

A Traditional Mediterranean Diet Decreases Endogenous Estrogens in Healthy Postmenopausal Women

Giuseppe Carruba, Orazia M. Granata, Valeria Pala, Ildegarda Campisi, Biagio Agostara, Rosanna Cusimano, Barbara Ravazzolo, and Adele Traina

Abstract: *Breast cancer incidence and mortality rates are markedly lower in the south than in the north of Europe. This has been ascribed to differences in lifestyle and, notably, dietary habits across European countries. However, little information exists on the influence of different dietary regimens on estrogens and, hence, on breast cancer risk. Here we report results of our MeDiet Project, a randomized, dietary intervention study aimed to assess the effect of a Mediterranean diet on the profiles of endogenous estrogens in healthy postmenopausal women. Out of the 230 women who initially volunteered to participate in the study, 115 were found to be eligible and were enrolled. Women were then randomly assigned into an intervention (n = 58) and a control (n = 57) group. Women in the intervention group adhered to a traditional, restricted Mediterranean diet for 6 mo, whereas women in the control group continued to follow their regular diet. Women in the intervention group changed their dietary regimen substantially, and this eventually led to a shift from a prevalent intake of animal fat and proteins to a prevalent intake of vegetable fat and proteins. Regarding urinary estrogens, no significant difference was observed between the intervention and control groups at baseline. After 6 mo, however, control women did not show any major change but women in the intervention group exhibited a significant decrease (over 40%) of total estrogen levels ($P < 0.02$). The largest part of this modification was based on a marked decrease of specific estrogen metabolites, including hydroxy- and keto- derivatives of estradiol or estrone. To our knowledge, this is the first report to show that a traditional Mediterranean diet significantly reduces endogenous estrogen. This may eventually lead to identify selected dietary components that more effectively decrease estrogens levels and, hence, provide a basis to develop dietary preventive measures for breast cancer.*

Introduction

Breast cancer is by large the most common malignancy in women, accounting for more than 30% of all female cancers and with an estimated 212,920 new cases in 2006 in the United States (1). However, breast cancer incidence and mortality rates vary greatly across European countries, and are markedly lower in the south than in the north of Europe (2). This variation may be ascribed to differences in lifestyle and, notably, dietary habits among different geographical areas. Early studies indicated that certain reproductive and lifestyle variables may be sufficient to explicate ethnic and international differences in breast cancer incidence (3). Migrant studies have shown clearly that breast cancer incidence changes dramatically following migration; in particular, studies comparing breast cancer risk in Asians migrating to United States and in their offspring reveal a major increase in risk between the first, second, and third generation (4).

Evidence from both epidemiologic and biomolecular studies clearly indicate that estrogens are implicated primarily in the development of human breast cancer (5–8). In particular, estrogens have been associated to mammary carcinogenesis as either initiating or promoting agents. Several studies have attempted to correlate plasma levels of circulating estrogens with breast cancer; in particular, it has been proposed that an increase in the biologically active estrogen fraction, including free and protein-bound estrogen, may be important for the development of breast malignancies (9,10). In such research, results obtained have been largely at variance and only small, though statistically significant, differences between normal subjects and breast cancer patients or a modest increase of breast cancer risk have been reported (11,12).

G. Carruba, O. M. Granata, I. Campisi, and B. Ravazzolo are affiliated with the Experimental Oncology and Clinical Oncology, Department of Oncology, ARNAS-Civico, Palermo, Italy. V. Pala is affiliated with the Department of Preventive and Predictive Medicine, Istituto Nazionale Tumori, Milan, Italy. Biagio Agostara, is affiliated with the Clinical Oncology, Department of Oncology, ARNAS-Civico, Palermo, Italy. R. Cusimano is affiliated with the Department of Public Health, Epidemiology and Preventive Medicine, A.S.L. 6, Palermo, Italy. A. Traina is affiliated with the Breast Cancer Registry, ARNAS-Civico, Palermo, Italy.

More recently, some cohort studies have revealed a strong relationship between endogenous estrogen levels and breast cancer risk (13,14). It should be emphasized that intratissue levels of estrogens in human breast have been reported to be several times (10 to 100) greater than the respective plasma values and that either normal or malignant breast tissues appear endowed with key enzyme activities of steroid metabolism (15–18). Therefore, circulating levels of individual estrogens could not be considered representative of the actual amounts of estrogen bioavailable to the tumor cells. In a recent study (19), we have assessed intratissue estrogens and their metabolic profiles in both normal and malignant human breast, also in relation to the severity of the disease. Our data clearly indicate that hydroxyestrogens represent the majority of intratissue estrogens in both normal and malignant breast, and that individual estrogen derivatives may have a distinct role in the development and clinical progression of breast cancer.

Early studies from our own group have revealed that metabolic profiles of urinary estrogens may be useful to better categorize breast cancer patients in relation to their estrogen-sensitive status, response to hormone treatment, and overall prognosis (20). In this respect, metabolic profiles of estrogens in urine samples appear to be comparable to those obtained by measurement of intratissue amounts of estrogens. This indirect evidence could suggest that the analysis of urinary estrogens can be considered as a helpful tool to measure endogenous estrogen levels.

Little information exists on the influence that different dietary components may have on estrogen levels and, hence, on breast cancer risk. In the present work we report results of our MeDiet Project, a randomized dietary intervention study aimed to evaluate the potential impact of a traditional Mediterranean diet on endogenous estrogens in healthy postmenopausal women.

Subjects and Methods

Subjects

Overall, 310 healthy female volunteers were contacted through a press campaign (newspapers and broadcasting advertisements). Out of the 310 women, all from the Palermo urban area, 230 were found to be eligible and were enrolled in the study. Among those, 115 (age range, 48–69 yr) were selected based on serum testosterone levels ≥ 0.14 mg/ml (median value). We selected women on the basis of their serum testosterone according to previous work where it has been shown to be a reliable predictor of breast cancer risk (14). Eligibility criteria also included the following: 1) 2 or more yr of postmenopause; 2) no history of bilateral ovariectomy; 3) no history of cancer; 4) no hormone replacement therapy regimen in the last 12 mo; 5) no adherence to a vegetarian or macrobiotic or any medically prescribed diet; and 6) no treatment for diabetes, thyroid disease, or bowel chronic disease. Women were then randomly assigned individually into a dietary intervention ($n = 58$) and a control ($n =$

Table 1. Dietary Intervention: Mediterranean Food and Biologically Relevant Agents

Food	Agent
Whole-grain cereals: wheat, barley	Lignans
Pumpkin, sesame, and sunflower seeds	
Garlic, onion, fennel, carrot	
Beans, chick peas, lupins, broad beans, peas, lentils	Isoflavonoids
Cauliflower, broccoli	Indol-3-carbinol
Tomato	Lycopene
Extra virgin olive oil	Oleic acid
	Oleuropein
Red grapes, figs, pomegranate, berries	Ellagic acid
Orange juice, red pepper chili	Ascorbic acid
Red wine	Resveratrol
Almonds, nuts, pistachios, olives	Tocopherols
Blue fish (sardines, mackerel, etc.)	Eicosanoids
Tuna fish, swordfish, anchovy	Ergosterol

57) group. Women in the intervention group adhered to a traditional, restricted Mediterranean diet for 6 mo, whereas women in the control group continued to follow their regular diet. In particular, intervention women attended a weekly “cooking course,” followed by a social dinner, and were trained by professional chefs in the correct use of natural ingredients of a traditional Mediterranean diet. Women were then instructed to consume the same food at home using a series of recipes based on a traditional Sicilian diet. This diet included whole cereals, legumes, seeds, fish, vegetables, and other Mediterranean seasonal food containing several biologically relevant anticancer agents (Table 1). Furthermore, women were asked to avoid the use of refined carbohydrates and additional animal fat, and to limit the use of salt.

The dietary intervention was intended to 1) reduce the intake of both refined sugar and saturated and total fat; 2) increase the consumption of mono- and polyunsaturated fat; 3) increase the intake of fruits and vegetables (notably cruciferous plants); and 4) increase the intake of food rich in phytoestrogens.

Before (baseline) and after the dietary intervention, women in both control and intervention groups compiled an adapted version of a validated food frequency questionnaire [developed for the European Prospective Investigation into Cancer and Nutrition (EPIC) study (21)]; fasting blood samples and 12-hour urine samples were collected to respectively assess hormone serum levels and profiles of urinary estrogens using a reversed-phase high-performance liquid chromatography (RP-HPLC) system with photodiode array detection (see **Methods**). Anthropometric measures, including height, weight, and waist and hip circumferences, were determined at the beginning and at the end of the study.

The design of the study is illustrated in Fig. 1.

Urine Collection and Estrogen Extraction

The 12-h (8 PM to 8 AM) urine samples were collected in plastic containers and supplemented with 1 g of ascorbic acid to protect estrogen compounds from oxidation. Urine vol-

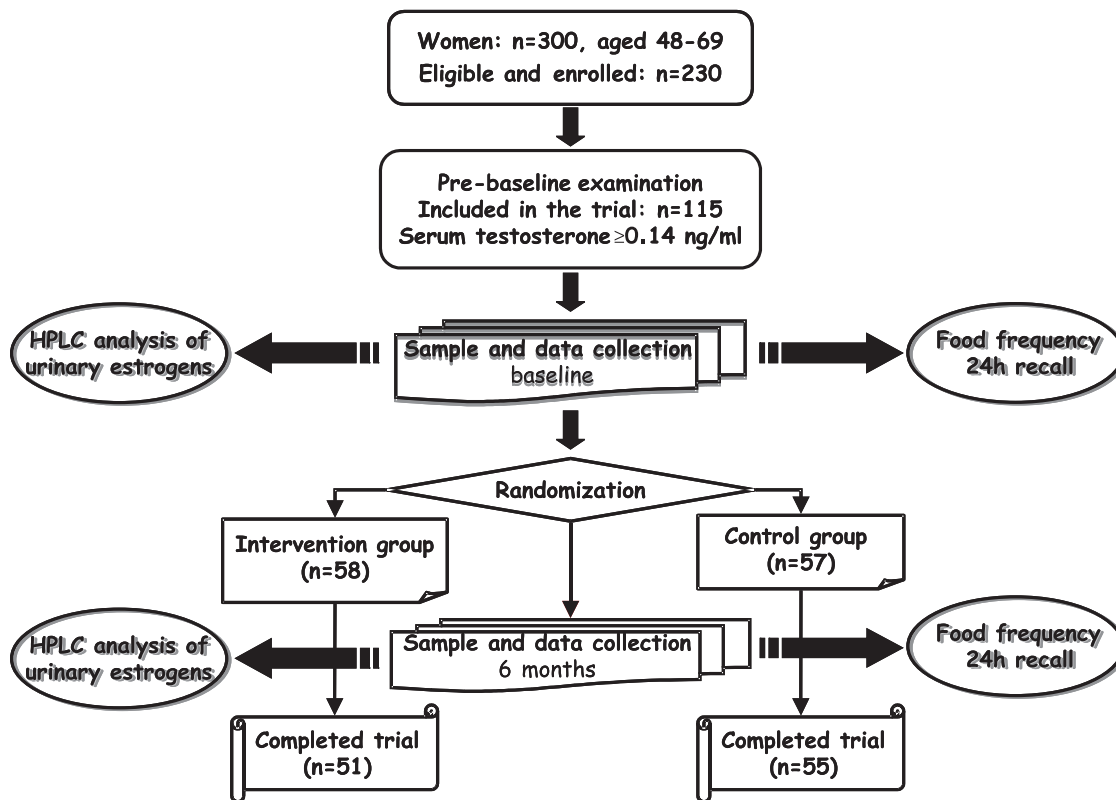


Figure 1. Flow diagram illustrating the design of MeDiet study. Abbreviation is as follows: HPLC, high-performance liquid chromatography.

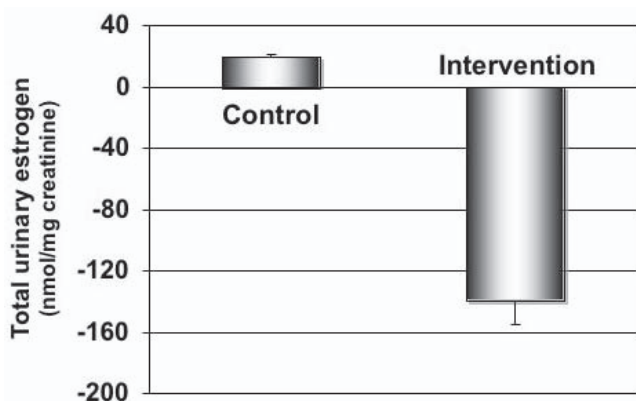


Figure 2. Changes of total urinary estrogens in control and intervention women after 6 mo observation. Values are expressed as mean \pm SEM.

umes were then measured and sodium azide was added at a 0.1% (wt/vol) concentration to prevent microbial contamination. Twenty-five-milliliter aliquots were collected and stored at -20°C until estrogen extraction. In this study, 10-ml urine aliquots were used after addition of 10 mg ascorbic acid and 10 μg equilin; the latter was used as an internal standard for the ensuing chromatographic analysis. Urine samples were centrifuged at 3,000 rpm for 5 min at 6°C . After centrifugation, both free and conjugated estrogens were desorbed from C18 cartridge (Sep-Pak; Waters, Milford, MA) using 4 ml methanol, according to a modification of the original solid-phase extraction method for estrogen extraction (22).

Samples were evaporated to dryness in a rotating evaporator, redissolved in 1 ml of acetate buffer (0.2 M, pH 5.0) containing 50 μl of β -glucuronidase/arylsulfatase (Glusulase; NEN, Wilmington, DE), and incubated overnight at 37°C for enzymatic hydrolysis of conjugated estrogens. Hydrolyzed estrogens were then processed as described above and eluted from a C18 cartridge using 4 ml of ethyl acetate. The estrogen fraction was washed with 1 ml of TRIS buffer (0.1 M, pH 8.6) and the organic layer evaporated to dryness, as before; the sample finally was frozen at -20°C until analysis. Recovery of free estrogens, assessed at three different concentrations from spiked urine samples, ranged from 92.1% to 103.5%. Creatinine levels were measured in each urine sample to adjust estrogen content.

Chromatographic Analysis

Urinary estrogens were analyzed using a HPLC system (Shimadzu, Tokyo, Japan), equipped with two pumps (LC-10ADvp model), an ultraviolet/visible photodiode array detector (SPD-M10Avp model), and an on-line electrochemical detector (5100A Coulochem model; ESA, Bedford, MA). The software package from the Shimadzu CLASS-VP2 chromatography data system was used to analyze the data acquired from both detectors. The separation of 15 estrogen derivatives was obtained using an RP column Spherisorb ODS2 5 μm (4.6×250 mm inner diameter; Waters) and acetonitrile–0.05 M citric acid, 35:65 (vol/vol) as mobile phase at a flow rate of 1 ml/min. Tests to validate this

photodiode array detector–based analytical method were performed in spiked urine samples to define both the response linearity (using detection limits of 40–300 ng in the column) and the reproducibility of area integration values, with coefficients ranging from 0.3% to 5.1% and 0.3% to 19.4% for intra-assay and interassay variation, respectively. The trueness of estrogen metabolite identity was supported, in addition to the relative retention time, by the spectral analysis and the high specificity and sensitivity for estrogens of electrochemical detection (23,24). To correct for variability in urinary dilution, the results were expressed per milligram of urinary creatinine.

Statistical Analysis

The 115 women selected initially for this study were assigned individually to an intervention ($n = 58$) or a control ($n = 57$) group using a blocked randomization method and were stratified for prebaseline parameters (including weight and serum testosterone) to achieve a balanced allocation of subjects in the two groups. Statistical analysis was conducted on the changes observed in urinary estrogen levels in both control and intervention groups at baseline and after 6 mo. The changes in concentrations of individual estrogens were also measured as the difference between values after 6 mo and at the beginning of the study comparing women of the two groups. The one-way analysis of variance (ANOVA) method was used to test the hypothesis that means are equal, as repeated measures within the group, and to compare means of the two groups, as for independent samples. Levels of urinary estrogens were expressed as mean \pm SEM of nanomoles per milligram creatinine. Because of the nonnormal and highly

skewed distribution of values, all of the values were transformed logarithmically to meet the statistical assumption of this parametric test. Multivariate ANOVA was also used to assess the impact of potentially confounding variables (including glycemia, cholesterol, testosterone, insulin, weight, and body mass index). Statistical analysis was performed using the SPSS software (SPSS, Inc., Chicago, IL). For all measurement, results were considered statistically significant at $P < 0.05$. The compliance with dietary recommendations, as well as changes in dietary habits in the two groups, were monitored using a food frequency questionnaire adapted from that originally developed for the EPIC study (21). A computer program, the Nutrition Analysis of Food Frequency Questionnaire (NAF), automatically converts questionnaire data into frequencies and average daily intake of foods, energy, and nutrients. The dietary intakes were expressed as means (\pm SD) of grams/day or as percent calories. All the analyses were performed using the STATA statistical package version 7.0 (STATA Corp., College Station, TX).

Results

After 6 mo, out of the 115 women initially enrolled, 106 women completed the study (51 in the intervention group and 55 in the control group).

Dietary Evidence

Intervention women changed their dietary regimen substantially, and this eventually led to a shift from a prevalent intake of animal fat and proteins to a prevalent intake of vegetable fat and proteins. As shown in Table 2, intervention

Table 2. Average Nutrient and Energy Intake in Intervention and Control Women^{a,b}

Nutrient	Intervention Group		Control Group	
	Baseline	6 Mo	Baseline	6 Mo
Protein, total	79.8 (29.5)	62.3 (19.9)*	73.3 (20.2)	67.1 (21.1)†
Animal	51.7 (23.4)	36.4 (13.5)*	47.0 (14.9)	43.9 (18.4)
Vegetable	28.1 (10.2)	25.9 (12.7)	26.3 (10.2)	23.2 (7.8)†
Lipid, total	82.2 (31.7)	67.9 (25.1)*	70.0 (21.0)	68.6 (23.9)
Animal	42.6 (20.8)	24.5 (10.6)*	35.2 (15.1)	33.6 (17.5)
Vegetable	39.6 (17.1)	43.4 (19.8)	34.8 (12.1)	34.9 (12.6)
Saturated	27.7 (11.9)	18.4 (6.7)*	23.4 (8.9)	22.6 (10.1)
Monounsaturated	40.7 (16.0)	37.2 (15.1)	34.1 (10.2)	34.3 (11.5)
Oleic	38.3 (15.2)	35.6 (17.7)	32.0 (9.7)	32.5 (10.9)
Polyunsaturated	9.1 (3.6)	8.6 (3.4)	8.2 (2.4)	7.7 (2.5)
Linoleic	7.1 (2.9)	6.6 (2.8)	6.4 (1.9)	6.0 (2.1)
α Linolenic	1.2 (0.4)	1.0 (0.4)	1.0 (0.3)	1.0 (0.3)
Other	0.9 (0.5)	0.9 (0.5)	0.8 (0.4)	0.6 (0.3)
Cholesterol	356.2 (159.7)	249.7 (125.8)*	283.2 (108.8)	282.5 (145.9)
Carbohydrates	230.5 (82.2)	192.3 (84.8)†	224.2 (87.1)	197.7 (69.8)†
Starch	136.3 (60.4)	113.8 (71.4)	120.5 (60.8)	106.8 (45.3)
Sugar	94.0 (37.5)	78.2 (28.7)‡	103.4 (46.4)	90.7 (38.0)
Dietary fiber	23.0 (8.0)	22.2 (9.1)	21.9 (7.9)	20.0 (6.7)
Alcohol	7.8 (10.4)	13.4 (26.5)	7.5 (14.5)	3.8 (5.2)
Energy (kcal)	1,978.0 (636.9)	1,676.4 (639.3)‡	1,818.2 (575.9)	1,654.3 (498.1)†

a: Values represent mean (\pm SD) of dietary intake in grams per day, as estimated from food frequency questionnaires at baseline and after 6 months.

b: Boldface indicates significant differences between control and intervention women. Statistical significance is as follows: * $P < 0.001$; † $P < 0.05$; ‡ $P < 0.01$.

women in fact reduced significantly the intake of both animal protein (by 30%) and fat (by >42%), notably saturated fat (by 34%). In addition, women in the intervention group showed a marked decrease of carbohydrate (especially sugar) intake, with a reduction in the overall energy intake of nearly 26%. Conversely, women in the control group did not show any significant change in their dietary nutrients, except a moderate decrease in both vegetable proteins and carbohydrate intake, with an overall reduction of caloric intake lower than 10%. Given that there were no major changes in body weight (−1.3 kg in the intervention group and −0.6 kg in the control group), both groups underestimated total caloric intake at 6 mo. A similar pattern of change, however, was obtained after correction for the energy intake (expressing nutrient intake as percent calories).

Hormone Evidence

Regarding urinary estrogens, it should be emphasized that, at baseline, a large proportion of the metabolites that could be detected was represented by hydroxy- and methoxy-derivatives of estradiol (E2) and estriol (E3), rather than by classical estrogens (E2, E3, and estrone), in both control (75%) and intervention (84%) groups. This evidence is in accordance with results of our early studies (11), indicating that in breast cancer patients, the majority of urinary estrogens were represented by hydroxy-derivatives of classical estrogens, referred to as “minor” or “unusual” metabolites. Although these compounds have been considered in the past as biologically unimportant products of estrogen metabolism, there is now clear-cut, converging evidence from our own and others’ studies that some estrogen metabolites may have a critical role in both mammary carcinogenesis and progression of human breast cancer (25–27). We have recently reported that classical estrogens (E1, E2, and E3) comprise

only a minor proportion of all estrogens in both nontumoral (5.2%) and malignant (4.3%) human breast tissues, whereas the majority of intratissue estrogens is represented by hydroxy- and/or methoxy-derivatives of either estradiol or estrone (19). In this respect, profiles of urinary estrogens appear to be comparable to patterns of intratissue estrogens and, hence, could be used potentially as indicators of tissue estrogen composition (28).

At baseline, no significant difference was observed in urinary levels of individual estrogens between intervention and control women, although total estrogen levels were greater in women from the intervention group (Table 3). This finding may also be a reflection of a relatively (but not significantly) higher proportion of overweight and obese women in the intervention group (60%) with respect to control group (53%). After 6 mo, control women did not show any major change, but women in the intervention group exhibited a significant decrease (more than 40%; $P < 0.02$) of total estrogen levels (Fig. 2). It is worth noting that most of this modification was based on a marked decrease of specific estrogen metabolites, including hydroxy- and keto-derivatives of E2 or E3. In particular, urinary levels of 2-hydroxy-E2 (2OHE2) and 17Epi-estriol (17EpiE3) decreased by 80% and 70%, respectively, whereas the concentration of 16keto-estradiol (16KetoE2) decreased by 27%. Overall, these three estrogen derivatives account for 57% and 68% of all urinary estrogens at baseline, respectively, in the control and intervention group. After 6 mo, this proportion decreased dramatically in the intervention women, whereas it was not changed significantly in control women. Conversely, E2 only, which represented a mere 0.2% of total urinary estrogens, showed a statistically significant increase in the intervention group; an even greater increase (not significant) was observed in the control group. Although this finding may appear in contradiction with other results, it may simply be a reflection of the

Table 3. Urinary Estrogens in Control and Intervention Women at Baseline and After 6 Months Dietary Intervention^a

Estrogen Type	Intervention Group			Control Group			Intervention vs Control
	Baseline	6 Mo	<i>P</i>	Baseline	6 Mo	<i>P</i>	<i>P</i>
E2	0.70 (0.23)	2.24 (0.90)	0.042	0.22 (0.05)	3.70 (1.70)	0.108	0.447
2OHE2	82.18 (26.49)	16.43 (5.27)	0.004	27.79 (9.81)	14.61 (6.79)	0.275	0.190
4OHE2	8.16 (2.12)	7.99 (1.57)	0.438	11.46 (2.17)	11.59 (2.58)	0.171	0.156
2MOE2	0.17 (0.05)	0.44 (0.28)	0.666	0.13 (0.03)	0.48 (0.20)	0.110	0.378
4MOE2	7.87 (7.46)	6.79 (5.82)	0.806	0.39 (0.08)	4.66 (3.99)	0.416	0.634
E1	0.99 (0.42)	0.23 (0.06)	0.261	0.44 (0.15)	0.55 (0.23)	0.764	0.293
2OHE1	2.71 (0.94)	3.05 (0.92)	0.855	1.19 (0.18)	1.32 (0.23)	0.653	0.638
4OHE1	3.91 (1.11)	8.46 (5.84)	0.886	2.38 (0.70)	4.06 (1.64)	0.626	0.824
2MOE1	0.34 (0.12)	0.13 (0.01)	0.146	0.25 (0.07)	0.34 (0.10)	0.634	0.181
4MOE1	0.85 (0.24)	0.71 (0.13)	0.834	0.51 (0.12)	0.55 (0.14)	0.643	0.899
16EpiE3	25.82 (8.37)	11.94 (3.23)	0.535	15.28 (3.52)	13.83 (3.86)	0.799	0.740
16KetoE2	81.57 (9.76)	59.33 (7.47)	0.046	60.82 (5.78)	65.88 (9.55)	0.763	0.201
16α OHE1	3.28 (0.74)	4.88 (1.62)	0.793	2.33 (0.52)	5.57 (2.66)	0.496	0.486
17EpiE3	60.17 (17.17)	18.95 (3.63)	0.021	25.92 (5.65)	28.35 (7.03)	0.866	0.196
E3	51.40 (7.46)	50.00 (9.28)	0.514	48.90 (8.38)	63.56 (11.84)	0.092	0.127
Total estrogen	330.13 (50.63)	191.56 (16.98)	0.011	198.92 (18.12)	218.15 (21.31)	0.358	0.009

^a: Values are expressed as mean (± SEM) of nanomoles per milligram of creatinine. Boldface indicates significant differences between control and intervention women. Abbreviations are as follows: E2, estradiol; OH, hydroxy; E1, estrone; MO, methoxy; E3, estriol.

markedly lower rates of E2 biotransformation into its hydroxylated derivatives (notably 2OHE2 and 16KetoE2) seen in the intervention group. In the latter group, the sum of 2OHE2 and 16KetoE2 decreased by half after 6 mo (54% reduction), whereas it remained nearly the same (91%) in the control group.

However, as reported in Table 3, when comparing the modifications seen in intervention and control groups after 6 mo, only total urinary estrogens remained markedly reduced, whereas changes observed in 2OHE2, 17EpiE3, or 16KetoE2, as for other detectable estrogen derivatives, were not significantly different between the two groups. This apparently conflicting evidence may be a result of the large, unpredictable variability of data and, hence, of the insufficient number of subjects in the two groups to identify statistically significant changes in levels of individual urinary estrogens.

Conclusions

Both nutritionists and epidemiologists have recognized that Mediterranean diet may have a protective role against cancer (29). In this work, women in the intervention group adhered to a traditional, restricted Mediterranean diet for 6 mo.

This traditional diet is comparable to that common in Southern Italy in the early 1960s; on this basis, it can be defined as a diet high in cereal (more than 60% of total energy intake), low in total fat (less than 30%), with moderate amounts of added fat (predominantly olive oil, which represents more than 70% of the total lipids), a high mono-saturated/saturated ratio (>2) and a moderate polyunsaturated/saturated ratio (about 0.4–0.5) (30). Unfortunately, during the last 30 yr, there has been a mounting “north-ernization” of dietary habits in populations of Southern Italy, especially in metropolitan area residents. This has eventually led to a marked increase in the consumption of milk and dairy products, meat, and animal fat, and to a corresponding decrease in the intake of cereals and unrefined carbohydrates. This significant change has been proposed as potentially responsible for an increased risk of developing coronary heart disease or cancer, regardless of other factors (31,32).

In the present work we report that a traditional Mediterranean diet markedly reduces endogenous estrogen levels in healthy postmenopausal women. In this study, the total of urinary estrogens (including classical estrogens and, especially, their hydroxy-derivatives) was significantly reduced (by more than 40%) in the intervention group ($n = 58$), in which women were randomly assigned to adhere to a restricted traditional Mediterranean diet for 6 mo. It is noteworthy that the majority of urinary estrogens measured at baseline in both control and intervention women was represented by hydroxy- and methoxy-derivatives of either E2 or E3. In addition, the significant decrease in total urinary estrogens we observe in the intervention group after 6 mo is largely based upon the reduction of selected estrogen metabolites, notably 2OHE2, 17EpiE3, and 16KetoE2, whereas

levels of classical estrogens, including E2 and E1 (a mere 0.5% of total as a sum), were not changed or even increased. Once again, this evidence emphasizes the potential implication of 2-, 4- and 16 α /16 β -hydroxylated estrogens in development of human breast cancer, as suggested by our previous study on metabolic profiles of intratissue estrogens in normal and malignant human breast.

To our knowledge, this is the first report to show that a traditional Mediterranean diet significantly reduces estrogen levels in healthy women. This may eventually lead to the identification of selected dietary components that more effectively decrease endogenous estrogens and, hence, provide a basis for the development of dietary preventive measures for breast cancer.

Acknowledgments and Notes

We thank Dr. Franco Berrino for his continuous support and assistance in reviewing data. Address correspondence to G. Carruba, Experimental Oncology, Department of Oncology, ARNAS-Civico, Piazzale N. Leotta 2 – 90127 Palermo, Italy. Phone: +39 091 66–64348. FAX: +39 091 66–64352. E-mail: lucashbl@unipa.it.

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