Dietary Enterolactone Affects Androgen and Estrogen Levels in Healthy Postmenopausal Women

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In this randomized dietary intervention study (DI) we analyzed levels of androgens, phytoestrogens, and estrogens in 12-h urine samples of 69 healthy postmenopausal women, 37 of whom followed a traditional Mediterranean diet for 6 months (intervention group) as compared to 32 women who followed their regular diet (control group). Circulating levels of both insulin and testosterone (T) were also assayed. Overall, enterolactone (ENL) was the most prominent phytoestrogen in urines of both control and intervention women, and its levels increased by a 20% after DI. At the baseline the ENL levels were seen to be significantly associated with both the total androgens (TOT-A) ($r = 0.371$, $P = 0.002$) and the TOT-A/total estrogen (TOT-E) ratio ($r = 0.351$, $P = 0.005$) in all 69 postmenopausal women. Furthermore, the DI resulted in a more pronounced negative association of both ENL with insulin ($r = -0.321$, $P = 0.05$) and insulin with TOT-A ($r = -0.421$, $P = 0.01$). Regarding urinary androgens, ENL associated with both 3α-androsterone (5α-androgen, $r = 0.363$, $P = 0.002$) and 3α-etiocolanolone (5β-androgen, $r = 0.295$, $P = 0.01$) at baseline, while after DI, circulating insulin and T exhibited a significant negative association with the 5β-androgen metabolite etiocolanolone ($r = -0.487$, $P = 0.002$; and $r = -0.336$, $P = 0.042$, respectively). We conclude that lignan components of the Mediterranean diet, notably ENL, are associated with urinary levels of products of androgen metabolism, including both 5α- and 5β-reductase enzymes, in healthy postmenopausal women. Further studies are necessary to better understand the interplay of sex hormones with dietary phytoestrogens.

Key words: **Mediterranean diet; enterolactone; insulin; testosterone; androsterone; etiocolanolone; dietary intervention**

Introduction

Soy-consuming Asian populations typically have high intake of isoflavones, whereas lignans are more important sources of phytoestrogens in the diets of other populations.[1]

The mammalian lignan enterolactone (ENL) is produced by intestinal bacterial flora from the glycoside form of metairesinol, contained mainly in flax, sesame, pumpkin, sunflower, and other seeds, as well as in dietary components having low glycemic index, such as whole grains and their derivatives, as well as in apricots, broccoli, and garlic.[2,3]

ENL has been associated with a substantial reduction of breast cancer risk, particularly of ER-negative breast tumors in both pre- and postmenopausal women in eastern Finland.[4–6] Furthermore, an Italian retrospective study on the relationship between serum ENL and the occurrence of breast cancer in

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women with palpable mammary cysts confirmed that a higher ENL concentration means a lower risk of breast malignancies.[7]

These outcomes may be due to the inhibition of aromatase induced by ENL,[8] activity that eventually leads to reduced local synthesis of bioactive estrogens.

In a previous randomized dietary intervention (DI) study (MeDiet), we demonstrated for the first time that the adoption of a traditional Mediterranean diet—implying a significant reduction of animal proteins and fat, of refined sugar, and a substantial increase in the intake of legumes, garlic, onion, nuts, fruits, and olives—induces a significant reduction of endogenous estrogens and, hence, is likely to decrease breast cancer risk in healthy postmenopausal women.[9]

Using both urine and blood samples collected in the same MeDiet study, in the present work we evaluated the potential correlation of ENL, a mammalian lignan produced from common Mediterranean dietary components, with urinary androgens; we evaluated sex hormones, also in relation to circulating insulin and testosterone (T), as indicators of breast cancer risk.

Subjects and Dietary Intervention

In this study we included healthy postmenopausal women who participated in the MeDiet project, a randomized, dietary intervention study. In this project, dietary intervention aimed to: (1) reduce the intake of refined sugar and saturated and total fat; (2) increase the consumption of mono- and polyunsaturated fat; (3) increase the intake of food rich in phytoestrogens; (4) increase the intake of fruits and vegetable (notably cruciferous plants). This was achieved through a controlled diet that included whole cereals, legumes, seeds, fish, vegetables, and other Mediterranean seasonal foods. Eligibility criteria, study design, and changes in the average intake of nutrients and energy with the dietary intervention were extensively reported in our previous work.[9] Overall, 69 women were included, 37 in the dietary intervention group and 32 in the control group.

Methods

Fasting blood samples and 12 h-urine supplemented with 1 g of ascorbic acid were collected at the baseline and after 6 months and aliquots stored at −80°C and −20°C, respectively. Double extraction was performed on the 10-ml aliquots of each urine sample after addition of 10 mg of ascorbic acid and 10 μg of equilin as internal standard to profile estrogen patterns by high performance liquid chromatography (HPLC) with ultraviolet photodiode array (UV-PDA) detector, and androgen and phytoestrogen patterns by gas chromatography-mass spectrometry (GC-MS). After centrifugation at 3000 rpm for 5 min at 6°C, free and conjugate hormones were desorbed from C18 cartridge (Sep-Pak; Waters, Milford, Massachusetts) using 4 ml methanol. Extracts were evaporated to dryness and hydrolyzed in 1 ml of acetate buffer (0.2 M, pH 5.0) containing 50 μl of β-glucuronidase/arylsulfatase (Glusulase; NEN) for 18 h at 37°C. The hydrolyzed samples were then processed as described above and eluted from C18 cartridges using 3 ml of ethyl acetate plus 1 ml of hexane. After washing with 1 ml of Tris buffer (0.1 M, pH 8.6), the organic layer was evaporated to dryness, as before, and sample extracts were frozen at −20°C until chromatographic analysis. Recovery assessed at three different concentrations from spiked urine samples ranged from 92.1% to 103.5% for estrogens, from 90.0% to 99.7% for androgens, and from 98.9% to 110.0% for phytoestrogens.

HPLC–UVvis Photodiode Array

Urinary estrogens were analyzed as reported previously[9] using an LC-10A HPLC system with an SPD-M10A ultraviolet/visible (UVvis) photodiode array detector equipped with a software CLASS-VP2 (Shimadzu, Tokyo, Japan).

The separation of 15 estrogen derivatives was obtained using a column Spherisorb ODS 5 μm (250 mm × 4.6 mm i.d.; Waters, Milford, Massachusetts) and acetonitrile-0.05 M citric acid, 35:65 (v/v) at isocratic conditions with a flow rate of 1 ml/min.

GC-MS

A Trace CG 8000 series gas chromatographer interfaced with a VG TRIO-2000 mass spectrophotometer (Fison Instruments, now known as Thermo Finningam, San Jose, California) was used for GC-MS analysis of urinary androgens and phytoestrogens. Data acquisition and processing were carried out using MassLynx NT 3.2 software (Thermo Finningam). An Equity-55%diphenyl-95%dimethylpolysiloxane column (30 m × 0.25 mm i.d., 0.25-μm film thickness; Supelco, Bellefonte, Pennsylvania) was used for all analyses. Operating conditions were as follows: injector port temperature 250°C; injection volume 1 μl in splitless mode; carrier gas He at constant pressure of 35 KPa; oven program temperature 200°C increased after 5 min, at 5°C/min to 240°C, and after 1 min, increased at 2°C/min to 290°C; GC-MS interface temperature 280°C; source temperature 200°C; ionization by electron ionization 150 μA. Analysis was performed in the selected ion monitoring (SIM).

Circulating serum levels of the insulin and T were assayed using IRMA (Immunotech) and the Spectria testosterone (ORION) methods, respectively.

Statistical analyses were performed using parametric Student's *t*-test and Pearson correlation test on logarithmically transformed values.

Results and Discussion

The use of a traditional Mediterranean diet, featured by a reinforced daily consumption of whole grains and their derivatives (such as traditional whole wheat bread with sesame seeds), along with a higher intake of garlic, cruciferous vegetables, olive oil, nuts, and certain fruits, provided a consistent consumption of lignans in our dietary intervention (DI) group.[2]

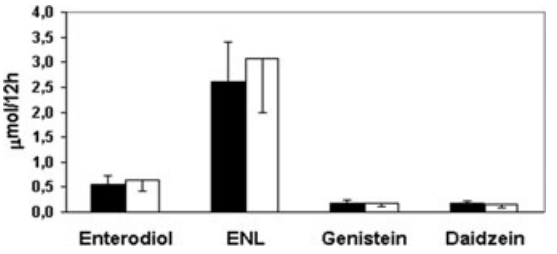

Figure 1. Urinary lignan and isoflavone levels (±SEM) in healthy postmenopausal women before (■) and after (□) DI.

The ENL was in fact the most prominent urinary phytoestrogen, as it occurs in western populations.[1]

DI increased, though not significantly, ENL levels by 20% (2.62 ± 0.81 vs. 3.07 ± 1.71 μmol/12 h), similar to what observed for the other lignan enterodiol (0.55 ± 0.17 vs. 0.65 ± 0.41 μmol/12 h), while the urine levels of the two isoflavones genistein (0.18 ± 0.06 vs. 0.18 ± 0.09 μmol/12 h) and daidzein (0.17 ± 0.07 vs. 0.14 ± 0.05 μmol/12 h) remained 15 times lower than those of ENL (Fig. 1).

As previously reported,[9] DI was very effective in lowering the urinary levels of total estrogens (TOT-E) (from 0.136 ± 0.037 to 0.098 ± 0.024 μmol/12 h, $P = 0.03$), and even though the total androgens (TOT-A) were only slightly increased, TOT-A to TOT-E ratio values rose by over 40% (from 69.683 ± 21.020 to 99.868 ± 45.920) (Fig. 2).

As reported in Figure 3, we observed a strong positive association between circulating levels of insulin and T (Pearson $r = 0.301$ and $P = 0.01$) at the baseline. This association, that has been postulated by several authors to represent the basis of an increased risk of breast cancer[10–12] was abrogated by the DI in favor of a negative correlation between circulating insulin and the urinary TOT-A levels (Pearson $r = -0.421$ and $P = 0.01$).

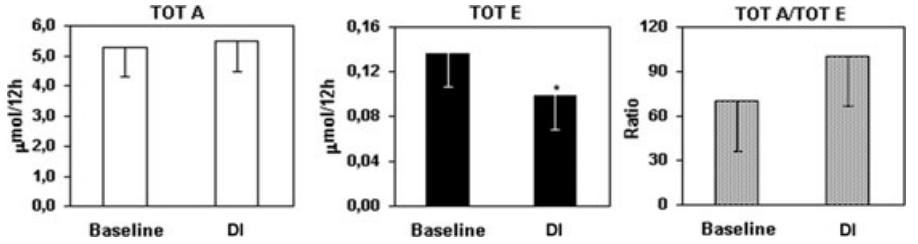

Figure 2. Changes in urinary androgen and estrogen levels and their ratio (±SEM) in healthy postmenopausal women before and after DI. (*) Student's *t*-test: $P = 0.036$.

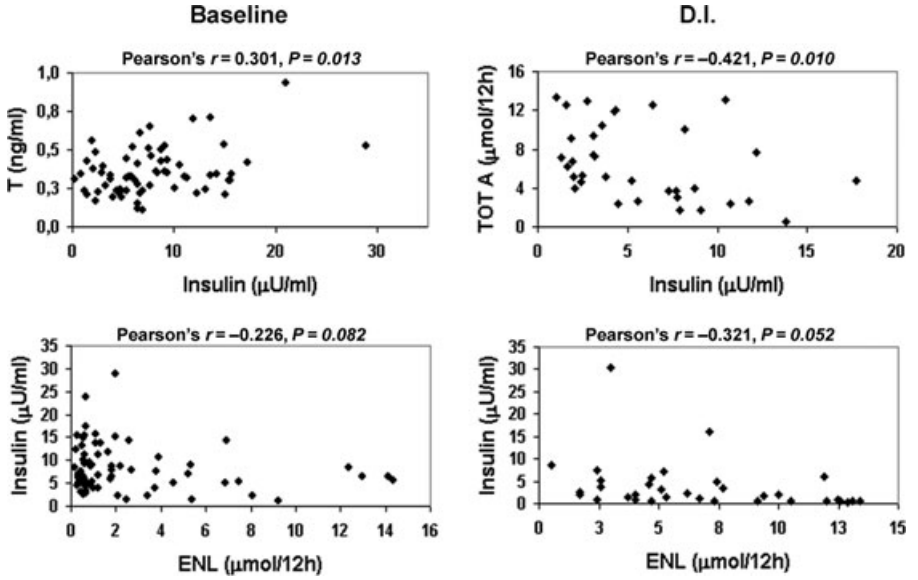

Figure 3. Correlation of circulating insulin and urinary androgens and ENL in healthy postmenopausal women at baseline and after DI.

As expected (Fig. 3), the higher the levels of the ENL, associated with the intake of low glycemic index foods,[2,3] the lower the levels of serum insulin. This negative correlation was reinforced after DI (Pearson $r = -0.321$ and $P = 0.05$).

At the baseline the ENL is seemingly associated with both the urinary levels of TOT-A ($r = 0.371$, $P = 0.002$) and the TOT-A to TOT-E ratio values ($r = 0.351$, $P = 0.005$) (data not shown).

Furthermore, as shown in (Fig. 4), ENL levels were positively correlated with both 3α-androsterone ($r = 0.363$, $P = 0.002$), the 3α-hydroxylated product of the 5α-reductase, and 3α-etiocolanolone ($r = 0.295$, $P = 0.01$), the major androgen derivative of the 5β-reductase pathway, at baseline.

After DI, however, both insulin and T became significantly and inversely related to 3α-etiocolanolone (insulin: $r = -0.487$, $P = 0.002$; T: $r = -0.336$, $P = 0.042$).

The 5β-reductase enzyme became more actively involved in the production of 3α-etiocolanolone as a consequence of DI, presumably because it reduces its activity in lipid and cortisol metabolism.[13,14]

In conclusion, urinary levels of androgen metabolites, including products of both 5α- and 5β-reductase activities, are associated with

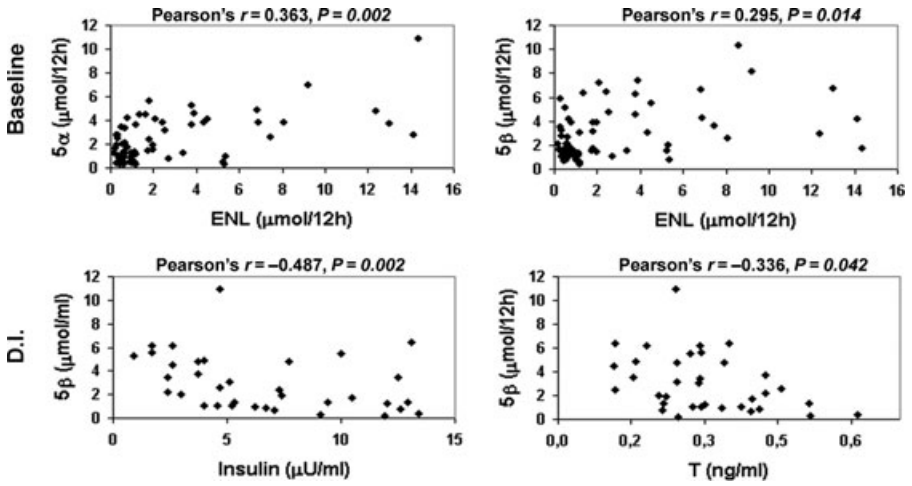

Figure 4. Correlation of 5α- or 5β-androgens and both urinary ENL and circulating insulin and T in healthy postmenopausal women at baseline and after DI.

lignan components of a Mediterranean diet, notably ENL. Further studies are necessary to better understand the complex interaction of sex hormones with dietary phytoestrogens.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Adlercreutz, H. 1998. Epidemiology of phytoestrogens. In *Phytoestrogens*, H. Adlercreutz, Ed.: 605–624. Bailliere Tindall. London, UK.
2. Milder, I.E.J., I.C.W. Arts, *et al*. 2005. Lignan contents of Dutch plant foods: A database including lariciresinol, pinoresinol, secoisolariciresinol and metairesinol. *Br. J. Nutr.* **93:** 393–402.
3. Foster-Powell, K., S.H.A. Holt & J.C. Brand-Miller. 2002. International table of glycemic index and glycemic load values: 2002. *Am. J. Clin. Nutr.* **76:** 5–56.
4. Pietinen, P., K. Stumpf, *et al*. 2001. Serum enterolactone and risk of breast cancer: A case-control study in eastern Finland. *Cancer Epidemiol. Biomark. Prev.* **10:** 339–344.
5. Piller, R., E. Verla-Tebit, *et al*. 2006. Cyp17 genotype modifies the association between lignan supply and premenopausal breast cancer risk in human. *J. Nutr.* **136:** 1596–1603.
6. Overvad, K., S. Moller, & A. Tjonneland. 2004. Plasma enterolactone and breast cancer incidence by estrogen receptor status. *Cancer Epidemiol. Biomark. Prev.* **10:** 339–344.
7. Boccardo, F., G. Lunari, *et al*. 2004. Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. *Eur. J. Cancer* **40:** 84–89.
8. Brooks, J.D. & L.U. Thompson. 2005. Mammalian lignans and genistein decrease the activities of aromatase and 17beta-hydroxysteroid dehydrogenase n MCF-7 cells. *J. Steroid Biochem. Mol. Biol.* **94:** 461–467.
9. Carruba, G., O.M. Granata, *et al*. 2006. A traditional Mediterranean diet decreases endogenous estrogens in healthy postmenopausal women. *Nutr. Cancer* **56:** 253–259.
10. Nestler, J.E. & D.J. Jakubowicz. 1996. Decreases in ovarian cytochrome p450c17αactivity and serum free testosterone after reduction of insulin secretion in polycystic ovary syndrome. *N. Engl. J. Med.* **335:** 617–623.
11. Kaaks, R., C. Bellati, *et al*. 2003. Effects of dietary intervention on IGF-I and IGF-binding proteins, and related alterations in sex steroid metabolism: The diet and androgens (DIANA) randomized trial. *Eur. J. Clin. Nutr.* **57:** 1079–1088.
12. Sieri, S., V. Paola, *et al*. 2007. Dietary glycemic index, glycemic load, and cancer risk in an Italian prospective cohort study. *Am. J. Clin. Nutr.* **86:** 1160–1166.
13. Charbonneau, A. & V. Luu-The. 2001. Genomic organization of a human 5β-reductase and its pseudogene and substrate selectivity of the expressed enzyme. *Bioch. Biophy. Acta.* **1517:** 228–235.
14. Westerbacka, J., H. Yki-Jarvinen, *et al*. 2003. Body fat distribution and cortisol metabolism in healthy men: Enhanced 5beta-reductase and lower cortisol/cortisone metabolite ratios in men with fatty liver. *J. Clin. Endocrinol. Metab.* **88:** 4924–4931.