prokopczyk B, Citron ML, Stellman SD (1994) Assessment of chlorinated pesticides and polychlorinated biphenyls in adipose breast tissue using a supercritical fluid extraction method. *Carcinogenesis* **15**: 2581–2585.
5. Davis DL, Bradlow HL, Wolff M, Woodruff T, Hoel DG, Anton-Culver H (1993) Medical hypothesis: xenoestrogens as preventable causes of breast cancer. *Environ Health Perspect* **101**: 372–377.
6. Wolff MS, Toniolo PG, Lee EW, Rivera LM, Dubin N (1993) Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst* **85**: 648–652.
7. Dewailly E, Dodin S, Verreault R, *et al.* (1994) High organochlorine body burden in women with estrogen receptor-positive breast cancer. *J Natl Cancer Inst* **86**: 232–234.
8. Olaya-Contreras P, Rodriguez-Villamil J, Posso-Valencia HJ, Olaya-Contreras P, Cortez JE (1998) Organochlorine exposure and breast cancer risk in Colombian women. *Cad Saude Publica* **14**: 125–132.
9. Romieu I, Hernández-Avila M, Lazcano-Ponce E, Weber JP, Dewailly E (2000) Breast cancer, lactation history, and serum organochlorines. *Am J Epidemiol* **152**: 363–370.
10. Falck F, Ricci A, Wolff MS, Godbold J, Deckers P (1992) Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. *Arch Environ Health* **47**: 143–146.
11. Hoyer AP, Grandjean P, Jorgersen T, Brock JW, Hartvig HB (1998) Organochlorine exposure and risk of breast cancer. *Lancet* **352**: 1816–1820.
12. Hoyer AP, Jorgensen T, Brock JW, Grandjean P (2000) Organochlorine exposure and breast cancer survival. *J Clin Epidemiol* **53**: 323–330.
13. Hoyer AP, Jorgensen T, Grandjean P, Hartvig HB (2000) Repeated measurements of organochlorine exposure and breast cancer risk (Denmark). *Cancer Causes Control* **11**: 177–184.
14. Aronson KJ, Miller AB, Woolcott ChG, Sterns E, McCredy DR, Lickley LA (2000) Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **9:** 55–63.
15. Dorgan JF, Brock JW, Rothman N, *et al.* (1999) Serum organochlorine pesticides and PCBs and breast cancer risk: results from a prospective analysis (USA). *Cancer Causes Control* **10**: 1–11.
16. Demers A, Ayotte P, Brisson J, Dodin S, Robert J, Dewailly E (2000) Risk and aggressiveness of breast cancer in relation to

plasma organochlorine concentrations. *Cancer Epidemiol Biomarkers Prev* **9**: 161–166.
17. Henderson AK, Rosen D, Miller GL, *et al*. (1995) Breast cancer among women exposed to polybrominated biphenyls. *Epidemiology* **6**: 544–546.
18. Hardell L, Lindström G, Liljegren G, Dahl P, Magnuson A (1996) Increased concentrations of octachlorodibenzo-*p*-dioxin in cases with breast cancer-results from a case–control study. *Eur J Cancer Prev* **5**: 351–357.
19. Hunter D, Hankinson S, Laden F, *et al*. (1997) Plasma organochlorine levels and the risk of breast cancer. *N Engl J Med* **337**: 1253–1258.
20. Lopez-Carrillo L, Blair A, Lopez-Cervantes M, *et al*. (1997) Dichlordyltrichloroethane serum levels and breast cancer risk: a case–control study from Mexico. *Cancer Res* **57**: 3728–3732.
21. Helzlsouer KJ, Alberg AJ, Huang HY, *et al*. (1999) Serum concentrations of organochlorine compounds and subsequent development of breast cancer. *Cancer Epidemiol Biomarkers Prev* **8**: 525–532.
22. Mendoca GA, Eluf-Neto J, Andrada-Serpa MJ, *et al*. (1999) Organochlorines and breast cancer: a case–control study in Brazil. *Int J Cancer* **83**: 596–600.
23. Krieger N, Wolff MS, Hiatt RA, Rivera M, Vogelman J, Orentreich N (1994) Breast cancer and serum organochlorines: a prospective study among white, black, and Asian women. *J Natl Cancer Inst* **86**: 589–599.
24. Laden F, Collman G, Iwamoto K, *et al*. (2001) 1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethylene and polychlorinated biphenyls and breast cancer: combined analysis of five US studies. *J Natl Cancer Inst* **93**: 768–776.
25. van't Veer P, Lobbezoo IE, Martin-Moreno JM, *et al*. (1997) DDT (dicophane) and postmenopausal breast cancer in Europe: case–control study. *BMJ* **315**: 81–85.
26. Stellman SD, Djordjevic MV, Britton JA, *et al*. (2000) Breast cancer risk in relation to adipose concentrations of organochlorine pesticides and polychlorinated biphenyls in Long Island, New York. *Cancer Epidemiol Biomarkers Prev* **9**: 1241–1249.
27. Wolff MS, Zeleniuch-Jacquotte A, Dubin N, Toniolo P (2000) Risk of breast cancer and organochlorine exposure. *Cancer Epidemiol Biomarkers Prev* **9**: 271–277.
28. Zheng T, Holford TR, Taylor Mayne S, *et al*. (2000) Risk of female breast cancer associated with serum polychlorinated biphenyls and 1,1-dichloro-2,2'-bis(*p*-chlorophenyl)ethylene. *Cancer Epidemiol Biomarkers Prev* **9**: 167–174.
29. Laden F, Hankinson SE, Wolff MS, *et al*. (2001) Plasma organochlorine levels and the risk of breast cancer: an extended follow-up in the Nurses' Health Study. *Int J Cancer* **91**: 568–574.
30. Gammon MD, Wolff MS, Neugut AI, *et al*. (2002) Environmental toxins and breast cancer on Long Island.II. Organochlorine compound levels in blood. *Cancer Epidemiol Biomarkers Prev* **11**: 686–697.
31. Safe SH (1997) Xenoestrogens and breast cancer. *N Engl J Med* **337**: 1303–1304.
32. Laden F, Hunter DJ (1998) Environmental risk factors and female breast cancer. *Annu Rev Public Health* **19**: 101–123.
33. Snedeker S (2001) Pesticides and breast cancer risk: a review of DDT, DDE, and Dieldrin. *Environ Health Perspect* **109**: 35–47.
34. Laden F, Ishibe N, Hankinson SE, *et al*. (2002) Polychlorinated biphenyls, cytochrome P450 1A1, and breast cancer risk in the nurses' health study. *Cancer Epidemiol Biomarkers Prev* **11**: 1560–1565.
35. Sasco AJ (2001) Epidemiology of breast cancer: an environmental disease? *APMIS* **109:** 321–332.
36. Soto A, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Olea-Serrano MF (1995) The E-SCREEN Assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* **103**: 113–122.
37. Soto A, Fernández MF, Luizzi M, Oles Karasko AS, Sonnenschein C (1997) Developing a marker of exposure to xenoestrogen mixtures in human serum. *Environ Health Pespect* **105**: 647–663.
38. Shekhar PVM, Werdell J, Basrur VS (1997) Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human cells lines. *J Natl Cancer Inst* **89**: 1774–1782.
39. Payne J, Scholze M, Kortenkamp A (2001) Mixtures of four organochlorines enhance human breast cancer cell proliferation. *Environ Health Perspect* **104**: 391–397.
40. Sonnenschein C, Soto A, Fernández MF, Olea N, Olea-Serrano MF (1995) Development of a marker of estrogenic exposure in human serum. *Clin Chem* **41**: 1888–1895.
41. Pazos P, Pérez P, Rivas A, *et al*. (1998) Development of a marker of estrogenic exposure in breast cancer patients. *Adv Exp Med Biol* **444**: 29–40.
42. Rivas A, Olea N, Olea-Serrano MF (1997) Human exposure to endocrine-disrupting chemicals: assessing the total estrogenic xenobiotic burden. *TRAC* **16**: 613–619.
43. Rivas A, Fernández MF, Cerrillo I, *et al*. (2001) Human exposure to endocrine disrupters: standardisation of a marker of estrogenic exposure in adipose tissue. *APMIS* **109**: 185–197.
44. Martínez Vidal JL, Moreno M, Garrido A, Olea-Serrano MF, Olea N (2000) Trace determination of alpha and beta endosulfan and three metabolites in human serum by GC-MS. *Rapid Comm Mass Spectrometry* **14**: 939–946.
45. Hernandez F, Pitarch E, Serrano R, Gaspar JV, Olea N (2002) Multiresidue determination of endosulfan and metabolic derivatives in human adipose tissue using automated liquid chromatographic cleanup and gas chromatographic analysis. *J Anal Toxicol* **26**: 94–103.
46. Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, Pedraza V (1995) The E-SCREEN assay: comparison among different MCF7 cell stocks. *Environ Health Perspect* **103**: 844–850.
47. SPPS Inc. (1999) SPSS 9.0 Base Sintax Reference Guide. Chicago: SPSS Inc.
48. Haftenberger M, Lahmann PH, Panico S, *et al*. (2002) Overweight, obesity and fat distribution in 50- to 64-year-old participants in the European Prospective Investigation into Cancer and Nutrition (EPIC). *Public Health Nutr* **5**: 1147–1162.
49. Wolf MS, Anderson HA (1999) Correspondence re: J.M. Schildkrauut *et al*., Environmental contaminants and body fat distribution. Cancer Epidemiol. Biomark. Prev. 8: 179–183, 1999. *Cancer Epidemiol Biomarkers Prev* **8**: 951–952.
50. Kohlmeier L, Kohlmeier M (1995) Adipose tissue as a medium for epidemiologic exposure assessment. *Environ Health Perspect* **103**: 99–106.
51. Agarwal VR, Takayama K, Van Wyk JJ, *et al*. (1998) Molecular basis of severe gynecomastia associated with aromatase expression in a fibrolamellar hepatocellular carcinoma. *J Clin Endocrinol Metab* **83**: 1797–1800.
52. Larner JM, Shackleton CHL, Roitman E, Schwartz PE, Hochberg RB (1992) Measurement of estradiol-17 fatty acid-esters in human tissues. *J Clin Endocrinol Metab* **75**: 195–200.
53. Moysich KB, Ambrosone CB, Mendola P, *et al*. (2002) Exposures associated with serum organochlorine levels among postmenopausal women from Westren New York State. *Am J Ind Med* **41**: 102–110.

54. Bigsby RM, Caperell-Grant A, Madhukar BV (1997) Xenobiotics released from fat during fasting produce estrogenic effects in ovariectomized mice. *Cancer Res* **57**: 865–869.
55. Traina ME, Rescia M, Urbani E, *et al*. (2003) Long-lasting effects of lindane on mouse spermatogenesis induced by in utero exposure. *Reprod Toxicol* **17**: 25–35.
56. Schecter A, Toniolo P, Dai LC, Thuy LT, Wolff MS (1997) Blood levels of DDT and breast cancer risk among women living in the north of Vietnam. *Arch Environ Contam Toxicol* **33**: 453–456.