REVIEW

Estrogen metabolite ratio: Is the 2-hydroxyestrone to 16α-hydroxyestrone ratio predictive for breast cancer?

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Abstract: Experimental studies have shown that two main estrogen metabolites hydroxylated by CYP1A1 and CYP1B1 in the breast differentially affect breast cell proliferation and carcinogenesis. Although 16α-hydroxyestrone (16αOHE1) exerts estrogenic activity through covalent estrogen receptor (ER) binding, 2-hydroxyestrone (2OHE1) presumably has antiestrogenic capabilities. The ratio of 2OHE1 to 16αOHE1 represents the relative dominance of one pathway over the other and is believed to be modifiable by diet. It was hypothesized that women with or at high risk of breast cancer have a lower estrogen metabolite ratio (EMR) compared with women without breast cancer. We conducted a systematic review on the EMR as a predictor for breast cancer. A total of nine studies (six prospective and three retrospective) matched our inclusion criteria, comprising 682 premenopausal cases (1027 controls) and 1189 postmenopausal cases (1888 controls). For the highest compared with the lowest quantile of urinary EMR, nonsignificant associations suggested at best a weak protective effect in premenopausal but not in postmenopausal breast cancer (range of odds ratios: 0.50–0.75 for premenopausal and 0.71–1.31 for postmenopausal). Circulating serum/plasma EMR was not associated with breast cancer risk. Associations were inconclusive for receptor subtypes of breast cancer. Uncontrolled factors known to be involved in breast carcinogenesis, such as 4-hydroxyestrone (4OHE1) concentration, may have confounded results for EMR. Results of the prospective studies do not support the hypothesis that EMR can be used as a predictive marker for breast cancer risk. Future research should concentrate on profiles of estrogen metabolites, including 4OHE1, to gain a more complete picture of the relative importance of single metabolites for breast cancer.

Keywords: estrogen metabolite ratio, 2-hydroxyestrone, 16α-hydroxyestrone, breast cancer, predictive marker, review

Introduction

Hypothesis (the extended hormonal risk factor hypothesis)

It is well known that endogenous estrogens have the potential of inducing and promoting cell proliferation and inducing tumor growth in breast tissue. Circulating estrogen levels correlate with breast cancer risk in postmenopausal women.[1,2] For premenopausal women, the estrogen–breast cancer association is not as well established. Some evidence for an association was found in one prospective study[3] but was not statistically significant in five other studies,[4] probably due to the much higher intra- and interindividual variation through menstrual cycle and reproductive lifetime.[5,6]

Since the early 1980s, a growing number of studies examined whether not only native estrogens but also their hydroxylated estrogen metabolites have potential carcinogenic properties in breast tissue. Experimental studies in breast cancer cells

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and animal models established the hypothesis of a differential estrogenic activity of the two main estrogen metabolites, 2-hydroxyestrone (2OHE1) and 16α-hydroxyestrone (16αOHE1). Because 2OHE1 has a weak binding capacity to the ER and has been associated with normal cell differentiation and apoptosis,[7–9] it is assumed to have antiestrogenic properties.[10] In contrast, the other major metabolite, 16αOHE1, can bind covalently to the ER and has been shown to induce abnormal cell proliferation.[11,12]

Both 2- and 16α-estrogen hydroxylating pathways are mutually exclusive and irreversible. Thus, the metabolite ratio of 2OHE1 to 16αOHE1 reflects the relative dominance of one pathway over the other. Since several relatively small-scale observational studies and human breast tissue studies found lower ratio levels accompanied by higher 16αOHE1 concentrations in breast cancer cases compared with women without breast cancer,[13–18] it has been suggested that this ratio may be used as a marker for the assessment of breast cancer risk. Subsequent studies yielded inconsistent results as to whether a lower ratio is associated with a higher risk for breast cancer, both in premenopausal and in postmenopausal women.

A concurrent hypothesis postulates that the catecholestrogens 2OHE1/2OH-estradiol and especially the 4-hydroxylated estrogens 4-hydroxyestrone (4OHE1) and 4-hydroxyestradiol may play an important role in breast carcinogenesis because of their ability to induce DNA depurination independent of ER binding.[19,20] Although experimental studies have demonstrated the carcinogenic potential of 4OHE1,[21,22] little attention has been given to 4OHE1 in epidemiological studies. Therefore, we focus here on the estrogen metabolite ratio (EMR) of 2OHE1 to 16αOHE1.

Metabolism of endogenous estrogens, genetic polymorphisms

In a first step, native 17β-estradiol (E2) and its oxidized form, estrone (E1), are metabolized to various hydroxylated estrogens by cytochrome P450-dependent pathways. The catecholestrogens 2OHE1/E2 and 4OHE1/E2 are characterized by two neighboring hydroxyl groups, which are rapidly methylated by the enzyme catechol-*O*-methyltransferase (COMT) to form 2-Meth-O-E1/E2 or 4-Meth-O-E1/E2. Further oxidation of unmethylated hydroxyestrogens may generate semiquinones and quinones, which on one hand can function as substrate for redox cycling processes, leading to reactive oxygen species (ROS), and on the other hand can bind to DNA to form stable or depurinating adducts with adenine or guanine. Subsequently, quinones and

hydroxylated estrogens, including 16αOHE1, are sulphatized or glucuronidated before excretion.[23] The tissue-specific hydroxylases are encoded by CYP1A1, CYP1B1 (breast), and CYP1A2 (liver) for the 2-hydroxylation of E1 to form 2OHE1/E2 and CYP1A1, CYP3A5 (breast),[24] CYP3A4, and CYP3A7[25] for 16α-hydroxylation. A smaller fraction of estrogens is hydroxylated to 4OHE1/E2 by CYP1B1 (breast) and CYP1A2 (liver) enzymes.[22] Tissue concentrations of the CYP enzymes vary considerably, and many of them are genetically polymorph, which implicates differences in activity levels of the hydroxylases.[26,27] Polymorphisms have also been described for COMT, leading to slower methylation of catecholestrogens.[28,29]

Menopausal status

To date, it is not well established whether the menopausal transition is accompanied by a general shift of the EMR to favor 16αOHE1, as has been previously discussed.[30] Although levels of estrogen metabolites are several times higher in premenopausal compared with postmenopausal women, a woman's metabolite ratio seems to be relatively stable throughout her life.[31] Most of the earlier studies on the EMR reported no significant mean differences between pre- and postmenopausal control groups without breast cancer.[32] Because the limited available data do not provide clear indication of an important influence of menopausal status on the EMR, both pre- and postmenopausal women were considered in this review.

The purpose of the present review was to evaluate the evidence for an inverse relation between EMR and breast cancer risk and whether this ratio can potentially serve as a predictive marker.

Methods
Selection of studies

A PubMed/MEDLINE search on epidemiologic studies of estrogen metabolites and breast cancer was conducted up to February 2010. The search strategy consisted of a combination of MeSH terms ("hydroxyestrones" and "breast neoplasms") and key words in titles and abstracts ("estrogen metabolites", "2-hydroxylation", and "breast cancer"). References from resulting articles were screened for missed studies. If only abstracts of publications were available, they were excluded from the review.

Inclusion criteria

Studies were included in this review if risk estimates and confidence intervals (CIs) of the urinary or circulating EMR

(2OHE1/16αOHE1) were reported for subjects not taking oral contraceptives, hormones, or tamoxifen and not pregnant at the time of blood donation or urine collection. Further requirements included frequency or individual matching of controls to cases by age and stratification by or adjustment for menopausal status to assess potentially differential evidence for pre- and postmenopausal women.

Measurement of the 2OHE1/16αOHE1 EMR

All but one study,[33] which employed an earlier version, used an improved enzyme immunoassay[34] (ELISA; ESTRAMET, Immunacare Corporation, Bethlehem, PA, USA) developed by Klug et al[35] for simultaneous urinary metabolites assessment. The assay measures three of at least 15 metabolites and parent estrogens, ie, 2OHE1, 2-hydroxyestradiol (2OHE2), and 16αOHE1. Therefore, the EMR reflects the 2OHE1 + 2OHE2/16αOHE1 ratio. The EMR is independent of urinary creatinine concentration.

Data extraction

To compare the median/mean EMR across studies, the third quintile's midpoint of EMR in pre- and postmenopausal controls has been derived as an approximate value for Muti et al[36] and the numbers in each tertile in the study by Meilahn et al[33] were deduced from given percentages. Risk estimates for quantiles and 95% CIs of the EMR were extracted from the tables of the publications and analyzed. To test for overall heterogeneity and heterogeneity by specimen type (urine, serum/plasma), risk estimates were weighted and pooled, and Cochran's Q statistic[37] was calculated.

Results

Overall, 21 studies were identified that dealt with any kind of estrogen metabolites and associated breast cancer risk, of which nine were included in this review (Table 1). One study included only premenopausal women[38] and four studies only postmenopausal women,[39–42] and the remaining four studies matched for menopausal status or analyzed data stratified by menopausal status.[33,36,43,44]

Excluded studies

The main reasons for exclusion were nonavailability of risk estimates, including CIs of the association between EMR and breast cancer,[13,14,18,45–51] and recruitment of control subjects from convenient samples that were not individually matched to cases by age.[13,14,16–18,45,46,48,51] Almost all of these small case-control studies, comprising, in total, 401 cases and

429 controls, found lower mean EMRs in cases compared with controls (Supplementary Table). Kabat et al[17] reported for a small hospital-based study an extreme risk reduction for the third versus first tertile of urinary EMR in postmenopausal women (adjusted odds ratio [OR]: 0.03, 95% CI: 0.003–0.29), but not in the combined group of pre- and postmenopausal women (OR: 0.51, 95% CI: 0.17–1.56). Similarly, Ho et al[16] who used controls with biopsies for benign breast disease, found at a urinary EMR median cut-point of 0.9 a strong inverse association independent of menopausal status (OR: 0.16, 95% CI: 0.05–0.49).

Included studies

The nine studies included were published between 1998 and 2009. Study characteristics are summarized in Table 1. In total, these studies comprised 682 premenopausal cases and 1027 premenopausal controls (five studies) and 1189 postmenopausal cases and 1888 postmenopausal controls (seven studies). Six studies were nested case-control studies within prospective cohorts,[33,36,38,40–42] and three were retrospective population-based case-control studies[39,43,44] with determination of the metabolites after disease onset. Studies were conducted in China,[44] Italy,[36] the UK,[33] Denmark,[41] and the US.[38–40,42,43] The baseline age distribution varied naturally across studies according to menopausal status, with the minimum age ranging from 25 years[44] to 65 years.[40] All studies were matched for age, and prospective but not retrospective studies in premenopausal women were additionally matched for phase of menstrual cycle at time of specimen sampling (follicular or luteal[33,38] and only luteal).[36] Median follow-up time between specimen sampling and diagnosis of breast cancer (approximately equal to time between sampling and laboratory analysis) ranged from 2.4 years in the Danish cohort[41] to 12–18 years in one of the US cohorts.[38] The retrospective studies collected urine prior to any therapy for several months,[44] a median of 3 months,[43] or several years[39] after diagnosis.

Six studies investigated urinary excretion of estrogen metabolites, and three of the more recent studies examined serum/plasma concentrations.[38,40,42] Among controls, mean urinary EMR values were consistently above 1, whereas means of circulating EMR were all below 1. In Western countries, urinary mean EMR varied between 1.9 and 2.57 in controls, whereas in the Shanghai study by Fowke et al[44] the mean EMR was around 50% lower. A significant difference between pre- and postmenopausal controls in mean urinary EMR has been found only in the UK cohort.[33] Studies of serum/plasma EMR examined either pre- or postmenopausal women.

Table 1 Characteristics of included studies on EMR and breast cancer risk estimates

Authors	Country	Design	Medium	Age (range and/or mean (SD))	Cases/controls (total)	Cases/controls (in models)
Premenopausal						
Meilahn et al[33]	UK	Prospective nested case-control	Spot urine	>34 (40.5 (4.2))	60/184	21/62 22/61 17/61
Muti et al[36]	Italy	Prospective nested case-control	12-h urine	35–57	67/264	19/54 14/51 11/52 12/54 11/53
Kabat et al[43]	USA	Population-based case-control	Spot urine	<50–>60	105 (70 is)/129	44/46 35/42 26/41
Arslan et al[38]	USA	Prospective, nested case-control	Serum	35–65 44.3 (4.8)	377/377	Not given for quartiles
Mixed menopausal						
Fowke et al[44] (presurgical)[a]	China	Population-based case-control	Spot urine	25–65	78/78 presurgical	30/20 25/27 23/31
Fowke et al[44] (postsurgical)[a]	China	Population-based case-control	Spot urine	25–65	32/32 postsurgical	5/14 10/11 17/7
Postmenopausal						
Meilahn et al[33]	UK	Prospective, nested case-control	Spot urine	>50 59 (6.2)	42/139	15/47 16/46 13/46
Ursin et al[39]	USA	Population-based case-control	Spot urine	50–70	66/76	29/26 11/25 26/25
Muti et al[36]	Italy	Prospective, nested case-control	12-h urine	43–70	71/274	12/54 16/55 17/54 12/57 14/54
Wellejus et al[41]	Denmark	Prospective, nested case-control	Spot urine	50–64	197/197	197/197
Kabat et al[43]	USA	Population-based case-control	Spot urine	<50–>60	164 (88 is)/197	70/64 62/52 65/48
Cauley et al[40]	USA	Prospective case-cohort	Serum	70 (5) >65	272/291	69/70 97/72 64/75 72/74
Eliassen[42]	USA	Prospective, nested case-control	Plasma	61.5 (4.7) 43–69	340/677	74/169 89/168 87/170 90/168

EMR (2OHE1/16αOHE1)	Adjusted OR (95% CI)	P_{trend}	Adjustment	Notes (median, mean or gmean in controls)
<1.72 1.72–<2.44 ≥2.44	I 0.99 (0.48–2.08) 0.75 (0.35–1.62)	NA	Age + menstrual phase matching; parity, others not exactly specified	Median follow-up 9.5 years, median EMR 2.1
<1.8 1.8–2.3 2.3–2.72 2.72–3.29 ≥3.29	I 0.76 (0.34–1.69) 0.60 (0.25–1.44) 0.62 (0.27–1.45) 0.55 (0.23–1.32)	NA	Age + menstrual phase matching, BMI, waist-to-hip, reproductive variables	Mean follow-up 5.5 years, median EMR 2.5
≤1.4 1.5–2.2 ≥2.3	I 0.63 (0.33–1.23) 0.50 (0.25–1.01)	0.05	Age matching, parity/first birth age, family history, BMI, education, medication (48 h prior urine collection)	In situ Ca separately; smoking, alcohol, diet prior tested; mean EMR (SD) 1.9 (1.0)
<0.45 0.453–0.635 0.636–0.936 >0.936	I 0.83 (0.53–1.31) 1.04 (0.65–1.65) 1.13 (0.68–1.87)	0.51	Age + menstrual phase matching; family history, ever smoking, BMI, menarche age, parity/first birth age	12–18 years of follow-up, mean EMR (SD) 0.76 (0.49)
≤0.69 0.70–1.22 ≥1.22	I 0.5 (0.2–1.3) 0.5 (0.2–1.3)	0.17	Matched for age and menopause; family history, physical activity, BMI, age at menarche, parity/first birth age, fibroadenoma history	Separate models for presurgical and postsurgical; presurgical included 19/19 postmenopausal; median EMR 1.0
≤0.69 0.70–1.22 ≥1.22	I 3.1 (0.4–23.6) 8.1 (1.2–54.6)	0.02	Matched for age and menopause; family history, physical activity, BMI, age at menarche, parity/first birth age, fibroadenoma history (but not family history, menarche age)	Postsurgical included 18/18 postmenopausal; median EMR 0.6
<1.39 1.39–<2.09 ≥2.09	I 0.99 (0.48–2.08) 0.75 (0.35–1.62)	NA	Age matching; Parity, others not exactly specified	Median follow-up 9.5 years; median EMR 1.7
≤1.16 1.17–1.73 >1.73	I 0.34 (0.12–0.98) 1.13 (0.46–2.78)	0.96	Age, family history, SES, education, menarche age, parity, weight	Low stage tumors 3–7 years after diagnosis; gmean EMR 1.76 (95% CI : 1.60–1.93)
<1.77 1.77–2.26 2.26–2.80 2.80–3.66 >3.66	I 1.42 (0.60–3.33) 1.41 (0.60–3.33) 1.02 (0.41–2.53) 1.31 (0.53–3.18)	NA	Age, BMI, waist-to-hip, reproductive variables	Mean follow-up 5.5 years; median EMR 2.6
log-2-transformed, OR for doubling of EMR	0.94 (0.71–1.43)	NS	Age matching only; education, parity/first birth age, BMI, alcohol, past HT prior tested	Median follow-up 2.4 years (P5–P95 0.2–4.9 years); median EMR 1.6, P5–P95 0.6–3.5
≤1.4 1.5–2.2 ≥2.3	I 0.97 (0.57–1.64) 0.78 (0.46–1.33)	0.37	Age, first birth age, family history, BMI, education, alcohol use and medication (48 h prior to urine collection)	In situ Ca separately; mean EMR (SD) 2.0 (1.0)
≤0.576 0.577–0.749 0.750–0.923 >0.923	I 0.98 (0.62–1.57) 0.89 (0.55–1.42) 1.17 (0.73–1.87)	NA	Age, BMI; Family history, past HT, education prior tested	Mean follow-up 8.7 years, 39 in situ Ca included; gmean EMR 0.73 (95% CI 0.70–0.76)
<0.28 0.28–<0.37 0.37–<0.48 ≥0.48	I 1.33 (0.89–1.99) 1.24 (0.83–1.85) 1.30 (0.87–1.95)	0.35	Age matching; BMI at age 18, family history, menarche age, parity/age at first birth, menopause age, duration of past HT use	Follow-up 10–11 years, 63 in situ Ca included; median EMR 0.37

Notes: [a]Fowke et al matched for menopausal status (presurgical: 59 premenopausal and 19 postmenopausal pairs, and postsurgical: 14 pre- and 18 postmenopausal pairs), thus estimates refer to both pre- and postmenopausal women.
Abbreviations: BMI, body mass index; Ca, carcinoma; CI, confidence interval; EMR, estrogen metabolite ratio; gmean, geometric mean; HT, hormone therapy; is, in situ; NA, not available; NS, not significant; P5–P95, 5th percentile to 95th percentile; SD, standard deviation; SE, standard error; SES, socioeconomic status.

All studies but one[41] used percentile distributions of the EMR among controls to assess the association with breast cancer risk by comparing the highest with the lowest group (tertiles, quartiles, or quintiles) with conditional or unconditional logistic regression or Cox's[40] regression analysis. Wellejus et al[41] applied log-2-transformed EMR values that reflect a doubling of concentration per unit change. Adjustment for potentially confounding factors differed slightly between studies. Characteristics that were included in most models were age, body mass index (BMI) or waist-to-hip ratio, and reproductive variables known to be risk factors for breast cancer. Wellejus et al[41] did not adjust their analysis apart from matching for exact age, and Cauley et al[40] adjusted their analysis only for age and BMI. Of the four studies that included both pre- and postmenopausal women, three analyzed pre- and postmenopausal women separately.[33,36,43]

The Shanghai study reported combined estimates based on analyses stratified by menopausal status and time of urine collection relative to surgery/treatment status.[44]

Main study results (risk estimates, 95% CI) are presented by specimen and menopausal status in Table 1 and Figure 1.

Urinary EMR
Premenopausal breast cancer
In the UK study (60 cases), the EMR was associated with a nonsignificant 25% risk reduction (first vs third tertile: OR = 0.75, 95% CI = 0.35–1.62).[33] The Italian study[36] (67 cases) reported nonsignificantly decreased ORs for the second to fifth quintile (ORs: Q2–Q5 = 0.76–0.55). A 50% risk reduction (95% CI: 0.25–1.01) for the highest tertile of the EMR was found in the retrospective study by
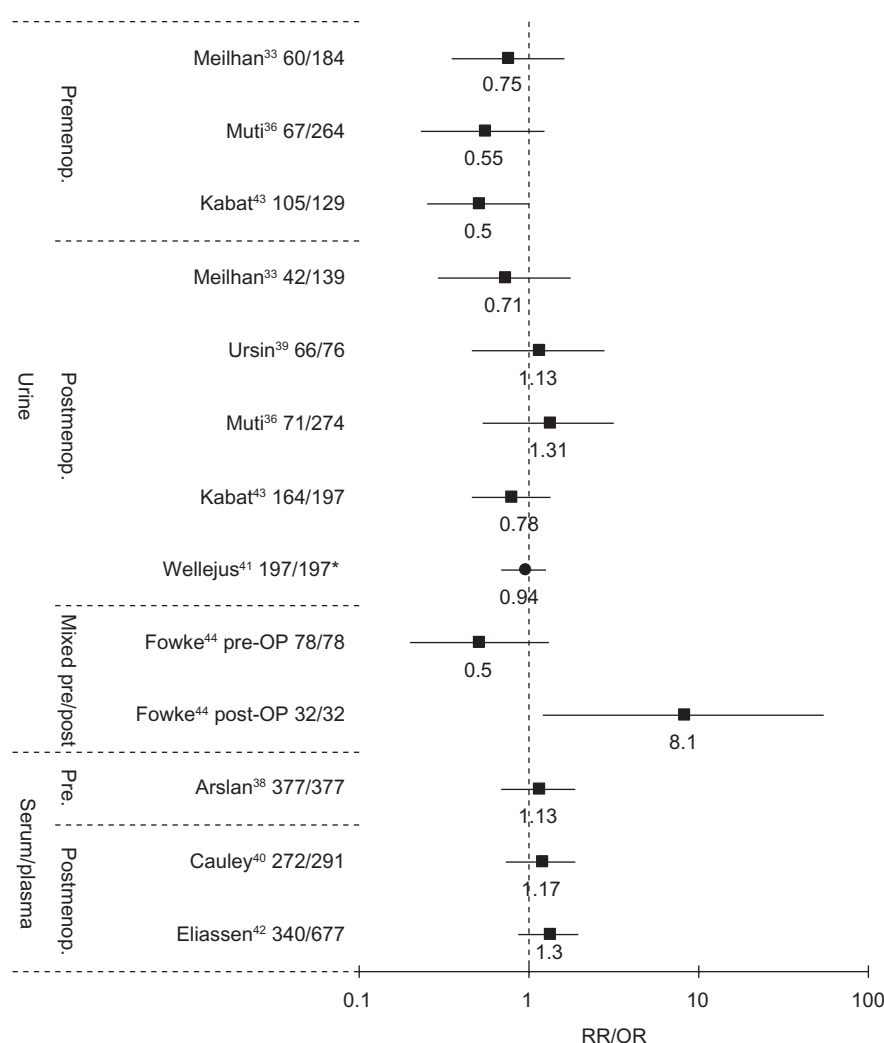
Figure 1 OR/RR for highest versus lowest quantile of EMR in studies on pre- and postmenopausal breast cancer.
Note: Wellejus used log-2-transformed EMR, representing a doubling of EMR per unit increase.
Abbreviations: EMR, estrogen metabolite ratio; OR, odds ratio; RR, risk ratio.

Kabat et al.[43] Analysis repeated for cases prior to and after chemotherapy showed no substantial differences in risk estimates. Fowke et al[44] reported results for 78 presurgical (the majority being premenopausal) and 32 postsurgical pairs (14 pairs being premenopausal). For presurgical pairs, the tertiles of the EMR were nonsignificantly associated with a 50% risk reduction, whereas a significantly positive association was present postsurgically (OR for third vs first tertile: 8.1, 95% CI: 1.2–54.6). Only Kabat et al[43] observed an inverse dose–response relationship over tertiles of EMR ($P_{trend} = 0.05$), whereas other studies found no trends[38,44] or did not provide a trend test.[33,36]

Postmenopausal breast cancer

Meilahn et al[33] (42 cases) and Kabat et al[43] (164 cases) reported nonstatistically significant inverse associations for the highest versus lowest tertile of the EMR (respective ORs 0.75 and 0.78), whereas Muti et al[36] (71 cases) and Ursin et al[39] (66 cases) observed nonsignificant risk elevations. Wellejus et al[41] found no significant relationship among 197 case-control pairs for a doubling of EMR per increment (OR: 0.94, 95% CI: 0.71–1.43).

In summary, none of the urinary studies reported a statistically significant inverse association between EMR and breast cancer risk, although the premenopausal study by Kabat et al[43] showed an upper confidence limit close to 1.

Serum/plasma EMR
Premenopausal breast cancer

Arslan et al[38] found no association between the serum EMR and premenopausal breast cancer risk among 377 case-control pairs, the OR for the top versus bottom quartile being 1.13 (95% CI: 0.68–1.87). Repeated analyses according to menstrual cycle phase (follicular pairs and luteal pairs) or after excluding cases diagnosed within 5 or 10 years after enrollment did not yield different results.

Postmenopausal breast cancer

Eliassen et al[42] and Cauley et al[40] included in situ cases in their analyses of 340 and 272 cases, respectively. Both studies found nonsignificant risk elevations for the highest compared with the lowest quartile of circulating EMR levels. Thus, the serum/plasma EMR was not significantly associated to pre- or postmenopausal breast cancer.

Heterogeneity testing

The test for heterogeneity across the combined pre- and postmenopausal urinary studies ($P_{heterogeneity} = 0.86$) and the test for circulating EMR studies ($P_{heterogeneity} = 0.86$) were negative. Additionally, the test for heterogeneity across all nine urinary and circulating EMR studies revealed no significant inconsistency between studies ($P_{heterogeneity} = 0.61$).

Breast cancer subtypes: receptor status and in situ cancer

Kabat et al found the urinary EMR to be statistically, significantly, and inversely related to ER-positive premenopausal tumors (third vs first tertile OR: 0.32, 95% CI: 0.12–0.84) and nonsignificantly related to ER-negative tumors in postmenopausal women (OR: 0.38, 95% CI: 0.15–1.01).[43] A nonsignificant inverse association in ER-negative cases (OR for doubling of urinary EMR: 0.78, 95% CI: 0.42–1.48) was also reported by Wellejus et al.[41] In contrast, serum/plasma levels of EMR were increased in ER-negative postmenopausal cases (OR: 3.7, 95% CI: 1.24–11.09, $P_{trend} = 0.004$, $P_{heterogeneity} = 0.005$) in one study[42] and in ER-positive premenopausal cases in the other study (OR: 2.15, 95% CI: 0.88–5.27).[38] Apart from Eliassen et al[42] none of the studies tested for heterogeneity by ER status.

Kabat et al[43] separately investigated 70 premenopausal and 88 postmenopausal cases with carcinoma in situ of the breast. No association of EMR was observed in both groups. Likewise, from their overall negative results, Eliassen et al[42] reported no differential relationship for postmenopausal in situ breast cancer.

Discussion
Summary of main results

Overall, nine studies that fulfilled our inclusion criteria investigated the association between the EMR of 2- to 16α-hydroxyestrone and breast cancer risk, six of which examined urinary and three circulating EMR. Studies on urinary EMR found ORs between 0.50 and 0.75 (except for the postsurgical OR in Fowke et al)[44] in premenopausal women and between 0.71 and 1.31 in postmenopausal women, comparing the highest with the lowest quantiles or, in one study, a doubling of EMR. Although none of the studies reached statistical significance, the premenopausal arm of the retrospective study by Kabat et al was borderline significant, suggesting that an inverse association of urinary EMR might be confined to premenopausal breast cancer. However, there was no differential effect in all urinary studies combined ($P_{heterogeneity} = 0.86$), which contradicts a hypothesized modification by menopausal status. Similarly, the circulating EMR was not associated with breast cancer

risk in pre- or postmenopausal women (ORs ranging from 1.13 to 1.30, $P_{\text{heterogeneity}}$ = 0.86). There was also no significant heterogeneity across all combined urinary and serum/plasma studies ($P_{\text{heterogeneity}}$ = 0.61).

Receptor status and in situ carcinomas

Limited data on ER status with contradictory results do not allow a firm conclusion regarding heterogeneity in the relationship between EMR and breast cancer risk by ER status. The two studies, which investigated in situ carcinomas separately, did not find the EMR to be inversely associated with risk of in situ breast cancer.

Quality of evidence
Completeness of data

Exclusion of two earlier small studies,[16,17] which found strong inverse associations for EMR, may have led to certain bias toward null results, but these studies were not included due to flaws in design or reporting. A funnel plot (Figure 2), including these studies, suggested that there might have been publication bias in this early phase. Another larger excluded study that assessed only single estrogen metabolites in serum of postmenopausal hormone therapy nonusers did not report statistically significant associations with breast cancer risk.[47] Though not necessarily, it is likely that the EMR was also not associated with breast cancer in this study.

Study size and power

The earliest publications that reported an inverse association between EMR and breast cancer risk were based on small numbers and thus have a tendency to overestimate the effect. Many of the subsequently published studies also had relatively small study sizes[33,36,39,44] and consequently limited statistical power to detect a significant risk reduction of about 30% (likely effect magnitude based on the first cohort studies).[33,36] If tertiles of exposure are considered, at least 200 case-control pairs are needed to identify a 50% risk reduction and 400 pairs to detect a relative risk of 0.7 with 80% power at an alpha of 0.05.[52]

Study design

The studies were heterogeneously designed, including both prospective and retrospective studies, using urinary or circulating EMR measurements for exposure assessment, including diverse populations, and many lacking statistical power. These variations may partly explain the differences in risk estimates for EMR.

The EMR may be influenced by disease status (tumor-driven activity), stage, and treatment.[18,43,44,53] Thus, the hypothesized association between the EMR and breast cancer might be subject to reverse causation in retrospective case-control studies. Fowke et al[44] found a decreased EMR risk in presurgical cases and an increased EMR risk
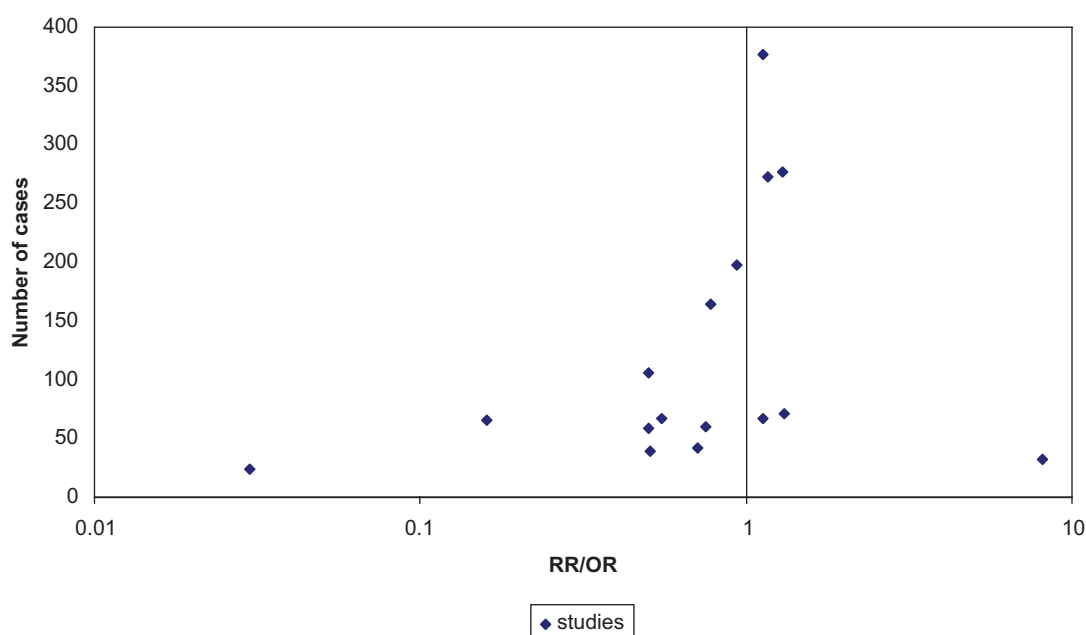

Figure 2 Funnel plot of risk estimates of nine included[33,36,38-44] and two excluded studies[16,17] (separate RR/OR for menopausal status, where available).
Abbreviations: RR, risk ratio; OR, odds ratio.

in postsurgical cases. If pre- and postsurgery subgroups were combined, this would have resulted in a crude OR for the EMR of 1.02 (95% CI: 0.72–1.25). Hence, an existing inverse association may be masked by a potential influence of treatment on EMR. No study actually assessed the intraindividual variability in EMR before and after a breast cancer diagnosis. Therefore, prospective studies of EMR will be required to elucidate a potential association of EMR with breast cancer.

Adjustment for confounders in the individual studies

Only studies that excluded current users of exogenous hormones were included in this review, as oral contraceptives and menopausal hormone therapy have been shown to increase[54–57] or decrease EMR.[41,47] Many of the known breast cancer risk factors, such as reproductive variables (ie, parity and age at first birth) and BMI, which have been found to be associated with the EMR,[42,58] were controlled in most included studies. Menstrual cycle phase in premenopausal women, a family history of breast cancer, and a history of benign breast disease were not consistently adjusted for. Although little evidence indicates that EMR is associated with a family history[59] or benign breast disease, circulating EMR has been shown to increase during the menstrual cycle.[60] Urinary estrogen metabolite levels vary strongly by ethnicity, showing lowest mean values in healthy African Americans followed by Asians and Caucasians.[32,45,61–64] Although ethnic variations in plasma EMR were less pronounced,[55] uncontrolled confounding could have masked, diluted, or even pretended a potential EMR effect in studies not adjusted for ethnicity depending on the combination of case-control pairs (even if percentages [other than for Caucasians] were as low as 12%–16%).[38,43] However, in some studies, adjusted risk estimates did not differ substantially from crude estimates, and EMR varied only slightly across categories of covariates.[33,36,40,41] Therefore, the risk estimates in these studies were considered as appropriate.

Possible modifiers of the association between EMR and breast cancer

Menopausal status did not strongly affect EMR in most studies, which is compatible with the observed overlapping distributions of EMR in pre- and postmenopausal women.[32] Genetic makeup and differential activity of metabolizing hydroxylases and other enzymes (eg, COMT) have been estimated to explain most of the variation in EMR.[65] In addition, estrogen hydroxylation pathways, particularly 2-hydroxylation, are believed to be modifiable through diet, physical activity, smoking, alcohol consumption, and caffeine intake. Clinical trials and observational studies generated inconsistent results regarding a potential influence of diet (eg, soy products, fat/fiber, and *Brassica* vegetables) and physical activity habits on EMR. Studies in the present review do not support that EMR may be associated with breast cancer risk in specific subgroups.

Reliability of measurements

Reliability of a single urinary EMR measurement in premenopausal women was rated sufficient in studies on variation in intraindividual EMR during the menstrual cycle.[31,66] Intraclass correlation coefficients between two urinary EMR measurements were 0.71 over a 1-year period[67] and 0.67[30] over a 6-month period, and between two plasma EMR measurements were 0.73 over a 3-year period.[42] All included studies, except for the study by Meilahn et al,[33] used a refined version of the ESTRAMET enzyme immunoassay to determine the EMR of $2OHE1/16\alpha OHE1$ validated against a gas chromatography/ mass spectrometry (MS) method. This assay could more sensitively detect estrogen metabolites in the lower postmenopausal range than the first version.[68] However, a recent study rated reliability of urinary estrogen metabolite determination of postmenopausal women by this enzyme immunoassay problematic compared with a new technique involving liquid chromatography (LC)–MS/MS.[69] Studies of postmenopausal women reported higher between-assay coefficients of variation than those of premenopausal women. Serum and plasma measurements are even more susceptible to errors because of the lower metabolite concentrations. Further, variation within and between studies may have resulted from differing specimens' sampling patterns, storage length, and time between sampling and diagnoses in prospective studies.

Findings that oppose the postulated hypothesis

Well-established risk factors for breast cancer that are considered to be related to estrogen exposure, eg, family history, age at menarche, and benign breast disease,[38] were not substantially or even inversely associated to the EMR, ie, a lower EMR with higher parity,[38] lower age at first birth,[58] and high breast density,[70] respectively. Furthermore, ethnic differences in the EMR do not correspond to breast cancer incidence patterns in Asians and Caucasians. Differences in breast cancer risk between Asians and African Americans were found to be better explained by urinary estradiol, E1, and estriol than by EMR in a cross-sectional study of healthy, nonestrogen-using women.[71]

Recommendations for future studies
Interrelationship of urinary, circulating, and tissue EMR

Urinary and circulating estrogen metabolites may not be representative of target tissue concentrations of unconjugated metabolites. A recent study of nine patients concluded that the urinary EMR is a good approximation for breast tissue EMR.[72] This result needs to be confirmed in women without breast cancer. Moreover, differences in median EMR of serum/plasma and urine samples raise the question of whether risk estimates derived from urinary EMR can be compared with those based on circulating EMR. A single study in young women not using oral contraceptives found fair correlation coefficients between urinary and plasma EMR ($r_s = 0.60$ in Caucasians).[60] Hence, further assessment of intraindividual correlations between serum/plasma and urinary EMR both in patients and in healthy individuals is warranted.

Association between EMR and subtypes of breast cancer

There is now ample evidence of etiologic heterogeneity for breast cancer subtypes.[73] Therefore, the potential for a differential role of EMR and other estrogen metabolites in subtypes of breast tumors (eg, by ER status, high grade) should be investigated in larger prospective studies with sufficient power.

Measurement of the catecholestrogens 4OHE1/4OHE2

The 4-hydroxylation of estrogens leading to the potentially carcinogenic catecholestrogens 4OHE1/4OHE2 is positively correlated to 2-hydroxylation and might be negatively correlated to 16αOHE1, depending on the extent of cross-reactivity of the CYP1A1/A2 pathway.[74] Studies in breast tissue have gained some insight into the relative and absolute amounts of estrogens and their metabolites. Although a higher EMR has been found in normal breast tissues,[75] supporting the hypothesized role of 16αOHE1, the most abundant metabolite in cancer tissues was 4OHE2. A study by Rogan et al[76] detected significantly higher tissue levels of 4OHE1 but not 16OHE1 in 28 cases compared with 46 controls. Additionally, higher concentrations of quinone conjugates derivatives were found in cancer tissues and interpreted as higher potential for quinones to react with DNA. Finally, in a small study, Gaikwad et al[49] focused on depurinating DNA adduct formation in relation to catecholestrogen concentration, expressed as a ratio. Higher ratio levels, particularly for 4OHE conjugates indicating a relatively higher DNA adduct formation, were seen in cases and in a high-risk group than in controls (confirmed in an extended group).[77] These preliminary findings may advise the future direction of research to include the detection of 4OHE1 and 4OHE2 in epidemiological studies of 2OHE1/16αOHE1 EMR.

Conclusion

All of nine properly designed epidemiological studies (six prospective case-control studies and three retrospective studies) failed to show a significant relationship between urinary or circulating EMR (2OHE1/16αOHE1) and breast cancer risk. Although premenopausal studies on urinary EMR have suggested a potentially weak inverse relationship, associations were not significantly different compared with postmenopausal or overall combined studies. Thus, at present, there is no evidence that the EMR can predict breast cancer risk. Larger prospective studies are needed to definitely assess a potential association of EMR with risk of breast cancer and risk by subtype (eg, by ER), adjusting for age, menstrual cycle phase, and ethnicity and menopausal status in cases of mixed study populations. A deeper knowledge of interrelationships between urinary, circulating, and tissue levels of estrogen metabolites would help to integrate studies with respect to target tissue values. The measurement of further estrogen metabolites by new LC–MS/MS methods to provide a more complete profile, particularly of 4-hydroxylated and methylated estrogens, may lead to more promising markers for breast cancer.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary table

Table S1 Characteristics of excluded studies on EMR and breast cancer risk

Authors	Country	Design	Medium (assay)	Menopausal status	Age (range or mean (SD))	Cases/controls	EMR (2OHE1/16αOHE1)	Notes
Excluded studies sorted by medium								
Adlercreutz et al[48]	Finland	Case series, controls	24-h urine (GC-MS)	Premenopausal	<50	10/11 vegetarian/12 omnivorous	Means not available	Unmatched, no risk estimate for EMR
Kabat et al[17]	USA	Case-control (hospital based)	Spot urine (ELISA)	Mixed	<50–>60	39/58 (premenopausal 16/30, postmenopausal 23/28)	Third vs first tertile: OR: 0.51, 95% CI: 0.17–1.56, postmenopausal OR: 0.03, 95% CI: 0.003–0.29	Combined model unadjusted for menopausal status; adjusted for age, race, parity, smoking, alcohol, chronic disease
Coker et al[45]	USA	Case-control (screening attendees)	Urine (ELISA)	Mixed	Unknown	74/58	Means cases/controls: white 1.91 vs 2.52, black 1.27 vs 2.23	Age, race, menopause adjusted difference, no risk estimate for EMR, only P = 0.008
Ho et al[16]	Singapore	Case-control (biopsies for benign disease)	Spot urine (ELISA)	Mixed	54 (SE 1.2)/54.8 (SE 0.9)	65/36 (premenopausal 23/12, postmenopausal 41/24)	Mean (SE) cases/controls 0.7 (0.1) vs 2.0 (0.3) Median > 0.9 vs < 0.9; OR: 0.16, 95% CI: 0.05–0.49	Unmatched, adjustment for age, menopausal status, parity, OC use
Zheng et al[18]	Shanghai	Case-control	Spot urine (ELISA)	Unknown	Unknown	20/20	Geometric means cases/controls: 1.16 vs 1.52, P = 0.046	Five-year age-group matching, no risk estimate for EMR
Alvarez-Vasquez et al[50]	USA	Case series, controls	Urine (ELISA)	Postmenopausal	Unknown	4/34	Mean cases/controls 1.35 (0.13)/2.71 (0.84)	Only unadjusted means
Gaikwad et al[49]	Italy/USA	Case series-high risk-controls	Urine (GC-MS)	Mixed	34–73	12/46/18 high risk	Means not available	2OHE2, 16αOHEI presented, age and ethnic differences, no risk estimate for EMR
Im et al[46]	USA	Cases, high risk and controls	Spot urine (ELISA)	Mixed	35–70	30/41/77 high risk	Means (SD) cases 1.29 (0.80), high risk 1.76 (2.33), controls 2.47 (1.14)	Menopausal status unclear, no risk estimate for EMR
Schneider et al[14]/Fishman et al[13]	USA	Case series, controls	Blood (radio-metric, indirect)	Postmenopausal	Ca. 43–74	24+ 9 perimenopausal/10	Means not available	Design error-prone, %-hydroxylation, no risk estimate for EMR

(*Continued*)

Table S1 (*Continued*)

Authors	Country	Design	Medium (assay)	Menopausal status	Age (range or mean (SD))	Cases/controls	EMR (2OHE1/16αOHE1)	Notes
Modugno et al,[47] WHI	USA	Prospective nested case-control	Serum (ELISA)	Postmenopausal	69.9 (6.5) cases	93/93 (no HT use)	Median cases/controls 0.43 vs 0.46	No risk estimate for EMR
Osborne et al[15]	USA	Case series, controls	In vivo tissue (radio-metric, indirect)	Premenopausal		4/4	Means not available	16-hydroxylation higher in cases
Castagnetta et al[75]	Italy	Case series, controls	In vivo tissue (HPLC; GC/MS)	Mixed	35–71	17/6	Means (SD) cases/controls 1.5 (0.11) vs 8.3 (4.6)	4OHE2 higher in cases

Abbreviations: EMR, estrogen metabolite ratio; GC–MS, gas chromatography–mass spectrometry; SD, standard deviation.