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Serum enterolactone levels and the risk of breast cancer in women with palpable cysts

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Abstract

Low levels of lignans, namely enterolactone, have been reported to be associated with an increased risk of breast cancer in the general female population. We assessed, retrospectively, the relationship between serum enterolactone concentrations and the occurrence of breast cancer in women with palpable cysts. The levels of enterolactone in cryopreserved serum aliquots, obtained from 383 women with palpable cysts at the time of their first cyst aspiration, were measured using a time-resolved fluoro-immunoassay (TR-FIA). After a median follow-up time of 6.5 years (range 0.5–12.75 years), 18 women were found to have developed an invasive breast cancer. Median values of serum enterolactone were significantly lower in women who subsequently developed breast cancer: 8.5 nM/l versus 16.0 nM/l: $P=0.04$. Odd Ratios (OR) for breast cancer were: 0.36 ($P=0.03$), 0.57 ($P=0.3$) and 0.38 ($P=0.25$) for 25th (8 nM/l), 50th (16 nM/l) and 75th (24 nM/l) percentile values, respectively. The receiver operating characteristic (ROC) analysis showed a satisfactory accuracy for enterolactone as a breast cancer risk indicator (area under the curve (AUC) = 0.64: $P=0.04$). Logistic regression analysis confirmed that the enterolactone concentration had a strong protective effect on the breast cancer risk. These findings may have important clinical implications with regard to interventional diet-focused chemo-preventive trials.

Keywords: Enterolactone; Breast cancer risk; Cyst type

1. Introduction

Isoflavonoids and lignans are plant compounds that have been shown to exert oestrogenic effects in different experimental conditions and thus are also known as phyto-oestrogens [1]. Main diet isoflavonoids (genistein, daidzein and glycitein) are mostly found in soybeans. The mammalian lignans, enterolactone and enterodiol, have a similar biphenolic structure, but are formed from precursors, that are contained as glycosides mainly in vegetables, whole grain products and berries, through the action of intestinal microflora [2]. Both isoflavonoids and lignans have been shown to have a protective effect against breast cancer, although results reported are still preliminary observations. In particular, the low breast cancer incidence in Asian women has been correlated with their high intake of soy-related phyto-oestrogens [3,4]. Lignans are probably the most important source of phyto-oestrogens in Western diets [5–7] and an inverse relationship between urinary enterolactone excretion or serum enterolactone levels and breast cancer risk has also been previously reported in Refs. [5,6].

The purpose of our study was to investigate whether a similar relationship between serum enterolactone concentration and breast cancer risk might be documented in women affected by palpable cysts. These women represent approximately 7% of women in the Western world and *are* recognised as a group of women with an increased risk of breast cancer [7].

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2. Materials and methods

2.1. Patient selection

A group of 383 women affected by palpable cysts were identified among the several hundreds attending our Department Clinics from 1985 to 1993, on the basis of the availability of properly cryopreserved serum aliquots drawn at the time of their first cyst aspiration. Women were commonly referred by their general practitioners (GPs) following the discovery of a breast lump. Most of the women, diagnosed with gross cyst disease of the breast, were followed regularly through clinical examination and mammography, usually on a yearly basis.

Information about the health status of women who failed to attend our outpatient clinics after one or more yearly visits was obtained through the Genoa Cancer Registry or by phone (for the years not covered by the Registry). These women represented 13% of the total number.

2.2. Serum evaluations

Serum enterolactone concentration was assayed using a time-resolved fluoroimmunoassay (TR-FIA), according to the method reported by Stumpf and colleagues in Ref. [8].

Briefly, thawed samples were centrifuged for 20 min at 4000 rotations per minute (rpm). For the hydrolysis, 200 μl of acetate buffer, pH 5.0, containing 2 mg/ml of β-glucuronidase (G-0751, Sigma) was added to tubes containing 200 μl of fluid. After mixing, the samples were incubated overnight at 37 °C. The following day, enterolactone was extracted twice with 1.5 ml of diethyl ether, the ether phases were transferred and combined in another tube and evaporated to dryness under a nitrogen stream. Assay buffer (200 μl) was added to the tubes and, after careful mixing, 20 μl of the solution, corresponding to 20 μl of the original sample, were taken for TR-FIA. TR-FIA was performed using the DELFIA Research Reagents for the measurement of enterolactone. This TR-FIA is based on competition. Goat anti-rabbit IgGs are immobilised to the walls of low fluorescence microtitre plates thus binding the anti-enterolactone antibody. Europium-labelled enterolactone and sample enterolactone compete for this antibody. Enhancement solution dissociates the europium ions from the labelled enterolactone into solution, where they form highly fluorescent chelates with components in the enhancement solution. Fluorescence from each sample is inversely proportional to the concentration of enterolactone in the sample. Fluorescence was measured through a Victor 1420 Multilabel Counter (Perkin-Elmer Life-Sciences) with time-resolved fluorometry parameters. A program was created for automatic measuring and the results were calculated by including recovery and dilution information. Recovery was estimated as 80% [8].

Intra-assay and interassay coefficients of variations between 4.6 and 6.0% and 5.5 and 9.9%, respectively, have been reported with this method [8]. Coefficients of variations in the same ranges were obtained in the preliminary assays performed by us before the formal analysis of the selected samples.

2.3. Statistical analysis

The risk of breast cancer was expressed as an Odds Ratio and differences between groups were estimated using the Pearson's Chi-square test with Yate's correction [9]. Logistic regression analysis [10] was performed to evaluate all possible interactions between enterolactone serum levels and variables potentially associated with the breast cancer risk, including patient age, family history of breast cancer (a positive history being defined by the presence of a breast carcinoma in first- and/or second−degree relatives) and cyst type (as defined through the Na/K ratio of the cyst fluid). A receiver operating characteristic (ROC) curve was constructed by plotting sensitivity versus 1− specificity for all possible cut-off points to evaluate the performance of enterolactone as a potential indicator of breast cancer risk. Performance, i.e. accuracy, was calculated on the basis of the area under the curve (AUC) [11].

In order to obtain a certain degree of 'blindness', the women's stories and health status were not known by the two people who performed the analysis of the samples.

3. Results

18 out of 383 patients were found to have developed an infiltrating breast cancer 5–98.5 months following their first cyst aspiration (median time from first cyst aspiration to breast cancer diagnosis: 53 months). The median age at the aspiration of the first cyst of the entire group of women was 46 years (25–79 years) and the median follow-up time (since cyst aspiration) was 6.5 years (range 0.5–12.75 years) for a total person/years equal to 2390.2. Family history of breast cancer was positive for 73 women. Cyst fluid cation content had previously been assessed through a selective ion-electrode technique [12] and was known for 302 women, of whom 218 had a Na/K ratio <3 (which defined type I cysts) and 84 had a Na/K ratio >3 (which defined type II cysts). For the purpose of present analysis, the 81 patients for whom cyst type was unknown, constituted a separate group with respect to this variable.

Enterolactone was detectable in variable amounts in the sera collected, varying from 1 to 140 nM/l with a median value of 16 nM/l and a mean value of 19.6 nM/l

(standard error of the mean (SEM)±0.98). Both the mean and median enterolactone concentrations were lower in women who had developed a breast cancer than in those who had not, the latter difference being statistically significant (mean concentrations±SEM: 14.7±4.25 nM/l versus 19.8±1.01 nM/l: $P=0.3$; median concentrations: 8.5 nM/l versus 16.0 nM/l: $P=0.04$). Table 1 shows the Odds Ratios according to the different percentile values. An inverse relationship was recorded between the risk of breast cancer and the serum concentration of enterolactone, irrespective of the percentile value considered. However, the difference between the ratios was statistically significant only for the 25th percentile value, which corresponded to a serum concentration of 8 nM/l. The ROC curve generated with all possible enterolactone cut-off points is shown in Fig. 1. The AUC was 0.64 (95% CI 0.51–0.77: $P=0.04$) which equals a satisfactory accuracy.

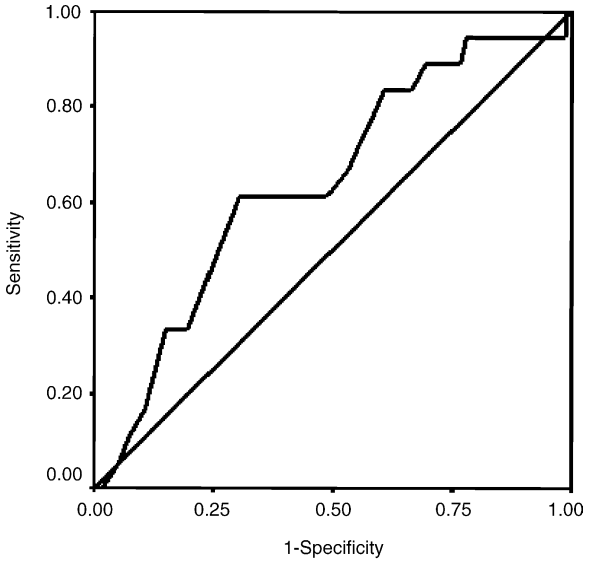

Fig. 1. Receiver operating characteristic (ROC) curve obtained by plotting sensitivity versus 1−specificity of all possible enterolactone cut-off points. (Area under the curve (AUC)=0.64: $P=0.04$; see text for further detail).

The cut-off value of 8 nM/l had a sensitivity of 50% and a specificity of 74%. Increasing the cut-off value resulted in an increasing sensitivity, but a lower specificity.

Relative risks (RR) of breast cancer according to the patient's age at aspiration, family history of breast cancer, cyst type and enterolactone concentration are shown in Table 2.

Serum enterolactone concentration was the only variable to be significantly correlated with the breast cancer risk, both after univariate and multivariate analyses.

4. Discussion

Higher risks of developing breast cancer, ranging from 1.7 to 7.5, have been reported in the literature for women with palpable cysts [13,14]. Several attempts have been made to identify women with palpable cysts

Table 2
Relative Risk (RR) of patients according to their age at aspiration, family history of breast cancer, cyst type and serum enterolactone concentration

	RR	(95% CI)	*P* value
Serum enterolactone (nM/l)			
⩽8	1.0		
>8	0.36	(0.14–0.93)	0.04
Age at first aspiration (years)			
⩽46	1.0		0.9
>46	0.96	(0.36–2.58)	
Family history of breast cancer			
Negative	1.0		0.7
Positive	1.27	(0.39–4.05)	
Cyst Type			
I (Na/k<3)	1.0		
II (Na/k>3)	0.45	(0.09–2.04)	0.5
Unknown	1.16	(0.38–3.54)	

Table 1
Odds Ratio (OR) of women according to the percentile value of the serum enterolactone concentration

	Person/years	No. of women developing breast cancer	OR	(95% CI)	*P* value
25th percentile value (nM/l)					
⩽8	662.3	9	1.0		
>8	1727.9	9	0.36	(0.14–0.925)	0.03
50th percentile value (nM/l)					
⩽16	1332.5	12	1.0		0.3
>16	1057.7	6	0.57	(0.21–1.54)	
75th percentile value (nM/l)					
⩽24	1847.7	16	1.0		0.25
>24	542.5	2	0.38	(0.085–1.67)	

95% CI, 95% Confidence Interval.

who may be at a higher risk on the basis of their age, family history of breast cancer or cyst type [7,14–21]

Dixon and colleagues studied 1374 women with palpable breast cysts and found an overall standardised incidence rate (SIR) of breast cancer of 2.81 (95% CI 2.17–3.59). The risk was particularly increased for women aged 45 years or less at the time of the cyst aspiration (RR = 5.54 (2.87–10.63)), but was independent of the cyst type [7]. In our analysis, age did not appear to correlate with the breast cancer risk, after either univariate or multivariate analyses, although we used a similar cut-off value for age of 46 years, which represented the median age of our series. It is not clear why younger women with palpable cysts should have an increased risk of breast cancer, since breast cancer risk is reported to increase with aging in the general population [15]. In accordance with Dixon's findings [17], but in contrast with the results of two previous studies [16,17], cyst type was not an independent predictor of breast cancer risk in our study, although a slightly decreased risk for women with type II cysts was demonstrated. There is no clear explanation for the decreased risk of breast cancer among women with type II cysts. However, profound differences in the distribution of sex hormones and growth factors have been previously reported in the two cyst types [18–21]. The concentration of sex hormones and mutagenic growth factors like the epidermal growth factor (EGF) is in fact substantially higher in type I cysts [18,19], while transforming growth factor β (TGFB), which is known to have inhibitory effects on the growth of breast epithelial cells, is preferably accumulated in type II cysts [20,21].

Interestingly, there were no appreciable differences in the serum enterolactone concentrations in our study based on cyst type (data not shown).

Family history of breast cancer is a recognised risk factor for breast cancer in the general population, with risks of 1.5–3 being reported in the literature [22,23]. In our study, family history had only a moderate effect on the breast cancer risk. However, our findings are probably not conclusive in this regard, due to the small number of patients with a positive family history, which precluded any further analysis according to the degree of positivity.

While we found no substantial effects for family history, cyst type and age at aspiration, we found a strong association between the serum enterolactone concentration and the breast cancer risk.

Circulating levels of enterolactone are determined by the consumption of whole grains products and by the intake of fruit and berries. In the women selected for our study, serum levels of enterolactone were extremely variable. Although these women represent just a subset of the female population, their serum enterolactone levels are likely to reflect those of the general population. Indeed, they do not differ from the levels previously described in an omnivorous Finnish population [24] or in women living in the New York area in the United States of America (USA) [25].

In particular, according to the estimations of Kikkinem and colleagues [24], serum enterolactone levels comparable to the average level found in the women selected for our study are consistent with the moderate/high consumption of vegetables and fresh fruits that characterises the Italian diet. However, a positive association with constipation has also been found, both in men and in women, strengthening the role for gut microflora [24]. Unfortunately, we had no information about the specific dietary habits or bowel movements of our study population. However, whatever the mechanism implied, there is no doubt that in our study circulating enterolactone levels of women who developed breast cancer were significantly lower than those recorded in women who did not.

Several factors might affect our findings, including the small size of our cohort of women and the relatively small number of cancers that occurred after cyst aspiration. However, our results match perfectly those of two previous case–control studies. Ingram and colleagues [5] have reported that a high urinary excretion of both equol and enterolactone was associated with a substantial reduction in the breast cancer risk, with significant trends through the quartiles. In spite of the different design and the different methodological approaches, it is noteworthy that the size of risk reduction related to urinary enterolactone levels in Ingram's study is quite comparable to that related to serum enterolactone levels in our own study. A significant association between breast cancer risk and serum enterolactone levels was also reported by Pietinen and colleagues in Ref. [6]. These investigators performed a case–control study, involving 184 breast cancer cases and 208 community-based controls, using exactly the same fluoroimmunoassay that was employed in our study. Again, higher levels were found in controls than in the cancer cases. Moreover, the risk for women with enterolactone levels above the 20th percentile value—which, interestingly enough, was comparable to the 25th percentile value of our cohort (6.0 and 8.0 nM/l, respectively)—was approximately 50% lower than in women with levels under this threshold.

One major objection that could be made against our study and the previous two case–control studies is that a single serum measurement may not be reliable. This point has been specifically addressed by Zeleniuch-Jacquotte and colleagues [25] who examined the distribution and long-term reliability of serum measurement of lignans and isoflavonoids in a group of 60 women enrolled in a Women's Health Study on sex hormones and breast cancer risk, conducted by New York University. While reliability coefficients for isoflavonoids were low, the reliability coefficient of a single measurement of

enterolactone was moderately high (0.55), suggesting that even a single serum measurement of this compound could be appropriate in prospective epidemiological studies.

The likely protective effect of lignans, namely of enterolactone, on mammary carcinogenesis appears to be supported by the growing body of knowledge on the biological effects exerted by lignans and isoflavonoids in multiple experimental systems. In addition to anti-oxidants properties [26], these compounds can actually interfere with oestrogen synthesis and bioavailability, by inhibiting human aromatases [27] and stimulating SHBG synthesis in the liver [28]. Moreover, they can exert anti-angiogenetic properties [29]. It has been demonstrated in animal models that a diet that is very rich in lignans can interfere with mammary tumour genesis and growth [30]. Although results achieved *in vitro* have been somewhat conflicting—both oestrogenic and anti-oestrogenic effects have been reported for enterolactone in breast cancer cells in culture [31,32], inhibitory effects for enterolactone and other phyto-oestrogens on 17-β-oestradiol and EGF-stimulated DNA synthesis have recently been reported when these compounds were added to the culture medium of MCF-7 cells in high concentrations [33].

In conclusion, the serum enterolactone concentration was inversely correlated with the risk of breast cancer in women with palpable cysts, irrespective of their age, cyst type or family history of breast cancer. Our results confirm the results of previous case–control studies. Altogether, these results suggest that very low levels of enterolactone may be associated with a significantly increased breast cancer risk and might thus have important clinical implications with regard to interventional diet-focused trials.

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