

Serum enterolactone levels and mortality outcome in women with early breast cancer: a retrospective cohort study

Pamela Guglielmini · Alessandra Rubagotti ·
Francesco Boccardo

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Abstract We previously demonstrated that high serum enterolactone levels are associated with a reduced incidence of breast cancer in healthy women. The present study was aimed at investigating whether a similar association might be found between serum enterolactone levels and the mortality of women with early breast cancer. The levels of enterolactone in cryopreserved serum aliquots obtained from 300 patients, operated on for breast cancer, were measured using a time-resolved fluoro-immunoassay. Levels were analyzed in respect to the risk of mortality following surgery. Cox proportional hazard regression models were used to check for prognostic features, to estimate hazard ratios for group comparisons and to test for the interaction on mortality hazards between the variables and enterolactone concentrations. The Fine and Gray competing risk proportional hazard regression model was used to predict the probabilities of breast cancer-related and breast cancer-unrelated mortalities. At a median follow-up time of 23 years (range 0.6–26.1), 180 patients died, 112 of whom died due to breast cancer-related events. An association between a decreased mortality risk and enterolactone levels ≥ 10 nmol/l was found in respect to both all-cause and breast cancer-specific mortality. The difference in mortality hazards was statistically significant, but it appeared to decrease and to lose significance after the first 10 years, though competing risk analysis showed that

breast cancer-related mortality risk remained constantly lower in those patients with higher enterolactone levels. Our findings are consistent with those of most recent literature and provide further evidence that mammalian lignans might play an important role in reducing all-cause and cancer-specific mortality of the patients operated on for breast cancer.

Keywords Serum enterolactone · Early breast cancer · Mortality

Introduction

Enterolactone and enterodiol, the most important lignans of western diets [1], are formed from precursors that are contained mainly in whole grain products, vegetables, fruits, berries and wine [2]. These compounds have shown a protective effect against hormone-dependent cancers [3]. However, results of studies relative to breast cancer are still contradictory [4, 5]. Our group has previously demonstrated an inverse relationship between serum enterolactone levels and the incidence of breast cancer in women affected by gross cystic disease of the breast [6]. In the same cohort of women, we have also demonstrated that enterolactone is accumulated in breast-cyst fluid and that, at the higher intracystic concentrations, it is inversely correlated with the incidence of breast cancer, though only in the women who also have higher intracystic concentrations of epidermal growth factor [7]. This correlation supports an additional biological mechanism through which lignans might affect breast carcinogenesis [8–11]. Very few studies so far have investigated the putative effects of lignans in women with an established diagnosis of breast cancer. Two studies have investigated the

P. Guglielmini · A. Rubagotti · F. Boccardo
Department of Oncology, Biology and Genetics,
University of Genoa, Genoa, Italy

A. Rubagotti · F. Boccardo (✉)
Department of Medical Oncology, IRCCS San Martino
and University Hospital, National Cancer Research Institute,
Largo Rosanna Benzi 10, 16132 Genoa, Italy
e-mail: fboccardo@unige.it

association between lignan dietary intake and breast cancer prognosis [12, 13], but only one of them demonstrated a decreased mortality risk in women diagnosed with breast cancer when the highest intake was compared to the lowest one [12]. The other study, in fact, showed a reduced mortality only for the higher intakes of flavones, isoflavones and anthocyanidins, but not of lignans [13]. However, studies on dietary intake are commonly believed to underestimate lignan effects, since dietary lignans are just simply precursors of the biologically active compounds which are formed in the gut through the action of intestinal microflora. For this reason, studies based on the direct measurement of lignans in biological fluids are thought to be more affordable. Two recent studies have investigated the association between enterolactone serum levels of women diagnosed with breast cancer and their mortality risk following surgery, both showing a strong mortality hazard reduction in the women with higher enterolactone levels [14, 15]. These findings prompted us to look at the mortality outcome of a cohort of breast cancer patients whose serum samples were previously measured relative to their enterolactone concentration.

Methods

Patients selection

300 patients who had an histologically confirmed diagnosis of breast cancer between 1984 and 1991 were selected. All the women were operated on and subsequently followed up in our Institute, having consented to giving blood samples and its cryopreservation. All were aware of the experimental nature of the studies which could be performed subsequently to blood drawing on the preserved material.

The main characteristics of the cohort patients are summarized in Table 1.

Serum evaluation

Serum samples were obtained before surgery, usually 1 month in advance, and preserved at -80°C until they were processed. Details have been published elsewhere [6]. Briefly, the enterolactone quantitative concentration was assayed using a time-resolved fluoro-immunoassay (TR-FIA) according to the method originally reported by Stumpf et al. [16]. TR-FIA was performed using the DELFIA research reagents (Perkin Elmer, Massachusetts, USA). Fluorescence from each sample, which is inversely proportional to the concentration of enterolactone, was measured by way of a Victor 1420 Multilabel Counter (Perkin Elmer, Massachusetts, USA) with time-resolved fluorometry parameters. A program was used for the

Table 1 Descriptive characteristics of cohort patients

Characteristics	No. pts = 300
Age at cancer diagnosis (range)	58.5 (31.0–90.0)
Menopausal status	
Premenopausal	88 (29.3%)
Postmenopausal	212 (70.7%)
Tumor size: cm in diameter	
≤ 2 cm	147 (49.0%)
>2 cm	125 (41.7%)
Missing	28 (9.3%)
Nodal status	
Node-Negative	157 (52.3%)
Node-Positive	115 (38.3%)
Missing	28 (9.4%)
Adjuvant chemotherapy	
No	180 (60.0%)
Yes	91 (30.3%)
Missing	29 (9.7%)
Adjuvant Tamoxifen	
No	220 (73.3%)
Yes	51 (17.0%)
Missing	29 (9.7%)

automatic measuring and the results were calculated by including recovery and dilution information. The intra-assay and inter-assay coefficients of variations between 4.6 and 6.0% and 5.5 and 9.9% respectively, which were originally reported by Stumpf et al. [16], had been confirmed by us in our previous studies [6]. Enterolactone serum concentration was expressed in nmol/l.

Study endpoints and statistical analysis

All-cause and breast cancer mortality were the major endpoints. Mortality data were obtained by consulting the patients' flow charts. Information about the women who were lost to follow-up was obtained by consulting the local Mortality Registry or the registry offices of the patients' place of residence. Whenever possible, causes of death were recorded in detail.

The deaths occurring after any well-documented breast cancer relapse were arbitrarily defined as to be "breast cancer-related". All other lethal events were considered to be "breast cancer-unrelated".

Mortality curves were constructed through the cumulative incidence function estimate by the Kaplan–Meier method and compared by way of the log-rank test [17]. Cox proportional hazard regression models were used to check for known prognostic features, to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for group comparisons, as well as to test for the interaction on mortality

hazards between variables and enterolactone concentrations [18]. Covariates included: menopausal status (pre vs post), tumor size (≤ 2 vs. > 2 cm), nodal status (negative vs. positive) and adjuvant chemotherapy or Tamoxifen (no vs. yes). The cumulative incidence function was used to describe cause-specific mortality and the Fine and Gray's test was used to investigate the cause-specific mortality differences [19]. Finally, the Fine and Gray competing risk proportional hazard regression model was used to predict the probabilities of breast cancer-related and breast cancer-unrelated mortality. All probability values were obtained from two-sided tests. All statistical tests were carried out using SPSS package (version 18.0) and STATASE10.

Results

Enterolactone concentrations varied from 1 to 145 nmol/l with a median value of 20 nmol/l. Although an inverse relationship was found between the risk of death and the enterolactone concentration, independently of the percentile value considered, only the 25th percentile value which corresponded to a concentration of 10 nmol/l, appeared to discriminate in a statistically significant way and thus, it was selected for analysis in the whole cohort and in the subgroups.

At a median follow-up time of 23 years (range 0.6–26.1), 180 deaths were recorded, of which 112 were breast cancer-related. As shown in Figs. 1a–d and 2a–d, Kaplan–Meier curves demonstrated lower all-cause and breast cancer-specific mortality probabilities among the patients with enterolactone levels ≥ 10 nmol/l. However, the difference in respect to the women with lower levels was statistically significant only during the first 5 (Figs. 1a; 2a) and 10 years (Figs. 1b; 2b), though in the latter case, the significance was lost after adjusting for all the covariates. In fact, in both cases, curves tended to converge afterwards (Figs. 1c, d and 2c, d). Subgroup analysis according to covariates showed that the protective effect associated with the higher pre-operative concentrations was evident in post-menopausal women, in those with tumors ≥ 2 cm, in node-negative women and in women not receiving adjuvant chemotherapy (Table 2). As previously recorded in the whole cohort, differences were evident mainly in the first 5 or 10 years. Finally, no major interaction between enterolactone levels and adjuvant Tamoxifen was evident. The effect of enterolactone concentration was further investigated in respect to the cause of death through competing risk analysis (Fig. 3a–c). Our results showed that, whilst there was no difference in breast cancer-unrelated mortality according to the enterolactone concentration, at least during the first 15 years (Fig. 3b),

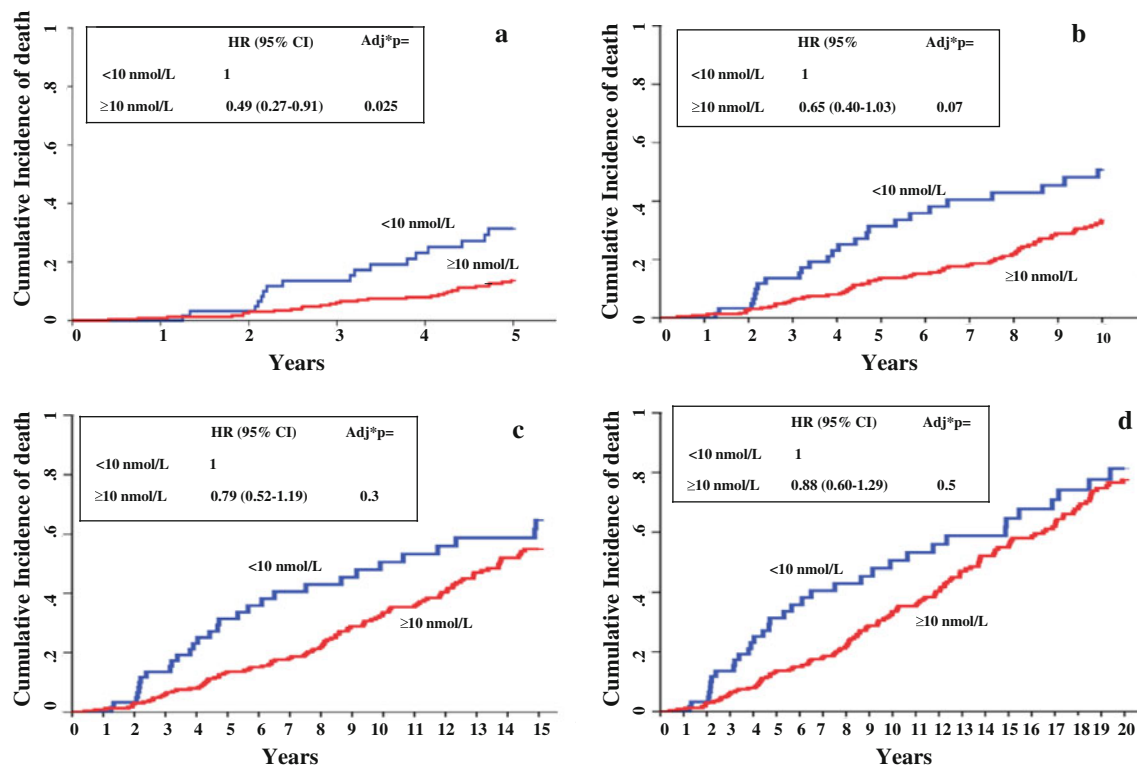


Fig. 1 Cumulative incidence of death at 5 (a), 10 (b), 15 (c) and 20 (d) years from surgery according to enterolactone levels. Comparisons were adjusted by menopausal status, tumor size, nodal status,

adjuvant chemotherapy and adjuvant Tamoxifen. *HR* hazard ratio, *95% CI* 95% Confidence Interval

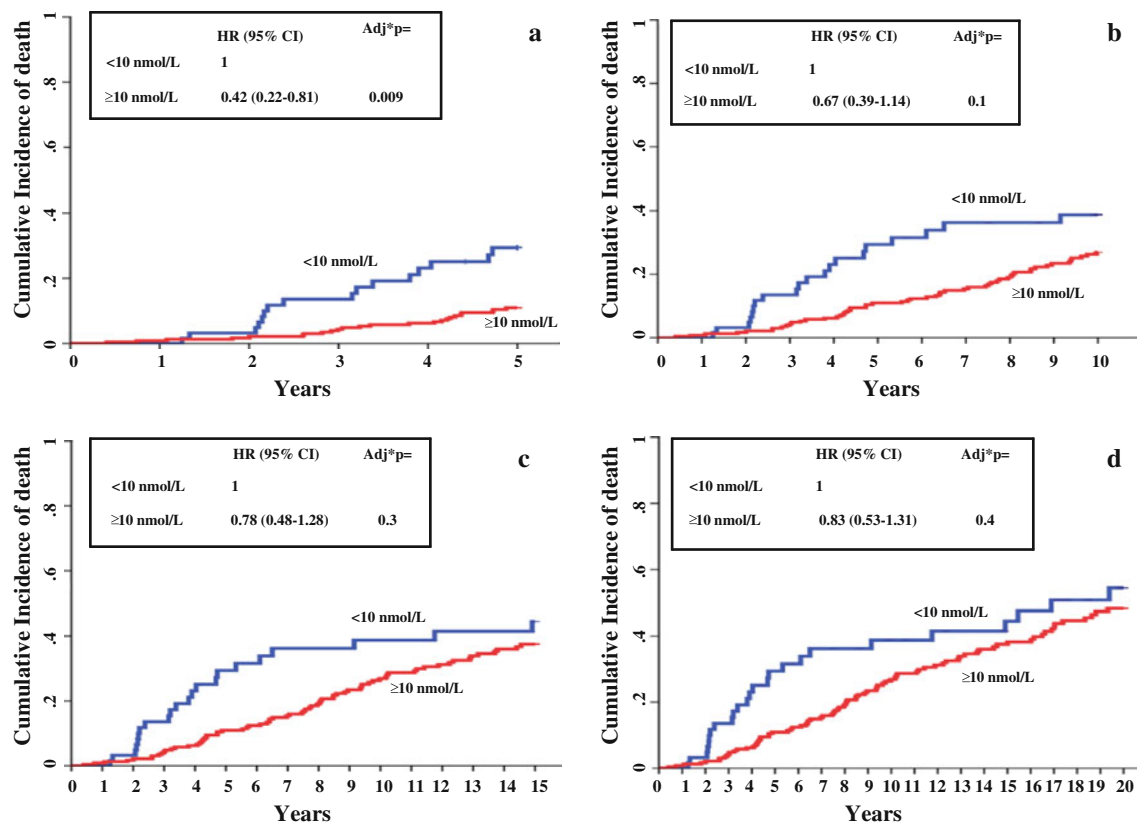


Fig. 2 Cumulative incidence of breast cancer-related death at 5 (a), 10 (b), 15 (c) and 20 (d) years from surgery according to enterolactone levels. Comparisons were adjusted by menopausal

status, tumor size, nodal status, adjuvant chemotherapy and adjuvant Tamoxifen. *HR* hazard ratio, *95% CI* 95% Confidence Interval

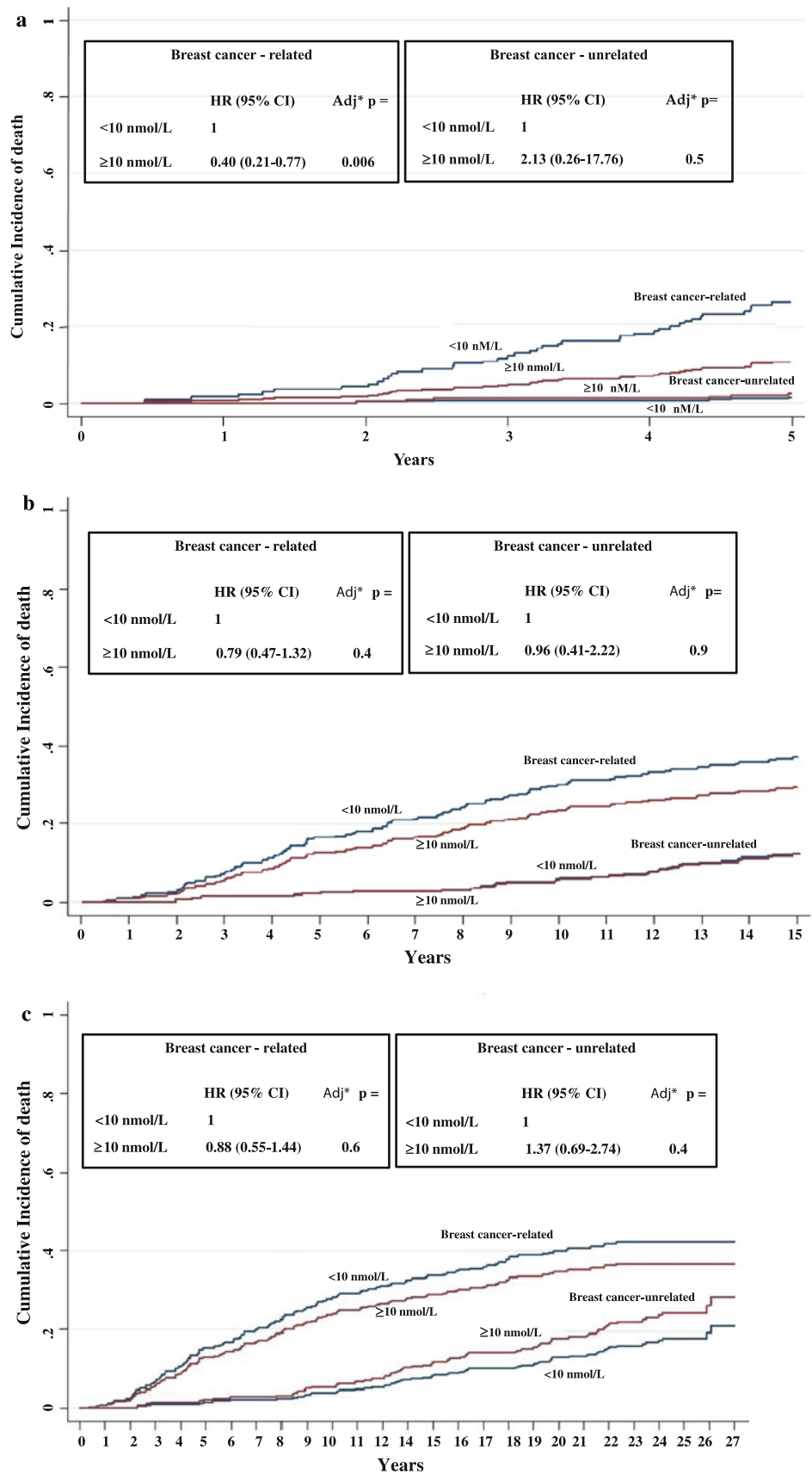
Table 2 Subgroup analysis of mortality according to covariates during the first 5 or 10 years

	Years 0–5						Years 5–10					
	All cause			Breast cancer-related			All cause			Breast cancer-related		
	HR	(95% CI)	Adj <i>P</i> =	HR	(95% CI)	Adj <i>P</i> =	HR	(95% CI)	Adj <i>P</i> =	HR	(95% CI)	Adj <i>P</i> =
Menopausal status												
Pre	1.34	(0.26–6.93)	0.7	1.14	(0.21–6.05)	0.9	1.85	(0.49–6.93)	0.4	1.77	(0.46–6.86)	0.4
Post	0.38	(0.19–0.76)	0.006	0.32	(0.15–0.66)	0.002	0.48	(0.28–0.82)	0.007	0.52	(0.29–0.94)	0.03
Tumor size												
≤ 2 cm	1.01	(0.11–8.94)	0.9	1.01	(0.11–8.94)	0.9	1.25	(0.36–4.30)	0.7	3.12	(0.41–23.9)	0.3
> 2 cm	0.43	(0.22–0.82)	0.01	0.36	(0.18–0.73)	0.004	0.55	(0.32–0.93)	0.025	0.54	(0.30–0.96)	0.035
Nodal status												
Negative	0.27	(0.09–0.82)	0.02	0.23	(0.07–0.72)	0.01	0.44	(0.21–0.94)	0.03	0.56	(0.22–1.43)	0.2
Positive	0.65	(0.30–1.38)	0.3	0.61	(0.26–1.38)	0.6	0.79	(0.43–1.48)	0.5	0.77	(0.39–1.49)	0.4
Adj CT												
No	0.37	(0.15–0.96)	0.04	0.26	(0.09–0.77)	0.015	0.72	(0.37–1.42)	0.3	0.92	(0.38–2.24)	0.9
Yes	0.71	(0.28–1.81)	0.5	0.65	(0.25–1.67)	0.4	0.89	(0.42–1.93)	0.8	0.84	(0.39–1.83)	0.7
Adj Tam												
No	0.54	(0.25–1.18)	0.1	0.43	(0.18–0.99)	0.049	0.72	(0.41–1.28)	0.26	0.80	(0.41–1.58)	0.5
Yes	0.47	(0.11–2.06)	0.3	0.46	(0.10–2.06)	0.3	0.96	(0.27–3.42)	0.9	0.94	(0.26–3.38)	0.9

HR Hazard ratio and *95% CI* for women in the ≥10 nmol/l group compared with women in the <10 nmol/l group (reference)

Adj adjusted for menopausal status, tumor size, nodal status, adjuvant chemotherapy and adjuvant Tamoxifen

Fig. 3 Cumulative incidence curves adjusting for competing risks. Results at 5 (a), 15 (b) and 25 (c) years from mastectomy. Comparisons were adjusted by menopausal status, tumor size, nodal status, adjuvant chemotherapy and adjuvant Tamoxifen. *HR* hazard ratio, *95% CI* 95% Confidence Interval



breast cancer-related mortality risk was consistently higher in the patients with enterolactone levels < 10 nmol/l (Fig. 3c), though the difference was more relevant and statistically significant only during the first 5 years (Fig. 3a).

Discussion

Present findings confirm that serum levels of enterolactone measured at the moment of breast cancer diagnosis are predictive of mortality hazard subsequent to surgery. In fact, consistent with the findings of Olsen et al. [14] and Buck et al. [15], we also were able to demonstrate that higher serum enterolactone levels are associated with a decrease in both all-cause and breast cancer-specific mortality. The three studies, i.e. our own, Olsen's [14] and Buck's [15] studies, had a comparable design, adopted the same fluorimetric method and were based on one single determination, immediately before or after the diagnosis of breast cancer. Noteworthy, is the fact that the median enterolactone level was almost comparable in the three studies (20, 20.5 and 20.8 nmol/l, respectively). Moreover, although the studies involved different numbers of women (300, 424 and 1140, respectively), in each of them the mortality analysis was based on a comparable number of deaths (180, 111 and 162, respectively) as they strongly differed in the median follow-up time (23.6, 10 and 6.7 years, respectively).

The three studies also shared the same criticisms. They are, in fact, based on one single serum determination, which at best, might reflect the serum levels of enterolactone of the previous 2 years, though with a relatively high reliability coefficient of 0.55 [20], but are lacking in periodic sequential determinations which might help in interpreting results, especially long-term. Indeed, several mechanisms might be at the basis of the protective effect shown by food lignans in these studies. Enterolactone levels might simply represent markers of a generally healthy diet and/or life style that, in itself, imply a lower mortality. However, this hypothesis appears to be weak in view of the fact that in all three studies, higher lignan serum levels showed a protective effect, not only on all-cause mortality, but specifically on breast cancer mortality. On the contrary, our findings and those of the other two previously mentioned studies [14, 15] appear to suggest a genuine effect of enterolactone on tumor progression, linked either to the possibility that the phenotype of tumors developed in women with higher levels might be "biologically" less aggressive or that continued exposure to higher lignan concentrations might interfere with tumor progression and dissemination. In regards to the former hypothesis, Buck's study [15] clearly shows that patients

with higher enterolactone levels tended to have a higher proportion of tumors of smaller sizes, lower tumor grades, hormone-receptor positive tumors and physician-detected tumors. Comparable trends also come out of our study, though differences were not statistically significant, probably due to the numbers being quite small. Olsen's study [14], on the other hand, did not analyze tumor characteristics according to actual enterolactone concentration and, therefore, cannot add any relevant information in this respect. In regards to the second hypothesis, unfortunately, neither our study nor the previously mentioned ones, had the possibility of checking for enterolactone serum levels following mastectomy so they cannot confirm or discard any putative effect of continuous exposure to high enterolactone concentrations during the years which followed breast surgery. However, it is clear that the same anti-oxidative, anti-neo-angiogenetic, anti-estrogenic and growth factor-modulating effects, which are commonly believed to represent the mechanisms through which mammalian lignans may affect breast carcinogenesis [8–11], might also play an important role in inhibiting the progression of established tumors. In fact, the findings of experimental studies on animal models are strongly supportive in this regard [21, 22]. Whatever the mechanisms involved, the three studies provide solid evidence that high pre- or post-diagnostic serum enterolactone levels have a definite protective effect on the mortality of the women diagnosed with breast cancer. The question that arises now though is why this protective effect was more evident in specific subgroups and why, as suggested by our findings, it tends to decrease over time.

In our study, the protective effect of higher enterolactone concentrations was evident in post-menopausal women but not in pre-menopausal ones. It is unclear why no associations were observed between the serum levels of enterolactone and mortality among premenopausal women. However, it is plausible that the lack of the protective effect in pre-menopausal women observed by us might be related to the known differences in the respective hormonal environments. Unfortunately, neither Olsen's [14] nor Buck's [15] studies included pre-menopausal women. Thus, they cannot help in confirming or discarding the trends observed by us in this regard.

The lack of an evident effect of higher enterolactone concentrations in node-positive patients is also intriguing. One reason might be that this group has such a poor prognosis that the enterolactone anti-tumoral effects, even in the presence of high lignan concentrations, do not show up. Should this be the reason though, one would expect a comparably reduced effect in patients affected by larger tumors, but this was not the case in our study. So, in interpreting the results of the analysis in the two nodal subsets, one should rather consider that, at the time the cohort patients were under study, no systemic adjuvant

treatment was commonly delivered to the node-negative patients, while node-positive patients were mostly treated with adjuvant chemotherapy and/or Tamoxifen. Therefore, the confounding effects of both therapies should be taken into account. In particular concerning the putative confounding effect of Tamoxifen, it cannot be ruled out that the anti-proliferative and pro-apoptotic effects of enterolactone might be “blunted” by those of this anti-estrogen, though neither any substantial interaction between the administration of Tamoxifen and the effect on the mortality hazard of the different enterolactone concentrations was seen in our study nor any clear-cut antagonism between the Tamoxifen and mammalian lignans was found in breast cancer cell lines *in vitro* [23]. Finally, it is not easy to explain why the protective effect of enterolactone tended to progressively decrease over time in our study. This trend might be simply correlated with the fact that the number of the patients on the study itself progressively decreased over time or relative to breast cancer-specific mortality only, with the fact that the probability of breast cancer-unrelated death was proportionately increasing more than the probability of dying due to breast cancer. However, it cannot be ruled out that patient ageing might simply imply substantial dietary modifications which could significantly reduce their lignan intake. In other cases, an increase in body weight, in constipation problems and in the consumption of drugs, including antibiotics which can interfere with the efficiency of gut microflora (all problems commonly encountered in elderly people) might all lead to inducing a progressive decrease in the levels of mammalian lignans, thus explaining the progressive weakening of the association between pre-diagnostic enterolactone levels and patient mortality. Lack of periodic enterolactone determinations does not allow us to consider this hypothesis and therefore we cannot put forward more than a mere speculation. Unfortunately, the median follow-up time of Olsen’s [14] or Buck’s [15] studies was considerably shorter than the median follow-up time of our study and so these studies cannot help either in confirming or in discarding the time-trend observed by us. This trend, however, is crucial both in explaining the effects of pre-diagnosis exposure to high enterolactone levels and in suggesting that patients should maintain a high intake in food lignans in order to avoid the risk of losing the benefit associated with high pre-diagnosis lignan intakes over time.

In conclusion, our findings possibly confirm that mammalian lignans might play an important role in the tertiary prevention of breast cancer that is, at least, comparable to that exerted in the primary prevention of this disease.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Reinli K, Block G (1996) Phytoestrogen content of foods—a compendium of literature values. *Nutr Cancer* 26:123–148
2. Mazur W (1998) Phytoestrogen content in foods. *Bailliere’s Clin Endocrinol Metab* 12:729–742
3. Adlercreutz H (2002) Phyto-oestrogens and cancer. *Lancet Oncol* 3:364–373
4. Boccardo F, Puntoni M, Guglielmini P et al (2006) Enterolactone as a risk factor for breast cancer: a review of the published evidence. *Clin Chim Acta* 365:58–67
5. Buck K, Zaineddin AK, Vrieling A et al (2010) Meta-analyses of lignans and enterolignans in relation to breast cancer risk. *Am J Clin Nutr* 92:141–153
6. Boccardo F, Lunardi G, Guglielmini P et al (2004) Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. *Eur J Cancer* 40:84–89
7. Boccardo F, Lunardi GL, Petti AR et al (2003) Enterolactone in breast cyst fluid: correlation with EGF and breast cancer risk. *Breast Cancer Res Treat* 79:17–23
8. Brooks JD, Thompson LU (2005) Mammalian lignans and genistein decrease the activities of aromatase and 17 β -hydroxysteroid dehydrogenase in MCF-7 cells. *J Steroid Biochem Mol Biol* 94:461–467
9. Bergman JM, Thompson LU, Dabrosin C (2007) Flaxseed and its lignans inhibit estradiol-induced growth, angiogenesis, and secretion of vascular endothelial growth factor in human breast cancer xenografts *in vivo*. *Clin Cancer Res* 13:1061–1067
10. Kitts DD, Yuan YV, Wijewickreme AN et al (1999) Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. *Mol Cell Biochem* 202:91–100
11. Chen J, Stavro PM, Thompson LU (2002) Dietary flaxseed inhibits human breast cancer growth and metastasis and down-regulates expression of insulin-like growth factor and epidermal growth factor receptor. *Nutr Cancer* 43:187–192
12. McCann SE, Thompson LU, Nie J et al (2009) Dietary lignan intakes in relation to survival among women with breast cancer: The Western New York Exposures and Breast Cancer (WEB) Study. *Breast Cancer Res Treat* 122:229–235
13. Fink BN, Steck SE, Wolff MS et al (2007) Dietary flavonoid intake and breast cancer survival among women on Long Island. *Cancer Epidemiol Biomarkers Prev* 16:2285–2292
14. Olsen A, Christensen J, Knudsen KE et al (2011) Prediagnostic plasma enterolactone levels and mortality among women with breast cancer. *Breast Cancer Res Treat* 128:883–889
15. Buck K, Vrieling A, Zaineddin AK et al (2011) Serum enterolactone and prognosis of postmenopausal breast cancer. *J Clin Oncol* 29:3730–3738

16. Stumpf K, Uehara M, Nurmi T et al (2000) Changes in the time-resolved fluoroimmunoassay of plasma enterolactone. *Am Biochem* 15:153–157
17. Kaplan EL, Meier P (1958) Non parametric estimation from incomplete observation. *J Am Stat Assoc* 53:457–481
18. Cox DR (1972) Regression models and life tables (with discussion). *J Royal Statist Soc B* 34:187–220
19. Gray RJ (1988) A class of k-sample tests for comparing the cumulative incidence of a competing risk. *Ann Stat* 16: 1141–1154
20. Zeleniuch-Jacquotte A, Adlercreutz H, Akhmedkhanov A et al (1998) Reliability of serum measurements of lignans and isoflavonoid phytoestrogens over a two-year period. *Cancer Epidemiol Biomarkers Prev* 7:885–889
21. Wang L, Chen J, Thompson LU (2005) The inhibitory effect of flaxseed on the growth and metastasis of estrogen receptor negative human breast cancer xenografts is attributed to both its lignan and oil components. *Int J Cancer* 116:793–798
22. Chen J, Wang L, Thompson LU (2006) Flaxseed and its components reduce metastasis after surgical excision of solid human breast tumor in nude mice. *Cancer Lett* 234:168–175
23. Chen J, Chen J, Thompson LU (2003) Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration in vitro. *Breast Cancer Res Treat* 80:163–170