

Annamari Kilkinen

SERUM ENTEROLACTONE

**DETERMINANTS AND ASSOCIATIONS
WITH BREAST AND PROSTATE CANCERS**

ACADEMIC DISSERTATION

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ABBREVIATIONS

ATBC	Alpha-Tocopherol, Beta-Carotene
ATC	Anatomical Therapeutic Chemical
BMI	body mass index
CI	confidence interval
CV	interassay coefficients of variation
CVD	cardiovascular disease
END	enterodiol
ENL	enterolactone
ER	estrogen receptor
FFQ	food frequency questionnaire
(ID-)GC-MS	(isotope-dilution)gas chromatography- mass spectrometry
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
MAT	matairesinol
OR	odds ratio
PSA	prostate-specific antigen
RR	risk ratio
SD	standard deviation
SE	standard error
SECO	secoisolariciresinol
SHBG	sex hormone-binding globulin
TR-FIA	time-resolved fluoroimmunoassay

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

- I** Kilkkinen A, Stumpf K, Pietinen P, Valsta LM, Tapanainen H, Adlercreutz H. Determinants of serum enterolactone concentration. *Am J Clin Nutr* 2001;73:1094-100.
- II** Kilkkinen A, Valsta L, Virtamo J, Stumpf K, Adlercreutz H, Pietinen P. Intake of lignans is associated with serum enterolactone concentration in Finnish men and women. *J Nutr* 2003;133:1830-3.
- III** Kilkkinen A, Pietinen P, Klaukka T, Virtamo J, Korhonen P, Adlercreutz H. Use of oral antimicrobials decreases serum enterolactone concentration. *Am J Epidemiol* 2002;155:472-7.
- IV** Kilkkinen A, Virtamo J, Vartiainen E, Sankila R, Virtanen MJ, Adlercreutz H, Pietinen P. Serum enterolactone concentration is not associated with breast cancer risk in a nested case-control study. *Int J Cancer* 2004;108:277-80.
- V** Kilkkinen A, Virtamo J, Virtanen MJ, Adlercreutz H, Albanes D, Pietinen P. Serum enterolactone concentration is not associated with prostate cancer risk in a nested case-control study. *Cancer Epid Biomark Prev* 2003;12:1209-12.

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1 ABSTRACT

Interest in the role of bioactive compounds present in plants has increased dramatically over the last decade. Many compounds have been discovered and intensively studied to evaluate their effects on health. Phenolic compounds, specifically lignan phytoestrogens, have received particular attention.

Enterolactone, the most abundant lignan in humans, is produced by intestinal microflora from dietary precursors found widely in plants. Enterolactone has been proposed to possess a broad spectrum of biological properties, giving it the potential to reduce risk of chronic diseases. Epidemiological evidence is, however, sparse and contradictory.

The aim of this study was to define the distribution of serum enterolactone concentration among Finnish adults and to examine its determinants, including selected background characteristics, dietary factors, and use of antimicrobials. Moreover, the association between serum enterolactone concentration and the risk of breast and prostate cancers was assessed.

Serum enterolactone concentration was analyzed among participants of the FINDIET survey carried out as part of the cross-sectional FINRISK survey in 1997. The range in serum enterolactone concentration was large (0-183 nmol/l), but 90% of subjects had a concentration under 38 nmol/l. The mean serum enterolactone concentration (nmol/l) of men and women was 16.9 (SD 13.8, median 13.4) and 19.6 (SD 16.8, median 15.6), respectively.

Serum enterolactone concentration was negatively associated with the use of antimicrobials and positively associated with self-reported constipation in both genders. In addition, it had a negative association with smoking and body mass index and a positive association with age in women, and a positive association with the length of time from last antimicrobial treatment in men. The mean daily lignan intake was low, <0.2 mg, and serum enterolactone concentration rather weakly reflected the intake of lignans but more strongly the consumption of lignan-containing foods, i.e. fruit, berries, and vegetables.

To examine the association between serum enterolactone concentration and risk of breast cancer, enterolactone concentrations were measured in serum collected in four independent cross-sectional FINRISK surveys (1982-1997) from 206 women with breast cancer diagnosed during follow-up (mean 8.0 years) and from 215 controls frequency-matched by study cohort, 5-year age group, and study area. The mean serum enterolactone concentration (nmol/l) did not differ between cases and

controls, 25.2 (SD 22.2) vs. 24.0 (SD 21.3) and no association was found between serum enterolactone concentration and risk of breast cancer (OR for the highest vs. the lowest quartile 1.30, 95% CI 0.73-2.31).

The association between serum enterolactone concentration and risk of prostate cancer was assessed among participants of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Enterolactone concentrations were measured in serum collected at trial baseline from 214 men with prostate cancer diagnosed during follow-up (median 6 years) and from 214 controls matched by age, date of baseline blood collection, intervention group, and local study area. No difference was present in mean serum enterolactone concentration (nmol/l) between cases and controls, 15.9 (SD 15.2) vs. 16.9 (SD 14.9) and no association was found between serum enterolactone concentration and risk of prostate cancer (OR for the highest vs. the lowest quartile 0.71, 95% CI 0.42-1.21).

In conclusion, a large range in serum enterolactone concentration (0-183 nmol/l) was observed, but 90% of subjects had a concentration under 38 nmol/l. Use of antimicrobials and self-reported constipation were the most important determinants of serum enterolactone concentration, supporting a central role of the gut environment in the bioavailability of lignans. Serum enterolactone concentration was not protectively associated with the risk of breast or prostate cancer.

2 INTRODUCTION

The great interest over the last several decades in diet and human cancer derives from the large variation present in rates of specific cancers among different populations of the world (Parkin et al. 1999, Pisani et al. 1999, Hsing et al. 2000). The highest rates of breast and prostate cancers have been observed in Western societies, where diets are typically high in fat and low in vegetables, while rates have been lower in Asian populations and developing countries, where mainly plant-based diets are consumed. Rates are, however, also increasing in developing countries, and offspring of migrants moving from countries with low breast and prostate cancer incidence to areas with a higher incidence adopt the rates of the new environment (Shimizu et al. 1991, Ziegler et al. 1993). This indicates that the differences in cancer rates are largely attributable to environmental and lifestyle factors rather than to genetics. Diet is a prominent environmental factor estimated to attribute to about one-third of cancer deaths (Doll & Peto 1981). In contrast to many other risk factors of cancer, diet is modifiable and therefore an area of much interest, both scientifically and among the public at large.

Of particular interest is the class of dietary compounds known as phytoestrogens. Based on their chemical structure, phytoestrogens are divided into three main classes, lignans, isoflavonoids, and coumestans. Almost any plant food may contain phytoestrogens, although the amounts and combinations of different compounds vary (Thompson et al. 1991, Mazur 1998, Liggins et al. 2000). The main dietary sources of isoflavonoids and coumestans are soybeans, and alfalfa sprouts and beans, respectively. Lignans are more widely distributed in plants, found, for example, in whole grains, berries, and seeds, and are therefore probably the most important phytoestrogens in Western populations, including Finland. Hence, lignans and their most abundant human metabolite, enterolactone, are the focus of this study.

Enterolactone, which is produced by intestinal microflora from dietary precursors, has been proposed to possess several biological activities (Adlercreutz 2002, Wang 2002), including but not limited to antioxidant activity and inhibition of several enzymes involved in steroid hormone metabolism, thus providing potential mechanisms for a preventive influence in hormone-dependent cancers and cardiovascular diseases. Epidemiological evidence is, however, limited and inconclusive. Some epidemiologic studies have reported protective associations between enterolactone exposure and chronic diseases (Ingram et al. 1997, Vanharanta et al. 1999, Pietinen et al. 2001, Dai et al. 2002), while other studies have not found these associations (den Tonkelaar et al. 2001, Stattin et al. 2002,

Peeters et al. 2003). The present work extends previous research by examining serum enterolactone concentration and its determinants in a large population sample. The associations between serum enterolactone concentration and breast and prostate cancers have also been assessed in two prospective design studies.

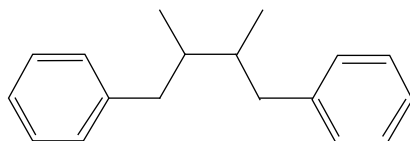
3 REVIEW OF THE LITERATURE

3.1 Dietary lignans

3.1.1 Origin and classification

Lignans are a large group of secondary plant metabolites found throughout the plant kingdom (Howarth 1936, Ayres & Loike 1990). They typically possess a dibenzylbutane structure, which is formed by the dimerization of two cinnamic acid residues (Figure 1). Nearly five hundred natural lignans have been identified in plants and plant parts (stems, leaves, seeds, fruits) (Ayres & Loike 1990, Ward 1993).

Figure 1. *Basic structure of lignans.*



A dimer of cinnamic acid

Due to the structural diversity of lignans, their chemical classification and nomenclature has been challenging (Ayres & Loike 1990). In medical literature, lignans have typically been divided into plant lignans and mammalian lignans, also called enterolignans, which are formed from plant lignans by the intestinal microflora. Here, we focus on the mammalian lignan enterolactone, but another mammalian lignan, enterodiol, as well as dietary precursors for both, mainly matairesinol and secoisolariciresinol, will also be discussed.

3.1.2 Food sources

Secoisolariciresinol and matairesinol are considered to be the major precursors of mammalian lignans. These precursors are widely distributed in the plant kingdom, especially in fiber-rich foods (Mazur 1998, Liggins et al. 2000). The richest source of lignans is flaxseed, but they are also found in other seeds, whole grain cereals, and various vegetables, fruit, and berries. Some examples of lignan content of foods have been collected in Table 1. The values are given per dry weight, and therefore,

many fruits and vegetables that have high water content may give the impression that they are richer sources of lignans than they actually are.

Apart from the above precursors, pinoresinol, lariciresinol, syringaresinol, isolariciresinol, artigenin, and hydroxymatairesinol have recently also been identified as precursors of mammalian lignans (Saarinen et al. 2000, Wang et al. 2000, Heinonen et al. 2001). The first three are present in cereals (Heinonen et al. 2001). Only a few preliminary analytical results have thus far been published.

3.1.3 Intake

Current information about typical dietary intake of lignans is limited (Table 2). Most of the studies have been carried out in the United States and a wide range in daily lignan intake, from 100 μg in women from California (Horn-Ross et al. 2002a, 2002b) to over 1 300 μg in men from Texas (Walcott et al. 2002), has been reported. The main sources of lignans are cereal products, fruit and berries, vegetables, coffee and tea, and alcoholic beverages. However, caution must be taken when comparing these reports because of differences in collection of food consumption data and development of phytoestrogen databases.

Table 1. Lignan content of selected foods ($\mu\text{g}/100\text{g}$ dry weight).

	SECO	MAT		SECO	MAT		SECO	MAT
Legumes			Nuts and seeds			Berries and fruits		
Kidney bean ^{1,2}	56-153	Tr	Almond ⁴	107	Tr	Apple ⁵	Tr	0
Lentil ³	0-7	Tr	Cashew nut ⁴	257	4	Avocado ⁵	77	16
Pea ³	3-13	Tr	Flaxseed ⁵	369-900	1087	Banana ⁴	10	0
Soybean ³	13-273	Tr	Hazelnut ⁴	119	4	Blueberry ⁴	835	0
			Peanut ^{1,2}	298-333	Tr	Black currant ⁴	388	10
Vegetables			Pistachio nut ⁴	96	0	Bramble ⁵	3718	23
Beetroot ⁴	100	Tr	Poppy seed ²	14	12	Cantaloupe ⁵	184	0
Broccoli ²	414	23	Sesame seed ⁴	90	608	Cloudberry ⁴	203	0
Cabbage ⁴	33	Tr	Sunflower seed ⁵	610	0	Cranberry ^{1,2}	1054-1510	0
Carrot ^{1,2}	192-370	Tr-3	Walnut ⁴	163	5	Lemon ⁵	61	0
Cauliflower ⁴	97	Tr				Lingonberry ⁴	1510	0
Chives ⁵	1254	Tr	Grains and cereals			Orange ⁵	77	0
Cucumber ⁵	25	Tr	Barley (whole grain) ²	58	0	Papaya ⁵	8	0
Eggplant ⁵	100	3	Barley bran ²	63	0	Plum ⁴	5	0
Garlic ^{1,2}	379-380	Tr-4	Crisp bread ⁶	28-42	42-62	Raspberry ⁴	139	0
Mushroom ²	8	0	Maize ⁵	16	0	Red currant ⁴	165	0
Onion ⁴	83	8	Oat bran ²	24	155	Strawberry ^{1,2}	1205-1500	5-78
Paprika, pepper ⁴	117	7	Oat meal ²	13	0			
Potato (peeled) ⁴	10	6	Rice ⁵	16	Tr	Beverages		
Pumpkin ⁴	3870	4	Rye (whole grain) ²	47	65	Black tea (brewed) ⁴	1050-2418	90-305
Radish ⁵	33	3	Rye bran ²	132	167	Green tea (brewed) ⁴	1794-2887	195-277
Red cabbage ⁴	141	Tr	Wheat (whole grain) ²	33	3	Red wine ⁵	686-1280	74-98
Tomato ⁵	52	7	Wheat bran ²	110	0	White wine ⁵	136-174	17-22
Zucchini ⁴	817	Tr	Wheat white meal ²	8	0			

MAT = matairesinol, SECO = secoisolarisiresinol, Tr = traces

¹Mazur & Adlercreutz 2000, ²Adlercreutz & Mazur 1997, ³Mazur et al. 1998, ⁴Mazur & Adlercreutz 1998, ⁵Mazur 1998, ⁶Mazur et al. 1996

Table 2. *Dietary intake of plant lignans ($\mu\text{g}/\text{day}$) based on the food frequency questionnaire in selected populations.*

Country	Population	Age (years)	Median intake of lignans	Sources	Reference
Germany	666 F ¹	<50	MAT + SECO 563	Nut and seeds, bread, wine, onion/garlic	Linseisen et al. 2004
The Netherlands	17 140 F	50-69	MAT 80 \pm 50 ² , SECO 1030 \pm 4 ²	Grain products, fruit, vegetables, coffee/tea, alcoholic beverages	Boker et al. 2002
USA	107 M ¹	60.6 \pm 6.9 ³	MAT 46, SECO 483	Black tea, flaxseed bread, cranberry juice/cranberries	Strom et al. 1999
USA	447 F	50-79	MAT 36 ⁴ , SECO 139 ⁴	Orange juice, coffee, sweet potatoes, rice, peaches, apricots	Horn-Ross et al. 2000
USA	1610 F ¹	35-79	MAT ~30 ⁵ , SECO ~122 ⁵	Not reported	Horn-Ross et al. 2001
USA	964 F	Postmenopausal	MAT 23 \pm 19 ² , SECO 622 \pm 357 ²	Fruit (no citrus), grain products, berries	de Kleijn et al. 2001
USA	558 F ¹	20-74	MAT ~34 ⁵ , SECO ~70 ⁵	Not reported	Horn-Ross et al. 2002a
USA	111 526 F	21-103	MAT 23 ⁴ , SECO 85 ⁴	Not reported	Horn-Ross et al. 2002b
USA	136 M ¹	18-55	MAT + SECO ~1355 ^{5,6}	Black tea, cranberry juice	Walcott et al. 2002
USA	470 F ¹	35-79	MAT 30, SECO 138	Not reported	Horn-Ross et al. 2003

M = male, F = female, MAT = matairesinol, SECO = secoisolarisiresinol

¹Controls

²Mean \pm SD

³Mean \pm SE

⁴Mean

⁵Values are rough estimates based on distributions presented by the author

⁶Median lignan intake in $\mu\text{g}/\text{d}$ has been estimated from median energy (1941 kcal) and lignan intake (698 $\mu\text{g}/1000$ kcal) intake

3.2 Lignans in humans

3.2.1 Metabolism

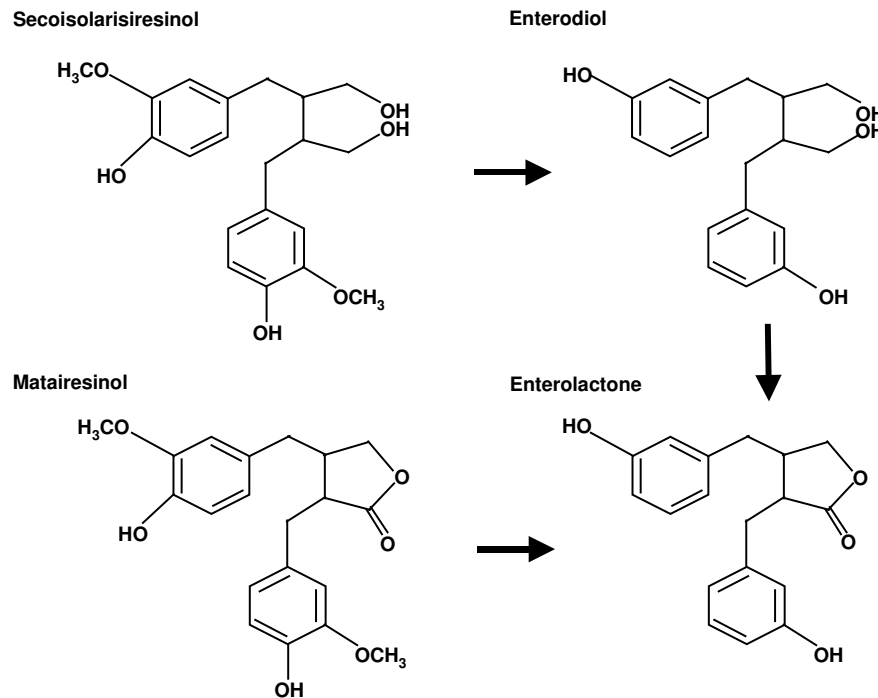
Some twenty years ago, two research groups independently characterized and reported the presence of enterolactone in human urine (Setchell et al. 1980, Stich et al. 1980). It was initially assumed to be a steroidal compound of ovarian origin due to enterolactone excretion following a menstrual cycle and occurring in relatively large quantities during early pregnancy (Stich et al. 1980). Soon afterwards, enterolactone (and enterodiol) was demonstrated to be a metabolic compound of the gut microflora (Axelson et al. 1982).

The best-known precursors of mammalian lignans, secoisolariciresinol and matairesinol, occur in plants as glycosidic conjugates which are hydrolyzed and further converted to mammalian lignans in the gut (Setchell et al. 1981a, 1981b, Axelson et al. 1982, Borriello et al. 1985). Secoisolariciresinol is transformed to enterodiol through reactions involving demethylation and dehydroxylation (Figure 2). Enterodiol can be further oxidized to enterolactone. Matairesinol is converted to enterolactone through demethylation and dehydroxylation.

After absorption, mammalian lignans are conjugated mainly with glucuronic acid and to a lesser degree with sulfates in the liver (Setchell et al. 1981a, 1981b, Axelson et al. 1982, Borriello et al. 1985). Like endogenous estrogens, lignans are located in the enterohepatic circulation and are excreted mainly in the urine but also in the feces (Axelson & Setchell 1981, Pettersson et al. 1996).

Studies in germ-free rats (Bowey et al. 2003), in humans administered antibiotics (Setchell et al. 1981a), and in ileostomy patients (Pettersson et al. 1996) have confirmed that production of mammalian lignans depends on the bacteria in the intestinal tract. However, bacterial strains responsible for the conversion of plant lignans to mammalian lignans have not been fully identified. *In vitro* studies have demonstrated that metabolism of lignans occurs under both anaerobic and aerobic conditions, indicating action by facultative bacteria (Borriello et al. 1985). Clostridia were suggested to be involved in the conversion (Setchell et al. 1981a), but this hypothesis is not supported by more recent studies (Borriello et al. 1985). Peptococcus and eubacterium strains have also been suggested to be involved in the metabolism of lignans (Wang et al. 2000). Although the conversion of plant lignans to mammalian lignans is thought to be efficient, detection of secoisolariciresinol and matairesinol in the urine (Bannwart et al. 1989) indicates that they may also be absorbed in unchanged form from the gastrointestinal tract.

Figure 2. *Metabolism of plant lignans to mammalian lignans.*



3.2.2 Bioavailability and pharmacokinetics

Limited studies with a small number of subjects and of brief duration - blood samples not collected beyond 24 h - have assessed the bioavailability and pharmacokinetic profile of lignans in human subjects. In these studies, no increase in plasma enterolactone concentration was observed until 8-9 h after lignan supplementation (Morton et al. 1997b, Nesbitt et al. 1999, Mazur et al. 2000), probably due to enterolactone being produced by gut microflora. The increase in enterolactone concentration continued up to 24 h in serum (Morton et al. 1997b, Mazur et al. 2000) and up to 35-36 h in urine (Mazur et al. 2000), although substantial variation was present in these timeframes as well as in the maximal levels achieved among participants. Variation in the recovery of plant lignans

determined as urinary mammalian lignans was also high, from -23% to 438% (Mazur et al. 2000).

No human studies have been performed to assess mammalian lignan disposition after supplementation, but in rats the highest levels were measured in tissues involved in lignan metabolism, i.e. intestinal, hepatic, and renal as well as certain estrogen-sensitive tissues, such as the uterus, but not the mammary gland (Rickard & Thompson 1998).

3.2.3 Concentrations in serum and urine

Table 3 presents information on plasma and serum concentrations of lignans in various populations. Mean plasma and serum lignan concentrations appear to be in the range of 6.6–191 nmol/l in individuals consuming typical diets; the highest levels were observed in postmenopausal women in Israel (Brzezinski et al. 1997) and the lowest in middle-aged Norwegian men (Stattin et al. 2002).

As with plasma levels, wide ranges in urinary lignans – from 3.4 μ mol/24-h to 1850 μ mol/24-h – have been observed (Table 3). However, caution must be taken when comparing these reports because of differences in specimen collection and the presentation of results.

Correlations between serum and urinary enterolactone measurements have been shown to vary from 0.84 (Valentin-Blasini et al. 2003) to 0.91 (Stumpf & Adlercreutz 2003). For enterodiols, a somewhat lower correlation (0.62) has been found (Valentin-Blasini et al. 2003).

In addition to urine, plasma, and serum, lignans have been detected in other biologic specimens, including amniotic fluid (Adlercreutz et al. 1999), cord plasma (Adlercreutz et al. 1999), feces (Adlercreutz et al. 1995), nipple aspirate fluid (Hargreaves et al. 1999), prostatic fluid (Morton et al. 1997a), saliva (Finlay et al. 1991), semen (Dehennin et al. 1982), and certain tissues, e.g. prostate tissue (Hong et al. 2002). Information on tissue concentrations is, however, limited. Based on preliminary studies, enterolactone concentrations in breast tissue (~3 nmol/l, Hargreaves et al. 1999), nipple aspirate fluid (~3 nmol/l, Hargreaves et al. 1999) and amniotic fluid (~10 nmol/l, Adlercreutz et al. 1999) are comparable with those in serum but higher than those in prostate tissue (93 vs. 28 nmol/l, Hong et al. 2002), prostatic fluid (68-549 vs. 13-21 nmol/l, Morton et al. 1997a), and breast cyst fluid (63 vs. 17 nmol/l, Boccardo et al. 2003).

Table 3. Serum, plasma, and urinary lignan concentrations in various populations.

Country	Population	Age (years)	Lignan ¹	Reference
<i>I. Plasmal/serum</i>				
Finland (North Karelia)	85 F + M	35-49	ENL 12.2 ³	Stumpf et al. 2000a
Finland	87 F	24-65	ENL 25.0 ± 16.6 ²	Uehara et al. 2000
Finland (North Karelia)	208 F ⁴	25-75	ENL 25.9 ± 21.9 ²	Pietinen et al. 2001
Finland	488 M ⁴	Middle-aged	ENL 15.5 ²	Stattin et al. 2002
Finland	62 F + 18 M	42.6-45.6 ⁵	ENL 30.0 ^{2,3,5}	Tarpila et al. 2002
Finland	100 M ⁴	59	ENL 16.6 ^{2,5}	Vanharanta et al. 2002b
Finland (North Karelia)	1889 M ⁴	42-60	ENL 17.1 ± 14.0 ²	Vanharanta et al. 2003
Italy	104 F	53	ENL 16.3 ^{3,5,6}	Albertazzi et al. 1999
Japan	111 F	40-60	ENL 13.3 ± 15.6 ²	Uehara et al. 2000
Japan	102 M	40-89	ENL 32.6 ± 58.7 ⁶	Morton et al. 2002
Japan	125 F	40-89	ENL 22.7 ± 31.3 ⁶	Morton et al. 2002
Norway	1720 M ⁴	Middle-aged	ENL 6.6 ²	Stattin et al. 2002
UK	43 M	Middle-aged	ENL 24.4 ± 24.5 ⁶	Morton et al. 1997a, 2002
USA	133 F	Middle-aged	ENL 18.7 ± 16.4 ⁶	Morton et al. 1994, 2002
USA	60 F	34-65	ENL 20.2 ⁶ , END 1.5 ⁶	Zeleniuch-Jacquotte et al. 1998
USA	115 F + 78 M	20-40	ENL 14.0 ± 16.0 ²	Horner et al. 2002
USA	208 F + M (61% F)	20-58	ENL 12.1 ^{3,7} , END 6.0 ^{5,7}	Valentin-Blasini et al. 2003

Sweden	492 F ⁴	51.2-58.1 ⁶	ENL 20.4-22.9 ^{2,5}	Hulten et al. 2002
Sweden	342 M ⁴	Middle-aged	ENL 13.8 ²	Stattin et al. 2002
II. Urine				
Australia	144 F ⁴	30-84	ENL 3.1 ⁸ , END 0.3 ⁸	Ingram et al. 1997
China	250 F ⁴	25-64	ENL 6.3 ± 8.6 ⁹ , END 0.9 ± 1.7 ⁹	Dai et al. 2002
Finland	126 F	24-65	ENL 4.9 ± 3.1 ¹⁰	Uehara et al. 2000
Korea	75 F	52-65	ENL 1.5 ± 1.1 ¹¹ , END 0.4 ± 0.5 ¹¹	Kim et al. 2002
The Netherlands	268 F ⁴	50-64	ENL 566 ± 354 ¹²	den Tonkelaar et al. 2001
USA	49 F + 49 M	18-37	ENL 3.6 ± 4.6 ⁸ , END 1.3 ± 3.4 ⁸	Lampe et al. 1999
USA	199 F + M (61% F)	20-58	ENL 1.72 ^{5,7} , END 0.2 ^{5,7}	Valentin-Blasini et al. 2003

25

M = male, F = female, ENL = enterolactone, END = enterodiol

¹Values are median or mean ± SD in nmol/l (plasma/serum) or µmol/24-h (urine)

²Serum/plasma lignans analyzed by TR-FIA

³Baseline value

⁴Controls

⁵Mean(s)

⁶Serum/plasma lignans analyzed by ID-GC-MS/GC-MS

⁷Serum/urinary lignans analyzed by HPLC, urinary lignans analyzed from spot sample and expressed in µmol enterolactone/l

⁸Urinary lignans analyzed by ID-GC-MS from 72-h urine samples

⁹Urinary lignans analyzed by LC-MS from overnight urine samples and results expressed in µmol enterolactone/g creatinine

¹⁰Urinary lignans analyzed by TR-FIA from 24-h urine samples

¹¹Urinary lignans analyzed by GC-MS from 24-h urine samples

¹²Urinary lignans analyzed by TR-FIA from overnight urine samples and values are expressed in µmol enterolactone/mol creatinine

3.2.4 Reliability of lignan measurements

Little work has been done to assess the reliability of serum and plasma or urinary lignan concentrations. The short-term reliability coefficient of serum enterolactone measurements has been reported to be 0.84 in samples collected on successive days (Horner et al. 2002), 0.79 in samples collected on five successive days within one week (Stumpf & Adlercreutz 2003), and 0.77 in samples collected on four Mondays within one month (Stumpf & Adlercreutz 2003). The long-term reliability of serum enterolactone measurements over a two-year period is somewhat lower, 0.55 (Zeleniuch-Jacquotte et al. 1998). Reliability of 24-h urinary enterolactone concentration is comparable with that of serum measurements (Stumpf & Adlercreutz 2003), and as expected, reliability of the overnight urinary enterolactone-creatinine ratio is poorer (den Tonkelaar et al. 2001, Stumpf & Adlercreutz 2003).

3.2.5 Factors associated with serum and urinary lignans

The content of plant lignans in the diet is often considered the most important determinant of serum and urinary lignan levels. Several small trials have shown that supplementation with flaxseed, the richest known source of mammalian lignans, causes a clear dose-dependent response in serum (Brzezinski et al. 1997, Morton et al. 1997b, Nesbitt et al. 1999, Tarpila et al. 2002) and urinary (Schultz et al. 1991, Lampe et al. 1994, Cunnane et al. 1995, Nesbitt et al. 1999, Hutchins et al. 2000) lignan concentrations. Depending on the level of intake and the compound sought, this increase has been up to 285-fold in urine (Nesbitt et al. 1999) and 6.6-fold in serum (Brzezinski et al. 1997). Moreover, not only flaxseed or other lignan-rich foods (Juntunen et al. 2000, Mazur et al. 2000, Pool-Zobel et al. 2000, Jacobs et al. 2002, Vanharanta et al. 2002a) but also a change in dietary habits towards a diet high in vegetables and fruit has caused an increase in serum (Stumpf et al. 2000a) and urinary (Hutchins et al. 1995) lignan levels. However, high interindividual variation in the response exists. The results of supplementation studies involving a minimum of 15 subjects were collected and are displayed in Table 4.

In larger studies, intake of fiber has been positively associated with serum and urinary level of lignans (Adlercreutz et al. 1982, 1987, Lampe et al. 1999, Rowland et al. 1999, Pietinen et al. 2001, Horner et al. 2002, Vanharanta et al. 2002b). Serum enterolactone concentration has also been positively associated with consumption of vegetables (Horner et al. 2002, Vanharanta et al. 2003) and negatively associated with intake of fat (Horner et al. 2002, Vanharanta et al. 2003). No consistent

association has been found for intake of alcohol (Horner et al. 2002, Vanharanta et al. 2003) or consumption of fruit (Lampe et al. 1999, Pietinen et al. 2001, Horner et al. 2002, Vanharanta et al. 2003). Lignan levels have tended to be higher in older persons (Rowland et al. 1999, Horner et al. 2002) and persons with a low-normal body mass index (BMI, Horner et al. 2002, Hulten et al. 2002, Vanharanta et al. 2003). Demographic characteristics and intake of fiber, alcohol, and caffeine accounted for 22% of variation in plasma enterolactone among young American volunteers (Horner et al. 2002), whereas in middle-aged Finnish men only about 10% of variation was explained by fiber, alcohol, saturated fatty acid, and vegetable consumption, BMI, constipation, and the number of bronchitis episodes diagnosed during the lifetime (Vanharanta et al. 2003).

Table 4. *Results of selected supplementation studies (n ≥15).*

Population	Age (years)	Supplementation	Duration	Lignans at baseline ¹	Change (%) ²	Reference
<i>I. Serum/plasma</i>						
78 F	43-65	Flaxseed ³	12 weeks	ENL 155.3 ± 43.3 ⁴ END 35.7 ± 20.0 ⁴	ENL 97 (62%) END 55 (155%)	Brzezinski et al. 1997
18 M + 21 F	M: 43 ⁵ F: 43 ⁵	Whole meal rye bread ⁶	4 weeks	M: ENL 28.1 ± 3.8 ⁴ F: ENL 39.3 ± 4.4 ⁴	M: ENL -3 (-9%) F: ENL -0.4 (-1%)	Juntunen et al. 2000
		White wheat bread ⁶	4 weeks		M: ENL -16 (-56%) F: ENL -25 (-62%)	
85 M + F	35-49	Diet high in vegetables and fruit	12 weeks	ENL 12.2 (10.4-19.3) ⁷	ENL 7 (60%)	Stumpf et al. 2000a
18 M + 62 F	M: 46 ± 10 ⁸ F: 43 ± 11 ⁸	Flaxseed food ⁹	4 weeks	ENL 26-30 ⁵	ENL 19-26 (63-200%)	Tarpila et al. 2002
15 F + M		Flaxseed food ⁹	4 months	ENL 33 ⁵	ENL 37 (112%)	
15 M	30-69	Rye bread high in phloem ¹⁰	4 weeks	ENL ~42 ⁵	ENL 27 (64%)	Vanharanta et al. 2002a
29 M	30-69	Rye bread low in phloem ¹⁰	4 weeks	ENL ~21 ⁵	ENL 25 (219%)	
29 M	30-69	Placebo rye bread without phloem ¹⁰	4 weeks	ENL ~38 ⁵	ENL 2 (5%)	

II. Urine

18 F	20-34	Flaxseed powder 10 g/d	3 months	ENL 3.2 ± 1.5 ¹¹ END 1.1 ± 1.1 ¹¹	ENL 25 (780%) END 18 (1689%)	Lampe et al. 1994
11 M + 9 F	Young	Soy diet	9 days	ENL 3.2 ± 0.5 ¹¹ END 0.5 ± 0.1 ¹¹	ENL -3 (-78%) END -0.4 (-80%)	Kirkman et al. 1995
		Carotenoid diet	9 days		ENL -1.7 (-113%) END 0 (0%)	
		Cruciferous diet	9 days		ENL -0.7 (-22%) END 0.9 (180%)	
31 F	52-82	Flaxseed 5 g/d	7 weeks	ENL 3.4 (2.9-4.1) ¹² END 0.4 (0.3-0.5) ¹²	ENL 21 (626%) END 1 (250%)	Hutchins et al. 2000
		Flaxseed 10 g/d	7 weeks		ENL 53 (1553%) END 3 (700%)	

M = male, F = female, ENL = enterolactone, END = enterodiol

¹Values are means ± SD in nmol/l (plasma/serum) or µmol/24-h (urine)

²Values are nmol/l (change in plasma/serum lignans) or µmol/24-h (change in urinary lignans)

³Diet included 2 teaspoons flaxseed/d (providing lignans ~4 mg/g) and soy products ~500 g/d

⁴Mean ± SEM

⁵Mean(s) without SD

⁶Mean consumption of whole meal rye bread was 219 g/d in men (providing lignans ~0.54 µmol/d) and 162 g/d in female (providing lignans ~0.40 µmol/d). Mean consumption of white wheat bread was 200 g/d in men (providing lignans ~0.06 µmol/d) and 153 g/d in female (providing lignans ~0.05 µmol/d)

⁷Median (95% CI)

⁸Mean ± SD

⁹Flaxseed foods provided lignans ~8 mg/d

¹⁰Daily amount of study bread was 70 g, providing lignans 20 773 nmol/d in high-phloem group, 12 331 nmol/d in low-phloem group, and 758 nmol/d in placebo group

¹¹Least-squares mean ± SE

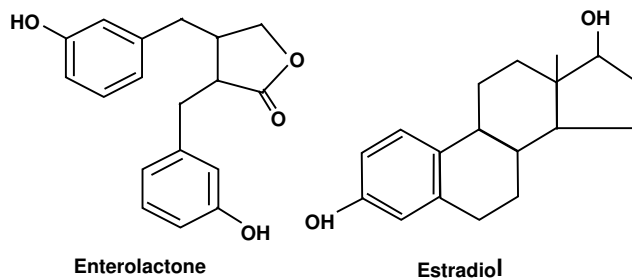
¹²Geometric mean (95% CI)

3.3 Biological effects of lignans

Numerous biological effects for dietary lignans have been proposed during the past twenty years. Evidence is, however, sparse and inconsistent and mostly limited to *in vitro* experiments.

Enterolactone has structural similarities to endogenous estrogens (Figure 3) suggesting possible estrogenic activity. Thus far, binding affinity of enterolactone to estrogen receptor (ER) has not been examined. In classical α - or β -type ER-mediated pathways, enterolactone has shown neither estrogenic nor antiestrogenic activity (Saarinen et al. 2000). However, enterolactone has exerted both weak estrogenic and weak antiestrogenic effects in human cell lines (Welshons et al. 1987, Hirano et al. 1990, Mousavi & Adlercreutz 1992, Sathyamoorthy et al. 1994, Wang & Kurzer 1997, 1998, Schultze-Mosgau et al. 1998, Sung et al. 1998). The concentrations tested have been many-fold than those measured in humans (Table 3). Moreover, no estrogenic activity of enterolactone has been observed *in vivo* (Setchell et al. 1981a, Waters & Knowler 1982, Saarinen et al. 2000, 2001), but some antiestrogenic effects have been reported in one preliminary study (Waters & Knowler 1982).

Figure 3. Chemical structure of enterolactone and estradiol.



Lignans have also been proposed to modulate production and bioavailability of sex hormones. An aromatase enzyme which converts testosterone and androstenedione to 17β -estradiol and estrone, respectively, has been inhibited *in vitro* by a relatively high concentration of enterolactone (Adlercreutz et al. 1993, Wang et al. 1994, Saarinen et al. 2002). Enterolactone has also inhibited both 5α -reductase, which catalyzes the synthesis of 5α -dihydrotestosterone from testosterone, and 17β -hydroxysteroid dehydrogenase, which converts testosterone to androstenedione (Evans et al. 1995). Furthermore, a high concentration of enterolactone has

stimulated the synthesis of sex hormone-binding globulin (SHBG) in the liver (Adlercreutz et al. 1992) and decreased the binding of steroid hormones to SHBG (Martin et al. 1996, Schottner et al. 1997, 1998). These effects, in theory, could induce lower lifetime exposure to sex hormones, which potentially could lower the risk of breast and prostate cancers. However, no aromatase inhibition was observed *in vivo* (Saarinen et al. 2002), and lignan supplementation has rather minor effects on serum sex hormones and SHBG in human clinical trials (Schultz et al. 1991, Phipps et al. 1993, Hutchins et al. 2001, Brooks et al. 2004).

On the basis of *in vitro* studies, enterolactone has also been suggested to possess antioxidant activity (Kitts et al. 1999, Pool-Zobel et al. 2000, Prasad 2000, Saarinen et al. 2000) and the ability to inhibit the Na⁺K⁺ pump (Braquet et al. 1986). The relevance of these findings in the understanding of enterolactone action *in vivo* is, however, unclear.

In animal models, some evidence of chemopreventive effects of lignans has emerged. A lignan-rich diet has retarded or reduced experimentally induced tumors in several tissues, including the mammary gland (Serraino & Thompson 1991, Thompson et al. 1996, Rickard et al. 1999, 2000, Tou & Thompson 1999, Saarinen et al. 2000, 2001, 2002, Ward et al. 2000, Dabrosin et al. 2002) and prostate (Zhang et al. 1997, Landstrom et al. 1998, Bylund et al. 2000). However, only a few studies (Thompson et al. 1996, Saarinen et al. 2000, 2002) used purified lignans, and thus, it is difficult to know whether the observed chemopreventive effects can be attributed directly to lignans rather than to other components of lignan-rich foods. Moreover, serum enterolactone concentrations measured in these experimental models have been very high compared with those measured in humans (Table 3). Therefore, in the absence of solid data from clinical and epidemiological studies, the question of whether lignans have chemopreventive effects in humans remains open.

3.4 Health effects of lignans

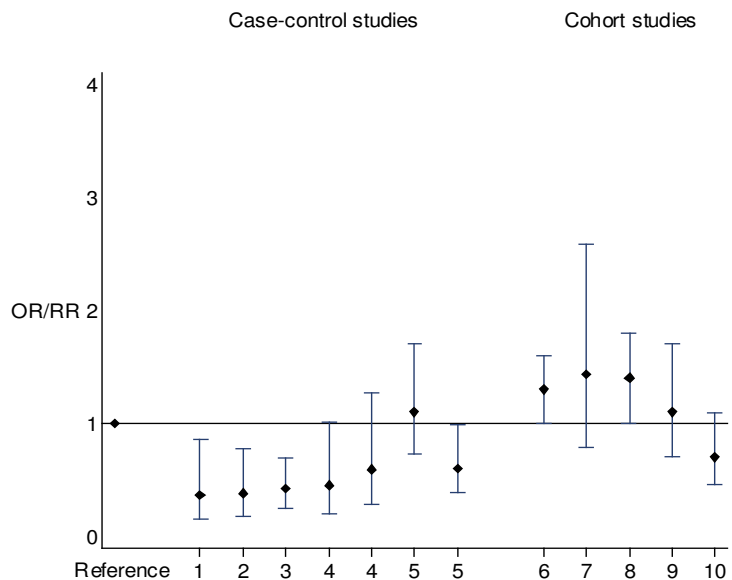
3.4.1 Breast cancer

Breast cancer is the most common cancer in women worldwide (Parkin et al. 1999). Established risk factors for breast cancer are associated with hormonal and reproductive factors (Kelsey et al. 1993, Key et al. 2001, Bianchini et al. 2002). However, these factors, including age, early menarche, nulliparity, late age at first pregnancy, late menopause, family history, and obesity in postmenopausal women, are estimated to explain less than half of breast cancer cases (Hankin 1993). Thus,

large gaps remain in current knowledge of etiology of the disease. Moreover, many of the known risk factors are relatively unmodifiable in a practical sense, thus limiting the means for primary prevention. Identification of new modifiable risk factors, such as dietary factors, has therefore been an area of much interest. Thus far, research concerning the effects of diet on risk of cancer has uncovered few definite effects and left much uncertainty (Willett 2001, Key et al. 2002, 2004).

In the past decade, lignans have received particular attention. The literature examining the association between lignan exposure and risk of breast cancer is, however, limited and inconclusive. A descriptive study with seven breast cancer cases and twenty controls was the first to detect lower urinary excretion of enterolactone in breast cancer patients than in controls (Adlercreutz et al. 1982). To date five epidemiological studies have assessed the association between blood or urinary enterolactone concentration and the risk of breast cancer (Table 5), and three of these studies have found an inverse association. In these case-control studies, risk reduction between high and low serum (Pietinen et al. 2001) or urinary (Ingram et al. 1997, Dai et al. 2002) enterolactone concentration has been approximately 60%. Findings from two prospective studies contradict those of the case-control studies, with no association being observed for urinary enterolactone excretion (den Tonkelaar et al. 2001) and only a marginal inverse association for serum enterolactone concentration (Hulten et al. 2002). Moreover, no associations have been found between dietary intake of plant or mammalian lignans and breast cancer risk in cohort studies (Horn-Ross et al. 2001, 2002b, Keinan-Boker et al. 2004). In case-control studies, a high intake of mammalian lignans was associated with a lower risk of premenopausal breast cancer (McCann et al. 2002, Linseisen et al. 2004) but not with a lower risk of postmenopausal breast cancer (McCann et al. 2002). All epidemiological studies in which the association between lignan or enterolactone exposure and risk of breast cancer has been examined have been collected in Figure 4. In addition to these studies, in a very small prospective study, breast cancer risk was inversely associated with serum enterolactone concentration (RR=0.36, 95% CI 0.14-0.93, Boccardo et al. 2004) but not with enterolactone concentration in cyst fluid (RR=0.70, 95% CI 0.22-2.27, Boccardo et al. 2003).

Figure 4. Association between lignan or enterolactone exposure and risk of breast cancer. OR/RR with 95% CI for the highest vs. lowest exposure groups is presented.



References: ¹Ingram et al. 1997, ²Pietinen et al. 2001, ³Dai et al. 2002, ⁴McCann et al. 2002, ⁵Linseisen et al. 2004, ⁶den Tonkelaar et al. 2001, ⁷Horn-Ross et al. 2001, ⁸Horn-Ross et al. 2002b, ⁹Hulten et al. 2002, ¹⁰Keinan-Boker et al. 2004

Table 5. *Epidemiological studies on the relationship between lignans and cancer.*

Age (years)	Medium	Study design	Number of cases	Number of controls/ cohort	Adjusted OR/RR ¹ (95% CI)	Reference
<i>I. Breast cancer</i>						
25-75	Serum	Case-control	194	208	ENL 0.38 (0.18-0.77)	Pietinen et al. 2001
51.2-58.1 ²	Plasma	Cohort	248	492	ENL 1.1 (0.7-1.7)	Hulten et al. 2002
54 ³	Urine ⁴	Case-control	144	144	ENL 0.36 (0.15-0.86)	Ingram et al. 1997
50-64	Urine ⁵	Cohort	88	268	ENL 1.43 (0.79-2.59) ⁶	den Tonkelaar et al. 2001
25-64	Urine ⁵	Case-control	250	250	ENL 0.42 (0.25-0.39)	Dai et al. 2002
35-79	Diet ⁷	Cohort	1 272	1610	SECO 1.3 (1.0-1.6) MAT 1.1 (0.89-1.5)	Horn-Ross et al. 2001
21-103	Diet ⁷	Cohort	711	111 526	SECO 1.4 (1.0-1.8) MAT 1.1 (0.8-1.4)	Horn-Ross et al. 2002b
Premenopausal Postmenopausal	Diet ⁷	Case-control	301 493	316 494	ENL + END 0.49 (0.32-0.75) ENL + END 0.72 (0.51-1.02)	McCann et al. 2002
49-70	Diet ⁷	Cohort	280	15 555	ENL + END 0.70 (0.46-1.09)	Keinan-Boker et al. 2004
< 50	Diet ⁷	Case-control	278	666	SECO 1.1 (0.73-1.7) MAT 0.58 (0.37-0.94) SECO + MAT 1.1 (0.72-1.7) ENL + END 0.61 (0.39-0.98)	Linseisen et al. 2004

II. Prostate cancer

Middle-aged and old	Serum	Cohort	794	2550	ENL 1.08 (0.83-1.39) ⁸	Stattin et al. 2002
60.6 ± 6.9 ⁹	Diet ⁷	Case-control	83	107	SECO 1.20 (0.65-2.21) MAT 0.89 (0.47-1.66)	Strom et al. 1999

III. Other cancers*Colorectal cancer*

M + F (age not reported)	Plasma	Cohort	117	232	ENL 0.99 (0.52-1.88)	Lundin 2001
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Endometrial cancer

35-79 F	Diet ⁷	Case-control	500	470	SECO 0.68 (0.21-0.85) MAT 1.6 (0.99-2.4)	Horn-Ross et al. 2003
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Ovarian cancer

40-85 F	Diet ⁷	Case-control	124	696	SECO + MAT 0.43 (0.21-0.85)	McCann et al. 2003
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Testicular cancer

18-55 M	Diet ⁷	Case-control	159	136	SECO + MAT 0.96 (0.11-8.09) ENL + END 0.73 (0.21-2.56)	Walcott et al. 2002
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Thyroid cancer

20-74 F	Diet ⁷	Cohort	608	558	SECO 0.56 (0.35-0.89) MAT 0.72 (0.46-1.10)	Horn-Ross et al. 2002a
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M = male, F = female, ENL = enterolactone, END = enterodiol, MAT = matairesinol, SECO = secoisolariciresinol

¹Highest vs. lowest group

²Mean(s)

³Median

⁴72-h urine samples

⁵Overnight urine samples

⁶Crude OR, after adjustment <10% change in crude OR

⁷Diet was assessed via a food frequency questionnaire

⁸Crude RR

⁹Mean ± SD

3.4.2 Prostate cancer

Prostate cancer is the leading cancer in males in most Western populations (Hsing et al. 2000). Despite this, its etiology remains largely unknown, with age, race, and family history of disease being the only established risk factors (Nomura & Kolonel 1991). The observations that the incidence of latent prostate cancer is similar worldwide but the rate of invasive disease varies markedly (Hsing et al. 2000) support an important role for environmental factors, including diet, in the progression and clinical manifestation of this malignancy.

Several dietary components have been studied with inconsistent findings (Giles & Ireland 1997, Schmitz-Drager et al. 2001, Key et al. 2004), emphasizing some of the current challenges in the epidemiology of prostate cancer. Due to their many biological actions, lignans have also been suggested to play a role in the prevention of prostate cancer. However, only a few epidemiological studies have been carried out (Table 5). High circulating enterolactone has not been associated with reduced risk of prostate cancer in a large prospective study based on population cohorts from Finland, Sweden, and Norway (Stattin et al. 2002), and no relationship between dietary intake of lignans and prostate cancer was observed in a case-control study conducted in the United States (Strom et al. 1999).

3.4.3 Other types of cancer

Some research has investigated the role of lignans in other cancers (Table 5). A high intake of lignans was associated with a reduced risk of endometrial (Horn-Ross et al. 2003), ovarian (McCann et al. 2003) and thyroid (Horn-Ross et al. 2002a) cancers but no relationship was observed with testicular cancer (Walcott et al. 2002). Neither was an association observed between serum enterolactone and risk of colorectal cancer in a prospective study in Sweden (Lundin 2001). In addition, in a recent study of patients with hepatocellular carcinoma and cirrhosis in Italy, no differences in lignan intake between these two groups were found (Lei et al. 2002).

3.4.4 Other diseases

Due to several biological activities, lignans are hypothesized to also protect against other chronic diseases. Intervention studies assessing the effects of a lignan-rich diet on risk factors of cardiovascular disease (CVD) have been few, but they have consistently reported a reduction in serum lipids (Jenkins et al. 1999, Lucas et al. 2002). Consumption of lignans has also been inversely associated with both plasma

triglycerides and CVD risk metabolic score in middle-aged women in the United States (de Kleijn et al. 2002). In a prospective study of 167 middle-aged Finnish men with acute coronary event and their matched controls, men in the highest quartile of serum enterolactone concentration had a 59% (95% CI 24%-78%) lower risk of acute coronary event than men in the lowest quartile (Vanharanta et al. 1999). High serum enterolactone was also associated with reduced risk of coronary heart disease mortality (RR=0.44, 95% CI 0.20-0.96) and CVD-related mortality (RR=0.55, 95% CI 0.29-1.01) but had a weaker association with all-cause mortality (RR=0.76, 95% CI 0.52-1.12) (Vanharanta et al. 2003).

Lignans have been hypothesized to exert hormonal effects, thereby playing a role in bone metabolism. However, results of two human studies are inconclusive; high urinary enterolactone excretion had a positive association with bone mineral density in Korean postmenopausal women (Kim et al. 2002), but it was associated with a higher rate of bone loss in postmenopausal women in the Netherlands (Kardinaal et al. 1998). Moreover, flaxseed supplementation has not had a beneficial effect on biomarkers of bone metabolism in animals (Ward et al. 2001a, 2001b) or humans (Lucas et al. 2002).

The potential for phytoestrogens to alleviate symptoms associated with menopause, particularly hot flashes, is an area of active research. However, most of the attention has focused specifically on isoflavonoids (Albertazzi & Purdie 2002). In two studies in which supplementation included a combination of flaxseed and soy, no consistent alleviation of menopause symptoms was observed (Brzezinski et al. 1997, Dalais et al. 1998).

4 AIMS OF THE STUDY

The general aim of this study was to examine the determinants of serum enterolactone concentration and to assess the association between serum enterolactone concentration and the risk of breast and prostate cancers.

Specific aims were as follows:

1. To define the distribution of serum enterolactone concentration among Finnish adults.
2. To examine the cross-sectional association between serum enterolactone concentration and dietary factors, including consumption of lignan-containing foods and intake of lignans.
3. To examine the cross-sectional association between serum enterolactone concentration and selected background characteristics.
4. To investigate the cross-sectional association between serum enterolactone concentration and recent use of antimicrobials.
5. To assess the relationship between serum enterolactone concentration and the risk of breast and prostate cancers.

5 MATERIALS AND METHODS

The material of this work came from two large studies. The cross-sectional FINRISK surveys were used to define the distribution of serum enterolactone concentration and to examine its determinants (Studies I-III) as well as to assess the association between serum enterolactone concentration and risk of breast cancer (Study IV). The Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) study was used to assess the association between serum enterolactone concentration and risk of prostate cancer (Study V).

5.1 The FINRISK and FINDIET surveys

The comparable cross-sectional FINRISK surveys have been carried out every fifth year since 1972 to assess the levels and trends of CVD risk factors in Finland (Vartiainen et al. 2000). The first two surveys were conducted in two eastern Finnish provinces, North Karelia and Kuopio. The surveys were expanded to southwestern parts of Finland in 1982, to the Helsinki metropolitan area in 1992, and to the northern province of Oulu in 1997. The target population consisted of men and women aged 25-64 years living in the survey areas. In 1997, the sample also included people aged 65-74 years. For each survey, an independent random sample stratified by 10-year age group, area, and sex (at least 250 subjects per cell) was drawn from the population register. As part of the FINRISK survey, the FINDIET dietary survey has been conducted since 1982.

5.1.1 Study population

Studies I-III

Studies I-III comprised data collected in the FINDIET survey in 1997 (Figure 5), the protocol of which was approved by the Ethics Committee of the National Public Health Institute. For this dietary survey, a subsample of 4000 subjects (40%) was randomly selected from the FINRISK participants, with 2862 (72%) subsequently participating in the survey (FINDIET 1997 Study Group 1998). A serum sample for enterolactone analysis was available for 2753 subjects, and of these, subjects who had used antimicrobials within the past three months (n=373) were excluded from Study I (n=2380). Intake of lignans was assessed among all FINDIET participants; ten subjects with extremely high intake (>17 mg/day) were subsequently excluded

from Study II (n=2852). All subjects for whom serum enterolactone concentration was available were included in the analysis of the association between intake of lignans and serum enterolactone concentration (n=2744). These analyses were done separately for those who had not used antimicrobials for one year prior to the examination (n=1784) and those who had (n=960). All FINDIET participants for whom information on serum enterolactone concentration and use of antimicrobials was available were included in Study III (n=2753). Some new analyses summarizing data from Studies I-III were performed. These analyses included subjects for whom information on serum enterolactone concentration, selected background variables, dietary factors, and use of antimicrobials was available (n=2539; n=1202 for men and n=1337 for women).

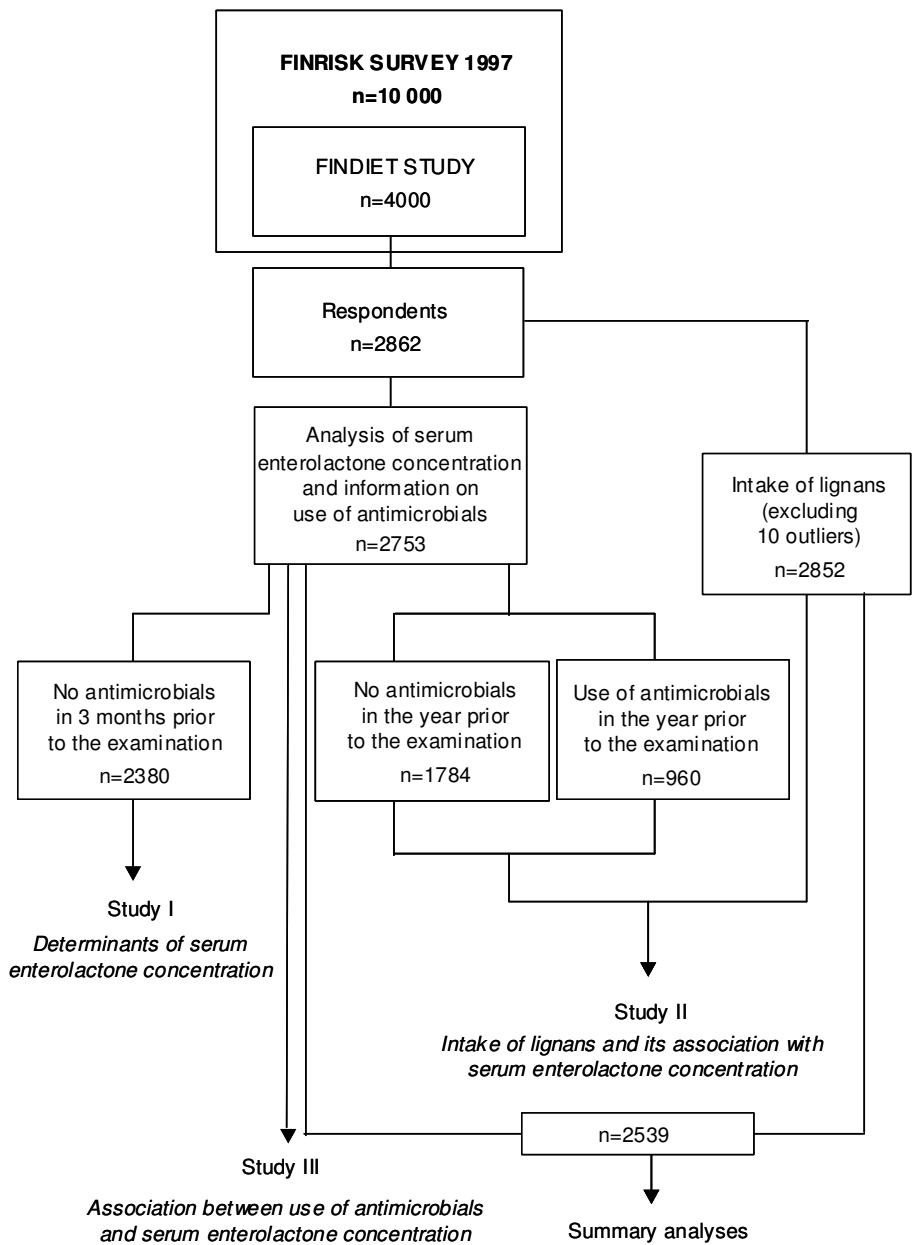
Study IV

Subjects of Study IV were participants of FINRISK surveys carried out between 1982 and 1997. A total of 19 000 women aged 25-74 years were included. The participation rate across cohorts varied from 76.2% to 85.0%, with the final sample comprising 15 497 women.

Incident cases of breast cancer (ICD-10 code C50) were identified through Finnish Cancer Registry. By the end of January 2002, 465 breast cancers had been identified among survey participants. For 16 subjects who had been diagnosed with breast cancer twice, only the first case was included, and the 13 cases who had participated in several surveys were included in only their first survey cohort. Those who had breast cancer diagnosed prior to participation in the survey (n=127) were excluded; thus, 322 breast cancer cases were eligible for our study.

For each case, a control frequency-matched to case by study cohort, age group (the same 5-year age group, i.e. from 25-29 years up to 70-74 years), and study area was selected. A serum sample for enterolactone analysis was available for 206 cases and 215 controls; the proportions of subjects providing a blood sample in the 1982, 1987, 1992, and 1997 cohorts were 58%, 61%, 92%, and 66%, respectively. The corresponding numbers of cases and controls from these cohorts were 88 and 95, 47 and 52, 49 and 49, and 22 and 19. Breast cancer subjects aged 51 years or less at the time of diagnosis were classified as premenopausal (n=69) and those over 51 years as postmenopausal (n=137, Luoto et al. 1994).

Figure 5. *Flow chart of study participants (Studies I-III and summary analyses).*



5.1.2 Data collection

Health examination

Subjects were invited to a health examination at a local health care center, where a fasting (minimum 4 h) venous blood sample was drawn. In addition, weight and height were measured, and BMI was computed as weight (kg) divided by height squared (m^2). The following cut-off points for BMI were used in Studies I-II: under $20.1 \text{ kg}/m^2$ underweight, $20.1\text{-}25.0 \text{ kg}/m^2$ normal weight, $25.1\text{-}30.0 \text{ kg}/m^2$ overweight, $30.1\text{-}35.0 \text{ kg}/m^2$ obese, and over $35.0 \text{ kg}/m^2$ severely obese. In Studies III-IV, BMI was used as a continuous variable.

Health and lifestyle questionnaire

In each survey, information on health, lifestyle, and socioeconomic factors was collected by a self-administered questionnaire completed at the examination. Educational levels were formed by dividing subjects born in the same year into tertiles based on their total years of education. Smoking history was assessed using a standard set of questions, and according to responses, participants were classified into three groups: those who had never smoked (nonsmokers), those who had quit smoking (ex-smokers), and those currently smoking (current smokers).

In Study I, alcohol consumption was calculated from the questionnaire, covering the frequency of use and the average portion of beer, wine, and spirits consumed during the previous 12 months. The following cut-off points for weekly ethanol intake were used for men: 0 g none, 1-139 g low, 140-280 g moderate, and over 280 g heavy. The corresponding values for women were 0 g, 1-104 g, 105-190 g, and over 190 g. Subjects were also divided into three categories (poor, satisfactory, good) by self-reported physical status. Furthermore, participants were asked whether they had suffered from constipation during the preceding month (30 days); the response alternatives were “yes” and “no”.

Dietary assessment

A food frequency questionnaire (FFQ) including 38 food items was used in the 1997 survey as part of the self-administered questionnaire. The consumption of whole-grain products was quantified from the FFQ by summing up the frequency of consumption as servings per month of rye bread, crisp bread, porridge, and cereals. Consumption of vegetables was calculated by summing up of the frequency of consumption of salad vegetables, roots, legumes, and vegetable dishes, and consumption of fruit and berries by summing up the frequency of consumption of fruit, berries, and fruit and berry juices. For the analyses of Study I, subjects were divided into tertiles by frequency of consumption of whole-grain products,

vegetables, and fruit and berries. In Study III, the frequencies of consumption of all lignan-containing foods, i.e. whole-grain products, vegetables, and fruit and berries, were summed.

In Study II, dietary intake was assessed using a 24-h recall. To estimate validity and repeatability of this 24-h recall, a 48-h dietary recall and a 3-day food record were collected from a randomly chosen subpopulation of the same study (n=223 and n=334, respectively). Intake of lignans, matairesinol, secoisolariciresinol, and their sum was quantified by a recently developed phytoestrogen database (Valsta et al. 2003).

Data on use of antimicrobials

Data on the use of antimicrobials (Study III) were based on the nationwide prescription register of the Social Insurance Institution. Since 1995, all prescriptions reimbursed by the National Sickness Insurance Scheme have been registered and classified according to the Anatomical Therapeutic Chemical (ATC) system. Oral antibacterials included tetracyclines, amoxicillin and other penicillins with an extended spectrum, phenoxymethylpenicillin and other beta-lactamase-sensitive penicillins, cloxacillin and other beta-lactamase-resistant penicillins, combined penicillins, cephalosporins, sulfonamides and trimethoprim, macrolides and lincosamides, fluoroquinolone antibacterials, and steroid antibacterials. Based on the total number of antimicrobial purchases, subjects were divided into five groups (none, 1, 2, 3, and at least 4), and based on time from last purchase into seven groups (under 2 months, 2-4 months, etc., to over 12 months).

5.2 The Alpha-Tocopherol Beta-Carotene Cancer Prevention study

The ATBC study was a randomized, double-blind, placebo-controlled trial aimed at determining whether daily supplementation with α -tocopherol, β -carotene, or both, would reduce the incidence of lung cancer and other cancers (The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group 1994). The ATBC study was a joint project between the National Public Health Institute of Finland and the National Cancer Institute of the United States. The institutional review boards of these two institutions approved the study protocol. Written informed consent was obtained from all study participants at baseline.

Participants were screened by a postal questionnaire from amongst all men aged 50-69 years and living in the southwestern part of Finland (n=209 406). Men who were

current smokers (smoked at least five cigarettes per day) and willing to participate were invited to undergo baseline examinations (n=42 957). Exclusion criteria were previous malignancy other than nonmelanoma skin cancer, severe angina, chronic renal insufficiency, cirrhosis of the liver, chronic alcoholism, anticoagulant therapy, use of supplements containing vitamin E (>20 mg/d), vitamin A (>20 000 IU/d), or β -carotene (>6 mg/d), and other medical problems, such as psychiatric disorders or physical disability, that might limit long-term participation. A total of 29 133 men eligible for the trial were randomized into one of four intervention regimens: α -tocopherol (50 mg/d), β -carotene (20 mg/d), both, or neither (placebo). Recruitment began in April 1985 and continued until June 1988. Participants were followed until death or the end of the trial (April 30, 1993, median follow-up 6.1 years).

5.2.1 Study population

A total of 246 prostate cancers were identified among ATBC study participants between May 1985 and April 1993, primarily through the Finnish Cancer Registry and the Register of Causes of Death. Medical records were reviewed centrally by two study oncologists to confirm diagnoses. Prostate cancers with available histology or cytology (98%) were also reviewed by pathologists. For each case, a control matched by age (± 1 year), date (± 28 days) of baseline blood collection, intervention group, and local study area was selected. A blood sample for serum enterolactone analysis was available for 233 cases and 222 controls, leaving 214 case-control pairs for analysis.

5.2.2 Data collection

Before randomization, each participant attended baseline examinations where fasting venous samples were taken and height and weight were measured. Information on medical history, smoking habits, education, physical activity, and dietary history was collected by questionnaires completed at the examinations.

5.3 Assay of serum samples

Fasting venous samples were drawn from the subjects at the examinations. Serum was separated, divided into 1-ml aliquots, and stored in glass vials either at -20°C (Studies I-IV) or at -70°C (Study V) until analysis of enterolactone.

Enterolactone analysis was performed by time-resolved fluoroimmunoassay (TR-FIA, Adlercreutz et al. 1998) with slight modifications (Stumpf et al. 2000b). A more rapid modification was used in Studies I-III because of the high number of serum samples.

The TR-FIA method used in Studies IV-V was briefly as follows: 250 μ l (Study IV) or 150 μ l (Study V) of serum was incubated with 250 μ l (Study IV) or 150 μ l (Study V) of hydrolysis reagent containing 2 U/ml sulfatase and 0.2 U/ml β -glucuronidase overnight at 37°C. After hydrolysis, the free enterolactone and hydrolyzed conjugates were extracted twice with 1.5 ml of diethyl ether. Diethyl ether was evaporated to dryness in a water bath, after which the dry residue was dissolved in 250 μ l (Study IV) or 150 μ l (Study V) of assay buffer. A sample was divided into two subsamples, and enterolactone of both subsamples was analyzed by TR-FIA using the VICTOR 1420 multilabel counter (Wallac Oy, Turku, Finland). Subsamples were analyzed in the same laboratory batch, and the mean value of these two measurements was used. Matched case-control sets of Study V were also analyzed in the same laboratory batch, and laboratory personnel were blinded to the case-control status of samples. All of the batches were analyzed with two quality control samples going through the whole method. The concentrations of quality control samples and their respective interassay coefficients of variation (CV) in Study IV were 8.9 nmol/l and 11.5%, and 20.2 nmol/l and 7.0%, and in Study V 3.2 nmol/l and 16.9%, and 12.4 nmol/l and 12.5%. One additional quality control sample with a concentration of 48.2 nmol/l and a CV of 9.3% in Study IV, and 110 nmol/l and 8.2% in Study V controlled for TR-FIA.

The more rapid modification of TR-FIA used in Studies I-III is briefly as follows: 50 μ l of serum was incubated with 50 μ l of hydrolysis reagent containing 2 U/ml sulfatase and 0.2 U/ml β -glucuronidase overnight at 37°C. Unlike standard TR-FIA, no extraction was performed, instead enterolactone was directly measured. The concentrations of quality control samples and their respective CVs were 4 nmol/l and 16.8%, 14 nmol/l and 10.1%, and 59 nmol/l and 13.1%.

The more rapid modification of TR-FIA tends to yield 15-30% higher results than the TR-FIA method with extraction. To enable comparison of results with previous studies, an equation between the modified TR-FIA and the standard TR-FIA with extraction was formed by analyzing 92 with both methods. The final results were calculated accordingly as follows: Final concentration = measured concentration \times 0.934 - 11.03.

In Study V, prostate-specific antigen (PSA) was determined by an immunometric method (Pettersson et al. 1995).

5.4 Statistical methods

5.4.1 Studies I-III and summary analyses

Dependence of serum enterolactone concentration on dietary factors, selected background variables, and use of antimicrobials was assessed by analysis of variance (Studies I-III). A mixed model for measurement error was used to test differences in intake of lignans between genders and in other background categories (Study II). In addition to 24-h dietary data, the model used a 48-h dietary recall and a 3-day food record; the attenuated regression coefficient (variance of 24-h recall data divided by variance of 3-day record data) was 1.18. T-test was used to analyze differences in means of log-transformed serum enterolactone concentrations between those who had not used antimicrobials and those who had (Study III).

To summarize the determinants of serum enterolactone concentration, some new analyses combining data from Studies I-III were performed. In these analyses, similar categories of age, BMI, constipation, consumption of alcohol, education, physical status, smoking, and use of antimicrobials were used as in the original studies (see above). However, FFQ-based consumption of lignan-containing foods (i.e. whole-grain products, vegetables, and fruit and berries) and 24-h recall-based intake of lignans were used as continuous rather than categorical variables because of their linear associations with serum enterolactone concentration. To identify the most important determinants of serum enterolactone concentration, analysis of variance was used. All variables were initially included in the model, and nonsignificant variables were then one by one eliminated. In the final model, only the significant ($p < 0.05$) variables were included. Because the distribution of serum enterolactone was skewed towards higher values, log-transformed enterolactone values were used, and all analyses were done separately for men and women.

5.4.2 Studies IV-V

The association between serum enterolactone concentration and risk of breast and prostate cancers was analyzed by conditional logistic regression using odds ratios (OR) with 95% confidence intervals (95% CI). Subjects were stratified into quartiles based on their serum enterolactone concentrations relative to the distribution in controls, and ORs were tested for linear trends across the quartiles using the Wald test with linear contrasts. Some subgroup analyses were conducted using serum enterolactone tertiles because of the smaller number of subjects in these subgroups.

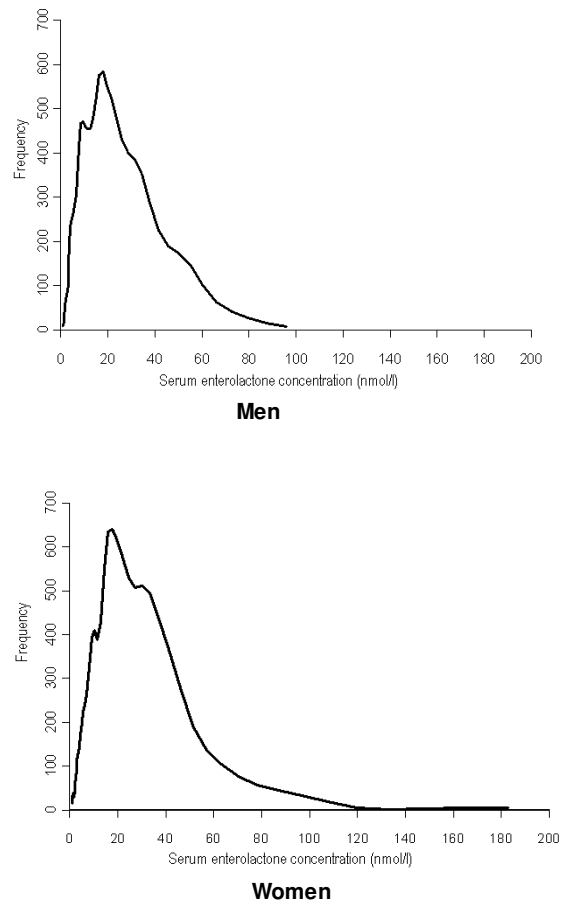
6 RESULTS

6.1 Serum enterolactone concentration and determinants

6.1.1 Lifestyle factors (I)

The distributions of serum enterolactone concentrations among study participants who had not used antimicrobials within the past three months are presented in Figure 6. The mean enterolactone concentrations (nmol/l) of men and women were 17.3 (median 13.8) and 20.5 (median 16.6), respectively.

Figure 6. *Distribution of serum enterolactone concentrations (nmol/l) in men and women.*



In men, serum enterolactone concentration was positively associated with self-reported constipation and consumption of whole-grain products and fruit and berries. In women, serum enterolactone concentration was positively associated with consumption of vegetables, age, and self-reported constipation and negatively associated with smoking. Moreover, female participants of normal weight had significantly higher serum enterolactone concentrations than their underweight or obese peers.

6.1.2 Intake of lignans (II)

The distribution of lignan intake was very skewed to the right; 34% of subjects had intakes less than 100 µg/d, and intake exceeded 0.5 mg/d in only 1.8%. The mean daily intake of lignans among men was 173 µg (19 µg/MJ), with a range of 0-1044 µg. The corresponding values for women were 151 µg (23 µg/MJ) and 0-874 µg. Thus, total lignan intake was higher in men than in women ($p < 0.0001$), but women had a higher lignan density in their diet than men ($p < 0.0001$).

Secoisolariciresinol made up most of the lignan intake, over 70% in men and about 80% in women. The main sources of secoisolariciresinol were fruit and berries (34% in men and 47% in women), followed by cereals (34% in men and 24% in women). Intake of matairesinol was derived mainly from cereals (90% in men and 85% in women), with rye intake accounting for over 80% of the total in both genders.

Among those who had not used antimicrobials during the preceding year, serum enterolactone concentration was positively associated with intake of lignans; enterolactone concentration was 50% higher in the highest quintile of lignan intake than in the lowest.

6.1.3 Use of antimicrobials (III)

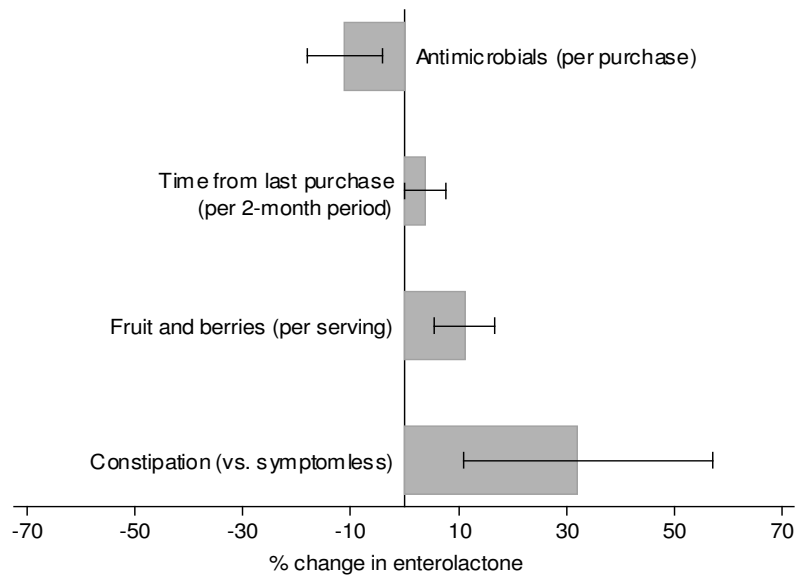
The mean serum enterolactone concentration (nmol/l) was significantly lower among those who had used oral antimicrobials up to 12-16 months before serum sampling than among nonusers, 16.4 (SD 14.3) vs. 19.3 (SD 16.1). Serum enterolactone concentration was negatively associated with the number of purchases and positively associated with the length of time from last purchase. Modest differences were also present between various antimicrobials, with macrolides tending to cause the strongest suppression in serum enterolactone concentration.

6.1.4 Summary analyses

The range in serum enterolactone concentration among subjects included in the summary analyses was 0-183 nmol/l. Distributions of the concentrations were very skewed to the right in both genders, and 90% of subjects had a concentration under 38 nmol/l. The mean serum enterolactone concentration (nmol/l) of men and women was 16.9 (SD 13.8, median 13.4) and 19.6 (SD 16.8, median 15.6), respectively.

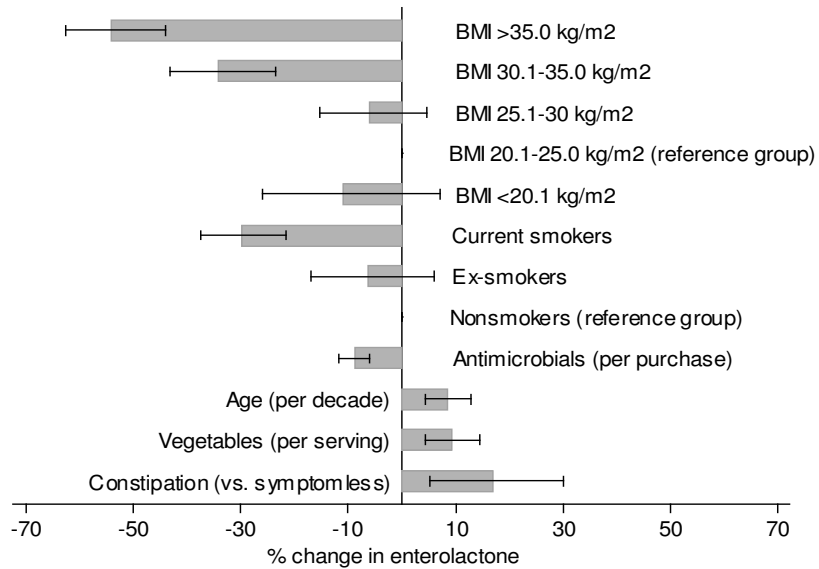
The results of summary analyses are presented in Figures 7 and 8. In men, log-transformed serum enterolactone concentrations were 32% higher among subjects who had reported suffering from constipation, and increasing 11% when daily consumption of fruit and berries was increased by one serving. Concentrations also increased by 4% for each two-month period since the last antimicrobial purchase and decreased by 11% for each antimicrobial treatment. However, only 5% of the variation in concentration could be accounted for by including these four variables in the final model.

Figure 7. *Mean percentage change with 95% CI in log-transformed serum enterolactone concentrations in men. The model includes only significant determinants.*



In women, log-transformed serum enterolactone concentrations were 18% higher among subjects who had reported suffering from constipation. Concentrations also increased 9% for each decade of age and 11% for daily consumption of vegetables being increased by one serving. Among smokers, log-transformed serum enterolactone concentrations were 29% lower than among nonsmokers. Each antimicrobial treatment also lowered serum enterolactone concentration by 11%. As BMI reached 30 kg/m² or 35 kg/m², log-transformed serum enterolactone concentrations were below those of normal BMI (BMI 20-25 kg/m²) by 33% and 54%, respectively. Together these six variables in the final model explained 13% of the variation in serum enterolactone concentrations in women.

Figure 8. *Mean percentage change with 95% CI in log-transformed serum enterolactone concentrations in women. The model includes only significant determinants.*



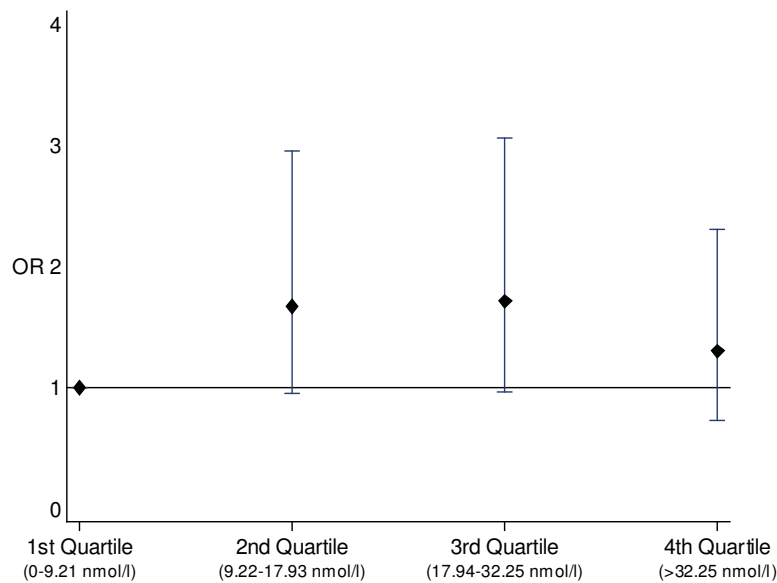
BMI = body mass index

6.2 Serum enterolactone concentration and risk of cancer

6.2.1 Breast cancer (IV)

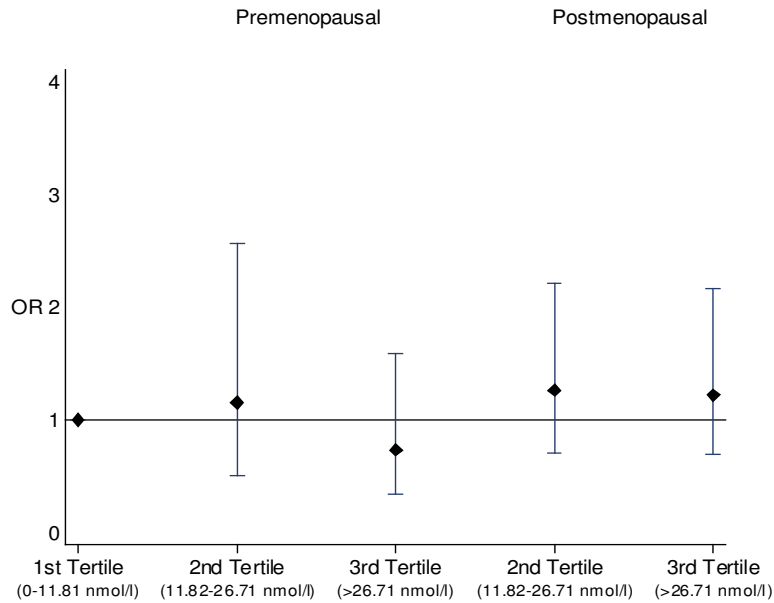
The mean serum enterolactone concentration (nmol/l) did not differ between breast cancer cases and controls, 25.2 (SD 22.2) vs. 24.0 (SD 21.3) ($p=0.52$). No significant association between serum enterolactone concentration and risk of breast cancer was found (Figure 9); the OR for the highest quartile was 1.30 (95% CI 0.73-2.31) and p for trend was 0.48. Adjustment for alcohol consumption, BMI, physical activity, smoking, and years of education did not substantially affect the results (<7% change in ORs). Nor were any consistent associations observed when the analysis was restricted to subjects diagnosed at least 2 years after blood collection or when data were analyzed according to years of follow-up.

Figure 9. *Risk of breast cancer in serum enterolactone quartiles.*



Two-thirds of cases had postmenopausal breast cancer. No consistent association was present between serum enterolactone concentration and risk of breast cancer when data were analyzed according to menopausal status (Figure 10).

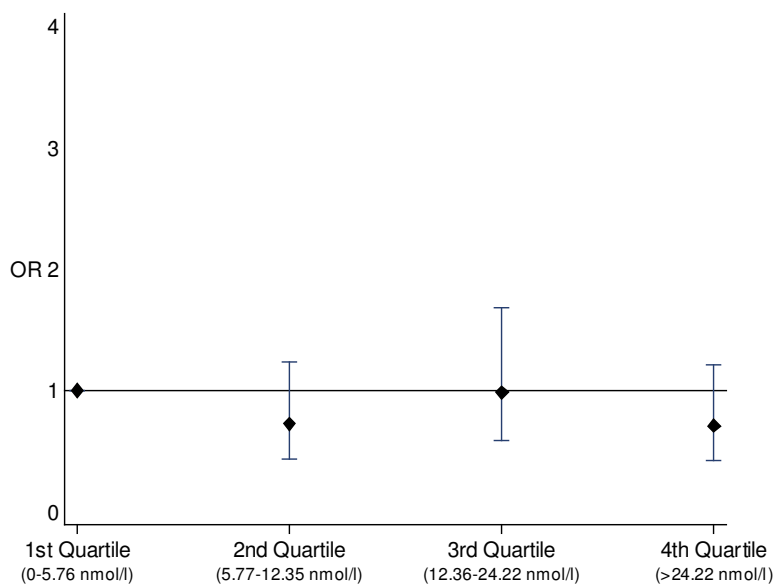
Figure 10. *Risk of premenopausal and postmenopausal breast cancer in serum enterolactone tertiles.*



6.2.2 Prostate cancer (V)

The mean serum enterolactone concentration (nmol/l) did not differ between prostate cancer cases and controls, 15.9 (SD 15.2) vs. 16.9 (SD 14.9), ($p=0.42$). No obvious association was observed between serum enterolactone concentration and risk of prostate cancer; the OR for the highest quartile was 0.71 (95% CI 0.42-1.21), and p for trend was 0.37 (Figure 11). Adjustment for age, area of residence, BMI, consumption of lignan-containing foods, education, history of prostatomegaly, or smoking did not substantially change the results. Neither did the trial supplementation significantly modify the enterolactone-prostate cancer association (p for interaction 0.50).

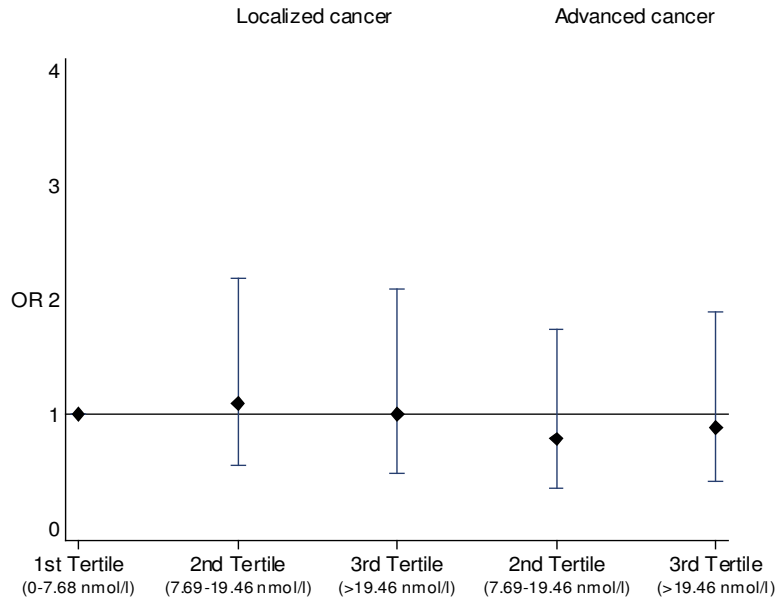
Figure 11. *Risk of prostate cancer in serum enterolactone quartiles.*



The mean time from blood collection to diagnosis was 3.9 (range 0.1-7.2) years. The serum enterolactone-prostate cancer association was not altered when the analysis was restricted to men diagnosed at least 2 years after blood collection. Exclusion of pairs where either the case or the control, or both, had elevated PSA (PSA >4 ng/ml) or had failed to provide a blood sample for PSA analyses also did not produce substantially different results from those including the entire study population.

Half of the cases had localized (stage 1 or 2) disease confined to the prostate. No consistent association was present between serum enterolactone concentration and risk of prostate cancer when data were analyzed according to extension of cancer (Figure 12).

Figure 12. *Risk of prostate cancer according to extension in serum enterolactone tertiles.*



7 DISCUSSION

7.1 Methodological considerations

7.1.1 Serum enterolactone concentration

Serum enterolactone concentrations were analyzed by TR-FIA in all studies. HPLC or GC combined with MS has typically been used for measuring lignans in body fluids, but these methods are expensive and time-consuming and are therefore not suitable for epidemiological studies involving a large number of samples. The immunoassay method has a relatively good reliability (CV% 7.0-16.9), and the correlations between the results obtained by TR-FIA and ID-GC-MS (a reference method) have been confirmed to be high (Adlercreutz et al. 1998, Stumpf et al. 2000b, Uehara et al. 2000). However, some crossreactivities may occur with TR-FIA, i.e. other metabolites in addition to the target compound might be measured.

Serum samples collected at baseline were stored at -20°C (Studies I-IV) or -70°C (Study V), and the time period from blood collection to enterolactone analysis varied from one to 20 years. It could be argued that the different storage temperatures and long storage time might affect the results, thus presenting a source of bias. Interestingly though, comparing the mean serum enterolactone concentrations between male participants of the cross-sectional survey (Studies I-III) and controls of the prostate cancer study (Study V), revealed no difference. Neither were significant differences in concentrations found between the four different study cohorts in the breast cancer study (Study IV).

The possibility of measuring the exposure variable in serum involves some benefits in comparison with dietary assessment. Biomarkers, such as enterolactone, provide objective measures that are independent of reporting bias and provide an index of intake and subsequent metabolism. They therefore serve as a measure of bioavailability, which is relevant when nutritional significance and potential systemic effects of a compound are being considered. On the other hand, when only a single blood sample per subject is available, as in all of the present studies, it can be questioned whether blood enterolactone concentration at a single time-point reflects recent exposure rather than long-term exposure. The reliability coefficient of a single measurement of enterolactone appears, however, to be moderately high, from 0.84 for two days (Horner et al. 2002) to 0.55 for two years (Zeleniuch-

Jacquotte et al. 1998), suggesting that a single serum enterolactone measurement is sufficient in epidemiological studies.

7.1.2 Study populations and designs

Studies I-IV were based on the cross-sectional FINRISK surveys. In each survey, a large, representative, population-based random sample was drawn. Despite a decline in the response rate over time, the participation rate remained relatively high (Vartiainen et al. 2000), supporting the generalizability of the results. However, more well-to-do population groups may be more likely to participate in these kinds of surveys, leading to some bias in results. The prostate cancer study was based on the ATBC trial, which was aimed at evaluating the effects of supplementation on the incidence of lung cancer. All participants were, therefore, current male smokers, restricting the generalizability of these results. Moreover, the in breast cancer study, the proportions of women with a blood sample available for enterolactone analysis varied between 58% and 92% in study cohorts; however, women who did not provide a blood sample were similar to those who did for all background variables studied.

The cross-sectional design of the FINRISK surveys does not allow the temporality of associations to be considered in Studies I-III and also limits determination of how changes in variables actually affect serum enterolactone concentration. The prospective study design in the breast and prostate cancer studies ensured identical collection and handling of blood samples from case and control subjects, and matching for date of blood collection to eliminate possible bias due to degradation of enterolactone during storage. Moreover, the blood samples used were obtained years prior to the diagnoses of cancer, making the presence of clinical cancer at the time of blood donation unlikely.

7.1.3 Dietary assessments and the phytoestrogen database

Both FFQ and 24-h recall were used to assess diet. The FFQ used in Studies I and III had not been designed to assess the consumption of lignan-containing foods, and therefore, some foods with a high content of lignans, like seeds and nuts, were not included in the questionnaire. Moreover, the questionnaire assessed the diet during the previous 12 months, whereas enterolactone probably has a relatively short half-life in the body (Morton et al. 1997b, Nesbitt et al. 1999, Mazur et al. 2000). This is, of course, only a problem in situations where dietary habits vary widely.

The dietary data in Study II were collected by a 24-h dietary recall. The main concern with this method is whether diet in one day represents the usual diet. In the

case of lignan intake, the 24-h recall method could be deemed suitable because lignans are widely distributed in plant foods (Mazur 1998, Mazur & Adlercreutz 1998, Liggins et al. 2000), and these foods are most likely consumed daily. However, some foods with very high amounts of lignans, such as flaxseed, are probably consumed not on a daily but on a weekly or a monthly basis, and this may lead to some misclassification. Naturally, a more representative dietary data by, for example, collecting a 24-h recall during all four seasons would have been advantageous.

The dietary questionnaire used in the ATBC study has been validated, and its reproducibility and validity were found to be satisfactory (Pietinen et al. 1988).

The recently developed phytoestrogen database (Valsta et al. 2003) was used to estimate intake of lignans (Study II). A strength of the database is that most of the food analyses have been performed in Finland using typical Finnish foods. However, in most cases, only a small number of samples were available, which is a cause for concern because lignan content of a food can vary substantially according to breed, crop season and location, processing method, and other variables. Moreover, several new and abundant enterolactone precursors, not included in our database, have recently been found (Saarinen et al. 2000, Wang et al. 2000, Heinonen et al. 2001).

7.1.4 Register-based study variables

Data on antimicrobial purchases (Studies I-III) were based on a nationwide drug register. At the time of the study, the register included over 95% of Finnish pharmacies with the only pharmacies excluded being those without a computer system for handling prescriptions. Moreover, we had no data on antimicrobials received in hospitals or antimicrobials that were not included in the reimbursement system due to low price (under 50 FIM, ~8.5€). However, only 13% of all antimicrobials were used in hospitals, and most of the inexpensive drugs are phenoxymethylpenicillin, sulpha-trimethoprim, and nalidixic acid, which all have been suggested to have a minor impact on microflora (Nord et al. 1986, Hooker & DiPiro 1988).

Incident cases of breast and prostate cancers were identified primarily through the Finnish Cancer Registry. The register covers over 99% of cancers identified in Finland, and its accuracy is high (Teppo et al. 1994, Korhonen et al. 2002). In addition, to confirm diagnoses of prostate cancer, medical records were reviewed centrally by two study oncologists, and cancers with available histology or cytology (98%) were also reviewed by pathologists.

7.1.5 Other study variables

Data on weight and height were based on measurements taken by specially trained study nurses, whereas most of the other variables used in this work were self-reported. According to other population studies, self-reported smoking, for instance, is considered rather reliable (Patrick et al. 1994). The increasing social unacceptability of smoking could, however, have led to underreporting (Jarvis et al. 1987). There is also considerable underestimation in the assessment of alcohol consumption in population surveys; in Finland, the population survey-based estimate accounts for only 40% of total alcohol consumption as measured by sales statistics (Simpura et al. 1995). Thus, the way in which data are collected must be kept in mind when interpreting the results.

7.2 Serum enterolactone concentration and determinants

7.2.1 Enterolactone concentrations

Although a large range in serum enterolactone concentration was evident (0-183 nmol/l), 90% of subjects had a concentration under 38 nmol/l. Overall, the mean enterolactone concentration was of the same order of magnitude as previous reports in Finland (Uehara et al. 2000, Pietinen et al. 2001, Stattin et al. 2002, Vanharanta et al. 2002b, 2003). In the men, concentrations were higher than in Norwegian men (Stattin et al. 2002) but lower than in Japanese (Morton et al. 2002) or American (Morton et al. 1997a, 2002) men. The average serum enterolactone concentrations of women were slightly higher than concentrations observed in Japanese (Uehara et al. 2000) and Italian women (Albertazzi et al. 1999) and comparable with those previously measured in American (Zeleniuch-Jacquotte et al. 1998) and Swedish women (Hulten et al. 2002). Due to differences in study populations, sample sizes, and methods of analysis between these studies, further investigations in representative populations are needed for reliable comparisons.

7.2.2 Determinants

The number of antimicrobial purchases and self-reported constipation were the most important predictors of serum enterolactone concentration. In addition, consumption of fruit and berries and time from last antimicrobial treatment were positively associated with serum enterolactone concentrations in men, whereas in women concentrations were positively associated with age and consumption of vegetables,

and negatively associated with smoking and BMI. However, these variables explained only a small part of the variation in serum enterolactone concentration.

The traditional Finnish diet is believed to be richer in lignans than typical Western diets because of relatively high intake of whole-grain products, particularly rye bread, and berries. However, the mean daily intake of lignans in our population was very low, <0.2 mg. Current information on lignan intake in other populations is limited, and comparisons of published reports are complicated by, for example, differences in the way food consumption data were collected and phytoestrogen databases were developed. Nevertheless, the mean daily intake of lignans in Finland appears to be comparable with, or even lower than, intakes in the United States, whereas the main sources of lignans were quite similar among both populations, i.e. grain products, vegetables, and fruit and berries (Strom et al. 1999, Horn-Ross et al. 2000, 2001, 2002a, 2002b, 2003, de Kleijn et al. 2001, Walcott et al. 2002).

Serum enterolactone concentration rather weakly reflected the actual intake of lignans, which can at least partly be explained by incompleteness of our database, e.g. recently discovered new precursors of enterolactone were missing. Serum enterolactone concentration had, however, a stronger association with the usual consumption of lignan-containing foods, i.e. fruit and berries in men and vegetables in women. In most previous studies, serum enterolactone concentration has been positively associated with consumption of vegetables ($r=0.16-0.24$, Adlercreutz et al. 1991, Horner et al. 2002, Vanharanta et al. 2002b, 2003), but no consistent association with consumption of fruit and berries ($r=0.08-0.25$) has been found (Horner et al. 2002, Vanharanta et al. 2003).

Regarding other lifestyle factors, our findings support earlier observations that younger female subjects (Rowland et al. 1999, Horner et al. 2002), female smokers (Hulten et al. 2002), and women with high BMI (Horner et al. 2002, Hulten et al. 2002, Vanharanta et al. 2003) have lower serum enterolactone concentration. There are no obvious reasons for these associations. However, enterolactone has been shown to pass through the preadipocyte's cell membrane (Adlercreutz et al. 1993, Wang et al. 1994), which means that in subjects with high BMI enterolactone may be "diluted" due to rapid transport into the cells, resulting in lower serum levels. Moreover, although the self-reported consumption of lignan-containing foods was almost the same in normal-weight women as in their under- or overweight counterparts, overweight individuals may overestimated their consumption of these healthy products, a finding described previously (Hirvonen et al. 1997). The diet of smokers has also been shown to be more unhealthy from that of nonsmokers (Dallongeville et al. 1998, Birkett 1999); here, smokers consumed less whole-grain products, vegetables, and fruit and berries than nonsmokers. This may explain why smokers had lower serum enterolactone concentrations than nonsmokers. Moreover,

serum estrogen concentrations during oral hormone administration but not during percutaneous administration seem to be lower in postmenopausal smokers than in nonsmokers (Jensen et al. 1985, Jensen & Christiansen 1988), which implies that the effects of smoking on sex hormones occur during absorption or in hepatic metabolism of hormones. The structural similarity of enterolactone to estrogens may enable smoking to also affect the metabolism of enterolactone. Further, cigarette smoke increases oxidative stress (Frei et al. 1991) and, like all phenolic compounds, enterolactone is likely to have antioxidant properties (Prasad 1997, Kitts et al. 1999). Enterolactone might therefore be consumed to eliminate free radicals, leading to lower serum enterolactone concentrations in smokers than in non-smokers.

Probably the most important factor affecting serum enterolactone concentration is the colonic environment. After ingestion, the precursors of enterolactone are metabolized by the action of the intestinal microflora (Borriello et al. 1985). The essential role of intestinal bacteria in the metabolism of mammalian lignans has previously been suggested by studies in germ-free rats (Bowey et al. 2003) and in ileostomy patients (Pettersson et al. 1996) as well as by a small-scale human study showing that oral administration of metronidazole or wide-spectrum oxytetracycline reduces urinary excretion of lignans (Setchell et al. 1981a). These results are supported by our findings that use of antimicrobials was inversely and in a dose-dependent manner associated with serum enterolactone. However, the specific bacteria involved in the conversion of plant lignans to mammalian lignans have not been fully identified. We observed the greatest suppression to be caused by macrolides, but the broad antibacterial spectrum of macrolides limits the speculation of the specific bacterial strains involved in the metabolism of lignans.

The important role of the colonic environment and metabolism is also supported by our findings that subjects who had reported suffering from constipation had significantly higher serum enterolactone concentration than their symptomless peers. Subjects with constipation probably have slower intestinal motility, and thus, the metabolism and absorption of lignans may be more complete than in asymptomatic subjects. Moreover, subjects suffering from constipation may increase their consumption of foods rich in fiber to prevent symptoms of constipation. However, here, the consumption of lignan-containing foods was the same in both asymptomatic and symptomatic subjects.

In summary, the hypothesis that serum enterolactone concentration is determined not only by intake levels but also by action of microflora is strongly supported by our findings. Moreover, the modest proportion of serum enterolactone concentration explained by the determinants examined here suggests the existence of other, still unknown, determinants.

7.3 Serum enterolactone concentration and risk of cancers

7.3.1 Breast cancer

The results of the nested case-control study (IV) do not support the hypothesis that high serum enterolactone concentration is associated with reduced risk of breast cancer. Overall, our findings are in accord with those of previous prospective studies where risk of breast cancer has not been associated with urinary enterolactone excretion (den Tonkelaar et al. 2001) and has only shown a marginal inverse association with serum enterolactone concentration (Hulten et al. 2002). Contrary to these findings, in three case-control studies, risk reductions between high and low serum (Pietinen et al. 2001) or urinary (Ingram et al. 1997, Dai et al. 2002) enterolactone concentration have been approximately 60%. Similar results have also been obtained in studies where intake of lignans was the focus: no association between intake of lignans and breast cancer risk was observed in prospective studies (Horn-Ross et al. 2001, 2002b, Keinan-Boker et al. 2004), while in case-control studies intake of mammalian lignans were inversely associated with risk of breast cancer, mostly in premenopausal women (McCann et al. 2002, Linseisen et al. 2004). In case-control studies, because enterolactone concentrations and intake of lignans have been assessed after breast cancer occurrence, causal interpretation of the results is seriously limited.

One potential source of bias in the present study is the lack of information on participants' reproductive histories and other risk factors. However, in previous studies, adjustment for these factors has not affected results substantially (den Tonkelaar et al. 2001, Horn-Ross et al. 2001, Pietinen et al. 2001, McCann et al. 2002). Thus, lack of this information is unlikely to have affected our results considerably. Another potential limitation is the absence of information on antibiotic use by study participants. We have no reason to assume, however, that use of antimicrobials differed between cases and controls. Finally, this study was limited by the range of enterolactone exposure (interquartile range 9-32 nmol/l) and concentrations being low compared with values observed to have beneficial effects in experimental studies.

7.3.2 Prostate cancer

The results of the case-control study (V) nested within a trial-based cohort of more than 29 000 Finnish men do not support the hypothesis that high levels of circulating enterolactone are associated with reduced risk of prostate cancer. Our findings are in agreement with the only published epidemiological study on circulating

enterolactone and prostate cancer (Stattin et al. 2002). In this nested case-control study based on population cohorts from Finland, Sweden, and Norway, no significant association between serum enterolactone and risk of prostate cancer was found (OR for the highest quartile 1.08, 95% CI 0.83-1.39). Moreover, no relationship between dietary intake of lignans and prostate cancer was observed in a case-control study conducted in the United States (Strom et al. 1999).

Men in our study were aged 50-69 years at blood collection, and among men in this age bracket an occult malignancy of the prostate has been observed to occur at high frequencies (Pienta & Esper 1993). In fact, 67% of cases and 10% of controls had a baseline PSA level >4 ng/ml, which is associated with a >20% risk of prostate cancer (Stenman et al. 1999). Exclusion of these subjects from the analyses did not, however, produce substantially different results. Nor did restriction of analyses to subjects with at least two years of prostate cancer-free follow-up alter the enterolactone-prostate cancer association. Therefore, the lack of inverse association between serum enterolactone and prostate cancer cannot be due to a high prevalence of subclinical prostate cancers at baseline.

Trial supplementation did not modify the serum enterolactone-prostate cancer association. Smoking, however, has been associated with a greater risk of fatal prostate cancer (Hsing et al. 1990, 1991), and tobacco may increase the virulence of tumors (Visakorpi et al. 1992). Because the ATBC study did not include nonsmokers, we cannot directly evaluate whether smoking modified the serum enterolactone-prostate cancer association. Although serum enterolactone concentration correlated negatively with the number of cigarettes smoked per day and positively with the number of smoking years in the ATBC study controls, the mean enterolactone levels in this study were comparable with other reports (Uehara et al. 2000, Stattin et al. 2002, Vanharanta et al. 2002b, 2003). It should also be noted that while information on use of antimicrobials was unavailable in this study there is no reason to assume that use differed between cases and controls. Finally, in our population, the exposure range was limited (interquartile range 6-24 nmol/l) and serum enterolactone concentrations were rather low compared with values shown to be beneficial in experimental studies.

8 CONCLUSIONS

Based on the main findings, the following conclusions were drawn:

1. The range in serum enterolactone concentration among the Finnish population was large (0-183 nmol/l), but 90% of subjects had a concentration under 38 nmol/l.
2. Lignans, especially secoisolariciresinol, were common components of the Finnish diet, but the mean daily lignan intake, <0.2 mg, was much lower than doses shown to be beneficial in experimental studies. Serum enterolactone concentration rather weakly reflected recent intake of lignans but more strongly the consumption of lignan-containing foods, i.e. fruit and berries and vegetables.
3. Serum enterolactone concentration had a positive association with self-reported constipation in both genders. In addition, it was positively associated with age and negatively associated with smoking and BMI in women. The modest proportion of serum enterolactone concentration explained by the variables examined suggests the existence of other, still unknown, determinants.
4. Serum enterolactone concentration was negatively associated with the number of antimicrobial treatments in both genders. In addition, it had a positive association with the length of time from last antimicrobial treatment in men. These findings support the central role of gut microflora in the bioavailability of lignans.
5. Serum enterolactone concentration was not protectively associated with risk of breast or prostate cancers.

9 FUTURE DIRECTIONS

Large gaps remain in the knowledge of lignans and their impact on human health. The following topics should be considered for future research:

1. The metabolism of lignans is still poorly understood, and therefore, studies on lignan bioavailability and stability in plasma, their distribution in the human body, and their concentrations in different tissues are needed.
2. The normal ranges of serum enterolactone concentration in different populations should be studied in large representative population samples.
3. Uncovering new determinants of serum enterolactone concentration requires further studies. This also includes updating lignan databases to include new enterolactone precursors.
4. Limited understanding of biological effects and mechanisms of action of enterolactone warrant more experimental studies, especially at concentrations of enterolactone commonly measured in populations. This should also include dose-response studies to clarify the concentration needed to achieve the desired effect.
5. More prospective epidemiological studies are required to clarify the association between serum enterolactone concentration and risk of hormone-dependent cancers and other chronic diseases.
6. If convincing evidence of the association between high lignan exposure and reduced risk of chronic diseases is obtained, clinical trials in humans are warranted to establish the beneficial effects of lignans on these diseases.

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