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Case-control study of phyto-oestrogens and breast cancer

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Summary

Background Phyto-oestrogens are a group of naturally occurring chemicals derived from plants; they have a structure similar to oestrogen, and form part of our diet. They also have potentially anticarcinogenic biological activity. We did a case-control study to assess the association between phyto-oestrogen intake (as measured by urinary excretion) and the risk of breast cancer.

Methods Women with newly diagnosed early breast cancer were interviewed by means of questionnaires, and a 72 h urine collection and blood sample were taken before any treatment started. Controls were randomly selected from the electoral roll after matching for age and area of residence. 144 pairs were included for analysis. The urine samples were assayed for the isoflavonic phyto-oestrogens daidzein, genistein, and equol, and the lignans enterodiol, enterolactone, and matairesinol.

Findings After adjustment for age at menarche, parity, alcohol intake, and total fat intake, high excretion of both equol and enterolactone was associated with a substantial reduction in breast-cancer risk, with significant trends through the quartiles: equol odds ratios were 1·00, 0·45 (95% CI 0·20, 1·02), 0·52 (0·23, 1·17), and 0·27 (0·10, 0·69)—trend p=0·009—and enterolactone odds ratios were 1·00, 0·91 (0·41, 1·98), 0·65 (0·29, 1·44), 0·36 (0·15, 0·86)—trend p=0·013. For most other phytoestrogens there was a reduction in risk, but it did not reach significance. Difficulties with the genistein assay precluded analysis of that substance.

Interpretation There is a substantial reduction in breast-cancer risk among women with a high intake (as measured by excretion) of phyto-oestrogens—particularly the isoflavonic phyto-oestrogen equol and the lignan enterolactone. These findings could be important in the prevention of breast cancer.

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See Commentary page 971

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Introduction

There is strong epidemiological evidence that diet has a role in the development of breast cancer. This evidence initially came from population and migration studies, the subsequent cohort and case-control studies in human beings, and from animal experiments. The bulk of this research is based on the hypothesis that a diet rich in fat predisposes a woman to breast cancer. The results of large cohort studies, however, do not support this hypothesis,[1,2] and interest has moved to other dietary factors.

Phyto-oestrogens are a group of biologically active compounds that have recently attracted attention. They are a diverse group of substances, with a chemical structure similar to that of steroidal oestrogens, and are found in many edible plants. The two principal varieties are isoflavonoids and lignans. When consumed, the plant isoflavonoids and lignans undergo metabolism by bowel microflora, and both the metabolites and the parent compounds are absorbed to a variable extent.[3,4] Oral intake of foods rich in phyto-oestrogens is followed by a peak urinary excretion in the subsequent 24 h; excretion returns to its previous rate in 48–72 h.[5,6] More than 15 phyto-oestrogens have so far been identified in human urine.[7]

Phyto-oestrogens have several potentially anticarcinogenic biological activities, and could thus have a role in the dietary aetiology of breast cancer. Phyto-oestrogens have antiangiogenic,[8] oestrogenic, and antioestrogenic properties,[9] and can also inhibit enzymes.[10,11] Cell-culture studies and animal experiments show that these compounds are tumour inhibiting.[12,13] Although human studies are scarce, Asian populations that consume large amounts of phyto-oestrogens derived from a soy-rich diet have a lower frequency of breast and prostate tumours than western populations that consume much lower quantities of phyto-oestrogens.[13-15] Our case-control study investigated the role of phyto-oestrogens in human breast cancer.

Methods

Study population

Women referred for management of confirmed breast cancer at a single private clinic (DI) or the outpatient clinic of Sir Charles Gairdner Hospital (Perth, Western Australia), were recruited for the study between December, 1992, and November, 1994. Eligible cases were aged between 30 and 84 years and were residents of the Perth area. Exclusion criteria were: pregnancy; antibiotic treatment in the preceding 6 weeks; a previous history of breast cancer; inability to speak or read sufficient English; planned surgery within 72 h of diagnosis; and no definite diagnosis of breast cancer before surgery.

Cases were individually matched, according to 5-year age group, to women selected randomly from the 1993 Perth electoral roll, living in the same postal-code area. Matched-controls were invited by letter to participate in a dietary study, with no mention of breast cancer. A follow-up letter was sent if no reply was received after 2 weeks; in the event of a second non-response, an attempt was made at telephone contact. If this was not possible, or if the woman declined to take part, the procedure was repeated until a suitable participant was found. The exclusion criteria for controls were the same as those for cases, except that controls with recent antibiotic use were included, provided they delayed their urine collection until at least 6 weeks after use of antibiotics stopped. Women who reported a personal history of breast cancer were not eligible as controls.

Data collection

All participants were informed of the nature and requirements of the study and gave written consent. Cases and controls were interviewed by one of three research assistants with a standard questionnaire to elicit information about demographic, reproductive, and lifestyle characteristics. In most instances, the same researcher interviewed both the case and the matched control. The procedure for collection of a 72 h urine sample was explained to each woman. A single blood sample was drawn by venepuncture. For cases, the urine specimen was collected before admission to hospital for surgery. The control women provided a urine sample at their earliest convenience. A second appointment was made to collect the urine sample and the food-frequency questionnaire; the latter was examined for unclear or missed responses.

Collection of samples

Each woman collected three consecutive 24 h urine specimens in separate plastic bottles containing 2 g ascorbic acid. The bottles were kept cool after collection. The three specimens were pooled, mixed, and the total urine volume measured. Three samples from the pooled urine of each woman were stored at −20°C in 50 mL disposable plastic tubes, containing 0·1% (g/L) sodium azide, until analysis for lignans and isoflavonoid phyto-oestrogens. Serum was separated from the blood sample and stored in 1 mL glass vials at −20°C.

Assay of samples

The urinary excretion rates of lignans, enterodiol, enterolactone and matairesinol and isoflavonoid phyto-oestrogens, equol, daidzein and genistein, were measured by isotope-dilution gas chromatography-mass spectrometry (GC-MS) in the select ion monitoring mode used by Adlercreutz and colleagues.[16] Roughly 1/1000 of the total urine volume was extracted on a Sep-Pak c_{18} cartridge (Waters Associates, Milford, MA, USA), the conjugated fractions of lignans and isoflavonoids isolated by chromatography on diethylaminoethyl-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) columns in acetate form, and known amounts of deuterated internal standard of all compounds added to the eluate. Enzymatic hydrolysis (glucuronidase/sulphatase from *Helix pomatia;* Boehringer-Mannheim, Germany) was done, followed by Sep-Pak extraction, and chromatography on Diethyl(2-hydroxypropyl)aminoethyl [QAE]-Sephadex A-25 columns in acetate form. The chromatography resulted in two fractions: fraction 1, which contained equol, enterolactone, enterodiol, matairesinol, and oestrogens; and fraction 2, which contained daidzein and genistein. Fraction 1 underwent further purification to eliminate the oestrogens by chromatography on the carbonate form of QAE-Sephadex. The two fractions containing the lignans and isoflavonoid phyto-oestrogens and their deuterated internal standards were converted to their trimethylsilyl ether, and quantified by GC-MS with select ion monitoring. The measurements were done on a Saturn II GC-MS (Varian Chromatography Systems, Walnut Creek, CA, USA) equipped with an automatic injector (Series 8100) and computer interface (Saturn Software Revision C). We calculated lignan and phyto-oestrogen content by comparing the ratio of the ions for the urinary compounds and deuterated internal standards with the same ratios of the standards forming the standard curve.

The samples were analysed in 30 batches over a 1-year period. Samples from matched cases and controls were analysed with the same assay batch. The between-assay coefficient of variation for the control-urine pool samples for enterolactone, enterodiol, equol, and daidzein was 10·9%, 15·1%, 20·5%, and 21·8%, respectively. The between-assay coefficient of variation for matairesinol at a mean concentration of 11·0 ng/mL was 42·6%. The instability of the trimethylsilyl-ether derivative of genistein, together with persistent interference from an unknown compound, prevented us measuring this isoflavonoid reliably; therefore no data for genistein are given.

One sample of urine from each participant was assayed for urea and ammonia so that a measure of total nitrogen excretion over the 72 h study period could be obtained as an index of total food intake.[17] Urinary urea was measured by an enzymatic-rate method on an automated Hitachi 747 Analyser (Boehringer Mannheim, Australia). We measured urinary ammonia by a glutamate dehydrogenase enzymatic method (Roche Cobas Bio; Roche, Australia).

Entry and analysis of data

Data collected with the questionnaire were coded, categorised, and edited with SPSS for Windows Base System (Release 6.0, 1993), and descriptive statistics were obtained.

Variable	Median (IQR)			
	Cases		Controls	
Age (years)	54·0	(45·0–62·5)	54·0	(45·5–63·0)
Reproductive variables				
Age at menarche (years)	13·0	(12·0–14·0)	13·0	(12·0–14·0)
Parity	2·0	(2·0–3·50)	3·0	(2·0–4·0)
Age first-term birth*	24·0	(21·0–27·0)	25·0	(21·0–27·0)
Lactation (months)*	4·5	(0·5–15·0)	7·5	(1·8–19·0)
Age at menopause	50·0	(47·0–53·0)	50·0	(45·0–53·0)
Anthropometry				
Weight (kg)	65·0	(59·0–71·5)	65·0	(58·0–74·0)
Height (cm)	162·0	(157·0–165·0)	162·0	(157·0–165·0)
Body-mass index (kg/m^2)	24·8	(22·6–27·8)	25·0	(22·0–28·2)
Nutritional variables				
Alcohol (g/day)	2·6	(0·0, 14·1)	2·9	(0·3, 9·4)
Energy intake (kJ/day)	7988	(6149, 9612)	8342	(6830, 9810)
Energy intake (percent fat)	33·6	(29·0, 37·1)	33·2	(30·1, 36·5)
Fat intake (g/day)	69·9	(51·3, 92·1)	72·0	(60·0, 88·2)

*Includes only those women who progressed to ⩾20 weeks' gestation.

Table 1: **Descriptive characteristics of study**

Reproductive variables, including age at menarche, age at first full-term birth, parity, months of lactation, and age at menopause, were categorised according to published classifications.[18] Hormone replacement therapy was categorised as "current" or "not current" use. Family history of breast cancer (first and second degree relatives), benign breast disorders, menopausal status, and abortions were classified "yes" or "no". For women whose menopausal status was unclear, the serum concentrations of follicle-stimulating hormone and oestradiol were measured by radioimmunoassay to allow correct categorisation. Phyto-oestrogen values were divided into quartiles according to their distributions in the control population.

Since women were matched by age and residential area, we did the analysis with conditional logistic regression in the statistical package EGRET (version 1.02.01). An odds ratio was used to represent the relative risk, and its 95% CI was assessed for each exposure variable, as well as any potential confounding factor associated with the variable under study. Whether a variable had a significant effect on breast-cancer risk was judged by the likelihood-ratio p values (two-sided), obtained when terms were added either as factored or unfactored variables. If the p value was less than or equal to 0·05, the effect was deemed significant. We did multivariate logistic regression to assess the association between breast-cancer risk and the respective lignan and isoflavonoid phyto-oestrogen excretion rates. Tests for linear trend, representing potential dose-response effects, were done by the fitting of a continuous variable.

An initial analysis of risk factors in the participants showed that age at menarche, parity, and dietary fat intake are associated with breast-cancer risk. Hence, we deemed these variables relevant for control, and included them as confounding variables. Since some studies have shown that alcohol consumption has a weak or modest association with breast-cancer risk,[19,20] and since a dietary constituent had potential to interfere with phyto-oestrogen metabolism, alcohol intake was also included as a confounding variable. Thus, the final regression model included these four variables.

Results

Study population

341 women were diagnosed with breast cancer during the study period. A large number of women did not meet the study criteria, mainly because their surgery was scheduled within 3 days of diagnosis, or the diagnosis was not confirmed until the time of their operation. Only a few women declined to participate. Of the 149 women who agreed, and who met the eligibility criteria, two changed their minds, and one refused to complete the food-frequency questionnaire. An error in age-matching resulted in the subsequent exclusion of another case. Of the 441 control women randomly chosen for the study, 249 did not wish to participate, and 45 could not be contacted. One was excluded because of pregnancy. 144 pairs remained for analysis.

	Cases	Controls	Crude OR (95% CI)	Adjusted OR (95% CI)
Daidzen				
⩽600·00†	51	31	1·00	1·00
600·01–900·00	29	36	0·49 (0·24, 0·99)	0·60 (0·27, 1·33)
900·01–1300·00	29	35	0·59 (0·30, 1·16)	0·80 (0·36, 1·80)
⩾1300·01	24	32	0·38 (0·16, 0·91)	0·47 (0·17, 1·33)
Test for homogeneity			p=0·081	p=0·411
Test for trend			p=0·033	p=0·241
Equol				
⩽70·00†	47	35	1·00	1·00
70·01–110·00	37	37	0·76 (0·40, 1·43)	0·45 (0·20, 1·02)
110·01–185·00	35	36	0·69 (0·36, 1·34)	0·52 (0·23, 1·17)
⩾185·01	24	36	0·41 (0·19, 0·90)	0·27 (0·10, 0·69)
Test for homogeneity			p=0·154	p=0·035
Test for trend			p=0·029	p=0·009
Enterodiol				
⩽170·00†	41	32	1·00	1·00
170·01–300·00	35	37	0·72 (0·36, 1·42)	0·92 (0·40, 2·09)
300·01–480·00	30	36	0·66 (0·34, 1·27)	0·62 (0·28, 1·37)
⩾480·01	38	39	0·74 (0·38, 1·46)	0·73 (0·33, 1·64)
Test for homogeneity			p=0·631	p=0·602
Test for trend			p=0·380	p=0·288
Enterolactone				
⩽1450·00†	51	36	1·00	1·00
1450·01–3100·00	44	36	0·80 (0·41, 1·55)	0·91 (0·41, 1·99)
3100·01–5250·00	30	36	0·51 (0·25, 1·03)	0·65 (0·29, 1·44)
⩾5250·01	19	36	0·36 (0·17, 0·75)	0·36 (0·15, 0·86)
Test for homogeneity			p=0·024	p=0·074
Test for trend			p=0·002	p=0·013
Matairesinol				
⩽17·00†	30	37	1·00	1·00
17·01–30·00	45	36	1·83 90·81, 4·13)	2·38 (0·89, 6·32)
30·01–42·00	31	32	1·47 (0·61, 3·50)	1·95 (0·67, 5·74)
⩾42·01	38	39	1·43 (0·64, 3·24)	2·18 (0·83, 5·76)
Test for homogeneity			p=0·528	p=0·334
Test for trend			p=0·759	p=0·308

*Adjusted for age at menarche, alcohol intake, and total fat intake. †Reference group.

Table 2: **Crude and adjusted odds ratios for risk of breast cancer associated with intake of phyto-oestrogens**

The characteristics of cases and controls were similar (table 1). There were no significant differences between the groups for age, age at menarche or menopause, parity, age at first full-term birth, duration of lactation, anthropometric variables, or the nutritional variables (ie, alcohol intake, total energy, total fat, or the energy percentage from fat).

Odds ratios

The unadjusted odds-ratio estimates showed that increasing excretion of daidzein, equol, and enterolactone was associated with a significant reduction in risk of breast-cancer development (table 2). This effect was particularly clear for equol—the risk for the highest quartile of excretion after adjustment for confounding variables was one quarter that of the lowest quartile of excretion (adjusted odds ratio 0·27 [95% CI 0·10–0·69]); this represents a four-fold reduction in risk. The test for trend through the quartiles was also significant (p=0·009). The lignan enterolactone showed a three-fold reduction in risk for the highest compared with the lowest quartile of excretion, even after adjustment for confounding variables (adjusted odds ratio 0·36 [0·15–0·86]). Again, the trend through the quartiles was significant (p=0·013). The crude odds ratio for daidzein

	Median (IQR)			
	Cases		Controls	
Phyto-oestrogen (nmol/24 h)				
Daidzen	782·9	(462·8, 1180·1)	913·4	(611·5, 1274·1)
Equol	97·2	(63·5, 162·2)	108·6	(70·4, 180·8)
Enterodiol	282·0	(157·0, 487·5)	316·5	(172·1, 481·3)
Enterolactone	1973·4	(991·9, 3869·7)	3097·7	(1484·8, 5149·8)
Matairesinol	28·9	(18·8, 47·4)	29·3	(16·6, 42·4)
Total nitrogen (g/24 h)	14·8	(11·4, 18·1)	15·9	(12·2, 19·8)

*Does not include those women with missing values.

Table 3: **Excretion rates of lignans, isoflavonoid phyto-oestrogens, and total nitrogen***

also showed a three-fold reduction in risk for the highest quartile of excretion, but this association ceased to be significant after control for confounding variables. The data were analysed separately for premenopausal and postmenopausal women, and there were similar trends for each group.

Median excretion of phyto-oestrogens

Because the distributions of the lignan and phyto-oestrogen excretion rates were skewed, we have reported the medians and IQRs rather than the means and SDs (table 3). For all phyto-oestrogens, the control women had higher median excretion rates than the cases; for enterolactone, the control median excretion was 50% higher, though the differences were smaller for the other phyto-oestrogens. We found little difference between cases and controls in the excretion of total nitrogen per 24 h, which suggests that differences in phyto-oestrogen excretion reflect differences in the types of foods consumed, and not just a general reduction in food intake.

Discussion

Our study shows that increased excretion of some phyto-oestrogens is associated with a substantial reduction in breast-cancer risk. This finding supports previous observational studies[13-15] that reported higher phyto-oestrogen excretion among populations with a low frequency of breast cancer. A case-control study of Singapore Chinese women found that soya consumption protected against breast cancer, though there were other significant dietary influences at work including β-carotene as a protective substance.[21] Other studies do not support this property of soya consumption.[22] The lower excretion of enterolactone by breast-cancer patients in our study accords with the findings of a previous small study (seven cases) in which enterolactone excretion was significantly lower in postmenopausal breast-cancer patients than in omnivorous and vegetarian controls.[23] Isoflavonic phyto-oestrogens, especially the unfermented forms,[6,24,25] are found predominantly in soya products, whereas lignans are found in the fibre present in such foods as whole grains, berries, fruit and vegetables, and, particularly, flax seed.[26–29] Cow's milk has also been identified as a source of equol;[30] this may be particularly important in Western Australia, where the pastures contain clover high in oestrogens. Some researchers report that consumption of milk products can protect against breast cancer.[31,32]

Given the size of the risk reduction in our study, the clear step-wise trend, and the confidence intervals (which did not include one for the lowest compared with the highest quartile), it is unlikely that our findings result from chance. Nevertheless, we have looked for potential bias. The cases were predominantly from a private clinic, and the controls from the electoral roll. It is thus possible that cases came from a wealthier socioeconomic group with differences in dietary intake. Matching for residential area, however, should counteract this bias. Another potential bias was the recruitment procedure. Cases were asked by the attending specialist at the time of their appointment to participate, and few declined. By contrast, controls were recruited from the community by a letter from the specialist, which resulted in a much lower participation rate. It is possible that women with an interest in their health and diet would be more likely to volunteer, though the effect of this self-selection process on our findings is not clear. Finally, the timing of urine collection may also have introduced bias. There is no ideal time to study cases, but immediately after diagnosis was preferable to any later period, so that factors such as admission to hospital, surgery, medications, and an increased awareness of the role of diet in breast cancer would have little influence on the women's usual diet. The period immediately after diagnosis is very stressful, and it is possible that these women ate less during the time of urine collection, though they were asked to continue with their usual eating habits. We attempted to measure this potential bias by assaying the urine samples for total nitrogen excretion;[33] since there was no significant difference between cases and controls in total nitrogen excretion, this bias was probably not important.

An advantage of our study over other studies of nutrition and breast cancer is that it did not rely solely on dietary recall or records. The direct measurement of phyto-oestrogen excretion in urine provides not only an index of intake and subsequent metabolism by the gut flora, but also an indication of bioavailability.[34] This is an important factor in the analysis of the mechanisms by which phyto-oestrogens might influence breast-cancer development.

Several laboratory studies have shown antiproliferative effects of phyto-oestrogens on human breast-cancer cell lines, and in animal experiments.[13] Several possible mechanisms have been proposed. First, phyto-oestrogens may influence breast-cancer development by alteration of sex-hormone metabolism. The diphenolic structure of the isoflavonic phyto-oestrogens is similar to that of synthetic oestrogens, and all are weakly oestrogenic.[9] Some investigators have suggested that isoflavonic phyto-oestrogens may also act as antioestrogens by competing with oestradiol for nuclear oestrogen-binding sites, and thereby inhibit the growth and proliferation of hormone-dependent cells.[9] There is evidence that lignans and isoflavonic phyto-oestrogens may stimulate sex-hormone-binding globulin in the liver,[26] and thus reduce the percentage of free, biologically active oestradiol in the plasma. Furthermore, several lignans and isoflavonic phyto-oestrogens inhibit aromatase—the enzyme that converts androstenedione to oestrone[11]—and thus may reduce the amount of circulating oestrogen.

Our findings have implications for the control of breast cancer. Early detection by screening mammography and adjuvant systemic therapy both reduce breast-cancer mortality, but these techniques do not prevent the occurrence of cancer in the first place. They do little, therefore, to reduce the enormous emotional and physical suffering the disease causes—nor do they reduce the massive financial cost to the community. Prevention is the only way to reduce this suffering and cost. The indication in our study that phyto-oestrogen consumption

reduces breast-cancer development provides a potential dietary mechanism for control. However, the association between breast-cancer risk and phyto-oestrogen excretion is not necessarily causal, and may merely result from some other dietary characteristic. Nevertheless, we are aware of no previously investigated preventive factor that has shown a degree of risk reduction similar to that found for some phyto-oestrogens in this study; and none has equal potential as a simple intervention as phyto-oestrogens. A cultural movement towards increased consumption of phyto-oestrogen-containing foods is taking place, encouraged by magazines and other lay media. Our findings go some way towards providing a rationale for these changes.

Contributors

David Ingram designed the study, secured funding, and coordinated the study. Kathy Sanders interviewed the women, collected samples, and set up and undertook the urinary assays. Marlene Kolybaba interviewed the women, collected samples, and undertook statistical analyses. Derrick Lopez assisted with the laboratory assays and data analysis. All authors contributed to the writing of the paper.

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