Enterolactone concentrations and prognosis after postmenopausal breast cancer: Assessment of effect modification and meta-analysis

Petra Seibold[1], Alina Vrieling[1,2], Theron S. Johnson[1], Katharina Buck[1], Sabine Behrens[1], Rudolf Kaaks[1], Jakob Linseisen[3], Nadia Obi[4], Judith Heinz[4], Dieter Flesch-Janys[4] and Jenny Chang-Claude[1]

[1] Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany
[2] Department for Health Evidence, Radboud University Medical Centre, Nijmegen, The Netherlands
[3] Institute of Epidemiology, German Research Center for Environmental Health, Munich, Germany
[4] Department of Cancer Epidemiology/Clinical Cancer Registry, University Cancer Center Hamburg (UCCH) and Department of Medical Biometrics and Epidemiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

We previously reported that high concentrations of enterolactone, a lignan metabolite, are associated with lower mortality in 1,140 breast cancer patients from Germany. Using an extended set of 2,182 patients aged 50–74 years at diagnosis (2001–2005) and prospectively followed up until 2009, we investigated whether the association with mortality differs by lifestyle factors and tumor characteristics. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated using multivariable Cox regression. Potential differential effects by tumor characteristics and lifestyle factors were assessed and a meta-analysis of five studies addressing lignan exposure and breast cancer prognosis was performed to summarize evidence. Median enterolactone concentrations were 17.4 (±30.5 standard deviation) and 22.9 nmol L^{-1} (±44.8), respectively, for 269 deceased and 1,913 patients still alive. High enterolactone concentrations were significantly associated with lower all-cause mortality (per 10 nmol L^{-1}: HR 0.94, 95% CI 0.90–0.98), breast cancer-specific mortality (HR 0.94, 0.89–0.99), and distant disease-free survival (HR 0.94, 0.90–0.98). Associations were found for stage 0–IIIA but not for stage IIIB–IV disease (p_{het} = 0.01) and were stronger in patients with BMI <25 kg m^{-2} than those with BMI ≥25 (p_{het} = 0.04). In patients with healthy lifestyle (BMI <25, nonsmoker, physically active), the inverse association with all-cause mortality was still apparent (HR 0.92, 0.85–0.99). The meta-analysis yielded significant associations both for all-cause (HR 0.57, 0.42–0.78) and breast cancer-specific mortality (HR 0.54, 0.39–0.75). Our findings show that high lignan exposure is associated with reduced mortality in breast cancer patients. The inverse association observed in this study cannot be entirely explained by a healthy lifestyle.

In Western populations, plant-derived lignans are usually the main contributor to the intake of foods rich in poly-phenolic compounds, rather than isoflavonoids (*e.g.*, from soy) for Asian population.[1,2] For example, plant lignans such as pinoresinol, lariciresinol, secoisolariciresinol and matairesinol are found in seeds (sunflower, pumpkin, sesame, flaxseeds), whole-grain cereals and (fiber rich) vegetables.[1] They are metabolized by the gut microflora to the biologically active forms enterolactone and enterodiol.[3] Because of structural similarities, lignans can bind to the estrogen receptor and thus exert both estrogenic and anti-estrogenic effects.[3,4] Enterolactone has been shown to be positively correlated with sex hormone binding globulin which binds free estradiol, which may lead to lower concentrations of circulating sex hormones.[4] Lignans can also act *via* estrogen-independent anti-carcinogenic activity and were shown to

Key words: phytoestrogens, lignans, enterolactone, breast cancer mortality

Abbreviations: BMI: body mass index; CI: confidence interval; EGFR: epidermal growth factor receptor; ENL: enterolactone; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; HH: Hamburg study region; HR: hazard ratio; ICD-10: the 10th revision of the international statistical classification of diseases and related health problems; IQR: interquartile range; MET: metabolic equivalent; MHT: menopausal hormone therapy; NACT: neoadjuvant chemotherapy; p het: *p* value for heterogeneity; PR: progesterone receptor; RNK: Rhein-Neckar-Karlsruhe study region; TR-FIA: time-resolved fluoroimmunoassay

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Correspondence to: Prof. Dr. Jenny Chang-Claude, Division of Cancer Epidemiology (C020), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 581, 69120 Heidelberg, Germany. E-mail: j.chang-claude@dkfz.de

What's new?

Plant-derived lignans are known to exert anticancer activity in an estrogen-independent manner, suggesting that they may be of value in the prevention of breast cancer. Here, in a population of 2,182 breast cancer patients, enterolactone, a lignan metabolite, was found to be associated with reduced all-cause mortality and breast cancer-specific mortality. The inverse association was restricted to early stage breast cancer and to patients with normal weight. A meta-analysis of five cohorts yielded consistent evidence that high lignan exposure is linked to lower mortality in breast cancer patients.

inhibit proliferation, increase apoptosis and reduce expression of human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR).[5] Women with a greater lignan intake may have a lower breast cancer risk.[6] Breast cancer patients with high lignan intake were less likely to develop advanced breast tumors.[7–9]

We previously reported that high concentrations of postdiagnostic serum enterolactone as a biomarker of lignan intake were associated with lower mortality in 1,140 postmenopausal breast cancer patients.[9] These findings were confirmed by two other studies assessing post-[10] and prediagnostic[11] circulating enterolactone concentrations. The results are consistent with those from two studies on dietary lignan intake and breast cancer survival[12,13] but not with those from another study.[14]

Given that the major dietary sources of lignans are fiber from whole grains, seeds, fruits and vegetables, it has been suggested that the beneficial effect of enterolactone or plant lignans may be a biomarker of a healthy lifestyle.[4,15] To provide more insight into the association between enterolactone and breast cancer prognosis, we investigated in an extended dataset of 2,182 postmenopausal breast cancer patients whether enterolactone concentrations are affected by other lifestyle factors and tumor characteristics and whether the association with prognosis might be restricted to subgroups defined by these factors.

Methods

Study population

Breast cancer patients recruited in the population-based case-control study MARIE (Mammakarzinom–Risikofaktoren–Erhebung),[16] were prospectively followed up until end of 2009.[17] Patients were aged 50–74 years at confirmed diagnosis of primary breast cancer (stage 0–IV) between January 2001 and September 2005 in two study regions in Germany (Hamburg (HH) and Rhein–Neckar–Karlsruhe (RNK)). Comprehensive information on pre-diagnostic lifestyle factors was collected in a standardized face-to-face interview. Vital status was assessed in 2009 *via* the population registries (100% completeness) and causes of death obtained from death certificates and coded according to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). Information on the clinical course and treatment was abstracted from medical records to verify clinical events either self-reported in a telephone follow-up interview conducted in 2009 or reported by treating physicians. Postdiagnostic serum or plasma samples were available for 73% of the 3,464 postmenopausal patients. Information on dietary habits (*e.g.*, of fiber intake) was available for 85% patients who filled out a validated food frequency questionnaire assessing dietary intake in the year prior to the breast cancer diagnosis. All patients gave written informed consent. Ethical approvals were obtained from the ethics committee of the University of Heidelberg, the Hamburg Medical Council, and the Medical Board of the State of Rheinland-Pfalz. The study was conducted in accordance with the Declaration of Helsinki.

Investigated endpoints

The primary endpoint to be assessed was all-cause mortality (including death from any cause), secondary endpoints were breast cancer-specific mortality (including death due to breast cancer; other deaths were censored), distant disease-free survival (including distant recurrence and death from any cause) and risk of recurrence (ipsilateral, contralateral, regional, distant recurrence; deaths were censored).

For mortality analyses, 2,182 postmenopausal patients with available enterolactone measurement and without previous non-breast tumors were considered. For the analysis on risk of recurrence, patients without information on recurrence ($N = 40$), with neoadjuvant chemotherapy ($N = 76$) or with stage IIIB to IV disease ($N = 155$) were excluded, resulting in 1,911 patients with stage 0 to IIIA disease.

Enterolactone measurements

Postdiagnostic blood samples were stored at −80°C. Enterolactone concentrations were measured for patients with available nonfasting serum (Rhein–Neckar–Karlsruhe region, 02/2010-05/2010) or plasma (Hamburg region, 06/2012-07/2012) samples using time-resolved fluoroimmunoassay (TR-FIA)[18] according to manufacturer's instructions (Labmaster, Turku, Finland) as previously described.[9] Assays were performed blinded to vital status. Results were calculated based on a standard curve fitted with a spline smoothed algorithm for duplicate standard measurements. Median time between diagnosis and blood collection was 140 days (standard deviation 359 days). For 68% of the patients, blood collection was either before start of chemotherapy or they did not receive any chemotherapy at all. For 25% of the patients, blood draw was after start of chemotherapy. Timing of blood collection with respect to chemotherapy was unknown for 13%. For 98 samples below the lowest standard of 0.5 nmol L^{-1},

enterolactone concentration values were set to 0.001 nmol L^{-1} (these were excluded in the previous publication)[9]; for ten samples above the upper detection limit of 300 nmol L^{-1}, enterolactone values were set to 300 nmol L^{-1}. Interassay coefficients of variation were 10.3% for the HH region and 14.6% for the RNK region. Because of different time points of measurement, we remeasured enterolactone concentrations in a subsample of the RNK region ($n = 107$) to examine comparability of the two batches and measurements in serum versus plasma samples. Pearson correlation coefficient between serum and plasma concentrations of the RNK region was 0.95. Because no significant statistical heterogeneity was found by study region (p for heterogeneity >0.05), we carried out pooled analysis of the two study regions, adjusting for time between blood draw and enterolactone measurements.

Tumor subtype definitions

We defined four tumor subtypes according to histopathological surrogate parameters[19]: luminal A (estrogen receptor (ER) and/or progesterone receptor positive (PR), HER2/neu negative, histological grade 1 or 2), luminal B (ER+ and/or PR+, HER2/neu positive or negative, grade 3), non-luminal HER2 positive (ER- and PR-, HER2 positive), and triple negative tumors (ER- and PR-, HER2 negative). Subtype classification was possible for 74% of the tumors. Patients who received either neoadjuvant chemotherapy or had *in situ* disease and therefore without valid ER/PR/HER2 data accounted for 40% of the patients for whom subtype classification was not possible. In terms of other tumor characteristic or lifestyle factors, the two groups of patients with or without subtype classification were comparable.

Statistical analysis

Time-to-event analysis was performed using the procedure PHREG in SAS 9.2. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated using delayed entry Cox proportional hazards models with follow-up time from interview to event/censoring. Enterolactone concentrations were analyzed both in quartiles (with lowest quartile, Q1, as reference) and per 10 nmol L^{-1} increment. Pooled analysis of the two study regions were stratified by study region and age at diagnosis in 5-year intervals. The basic multivariable model (complete-case analysis) included the following prognostic adjustment factors considered to be relevant: tumor size ($\leq$2, $>$2–5, $>$5 cm, growth into chest wall, neoadjuvant chemotherapy/NACT), nodal status (0, 1–3, 4–9, $\geq$10 regional lymph nodes affected, NACT), metastasis status at baseline (no metastases, metastases), ER/PR status (ER+PR+, ER-PR+ or ER+PR-, ER-PR-, NACT), histological grading (grade 1+2, grade 3+4, NACT), mode of detection (self-detected, routinely detected), prediagnostic leisure time physical activity in metabolic equivalent (MET) hours per week ($<$28, $\geq$28 METhours/week according to WCRF criteria) and usual adult body mass index ($<$18.5, 18.5–$<$25 as reference, 25–$<$30, $\geq$30 kg m^{-2} according to WHO criteria). In addition, time between blood draw and ENL measurement (continuous) was included to account for possible temporal differences in measurements of the two batches. Further adjustment variables were selected by backward selection ($p \leq 0.05$): use of menopausal hormone therapy (MHT) at diagnosis (never/past, current), smoking status at diagnosis (never/past, current), and radiotherapy (no, yes). Time between diagnosis and blood draw, surgery type, cardiovascular disease, diabetes, HER2 status, education, occupation and chemotherapy were not significant in the model and were not confounders, *i.e.*, did not change the association with enterolactone by 10% or more. An adjusted survival plot was generated by using the plot option in the PHREG procedure. The proportional hazard assumption was tested by using martingale residuals with SAS option ASSESS PH.[20] Two-sided p values <0.05 were considered significant.

Potential differential effects by tumor characteristics and lifestyle factors such as BMI, physical activity and smoking, were assessed using a multiplicative interaction term (continuous enterolactone concentrations $\times$ binary covariate) and comparing models with and without interaction term by the likelihood ratio test.

Meta-analysis

In May 2013, we performed a systematic search on epidemiological studies in humans investigating association of lignans with breast cancer prognosis, using the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed). Combinations of the following search terms were used: "lignans," "enterolactone," "breast cancer," "breast neoplasms," "prognosis," "survival," "mortality." To identify further potential articles that may have been missed, cited references in the retrieved articles were additionally checked. In total, 49 articles were identified. After removal of four articles on duplicate study populations (including two articles were based on our own study population), we excluded further 38 articles on *in vitro* studies, other endpoints or without association with prognosis. Full text was reviewed from seven studies. Including the present study, we thus identified five independent study populations investigating the association of lignans with breast cancer mortality, measured either as concentrations of the biomarker enterolactone or based on dietary assessment. A PRISMA ("Preferred Reporting Items for Systematic Reviews and Meta-Analyses") flowchart[21] can be found in Supporting Information Figure S1.

To summarize evidence from published studies on lignans and breast cancer prognosis, we extracted hazard ratios from the identified studies and conducted a meta-analysis using random effects models[22] using the package "meta" in R (version 2.15). Epidemiological studies evaluating this association were identified using a systematic MEDLINE search (http://www.ncbi.nlm.nih.gov/pubmed). The hazard ratio and corresponding 95% confidence intervals of the highest compared to the lowest quantile or—if not applicable—the risk estimate

Table 1. Descriptive covariates of 2,182 postmenopausal breast cancer patients according to enterolactone concentrations

Characteristics	Total population (N)	(%)	ENL [nmol/L] Median	IQR
All patients	2,182	100.0	22.3	36.6
Deaths overall	269	12.3	17.4	28.8
Deaths related to breast cancer	194	72.1	17.8	31.9
Recurrences (stage 0–IIIA, N=1,911)	207	10.8	17.8	34.9
Tumor size				
≤2 cm	1,127	51.7	23.9	38.1
>2–5 cm	711	32.6	21.1	34.0
>5 cm	73	3.4	19.5	33.5
Growth into chest wall	56	2.6	15.1	28.3
NACT	78	3.6	14.3	25.7
in situ	134	6.1	26.3	45.2
Nodal status				
0 affected lymph nodes	1,337	61.3	22.9	37.5
1 to 3	436	20.0	22.6	33.6
4 to 9	116	5.3	17.8	29.7
≥10	79	3.6	23.4	40.1
NACT	78	3.6	14.3	25.7
in situ	134	6.1	26.3	45.2
Metastasis				
no	1,984	90.9	22.1	36.4
yes	62	2.8	24.3	32.2
in situ	134	6.1	26.3	45.2
Tumor grade				
1 + 2	1,438	65.9	23.4	37.0
3 + 4	522	23.9	19.4	34.0
NACT	78	3.6	14.3	25.7
in situ	134	6.1	26.3	45.2
ER/PR status				
ER+/PR+	1,273	58.3	23.8	36.2
ER+/PR- or ER-/PR+	387	17.7	21.4	40.7
ER-/PR-	309	14.2	17.6	30.9
NACT	78	3.6	14.3	25.7
in situ	134	6.1	26.3	45.2
HER2/neu status				
positive	364	16.7	21.2	31.3
negative	1,413	64.8	22.4	38.0
NACT	78	3.6	14.3	25.7
in situ	134	6.1	26.3	45.2
MHT use at diagnosis				
past/never	1,199	54.95	20.7	35.7
current	972	44.55	24.4	38.4
Chemotherapy				
no	1,156	53.0	25.8	40.4
adjuvant/palliative	922	42.3	18.9	34.1
neoadjuvant	78	3.6	14.3	25.7
Radiotherapy				
no	506	23.2	23.1	39.1
yes	1,654	75.8	22.2	36.2
Tamoxifen use				
never	653	29.9	21.2	34.8
ever	1,431	65.6	23.4	37.2
Mode of detection				
self-detected	1,174	53.8	20.3	36.3
physician-detected	1,001	45.9	23.9	37.1
Surgery type				
Ablation	667	30.6	21.8	35.2
breast conserving	1,503	68.9	22.6	37.5
History of cardiovascular disease				
no	1,082	49.6	23.4	35.7
yes	1,100	50.4	21.3	37.2
History of diabetes				
no	1,975	90.5	22.2	36.0
yes	203	9.3	23.3	39.4
Smoking status at diagnosis				
never	1,219	55.9	23.5	36.4
past	573	26.3	22.6	38.3
current	390	17.9	17.3	35.9
Alcohol consumption (g/day)				
<0.5	585	26.8	20.0	35.8
0.5–<6	818	37.5	22.5	35.2
6–<12	329	15.1	23.9	38.0
≥12	448	20.5	23.3	36.1
BMI (kg/m^2)				
<18.5	57	2.6	31.5	50.9
18.5–<25	1,599	73.3	23.5	36.5
25–30	452	20.7	17.3	33.1
≥30	74	3.4	17.4	41.6
Leisure physical activity (at age 50)				
<28 MET hours/week	598	27.4	20.0	36.1
≥28 MET hours/week	1,564	71.7	23.1	36.7
Education				
low	1,297	59.4	19.9	35.0
medium	572	26.2	25.0	37.5
high	312	14.3	26.6	46.1

Table 1. Descriptive covariates of 2,182 postmenopausal breast cancer patients according to enterolactone concentrations (Continued)

	Total population		ENL [nmol/L]	
Characteristics	(N)	(%)	Median	IQR
Occupation				
low	831	38.1	19.3	34.6
medium	810	37.1	24.3	40.1
high	529	24.2	23.5	35.7
Age at diagnosis (years)				
50–54	163	7.5	14.6	30.6
55–59	471	21.6	19.3	36.9
60–65	698	32.0	23.1	34.9
65–70	595	27.3	22.8	39.0
70–74	255	11.7	28.5	42.4
Study region				
Hamburg	1,021	46.8	24.1	40.2
Rhein-Neckar-Karlsruhe	1,161	53.2	19.6	34.5

CI: confidence interval; ENL: enterolactone; ER: estrogen receptor; HR: hazard ratio; IQR: interquartile range; MET: metabolic equivalent; MHT: menopausal hormone therapy; NACT: neoadjuvant chemotherapy; PR: progesterone receptor.
Number might not add up to 100% due to missings.

from a dichotomized variable from the most fully adjusted model was abstracted from each study. Study heterogeneity was assessed using I-squared index, tau-squared heterogeneity estimator, and Q statistic[22,23] and possible publication bias examined using a funnel plot[24] and quantified by using weighted linear regression of the effect estimate on its standard error of the estimate.[25]

Results

Median enterolactone concentration was 22.3 nmol L^{-1} overall (24.1 nmol L^{-1} in HH study region, 19.6 nmol L^{-1} in RNK study region) and for deceased and censored patients, 17.4 and 22.9 nmol L^{-1}, respectively. Patients with higher compared to lower circulating enterolactone concentrations were diagnosed with tumors of smaller size or *in situ* tumors, lower grade, and positive hormone receptor status as presented in Table 1. They were also more likely to be current MHT users, higher educated, of older age, and from the Hamburg study region. Further, women with higher concentrations had a BMI <25 kg m^{-2}, had not received chemotherapy, used tamoxifen, had routinely detected tumors, and were less likely to be current smokers at diagnosis (Table 1). After a median follow-up time from recruitment to death or censoring of 5.4 years, 269 of the 2,182 patients had died, 194 due to breast cancer. In the 1,911 patients with stage 0 to IIIA ("early stage disease"), 207 events related to local, regional, or distant recurrence occurred.

Association between circulating enterolactone and breast cancer prognosis

Data from the Hamburg study region confirmed an inverse association of enterolactone concentrations with all-cause mortality (per 10 nmol L^{-1} increment: HR 0.91, 95% CI 0.85–0.97) (Supporting Information Table S1). As we did not observe statistically significant heterogeneity by study region (p for heterogeneity = 0.22), we present the results on all-cause, breast cancer specific mortality and risk of recurrence for the two study centers combined in Table 2. Overall, high circulating enterolactone concentrations were associated with a ~40% statistically significantly lower all-cause mortality in the multivariable model (Table 2: highest, Q4, *vs.* lowest quartile, Q1: HR 0.59, 95% CI 0.40–0.87). Per 10 nmol L^{-1} increment, HR was 0.94 (95% CI 0.90–0.98). In Supporting Information Table S2, the estimates effects of all variables included in the final multivariable all-cause mortality model are presented. The univariate association was similar: HR 0.93, 95% CI 0.90–0.97. An adjusted survival plot for all-cause mortality according to quartiles of enterolactone concentrations is shown in Supporting Information Figure S2. Associations were in a similar order of magnitude for breast cancer-specific mortality, for distant disease-free survival, and also when restricted to invasive tumors (data not shown). Higher enterolactone concentrations were not significantly associated with risk of recurrence.

Effect modification

We further investigated whether these associations differed by tumor characteristics (Table 3) or modifiable factors (Table 4). The inverse associations with enterolactone concentrations were significantly heterogeneous by stage of disease. Higher compared to lower circulating enterolactone concentrations were significantly associated with all-cause mortality only in patients with early (stage 0 to IIIA) breast cancer (per 10 nmol L^{-1}: HR 0.91, 95% CI 0.86–0.97, Table 3) but not in those with advanced (stage IIIB to IV) disease (HR 1.04, 95% CI 0.96–1.14, p for heterogeneity = 0.01). We did not find significant differences by tumor size, nodal status, hormone receptors status, HER2 status or tumor subtype.

Regarding modifiable factors, statistically significantly stronger associations were found in women with BMI < 25 kg m^{-2} (per 10 nmol L^{-1}: HR 0.92, 95% CI 0.87–0.97, Table 4) compared to overweight women (HR 0.99, 95% CI 0.93–1.06, p for heterogeneity = 0.04). We did not find any significant differences by physical activity, smoking, fiber intake or menopausal hormone therapy.

To mitigate the argument of enterolactone concentrations being a marker of a "healthy lifestyle" effect, we investigated the association between circulating enterolactone and breast cancer prognosis in the subgroup of patients with a healthy lifestyle, defined as being nonsmokers, BMI lower than 25 kg m^{-2} and physically active at diagnosis (≥28 METhours/week). The association of higher enterolactone concentrations with reduced all-cause mortality was still present in these 997 breast cancer patients with a healthy lifestyle, with a statistically significant 8% reduction per 10 nmol L^{-1} enterolactone (HR 0.92, 95% CI 0.85–0.99). In the remaining patients with

Table 2. Association of circulating enterolactone with postmenopausal breast cancer prognosis from multivariable pooled analysis from both study centers (Hamburg and Rhein-Neckar-Karlsruhe)

		HR (95% CI)[1]
All-cause mortality	ENL, median (nmol/L)	N = 2,107 (254 events)
Q1 lowest quartile	3.1	1.0 (Ref.)
Q2	14.9	0.86 (0.62–1.21)
Q3	31.1	0.88 (0.63–1.25)
Q4 highest quartile	72.1	0.59 (0.40–0.87)
Per 10 nmol/L		0.94 (0.90–0.98) $p < 0.01$
Breast cancer-specific mortality	ENL, median (nmol/L)	N = 2,107 (186 events)
Q1 lowest quartile	3.1	1.0 (Ref.)
Q2	14.9	0.77 (0.51–1.16)
Q3	31.1	1.00 (0.67–1.49)
Q4 highest quartile	72.1	0.59 (0.37–0.94)
Per 10 nmol/L		0.94 (0.89–0.99), $p = 0.02$
Distant disease-free survival	ENL, median (nmol/L)	N = 1,846 (214 events)
Q1 lowest quartile	3.5	1.0 (Ref.)
Q2	15.5	0.86 (0.61–1.22)
Q3	31.8	0.74 (0.51–1.07)
Q4 highest quartile	74.2	0.51 (0.34–0.77)
Per 10 nmol/L		0.94 (0.90–0.98), $p < 0.01$
Risk of recurrence	ENL, median (nmol/L)	N = 1,838 (188 events)
Q1 lowest quartile	3.5	1.0 (Ref.)
Q2	15.5	0.79 (0.53–1.18)
Q3	31.8	0.92 (0.62–1.36)
Q4 highest quartile	74.2	0.77 (0.51–1.16)
Per 10 nmol/L		0.99 (0.95–1.02), $p = 0.45$

ENL: enterolactone.
[1]Hazard ratios (HR) and 95% confidence intervals (95% CI) from multivariable complete-case analysis, stratified by age at diagnosis and study region, and adjusted for tumor size, nodal status, metastases status, histological grading, ER/PR status, BMI, radiotherapy, smoking, physical activity, MHT use, time between blood draw and enterolactone measurement.

a "less healthy lifestyle" (*i.e.*, smoker or BMI $\geq$25 kg m^{-2} or <28 METhours/week), higher enterolactone concentrations were associated with a nonsignificantly reduced all-cause mortality (HR 0.96, 95% CI 0.91–1.01, p for heterogeneity = 0.92).

As sensitivity analysis, we assessed whether effect modification by tumor characteristics and modifiable factors was different when limited to patients with early stage disease (Supporting Information Tables S3 and S4). Findings were comparable to the total study population. In addition, we assessed whether the observed associations were modified by timing of blood draw with respect to chemotherapy. Patients with blood draw before chemotherapy or who had not received any chemotherapy had higher median enterolactone concentrations compared to patients with blood draw after chemotherapy (23.8 *vs.* 17.5 nmol L^{-1}, respectively). However, there was no significant difference in associations found in patients with blood samples before chemotherapy and in those with blood samples after chemotherapy for all endpoints (p for heterogeneity = 0.56 for all-cause mortality) (Supporting Information Table S5).

Meta-analysis

We identified five independent epidemiological studies that investigated whether survival after breast cancer is associated with exposure to lignans, measured either as enterolactone concentrations or based on dietary assessment (Table 5). Three studies assessed circulating enterolactone concentrations in relation to breast cancer survival,[9–11] yielding with the present analysis a total of three independent studies as Buck et al. 2011a[9] was based on one study region of our study. Additionally, three publications reported on dietary intake of lignans in association with survival,[12–14] including one from our own study population (Buck et al., 2011b).[12] Three studies included both pre- and postmenopausal patients[10,13,14] but all provided estimates of association according to menopausal status. We performed a meta-analysis only for postmenopausal breast cancer because of

Table 3. Association between circulating enterolactone and all-cause mortality in postmenopausal breast cancer patients stratified by tumor characteristics

Subgroup analysis	N patients (events)	Per 10 nmol/L HR (95% CI)[1]	Q1 HR (95% CI)[1]	Q2 HR (95% CI)[1]	Q3 HR (95% CI)[1]	Q4 HR (95% CI)[1]	p_{het}
Tumor size							
T1, *in situ*	1,224 (76)	0.90 (0.83–0.98)	1.0 (Ref.)	0.96 (0.51–1.82)	0.82 (0.43–1.56)	0.51 (0.25–1.05)	0.20
T2–T4	808 (152)	0.96 (0.91–1.01)	1.0 (Ref.)	0.77 (0.49–1.20)	0.85 (0.54–1.33)	0.63 (0.38–1.04)	
N status							
N0	1,426 (98)	0.92 (0.85–0.99)	1.0 (Ref.)	0.69 (0.41–1.18)	0.58 (0.33–1.02)	0.35 (0.18–0.68)	0.34
≥N1	606 (130)	0.96 (0.91–1.02)	1.0 (Ref.)	0.94 (0.56–1.58)	1.05 (0.64–1.72)	0.89 (0.52–1.53)	
Tumor stage							
stage 0–IIIA	1,886 (156)	0.91 (0.86–0.97)	1.0 (Ref.)	0.80 (0.53–1.20)	0.71 (0.46–1.09)	0.42 (0.26–0.69)	0.01
stage IIIB–IV	148 (73)	1.04 (0.96–1.14)	1.0 (Ref.)	1.28 (0.59–2.78)	3.29 (1.53–7.11)	1.89 (0.85–4.19)	
Tumor grade							
grade 1+2	1,408 (121)	0.94 (0.89–1.00)	1.0 (Ref.)	1.07 (0.64–1.79)	1.07 (0.65–1.79)	0.65 (0.36–1.19)	0.66
grade 3+4	496 (105)	0.95 (0.89–1.02)	1.0 (Ref.)	0.72 (0.41–1.26)	0.70 (0.40–1.22)	0.59 (0.32–1.10)	
ER status (invasive only)							
positive	1,539 (159)	0.95 (0.91–1.00)	1.0 (Ref.)	1.01 (0.64–1.59)	1.04 (0.67–1.61)	0.76 (0.46–1.24)	0.63
negative	362 (67)	0.92 (0.83–1.02)	1.0 (Ref.)	0.72 (0.36–1.45)	0.87 (0.42–1.82)	0.37 (0.16–0.89)	
PR status (invasive only)							
positive	1,291 (125)	0.96 (0.91–1.01)	1.0 (Ref.)	0.86 (0.51–1.44)	0.86 (0.52–1.42)	0.74 (0.43–1.26)	0.29
negative	610 (101)	0.92 (0.86–1.00)	1.0 (Ref.)	1.14 (0.64–2.01)	1.02 (0.57–1.81)	0.53 (0.27–1.05)	
HER2 status (invasive only)							
positive	353 (51)	0.87 (0.77–0.98)	1.0 (Ref.)	1.59 (0.67–3.77)	1.15 (0.47–2.84)	0.52 (0.18–1.51)	0.42
negative	1,364 (153)	0.94 (0.89–0.99)	1.0 (Ref.)	0.86 (0.54–1.36)	0.88 (0.56–1.38)	0.58 (0.36–0.95)	
Tumor subtypes (invasive only)							
luminal A	998 (82)	0.89 (0.82–0.97)	1.0 (Ref.)	0.96 (0.51–1.80)	0.76 (0.41–1.40)	0.44 (0.21–0.90)	0.69
luminal B	287 (53)	0.98 (0.89–1.08)	1.0 (Ref.)	0.96 (0.40–2.28)	0.84 (0.37–1.93)	1.21 (0.50–2.93)	
HER2 positives	101 (20)	0.66 (0.47–0.93)	1.0 (Ref.)	0.81 (0.08–7.99)	0.92 (0.07–13.0)	0.03 (0.00–0.98)	
triple negatives	176 (36)	0.95 (0.82–1.10)	1.0 (Ref.)	0.39 (0.10–1.48)	0.82 (0.29–2.31)	0.46 (0.13–1.61)	

ENL: enterolactone; p_{het}: p for heterogeneity: likelihood ratio test comparing model with multiplicative interaction term enterolactone (continuous) x variable of interest and model without interaction term.
[1]Hazard ratios (HR) and 95% confidence intervals (95% CI) from multivariable complete-case analysis, stratified by age at diagnosis and study region, and adjusted for tumor size, nodal status, metastases status, histological grading, ER/PR status, BMI, radiotherapy, smoking, physical activity, MHT use, mode of detection, time between blood draw, and enterolactone measurement, if the adjustment variable was not the subgroup analysis variable.
Median enterolactone concentrations: Q1: 3.1; Q2: 14.9; Q3: 31.1; Q4: 72.1 nmol/L.

the small sample sizes for premenopausal disease. We did not include results of the two other publications of the MARIE study, one of which reported on a subsample of patients[9] and the other on dietary lignan intake.[12] High exposure to lignans (*i.e.*, measured as enterolactone or dietary intake) was statistically significantly inversely associated with both all-cause and breast cancer-specific mortality of postmenopausal breast cancer with summary estimates of HR 0.57 (95% CI 0.42–0.78) and 0.54 (95% CI 0.39–0.75), respectively (Figs. 1*a* and 1*b*). There was no statistically significant study heterogeneity ($p = 0.08$ and 0.18 for all-cause and breast cancer-specific mortality, respectively). We also did not find evidence for publication bias ($p = 0.67$ and 0.25, respectively, Supporting Information Figure S3).

Discussion

On the basis of a large study sample of 2,182 breast cancer patients, we found that post-diagnostic circulating enterolactone concentrations were associated with significantly reduced all-cause and breast cancer-specific mortality as well as a better distant disease-free survival in postmenopausal breast cancer patients. Per 10 nmol L^{-1} increment, risk of dying was significantly lower by 6% for these three endpoints in our study. The lack of significance for risk of recurrence may indicate that the inverse association is mainly due to reduction in deaths, rather than due to local or distant recurrences. In the meta-analysis including five independent study populations, lignan exposure was significantly associated with both reduced all-cause and breast cancer-specific mortality.

Table 4. Association between circulating enterolactone and all-cause mortality in postmenopausal breast cancer patients stratified by modifiable factors

Subgroup analysis	N patients (events)	Per 10 nmol/L HR (95% CI)[1]	Q1 HR (95% CI)[1]	Q2 HR (95% CI)[1]	Q3 HR (95% CI)[1]	Q4 HR (95% CI)[1]	p_{het}
Usual adult BMI (kg/m^2)							
<25	1,602 (171)	0.92 (0.87–0.97)	1.0 (Ref.)	0.73 (0.48–1.11)	0.66 (0.43–1.02)	0.54 (0.34–0.87)	0.04
≥25	505 (83)	0.99 (0.93–1.06)	1.0 (Ref.)	1.00 (0.53–1.88)	1.06 (0.56–1.99)	0.68 (0.31–1.51)	
Physical activity (hours/week)							
<28 MET	579 (87)	0.99 (0.92–1.06)	1.0 (Ref.)	0.95 (0.50–1.82)	1.32 (0.69–2.54)	0.72 (0.36–1.47)	0.16
≥28 MET	1,528 (167)	0.91 (0.86–0.97)	1.0 (Ref.)	0.85 (0.56–1.29)	0.76 (0.49–1.17)	0.50 (0.30–0.82)	
Smoking							
never/former smoker	1,732 (193)	0.94 (0.90–0.99)	1.0 (Ref.)	1.12 (0.75–1.67)	0.98 (0.65–1.47)	0.71 (0.45–1.11)	0.97
current smoker at diagnosis	375 (61)	0.93 (0.84–1.02)	1.0 (Ref.)	0.32 (0.14–0.71)	0.54 (0.24–1.20)	0.31 (0.13–0.78)	
Dietary fiber intake							
<median	934 (120)	0.93 (0.86–0.99)	1.0 (Ref.)	0.56 (0.33–0.94)	0.57 (0.34–0.94)	0.47 (0.26–0.85)	0.32
≥median	937 (92)	0.89 (0.82–0.97)	1.0 (Ref.)	1.19 (0.68–2.10)	0.88 (0.48–1.61)	0.46 (0.23–0.91)	
Menopausal hormone therapy							
never or former user	1,162 (184)	0.95 (0.91–1.00)	1.0 (Ref.)	0.83 (0.55–1.24)	1.05 (0.70–1.58)	0.65 (0.41–1.03)	0.20
current user at diagnosis	945 (70)	0.88 (0.79–0.97)	1.0 (Ref.)	1.08 (0.57–2.04)	0.57 (0.28–1.16)	0.42 (0.19–0.92)	
Lifestyle							
healthy (never/past smoker, BMI <25, and ≥28 MET hours/week)	997 (88)	0.92 (0.85–0.99)	1.0 (Ref.)	1.14 (0.62–2.07)	0.78 (0.43–1.42)	0.67 (0.35–1.28)	0.92
less healthy (smoker or BMI ≥25 or <28 MET hours/week)	1,117 (166)	0.96 (0.91–1.01)	1.0 (Ref.)	0.79 (0.52–1.20)	0.90 (0.58–1.41)	0.61 (0.37–1.01)	

ENL: enterolactone; p_{het}: p for heterogeneity: likelihood ratio test comparing model with multiplicative interaction term enterolactone (continuous) x variable of interest and model without interaction term.
[1]Hazard ratios (HR) and 95% confidence intervals (95% CI) from multivariable analysis, stratified by age at diagnosis and study region, and adjusted for tumor size, nodal status, metastases status, histological grading, ER/PR status, BMI, radiotherapy, smoking, physical activity, MHT use, mode of detection, time between blood draw and enterolactone measurement, if the adjustment variable was not the subgroup analysis variable.
Median enterolactone concentrations: Q1: 3.1; Q2: 14.9; Q3: 31.1; Q4: 72.1 nmol/L.

Our study results are consistent with two independent studies on circulating enterolactone. In a prospective Danish study investigating prediagnostic enterolactone concentrations in 424 postmenopausal breast cancer patients, risk of dying was reduced by 18 and 12% per 20 nmol L^{-1} increment for all-cause and breast cancer-specific mortality, respectively.[11] A retrospective Italian study reported stronger associations in postmenopausal women with HR of 0.38 and 0.32 when comparing enterolactone concentrations ≥10–<10 nmol L^{-1} on all-cause and breast cancer-specific mortality within 5 years after surgery, respectively.[10] The median levels were comparable across the three independent studies, with postdiagnostic concentrations of 22.3 nmol L^{-1} in this study and of 20.0 nmol L^{-1} in the Italian study,[10] as well as prediagnostic concentrations of 20.5 nmol L^{-1} in the Danish study.[11] Corroborating evidence from associations with dietary intake of lignans and estimated enterolignans can be derived from two independent studies[13,14] and our study population.[12] Lignan intake or estimated enterolactone levels was significantly associated with a 40–50% lower all-cause mortality and a 30–70% reduced breast cancer-specific mortality in the two studies including 2,653[12] and 804[13] postmenopausal breast cancer patients but not in a third study of 834 postmenopausal women.[14] The latter study, however, did not adjust for tumor characteristics. The authors reported that adjustment for potential confounders such as tumor size did not change the estimates by more than 10%. Although dietary lignan intake does not correlate strongly with biomarker measurement and different cutoff values were used across the studies, we included all existing studies to assess the strength of current evidence for an effect of high lignan compared to low lignan exposure on prognosis of breast cancer. We found that high lignan exposure is significantly associated with a reduced mortality in postmenopausal breast cancer patients. The absence of study heterogeneity provides some assurance that the association is robust despite heterogeneous patient samples and different follow-up time across studies.

There is at present no clear evidence that the inverse association of enterolactone concentrations with mortality is

Table 5. Summary of epidemiological studies evaluating association of enterolactone/lignan with breast cancer prognosis

Study, publication year (Ref.)	Country, years of diagnosis	N_{total} (all deaths/ due to BC)	Tumor stage	Follow-up time (median)	Exposure	Menopausal status ($N_{subgroup}$/deaths)	Enterolactone categories	All-cause mortality HR (95% CI)	Cancer-specific mortality HR (95% CI)	Adjustment factors
Circulating enterolactone										
Buck 2011a (9)[1,2]	Germany (RNK), 2002–2005	1,140 (162/124)	0–IV	6.1 yrs	Serum enterolactone, postdiagnosis	postmenop. (all)	Q4 (≥42.3) *vs.* Q1 (≤7.8 nmol/L)	0.58 (0.34–0.99)	–	TNM, grade, ER/PR, mode of detection, diabetes, use of menopausal hormone therapy, BMI, physical activity (strata: age, study region)
							per 10 nmol/L	0.94 (0.88–1.00)		
Guglielmini 2012 (10)[M]	Italy, 1984–1991	300 (180/112)	early disease	5 yrs[3]	Serum enterolactone, postdiagnosis	premenop. (88/NA)	≥10 *vs.* <10 nmol/L, within 5 yrs	1.34 (0.26–6.93)	1.14 (0.21–6.05)	tumor size, nodal status, adjuvant chemotherapy, adjuvant tamoxifen
						postmenop. (212/NA)	≥10 *vs.* <10 nmol/L, within 5 yrs	0.38 (0.19–0.76)	0.32 (0.15–0.66)	
Olsen 2012 (11)[M]	Denmark, 1993–1997	424 (111/80)	NA	10 yrs	Plasma enterolactone, prediagnosis	postmenop. (all)	>*vs.* ≤ 20.5 nmol/L (median)	0.47 (0.32–0.68)	0.56 (0.36–0.87)	grade, alcohol intake, menopausal hormone therapy
							per 20 nmol/L	0.82 (0.70–0.96)	0.88 (0.75–1.03)	
This study[1,M]	Germany, 2001–2005	2,182 (269/194)	0–IV	5.4 yrs	Plasma enterolactone, postdiagnosis	postmenop. (all)	Q4 (>45.1) *vs.* Q1 (≤8.5 nmol/L)	0.59 (0.40–0.87)	0.59 (0.37–0.94)	TNM, grade, ER/PR, mode of detection, physical activity, time between blood draw and enterolact one measurement, menopausal hormone therapy, BMI, smoking, radiotherapy (strata: age, study region)
							per 10 nmol/L	0.94 (0.90–0.98)	0.94 (0.89–0.99)	
Dietary lignan intake										
Buck 2011b (12)[1]	Germany, 2001–2005	2,653 (321/235)	0–IV	6.4 yrs	Estimated enterolactone, prediagnosis	postmenop. (all)	Q5 (median 502.0) *vs.* Q1 (median 146.0 μg/d)	0.60 (0.40–0.89)	0.69 (0.43–1.10)	TNM, grade, ER/PR, mode of detection, diabetes, use of menopausal hormone therapy, study region, energy intake (strata: age)
Fink 2007 (14)[M]	US, 1996–1997	1,210 (173/113)	invasive	5 yrs	Estimated dietary lignans, prediagnosis	premenop. (376/43)	Q5 (≥9) *vs.* Q1 (≤2.2 mg/d)	1.27 (0.63–2.54)	1.16 (0.52–2.58)	age, energy intake
						postmenop. (834/130)	Q5 (≥9) *vs.* Q1 (≤2.2 mg/d)	0.98 (0.63–1.54)	0.87 (0.49–1.55)	
McCann 2010 (13)[M]	US, 1996–2001	1,122 (160/94)	0–IV	87 mo (mean)	Estimated dietary lignans, prediagnosis	premenop. (315/44)	Q4 (>257) *vs.* Q1 (<128 μg/d)	2.14 (0.82–5.56)	1.84 (0.65–5.27)	age, race, total energy, stage, BMI, education
						postmenop. (807/116)	Q4 (>318) *vs.* Q1 (<155 μg/d)	0.49 (0.26–0.91)	0.29 (0.11–0.76)	

[1]Overlapping study populations, therefore Buck et al. 2011a and 2011b not included in the meta-analysis.
[2]Includes patients from only one of the two study region of the German MARIE study.
[3]Restricted to 5 years (median follow-up time of whole study population: 23 yrs). M: included in the meta-analysis.
ENL: enterolactone; HR (95% CI): Hazard ratios (HR) and 95% confidence intervals (CI) from most adjusted models; NA: not available; Q1: lowest quantile; Q4: highest quartile; Q5: highest quintile; RNK: Study region Rhein-Neckar-Karlsruhe; TNM: tumor size, nodal status, metastases.

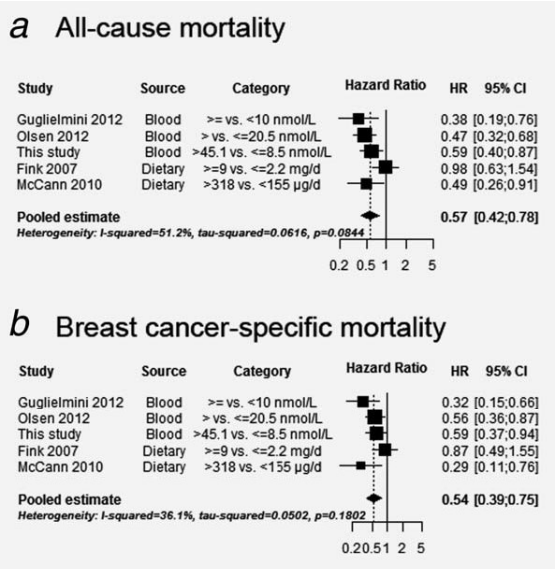

Figure 1. Meta-analysis: Association between lignan exposure and breast cancer prognosis in postmenopausal women. Results for all-cause mortality (*a*) and breast cancer-specific mortality (*b*) are presented. Fully adjusted hazard ratios (HR) from individual studies are displayed by filled boxes with relative sample sizes represented by the sizes of symbols. Horizontal lines indicate 95% CIs for the respective HR. Pooled estimates with 95% CIs from random-effects models are represented by a diamond. As the study populations of Buck et al. 2011a and Buck et al. 2011b overlap with this investigation, we did not include the Buck et al. 2011a and Buck et al. 2011b publications in the meta-analysis.

restricted to a subgroup of breast tumors or patients with certain characteristics. All previous studies included all stages of disease and none thoroughly investigated differential effects by tumor characteristics and lifestyle factors. We found an inverse association only for early stage breast cancer. While this was not examined by other studies, our findings are consistent with other reports that lignan exposure before breast cancer diagnosis may be associated with more favorable tumor characteristics, such as hormone receptor positivity, no lymph node involvement, and smaller tumor size.[7,8] Estrogen receptor status was not found to significantly modify the association in our study and the Danish study.[11] These observations are supported by experimental findings showing that besides antiestrogenic properties, lignans have estrogen-independent anticarcinogenic activity through inhibiting proliferation, stimulating apoptosis and reducing expression of HER2 and EGFR.[4,26] In addition, observations from a recent *in vitro* study suggested that anti-metastatic properties of enterolactone may be attributed to downregulation of certain matrix metalloproteinases, which are crucial for the development of metastases.[27] These results are also in line with findings from a randomized trial with 32 breast cancer patients of a reduction in proliferation (Ki-67) and HER2 expression as well as an increase in apoptosis after flaxseed intervention.[28]

Higher enterolactone concentrations and lignan intake have been associated with older age, nonsmoking and BMI <25 kg m^{-2} in women, both in our and in other studies.[29,30] It has been suggested that lower biomarker concentrations in obese women might be due to dilution as a result of rapid transport of enterolactone into preadipocytes.[29] This may in part explain the stronger inverse associations of enterolactone concentrations with mortality in patients with BMI <25 kg m^{-2}. However, there was still a reduced HR associated with high enterolactone concentrations (Q4 *vs.* Q1) in patients with BMI ≥ 25 kg m^{-2}, albeit nonsignificant.

It is not entirely established whether the observed associations with enterolactone concentrations indicate a direct role of lignans. Our results suggest that enterolactone is not simply a biomarker of a healthy lifestyle. We carefully adjusted for all possible confounding factors although we cannot exclude residual confounding. Among patients with a healthy lifestyle, as defined by normal weight, nonsmoking and physical activity, there was still a significant inverse association between high enterolactone concentrations and mortality. Confounding by socioeconomic status is unlikely, as neither education nor occupation changed the association of enterolactone with overall mortality substantially. Foods containing lignans may contain a mixture of healthy food ingredients, including fiber and unsaturated fatty acids, for example, which exert a beneficial effect in concert.[4] In our study population, estimated enterolignans were positively correlated with dietary intake of sunflower-, pumpkinseed, sesame and flaxseed as well as fiber intake.[12] The association with enterolactone concentrations is, however, not likely to be entirely explained by fiber intake as we did not observe differential effects by level of dietary fiber intake. On the other hand, the known relatively weak correlation between enterolactone concentration and estimated dietary lignans intake from food frequency questionnaires indicates the importance of gut microflora for bioactivation of lignans. Indeed, a recent study found that rats with lignan-metabolizing bacteria had a lower tumor burden with lower tumor size and proliferation after a lignan-rich diet with flaxseeds, although the overall tumor incidence was not different from control animals.[31] A better understanding of the extent to which gut microbiota affects enterolactone concentrations and how lifestyle factors affect colonic environment would be important for planning future dietary intervention studies.

One potential limitation of our study is that the enterolactone measurements—like in the other observational studies—were based on one single time point. However, it was previously shown that enterolactone measurements were quite stable over a 2-year period[32] but may not reflect long-term exposure. Furthermore, we do not have any information on antibiotics use before blood draw or on constipation which may have influenced metabolization of lignans. We were not able to account for changes in lifestyle factors after diagnosis. A limitation of the meta-analysis is the different quantile ranges the studies used to categorize their data. We recognize

that these increments may not be comparable. However, we were interested in the comparison of higher levels to lower levels rather than the absolute quantities. Indeed, we did not observe significant heterogeneity of results across studies. Major strengths of our study include the large sample size, restriction to postmenopausal women, the (nearly) complete follow-up information with verified endpoints, and comprehensive clinical data and lifestyle information.

In conclusion, there is good evidence that high lignan exposure may be associated with reduced mortality after breast cancer. Our study results suggest that enterolactone concentrations are not simply a biomarker of a healthy lifestyle pattern. Further studies are warranted to elucidate the underlying mechanisms.

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