Serum enterolactone and postmenopausal breast cancer risk by estrogen, progesterone and herceptin 2 receptor status

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Lignans are a group of estrogenic compounds present in plants. Several epidemiological studies proposed that lignans may protect against breast cancer by exerting anticarcinogenic activity. Levels of enterolactone were determined in serum samples of 1,250 cases and 2,164 controls from a large population-based case–control study. We assessed the association between serum enterolactone and postmenopausal breast cancer risk using conditional logistic regression accounting for potential risk and confounding factors. Fractional polynomials were used to determine the function that best fitted the data. Moreover, we assessed heterogeneity by estrogen/progesterone/herceptin (ER/PR/HER2) status of the tumor. Additionally, a meta-analysis with seven further studies addressing enterolactone concentrations and breast cancer risk was performed. Postmenopausal breast cancer risk decreased with increasing serum enterolactone levels [highest compared to lowest quintile: [odds ratio = 0.65; 95% confidence interval (CI) 0.52–0.83, p_{trend} = <0.0001]. A significant inverse association for ER+/PR+ as well as ER−/PR− tumors was observed, with a significantly stronger association for ER−/PR− tumors ($p_{heterogeneity}$ = 0.03). The association for ER−/PR− tumors did not differ by expression of HER2 ($p_{heterogeneity}$ = 0.3). The meta-analysis yielded a significant reduced pooled risk estimate of: 0.66; 95% CI: 0.55–0.77) comparing the highest to the lowest quantiles of enterolactone levels. We found strong evidence for a significant inverse association between serum enterolactone and postmenopausal breast cancer risk, which was stronger for ER−PR− than for ER+PR+ tumors but not differential by further expression of HER2. The overall evidence together with other studies supports an inverse association between higher serum enterolactone levels and postmenopausal breast cancer risk.

Phytoestrogens are a group of biologically active compounds that have been shown to protect against hormone-dependent cancers, in particular, breast cancer.[1–4] Their protective inverse association with breast cancer may be due to either estrogenic or antiestrogenic properties.[5–8] Because of their chemical structure, phytoestrogens can compete with endogenous estrogen for binding to the estrogen receptor (ER) of the tumor, thereby plausibly reducing the hormonal effect of the endogenous estrogens.[9] In addition to this, phytoestrogens also have antioxidative and antiproliferative properties and thereby may also reduce cancer risk.[10] The two main groups of phytoestrogens are the isoflavones and the lignans. Isoflavones, in particular, genistein, are mostly found in soy products. Only a few nonsoy plants contain isoflavones (*e.g.*, legumes, grapefruit and raisins); but, at lower concentration, isoflavones do not importantly contribute to phytoestrogen intake in Western diets.[11,12] Plant lignans are widely present in whole grain, berries, vegetables, fruits, flaxseeds and other types of seeds and are the main source of phytoestrogens in Western populations.[13] Plant lignans (lariciresinol, pinoresinol, secoisolariciresinol and matairesinol) are metabolized into mammalian lignans (enterolignans: enterolactone, enterodiol) in the human gut. Serum or urinary levels of enterolignans can serve as an alternative indicator of individual dietary lignan intake. The advantage of these biomarkers is that they do not rely on self-reported dietary intake or on dietary databases for lignan quantification.

Several studies in Western populations have investigated the association of either dietary lignan intake or lignan

Key words: lignans, enterolactone, postmenopausal women, breast cancer

Abbreviations: BMI: body mass index; CI: confidence interval; ER: estrogen receptor; FFQ: Food Frequency Questionnaire; HER2: herceptin receptor; HRT: hormone replacement therapy; OR: odds ratio; PR: progesterone receptor; TR-FIA: time-resolved fluoroimmunoassays; RE: risk estimates

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biomarkers with pre- or postmenopausal breast cancer risk, with inconsistent results.[14–21] Two recently published meta-analyses (including our own) showed that high serum or urinary enterolactone levels were associated with a nonsignificant reduced postmenopausal breast cancer risk, whereas high consumption of dietary lignans was associated with a significant reduced postmenopausal breast cancer risk.[22,23]

The objective of our study was to examine the association of the major serum enterolignan (enterolactone) with the risk of postmenopausal breast cancer in a large German study population with Western dietary habits. Additionally, we investigated whether this association differs by ER, progesterone receptor (PR) or herceptin receptor (HER2) status of the tumor.

Material and methods

Study population

A population-based case–control study Mammary carcinoma Risk factor Investigation was carried out in two study regions in Germany (Hamburg and Rhine–Neckar–Karlsruhe). German-speaking women aged 50–74 years, diagnosed with histologically confirmed primary invasive or *in situ* breast cancer between January 1, 2001 and September 30, 2005 in Hamburg and between August 1, 2002 and July 31, 2005 in the Rhine–Neckar–Karlsruhe region, were identified through participating clinics and the Hamburg Cancer Registry.

For each patient, two controls matched for birth year and study center were randomly selected using resident registries. In total, 3,464 postmenopausal cases and 6,657 postmenopausal controls were participated in this study. Women were defined as postmenopausal if they reported their last natural menstrual bleeding at least 12 months before the reference date, or a bilateral oophorectomy, or cessation of menses due to treatment of disease other than breast cancer by either radiation or chemotherapy. Those above age 55 years whose menopausal status was unclear because of hysterectomy or hormone use were also considered postmenopausal but assigned an unknown age at menopause. More detailed information on the study population can be found elsewhere.[24] Face-to-face interviews were carried out by trained interviewers using a standardized questionnaire to collect information on sociodemographic characteristics as well as putative risk factors for breast cancer, including reproductive history, use of hormone replacement therapy (HRT), family history of breast cancer, physical activity, smoking and alcohol consumption.

Furthermore, a self-administered Food Frequency Questionnaire (FFQ) was applied to collect information on nutritional habits in the year before diagnosis of breast cancer or the year before FFQ completion for controls.

All participants gave written confirmed consent. The study was approved by the ethics committees of both the University of Heidelberg and the University of Hamburg and conducted in agreement with the Helsinki declaration.

Handling of blood samples

Nonfasting serum samples were obtained from participants in the Rhine–Neckar–Karlsruhe region and stored at −80°C. Hemolytic samples were excluded from the measurements (143 cases and 236 controls), resulting in 1,250 cases (including 78 *in situ* cancers) and 2,164 controls. For cases, median time between diagnosis and blood collection was about 3 months. The range for blood collection was 0–37 months after diagnosis; nevertheless, for 98.6% of participants, blood collection was within 1 year of diagnosis.

Biochemical analyses

Enterolactone was measured by time-resolved fluoroimmunoassays (TR-FIA) according to the methods developed and validated by Adlercreutz *et al.* and Wang *et al.*[25,26] The measurements were conducted using the enterolactone kit as described by the manufacturer *Labmaster* Laboratory in Turku Finland. In brief, 150 μl of serum was incubated overnight at 37°C with 150 μl of 100 mM acetate buffer (pH 5.0, containing 0.2 U/ml of β-glucuronidase and 2 U/ml of sulfatase), mixed with 150 μl aliquot of serum and incubated overnight at 37°C. Diethyl-ether was used to extract unconjugated phytoestrogen after hydrolysis. Two diethyl-ether phases were combined and evaporated to dryness using a water bath. Finally, the dried residues were dissolved in assay buffer and measured using (europium-labeled) TR-FIA. All batches were mixed and measured blinded with respect to case–control status and included two quality controls at varying positions on the assay plate. Single measurements were performed for all samples. For the quality controls for enterolactone in 33.1 nmol sample volume, the mean intra- and interassay coefficient of variation were 10.8 and 19.2%, respectively.

ER, PR and HER2 determination

Clinical and pathologic tumor characteristics were abstracted from hospital records and pathology reports. Hormone receptor status was ascertained using immunohistochemical testing, with immunoreactive scoring according to Remmele and Stegner.[27] The Remmele scoring system combines both the intensity of staining and the percentage of positive cells. The staining intensity is categorized into four groups (0 = no staining (negative), 1 = weak staining, 2 = moderate staining and 3 = strong staining). The percentage of positive cells was categorized into five groups (0% = 0, <10% = 1, 10–50% = 2, 51–80% = 3 and >80% = 4). A cut-off point of 0–2 was interpreted as negative tumors, and ≥3 was interpreted as positive tumors. HER2 was assessed using standardized immunohistochemical testing (graded from 0 to 3, 0–1 means that a normal amount of the HER2 protein is present and the result is HER2-negative, 2 means that a moderate amount of the HER2 protein is present, 3 means that there is a higher than normal level of HER2 protein and the result is HER2-positive), followed by a fluorescent *in situ* hybridization test (FISH) in the case of weak positive reaction. The

scoring is either FISH-negative when normal levels of the gene are present or FISH-positive when excessive amounts of the gene are present.[27,28]

Statistical analyses

The association between serum enterolactone and risk of postmenopausal breast cancer was assessed using conditional logistic regression. Odds ratios (ORs) and their corresponding 95% confidence intervals (95% CI) were calculated using the PROC LOGISTIC procedure of the SAS statistical software package, version 9.2 (SAS institute, Cary, NC).

Fractional polynomials were used to determine the function that best fitted the data.[29] The continuous variable for enterolactone concentration was entered into the multivariate logistic regression models *via* a set of defined transformations [x^{-2}, x^{-1}, $x^{-0.5}$, $x^{0.5}$, x^{1}, x^{2}, x^{3} and $\log(x)$], allowing for a maximum of two terms in the model. Using fractional polynomials, we found that the log-transformed model best fitted our data, indicating a nonlinear relationship between serum enterolactone concentrations and postmenopausal breast cancer risk ($p_{\text{nonlinearity}} = 0.01$; Supporting Information Fig. 1). Based on this, serum enterolactone levels were logarithmically transformed to achieve normality. The value 0.001 was assigned to phytoestrogen levels that were below detection limit (26 cases and 28 controls), and the value 300 nmol/l was assigned to values that were above quantification level (4 cases and 11 controls).

Quintiles were calculated using the distribution of serum concentrations in the controls. Crude and multivariate analyses were stratified by year of birth. For multivariate analyses, participants with missing information on any of the covariates were excluded from the analyses (5 cases and 18 controls). All multivariate analyses were adjusted for breast cancer risk factors and potential confounding factors that changed the risk estimate (RE) by $\geq$10%. The adjustment factors were given as follows: menopausal induction (natural with/without HRT, induced menopause, hysterectomy, start of HRT and other), body mass index (BMI) ($<$22.5, 22.5–25, 25–30 and $\geq$30 kg/m^2), occupational level (blue collar worker, simple employee, medium employee, higher employee and leading position), first-degree family history of breast cancer (yes, no and unknown), history of benign breast disease (yes and no), number of pregnancies ($\geq$28th week) (0, 1, 2 and $\geq$3), age at menarche ($<$12, 12–14 and $\geq$15), breastfeeding history (ever and never), total number of mammograms (0, 1–4, 5–9 and $\geq$10, unknown), smoking habit (never, past and current), alcohol consumption (g/day) (0, 0–18.9 and $\geq$19) and use of phytoestrogen supplements (yes or no). Physical activity, fruit, vegetable and fiber intake did not substantially change the REs and were therefore excluded from the adjusted model. To test for trend, the log-transformed continuous serum concentration of enterolactone was used in the logistic regression model. The significance level for the trend analyses was 0.05.

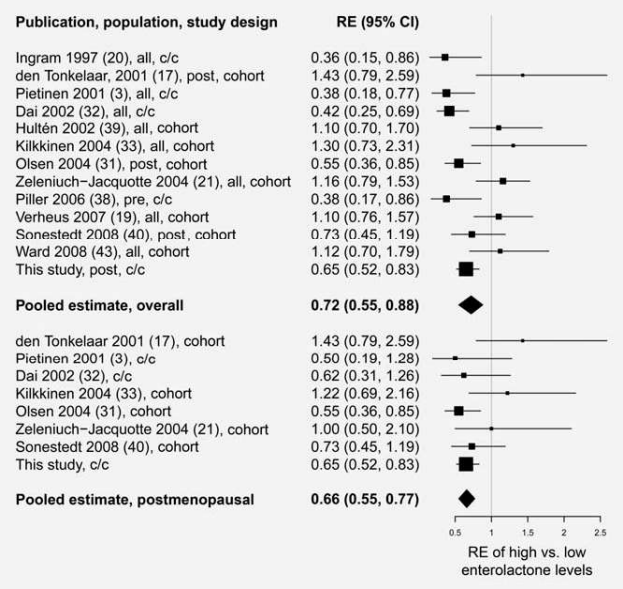

Figure 1. Association between serum, plasma and urine enterolactone biomarkers with breast cancer risk according to menopausal status. Risk estimates (REs) from individual studies are represented by black-filled boxes. REs from fixed-effects models are represented by filled black diamonds. Relative sample sizes are represented by the sizes of symbols. Horizontal lines indicate 95% CIs for the respective RE. Premenopausal women (pre), postmenopausal women (post), and a combination of pre- and postmenopausal women (all) are shown. Prospective cohorts/nested case–control studies (cohorts) and case–control studies (c/c) are shown.

Polytomous logistic regression analysis was used to assess the association of serum enterolactone with postmenopausal breast cancer risk by ER and PR status of the tumor. We further examined the association between serum enterolactone and the risk of breast cancer according to the combined ER/PR status of the tumor. Moreover, we assessed the association of serum enterolactone by combined ER/PR/HER2 status.

Because adjuvant chemotherapy might have an effect on the levels of phytoestrogen biomarkers, we performed a sensitivity analysis by dividing our study population into two groups; the first group had blood collection before start of chemotherapy or did not undergo chemotherapy, and the second group had blood collection after start of chemotherapy. Additionally, we performed a sensitivity analysis to investigate if the association was different according to timing of blood collection before or after median time between diagnosis and blood collection (3 months). Tests for heterogeneity were performed using the Q statistic with a significance level of 0.05.

Meta-analysis of epidemiologic studies addressing enterolactone concentrations and breast cancer risk, including the present study, was carried out using the "meta" and "rmeta" packages of the statistical software environment R (version

2.9.2). To select epidemiological studies that reported on the association of serum/urinary lignans on breast cancer risk, a systematic Medline search was performed for publications between 1997 and December 2010. We used a combination of key words such as breast, phytoestrogens, lignans, enterolactone, serum, plasma, urine and epidemiological studies. Moreover, cited references in the retrieved articles were carefully reviewed to identify possible additional articles that might have been missed in the initial search. Suitable publications were assessed by three authors independently (AKZ, KB and AV). The most fully adjusted REs and CIs for the highest quantile compared to the reference quantile (the lowest quantile) from each study were used for the meta-analyses. The statistical analyses included a test of heterogeneity to determine whether the study results (of groups) were significantly heterogeneous. Fixed-effect models were used when heterogeneity was low ($p > 0.1$). Otherwise, random effects models were used.[30] The individual study results and combined pooled estimates were illustrated in forest plots. Analyses were conducted for all women combined (pre and postmenopausal) and for postmenopausal women separately. Moreover, analyses were stratified by study design for studies on postmenopausal breast cancer risk.

Results

The mean age of the cases and controls was 63 years. Cases were more likely than controls to have an earlier age at menarche, a lower number of pregnancies, no breast feeding history, a history of benign breast disease, a higher number of mammograms, a first degree family history of breast cancer and to have never smoked and were less likely to use phytoestrogen supplements (Table 1). There was no difference between cases and controls with respect to menopausal induction, BMI, occupational class and alcohol consumption. The median serum enterolactone concentration was 19.5 nmol/l in cases and 22.8 nmol/l in controls (p-value = 0.005).

The crude OR for the highest *versus* lowest quintile of serum enterolactone in relation to postmenopausal breast cancer risk was 0.66 (95% CI: 0.53–0.87; p for trend = 0.001; Table 2). After adjustment for breast cancer risk factors and potential confounding factors, the estimates did not change substantially (OR = 0.65; 95% CI: 0.52–0.83; p for trend $\leq$ 0.0001). Significantly reduced ORs were also observed for the second, third and fourth quintile compared to the lowest quintile (Table 2). Restriction to invasive cases also did not change results substantially (for highest quintile OR = 0.61; 95% CI: 0.47–0.78; p for trend $\leq$ 0.0002).

We investigated the association of serum enterolactone with postmenopausal breast cancer risk by ER and PR status of the tumor (Table 3). Enterolactone levels were associated with both ER+ tumors (highest *vs.* lowest quintile: OR = 0.73; 95% CI: 0.56–0.95; p for trend = 0.01) and ER-tumors (highest *vs.* lowest quintile: OR = 0.39; 95% CI: 0.25–0.59; p for trend $\leq$ 0.0001). However, the association was stronger for ER-tumors (p for heterogeneity = 0.01). We also observed a significant inverse association in both PR+ tumors (highest *vs.* lowest quintile: OR = 0.68; 95% CI: 0.52–0.90; p for trend = 0.001) and PR− tumors (highest *vs.* lowest quintile: OR = 0.51; 95% CI: 0.33–0.73; p for trend = 0.0004), but without significant effect heterogeneity (p for heterogeneity = 0.2).

Analyses by combined ER/PR status of the tumor yielded the strongest inverse association for the ER−/PR− tumors, with an OR for the highest compared to the lowest quintile of 0.36 (95% CI: 0.22–0.60; p for trend 0.003), with significant heterogeneity between ER−/PR− and ER+/PR+ tumors (p for heterogeneity = 0.03).

The additional consideration of HER2 expression for tumor subtypes did not indicate effect heterogeneity. There was a significant inverse association of enterolactone levels with breast cancer for ER−/PR−/HER2+ (71 cases, p for trend = 0.05) as well as ER−/PR−/HER2− (triple negative) tumors (130 cases and p for trend = 0.004). For ER+/PR+/HER− tumors (540 cases), a significant association was observed only for the highest quintile (OR = 0.68; 95% CI: 0.50–0.94, p for trend = 0.003). There was no association with ER+/PR+/HER+ tumors (87 cases and p for trend = 0.5). However, results by HER2 expression should be interpreted with caution due to the relatively small sample size in these subgroups.

Median enterolactone levels were 22.0 and 21.5 nmol/l for blood donation before and after chemotherapy, respectively. The association between serum enterolactone and postmenopausal breast cancer risk did not differ by blood collection before or after start of chemotherapy (p for heterogeneity = 0.5) nor by blood collection within or after the median time between diagnosis and blood collection (p for heterogeneity = 0.7).

The breast cancer REs of studies included in the meta-analysis are shown in (Fig. 1). Overall, a significant association with breast cancer risk was observed, with a pooled RE of 0.72 (95% CI: 0.55–0.88) comparing the highest quantile to the reference (lowest) quantile of enterolactone. For postmenopausal women, the highest quantile was associated with a significantly reduced pooled RE of 0.66 (95% CI: 0.55–0.77). When stratifying the analysis for studies on postmenopausal breast cancer risk by study design, we observed a significantly reduced postmenopausal breast cancer risk both in prospective cohort/nested case–control studies and in case–control studies with pooled RE of 0.70 (95% CI: 0.52–0.88) and pooled RE of 0.64 (95% CI: 0.49–0.78), respectively (Fig. 2).

Discussion

We found that circulating enterolactone concentrations in the highest compared to the lowest quintile were associated with statistically significantly reduced risk of postmenopausal breast cancer. Moreover, the relationship between serum enterolactone and postmenopausal breast cancer risk was

Table 1. Distribution of sociodemographic variables and risk factors for postmenopausal breast cancer cases ($n = 1,250$) and controls ($n = 2,164$)

	Cases		Controls		
	n	%	*n*	%	*p*-value[1]
Induction of menopause					0.10
Natural with/without HRT**	659	52.7	1173	54.2	
Induced (bilateral oophorectomy/chemotherapy/radiation)	90	7.2	171	7.9	
Hysterectomy	318	25.4	563	26.0	
Start of HT	160	12.8	225	10.4	
Other	23	1.9	32	1.5	
Age at menarche (years)					0.01
<12	114	9.1	179	8.3	
12–<15	840	67.2	1392	64.3	
15+	296	23.7	587	27.1	
Missing	–	–	6	0.3	
Number of pregnancies ≥ 28 weeks					0.001
0	178	14.2	250	11.6	
1	343	27.4	527	24.4	
2	453	36.2	853	39.4	
3+	276	22.2	534	24.6	
Breastfeeding history					
No	490	39.2	725	33.5	0.0004
Yes	760	60.8	1439	66.5	
History of benign breast disease					<0.0001
No	758	61.6	1504	69.5	
Yes	487	38.0	650	30.0	
Missing	5	0.4	10	0.5	
Number of mammograms					<0.0001
No	170	13.6	278	12.9	
1–4	533	42.6	1132	52.3	
5–9	306	24.5	499	23.1	
10+	226	18.1	238	11.0	
Unknown number	15	1.2	17	0.7	
BMI (kg/m^2)					
<22.5	486	38.9	868	40.1	0.87
22.5–<25	430	34.4	693	32.0	
25–<30	282	22.5	514	23.8	
≥30	52	4.2	89	4.1	
First-degree family history of breast cancer					0.001
No	986	78.9	1786	82.5	
Yes	210	16.8	285	13.2	
Unknown	54	4.3	93	4.3	
Occupation					0.20
Blue collar worker	236	18.9	378	17.5	
Simple employee	296	23.7	576	26.6	
Medium employee	425	34.0	763	35.3	

Table 1. Distribution of sociodemographic variables and risk factors for postmenopausal breast cancer cases ($n = 1,250$) and controls ($n = 2,164$) (Continued)

	Cases		Controls		
	n	%	*n*	%	*p*-value[1]
Higher employee	235	18.8	377	17.4	
Leading position	50	4.0	60	2.8	
Unknown	8	0.6	10	0.4	
Smoking					0.02
Never	793	63.5	1269	58.5	
Former	283	22.6	590	27.2	
Current	174	13.9	304	14.2	
Missing	–	–	1	0.1	
Alcohol consumption (g/day)					0.70
0	227	18.2	355	16.4	
0–<19	868	69.4	1554	71.8	
≥19	155	12.4	254	11.6	
Missing	–	–	1	0.2	
Phytoestrogen supplement use					0.03
No	1243	99.4	2135	98.7	
Yes	7	0.6	29	1.3	

[1]Chi-square-test (categorized variables). Abbreviations BMI: body mass index; HRT: hormone replacement therapy.

Table 2. Crude and adjusted odds ratios[1] (ORs) and 95% confidence intervals (95% CIs) for serum enterolactone concentrations and postmenopausal breast cancer risk

	Quintiles					
	I	II	III	IV	V	*p*-trend[2]
			Enterolactone			
Median nmol/l	3.1	11.9	22.8	39.1	80.9	
Quintile range nmol/l	≤7.2	>7.2–16.1	>16.1–29.9	>29.9–54.3	>54.3	
Cases/controls[3]	310/432	238/434	246/432	248/434	208/432	
Crude OR (95%CI)	1.00	0.76 (0.62–0.95)	0.79 (0.64–0.98)	0.78 (0.64–0.98)	0.66 (0.53–0.87)	0.001
Adjusted[1] OR (95% CI)	1.00	0.74 (0.59–0.93)	0.77 (0.62–0.97)	0.76 (0.61–0.96)	0.65 (0.52–0.83)	<0.0001

[1]Calculated using log-transformed values of enterolactone and adjusted for induction of menopause, BMI, education level, first-degree family history of breast cancer, history of benign breast disease, number of pregnancies (≥28th week), age at menarche, breastfeeding history, total number of mammograms, smoking habit, alcohol consumption and use of phytoestrogen supplements. [2]Log transformed continuous serum concentration was used as an ordinal variable in the logistic regression model. [3]Number of participants in the crude OR and 95% CI, for the adjusted analysis 5 cases and 18 controls were excluded due to missing values in the covariates.

nonlinear. The inverse associations with serum enterolactone were significantly stronger for ER− compared to ER+ tumors but did not differ by PR status of the tumor. The risk reduction associated with high enterolactone levels was also significantly stronger for ER−/PR− compared to ER+/PR+ tumors and did not further differ by HER2 expression.

Our findings of a significant inverse association of serum enterolactone with postmenopausal breast cancer risk corroborate the results of a relatively large Danish prospective cohort study (381 cases and 381 controls).[31] Several previous studies on serum, plasma and urine enterolactone concentrations in postmenopausal women found inverse but not statistically significant associations with breast cancer risk[3,32] or no association.[17,33–35] This could have been in part due to small sample sizes of each of these studies and corresponding limited statistical power to detect a possible inverse association of enterolactone. Our previous meta-analysis including seven published studies showed that high serum or urinary levels of enterolactone are negatively but not significantly associated with postmenopausal breast cancer risk.[22] Inclusion of the present study yielded a significantly increased pooled RE for an association of higher enterolactone levels with reduced postmenopausal breast cancer risk. This change in the pooled RE emphasizes the need for large studies to provide substantive evidence for an inverse association of lignan exposure with postmenopausal breast cancer risk.

Table 3. Adjusted odds ratios (ORs) and 95% confidence interval (95% CI) for serum enterolactone concentrations and postmenopausal breast cancer risk by ER, PR and HER2 status of the tumor

	Quintiles					
	I	II	III	IV	V	
Tumor Receptor Status	*n* cases OR (95% CI)[2]	*n* cases OR (95% CI)[2]	*n* cases OR (95% CI)[2]	*n* cases OR (95% CI)[2]	*n* cases OR (95% CI)[2]	*p*-trend[1]
ER+[3]	198	164	180	186	151	0.01
	1	0.79 (0.62–1.03)	0.87 (0.67–1.12)	0.89 (0.69–1.16)	0.73 (0.56–0.95)	
ER−	86	62	50	50	33	<0.0001
	1	0.70 (0.48–1.02)	0.59 (0.40–0.85)	0.54 (0.37–0.81)	0.39 (0.25–0.59)	
PR+	171	148	158	158	121	0.001
	1	0.83 (0.64–1.08)	0.89 (0.69–1.17)	0.92 (0.71–1.20)	0.68 (0.52–0.90)	
PR−[3]	113	78	72	73	62	0.0004
	1	0.69 (0.50–0.95)	0.62 (0.44–0.86)	0.64 (0.46–0.89)	0.51(0.33–0.73)	
ER+/PR+[3]	155	133	149	152	113	0.001
	1	0.80 (0.61–1.06)	0.91 (0.69–1.19)	0.92 (0.70–1.21)	0.69 (0.52–0.93)	
ER+/PR− or ER−/PR+	57	46	40	38	45	0.08
	1	0.77 (0.49–1.18)	0.68 (0.43–1.06)	0.66 (0.41–1.03)	0.73 (0.47–1.13)	
ER−/PR−	71	47	41	41	25	0.003
	1	0.66 (0.44–0.99)	0.58 (0.38–0.89)	0.57 (0.37–0.88)	0.36 (0.22–0.60)	
ER+/PR+/HER2−	122	99	108	121	90	0.003
	1	0.75 (0.55–1.02)	0.83 (0.61–1.13)	0.94 (0.70–1.30)	0.68 (0.50–0.94)	
ER+/PR+/HER2+[3]	18	20	20	16	13	0.5
	1	1.14 (0.57–2.26)	1.23 (0.61–2.49)	0.77 (0.35–1.69)	0.82 (0.38–1.77)	
ER−/PR−/HER2+	24	15	14	12	6	0.05
	1	0.69 (0.35–1.37)	0.59 (0.29–1.20)	0.44 (0.20–0.95)	0.24 (0.10–0.61)	
ER−/PR−/HER2−	42	25	23	23	17	0.004
	1	0.57 (0.33–0.97)	0.56 (0.32–0.97)	0.59 (0.34–1.02)	0.43 (0.24–0.78)	

[1]Log transformed continuous serum concentration was used as an ordinal variable in the logistic regression model. [2]Calculated using log transformed values of enterolactone and adjusted for induction of menopause, BMI, education level, first-degree family history of breast cancer, history of benign breast disease, number of pregnancies (≥28th week), age at menarche, breastfeeding history, total number of mammograms, smoking habit, alcohol consumption and use of phytoestrogen supplements. [3]P for heterogeneity were as following: between ER+ and ER− = 0.01, between PR+ and PR- = 0.2, between ER+/PR+ and ER−/PR− = 0.03 between ER−/PR−/HER2+ and ER−/PR−/HER2− = 0.3.m

To assess whether study design had an effect on the association of serum enterolactone on postmenopausal breast cancer risk, we also performed a meta-analysis by study type. Although not all prospective studies reported (nonsignificant) inverse associations, the pooled REs did not indicate differential effects by study type. Therefore, the overall evidence up to date supports an inverse association between serum enterolactone levels and postmenopausal breast cancer risk. Overall, the association was weaker in the prospective-cohort studies than in the case–control studies. Because blood withdrawal is before diagnosis in prospective cohort studies and generally after diagnosis of breast cancer in case–control studies, it is possible that serum concentrations are influenced by the diagnosis and therapy of breast cancer and therefore bias results in case–control studies. In addition, the potential for selection bias and residual confounding in case–control studies could also lead to bias. Nevertheless, no heterogeneity was observed in the pooled REs of postmenopausal breast cancer risk when stratified by study design.

Our study was conducted in a large German study population, with a diet relatively high in whole-grain products, berries and vegetables that are rich sources of lignans. In addition, participants were mostly nonsmokers, and their alcohol consumption was moderate. It is known that smoking is inversely correlated with serum enterolactone levels and that moderate alcohol consumption is positively correlated with serum enterolactone levels.[36,37] This may partially explain the relatively high median serum enterolactone levels in our study population compared to other studies.[3,33,34,38,39] In our study population, a reduction in OR for breast cancer was already apparent in the second quintile although the ORs decreased further with higher enterolactone concentrations. The median or range of our lowest quintile is comparable to several studies that also reported a negative yet

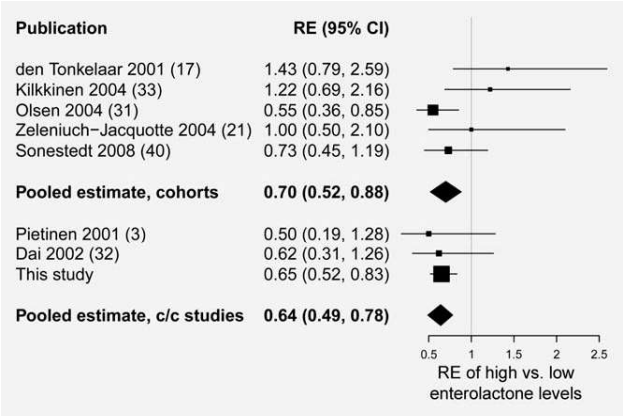

Figure 2. Association between serum, plasma, and urine enterolactone biomarkers with postmenopausal breast cancer risk according to study design. Risk estimates (REs) from individual studies are represented by black-filled boxes. REs from fixed-effects models are represented by filled black diamonds. Relative sample sizes are represented by the sizes of symbols. Horizontal lines indicate 95% CIs for the respective RE. Prospective cohorts/nested case–control studies (cohorts) and case–control studies (c/c) are shown.

nonsignificant association with postmenopausal breast cancer risk.[3,33,34] Moreover, significant inverse association was observed in two studies with generally high-serum enterolactone levels comparable to our study but only when considering the second quantile as reference group.[31,40] Compared to the higher quintiles, there were more participants with BMI above 30 and more smokers in the lowest quintile in our study. These variables were accounted for in the multivariable logistic models; however, we cannot entirely exclude residual confounding. Lignans are present in high amounts in fiber rich food (whole grain), fruits and vegetables. We assessed potential confounding by these food groups in multivariable logistic models but observed no substantial change in the REs and therefore did not include them in the final models. In addition, adjustment for energy intake did not influence the REs.

Our observation of an inverse association with circulating enterolactone concentrations independent of ER and PR status is in line with postulated antiproliferative and anticarcinogenic properties of enterolactone, in addition to antiestrogenic properties.[7,41] The effect was stronger among ER– tumors that could be attributable to anticarcinogenic properties of lignans that do not involve ERs, such as their influence on enzymes, proteins, angiogenesis and cell differentiation.[10,42]

The literature regarding the association of serum enterolignans with postmenopausal breast cancer risk by ER or PR status of the tumor is limited. A prospective study conducted in the United Kingdom reported no difference by ER status of the tumor in a population of predominantly postmenopausal women.[43] However, an association of plasma enterolactone with a decreased risk of ER− but not ER+ postmenopausal breast cancer was observed in a Danish study.[31] A Swedish study among postmenopausal women observed an inverse association of plasma enterolactone for ERα (positive) and ERβ (negative) tumors, with significantly different REs for ERβ (negative) and ERβ (positive) tumors.[40] When combining our findings by ER status with those of the first two published studies[31,43] included in our recently published meta-analysis,[22] the pooled estimates for ER+ tumors and ER− tumors were 0.72 (0.65–0.80) and 0.47 (0.21–0.73), respectively, nevertheless, without significant heterogeneity between the estimates (p for heterogeneity 0.07). Therefore, further large studies will be required to confirm whether the association is differential by ER status.

The association between measured enterolignans and breast cancer risk by PR status has not been investigated previously. A large prospective study investigated the effect of dietary enterolignans (estimated from FFQs), by ER and PR status of the tumor and reported an inverse association of dietary enterolactone for both ER− and PR+ tumors.[44]

We observed an inverse association of enterolactone for both ER+/PR+ and ER−/PR− tumors, but the association was stronger in ER−/PR−. To our knowledge, the effect of serum enterolignans has not been previously assessed according to combined ER/PR tumor status. Only one study investigated association of calculated enterolignans with breast cancer risk by combined ER/PR status and reported an inverse association for ER+/PR+ but not for ER−/PR− tumors.[44]

This is the first study to consider heterogeneity of the risk association of serum enterolactone also by HER2 expression. Our data, suggesting no differential risk by HER2 status, are corroborated by the absence of other lines of evidence for an effect of enterolactone on HER2 expression. Sample sizes in these subgroups were limited, and further studies with larger number of participants are needed to confirm these results.

Our study has several methodological advantages. To our knowledge, it is the largest study with sufficient power to investigate the association of serum enterolactone with postmenopausal breast cancer risk. We restricted our analyses to postmenopausal women and investigated risk associations by ER and PR status and, additionally, by HER2 status. We used fractional polynomials to identify the best-fitted model for the data. Moreover, two sensitivity analyses were performed to investigate whether time of blood collection (prior or after median time of diagnosis) or chemotherapy (prior of after start of chemotherapy) influence the association with serum enterolactone levels, and no heterogeneity was observed.

Similar to most epidemiological studies, only one measure of enterolactone in nonfasting serum samples was performed, which might not reflect long-term exposure to lignan-rich diets. Nevertheless, feeding studies showed that serum enterolactone levels do not decrease rapidly.[13] In addition, another study showed that the reliability coefficient of a single measurement of enterolactone was relatively high, *i.e.*, 0.6 for three repeated measurements during 2 years.[45] Antibiotic use

was not recorded that might have influenced the bioavailability of phytoestrogens.[18] Moreover, biomarker measurements provide more precise data than dietary information, reflecting the bioavailable amount of ingested lignans. Furthermore, serum levels may have been also influenced by the disease status as blood samples were collected after breast cancer diagnosis, thereby not representing prediagnosis levels.

In conclusion, results of our study suggest that high enterolactone levels are inversely associated with postmenopausal breast cancer risk in this Western population. The inverse association was stronger for ER−/PR− than for ER+/PR+ tumors, but did not appear to be different from further stratification according to expression of HER2. These findings may have important health implications for women in Western populations with very low phytoestrogen consumption. Further prospective studies in large populations with large range in intake levels of lignans are needed to define the association between circulating enterolactone levels and the subsequent risk of breast cancer according to menopausal status and ER/PR status of the tumor.

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