Reducing Bioavailable Sex Hormones through a Comprehensive Change in Diet: the Diet and Androgens (DIANA) Randomized Trial[1]

Franco Berrino,[2] Cristina Bellati, Giorgio Secreto, Edgarda Camerini, Valeria Pala, Salvatore Panico, Giovanni Allegro, and Rudolf Kaaks

Unit of Epidemiology (F. B., C. B.,V. P.), Unit of Nuclear Medicine (G. S.), Unit of Laboratory Medicine (E. C.), Istituto Nazionale Tumori, 20133 Milan, Italy; Department of Clinical and Experimental Medicine, Federico II University, 80131 Naples, Italy (S. P.); Association Le Cinque Stagioni, 10018 Ivrea, Italy (G. A.); and Nutrition and Cancer Unit, International Agency for Research on Cancer, 69372 Lyon, France (R. K.)

Abstract

High serum levels of testosterone and estradiol, the bioavailability of which may be increased by Western dietary habits, seem to be important risk factors for postmenopausal breast cancer. We hypothesized that an *ad libitum* diet low in animal fat and refined carbohydrates and rich in low-glycemic-index foods, monounsaturated and n-3 polyunsaturated fatty acids, and phytoestrogens, might favorably modify the hormonal profile of postmenopausal women. One hundred and four postmenopausal women selected from 312 healthy volunteers on the basis of high serum testosterone levels were randomized to dietary intervention or control. The intervention included intensive dietary counseling and specially prepared group meals twice a week over 4.5 months. Changes in serum levels of testosterone, estradiol, and sex hormone-binding globulin were the main outcome measures. In the intervention group, sex hormone-binding globulin increased significantly (from 36.0 to 45.1 nmol/liter) compared with the control group (25 *versus* 4%,; $P < 0.0001$) and serum testosterone decreased (from 0.41 to 0.33 ng/ml; −20 *versus* −7% in control group; $P = 0.0038$). Serum estradiol also decreased, but the change was not significant. The dietary intervention group also significantly decreased body weight (4.06 kg *versus* 0.54 kg in the control group), waist:hip ratio, total cholesterol, fasting glucose level, and area under insulin curve after oral glucose tolerance test. A radical modification in diet designed to reduce insulin resistance and also involving increased phytoestrogen intake decreases the bioavailability of serum sex hormones in hyperandrogenic postmenopausal women. Additional studies are needed to determine whether such effects can reduce the risk of developing breast cancer.

Introduction

Recent prospective studies have provided strong evidence that the risk of developing breast cancer in postmenopausal women is increased by high serum levels of testosterone and estradiol, low levels of sex hormone-binding globulin, and, hence, high circulating levels of free steroid sex hormones (1–7). Evidence is accumulating that Western dietary habits contribute this high-risk hormonal profile, but the efficacy of changes in diet in reducing the availability of sex hormones has not been sufficiently investigated.

Chronic hyperinsulinemia may be a key link between nutrition-related life-style factors, development of a high-risk steroid hormone profile, and increased breast cancer incidence (8). Insulin inhibits the hepatic production of sex hormone-binding globulin (9) and stimulates the ovarian production of androgens (10, 11). Women who are overweight, especially those with large intra-abdominal fat stores, which in postmenopausal women are often associated with increased risk of breast cancer (12), often have insulin resistance (9, 13), low serum levels of sex hormone-binding globulin (14), and high sex hormone levels (15). Epidemiological studies suggest an association of breast cancer risk with increased serum levels of insulin (16) and also with increased activity of insulin-like growth factor-I (17).

The availability of steroid sex hormones in the blood may also be reduced by the dietary intake of phytoestrogens (18–21), plant-derived diphenolic compounds that display both estrogenic and antiestrogenic activities and may protect against breast cancers (22). Phytoestrogens include isoflavones from soy (23), lignans from flax and other seeds and fiber-rich vegetables (24, 25), and coumestrol from alfalfa sprouts and other legumes (26). Indole-3-carbinol, a compound that occurs in cruciferous plants, also exhibits antiestrogenic activity (27).

Among women from low-cancer-risk Asian populations, characterized by the consumption of fairly large quantities of soy products, serum levels of testosterone and estradiol have been found to be 20–50% lower than in Western women (28–31) and inversely related to the consumption of soy products (32). Furthermore, in two (29, 33) of four studies (28, 29, 31, 33), levels of serum sex hormone-binding globulin were higher among Asian women. Epidemiological studies have suggested a lowered risk of breast cancer with increased urinary excretion of phytoestrogens (34, 35) but have not consistently found a negative association with increased consumption of soy products (36).

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[2] To whom requests for reprints should be addressed, at Epidemiology Unit, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-02-70-60-18-53 or 39-02-70-63-83-98; Fax: 39-02-23-90-762; E-mail: berrino@istitutotumori.mi.it.

We present here the results of the DIANA[3] study. This was a randomized dietary intervention in postmenopausal women with high plasma levels of testosterone. The hypothesis of the study was that levels of testosterone and estradiol might be lowered, and levels of sex hormone-binding globulin increased, by a radical change in diet. The new diet was *ad libitum* and had two overlapping dimensions: (*a*) increasing phytoestrogen intake and (*b*) other changes designed to reduce plasma insulin levels.

Increased phytoestrogen intake was ensured by increasing the consumption of soy products, other legumes, whole-grain cereals, flax and other seeds, seaweed, berries, crucifers, and other vegetables (23, 24, 26).

The plasma insulin-lowering aspect involved reducing total fat intake, so as to help reducing body mass index and waist circumference, which are major determinants of insulin resistance (8, 37–39); increasing the proportion of n-3 polyunsaturated and monounsaturated fatty acids, which may improve insulin sensitivity (40–43); reducing foods rich in sugar or highly refined carbohydrates, which lead to high postprandial glycemic and insulinemic responses (44) and to insulin resistance (45); and increasing consumption of low-glycemic-index foods such as unrefined cereals, legumes, and vegetables (43–46).

The ultimate aim of the study was to determine whether such a diet might be worth investigating in long-term trials designed to reduce the risk of breast cancer.

Subjects and Methods

Subjects. Three hundred and twelve healthy women, ages 50–65 years, from the Milan area (northern Italy) volunteered to take part in the study after advertisements had been placed in the local media. Eligibility criteria were: (a) postmenopausal for at least 2 years; (b) presence of at least one ovary; (c) not on hormonal replacement therapy for at least the previous 6 months; (d) no history of cancer; (e) not following vegetarian, macrobiotic, or other medically prescribed diet; and (f) not receiving treatment for diabetes.

Written informed consent was obtained from all of the women, and the Scientific and Ethical Committee of the Milan Cancer Institute approved the study.

Study Design. Levels of testosterone in the serum of the volunteers were determined (prebaseline), and the 104 women in the upper tertile (testosterone, >0.38 ng/ml) were selected for the study. With the exception of two close friends, who were allocated to the same group, these women were individually randomized to the intervention and control groups (52 women each), stratified for age (above or under the median of 58 years), prebaseline serum testosterone (three levels), and prebaseline fasting insulin (three levels). We selected women on the basis of the serum testosterone level because its measurement is highly reliable (47), and it has been shown to predict breast cancer risk not less than estrogen levels (1, 2). The women in the intervention group agreed to adhere to the diet described below for 4.5 months. The control women were not given any information about this diet, nor any dietary instruction**,** but were advised to increase their consumption of fruit and vegetables according to the cancer prevention decalogue of the Europe against Cancer program, a leaflet largely available to the general population.

Before the start and at the end of the intervention, fasting blood samples and 24-h urine samples were taken and stored at −30°C for hormone assays. An oral glucose tolerance test was also performed, involving collection of blood samples 1, 2, and 3 h after the ingestion of 100 g of glucose.

Taking into account the intraindividual variation in hormone levels (48), we estimated that the study had a statistical power of more than 90% for detecting a 20% change in the main outcome variables.

Dietary Intervention. Women in the intervention group were invited for common meals and cooking classes twice a week for 18 weeks. On each occasion the menu was different, but mainly based around Mediterranean vegetarian and macrobiotic recipes. The foods used are described in the Appendix. We recommended that the same foods should be consumed on a daily basis at home, but we did not prescribe menus. However, we provided written instructions that indicated how to substitute meat, eggs, and dairy products with vegetable sources of essential amino acids, vitamins, and minerals; recommended that meat, eggs, and dairy products should not be eaten more than once a week; urged reducing the consumption of refined carbohydrates (sucrose, white bread, refined flour), substituting whole-grain cereal products, using fruit or fermented cereal as edulcorants; and recommended cooking with little added fat and salt.

The women were also encouraged to eat at least one portion of a soy product (soy milk, miso soup, tofu, tempeh, or soy beans) every day, to season moderately with unrefined olive oil and various seeds but not dairy fats, and to consume fish and seaweed.

Every week, each woman received a 1-kg loaf of bread made from whole wheat flour and 8% flax seed (half whole seeds and half milled), occasionally mixed with oats or rye, and also a free pack of other recommended products that are not a normal part of the northern Italian diet.

In the first month of the study, participants were asked to change their habits gradually to prevent adverse reactions due to excessive fermentation in the bowel. The diet was *ad libitum*, and no advice was given to reduce total food intake or to count calories.

Assessment of Dietary Intake and Anthropometric Measurements. Before randomization, all of the women compiled a food frequency questionnaire developed for EPIC (49). During the study, compliance with dietary recommendations was monitored by 24-h food frequency diaries, which were filled in 24 times by the intervention group and 10 times by the control women. In the 4th month of the study, all of the women were interviewed and asked to recall everything they had eaten in the preceding 24 h, including quantities. Data were collected with the computerized EPIC 24-h dietary recall system (50), which was then used to estimate absolute intakes of nutrients and energy in the two groups. The system makes use of the Italian food composition database (51), which also includes several foods used in macrobiotic recipes. Average consumption of isoflavonoids and lignans by the intervention and the control groups were estimated from available databases on the phytoestrogen content of foods (23–26, 52, 53) and from the food frequency diaries, using as standard portion sizes those derived from the interviews.

Height, weight, waist circumferences (at natural waist when clearly identifiable or midway between lower rib and iliac crest), and hip circumference (at crotch) were measured at the beginning and at the end of the study.

[3] The abbreviations used are: DIANA, diet and androgens; EPIC, European Prospective Investigation into Cancer and Nutrition; IRMA, immunoradiometric assay; MEIA, microparticles enzyme immunoassay.

Laboratory Analyses. Circulating hormones were measured using commercial kits: RIA kits from ORION Diagnostic (Turku, Finland) for testosterone and estradiol; IRMA kits from Farmos (Oulunsalo, Finland) for sex hormone-binding globulin; and MEIA kits from ABBOTT (Abbott Park, IL) for insulin. The coefficients of intra- and interassay variation in eight replicates were, respectively: 4.2 and 12.5% for a testosterone value of 0.420 ng/ml; 5.2 and 11.1% for an estradiol concentration of 10 pg/ml; 3.5 and 6.7% for a sex hormone-binding globulin value of 34.0 nmol/liter; and 2.5 and 4.6% for an insulin value of 14.2 μIU/ml. For insulin, samples were analyzed within 2 weeks of collection. To reduce the effects of interassay variability, for sex hormone-binding globulin, testosterone, and estradiol, baseline and final serum samples of the same woman were analyzed in the same batch. We have previously shown that both estradiol and testosterone are stable in serum preserved at low temperature (47).

We measured urinary daidzein and its metabolite equol by gas chromatography after solid-phase extraction and high-performance liquid chromatography purification. Coefficients of variation were 7.5% for low (14 ng/ml) and 10.7% for high (9982 ng/ml) daidzein concentrations, and 4.0% for low (80 ng/ml) and 2.9% for high (10,500 ng/ml) equol concentrations. All of the blood and urinary samples were analyzed blind to intervention-control status.

Compliance and Subjects Excluded from Statistical Analysis. Fifty of the 52 women of the intervention group followed the whole dietary program. Two women followed only about half of the program but were included in all of the analyses. Only five women were absent more than five times from the 36 lessons and common meals. Urinary daidzein and equol levels were used as indicator of compliance with soy consumption. Two women from the intervention group and one woman from the control group were excluded because they received hormonal drugs during the study period. Two other women from the control group were excluded because they did not attend the final examination. A total of 99 women were analyzed: 50 in the intervention group and 49 controls. Of these, four (two in the intervention group and two controls) had missing values for fasting insulin, and five (one in the intervention and four in the control group) had missing values for the oral glucose tolerance.

Statistical Methods. The statistical analysis focused on changes in hormonal and other relevant variables, calculated as the difference between end of study and baseline values for each woman. Hormone values were log-transformed to obtain approximately normal frequency distributions. The statistical significance of mean changes in the intervention group compared with controls was assessed by ANOVA. Multivariate ANOVA was used to perform an *omnibus* test for simultaneous changes in the main hormonal variables, circumventing the problem of significance testing with multiple, partially independent comparisons for each parameter. All of the ANOVA were stratified according to the blocking scheme used for the randomization. Interaction terms were used to test whether the magnitude of the effect of the dietary intervention differed for women with different baseline values of testosterone or insulin. Because the numbers of observations within the various blocks were not equal, all of the ANOVA used generalized linear models, using the SAS statistical software package (54). Finally, Spearman correlation coefficients were computed to evaluate cross-sectional relations between anthropometric and hormonal variables at baseline and longitudinal relations between the changes in the different variables. All of the *P*s are two-tailed.

Results

The diet of the participating women before randomization, as estimated from the food frequency questionnaire, was typical of northern Italy, with 42% of calories obtained from carbohydrates (mainly bread and pasta) and 37% from fat (mainly meat, dairy products, and olive oil), without significant differences between women eventually randomized in the intervention group and in the control group (Table 1). The diet-recall interviews in the 4th month of intervention slightly underestimated the total caloric intake with respect to energy requirement (55) but showed a lower total energy intake in the intervention group than in the control group, about 250 kcal per day on average, mainly caused by a lower intake of total and saturated fat. Intervention women also shifted from animal to vegetable sources of protein and fat and from simple to complex carbohydrates, and consumed more vegetable fibers (Table 1). According to the food frequency diaries compiled during the study, the intervention women consumed meat or meat products twice a week against once a day in the control women, but consumed fish more often (3 times a week *versus* 1.5 in controls). Milk and cheese consumption was cut by half (0.4 *versus* 1.0 servings per day) and butter was virtually eliminated. A soy product was consumed on average 1.7 times per day (SD, 0.6); flax seeds, either in bread or cookies or as such were eaten every day (about 8 g per day), and seaweed was used every other day as ingredients of various dishes. The control women rarely, if ever, consumed any of these food items. Intervention women also consumed the following much more often than controls: whole rice or other whole grain or whole-meal cereal products (2.5 *versus* 0.5 per day), walnuts, almonds, sesame and other seeds (1.2 *versus* 0.05), legumes (0.5 *versus* 0.1), cruciferous vegetables (0.8 *versus* 0.1), and berries (0.4 *versus* 0.1). Other vegetables and fruits were consumed almost as frequently by the control group as by the intervention group (2.2 and 2.3 times a day, respectively).

We estimated that women in the intervention group consumed on average of ~38–45 mg of isoflavonoids per day. The estimated average daily intake of lignans was more uncertain (9–32 mg) because of large inconsistencies between different methods of chemical assay in food (24, 25). The corresponding estimates for controls, however, were much lower (about 2 mg/day isoflavonoids and 1 mg/day lignans). The high intake of isoflavone-rich food by the intervention group was confirmed by assay of daidzein and its metabolite equol in 24-h urine samples collected toward the end of the study period. Mean cumulative excretion was 5,32 mg/24 h in the intervention group (range, 0.02–10.18) *versus* 0.17 mg/24 h in controls (range, 0.01–1.09). In the control group, only one woman had values above 1 mg/24 h, and 29 values were under 0.1 mg/24 h; in the intervention group, nine women had values under 1 mg/24 h and only three under 0.1 mg/24 h, including the two women who did not complete the intervention.

The high compliance of the intervention women with dietary recommendations was confirmed by the analysis of changes in serum cholesterol levels and anthropometric variables. Total cholesterol levels decreased from 240.0 to 206.5 mg/dl in the intervention group (−14%) *versus* 240.6 to 230.4 in the control group (−4%; $P = 0.0005$). Intervention women lost more weight ($P < 0.0001$) than control women: 4.06 kg (range, −0.6 to −8.8 kg) *versus* 0.54 kg (range, +2.2 to 5.3 kg; Table 2); with similar differences in waist circumference ($P <$

Table 1 Average energy and nutrient intake of intervention and control women as estimated before the start and towards the end of the study

Nutrients	Dietary intake before randomization (from food frequency questionnaire)			Intake in the 4th month of the study (24-h recall interview)			
	Intervention group g/day (SD)	Control group g/day (SD)	Both groups % calories	Intervention group g/day	% calories	Control group g/day	% calories
Protein, total	93.3 (24.8)	89.3 (34.4)	16.6	61.0	15.7	68.4	15.2
Animal	63.1 (18.6)	60.7 (25.5)	11.4	17.9	4.6	41.3	9.2
Vegetable	30.2 (11.8)	28.5 (12.6)	5.2	43.0	11.1	26.9	6.0
Lipid, total	93.8 (30.4)	90.1 (36.4)	37.1	53.0	30.8	65.9	33.0
Animal	47.2 (18.3)	48.2 (24.6)	19.1	9.7	5.6	28.4	14.2
Vegetable	46.7 (20.2)	41.9 (20.0)	18.0	43.0	25.0	36.8	18.3
Saturated	32.1 (11.9)	31.5 (14.5)	12.7	10.6	6.2	20.2	10.1
Monounsaturated	45.2 (14.8)	42.6 (18.5)	17.8	25.1	14.6	31.8	15.9
Oleic	42.7 (14.2)	40.2 (17.6)	16.8	23.7	13.8	29.8	14.9
Polyunsaturated	11.4 (6.8)	10.9 (4.9)	4.5	12.8	7.4	10.0	5.0
Linoleic	9.3 (6.4)	8.9 (4.4)	3.7	9.5	5.5	7.8	3.9
α-linolenic	1.4 (0.5)	1.3 (0.5)	0.5	2.4	1.4	1.1	0.5
Other	0.7 (0.3)	0.6 (0.3)	0.3	0.7	0.4	0.9	0.4
Cholesterol (mg/day)	375 (113)	352 (152)		73.8		235.9	
Carbohydrates	257.0 (89.4)	246.5 (99.8)	42.3	212.1	51.3	225.9	47.0
Sugar	106.6 (44.0)	98.5 (46.0)	17.4	71.1	17.2	98.6	20.5
Starch	150.1 (63.8)	147.6 (73.9)	24.9	135.2	32.7	126.7	26.3
Dietary fiber	23.4 (8.1)	21.3 (8.3)		35.5		23.3	
Alcohol	11.3 (11.8)	13.8 (16.4)	4.0	4.8	2.2	12.5	4.8
Energy (kcal/day)	2261.7 (647.4)	2190.1 (818.0)	100	1550.0	100	1805.0[a]	100

[a] The estimated mean energy requirement, calculated from body weight measured at time of the 24-h recall interview and assuming a sedentary life style (55), was 1955 and 1979 kcal/day, respectively, for intervention and control women, suggesting that the frequency questionnaire overestimated and that the 24-h recall interview underestimated energy intake by about 10%.

Table 2 Mean values of anthropometric variables in intervention and control women before and after dietary intervention

		n	Mean		Change	*P*
			January	June		
Weight (kg)	Intervention	50	67.14	63.08	−4.06	
	Control	49	66.85	66.31	−0.54	0.0001
Body mass index (kg/m^2)	Intervention	50	26.88	25.26	−1.62	
	Control	49	27.36	27.14	−0.22	0.0001
Waist (cm)	Intervention	50	84.02	80.15	−3.88	
	Control	49	83.43	82.94	−0.49	0.0001
Hip (cm)	Intervention	50	102.34	99.87	−2.47	
	Control	49	102.67	102.87	0.20	0.0001
Waist:Hip ratio	Intervention	50	0.82	0.80	−0.02	
	Control	49	0.81	0.80	−0.01	0.0045

0.0001), hip circumference ($P < 0.0001$), and waist:hip ratio ($P = 0.0045$; Table 2).

Using multivariate ANOVA, we found a statistically significant change ($P < 0.0001$) in the intervention group compared with controls for the five major hormonal and metabolic outcomes combined (sex hormone-binding globulin, testosterone, estradiol, fasting insulin, and fasting glycemia). The change was also significant ($P < 0.0002$) when the first three of these variables were combined with area under insulin curve and area under glucose curve, instead of fasting insulin and fasting glucose levels.

Serum sex hormone-binding globulin levels increased (+25.2%) and serum levels of testosterone and estradiol decreased (−19.5% and −18.0%) in the intervention women (Table 3). In the control group, there were also small changes in sex hormone-binding globulin (+3.6%), testosterone (−7.1%), and estradiol (−5.5%) levels, in the same direction as in the intervention group. The changes in sex hormone-binding globulin and testosterone levels were significantly larger in the intervention than in the control group ($P < 0.0001$ and $P = 0.0038$, respectively) whereas the changes in estrogen did not differ significantly between the groups ($P = 0.13$). The ratio of testosterone:sex hormone-binding globulin decreased in all except two of the intervention women ($P < 0.001$; Table 4). Fasting glycemia and the total area under the insulin curve during the glucose tolerance test also decreased significantly in the intervention group compared with controls ($P = 0.0260$ and $P = 0.0404$, respectively); however, the change in fasting insulin was not significant (Table 3).

At baseline, body mass index correlated strongly with serum estradiol levels (Spearman coefficient of correlation *r*, 0.60) and negatively with sex hormone-binding globulin levels (*r*, −0.53), but not with testosterone levels (*r*, 0.19). In the intervention group, changes in body weight were significantly correlated with changes in serum levels of sex hormone-binding globulin (*r*, −0.33) but not with changes in levels of insulin (*r*, 0.20), testosterone (*r*, 0.19), and estradiol (*r*, 0.09). The ratio of testosterone:sex hormone-binding globulin decreased markedly in women who lost over 4.5 kg of body weight but decreased also in women who lost less than 3 kg (Table 4). After adjustment for weight changes, however, the differences between changes in hormonal levels in the intervention and in the control group were no more statistically significant (Table 3), which suggests that the hormonal effects of dietary intervention could be largely mediated through changes in body weight. Among women who initially had high testosterone levels, the dietary intervention caused a larger decrease in testosterone levels than in women with initially low levels, but the interaction was not significant ($P = 0.0849$).

Table 3 Mean values of hormonal variables in intervention and control women before and after dietary intervention

	n	Geometric mean		% change	*P*	*p*[a]
		January	June			
SHBG[b] (nmol/liter)						
Intervention	50	36.03	45.10	25.2		
Control	49	36.32	37.61	3.6	0.0001	0.28
Testosterone (ng/ml)						
Intervention	50	0.41	0.33	−19.5		
Control	49	0.42	0.39	−7.1	0.0038	0.30
Estradiol (pg/ml)						
Intervention	50	8.62	7.07	−18.0		
Control	49	8.30	7.84	−5.5	0.1293	0.85
Fasting insulin (μIU/ml)						
Intervention	48	4.82	4.31	−10.6		
Control	47	4.63	4.87	5.2	0.1441	0.72
Insulin area (μIU/ml × 180 min)						
Intervention	47	7300	6740	−7.7		
Control	43	6879	7525	9.4	0.0404	0.66
Fasting glucose (mg/dl)						
Intervention	50	91.66	86.47	−5.7		
Control	49	93.55	92.44	−1.2	0.0260	0.05
Glycemic area (mg/dl × 180 min)						
Intervention	47	20000	20968	4.8		
Control	43	20291	21474	5.8	0.8465	0.24

[a] Value after additional adjustment for effects related to weight change.
[b] SHBG, sex hormone-binding globulin.

Table 4 Distribution of participating women according to change in the testosterone:SHBG[a] ratio from baseline to the end of the dietary intervention

	Increased	Decreased by		Total
		1–33%	>33%	
Control group	20	24	6	49
Intervention group	2	21	27	50
Intervention group by weight reduction				
4.5 kg or more (average, 5.8)		4	14	18
3.4–4.4 kg (average, 4)		7	9	16
3 kg or less (average, 2.2)	2	10	4	16

[a] SHBG, sex hormone-binding globulin.

Discussion

We observed significant and favorable changes in hormonal indicators of breast cancer risk in a group of postmenopausal women living in northern Italy, initially with high serum levels of testosterone**,** who followed an *ad libitum* diet of radically modified composition for 4.5 months. The main results were that serum sex hormone-binding globulin levels were increased and serum testosterone and estradiol levels were decreased. We also found decreased body weight, decreased insulinemic response to oral glucose, decreased fasting glucose, and decreased cholesterol: all of these changes were anticipated by the study hypothesis. Minor changes in the same direction were observed also among women in the control group, who were blind to the dietary strategy of the study but may have slightly changed their diet following publicly available cancer prevention guidelines.

These results suggest that the multifactorial dietary intervention applied in this study may prevent breast cancer if continued in the long term. An intrinsic limitation, however, is that multifactorial intervention precludes estimation of the contributions of individual factors to the overall effect. It is of interest, therefore, to examine our results in relation to published, mostly unifactorial, intervention studies.

The observed weight reduction is consistent with the results of previous randomized controlled studies of low-fat *ad libitum* diet, which showed that weight can be lost merely by reducing the fat content of the diet without restricting food intake (56–58), which would compromise satiety, quality of life, and, in the long run, compliance. A drop in body weight of 3.52 kg (4.06 in the diet group *minus* 0.54 in the control group) corresponds to a cumulative energy deficit of about 26,400 kcal (7.5 kcal per gram of adipose tissue) and, hence, to an average reduction of about 200 kcal per day over 4.5 months, which fits well with the estimated difference in energy intake between the intervention and control groups: 255 (1805 − 1550) kcal per day measured close to the end of the study period when the intervention was being fully implemented (Table 1). This reduced energy intake was achieved through increased consumption of highly satiating bulky food with low-energy density, which implies reducing both total energy and the proportion derived from fat. The effect of the consumption of sugars on appetite and food intake is controversial (59), but we suspect that the reduction of the glycemic load may have contributed to weight reduction.

The observed decrease in the quantity of insulin required to deal with a standard glucose load after overnight fast indicates that we succeeded in improving insulin sensitivity. Several observational studies have shown a direct relationship between total or saturated fat intake on the one hand and indices of insulin resistance and development of glucose intolerance on the other (42, 43, 60, 61), but previous intervention studies that reduced dietary fat content showed only weak or no effect (39). In most of these studies, however, energy intake was held constant to maintain body weight (isocaloric substitution of carbohydrates for fats), and the substituting carbohydrates had relatively high glycemic indices and the intervention periods were short (1–3 weeks). The improvement in insulin sensitivity observed in the present study may therefore be attributable not only to the decrease in total fat and energy intake and subsequent body weight loss (39) but also to the increased proportion

of unsaturated fats (40–43) and lower glycemic index of carbohydrate-rich foods (44–46; Table 1).

The changes in sex hormone-binding globulin and sex hormones could also have been attributable to the combined effects of lowered total energy intake and increased fiber and phytoestrogen consumption. The study design did not allow us to disentangle a possible aspecific effect of weight loss from the effect of specific changes in dietary composition. Weight reduction was part of the intervention strategy, which aimed at reducing body mass index and waist:hip ratio to reduce insulin resistance. However, the observation that hormonal changes lost statistical significance after additional adjustment for weight change does not imply that they are entirely mediated by this intermediate variable. Energy-restriction trials to reduce weight in obese women have consistently shown increased serum sex hormone-binding globulin levels (62–67) and corresponding decreases in free testosterone (64–66) but generally without reductions in total serum estradiol (62, 63) or total testosterone [Refs. 63, 65, 67; although energy restriction may reduce total testosterone in obese women with polycystic ovaries (68)]. By contrast low-fat interventions, mostly in nonobese women (56, 69–71), have shown no increase in plasma sex hormone-binding globulin levels, although in some of these experiments (56, 70), average body weight losses were similar to those in the present study. We speculate that the lack of effect of low-fat diets on sex hormone-binding globulin levels may have been attributable to increased intake of carbohydrate-rich foods with high glycemic indices, so that there would be no improvement in insulin sensitivity; however, the studies cited do not give details of the food consumed or recommended.

A recent review of 13 dietary intervention studies suggested that low-fat diets (10–25% of total calories) could significantly reduce plasma estradiol concentrations. The mean figures cited were −7.4% before menopause (9 studies) and −23.0% after menopause (4 studies; Ref. 72). However, in most of these studies, the intake of fiber-rich foods also increased significantly. We obtained a similar reduction of serum estradiol (18%) with a much lower reduction of fat intake (from about 37 to 31% of total calories) but with a major shift from animal to vegetable fat and from high- to low-glycemic-index carbohydrates.

Intervention studies in which particular types of dietary fiber (73–76) or fiber-rich food (77, 78) were supplemented found no significant increases in plasma sex hormone-binding globulin levels, although plasma estradiol levels were usually [but not always (75)] reduced, an effect that may be attributable to fiber inhibition of steroid reabsorption from the gut (79). A lack of effect of wheat fiber supplementation on plasma sex hormone-binding globulin is consistent with the lack of effect of single-fiber-type supplementation on postprandial and fasting plasma insulin levels (80), in contrast to whole-grain food (81). In the present study, women were requested to rely on the recommended foods and to avoid fiber or other supplements.

In vitro, several phytoestrogens inhibit enzymes involved in the synthesis of endogenous steroid sex hormones (18, 20, 21) and stimulate the liver synthesis of sex hormone-binding globulin (19). *In vivo*, the possibility that phytoestrogen intake can affect the bioavailability of endogenous sex hormones has been examined using various study designs, end points, and dietary or supplemental strategies. Cross-sectional observational studies (82, 83) suggest that the consumption of lignans is associated with reduced total and free sex hormones but do not show a consistent relationship with sex hormone-binding globulin. Before menopause, phytoestrogen supplementation with soy protein isolates, soy milk or flax seeds, usually results in prolongation of the menstrual cycle but has no effect on serum sex hormone-binding globulin (84–87) or testosterone (87, 88); however, estradiol serum levels react more erratically, being reduced after soy milk (84, 86) and a variety of soy food (89) but not with the introduction of soy protein isolates (85, 87, 90) or flax seeds (88). In postmenopausal women, supplementation with soy protein did not increase sex hormone-binding globulin (91–94), but isocaloric substitution of 25% of the dietary calories with a variety of soy foods did (95). This pattern suggests that several phytoestrogen-rich foods may be more effective than soy protein isolate, which is consistent with the results of the present study. However a study that compared the effects of soy powders containing very high levels of isoflavones (2 mg/kg/day) with those containing low levels (0.1 mg/kg/day), showed a modest but significant decrease in serum estradiol (−12%) and a small increase in sex hormone-binding globulin (+4%) in postmenopausal women who consumed high isoflavone powders (92).

The concentrations of phytoestrogens that have a significant metabolic effect on steroid hormone synthesis *in vitro* are higher than those in human blood after intake of phytoestrogen-rich foods. However, significant *in vitro* effects can also be obtained by accumulating various lignans and isoflavonoids, each in concentrations similar to those observed in the plasma of Japanese (whose diet is rich in isoflavonoids) or of Western vegetarians (whose diet is rich in lignans; Ref. 18). The effect of phytoestrogens in our study may have been substantially higher than in previous studies in which the usual diet was supplemented with a single phytoestrogen source. Furthermore, the bioavailability of phytoestrogens may have been higher in our study because of changes in the intestinal microflora. Phytoestrogens are present in food as glycosides, which must be hydrolyzed by the gut microflora to produce absorbable aglycones. Compared with the usual Western microflora, the gut of macrobiotic or vegetarian subjects may be richer in lactobacilli and bifidobacteria, which can hydrolyze numerous plant glycosides present in the human diet, and poorer in clostridia, which degrade diphenolic to monophenolic compounds (96). Dietary supplementation with isolated phytoestrogen rich products, therefore, may be less effective than a comprehensive dietary change, which may also modify bowel function and microflora.

In the present study, the effects of dietary intervention on hormonal levels were clearer than those of previous trials involving a single factor intervention, *e.g.*, reducing total fat intake or supplementing with cereal fibers, soy protein, or flaxseed. We suggest that these favorable changes are to be attributed to the cumulative effects of a comprehensive dietary strategy that combines lowered total fat intake, lowered proportion of saturated fatty acids, and lowered consumption of high-glycemic-index foods with increased intake of dietary fibers from cereals, legumes, and vegetables, and a high cumulative dose of diverse phytoestrogens from various food sources. The very high compliance obtained in this study, however, required about 150 h of teaching and counseling sessions over 4.5 months, which would not be feasible in large-scale public health intervention programs and may not be sustainable in the long run. Additional studies are needed to establish strategies for successful long-term dietary changes in the general population.

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Appendix

Foods recommended for home consumption and used to prepare common meals for the intervention group in the DIANA study

The cumulative consumption over 36 meals/lessons is also given (g per woman).

Food group	Rationale[1]	Actual food items (g per woman)
Soy products	Isoflavonoids, lignans, linolenic acid, fiber, LGIC[a]	Tofu (598), Soy milk (577), Miso (171), soy sauce (Shoyu or Tamari) (110), soy ice cream (98), soybeans or tempeh (75)
Other legumes	Lignans, Coumestrol, fiber, LGIC	Azuki beans, mung beans or black soy beans (143), green-peas (160), other beans (115), lentils (100), alfalfa sprouts (68), chick-peas (56)
Cereal products	Lignans, fiber, LGIC, vitamins, minerals	Whole bread (1884), flour or pasta of unrefined wheat (1039), whole rice (648), seitan (wheat gluten) (346), corn or corn flour (283), whole wheat (115), oat or oatmeal (76), couscous (72), millet (70), buckwheat (70), barley (60), spelt (60), rice flour or pasta (32)
Seeds and nuts	Lignans, fibers, PUFA, vitamins, minerals	Flaxseeds (187), sesame or tahini (141), almonds (103), walnuts (43), hazelnuts (31), sunflower (58), coconut (19), pistachio, pine, pumpkin, or mustard seeds (27)
Unrefined oils	MUFA, PUFA	Olive oil (282), corn, sesame, or sunflower oil (59)
Cruciferae	Indole-3-carbinol, coumestrol, fiber, LGIC	White, red, or savoy cabbage (287), cauliflower (126), brussels sprouts (80), broccoli (65), daikon or radish (258), turnip (135), rocket (94), water-cress (75)
Other vegetables	Phytoestrogens, essential fatty acids, fiber, LGIC, vitamins, minerals	Carrot (1557), onion or leek (1449), green leafy vegetables (912), squash (468), potato (260), pepper (130), parsley (118), string bean (86), dried Shitake or other mushrooms (64), ginger (41), purslane (27), garlic (16), other vegetables (468)
Fruit[b] and edulcorants	Phytoestrogens, vitamins, trace elements, sugar replacement	Apple (661), strawberry (420), bilberry or other berries (273), naturally fermented rice or barley malt (240), apple juice (236), citrus fruit (183), amasake (fermented rice) (100), maple, black-currant, or apple syrup (68), raisin (56), dried apricots (33)
Seaweed	Lignans, minerals, linolenic acid	Kombu (56), wakame (28), hijiki (27), arame (23), ohers (18)
Fish and shellfish	n-3 fatty acids, vitamins, minerals	Trout (168), cod (190), anchovies (135), other sea fish (215), clams (78)
Miscellaneous		Yogurt (40), vinegar (32), arrow root (29), umeboshi (19), wine (13), brewer's yeast (13), egg (8), cocoa (5), unrefined sea salt, cinnamon, oregano, sage, saffron

[a] LGIC, low glycemic index carbohydrates; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b] Fruit was not usually served as such but was recommended for consumption between meals.

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