SERUM ENTEROLACTONE AS A BIOLOGICAL MARKER AND IN BREAST CANCER: FROM LABORATORY TO EPIDEMIOLOGICAL STUDIES

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ACADEMIC DISSERTATION

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To Erik and Oskar

List of original contributions

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

I   Stumpf K, Uehara M, Nurmi T, Adlercreutz H. Changes in the time-resolved fluoroimmunoassay of plasma enterolactone (2000). Anal Biochem 284(1): 153-157.

II   Stumpf K, Adlercreutz H. Short-term variations in enterolactone in serum, 24-hour urine, and spot urine and relationship with enterolactone concentrations (2003). Clin Chem 49(1): 178-181.

III*   Kilkkinen A, Stumpf K, Pietinen P, Valsta LM, Tapanainen H, Adlercreutz H. Determinants of serum enterolactone concentration (2001). Am J Clin Nutr 73(6): 1094-1100.

IV   Stumpf K, Pietinen P, Puska P, Adlercreutz H. Changes in serum enterolactone, genistein, and daidzein in a dietary intervention study in Finland (2000). Cancer Epidemiol Biomarkers Prev 9(12): 1369-1372.

V   Pietinen P, Stumpf K, Männistö S, Kataja V, Uusitupa M, Adlercreutz H. Serum enterolactone and risk of breast cancer: a case-control study in eastern Finland (2001). Cancer Epidemiol Biomarkers Prev 10(4): 339-344.

In addition, some unpublished data are included.

*This article also appears in the thesis of Annamari Kilkkinen.

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Contents

Abbreviations

BMI   Body mass index
BSA   Bovine serum albumin
CEAD   Coulometric electrode array detection
CI   Confidence interval
CV   Coefficient of variation
DMBA   7,12-dimethylbenzanthracene
EGF   Epidermal growth factor
END   Enterodiol (lignane-3,3',9,9'-tetraol)
ENL   Enterolactone (3,3'-dihydroxylignano-9,9'-lactone)
FFQ   Food frequency questionnaire
GC   Gas chromatography
HMR   7'-Hydroxymatairesinol (4,4',7'-trihydroxy-3,3'-dimethoxylignano-9,9'-lactone)
HPLC   High-performance liquid chromatography
ICC   Intra-class correlation coefficient
IGF-I   Insulin-like growth factor I
IMS   Ion mobility spectrometry
LAR   Lariciresinol (3,3'-dimethoxy-7,9'-epoxylignane-4,4',9-triol)
MS   Mass spectrometry
MAT   Matairesinol (4,4'-dihydroxy-3,3'-dimethoxylignano-9,9'-lactone)
NADPH   Nicotinamide adenine dinucleotide phosphate (reduced form)
NMU   N-methyl-N-nitrosourea
OR   Odds ratio
PIN   Pinoresinol (3,3'-dimethoxy-7,9':7',9-diepoxylignane-4,4'-diol)
SEC   Secoisolariciresinol (3,3'-dimethoxy-lignane-4,4',9,9'-tetraol)
SHBG   Sex hormone binding globulin
SYR   Syringaresinol (3,3',5,5'-tetramethoxy-7,9':7',9-diepoxylignane-4,4'-diol)
TR-FIA   Time-resolved fluoroimmunoassay

Abstract

Objective:
Enterolactone, a mammalian lignan, is produced by colonic microflora from its precursors present widely in food plants. Experimental studies in animals and *in vitro* suggest that enterolactone has anticarcinogenic activity. Here, a revised methodology for serum enterolactone analysis is presented. In addition, the role of enterolactone as a biological marker and the association between serum enterolactone and breast cancer risk in a case-control study of Finnish women were investigated.

Time-resolved fluoroimmunoassay for serum enterolactone:
In its previous version, excessive amounts of enzyme in hydrolysis disturbed the assay. This study presents a new version of the assay, which exhibits a good correlation with the reference method of gas chromatography-mass spectrometry. This revised method proved to be a functional tool in epidemiological studies. A fully automated short time-resolved fluoroimmunoassay for serum enterolactone using minute sample amounts is also described.

Enterolactone as a biological marker:
In our cross-sectional survey of 2383 Finnish adults, we found a large between-subject variation in serum enterolactone concentration 0-180 nmol/L. The median serum enterolactone concentration in men was 14 nmol/L, and in women 17 nmol/L.

The short-term within-subject variation in enterolactone was studied in serum, in 24-hour urine, and in the spot urine enterolactone:creatinine ratio in 19 volunteers. The variation was large in both serum and urine enterolactone measurements. To estimate enterolactone concentration within ±50% with 80% confidence requires three serum or 24-hour urine samples, but 10 spot urine samples.

In women, serum enterolactone concentration was positively and independently associated with vegetable consumption, age, and constipation, and negatively associated with smoking. In men, multiple regression analyses showed positive, independent associations with consumption of whole-grain products, fruit, and berries, and with constipation. However, diet and health-related variables appeared to explain only a small part of the vast variation between subjects. A 12-week dietary intervention study with 85 subjects was able to raise the median concentration of enterolactone from 12 to 20 nmol/L (p = 0.002) with the aid of a low-fat, high-vegetable diet.

Enterolactone in breast cancer:

In a Finnish case-control study of 194 breast cancer cases and 208 controls, serum enterolactone was inversely associated with breast cancer risk. The odds ratio in the highest quintile of enterolactone concentrations adjusted for all known risk factors for breast cancer was 0.38 (95% CI 0.18-0.77, p=0.03) compared with the lowest quintile.

Conclusions:

Time-resolved fluoroimmunoassay is a useful method for serum enterolactone analysis. To obtain reliable data epidemiological studies should measure enterolactone in multiple serum samples. Due to numerous confounding factors, the value of serum enterolactone measurement as a biological marker of diet is limited. In this case-control study serum enterolactone was inversely associated with breast cancer risk.

Tiivistelmä (Finnish abstract)

Enterolaktoni on lignaaneihin kuuluva yhdiste. Monet ravintona käytettävistä kasvikunnan tuotteista sisältävät enterolaktonin esiasteita, joista suoliston bakteerikanta tuottaa enterolaktonia. Kokeelliset eläin- ja solukokeet osoittavat, että enterolaktonilla saattaa olla syövältä suojaavia vaikutuksia. Tässä tutkimuksessa kehitettiin määritysmenetelmää seerumin enterolaktonipitoisuudelle, tutkittiin enterolaktonipitoisuuksien vaihtelua ja pitoisuutta selittäviä tekijöitä suomalaisväestössä, ja selvitettiin seerumin enterolaktonipitoisuuden yhteyttä rintasyöpäriskiin.

Seerumin enterolaktonin aikarajoitteinen fluoroimmunomääritys:
Tässä työssä esitettiin aikarajoitteisen fluoroimmunomäärityksen uusi versio. Uuden version tulokset korreloivat hyvin viitemenetelmän (kaasu kromatografia yhdistettynä massaspektrometriaan) kanssa. Lisäksi työssä osoitettiin, että edeltävän version virheelliset tulokset johtuivat hydrolyysissä käytetystä suuresta entsyymimäärästä, joka häiritsi määritystä. Uusi menetelmä sopii hyvin epidemiologisiin tutkimuksiin, joissa näytteiden lukumäärä usein on suuri. Työssä esitettiin myös täysin automatisoitu pikamenetelmä, jota voidaan käyttää hyvin pienillä näytemäärillä.

Enterolaktoni biologisena merkkiaineena:
Yli 2000 suomalaisen aikuisen poikkileikkaustutkimus osoitti, että seerumin enterolaktoni pitoisuudella on suuri vaihteluväli, 0-180 nmol/l. Enterolaktonin mediaanipitoisuus miehillä oli 14 nmol/l, ja naisilla 17 nmol/l. Naisilla seerumin enterolaktonipitoisuuteen vaikuttavia tekijöitä olivat ikä, ummetus ja tupakointi, sekä ravintotekijöistä vihannesten kulutus. Miehillä pitoisuuteen vaikuttivat täysjyvätuotteiden ja marjojen kulutus sekä ummetus. Yllätävää oli, että ruokavaliolla ja terveyteen liittyvillä muuttujilla pystyttiin selittämään vain pieni osa seerumin enterolaktonipitoisuuksien laajasta vaihtelusta.

Enterolaktonin seerumipitoisuuden, erityksen vuorokausivirtsaan ja kertavirtsanäytteen enterolaktoni:kreatiniini suhteen lyhyen aikavälin yksilönsisäistä vaihtelua tutkittiin 19 vapaaehtoisella. Vaihtelu oli huomattavaa kaikissa näytemuodoissa. Vallitsevan pitoisuuden arviointi ±50% tarkkuudella ja 80% varmuudella vaati kolme seerumi- tai vuorokausivirtsanäytettä, mutta kymmenen kertavirtsanäytettä.

Ruokavalion muuttaminen vähentämällä rasvan osuutta ja lisäämällä kasvisten kulutusta nosti seerumin enterolaktonin mediaanipitoisuutta 85 koehenkilöllä 12:sta 20:een nmol/l (p = 0.002).

Enterolaktoni ja rintasyöpä:
Seerumin enterolaktonipitoisuuden ja rintasyövän välistä yhteyttä tutkittiin tapaus-verrokki tutkimuksessa, johon osallistui 194 rintasyöpään sairastunutta naista ja 208 tervettä suomalaisnaista. Tutkimus osoitti, että niillä naisilla, joilla seerumin enterolaktonipitoisuus oli matala, oli suurentunut riski sairastua rintasyöpään.

Päätelmät:
Seerumin enterolaktonipitoisuuden aikarajoitteinen fluoroimmunomääritys on käyttökelpoinen menetelmä seerumin enterolaktonin analytiikassa. Koska enterolaktonipitoisuus vaihtelee suuresti päivästä toiseen, luotettavien tulosten saamiseksi pitäisi epidemiologisissa tutkimuksissa jokaisesta koehenkilöstä olla käytettävissä useita seeruminäytteitä. Lukuisista sekoittavista tekijöistä johtuen seerumin enterolaktonipitoisuus ei ole käyttökelpoinen ravinnonkäytön merkkiaine. Tapausverrokki tutkimuksessa matalaan seerumin enterolaktonipitoisuuteen liittyi suurentunut rintasyöpäriski.

Introduction

Breast cancer is the predominant cancer type in women in Finland and in other industrialized countries. Since the 1950s, the age-adjusted incidence of breast cancer has increased approximately 1% per year. In 2004, over 3800 women were predicted to be diagnosed with breast cancer (Finnish Cancer Registry 2004). Several factors have been associated with an increased risk of breast cancer. Family history accounts for about 10% of all cases. Both endogenous and exogenous hormonal factors, including early menarche and late menopause, nulliparity or late age at first full-term pregnancy, postmenopausal obesity, and hormone replacement therapy, as well as a low level of physical activity increase the risk of breast cancer. However, in at least 50% of breast cancer cases, none of these risk factors applies, and the etiology remains obscure (Johnson-Thompson *et al.* 2000). Diet could be an important component in explaining the unknown etiology. Alcohol intake is the best-established specific dietary risk factor for breast cancer; even moderate alcohol intake increases the risk of the disease. Results for the alleviating impact of vegetables, fruit, and dietary fiber are inconsistent, and at most suggest a modest reduction in the risk of breast cancer (Willett 2001).

Lignans, plant-derived compounds of dietary origin, are mainly found in wholegrains, berries, and some fruits and vegetables (Mazur *et al.* 1998). Already in the early 1980s, excretion of enterolactone (ENL, 3,3'-dihydroxylignano-9,9'-lactone), the most abundant lignan in humans, was observed to be lower in women with diagnosed breast cancer than in healthy women (Adlercreutz *et al.* 1982). Two hypothesis were presented: lignans are the protective compounds in wholegrains, berries, and some fruits and vegetables (Adlercreutz *et al.* 1982), and ENL is a biological marker of a healthy vegetable- and grain-based diet (Adlercreutz 1984). This work searched for answers to either support or oppose these hypotheses.

The first step here was to establish a method suitable for the analysis of ENL in large epidemiological studies. Time-resolved fluoroimmunoassay (TR-FIA) is a technique that offers specificity, a high rate of sample throughput, and comparatively low costs (Hemmilä 1988, Dickson *et al.* 1995). This work is a continuation of the earlier effort to establish a functioning TR-FIA for serum ENL (Adlercreutz *et al.* 1998).

After a suitable method was devised, the characteristics of ENL as a biological marker were examined. This included analyses of the biological variation. Knowledge of the magnitude of biological variation is essential when conducting epidemiological studies because analytes with large within-subject variation almost always show weak associations with any given disease (blurring = bias towards the null) (Vineis 1997). Before the present study, no information on the biological variation of ENL measurements in Finnish subjects existed. In

addition, this work studied the role of diet on serum ENL levels in cross-sectional and intervention studies. Cross-sectional analysis of the determinants of serum ENL concentration reveals, whether ENL serves as a biological marker of any food item(s) and uncover possible confounding factors. In light of low serum ENL concentration being a risk factor of many diseases, it is important to determine whether dietary changes can raise ENL levels in a population. This was examined by the analyzing serum ENL in a dietary intervention study of a Finnish population.

Finally, the relationship between ENL and the risk of breast cancer was elucidated in a case-control study of a population in northeastern Finland.

Review of the literature

Lignans

Definition

The term "lignan" was introduced in 1936 for a class of compounds with a common feature of two C_6C_3 (phenyl propane) units linked at the central carbon atom of the side chain (Howarth 1936). Lignan structures can be divided into dibenzylbutanes, bibenzylbutyrolactones, tetrahydrofurans, arylnaphtalenes, furo-furans, and tetrahydrofurans (Ayres *et al.* 1990). Lignans are often classified as mammalian or plant lignans. Mammalian lignans have been identified in mam-malians only and they are formed from plant lignans by the intestinal microflora.

Plant lignans

In plants, lignans are involved in plant defense, having antioxidant, antifungal, antibacterial, antiviral, insect antifeedant, phytotoxic, and cytotoxic properties (Kwon *et al.* 2001). Many drugs in traditional oriental medicine contain lignans in high quantities (Nishibe 1993). Some natural lignans have clinical use also in Western medicine, such as the antiviral podophyllotoxin for the treatment of venereal warts (Ayres *et al.* 1990).

Lignans occur widely in nature (Davin *et al.* 2000) and are therefore ubiqui-tous in the human diet. Matairesinol (MAT, 4,4'-dihydroxy-3,3'-dimethoxy-lignano-9,9'-lactone) and secoisolariciresinol (SEC, 3,3'-dimethoxy-lignane-4,4',9,9'-tetraol) have been quantified in numerous food items (Mazur *et al.* 1998). In Western diets, SEC is more abundant than MAT. In the Finnish diet the main sources of SEC are fruit and berries, whereas MAT mostly derives from whole grains, mainly rye (Kilkkinen *et al.* 2003a). The main sources of MAT and SEC for American women are fruits, grain products, and berries (de Kleijn *et al.* 2001), and for Dutch women grain products, fruit, and wines (Boker *et al.* 2002). The SEC content of flaxseed is exceptionally high (Mazur *et al.* 1998).

Knowledge of the occurrence of other lignans in foods is limited; whole grain is a rich source of pinoresinol (PIN, 3,3'-dimethoxy-7,9':7',9-diepoxylignane-4,4'-diol), syringaresinol (SYR, 3,3',5,5'-tetramethoxy-7,9':7',9-diepoxylignane-4,4'-diol), and lariciresinol (LAR, 3,3'-dimethoxy-7,9'-epoxylignane-4,4',9-tri-ol) (Heinonen *et al.* 2001), and PIN is present in high quantities in olive oil (Owen *et al.* 2000). Thus far, no study has identified ENL or enterodiol (END, lignane-3,3',9,9'-tetraol) in plants.

Mammalian lignans

ENL and END were the first lignans identified in mammals (Setchell *et al.* 1980b, Stitch *et al.* 1980). Instead of being endogenously synthesized, they are of

dietary origin, with plant lignans as their precursors (Axelson *et al.* 1982). No lignan biosynthesis is known to occur in mammals.

Dietary precursors of mammalian lignans

To date, five dietary lignans have been identified as ENL precursors (Fig. 1): SEC (Axelson *et al.* 1982), MAT (Borriello *et al.* 1985), LAR, SYR, and PIN (Heinonen *et al.* 2001). 7'-Hydroxymatairesinol (HMR, 4,4',7'-trihydroxy-3,3'-dimethoxylignano-9,9'-lactone) (Saarinen *et al.* 2000) and arctigenin (Heinonen *et al.* 2001) are converted to mammalian lignans, but they are not known to occur in plants commonly used as human foods.

Lignans and intestinal microflora

Plant lignans occur as glycosides and their bioavailability requires hydrolysis of the sugar moiety for uptake into the circulation. Hydrolysis may occur in the intestinal lumen by bacterial β-glucosidases or in enterocytes by cytosolic β-glucosidases (Setchell *et al.* 2002). In the intestine, plant lignans become substrates for the metabolism to mammalian lignans. According to studies in pigs, metabolism of plant lignan glucosides to END and ENL (Glitsø *et al.* 2000) and absorption of END and ENL occur in the large intestine (Bach Knudsen *et al.* 2003). Free plant lignans may also be absorbed unchanged, but they are present in urine only in small quantities, in contrast to mammalian lignans, which form the majority of circulating and urinary lignans (Bannwart *et al.* 1984, 1989, Nurmi *et al.* 2003a).

The conversions of plant lignans to END and ENL, and of END to ENL are not spontaneous, but require the presence and activity of gut bacteria (Axelson *et al.* 1981, Setchell *et al.* 1981, Borriello *et al.* 1985). END and ENL production occurs under both aerobic and anaerobic conditions (Borriello *et al.* 1985). Bacterial strains responsible for the conversion of plant lignans to ENL are not fully identified. One study suggested an essential role for Clostridia (Setchell *et al.* 1981), but this was not confirmed (Borriello *et al.* 1985). Subsequent studies have identified the Gram-positive anaerobic species *Peptostreptococcus* and *Eubacterium*, which are capable of transforming SEC to END (Wang *et al.* 2000), and *Enterococcus faecalis*, which is responsible for the transformation of (+)-PIN to (+)-LAR (Xie *et al.* 2003). The microflora influences the ratio of ENL to END formed in the gut (Setchell *et al.* 1981). In Western populations, ENL seems to be 3-12 times more abundant than END (Adlercreutz *et al.* 1986a, Kirkman *et al.* 1995, Lampe *et al.* 1999). In a study with five men, the production of END recovered more rapidly after antimicrobial treatment than that of ENL, and the relative abundance of END became greater (Setchell *et al.* 1981, Adlercreutz *et al.* 1986a).

Recovery of mammalian lignans

In a metabolic study with pigs, the conversion of mammalian lignan precursors to ENL was on average 48% and 60% with two different diets. The calculated

Fig. 1. Metabolism of plant lignans to END and ENL.

Pinoresinol   **Lariciresinol**   **Secoisolariciresinol**   **Matairesinol**

Syringaresinol   **Enterodiol**   **Enterolactone**

References: Heinonen *et al. 1999.*
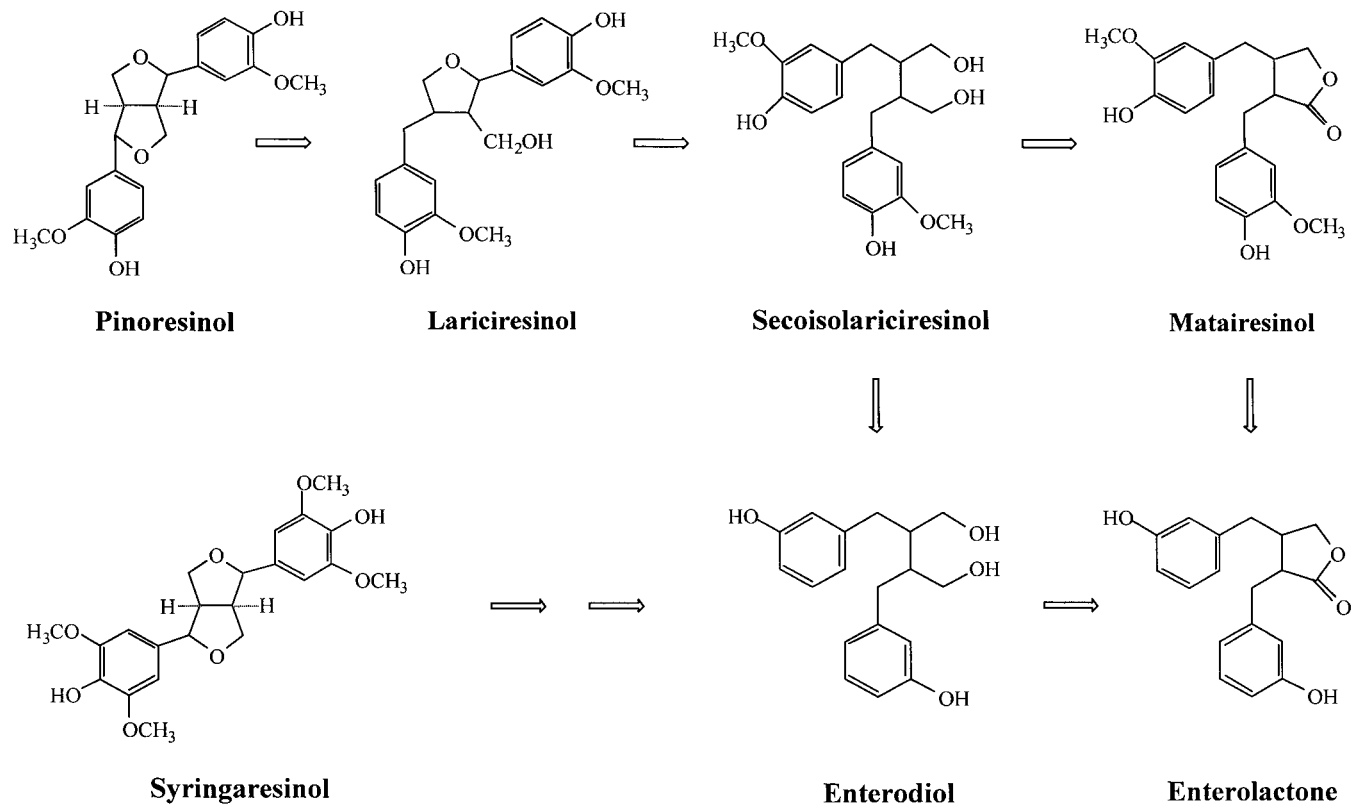
bioavailability of MAT and SEC was higher (75-77%) than that of LAR, PIN, and SYR (10-19%) (Bach Knudsen *et al.* 2003). In human subjects, the recovery of dietary lignans in urine varies widely (Nesbitt *et al.* 1999, Mazur *et al.* 2000). *In vitro*, after incubation of plant lignans for 24 h in human fecal inoculum, the recovery was dependent on the individual differences of the fecal flora and on the compound of interest, ranging from 4% (SYR) to 101% (LAR) (Heinonen *et al.* 2001). The recovery may also vary due to individual differences in phase I and II metabolism of lignans.

Serum enterolactone as a biological marker

What is a biological marker?

Definition

A biological marker, or commonly abbreviated to biomarker, is any substance or process that can be measured in the human body or in its products and may influence or predict the incidence of disease (Toniolo *et al.* 1997). A biological marker is not a diagnostic test but an indicator of an increased or decreased risk of disease (Grandjean 1995).

Application of biological markers in epidemiology

Laboratory studies form the basis for research of biological markers by creating a method for the measurement of the marker. Transitional studies provide a bridge between the use of biological markers in laboratory experiments and their use in cancer epidemiology (Schulte *et al.* 1997) (Table 1).

Table 1. Study design for application of biological markers in epidemiology.

STUDY DESIGN	AIM
1. Laboratory studies	To develop a method for measurement of the marker
2. Transitional studies	To determine
Developmental studies	Biological relevance in disease development
	Pharmacokinetics of the marker
	Optimal conditions for collecting and storing biological specimens
Characterization studies	Levels of marker in a population and its subgroups
	Components of variation and the reliability of the measurement
	Factors confounding and modifying the level of the marker
Applied transitional studies	Relationship between the marker and exposure
	Whether intervention is efficacious in changing the level of the marker
3. Etiological studies:	To determine
Observational studies	
Ecological	Geographical differences
	Timetrends in the level of the marker and in disease prevalence and incidence
Case-control and cohort	Association between marker and risk of disease
Experimental studies	Causality
4. Public health applications	To affect policy-making decisions
	To incorporate biomarkers in
	Screening
	Clinical prevention

References: Rothman et al. 1995, Toniolo et al. 1997

Laboratory studies – methods available for serum enterolactone measurements

Chromatographic methods

Several chromatographic methods have been developed for the analysis of ENL in plasma. The first methods were based on gas chromatography (GC) with mass (MS) or ion mobility (IMS) spectrometry. These methods face the problem of complex sample pretreatment and require a large plasma volume for the analysis. Later methods that utilize high-performance liquid chromatography (HPLC) combined with coulometric electrode array detection (CEAD) or MS have been able to escape these problems. The available methods and their sample volume requirements are summarized in Table 2.

Table 2. Chromatographic methods for the analysis of ENL in serum/plasma.

Reference	Instrumentation	Sample volume required (mL)
Setchell *et al*. 1983	GC-MS[1]	5.0-10
Adlercreutz *et al*. 1993b	GC-MS/SIM	4.0
Atkinson *et al*. 1993	GC-IMS	1.0
Gamache *et al*. 1998	HPLC-CEAD	0.20
Nurmi *et al*. 1999	HPLC-CEAD	0.50
Valentin-Blasini *et al*. 2000	HPLC-MS/MS	1.0
Franke *et al*. 2002	HPLC-DAD-MS	0.45
Grace *et al*. 2003	HPLC-MS/MS	0.20
Smeds *et al*. 2003	HPLC-MS/MS	0.30

Abbreviations: GC, gas chromatography; MS, mass spectrometry; SIM, selected ion monitoring; IMS, ion mobility spectrometry;
HPLC, high-performance liquid chromatography; CEAD, coulometric electrode array detection; DAD, diode array detection

Immunological methods

Immunological methods allow the generation of highly automatic and rapid yet specific analyses. The only available immunological method for measuring ENL in serum is TR-FIA (Adlercreutz *et al*. 1998). TR-FIA combines the advantages of nonradioisotopic assays, high sensitivity, and low background interference (Hemmilä 1988, Dickson *et al*. 1995). In the assay, a synthetic ENL derivative coupled with a Europium label competes for binding to the antiserum with the ENL present in the sample. After the reaction is completed, the bound Europium ions are dissociated from the chelates by enhancement solution. In solution, Europium ions form highly fluorescent complexes that can be measured by fluorometry.

TR-FIA for serum ENL had already been published (Adlercreutz *et al.* 1998) and taken into use by the authors before this work. The publication describes the early, original version of the assay, although this was no longer in use at the time of publication. Already before the publication, creation of a sample pretreatment that is convenient in practical work became necessary. This was achieved by reducing the volume of the hydrolysis reagent, which enabled hydrolysis and ether extraction to be performed in disposable glass tubes. The volume of diethyl ether was simultaneously reduced, shortening the time required for the extraction. This work started with the unexpected finding that these two versions of the assay gave unequal results.

Transitional studies

Metabolism of enterolactone

Kinetics
The elimination half-life of ENL in the human body is unknown. This may partly depend on the lack of sufficiently large amounts of pure ENL. A single dose of lignan-rich food increased ENL concentration in plasma continuously, with no peak, up to the last time-point (24 h) (Mazur *et al.* 2000, Morton *et al.* 1997a, Nesbitt *et al.* 1999). In fecal incubations of SEC diglycoside, demethylated and dehydroxylated metabolites first appeared after 48 h (Wang *et al.* 2000). In pigs, serum ENL concentration reached a steady state of ~400 nmol/L three days after the introduction of a lignan-rich diet (Bach Knudsen *et al.* 2003).

Phase I metabolism
Phase I metabolism of ENL occurs before conjugation (Phase II metabolism) in microsomal enzymes, which are present in the highest amounts in the liver and gastrointestinal tract. Several oxidative metabolites of ENL and END have been detected in human urine (Fig. 2) (Jacobs *et al.* 1999a). *In vitro*, human microsomes were unable to produce two of the compounds (4-OH-ENL and 4'-OH-ENL), and these seem to be intermediates of the bacterial conversion of SEC and/or MAT to ENL (Jacobs *et al.* 1999b). The proportion of the Phase I metabolites of ENL and END is estimated to be <5% of all lignans present in urine (Jacobs *et al.* 1999a). The microsomal incubation of END did not yield ENL, indicating that this oxidation only occurs via intestinal bacteria.

Phase II metabolism
Free, absorbed lignans are readily conjugated to glucuronides and sulfates, which increases their water solubility and decreases the passive penetration of cell membranes. The conjugation is achieved with specific enzymes: cytosolic sulfotransferases and glucuronosyltransferases (Ritter 2000, Glatt *et al.* 2001). The enzymes are present ubiquitously in tissues but are enriched in the intestinal wall and in the liver. *In vitro*, HepG2 hepatic cancer cells as well as MCF-7 breast

Fig. 2. Phase I metabolism of END and ENL.

Enterodiol

Hydroxylated
aliphatic
metabolites

Enterolactone
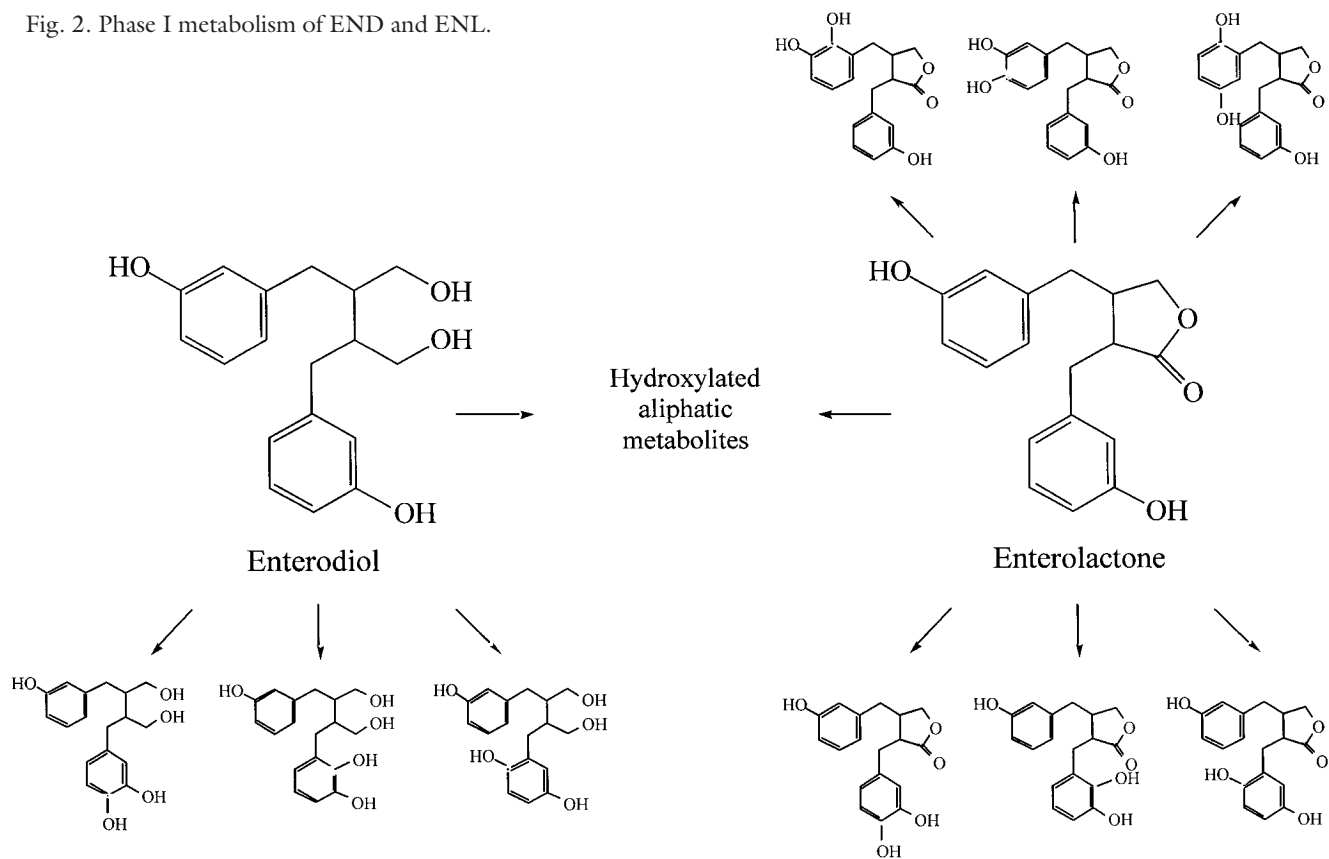
References: Jacobs et al. 1999a, 1999b.

cancer cells conjugate ENL effectively (Adlercreutz *et al.* 1992, Mousavi *et al.* 1992). The activity of the subtypes of sulfotransferases and glucuronosyltransferases shows marked individual variation (Glatt *et al.* 2001), resulting in differences in the proportions of ENL glucuronide and sulfate conjugates in human plasma (Adlercreutz *et al.* 1993b) and urine (Adlercreutz *et al.* 1995b).

Distribution

After absorption and first-pass metabolism in the intestinal tract and in liver, lignans are distributed throughout the body via circulation. In plasma, 55-95% (mean 79%, n=28) of ENL occur as glucuronides, and 5-45% (mean 21%) are present in free form or as sulfates (Adlercreutz *et al.* 1993b). In circulation, ENL may bind to plasma proteins. *In vitro,* free ENL and END in >1 µmol/L concentrations displace endogenous steroid hormones from sex hormone binding globulin (SHBG) (Martin *et al.* 1996, Schottner *et al.* 1998). From blood vessels, lignans may enter several tissues. ENL in human subjects occurs in some tissues in relatively high quantities: prostate tissue 93±25 nmol/L (mean±standard error), n=10 (Hong *et al.* 2002), prostatic fluid 230 (0-2100) nmol/L (mean (range)), n=83 (Morton *et al.* 1997a), and semen 180 (63-560) nmol/L (mean (range)), n=6 (Dehennin *et al.* 1982). ENL is also present in breast tissue (mean 20 pmol/g, n=14) (Hargreaves *et al.* 1999). It accumulates in breast cyst fluid. In type I cysts, the mean ENL concentration was 140 nmol/L (standard error 12) nmol/L, n=141, in type II cysts 17 (5.7) nmol/L, n=50 (Boccardo *et al.* 2003). The disposition of the metabolites of ^{3}H-SEC diglycoside has been studied in rats; the main targets were the liver, kidney, gastrointestinal tract, uterus, and ovaries. Chronic (>10 d) exposure to lignans resulted in increased lignan disposition in liver and adipose tissue compared with acute exposure (Rickard *et al.* 1998).

Excretion

Conjugated lignans can be excreted to either urine or bile. Of the urinary lignans, 84-99% (mean 92%, n=6) occur as glucuronides (Adlercreutz *et al.* 1995b). Direct evidence on the excretion of conjugated ENL and END into the bile exists only for rats, in which the amounts of ENL excreted into bile and urine are almost equal (Axelson *et al.* 1981). In the intestine, the excreted lignans can be reabsorbed (enterohepatic circulation) or excreted in feces. In rats treated with ^{3}H-SEC diglycoside, >50% of radioactivity was found in feces and 28-32% in urine at 48 h (Rickard *et al.* 1998). Of the fecal lignans, the majority occur as plant lignans and END (Adlercreutz *et al.* 1995a).

Potential mechanisms of action of enterolactone, *in vitro* and *in vivo*

Estrogenic and antiestrogenic effects in vitro

1. Activation of estrogen receptors

Most *in vitro* studies have examined the estrogenicity of ENL by a somewhat unspecific measurement, by studying the effect of ENL on the growth of

estrogen-dependant breast cancer cells. The results are diverse, and here this issue is simplified by considering only the results obtained with ENL in possibly physiological ≤1 µM concentrations. At these concentrations, ENL alone had either a stimulatory or no effect on cell growth on estrogen dependant cell lines MCF-7 and T47D (Welshons *et al.* 1987, Mousavi *et al.* 1992, Wang *et al.* 1998a). In the presence of estradiol, ENL either inhibited (Mousavi *et al.* 1992) or had no effect (Wang *et al.* 1998a) on cell growth. ENL mildly increased estrogen-responsive pS2 protein expression, a measurement perhaps more specific to estrogen receptor activation (Sathyamoorthy *et al.* 1994). A later study, by contrast, found no transcriptional response of either human estrogen receptor α or β. The presence of estradiol did not alter the results (Saarinen *et al.* 2000).

2. Inhibition of estrogen synthetase (aromatase)

Aromatase belongs to the cytochrome P450 enzyme system and catalyzes the conversion of androgens to estrogens (androstenedione to estrone and testosterone to estradiol). *In vitro* ENL has been able to inhibit human aromatase in placental microsomes (Adlercreutz *et al.* 1993a) and several cell lines (Adlercreutz *et al.* 1993a, Wang *et al.* 1994, Saarinen *et al.* 2002a). Concentrations needed for 50% enzyme inhibition were 14 µM in placental microsomes (Adlercreutz *et al.* 1993a), 74 µM in human preadipocytes (Wang *et al.* 1994), and 8.9 mM in embryonic kidney cells (Saarinen *et al.* 2002a). At a 1 µM concentration, ENL reached 22-27% inhibition of aromatase in human chorion carcinoma cells (Adlercreutz *et al.* 1993a), 3% inhibition in preadipocytes (Wang *et al.*1994), and 6% inhibition of placental aromatase (Adlercreutz *et al.* 1993a).

3. Inhibition of 17β-hydroxysteroid dehydrogenase

17β-hydroxysteroid dehydrogenase is the enzyme responsible for the conversion of estrone to estradiol and androstenedione to testosterone. At 100 µM, ENL showed a 98% inhibition of the enzyme in human genital skin fibroblasts (Evans *et al.* 1995). No information exists on the inhibitory effect of ENL at lower, physiological concentrations.

4. Stimulation of SHBG synthesis

ENL stimulated SHBG synthesis in HepG2 cells in a dose-dependant manner at all concentrations tested (starting at 1 µM) (Adlercreutz *et al.* 1992).

Estrogenic and antiestrogenic effects in vivo

1. Animal studies

The estrogenic activity of ENL has been assessed in animal studies by its impact on uterine weight. In immature female mice and rats, neither subcutaneous ENL nor oral HMR demonstrated any detectable effect on uterine growth (Setchell *et al.* 1981, Saarinen *et al.* 2001). In contrast, chronic treatment with ENL showed antiestrogenic effects in rats by reducing relative uterine weight (Saarinen *et al.* 2002a). The mechanism behind this finding remains unclear. In a short-term experimental study by the same authors, ENL and other lignans showed no aromatase inhibition (Saarinen *et al.* 2002a).

2. Human studies

In a cross-sectional study with 34 premenopausal women, urinary ENL excretion correlated negatively with plasma percentage of free estradiol, and END with plasma free estradiol. Plasma SHBG had a positive correlation with urinary ENL excretion (Adlercreutz *et al.* 1987). In postmenopausal women, a dietary intervention with 5 or 10 g of flaxseed per day reduced serum concentrations of 17β-estradiol and estrone sulfate (Hutchins *et al.* 2001). The changes in sex hormone levels may result from the effects of ENL on estrogen biosynthesis or from the increased fecal excretion of estrogens due to the dietary fiber associated with the lignans (Goldin *et al.* 1981). In premenopausal women, by contrast, 10 g of flaxseed per day did not affect sex hormone or SHBG levels (Phipps *et al.* 1993).

Antioxidativity
1. *In vitro*

Free ENL and END have shown antioxidant activity *in vitro* only at supraphysiological concentrations (Kitts *et al.* 1999, Pool-Zobel *et al.* 2000, Prasad 2000). When the potency of ENL to inhibit lipid peroxidation in rat liver microsomes was examined, a 50% inhibitory effect was achieved at a concentration of 16 μM (Saarinen *et al.* 2000).

2. *In vivo*

Research on the ability of ENL to function as an antioxidant *in vivo* is sparse. A study on ENL and F_2-isoprostanes, a measure of lipid peroxidation, suggested an antioxidant activity of ENL. A low serum ENL concentration was associated with increased plasma F_2-isoprostanes in a cross-sectional study of 100 Finnish men. The association between ENL and F_2-isoprostanes remained after adjustment for selenium and alcohol intake and plasma concentrations of homocysteine, alpha-tocopherol, β-carotene, ascorbic acid, and dietary folate (Vanharanta *et al.* 2002b). In contrast, a dietary intervention with rye crisp breads did not affect the antioxidative capacity of plasma. An explanation for the negative result may be that the average serum ENL concentrations were low and changed only slightly, from 8.8 to 10.7 nmol/L (Pool-Zobel *et al.* 2000).

Effects on other hormones, enzymes, and growth factors
1. Prolactin

In 28 postmenopausal women, consumption of 10 g of flaxseed for seven weeks increased serum prolactin concentration by an unknown mechanism (Hutchins *et al.* 2001). The same effect was not observed in premenopausal women (Phipps *et al.* 1993), nor was statistical significance reached in rats treated with peroral ENL (Saarinen *et al.* 2002a).

2. 5α-reductase

5α-reductase converts testosterone into a more potent androgen, 5α-dihydro-testosterone. ENL was shown to be an inhibitor of the enzyme in benign prostate hyperplasia tissue homogenate with an IC_{50} concentration of 14 μM (Evans *et al.* 1995).

3. Phase II detoxifying enzymes

Many potentially carcinogenic compounds become relatively inert after conjugation with detoxifying phase II enzymes, such as NADPH (reduced form of nicotinamide adenine dinucleotide phosphate):quinone reductase (Ross *et al.* 2000). ENL effectively induced NADPH:quinone reductase in human Colo205 colon carcinoma cells at concentrations ≥0.1 µM (Wang *et al.* 1998b).

4. Growth factors

High plasma levels of insulin-like growth factor I (IGF-I) have been linked to increased risk of several cancer types, including breast, prostate, and colorectal cancers (Giovannucci 1999). *In vivo*, an intake of flaxseed or purified SEC diglycoside in rats reduced plasma IGF-I concentrations (Rickard *et al.* 2000). In addition, dietary flaxseed was able to reduce the expression of IGF-I, epidermal growth factor (EGF) receptor, and extracellular levels of vascular endothelial growth factor in a primary tumor site of established human breast cancer in nude mice (Chen *et al.* 2002, Dabrosin *et al.* 2002). In MDA-MB-468 breast cancer cells, however, ENL demonstrated no effect on EGF transcription (Schultze-Mosgau *et al.* 1998).

Adverse effects and toxicity

1. Genotoxicity

In most studies, no signs of genotoxicity have been observed at ≤100 µM ENL (Setchell *et al.* 1981, Kulling *et al.* 1998, Pool-Zobel *et al.* 2000). One study did, however, show toxicity of ENL on human colon carcinoma cell growth at concentrations of ≥1 µM (Wang *et al.* 1998b).

2. Reproductive health

  a. Menstrual cycle

   A 10-g daily dose of flaxseed in healthy women caused longer luteal phase lengths and higher luteal phase progesterone/estradiol ratios and midfollicular phase testosterone concentrations than a control diet, but no anovulatory cycles (Phipps *et al.* 1993). By contrast, rats exposed to 10% flaxseed either at an early age, during pregnancy and lactation, or in adulthood had an increased risk of menstrual irregularities or persistent diestrus (Orcheson *et al.* 1998, Tou *et al.* 1998).

  b. Infertility

   No evidence of an association between human infertility and lignans exists. Although ENL is present in human semen in relatively high concentrations, no changes in the proportion of motile spermatozoa or in sperm motility of ejaculates occurred when mixed with 1 µM concentrations of synthetic ENL (Dehennin *et al.* 1982). In rats, flaxseed had no effect on spermatogenesis (Sprando *et al.* 2000).

  c. Fetal malformations

   Exposure of human subjects to lignans during pregnancy does not appear to pose a risk; no reports have emerged of any adverse effects. In rats, a 10%

flaxseed diet had no impact on pregnancy outcome other than lowering birth weight and suggesting estrogenic effects. In contrast, 5% flaxseed or an equivalent dose of SDG indicated antiestrogenic effects (Tou *et al.* 1998). Ingestion of flaxseed during lactation deemed safe (Ward *et al.* 2001).

Summary
In man, the evidence of ENL bioactivity is limited. The detectable actions of ENL *in vitro* occur mainly at supraphysiological concentrations, and the relevance of these findings on health is unclear. In addition, most studies examine the function of free ENL, which forms only a minor proportion of the circulating ENL. Future studies should thus examine the bioactivity of ENL conjugates as well. Our lack of knowledge about ENL bioactivity does not necessarily indicate that ENL is inactive in the body. The concentrations of ENL in tissues are mostly unknown and may be relatively high, particularly in the gastrointestinal tract and in the liver, which are the major sites for the synthesis of SHBG and growth factors. Because food is consumed daily, dietary compounds with even weak activity, may be of importance in human health and prevention of diseases.

Serum enterolactone concentrations in Finnish and other populations

The amounts of circulating ENL in different populations have been explored in several studies (Tables 3 and 4). Diversity is present in serum ENL concentrations in Finnish subjects. A likely explanation for this is that most studies have small subject pools, and thus, may not represent the group of interest well. In addition, differences in sex, age, and dietary habits may explain the disparities. Serum ENL concentrations also seem to differ between subjects of different nationalities. However, no studies have compared these differences using appropriate statistical methods. Several studies have examined ENL excretion in urine in different populations. For a review of these studies, please refer to the recent thesis by Niina Saarinen (2002b).

Table 3. Reported serum/plasma ENL concentrations in healthy Finnish adults.

Reference	Diet	Subjects	n	Method	Function	ENL (nmol/L)
Uehara *et al.* 2000a	habitual	females (F)	87	TR-FIA	median	21
Juntunen *et al.* 2000	habitual	F	21	TR-FIA	mean	39
	habitual	males (M)	18	TR-FIA	mean	28
Adlercreutz *et al.* 1993b	omnivore	premenopausal F	10	GC-MS	mean	28
	omnivore	postmenopausal F	4	GC-MS	mean	47
	vegetarian	premenopausal F	10	GC-MS	mean	90
	vegetarian	premenopausal F	4	GC-MS	mean	660

Table 4. Reported serum/plasma ENL concentrations in healthy adults in different countries.

Reference	Diet	Country	Subjects	n	Method	Function	ENL (nmol/L)
Brzezinski *et al.* 1997	habitual	Israel	F	78	GC-MS	mean	160
Morton *et al.* 1997b	habitual	Hong Kong	M	53	GC-MS	mean	21
	habitual	Portugal	M	50	GC-MS	mean	13
	habitual	US	M	36	GC-MS	mean	13
Zeleniuch-Jacquotte *et al.* 1998	habitual	US	F	60	GC-MS	median	23
Uehara *et al.* 2000a	habitual	Japan	F	111	TR-FIA	median	7.7
Franke *et al.* 2002	habitual	Hawaii	F&M	20	HPLC-MS	mean	19
Horner *et al.* 2002	habitual (high/low vegetable intake)	US	F&M	193	TR-FIA	geometric mean	14
Morton *et al.* 2002	habitual	UK	M	43	GC-MS	median	19
	habitual	UK	F	133	GC-MS	median	16
	habitual	Japan	M	102	GC-MS	median	11
	habitual	Japan	F	125	GC-MS	median	11
Grace *et al.* 2003	habitual	UK	F&M	300	HPLC-MS	median	14
Valentin-Blasini *et al.* 2003	habitual	US	F&M	208	HPLC-MS	mean	12

Reliability of enterolactone measurements in biological samples

The long-term (two years) reliability of serum ENL measurements proved not to be very good in one American population (intra-class correlation coefficient, ICC = 0.55) (Zeleniuch-Jacquotte *et al*. 1998). The long-term reliability of overnight urine ENL:creatinine ratio was studied in a Dutch population (den Tonkelaar *et al*. 2001); for four samples collected with a lag-time of 1–4.5 years the reliability was even lower, with the correlation coefficients varying between 0.27 and 0.58. Two studies have examined the short-term reliability of serum ENL. In American subjects, the Pearson correlation coefficient for ENL concentrations of two plasma samples collected on consecutive days was 0.84 (Horner *et al*. 2002). In Hawaiian volunteers, the ICC of three overnight urine samples collected eight days apart was 0.74 (Franke *et al*. 2002). No information on the reliability of ENL measurements in Finnish subjects is available.

The most common way to store the samples is at –20°C. At this temperature, serum ENL concentration is known to stay unchanged for at least ten years (Adlercreutz H., pers. comm.).

Only one recent study has examined and found a highly significant correlation of ENL levels in urine and serum samples from the same person (Valentin-Blasini *et al*. 2003). No studies appear to have compared the reliability of ENL measurement in blood and urine.

Applied transitional studies

Determinants of enterolactone

Several studies have reported an association between plasma or serum and urine ENL and intake of different dietary and other variables. Table 5 summarizes these results.

Factors confounding the relation between diet and ENL in serum or urine may have drastic effects on the specificity of ENL as a biological marker of dietary exposure. However, little is known about these non-dietary determinants. Oral antimicrobial treatment, as a result of the destruction of colon microflora, diminishes ENL formation in the gut. In a recent cross-sectional study of Finnish subjects, the strong association between serum ENL and dietary MAT and SEC intake disappears in users of antimicrobials (Kilkkinen *et al*. 2003a). In the same study population, oral antimicrobial treatment decreased serum ENL concentration for up to 12-16 months, and the fall in the concentrations was stronger after several treatments (Kilkkinen *et al*. 2002). By contrast, in an American multi-ethnic population, the intake of antibiotics during the preceding year had not influenced ENL urinary excretion. No information was, however, available on the recency of the intake (Horn-Ross *et al*. 1997). Another study showed highly elevated serum ENL concentrations in patients with severe diabetic nephropathy, which supports the important role of kidney function in elimination of ENL. Whether related to kidney function or not, age seems to be positively associated with ENL (Horner *et al*. 2002). In addition, a high body mass index (BMI)

Table 5. Reported determinants of serum/plasma and urine ENL[1]

Reference	Subjects	Diet	n	Dietary assessment
Adlercreutz *et al.* 1981	American F	omnivore vegetarian	12 14	food record
Adlercreutz *et al.* 1986	American F	omnivore vegetarian omnivore (breast cancer patients)	20 20 7	food record
Adlercreutz *et al.* 1987	Finnish F	habitual	48	food record
Adlercreutz *et al.* 1991	Japanese F+M	habitual	19	food record
Lampe *et al.* 1999	American F+M	low or high vegetable	98	5-d food record
Horner *et al.* 2002	American F+M	low or high vegetable	203	3-d food record
Hulten *et al.* 2002	Swedish F	habitual	740	
Vanharanta *et al.* 2002	Finnish M	habitual	100	4-d food record
Kilkkinen *et al.* 2003	Finnish M	habitual	428	
Vanharanta *et al.* 2003	Finnish M	habitual	1889	4-d food record
von Hertzen *et al.* 2004	Finnish type 1	habitual	400	

[1] Abbreviations: ENL, enterolactone; F, female; M, male; pos, positive; neg, negative; n.a., no association; FFQ,

Statistical method	Measurement	Determinant	Association	% of Variance explained
correlation	ENL 72-h urine excretion	fiber	pos (r=0.59)	
correlation between means of the groups	ENL 72-h urine excretion in four samples	grain intake	pos (r=0.996)	
Pearson correlation (effect of body weight and age eliminated)	ENL 72-h urine excretion in four samples	vegetable fiber berries and fruit fiber legume fiber total fiber	pos (r=0.32) pos (r=0.51) pos (r=0.31) pos (r=0.53)	
Pearson correlation	ENL 48-h urine excretion	green and yellow vegetables boiled soybeans	pos (r=0.53) pos (r=0.76)	

Pearson correlation	ENL 72-h urine excretion	vegetables
		fruit
		fiber
		   from vegetables
		   from fruit
		   from grains
			n.a.
			pos (r=0.20)
			pos (r=0.24)
			n.a.
			n.a.
			pos (r=0.22)	

linear regression model	Serum ENL	fiber
		   water-soluble
		   water-insoluble
		fruit
		vegetables
		fat
		caffeine
		alcohol
		age
		female sex
		BMI
			
			pos (r=0.35)
			pos (r=0.34)
			n.a.
			pos (r=0.17)
			n.a.
			pos (r=0.21)
			pos (r=0.24)
			pos
			pos
			neg
				22 %

comparison of medians	Serum ENL	smoking	neg	

linear regression model	Serum ENL	fiber
		   water soluble
		   water insoluble
		fruit and berries
		vegetables
		cereals
			
			pos (r=0.39)
			pos (r=0.28)
			pos (r=0.25)
			pos (r=0.24)
			pos (r=0.19)
				17 %

Spearman correlation	Serum ENL	age smoking number of smoking years BMI	pos neg pos (in control group) neg (in prostate cancer cases)	
linear regression model	Serum ENL	fiber vegetables alcohol saturated fatty acids constipation BMI number of bronchitis	pos pos neg neg pos neg neg	10 %
analysis of variance Pearson correlation	Serum ENL ENL 24-h urine excretion	severity of renal disease serum creatinine systolic blood pressure severity of renal disease	pos pos (r=0.60) pos (r=0.24) n.a.	

food frequency questionnaire

appears to be associated with low serum ENL concentration (Horner *et al.* 2002, Hulten *et al.* 2002, Vanharanta *et al.* 2003). Caffeine intake showed a strong positive correlation with plasma ENL concentration (Horner *et al.* 2002). The results on the effect of alcohol are conflicting; the association has been both positive (Horner *et al.* 2002) and negative (Vanharanta *et al.* 2003). Some early, small-scale studies suggested that ENL follows a cyclic pattern of excretion during the menstrual cycle (Setchell *et al.* 1979, 1980a, 1980b, Stitch *et al.* 1980). A later study with 18 women consuming flaxseed for three menstrual cycles found no differences in ENL or END excretions in three-day urine samples from follicular and luteal phases (Lampe *et al.* 1994).

Intervention studies

A number of studies have demonstrated an increase of serum or plasma ENL concentration in human subjects after supplementation of diet with flaxseed (Morton *et al.* 1994, 1997a, Nesbitt *et al.* 1999, Tarpila *et al.* 2002). In addition, incorporating strawberries (Mazur *et al.* 2000), rye bran bread (Bylund *et al.* 2003), or whole-grain products (Jacobs *et al.* 2002) into a controlled, low-lignan basal diet raised serum ENL concentrations. Similar changes were present in urinary ENL excretion in several intervention studies. For a review, please refer to the thesis by Niina Saarinen (2002b).

In a recent intervention study, a high-fiber rye diet raised serum ENL concentrations, whereas with a high-fiber wheat diet the concentrations remained unchanged compared with a low-fiber basal diet (McIntosh *et al.* 2003). Substitution of rye flour with phloem (pettu) powder effectively increased serum ENL concentration (Vanharanta *et al.* 2002a). Not all studies have indicated positive results; supplementation of a fiber-free diet with whole-grain rye bread for 12 volunteers had no impact on serum ENL concentration (Pool-Zobel *et al.* 2000). In another intervention in Finnish subjects, surprisingly, supplementation of the habitual diet with rye bread caused no changes in serum or urine ENL levels, although the intake of rye doubled. The authors suggested a plateau with intakes of 70-90 g rye bread/day, resulting in mean serum ENL concentrations of 26 nmol/L in men and 40 nmol/L in women. No dose-response correlation was present between rye bread intake and ENL in serum or urine (Juntunen *et al.* 2000). A common feature of all of the intervention studies was the notable between-subject differences in ENL production.

Enterolactone in breast cancer

Epidemiological studies

Enterolactone in body fluids and breast cancer risk

Case-control studies
Already in 1982, the first descriptive study suggested that ENL may protect against breast cancer. The mean urinary excretion of ENL in four 72-h urine collections was lower in 7 women with breast cancer than in 10 vegetarian and 10 omnivorous healthy women (Adlercreutz *et al.* 1982). Subsequent case-control studies have supported this finding. In an Australian study with 144 pairs, 72-h urinary excretion of ENL had an inverse association with risk of breast cancer. The odds ratio (OR) in the highest quintile of ENL excretion was 0.36 (95% confidence interval (CI) 0.15-0.86) after adjustments for age at menarche and alcohol and total fat intakes (Ingram *et al.* 1997). Similarly, urinary ENL:creatinine ratio was inversely associated with breast cancer risk in 250 Chinese breast cancer patients with individually matched controls. The OR in the highest tertile was 0.42 (95% CI 0.25-0.69) after adjustment for age, age at first live birth, a diagnosed fibroadenoma, total meat intake, and physical activity level (Dai *et al.* 2002). The authors, later, reanalyzed a subgroup of 117 pairs and found a stronger inverse association of urinary lignans with breast cancer among women with a high BMI or waist:hip ratio. However, no association existed after stratifying by plasma steroid or SHBG concentration (Dai *et al.* 2003).

Prospective studies
To date, only four prospective studies on serum or urine ENL and breast cancer risk are available. A recent follow-up study suggested that ENL has a strong protective effect on the breast cancer risk. It assessed the relationship between serum ENL concentrations and the occurrence of breast cancer in 383 Italian women with palpable cysts. After a median follow-up of 6.5 years, 18 women had developed invasive breast cancer. Median serum ENL concentrations were lower in women with breast cancer than in controls: 8.5 nmol/L versus 16 nmol/L. The OR in the lowest quintile (Serum ENL ≤8 nmol/L) was 0.36 (95% CI 0.14-0.93) (Boccardo *et al.* 2004). A nested case-control study with 248 Swedish cases and 492 controls from three cohorts showed an increased risk of breast cancer in women below the 12.5[th] percentile (mean plasma ENL 2.9 nmol/L) and, surprisingly, in two of the cohorts above the 87.5[th] percentile (mean plasma ENL 58 nmol/L). The authors suggested that this discrepancy may derive from pure chance, excessive intake of flaxseed, disturbed hormonal metabolism, or the effect of alcohol on ENL levels (Hulten *et al.* 2002). The other two nested case-control studies demonstrated negative results; serum ENL concentration was not associated with risk of breast cancer in a Finnish study with 206 women with breast cancer (both pre- and postmenopausal) and 215 controls (Kilkkinen *et al.*

2004), nor did a Dutch study with 88 postmenopausal breast cancer cases and 268 controls find any association between overnight urinary ENL:creatinine ratio and breast cancer risk (den Tonkelaar *et al.* 2001).

A recent, interesting follow-up study showed that ENL accumulates in breast cysts (Boccardo *et al.* 2003). The study included 191 women, 12 of whom developed breast carcinoma during the follow-up. Intracystic ENL did not correlate with breast cancer risk. However, it modulated the effect of intracystic EGF concentration, which is a strong predictor of breast cancer. ENL appeared to decrease the cancer risk of patients with high intracystic EGF concentration, while in patients with low intracystic EGF concentration ENL increased the breast cancer risk.

Dietary lignans and breast cancer risk

A case-control study with 207 women with breast cancer and 188 controls showed a reduced breast cancer risk in women in the highest tertile of dietary lignan intake. The ORs in the highest tertile were 0.45 for premenopausal (95% CI 0.20-1.01) and 0.59 (0.28-1.27) for postmenopausal women. The risk reduction was substantially higher in premenopausal women with at least one A2 allele of the cytochrome P450c17α gene leading to high estrogen levels (OR 0.12, 95% CI 0.03-0.50) (McCann *et al.* 2002). The opposite findings were observed in the second American case-control study with 1326 cases and 1657 controls, in which the intake of MAT and SEC was not associated with breast cancer risk (Horn-Ross *et al.* 2001). Similarly, two cohort studies, one with 111 526 Californian (Horn-Ross *et al.* 2002) and the other with 15 555 Dutch (Keinan-Boker *et al.* 2004) women, found no association between dietary lignan intake and breast cancer risk.

Dietary lignan intake may not be a suitable marker of body exposure to mammalian lignans because this measurement is unable to take into account the large between-subject differences in the intestinal conversion of plant precursors to mammalian lignans. In addition, there is insufficient knowledge occurrence of lignans in common foods, leading to an underestimation of plant lignan intake. Thus, the relevance of these studies in elucidating the role of ENL in preventing breast cancer is uncertain.

Experimental studies

In vitro

The *in vitro* evidence of (anti)estrogenity of ENL is summarized above. ENL was able to inhibit various steps of metastasis in two estrogen receptor-negative human breast cancer cell lines. At concentrations of 1-5 µM, the inhibition of cell adhesion and invasion was dose-dependant. When ENL, END, and tamoxifen were combined in a 1 µM dose, a greater inhibitory effect on cell adhesion and invasion was observed than with any of these compounds alone. ENL at doses of 0.1-10 µM reduced cell migration (Chen *et al.* 2003b). ENL may have an

antiproliferative effect on estrogen receptor-negative breast cancer cells (Hirano *et al.* 1990, Wang *et al.* 1997), but only at such high concentrations that the relevance of this finding is questionable.

In vivo

Animal studies

Experimental animal studies provide strong evidence of a beneficial action of lignans against mammary carcinoma at several stages. Orally administered ENL (Saarinen *et al.* 2002a), HMR (Saarinen *et al.* 2000, 2001), flaxseed (high in SEC diglycoside), or SEC diglycoside (Thompson *et al.* 1996a, 1996b) suppressed the growth of chemically (7,12-dimethylbenzanthracene, DMBA) induced mammary tumors. ENL and HMR were additionally shown to increase the proportion of stabilized and regressing tumors (Saarinen, *et al.* 2001, 2002a). The growth inhibition was observed when serum lignan concentrations were hundreds of nanomols. Flaxseed, by contrast, was less protective against N-methyl-N-nitrosourea (NMU) -induced mammary tumorigenesis in rats, and low doses even suggested tumor promotion. The authors considered the disparities to be related to differences in experimental design, different carcinogen, and protective effects of the alpha-linolenic acid present in the basal diet (Rickard *et al.* 1999). In a nude mice model, flaxseed reduced by 45% the incidence of metastasis in established estrogen receptor-negative human breast cancers (Chen *et al.* 2002). The authors stated that this effect was partly due to downregulation of IGF-I and EGF receptor expression. Developmental studies showed that exposure of rat pups to lignans during suckling enhances the differentiation of developing mammary gland structures (Ward *et al.* 2000), which subsequently suppresses DMBA-induced mammary tumorigenesis (Chen *et al.* 2003a).

Human studies

Experimental studies in women on the effect of lignans on breast cancer development are sparse. One randomized, double-blind, placebo-controlled study determined the effect of daily consumption of a placebo muffin versus a muffin containing 25 g of flaxseed from the time of diagnosis to the time of surgery. Analysis of tumor tissues indicated reduced tumor growth in the flaxseed group (Thompson *et al.* 2001).

Aims of the study

This study aimed to:

1. Develop a TR-FIA methodology for serum ENL
Specific aims were to:
a. elucidate the etiology for the differing results of the original and revised versions of the basic TR-FIA for serum ENL
b. validate the revised version of the basic method
c. develop a fully automated short method for serum ENL measurement

2. Study the characteristics of ENL as a biological marker
In this part of work, specific aims were to investigate:
a. the reliabilities of serum and urine ENL measurements
b. the associations between ENL concentrations in serum and urine
c. the serum concentrations of ENL in the Finnish population
d. the determinants of serum ENL concentration
e. whether serum ENL levels can be modified by diet at the population level

3. Investigate whether serum ENL is related to breast cancer risk in Finnish women

Materials and methods

Materials

Instrumentation

TR-FIA

The time-resolved fluoroimmunoassay for ENL was performed with the following equipment:

1. A VICTOR 1420 multilabel counter with software version 1.0 for fluorescence measurements, and a dissociation-enhanced lanthadine fluoroimmunoassay (DELFIA) plate washer and plate shaker (Wallac Oy, Turku, Finland) for immunoassay procedures (I, III, IV, V).
2. An AutoDELFIA 1235 Automatic Immunoassay System (Wallac Oy, Turku, Finland) (II).

Radioactivity counting

An LKB 1217 Rackbeta scintillation counter was used to calculate radioactivity (β-radiation) for recovery calculations.

GC-MS

Isotope dilution gas chromatography-mass spectrometry in the selected ion monitoring mode with synthesized deuterated internal standard was used as a reference method. The GC-MS instrument was a Fisons MD 1000 quadruple mass spectrometer combined with a Fisons GC 8000 gas chromatograph. Helium was used as the carrier gas.

HPLC-CEAD

The high-performance liquid chromatography system consists of two Model 580 ESA (ESA Inc., Chelmsford, MA, USA) solvent pumps, a Model 540 ESA autosampler with refrigeration, a thermal chamber for a column, and a detector, two detector cells each containing four measuring electrode pairs, a system control module, and a computer.

DCA 2000 analyzer

Urinary creatinine concentrations were determined using a DCA 2000 Analyzer (Bayer, Elkhart, IN, USA).

Statistical equipment

The statistical analyses were performed using SPSS package program version 9.0 (IV) or 10.0 (II) (SPSS Inc., Chicago, IL, USA) and SAS program Ver 6.12 (SAS Institute Inc., Cary, NC, USA) (III).

Standards, chemicals, and reagents

Standards

ENL, END, MAT, SEC (Bannwart *et al.* 1984, Adlercreutz *et al.* 1986b), ENL hydroxylation metabolites (Mäkelä *et al.* 2000, 2001), and 5-O-carboxymethoxy-ENL (Adlercreutz *et al.* 1998) were synthesized and PIN (Nishibe *et al.* 1990) was isolated as described.

Immunogen synthesis and immunization

The 5-O-carboxymethoxy-ENL was coupled to bovine serum albumin (BSA), and the conjugates were used for the immunization of rabbits. Immunogen synthesis and immunization have been described in detail elsewhere (Adlercreutz *et al.* 1998).

Fluorescence label

A Europium chelate of 5-O-carboxymethoxy-ENL served as the fluorescence label for TR-FIA (Adlercreutz *et al.* 1998).

Chemicals and reagents

Enzymes
β-glucuronidase (EC 3.2.1.31) (Boehringer-Mannheim, Mannheim, Germany; Cat. No. 1585665)
Sulfatase (EC 3.1.6.1) (Sigma, St. Louis, MO, USA; Cat. No. S9626)

Reagents
BSA, diethyl ether, methanol, ethanol (Merck AG, Darmstadt, Germany)
[6,7-^{3}H]Estradiol-17β-glucuronide (NET 1106, 250 μCi (9.25 MBq)) (NEN Lifescience Products)
Goat anti-rabbit IgG-coated microtitration strips (Wallac Oy, Turku, Finland)
DELFIA enhancement solution (Wallac Oy, Turku, Finland)
DELFIA wash concentrate (Wallac Oy, Turku, Finland)

Buffers
Hydrolysis buffer: 0.1 M acetate buffer, pH 5
Assay buffer: 50 mM Tris-BSA buffer, pH 7.8 containing 8.78 g of NaCl, 0.5 g of sodium azide, 5 g of BSA, and 0.1 g of Tween 40 per liter

Glassware
The samples were handled in disposable glass tubes (12 × 75 mm).

Human samples

Ethics
The study protocols were approved by the Ethics Committees of the hospital district of Helsinki and Uusimaa (II), the National Public Health Institute (III, IV), and the University of Kuopio (V).

Study II

Subjects
Twenty volunteers recruited among university students gave their written consent before entering the study. Exclusion criteria were age less than 18 years, antibacterial treatment during the preceding 3 months, any major illness, and any regular medication, except contraceptive pills. One female subject who regularly took an antidepressant reported this only at the end of the study. One subject dropped out because of antibacterial treatment for a urinary infection. One spot urine sample was missing for a female subject; this participant was subsequently excluded from that analysis.

Study design
The serum, spot urine, and 24-h urine samples were collected on 5 successive days (Monday to Friday, within-week variation) and on the following 3 Mondays (within-month variation).

Study III

Subjects
The participants of the national cross-sectional survey were 25-64 years old and lived in 5 areas: 1) Helsinki and Vantaa (the metropolitan area), 2) the cities of Turku and Loimaa as well as some rural communities in Loimaa, and the provinces of 3) North Karelia, 4) Kuopio, and 5) Oulu. An independent random sample (n=10 000) stratified by 10-year age groups, regions, and sex, was drawn from the National Population Register. For the dietary survey subsample, 4000 of these individuals were chosen and 72% subsequently participated. Subjects who had used antibiotics within the past 3 months (n=372) were excluded.

Study design
The venous samples were taken from the subjects at the clinical examination. Data on health, socioeconomic factors, physical status, smoking, alcohol consumption, and dietary habits were collected by a self-administered questionnaire. A food frequency questionnaire (FFQ) including 38 food items was used to assess dietary intake over the previous 12 months. The consumption of wholegrain products was quantified by summing up the consumption of rye bread, crisp bread, porridge, and cereals. Consumption of vegetables was calculated by summing up the consumption of salad vegetables, roots, legumes, vegetable dishes, and consumption of fruit and berries by summing up the consumption of fruit,

berries, and juices. For the analyses, subjects were divided into tertiles by frequency of consumption of whole-grain products, vegetables, and fruit and berries.

Studies IV and I

Subjects
The intervention study was carried out in two semi-rural communities (Kitee and Tohmajärvi) in North Karelia, Finland, in spring 1983. The original aim of the study was to investigate the effects of dietary modification on blood pressure and serum lipids (Kuusi *et al*. 1985, Puska *et al*. 1985). The participating families had been initially identified through the countrywide hypertension register or through local risk factor screenings. Persons aged 35-49 years with known borderline or mild hypertension and no antihypertensive treatment, as well as their spouses, were invited to participate. The volunteers gave their written informed consent, and, if no exclusion factors emerged, underwent a medical examination. One person with major health problems was excluded during the study, and the final study group comprised 85 middle-aged subjects.

Study design
The study consisted of a baseline period of 2 weeks, a 12-week intervention period, and a 5-week switchback period. A fasting venous blood sample was taken at the end of each period and in the middle of the intervention period (Kuusi *et al*. 1985, Puska *et al*. 1985).

During the baseline and switchback periods the families ate their usual diets. The local North Karelian diet has traditionally been high in saturated fat derived from whole-milk products and meat, and low in polyunsaturated fat and vegetables. During the intervention the subjects were given counseling to reduce the intake of fat and increase the consumption of grain products as a carbohydrate source, as well as vegetables, berries, and fruit. The families were randomly allocated into two groups with different polyunsaturated/saturated fatty acid ratios by providing the families with different fat spreads. The subjects kept a food consumption record for 7 days during the baseline, 12 days during the intervention, and 10 days during the switchback period. The study protocol concerning changes in foods was identical to an earlier study in the same area (Pietinen *et al*. 1984). The consumption of dairy products, cereal products, meat, and eggs did not change significantly, while the consumption of vegetables, fruit and berries, fish, and low-fat cheese increased.

Study V

Subjects
The subjects were participants in the Kuopio Breast Cancer Study. During the period from April 1990 to December 1995, all women living in the catchment area of Kuopio University Hospital who had breast symptoms or suspected a lump were referred for further examination and interview by a local physician. The age range of subjects was 25-75 years. Of women entering the hospital for

breast examination, only 3% refused to participate in the study. Of all participants, 24% were later diagnosed with breast cancer. A random sample of population controls was drawn from the National Population Register covering the same geographical area. The controls were matched to breast cancer cases by age (within ±5 years) and area of residence (rural/urban). The participation rate of the population controls was 72%. For ENL analyses, a subset of cases and controls was selected, covering the time period 1992-1995. After exclusions because of missing data, 194 cases and 208 controls were available for analysis.

Data collection
In cases, the venous blood sample was drawn before undergoing the hospital examinations for diagnosis. The study nurse interviewed the women with questionnaires on demographic, reproductive, and lifestyle characteristics, and guided the anthropometric measurements. The controls visited the hospital to give a blood sample and an interview. The diet over the preceding 12 months was assessed by a semiquantitive FFQ (Männistö *et al.* 1999).

Sample preparation
All venous samples were drawn after at leat 4 h of fasting. The serum aliquots used had been thawed either once (V, I+IV partly) or stored unthawed (II, III) until the analysis of ENL. Collection of the 24-h urine samples (II) began 24 h prior to the serum samples being drawn. They were collected in 2-L plastic containers containing 2 g of ascorbic acid. After the collection, the volumes of the 24-h urine samples were measured. Spot urine samples were collected at the end of each 24-h collection.

Methods

Analytical methods

TR-FIA for serum and urine enterolactone
Basic method for serum enterolactone (I, II, IV, V)
Pretreatment. First, 25 µg of ^{3}H-estradiol glucuronide was added to 250 µL of serum sample. The sample was hydrolyzed overnight with 250 µL of hydrolysis reagent containing hydrolysis buffer with 0.2 units/mL β-glucuronidase and 2 units/mL sulfatase. After hydrolysis, the serum samples were extracted twice with diethyl ether. After extraction and evaporation, 250 µL of assay buffer was added to the tubes to obtain the original sample volume. The recovery of the hydrolysis and extraction steps was measured by radioactivity counting.

   Enterolactone measurement. Twenty microliters of sample, 100 mL of assay buffer with antiserum, and 100 µL of assay buffer with the europium label were

pipetted in duplicate on antirabbit microtitration strips. The plates were incubated slowly on a plate shaker for 90 min, after which the strips were washed and shaken further with enhancement solution for 5 min, and then the fluorescence was measured.

Calculations. The final results were obtained by correcting the measured concentration for the average recovery of the samples.

Short method for serum enterolactone (I, III)
Pretreatment. Fifty microliters of serum samples were incubated with 50 µL of hydrolysis reagent containing sulfatase and β-glucuronidase overnight at 37°C. After hydrolysis, 150 µL of assay buffer was added to the samples.

Enterolactone measurement was performed equally as described for the basic method.

Calculations. To obtain results corresponding to the basic method, the final results were calculated according to the formula:

Final concentration = [measured concentration] × 0.934–11.03

TR-FIA for urine enterolactone (II)
Pretreatment. Fifty microliters of urine was hydrolyzed overnight with 450 µL of the same hydrolysis reagent as in serum ENL assay. After hydrolysis, the urine samples were diluted further with the assay buffer to obtain optimal pH and protein and analyte concentrations for the assay (Uehara *et al.* 2000b).

Enterolactone measurement was performed as for serum ENL measurements.

Calculations. The final results were calculated as follows:

Final concentration = [measured concentration] × 10 × dilution factor.

Quality control
The measurement was repeated if the results of the duplicate fluorescence measurements deviated more than ±15% from the mean value and 1.0 nmol/L from each other. The urine samples were diluted further with the assay buffer when the sample concentrations exceeded 100 nmol/L to reach the linear part of the standard curve.

The batches of serum samples included two (IV, V) or three (II, III) quality control samples with different ENL concentrations repeated once in every 40 samples. The urine samples (V) were analyzed with two urine quality control samples. The control samples were pooled serum samples either obtained from the Finnish Red Cross or collected from laboratory personnel.

Urine creatinine (II)
Urinary creatinine concentrations were determined by a colorimetric method with a modified Benedict-Behre reaction. The method is based on the reaction of 3,5-dinitrobenzoic acid with creatinine in a highly alkaline medium. Validation of the method has been presented elsewhere (Hiar 1997).

GC-MS (I)

GC-MS for plasma ENL was used as a reference method. The original method (Adlercreutz *et al.* 1993b) was slightly modified as described earlier (Adlercreutz *et al.* 1998).

HPLC-CEAD (I)

Study I measured ENL in serum after incubation of samples with varying enzyme amounts. The samples were otherwise pretreated as described above for basic TR-FIA for serum ENL. ENL measurements were performed by both TR-FIA and HPLC-CEAD. The chromatographic conditions were described in detail by Nurmi *et al* (1999).

Statistical methods

Intra- and inter-assay coefficients of variation (CV) were calculated as follows: $CV = \sigma/\text{average}$. The limit of detection in analysis was determined according to the definition by the International Union of Pure and Applied Chemistry (IUPAC) (limit of detection = 3(standard deviation of the blank) + signal from the blank) (McNaught *et al.* 1997).

ENL concentrations are expressed as median values ±95% CI or range. All calculations for statistical significance were performed on a logarithmic scale to obtain a normal distribution. The findings were considered significant at $p < 0.05$.

The reliability calculations (II) included ICC, between-subject variance (σ^2_B), within-subject variance (σ^2_W), analytical variance (σ^2_A), and total variance. σ^2_A was obtained by measuring the day-one samples in two aliquots. Individual variances (σ^2_I) were calculated by the following formula: $\sigma^2_I = \sigma^2_W - \sigma^2_A$. The reliabilities within a week (five consecutive days) and within a month (four consecutive Mondays) were estimated separately. Standard deviations (σ) were estimated as square roots of the respective variances, and CVs were calculated by the following formula: $CV = 100(e^{\sigma}-1)$ % (Bland *et al.* 1996). The number of repeated measurements (of samples collected in different weeks) needed to estimate the underlying homeostatic set point to within ± 50% with 80% confidence was calculated by the formula presented by Fraser and Harris (1989).

Pearson correlation coefficients with 95% CIs were calculated between ENL levels in urine and serum samples from the same person (Hopkins 2002). The serum:urine ENL ratios were also calculated separately for each person, and possible differences between subjects were tested by one-way ANOVA, Tanhame's test (not assuming equal variances) as a post hoc test to determine which individuals differed from each other.

The determinants of serum ENL concentration (III) were identified by a regression model. All variables (age, education, BMI, constipation, self-reported physical health, smoking, and consumption of alcohol, whole-grain products, vegetables, and fruit and berries) were examined as potential determinants of serum ENL concentration. In the final model, only the significant variables were included. The analyses were done separately for men and women.

The differences in median concentrations in Study IV were tested by repeated measures ANOVA with least significant difference as a post hoc test.

The association between serum ENL and risk of breast cancer (V) was determined with a logistic regression model. Group matching of cases and controls was used, and the quintiles were based on cases and controls combined. The regression model was adjusted for age, area, age at menarche and at first full-term pregnancy, first-degree family history of breast cancer, history of benign breast disease, education, current alcohol intake, smoking, leisure time physical activity, waist-to-hip ratio, and BMI. Data were analyzed separately for premeno-pausal and postmenopausal women. The effect of short-term variation in serum ENL concentrations on the odds ratio was calculated by the following equation: $OR_{observed} = e^{(lnORtrue \times ICC)}$ (White 1997).

Results

TR-FIA for serum enterolactone

Differences in serum enterolactone results (I)

This study compared for the first time the results of the original and the revised versions of the basic TR-FIA with each other and with the GC-MS reference method. Unexpectedly, a striking discrepancy was present in these results (Fig. 3).

Fig. 3. Linear correlations between S-ENL concentrations measured by GC-MS and a) the original and b) the revised versions of basic TR-FIA for serum ENL.
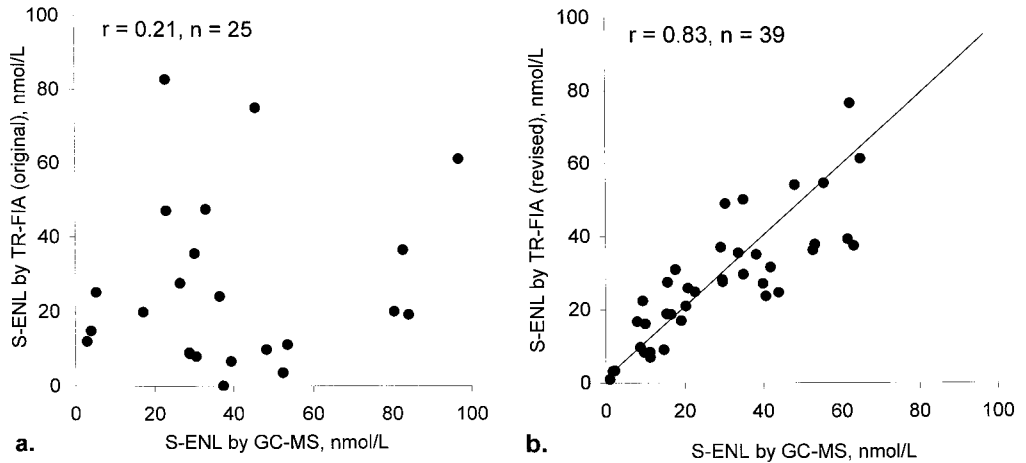
The analysis of 184 serum samples showed that the results of the two versions of TR-FIA associated rather poorly with each other. If reanalysis of the samples is impossible, the results of the original version of the basic method can be corrected by the following formula:

[result of the revised basic version] = $0.63 \times$ [result of the original version] – 6.8
R=0.60, p<0.001, n=184.

Explaining the differences in study results

Effect of enzymes (I)

Both versions of the method use a partially purified sulfatase manufactured from the snail *Helix Pomatia* (also with β-glucuronidase activity), and a specific β-glucuronidase produced in bacteria. Whether one or both of these enzymes caused the difference in the results was tested by changing the enzyme amounts one at a time.

β-glucuronidase
Hydrolysis of the glucuronides proved to be sufficient with the smaller amount of β-glucuronidase present in the revised version of the basic method (0.2 U/mL). Its increase did not change the results (Fig. 4).

Sulfatase
Increase in the amounts of sulfatase used in hydrolysis caused dramatic changes in the results. TR-FIA measured linearly increasing ENL concentrations when the

Fig. 4. Hydrolysis of serum (200 μL) with hydrolysis reagent (200 μL) with different β-glucuronidase [(EC 3.2.1.31, Cat. No. 1585665) from Boehringer-Mannheim] concentrations. ENL concentrations were measured by TR-FIA.
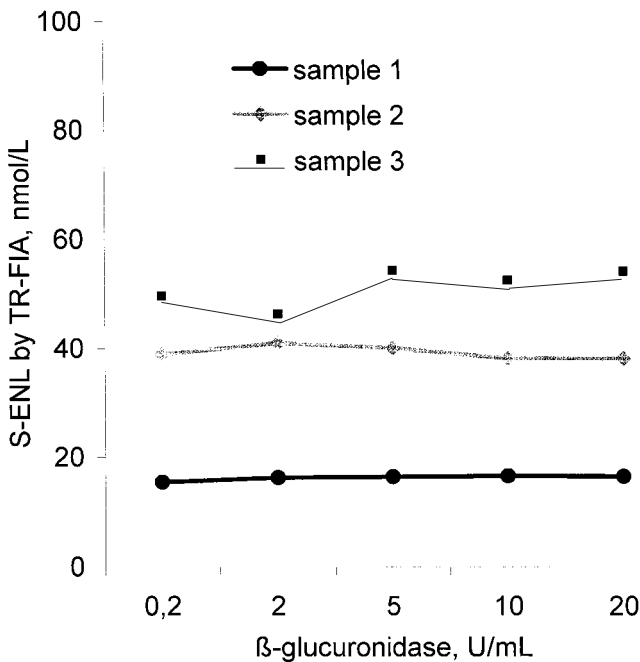
concentrations of sulfatase in the hydrolysis reagent rose (Fig. 5). HPLC-CEAD, by contrast, detected no differences between the samples. The results were similar when the amount of sulfatase was raised by increasing the amount of the hydrolysis reagent per sample. The results of TR-FIA and HPLC-CEAD were closest to each other with the sulfatase amount present in the revised version of the basic assay (200 µL hydrolysis reagent with sulfatase 2 U/mL).

Fig. 5. Hydrolysis of serum (200 µL) with hydrolysis reagent (200 µL) with different sulfatase [(EC 3.1.6.1, Cat. No. S926) from Sigma] concentrations. ENL concentrations were measured by TR-FIA and HPLC-CEAD.
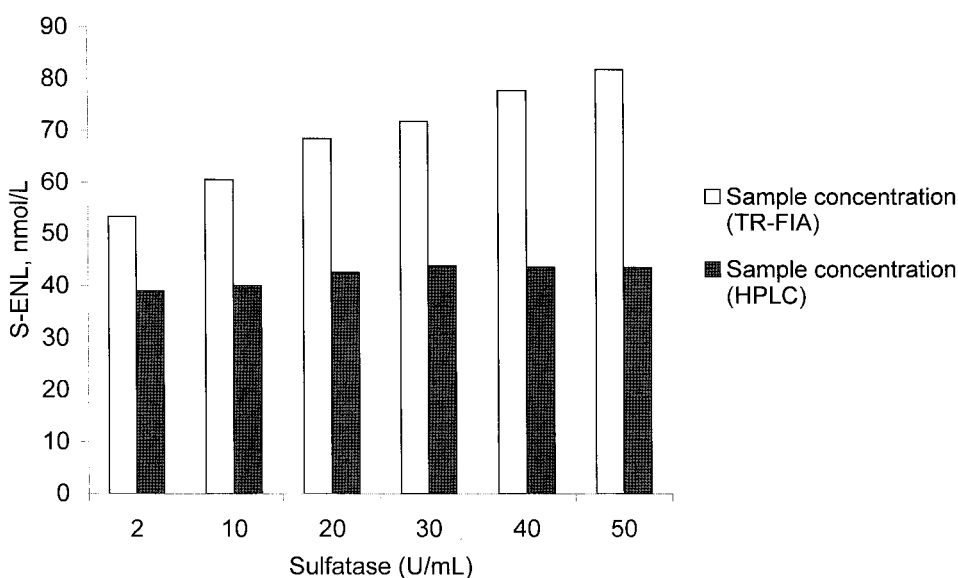
Effect of matrix (I)

With its strong brown color, sulfatase might itself cause fluorescence. This was, however, not the case; the enzyme alone caused no changes in fluorescence when hydrolysis was performed in buffer or ENL standard solution, or in serum without incubation. This led to the hypothesis that sulfatase disturbs the assay by releasing some compound(s) from the matrix.

Cross-reactions of antiserum

Compound(s) responsible for the disturbance of the assay may structurally be related to ENL. They may be liberated from plasma during hydrolysis and bind to the antiserum, and thus interfering with the results. ENL has several

structurally closely related metabolites. Unfortunately, not all of them were available as standards. Of the tested compounds, α-,β-desmethyl-β-deshydroxy MAT exhibited a significant cross-reactivity. The results from the cross-reactions of the related, available compounds are presented in Table 6.

Table 6. Cross-reactions of the antiserum and the structures of the 5-O-carboxymethoxy-ENL and the MAT derivatives.

Compound	Cross-reactivity
1. Present in large amounts in circulation	
daidzein	–
genistein	–
equol	–
quercetin	–
enterodiol	0.2%
2. Present in trace amounts or not present in circulation	
secoisolariciresinol	–
matairesinol	–
pinoresinol	–
lariciresinol	–
isolariciresinol	–
7'-OH-enterolactone[1]	0.7%
α-desmethyl-β-desmethoxy matairesinol[a2]	–
bis-desmethoxy matairesinol[a3]	–
bis-(desmethyl) matairesinol[a4]	*~0.1%*
α-,β-desmethyl-β-deshydroxy matairesinol[a5]	*~10%*

[1-5]Structures presented below (1-5)
[a]No quantitative standard solution available, cross-reactivity is only estimated

References: Stumpf K. *et al*. unpublished results, Adlercreutz *et al*. 1998

5-O-carboxymethoxy-ENL


1   2   3   4   5
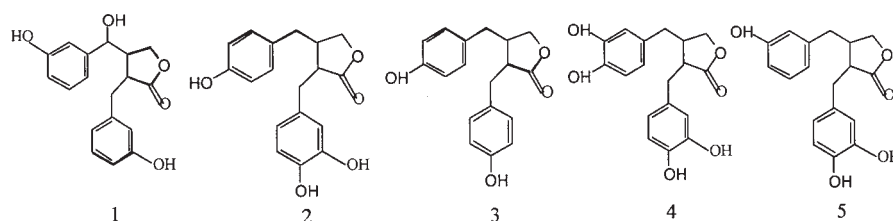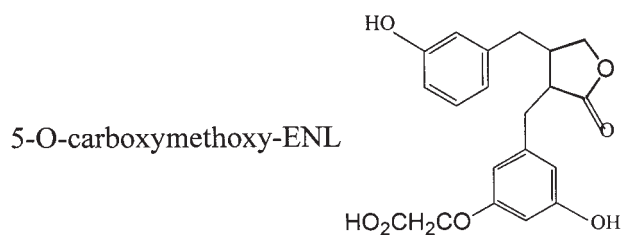
Short TR-FIA for serum enterolactone (I)

Development of the short TR-FIA aimed at an automated method for auto-DELFIA. Because only a minor portion of circulating ENL is in free form, its direct measurement in serum without any sample pretreatments failed to yield reliable results. Therefore, the second stage was to inspect an assay with hydrolysis but no extraction. Inclusion of hydrolysis freed all of the ENL conjugates for measurement, while exclusion of extraction eliminated the manual part of the method. Exclusion of extraction resulted in a higher mean serum ENL concentration (45 nmol/L) than the basic TR-FIA for serum ENL (29 nmol/L) or the GC-MS (28 nmol/L). Even so, the correlation between these methods was excellent (Fig. 6). The short method turned out to be useful, but the final results require a mathematical conversion by using the following equation:

[basic TR-FIA] = 0.87 × [short TR-FIA] − 11.3
r=0.94, p<0.001, n=262

Fig. 6. Correlation between the short and the basic TR-FIA for serum ENL.
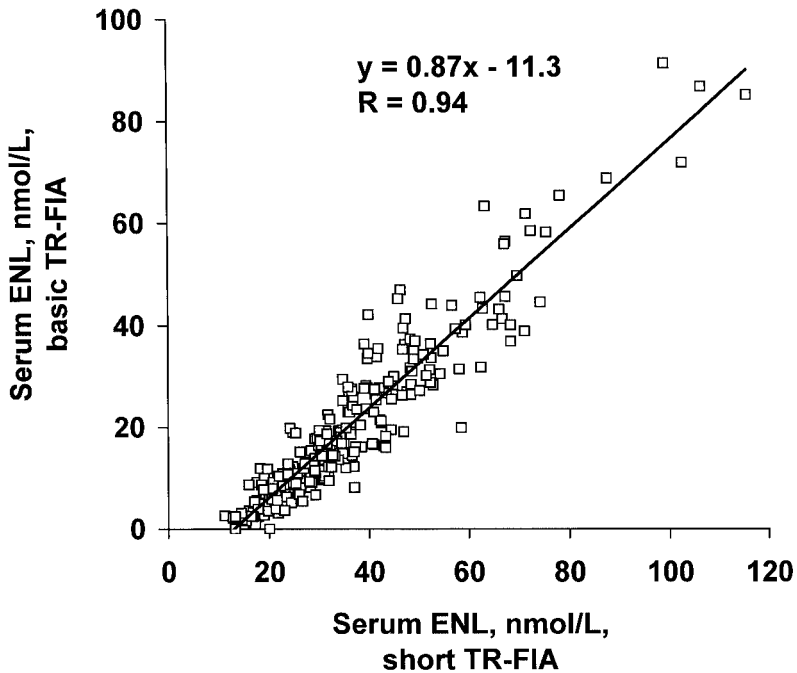
Validation of the method

Precision
The intra- and inter-assay CVs for the basic and the short TR-FIA are presented in Table 7.

Table 7. Intra- and inter-assay coefficients of variation (CV) for serum ENL by the basic (a) and short (b) TR-FIA.

a.			b.		
Concentration (nmol/L)	Number of samples	CV (%)	Concentration (nmol/L)	Number of samples	CV (%)
		Intra-assay			Intra-assay
13.2	10	4.7	30.7	10	4.0
38.0	10	6.0	58.5	10	6.1
79.5	10	3.3	106.7	10	3.1
		Inter-assay			Inter-assay
16.3	20	9.0	3.7	20	7.0
45.2	20	9.9	14.1	20	6.1
96.6	20	6.9	59.5	20	8.6

Comparisons with the reference method
The residual plots between the reference method GC-MS and the basic and the short TR-FIA for serum ENL are presented in Fig. 7.

Fig. 7. Residual plots between a) GC-MS and the basic TR-FIA for serum ENL, and b) GC-MS and the short TR-FIA for serum ENL.


a.

b.
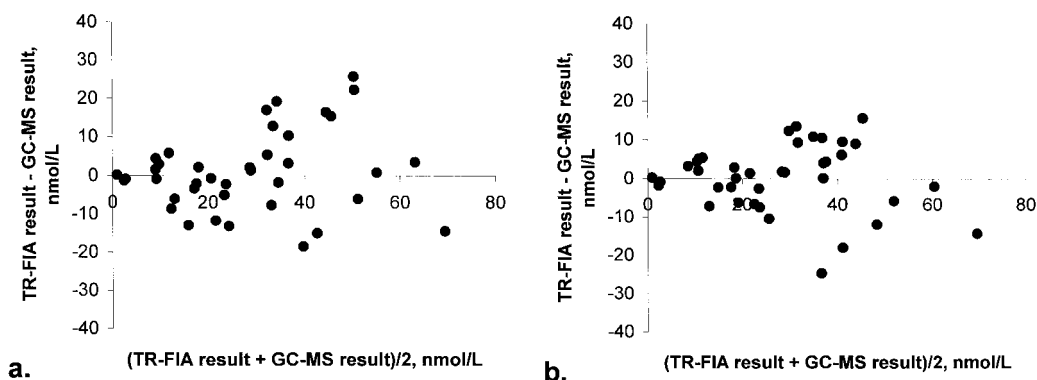
Sensitivity

Detection limit
Detection limit, expressed as the minimal amount of ENL distinguishable from the zero sample with 95% probability is 0.35 nmol/L.

Working range
Studies I and III-V used a standard curve with a working range of 1.5-540 nmol/L (Fig. 8, standard curve 1). The working range in the basic and the short method was identical. Because the distribution of serum ENL concentrations in the Finnish population was shown to be skewed toward zero (III), there was a need to widen the working range towards the low concentrations. Therefore, a more sensitive standard curve with a working range of 0.4-300 nmol/L (Fig. 8, standard curve 2) was developed and taken into use in Study II.

Fig. 8. The standard curves. The lines connect the lowest and highest points of the working range.
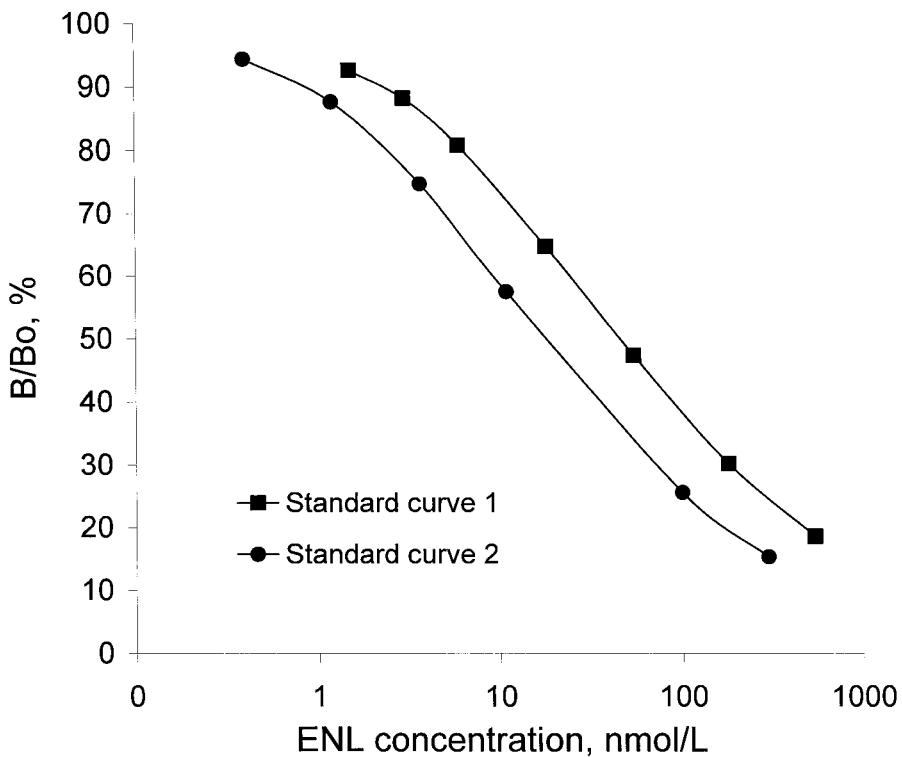
Serum enterolactone as a biological marker

Serum enterolactone concentrations in the Finnish population (II-V)

The median concentrations were 7.3 (range 0.4-25.7) (II), 15.3 (0-183) (III), 12.2 (1.1-71.9) (IV), and 19.3 (0.5-124) (V) nmol/L. The distribution of serum ENL was highly skewed and concentrated in the low values. Serum ENL concentrations were higher in women than in men and in older than in younger subjects (III).

Reliability of enterolactone measurements (II)

The ranges and median ENL concentrations of the eight samples collected from each subject are presented in Fig. 9. The median ENL serum concentration and urinary excretions, as well as the ICCs and the numbers of samples required to estimate the underlying homeostatic set point, are presented in Table 8.

Table 8. Overall median concentrations, intra-class correlation coefficients, and number of samples (on different weeks) required to estimate the underlying homeostatic set point.

|	Serum ENL[a]	24-h urine ENL	Spot urine ENL:creatinine
n[b]	19	19	18
Overall median	9.1 nmol/L	2460 nmol/24 h	289 μmol/mol
Intra-class correlation coefficient (95% CI[c]**)**			
within-week	0.79 (0.65-0.90)	0.80 (0.67-0.91)	0.72 (0.55-0.86)
within-month	0.77 (0.61-0.89)	0.79 (0.64-0.90)	0.67 (0.46-0.84)
Number of samples required to estimate the underlying homeostatic set point, within-month[d]	3	3	10

[a] enterolactone

[b] number of subjects included in analysis

[c] 95% confidence interval

[d] to within ±50% with 80% confidence

Fig. 9. Within-subject variations in (a) ENL serum concentration, (b) ENL urinary excretion, and (c) spot urine ENL:creatinine ratio. Lines indicate the ranges and vertical bars the median concentrations of eight samples collected in one month.
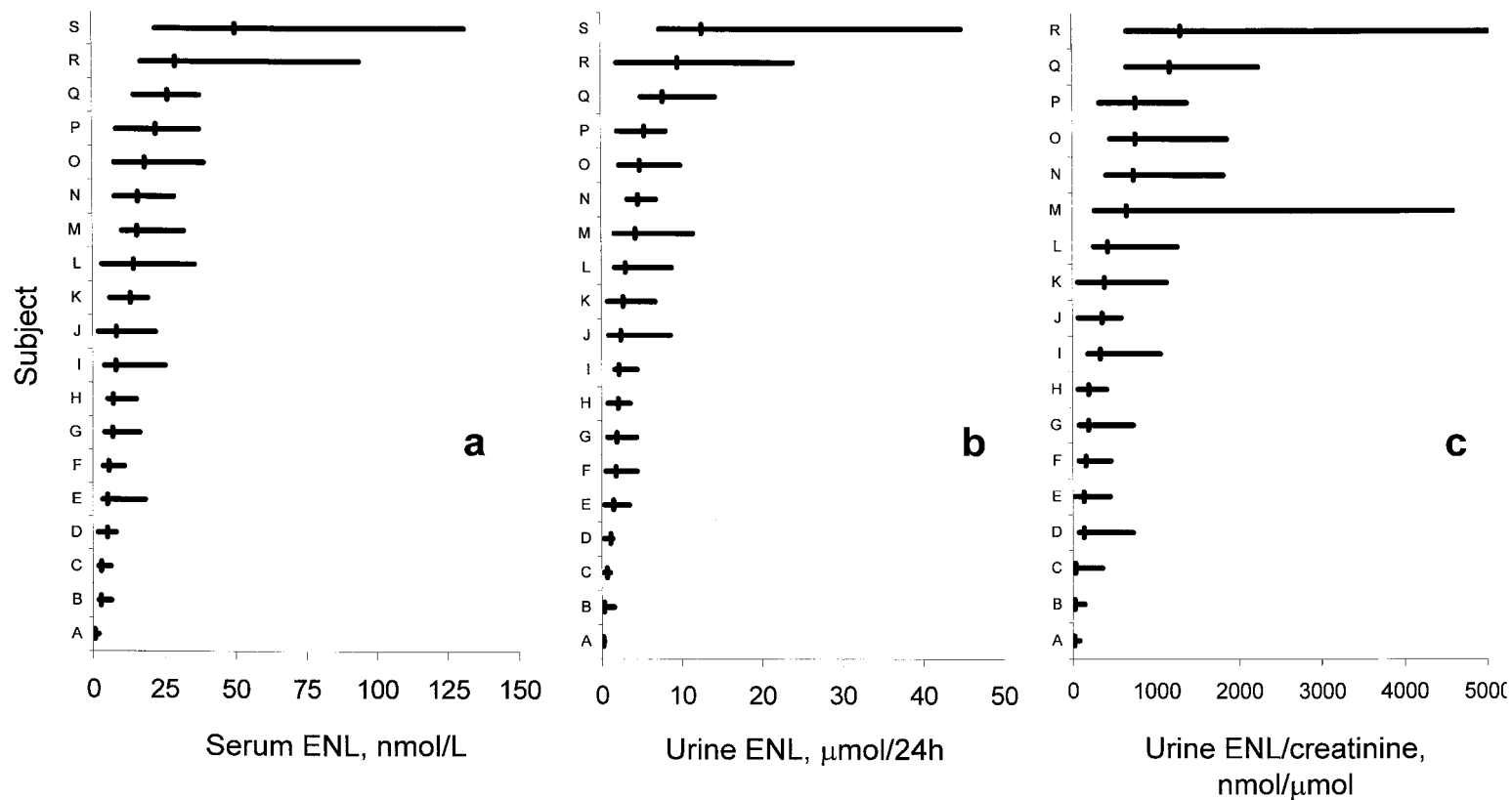
Association between enterolactone concentrations in serum and urine (II)

Linear correlations existed between ENL measurements in serum, 24-h urine, and spot urine (Fig. 10).

Determinants of serum enterolactone (III)

In men, multiple regression analysis showed a strong positive and independent association between serum ENL and consumption of whole-grain products (p=0.02) and fruit and berries (p=0.03), and constipation (p=0.02). Significant

Fig. 10. Correlations between (a) serum ENL concentration and 24-h urinary excretion, (b) 24-h urinary excretion and spot urine ENL:creatinine ratio, and (c) serum ENL concentration and spot urine ENL:creatinine ratio. Correlations were calculated between single samples (day 1). ENL serum concentration relative to urinary excretion of one subject (f) deviates from the 15 others (p<0.05).
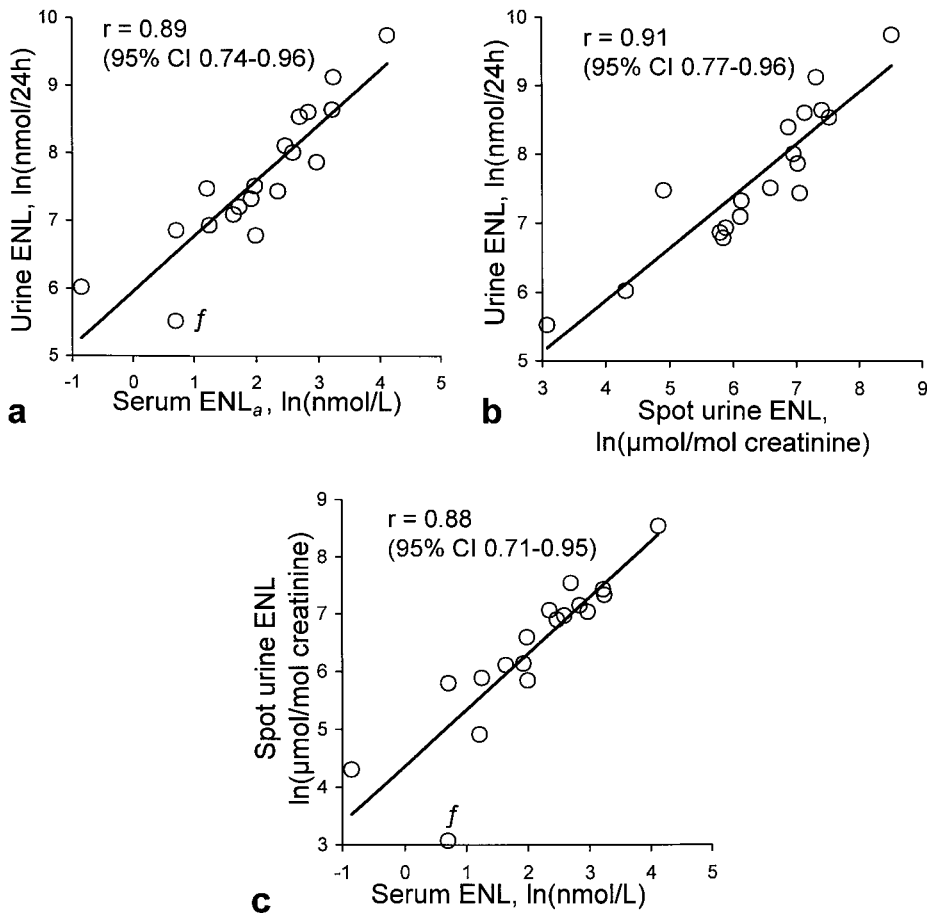
positive associations between serum ENL and years of education and age were also observed, but the associations did not remain significant after adjusting for other variables. Similarly, despite nonsmokers and ex-smokers having over 25% higher serum ENL concentrations than smokers, the association failed to remain significant after adjusting for other variables. Vegetable consumption was not an independent predictor of serum ENL, and no relationship was found between serum ENL and BMI, physical status, or alcohol consumption. The final model for men, including constipation and consumption of whole-grain products and fruit and berries, explained only 2.7% of the variation in serum ENL.

In women, multiple regression analysis revealed that BMI (p=0.0001), constipation (p=0.004), age (p=0.0003), and consumption of vegetables (p=0.0009) were independently and positively associated, and smoking (p=0.0001) negatively associated with serum ENL concentration. Other lignan-containing foods, as well as self-reported physical status and education, were associated with serum ENL; however, the associations did not remain significant after adjustment for other variables. No association was observed between serum ENL and alcohol consumption. Together, five variables (BMI, smoking, age, constipation, and consumption of vegetables) in the final model for women explained almost 14% of the variation in serum ENL.

Serum enterolactone in a dietary intervention study (IV)

The median concentration of serum ENL rose from 12.2 (95% CI 10.4-19.3) nmol/L at baseline to 19.5 (16.1-31.5) nmol/L (p=0.002) during the intervention period, being highest at the end of the intervention period. After the 5-week switchback period, the concentrations had decreased only slightly, to 17.6 (95% CI 14.6-26.5) nmol/L (p=0.18). Serum ENL was <10 nmol/L in 33% of subjects during the baseline period and in as many as 24% of subjects at the end of the intervention period. In 8% (7 subjects), serum ENL concentration was <10 nmol/L in all four samples.

Enterolactone in breast cancer

The mean serum ENL concentration was 20 nmol/L for all cases and 26 nmol/L for controls (p=0.003), 17 nmol/L for premenopausal cases and 21 nmol/L for controls (p=0.10), and 21 nmol/L for postmenopausal cases and 29 nmol/L for controls (p=0.01). The ORs adjusted for known risk factors for breast cancer are presented in Table 9 (V). This table also shows the ORs without the attenuating effect of within-subject variation in serum ENL (ICC=0.77).

Serum ENL was associated with intake of rye products, tea, dietary fiber (total and water-insoluble), and vitamin E. No association was seen with vegetables, fruit, or berries.

Table 9. Adjusted ORs of breast cancer in quintiles of serum ENL concentration, and the effect of the short-term within-subject variation on the results (V).

	Quintiles of serum ENL concentration					*P for trend*
All women	1	2	3	4	5	
S-ENL, nmol/L	< 6.2	6.2–14.1	14.2–24.3	24.4–34.8	> 34.8	
No. of cases	49	43	36	37	29	
OR[a] (95% CI)	1.00	0.60 (0.30–1.17)	0.57 (0.29–1.13)	0.53 (0.27–1.05)	0.38 (0.18–0.77)	0.03
Premenopausal women						
S-ENL, nmol/L	< 5.5	5.5–11.4	11.7–20.2	20.6–30.0	> 30.0	
No. of cases	17	14	13	13	11	
OR[a] (95% CI)	1.00	0.82 (0.22–3.09)	0.39 (0.09–1.78)	0.52 (0.14–2.00)	0.42 (0.10–1.77)	0.18
Postmenopausal women						
S-ENL, nmol/L	< 6.3	6.3 - 14.9	15.1 - 26.0	26.1 - 37.7	> 37.7	
No. of cases	32	29	23	24	18	
OR[a] (95% CI)	1.00	1.19 (0.46–3.07)	0.60 (0.24–1.49)	0.80 (0.32–2.02)	0.50 (0.19–1.28)	0.10

[a]ORs adjusted for age, area, age at menarche, age at first full-term pregnancy, use of oral contraceptives, use of estrogen replacement therapy, first-degree family history of breast cancer, history of benign breast disease, level of education, current alcohol intake, smoking habits, physical activity, waist:hip ratio, and BMI.

Effect of short-term variation (ICC=0.77) on the results, all women (additional analysis)

S-ENL, nmol/L	< 6.2	6.2–14.1	14.2–24.3	24.4–34.8	> 34.8
$OR_{observed}$ (95% CI)	1.00	0.60 (0.30–1.17)	0.57 (0.29–1.13)	0.53 (0.27–1.05)	0.38 (0.18–0.77)
OR_{true} (95% CI)	1.00	0.52 (0.21–1.23)	0.48 (0.20–1.17)	0.44 (0.18–1.07)	0.28 (0.11–0.71)

Discussion

Enterolactone as a biological marker

Both experimental and *in vitro* studies support the theory of the anticarcino-
genicity of lignans. Therefore, studying the characteristics of the most abundant
mammalian lignan, ENL, as a biological marker seemed justified.

Laboratory studies

The accuracy of the original version of basic TR-FIA for serum ENL was found
to be poor and a revised version of the method was described (I). The problems
had apparently originated from enzyme hydrolysis from too great a quantity of
sulfatase. The incubation of the matrix with the excessive enzyme led to a
disturbance of the assay. The revised version of the basic TR-FIA, by contrast,
proved to give reliable and repeatable results.

The exact mechanism for the interference remained unknown, and no other
groups have reported similar problems with hydrolysis. The antiserum for the
5-O-carboxymethoxy-ENL showed cross-reactions with some ENL metabolites
present in urine of human subjects. Could the cross-reactivity of lignan
metabolites with the ENL antiserum explain the problems in the methodology?
Probably not; although no data exist on the quantities of these metabolites in the
circulation, they are probably minimal in human subjects consuming their
habitual diet. A more likely explanation is that sulfatase releases some compound(s)
from the matrix. The compound(s) could act either by chelating the Europium
label or by binding to the antiserum. Nevertheless, the significance of the cross-
reactions of the lignan metabolites should be further studied when quantitative
standards of these compounds become available.

The TR-FIA for serum ENL is a feasible tool for epidemiological studies. The
advantages of TR-FIA in ENL analysis include its low costs, rapidness, and the
possibility of automation. Study III showed that the distribution of serum ENL
concentrations is skewed towards zero, which calls for sensitivity of the analytical
method. This is a major benefit of TR-FIA.

The short TR-FIA for serum without extraction reduces the costs of the method
even further. It is a useful alternative in large-scale studies or in studies with
limited sample quantity. The short TR-FIA gives higher results than the other
methods. However, in epidemiological studies, this should not be a problem. As
long as the assay is reliable, the ordering of the subjects by serum ENL
concentration is preserved. Because this is all that is required for studying a
marker-disease relationship, reliability, not accuracy, of the method is of primary
importance (Schulte *et al.* 1997). The results of the short TR-FIA can be
mathematically converted to correspond to the results of the basic TR-FIA. Both

serum and heparin plasma samples are suitable for ENL measurements by the short method. EDTA is a known interferent of TR-FIA because of its ability to chelate the Europium label. Extraction excludes EDTA from the extract, and EDTA plasma samples are thus available for the basic TR-FIA with extraction.

A disadvantage of TR-FIA, compared with the chromatographic methods, is its inability to measure other lignans, such as END, and their metabolites. In addition, TR-FIA is unable to separate the free ENL and the sulfate and glucuronide conjugates from each other. Therefore, other methods are more suitable for studies on the metabolism and kinetics of lignans.

Transitional studies

Because most cancers and many other chronic diseases take years or decades to manifest clinically, in epidemiology, the most useful biological markers are those with long elimination half-lives (Bernard 1995). The elimination half-life of ENL is probably relatively short (Morton *et al.* 1997a, Nesbitt *et al.* 1999, Mazur *et al.* 2000), and thus, the extent of within-subject variation is essential. In epidemiological studies, markers with large within-subject variation almost invariably show weak associations with any given disease (Vineis 1997). A substantial fraction of the absorbed mammalian lignans undergoes enterohepatic circulation, which stabilizes serum levels of all compounds, including ENL (Bach Knudsen *et al.* 2003). However, notable within-subject variation was evident within one week (II). Variation of such magnitude may cause attenuation of results in epidemiological studies and must be taken into account in design of future studies. To estimate the current ENL status within ±50% with 80% confidence requires three serum or 24-h urine samples or 10 spot urine samples. For greater confidence or precision, the number of samples must be higher. Study II did not explore the effect of fasting time on ENL. The only study available on this topic did not find any influence of fasting time between 0-12 h on serum ENL concentration (Hulten *et al.* 2002). However, in Studies II-V all of the samples had been collected after 4 h - overnight fasting.

Serum and 24-h urine ENL measurements demonstrated similar short-term within-subject variations. The advantages of serum, compared with urine samples, include the more specific immunoassay method, as the immunoassay of urine overestimates ENL excretion by 30% (Uehara *et al.* 2000b). In addition, drawing blood is much easier than collecting 24-h urine. Although the collection of spot urine samples is convenient, the spot urine ENL:creatinine ratio showed low reliability, and therefore, is less suitable for epidemiological studies than serum or 24-h urine samples. The spot urine samples were collected with no timing requirements, and an overnight or 4-h urine sample may have given better reliability.

The short-term reliability of serum ENL is quite similar in American and Finnish populations (II) (Horner *et al.* 2002), and the short-term reliability of overnight urine in Hawaiian volunteers resembles that of 24-h urine in Study II

(Franke *et al.* 2002). However, the long-term reliabilities of serum ENL in an American population (Zeleniuch-Jacquotte *et al.* 1998) and of overnight urine ENL:creatinine ratio in a Dutch population (den Tonkelaar *et al.* 2001) were poorer than the short-term ones in Study II. Whether the same is true in the Finnish population is unknown. The weakness of Study II was that the subjects represented a homogeneous group of similar age and lifestyle. Applying these results to the Finnish population at large should be done with caution.

Studies III-V were able to use only single measurements of serum ENL. Intraindividual variation greatly attenuated the results of Study V. A similar attenuation is probably present in Studies III and IV. These studies might have found larger differences in ENL concentrations between the different dietary groups had several serum samples from each subject been available.

Serum ENL concentrations vary markedly within populations and the distribution of the concentrations is strongly skewed towards zero. The median serum ENL concentrations here were 7.3 (II), 15.3 (III), 12.2 (IV), and 19.3 nmol/L (V). The results of Study III may explain the differences; serum ENL concentrations in aged females were the highest and in young males the lowest. Similarly, Study II with young student volunteers showed the lowest, and Study V with mainly postmenopausal females the highest median ENL concentration. The serum concentrations are in accordance with other large studies of Finnish subjects (median serum ENL 15-24 nmol/L) (Vanharanta *et al.* 1999, Uehara *et al.* 2000a, Stattin *et al.* 2002, Vanharanta *et al.* 2003, Kilkkinen *et al.* 2003b, 2004).

An early study suggested a very high correlation between grain intake and urine ENL excretion in groups of women with different diets (Adlercreutz *et al.* 1986a). Study III did not confirm this association. Only in males did a weak association exist between grain intake and serum ENL. The independent dietary determinants of serum ENL for men were consumption of whole-grain products and fruit and berries, and for women the consumption of vegetables. Two major differences between these two studies can partly explain the disparity. First, the former study analyzed four 72-h urine samples from each volunteer and compared the mean ENL of the groups. This methodology minimizes the great variation present in Study III. Second, the study population in the former study included vegetarians and breast cancer patients, and the differences both in grain intake and in ENL excretion between the extremities were huge. Study III, by contrast, compared ENL concentrations and dietary differences within the context of the typical Finnish diet, and did not therefore find such extreme differences. Thus, the explanatory role of diet on the vast variation in ENL appears to be minimal within an average population, in which nondietary confounding factors play a major role.

Study IV indicated that high intake of fruit, berries, and vegetables can increase median serum ENL concentration in a free-living population. This is in accordance with previous findings in controlled, experimental conditions (Hutchins *et al.* 1995, Kirkman *et al.* 1995). In Study IV, serum ENL concentrations were highest at the end of the intervention period, indicating that adapta-

tion of the microflora to high lignan intake takes at least three months. The importance of the adaptation time has been confirmed by other studies as well (Nesbitt *et al.* 1999, Jacobs *et al.* 2002, Tarpila *et al.* 2002). On the other hand, despite the dietary changes, serum ENL concentration stayed below 10 nmol/L in a quartile of the population even after 12 weeks of intervention.

The poor explanatory power of the diet for serum ENL concentrations in Studies III and IV suggests that the non-dietary (confounding) factors may determine individual ENL concentration reached. Study III indicated the four factors: age (in women), BMI (in women), smoking (in women), and constipation as subjective symptoms. All of these findings have later been confirmed by other studies. Serum ENL concentrations are higher in aged than in young subjects (III, Horner *et al.* 2002, Vanharanta *et al.* 2003). A possible explanation for this finding is that in aged subjects the elimination of foreign compounds, such as drugs, is slowed. In diabetic patients, serum ENL was positively associated with the severity of renal disease (von Hertzen *et al.* 2004). Serum ENL concentrations were higher in normal-weight than in under- or overweight women (III, Horner *et al.* 2002, Hulten *et al.* 2002) or men (Horner *et al.* 2002, Vanharanta *et al.* 2003). Although the difference in Study III could not be explained by diet, overweight individuals might have overestimated their consumption of healthy, lignan-containing food products. Further, a given lignan intake may result in lower serum ENL concentrations in obese than in normal-weight subjects because of the distribution of lignans to the fat tissue (Rickard *et al.* 1998). Smoking lowers serum ENL concentration (III, Hulten *et al.* 2002) by an unknown mechanism. Smoking can induce oxidizing enzymes and affect ENL catabolism similar to estrogens (Berta *et al.* 1992). In addition, cigarette smoke increases oxidative stress, and because ENL seems to function as an antioxidant (Pool-Zobel *et al.* 2000, Prasad 2000, Saarinen *et al.* 2000, Vanharanta *et al.* 2002b), the elimination of free radicals may consume ENL and lead to lower serum ENL concentrations in smokers than in nonsmokers. Volunteers with a subjective report of constipation show a higher serum ENL concentration than asymptomatic ones (III, Vanharanta *et al.* 2003). The physiological explanation for these findings remains obscure. Subjects with constipation may be speculated to have slower intestinal motility, and as a result, the metabolism and absorption of lignans are more complete than in asymptomatic subjects. Findings of the effect of alcohol intake on serum ENL are inconsistent. Studies III and V showed no association between alcohol intake and serum ENL, but reports on both positive (Horner *et al.* 2002) and negative (Vanharanta *et al.* 2003) associations are also available. This relation may depend on the type of alcohol consumed. Red wine, for example, is known to contain a significant amount of lignans (Mazur 1998, Nurmi *et al.* 2003b). Obviously, more studies on this subject are needed. The role of intestinal microflora, however, seems fundamental. The use of antimicrobial drugs may be one of the strongest confounders. Subjects receiving antimicrobial treatment during the past three months were excluded in Studies II and III. The effect of the antimicrobials may, however, last longer (Kilkkinen *et al.* 2002).

Most of the extensive differences in serum ENL concentrations between individuals remained unexplained. A better explanatory power between the determinants and serum lignans might have been achieved if 1) ENL was measured in several serum samples each subject, 2) the lignan measurement also included other circulating lignans, END being the most important, 3) the FFQ had been originally planned to assess plant lignan intake.

Enterolactone in breast cancer

Experimental animal studies provide strong evidence of a beneficial action of lignans against mammary cancer (Thompson *et al.* 1996a, 1996b, Saarinen *et al.* 2000, 2001, 2002a, Chen *et al.* 2003a). In Study V, serum ENL was associated with a reduced risk of breast cancer. This supports the findings of other case-control studies (Adlercreutz *et al.* 1982, Ingram *et al.* 1997, Dai *et al.* 2002). By contrast, only two of four later prospective studies found an inverse association between ENL and risk of breast cancer (den Tonkelaar *et al.* 2001, Hulten *et al.* 2002, Boccardo *et al.* 2004, Kilkkinen *et al.* 2004).

Numerous explanations for the inconsistency exist. A major weakness of Study V and the other case-control studies is the possibility that the disease process influenced the level of ENL. Catabolic diseases, including malignancies, are known to cause a decrease in serum total cholesterol (Iribarren *et al.* 1995). In a similar manner, the cancer process may affect the metabolism of lignans. No follow-up studies appear to have examined the effect of contracting cancer on serum ENL levels. The within-subject variation in ENL is another major concern. With one exception, all of the above studies measured ENL in only a single blood or urine sample. Den Tonkelaar *et al.* (2001) did, however, use the mean of two overnight urine samples. Nevertheless, the reliability in this study proved to be so poor that the authors themselves suggested a substantial attenuation of their results and questioned the significance of their findings. A serious weakness of all of the available studies on the association between ENL and risk of breast cancer is the lack of knowledge about recent antimicrobial treatments. Especially in studies with an ENL measurement from only one sample, the absence of this information may notably distort the results. Alcohol consumption is a known risk factor of breast cancer (Singletary *et al.* 2001) and may have an association with ENL concentration in serum (Horner *et al.* 2002, Vanharanta *et al.* 2003). Information on alcohol intake should therefore be available, which was only the case in Study V and in studies by Ingram *et al.* (1997) and Kilkkinen *et al.* (2004). In these three studies, alcohol intake was not, however, associated with either breast cancer or ENL.

The existing epidemiological studies on ENL and risk of breast cancer have several drawbacks, and whether lignans truly offer protection against breast cancer, remains an open question. The development of breast cancer, postmenopausally, in particular, is a process requiring years or even decades. An ENL measurement of a single serum or urine sample may not reflect the critical

period of time. Based on animal studies, the critical time during which lignans provide protection is childhood (Ward *et al.* 2000), which makes it very difficult to study.

Several theories on the potential protective mechanisms of action of ENL are available. Although it is unlikely that ENL acts through the estrogen receptors (Saarinen *et al.* 2000), it may show its antiestrogenity via SHBG. An increase in plasma SHBG concentration reduces the free circulating steroids, thus reducing the bioavailability of estrogens (Adlercreutz 1990). Furthermore, SHBG is not only a passive transporter of steroid hormones but also an active compound. SHBG receptors are present in breast tissue and in estrogen-dependant MCF-7 breast cancer cells (Fortunati *et al.* 1999), and the binding of the SHBG-estrogen complex to its receptor decreases estrogen-induced cell growth (Rosner *et al.* 1999). Other possible modes of action of ENL include the reduction of IGF-I (Rickard *et al.* 2000), antioxidative activity (Vanharanta *et al.* 2002b), and intracellular competition for steroid enzymes (Evans *et al.* 1995).

ENL is a product of intestinal metabolism of plant lignans. But is ENL the active molecule? A microflora capable of metabolizing lignans may also generate other active metabolic products. These possible "side-products" form a largely unknown area of research. One candidate for such a compound may be butyrate. It confers protection against NMU-induced mammary carcinoma in rats (Belobrajdic *et al.* 2000), and a dietary supplementation with rye was found to increase the production of both fecal butyrate and serum ENL (McIntosh *et al.* 2003). Regardless of the identity of the molecule(s), the increase in the risk of breast cancer following antimicrobial treatments (Knekt *et al.* 2000, Velicer *et al.* 2004) supports the hypothesis that the intestinal microflora plays a key role in mammary carcinogenesis.

Conclusions

Time-resolved fluoroimmunoassay proved to be a useful method for serum ENL analysis.

Dietary variables were unable to explain the vast variation in serum ENL concentrations in subjects consuming a typical Finnish diet. Nondietary factors, by contrast, seem to play an important role in determining serum ENL concentration. Serum ENL is therefore unsuited as a marker of dietary, including plant lignan, intake. It may more appropriately be used as a measurement of body exposure to mammalian lignans.

Of the known determinants of serum ENL, the gut microflora may be the most important. In a standard diet, ENL can express differences well between individuals in the ability of the microflora to convert plant lignans to mammalian lignans. The importance of the intestinal microflora for mammalian lignan formation leads to speculation that ENL may serve as a good marker of a functional intestinal microflora. The profound effect of antimicrobials on the END:ENL ratio suggests that this measurement may function as a biomarker of intestinal function even better than ENL alone.

The value of single serum or urine ENL measurements as a biological marker in epidemiology is limited. Single measurements face the problem of vast individual variation, which may lead to attenuation of results. Epidemiological studies should therefore measure ENL in several serum or urine samples.

Experimental animal studies strongly suggest a protective role for ENL against breast cancer. This work strengthens the hypothesis; serum ENL was inversely associated with breast cancer risk in a case-control study. However, since later prospective studies have given conflicting results, this question remains open.

Clinical chemistry has many things to offer to this research field in future. Important future tasks include:

1) finding a more stable marker for body exposure to mammalian lignans than serum ENL. ENL concentration may be more stable in adipose tissue, and therefore, adipose tissue ENL concentration could serve as a better biological marker in cancer epidemiology.
2) developing and characterizing a biological marker of functional intestinal microflora. This marker could, in addition to the END:ENL ratio, include other compounds besides lignans.

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References

Adlercreutz H, Fotsis T, Heikkinen R, Dwyer JT, Woods M, Goldin BR and Gorbach SL (1982). Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian postmenopausal women and in women with breast cancer. Lancet 2(8311): 1295-1299.

Adlercreutz H (1984). Does fiber-rich food containig animal lignan precursors protect against both colon and breast cancer? An extension of the "fiber hypothesis". Gastroenterology 86(4): 761-766.

Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Mäkelä T, Brunow G and Hase T (1986a). Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. J Steroid Biochem 25(5B): 791-797.

Adlercreutz H, Musey PI, Fotsis T, Bannwart C, Wähälä K, Makela T, Brunow G and Hase T (1986b). Identification of lignans and phytoestrogens in urine of chimpanzees. Clin Chim Acta 158(2): 147-154.

Adlercreutz H, Höckerstedt K, Bannwart C, Bloigu S, Hämäläinen E, Fotsis T and Ollus A (1987). Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG). J Steroid Biochem 27(4-6): 1135-1144.

Adlercreutz H (1990). Western diet and western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest 50(Suppl 201): 3-23.

Adlercreutz H, Mousavi Y, Clark J, Höckerstedt K, Hämäläinen E, Wähälä K, Mäkelä T and Hase T (1992). Dietary phytoestrogens and cancer: *in vitro* and *in vivo* studies. J Steroid Biochem 41(3-8): 331-337.

Adlercreutz H, Bannwart C, Wähälä K, Mäkelä T, Brunow G, Hase T, Arosemena P, Kellis J and Vickery L (1993a). Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J Steroid Biochem Molec Biol 44(2): 147-153.

Adlercreutz H, Fotsis T, Lampe J, Wähälä K, Mäkelä T, Brunow G and Hase T (1993b). Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas chromatography-mass spectrometry. Scand J Clin Lab Invest 53(Suppl. 215): 5-18.

Adlercreutz H, Fotsis T, Kurzer MS, Wähälä K, Mäkelä T and Hase T (1995a). Isotope dilution gas chromatographic-mass spectrometric method for the determination of unconjugated lignans and isoflavonoids in human feces, with preliminary results in omnivorous and vegetarian women. Anal Biochem 225(1): 101-108.

Adlercreutz H, van der Wildt J, Kinzel J, Attalla H, Wähälä K, Mäkelä T, Hase T and Fotsis T (1995b). Lignan and isoflavonoid conjugates in human urine. J Steroid Biochem Molec Biol 52(1): 97-103.

Adlercreutz H, Wang G, Lapcík O, Hampl R, Wähälä K, Mäkelä T, Lusa K, Talme M and Mikola H (1998). Time-resolved fluoroimmunoassay for plasma enterolactone. Anal Biochem 265(2): 208-215.

Atkinson DA, Hill HH and Shultz TD (1993). Quantification of mammalian lignans in biological fluids using gas chromatography with ion mobility detection. J Chromatogr 617(2): 173-179.

Axelson M and Setchell KD (1981). The excretion of lignans in rats — evidence for an intestinal bacterial source for this new group of compounds. FEBS Lett 123(2): 337-342.

Axelson M, Sjövall J, Gustafsson BE and Setchell KD (1982). Origin of lignans in mammals and identification of a precursor from plants. Nature 298(5875): 659-660.

Ayres D and Loike J (1990). Lignans. Chemical, biological and clinical properties. Cambridge University Press, Cambridge, UK.

Bach Knudsen KE, Serena A, Kjaer AK, Tetens I, Heinonen SM, Nurmi T and Adlercreutz H (2003). Rye bread in the diet of pigs enhances the formation of enterolactone and increases its levels in plasma, urine and feces. J Nutr 133(5): 1368-1375.

Bannwart C, Adlercreutz H, Fotsis T, Wähälä K, Hase T and Brunow G (1984). Identification of O-desmethylangolensin, a metabolite of daidzein, and of matairesinol, one likely plant precursor of the animal lignan enterolactone, in human urine. Finn Chem Lett: 120-125.

Bannwart C, Adlercreutz H, Wähälä K, Brunow G and Hase T (1989). Detection and identification of the plant lignans lariciresinol, isolariciresinol and secoisolariciresinol in human urine. Clin Chim Acta 180(3): 293-301.

Belobrajdic DP and McIntosh GH (2000). Dietary butyrate inhibits NMU-induced mammary cancer in rats. Nutr Cancer 36(2): 217-223.

Bernard AM (1995). Biokinetics and stability aspects of biomarkers: recommendations for application in population studies. Toxicology 101(1-2): 65-71.

Berta L, Frairia R, Fortunati R, Fazzari N and Gaidano G (1992). Smoking effects on the hormonal balance of fertile women. Horm Res 37(1-2): 45-48.

Bland JM and Altman DG (1996). Measurement error proportional to the mean. BMJ 313(7049): 106.

Boccardo F, Lunardi GL, Petti AR and Rubagotti A (2003). Enterolactone in breast cyst fluid: correlation with EGF and breast cancer risk. Breast Cancer Res Treat 79(1): 17-23.

Boccardo F, Lunardi G, Guglielmini P, Parodi M, Murialdo R, Schettini G and Rubagotti A (2004). Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. Eur J Cancer 40(1): 84-89.

Boker LK, Van der Schouw YT, De Kleijn MJ, Jacques PF, Grobbee DE and Peeters PH (2002). Intake of dietary phytoestrogens by Dutch women. J Nutr 132(6): 1319-1328.

Borriello SP, Setchell KD, Axelson M and Lawson AM (1985). Production and metabolism of lignans by the human fecal flora. J Appl Bacteriol 58(1): 37-43.

Brzezinski A, Adlercreutz H, Sahoul R, Rösler H, Shmueli A, Tanos V and Schenker J (1997). Short-term effects of phytoestrogen-rich diet on postmenopausal women. J North Am Menopause Soc 4(2): 89-94.

Bylund A, Lundin E, Zhang JX, Nordin A, Kaaks R, Stenman UH, Aman P, Adlercreutz H, Nilsson TK, Hallmans G, Bergh A and Stattin P (2003). Randomised controlled short-term intervention pilot study on rye bran bread in prostate cancer. Eur J Cancer Prev 12(5): 407-415.

Chen J, Stavro PM and Thompson LU (2002). Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. Nutr Cancer 43(2): 187-192.

Chen J, Tan KP, Ward WE and Thompson LU (2003a). Exposure to flaxseed or its purified lignan during suckling inhibits chemically induced rat mammary tumorigenesis. Exp Biol Med 228(8): 951-958.

Chen J and Thompson LU (2003b). Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration *in vitro*. Breast Cancer Res Treat 80(2): 163-170.

Dabrosin C, Chen J, Wang L and Thompson LU (2002). Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. Cancer Lett 185(1): 31-37.

Dai Q, Franke AA, Jin F, Shu XO, Hebert JR, Custer LJ, Cheng J, Gao YT and Zheng W (2002). Urinary Excretion of Phytoestrogens and Risk of Breast Cancer among Chinese Women in Shanghai. Cancer Epidemiol Biomarkers Prev 11(9): 815-821.

Dai Q, Franke AA, Yu H, Shu XO, Jin F, Hebert JR, Custer LJ, Gao YT and Zheng W (2003). Urinary phytoestrogen excretion and breast cancer risk: evaluating potential effect modifiers endogenous estrogens and anthropometrics. Cancer Epidemiol Biomarkers Prev 12(6): 497-502.

Davin LB and Lewis NG (2000). Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. Plant Physiol 123(2): 453-462.

Dehennin L, Reiffsteck A, Jondet M and Thibier M (1982). Identification and quantitative estimation of a lignan in human and bovine semen. J Reprod Fertil 66(1): 305-309.

den Tonkelaar I, Keinan-Boker L, Veer PV, Arts CJ, Adlercreutz H, Thijssen JH and Peeters PH (2001). Urinary phytoestrogens and postmenopausal breast cancer risk. Cancer Epidemiol Biomarkers Prev 10(3): 223-228.

Dickson EF, Pollak A and Diamandis EP (1995). Ultrasensitive bioanalytical assays using time-resolved fluorescence detection. Pharmac Ther 66(2): 207-235.

Evans BA, Griffiths K and Morton MS (1995). Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. J Endocrinol 147(2): 295-302.

Finnish Cancer Registry (updated 2 Mar 2004). Cancer Statistics at www.cancerregistry.fi, accessed 18 April 2004.

Fortunati N, Becchis M, Catalano MG, Comba A, Ferrera P, Raineri M, Berta L and Frairia R (1999). Sex hormone-binding globulin, its membrane receptor, and breast cancer: a new approach to the modulation of estradiol action in neoplastic cells. J Steroid Biochem Mol Biol 69(1-6): 473-479.

Franke AA, Custer LJ, Wilkens LR, Le Marchand LL, Nomura AM, Goodman MT and Kolonel LN (2002). Liquid chromatographic-photodiode array mass spectrometric analysis of dietary phytoestrogens from human urine and blood. J Chromatogr B Analyt Technol Biomed Life Sci 777(1-2): 45-59.

Fraser CG and Harris EK (1989). Generation and application of data on biological variation in clinical chemistry. Crit Rev Clin Lab Sci 27(5): 409-437.

Gamache PH and Acworth IN (1998). Analysis of phytoestrogens and polyphenols in plasma, tissue, and urine using HPLC with coulometric array detection. Proc Soc Exp Biol Med 217(3): 274-280.

Giovannucci E (1999). Insulin-like growth factor-I and binding protein-3 and risk of cancer. Horm Res 51(Suppl 3): 34-41.

Glatt H, Boeing H, Engelke CE, Ma L, Kuhlow A, Pabel U, Pomplun D, Teubner W and Meinl W (2001). Human cytosolic sulphotransferases: genetics, characteristics, toxicological aspects. Mutat Res 482(1-2): 27-40.

Glitsø LV, Mazur WM, Adlercreutz H, Wähälä K, Mäkelä T, Sandström B and Bach Knudsen KE (2000). Intestinal metabolism of rye lignans in pigs. Br J Nutr 84(4): 429-437.

Goldin BR, Adlercreutz H, Dwyer JT, Swenson L, Warram JH and Gorbach SL (1981). Effect of diet on excretion of estrogens in pre- and postmenopausal women. Cancer Res 41(9 Pt 2): 3771-3773.

Grace PB, Taylor JI, Botting NP, Fryatt T, Oldfield MF, Al-Maharik N and Bingham SA (2003). Quantification of isoflavones and lignans in serum using isotope dilution liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 17(12): 1350-1357.

Grandjean P (1995). Biomarkers in epidemiology. Clin Chem 41(12): 1800-1803.

Hargreaves D, Potten C, Harding C, Shaw L, Morton M, Roberts S, Howell A and Bundred N (1999). Two-week dietary soy supplementation has an estrogenic effect on normal premenopausal breast. J Clin Endocrinol Metab 84(11): 4017-4024.

Heinonen S, Nurmi T, Liukkonen K, Poutanen K, Wähälä K, Deyama T, Nishibe S and Adlercreutz H (2001). *In vitro* metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. J Agric Food Chem 49(7): 3178-3186.

Hemmilä I (1988). Lanthanides as probes for time-resolved fluorometric immunoassays. Scand J Clin Lab Invest 48: 389-400.

von Hertzen L, Forsblom C, Stumpf K, Pettersson-Fernholm K, Adlercreutz H and Groop P on behalf of FinnDiane Study Group (2004). Highly elevated serum phytoestrogen concentrations in patients with diabetic nephropathy. J Int Med 255(5):602-609.

Hiar CE (1997). Performance of the DCA 2000 microalbumin/creatinine system. Tarrytown, Bayer.

Hirano T, Fukuoka K, Oka K, Naito T, Hosaka K, Mitsuhashi H and Matsumoto Y (1990). Antiproliferative activity of mammalian lignan derivatives against the human breast carcinoma cell line, ZR-75-1. Cancer Invest 8(6): 595-602.

Hong SJ, Kim SI, Kwon SM, Lee JR and Chung BC (2002). Comparative study of concentration of isoflavones and lignans in plasma and prostatic tissues of normal control and benign prostatic hyperplasia. Yonsei Med J 43(2): 236-241.

Hopkins WG (2002). A new view of statistics. http://www.sportsci.org/resource/stats/index.html, accessed February 2002.

Horner NK, Kristal AR, Prunty J, Skor HE, Potter JD and Lampe JW (2002). Dietary determinants of plasma enterolactone. Cancer Epidemiol Biomarkers Prev 11(1): 121-126.

Horn-Ross PL, Barnes S, Kirk M, Coward L, Parsonnet J and Hiatt RA (1997). Urinary phytoestrogen levels in young women from a multiethnic population. Cancer Epidemiol Biomarkers Prev 6(5): 339-345.

Horn-Ross PL, John EM, Lee M, Stewart SL, Koo J, Sakoda LC, Shiau AC, Goldstein J, Davis P and Perez-Stable EJ (2001). Phytoestrogen consumption and breast cancer risk in a multiethnic population: the Bay Area Breast Cancer Study. Am J Epidemiol 154(5): 434-441.

Horn-Ross PL, Hoggatt KJ, West DW, Krone MR, Stewart SL, Anton H, Bernstei CL, Deapen D, Peel D, Pinder R, Reynolds P, Ross RK, Wright W and Ziogas A (2002). Recent diet and breast cancer risk: the California Teachers Study (USA). Cancer Causes Control 13(5): 407-415.

Howarth RD (1936). Natural resins. Ann Rep Prog Chem 33: 266-279.

Hulten K, Winkvist A, Lenner P, Johansson R, Adlercreutz H and Hallmans G (2002). An incident case-referent study on plasma enterolactone and breast cancer risk. Eur J Nutr 41(4): 168-176.

Hutchins AM, Lampe JW, Martini MC, Campbell DR and Slavin JL (1995). Vegetables, fruits, and legumes: effect on urinary isoflavonoid phytoestrogen and lignan excretion. J Am Diet Assoc 95(7): 769-774.

Hutchins AM, Martini MC, Olson BA, Thomas W and Slavin JL (2001). Flaxseed consumption influences endogenous hormone concentrations in postmenopausal women. Nutr Cancer 39(1): 58-65.

Ingram D, Sanders K, Kolybaba M and Lopez D (1997). Case-control study of phyto-oestrogens and breast cancer. Lancet 350(9083): 990-994.

Iribarren C, Reed DM, Chen R, Yano K and Dwyer JH (1995). Low serum cholesterol and mortality. Which is the cause and which is the effect? Circulation 92(9): 2396-2403.

Jacobs DR, Jr., Pereira MA, Stumpf K, Pins JJ and Adlercreutz H (2002). Whole grain food intake elevates serum enterolactone. Br J Nutr 88(2): 111-116.

Jacobs E, Kulling S and Metzler M (1999a). Novel metabolites of the mammalian lignans enterolactone and enterodiol in human urine. J Steroid Biochem Molec Biol 68(5-6): 211-218.

Jacobs E and Metzler M (1999b). Oxidative metabolism of the mammalian lignans enterolactone and enterodiol by rat, pig, and human liver microsomes. J Agric Food Chem 47: 1071-1077.

Johnson-Thompson MC and Guthrie J (2000). Ongoing research to identify environmental risk factors in breast carcinoma. Cancer 88(5 Suppl): 1224-1229.

Juntunen KS, Mazur WM, Liukkonen KH, Uehara M, Poutanen KS, Adlercreutz H and Mykkänen HM (2000). Consumption of wholemeal rye bread increases serum concentrations and urinary excretion of enterolactone compared with consumption of white wheat bread in healthy Finnish men and women. Br J Nutr 84(6): 839-846.

Keinan-Boker L, van Der Schouw YT, Grobbee DE and Peeters PH (2004). Dietary phytoestrogens and breast cancer risk. Am J Clin Nutr 79(2): 282-288.

Kilkkinen A, Pietinen P, Klaukka T, Virtamo J, Korhonen P and Adlercreutz H (2002). Use of oral antimicrobials decreases serum enterolactone concentration. Am J Epidemiol 155(5): 472-477.

Kilkkinen A, Valsta LM, Virtamo J, Stumpf K, Adlercreutz H and Pietinen P (2003a). Intake of lignans is associated with serum enterolactone concentration in Finnish men and women. J Nutr 133(6): 1830-1833.

Kilkkinen A, Virtamo J, Virtanen MJ, Adlercreutz H, Albanes D and Pietinen P (2003b). Serum enterolactone concentration is not associated with prostate cancer risk in a nested case-control study. Cancer Epidemiol Biomarkers Prev 12(11 Pt 1): 1209-1212.

Kilkkinen A, Virtamo J, Vartiainen E, Sankila R, Virtanen MJ, Adlercreutz H and Pietinen P (2004). Serum enterolactone concentration is not associated with breast cancer risk in a nested case-control study. Int J Cancer 108(2): 277-280.

Kirkman LM, Lampe JW, Campbell DR, Martini MC and Slavin JL (1995). Urinary lignan and isoflavonoid excretion in men and women consuming vegetable and soy diets. Nutr Cancer 24(1): 1-12.

Kitts DD, Yuan YV, Wijewickreme AN and Thompson LU (1999). Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. Mol Cell Biochem 202(1-2): 91-100.

de Kleijn MJ, van der Schouw YT, Wilson PW, Adlercreutz H, Mazur W, Grobbee DE and Jacques PF (2001). Intake of dietary phytoestrogens is low in postmenopausal women in the United States: the Framingham study(1-4). J Nutr 131(6): 1826-1832.

Knekt P, Adlercreutz H, Rissanen H, Aromaa A, Teppo L and Heliövaara M (2000). Does antibacterial treatment for urinary tract infection contribute to the risk of breast cancer? Br J Cancer 82(5): 1107-1110.

Kulling SE, Jacobs E, Pfeiffer E and Metzler M (1998). Studies on the genotoxicity of the mammalian lignans enterolactone and enterodiol and their metabolic precursors at various endpoints *in vitro*. Mutat Res 416(1-2): 115-124.

Kuusi T, Ehnholm C, Huttunen JK, Kostiainen E, Pietinen P, Leino U, Uusitalo U, Nikkari T, Iacono JM and Puska P (1985). Concentration and composition of serum lipoproteins during a low-fat diet at two levels of polyunsaturated fat. J Lipid Res 26(3): 360-367.

Kwon M, Davin LB and Lewis NG (2001). In situ hybridization and immunolocalization of lignan reductases in woody tissues: implications for heartwood formation and other forms of vascular tissue preservation. Phytochemistry 57(6): 899-914.

Lampe JW, Martini MC, Kurzer MS, Adlercreutz H and Slavin JL (1994). Urinary lignan and isoflavonoid excretion in premenopausal women consuming flaxseed powder. Am J Clin Nutr 60(1): 122-128.

Lampe JW, Gustafson DR, Hutchins AM, Martini MC, Li S, Wähälä K, Grandits GA, Potter JD and Slavin JL (1999). Urinary isoflavonoid and lignan excretion on a western diet: relation to soy, vegetable, and fruit intake. Cancer Epidemiol Biomarkers Prev 8(8): 699-707.

Martin ME, Haourigui M, Pelissero C, Benassayag C and Nunez EA (1996). Interactions between phytoestrogens and human sex steroid binding protein. Life Sci 58(5): 429-436.

Mazur W and Adlercreutz H (1998). Naturally occurring oestrogens in food. Pure & Appl Chem 70(9): 1759-1776.

Mazur WM, Uehara M, Wähälä K and Adlercreutz H (2000). Phyto-oestrogen content of berries, and plasma concentrations and urinary excretion of enterolactone after a single strawberry-meal in human subjects. Br J Nutr 83(4): 381-387.

McCann SE, Moysich KB, Freudenheim JL, Ambrosone CB and Shields PG (2002). The risk of breast cancer associated with dietary lignans differs by CYP17 genotype in women. J Nutr 132(10): 3036-3041.

McIntosh GH, Noakes M, Royle PJ and Foster PR (2003). Whole-grain rye and wheat foods and markers of bowel health in overweight middle-aged men. Am J Clin Nutr 77(4): 967-974.

McNaught AD and Wilkinson A (1997). IUPAC Compendium of Chemical Terminology. Royal Society of Chemistry, Cambridge, UK.

Morton MS, Wilcox G, Wahlqvist ML and Griffiths K (1994). Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. J Endocrinol 142(2): 251-259.

Morton MS, Matos-Ferreira A, Abranches-Monteiro L, Correia R, Blacklock N, Chan PS, Cheng C, Lloyd S, Chieh-ping W and Griffiths K (1997a). Measurement and metabolism of isoflavonoids and lignans in the human male. Cancer Lett 114(1-2): 145-151.

Morton MS, Chan PS, Cheng C, Blacklock N, Matos-Ferreira A, Abranches-Monteiro L, Correia R, Lloyd S and Griffiths K (1997b). Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the United Kingdom. Prostate 32(2):122-128.

Morton MS, Arisaka O, Miyake N, Morgan LD and Evans BA (2002). Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. J Nutr 132(10): 3168-3171.

Mousavi Y and Adlercreutz H (1992). Enterolactone and estradiol inhibit each other's proliferative effect on MCF-7 breast cancer cells in culture. J Steroid Biochem 41(3-8): 615-619.

Mäkelä TH, Wähälä KT and Hase TA (2000). Synthesis of enterolactone and enterodiol precursors as potential inhibitors of human estrogen synthetase (aromatase). Steroids 65(8): 437-441.

Mäkelä TH, Kaltia SA, Wähälä KT and Hase TA (2001). alpha,beta-Dibenzyl-gamma-butyrolactone lignan alcohols: total synthesis of (+/-)-7'-hydroxyenterolactone, (+/-)-7'-hydroxymatairesinol and (+/-)-8-hydroxyenterolactone. Steroids 66(10): 777-784.

Männistö S, Virtanen M, Mikkonen T, Pietinen P (1996). Reproducibility and validity of a food frequences questionnaire in a case-control study on breast cancer. J Clin Epidemiol 49(4): 401-409.

Nesbitt PD, Lam Y and Thompson LU (1999). Human metabolism of mammalian lignan precursors in raw and processed flaxseed. Am J Clin Nutr 69(3): 549-555.

Nishibe S, Kinoshita H, Takeda H and Okano G (1990). Phenolic compounds from stem bark of Acanthopanax senticosus and their pharmacological effect in chronic swimming stressed rats. Chem Pharm Bull 38(6): 1763-1765.

Nishibe S (1993). Lignans as bioactive compounds in traditional medicines. Polyphenolic phenomena. A. Scalbert, INRA Editions, Paris, France: 247-255.

Nurmi T and Adlercreutz H (1999). Sensitive high-performance liquid chromatographic method for profiling phytoestrogens using coulometric electrode array detection: application to plasma analysis. Anal Biochem 274(1): 110-117.

Nurmi T, Voutilainen S, Nyyssönen K, Adlercreutz H and Salonen JT (2003a). Liquid chromatography method for plant and mammalian lignans in human urine. J Chromatogr B Analyt Technol Biomed Life Sci 798(1): 101-110.

Nurmi T, Heinonen S, Mazur W, Deyama T, Nishibe S, Adlercreutz H (2003b) Lignans in selected wines. Food Chem 83(2): 303-309.

Orcheson LJ, Rickard SE, Seidl MM and Thompson LU (1998). Flaxseed and its mammalian lignan precursor cause a lengthening or cessation of estrous cycling in rats. Cancer Letters 125(1-2): 69-76.

Owen R, Mier W, Giacosa A, Hull W, Spiegelhalder B and Bartsch H (2000). Identification of lignans as major components in the phenolic fraction of olive oil. Clin Chem 46(7): 967-988.

Phipps WR, Martini MC, Lampe JW, Slavin JL and Kurzer MS (1993). Effect of flax seed ingestion on the menstrual cycle. J Clin Endocrinol Metab 77(5): 1215-1219.

Pietinen P, Dougherty R, Mutanen M, Leino U, Moisio S, Iacono J and Puska P (1984). Dietary intervention study among 30 free-living families in Finland. J Am Diet Assoc 84(3): 313-318.

Pool-Zobel BL, Adlercreutz H, Glei M, Liegibel UM, Sittlingon J, Rowland I, Wähälä K and Rechkemmer G (2000). Isoflavonoids and lignans have different potentials to modulate oxidative genetic damage in human colon cells. Carcinogenesis 21(6): 1247-1252.

Prasad K (2000). Antioxidant activity of secoisolariciresinol diglucoside-derived metabolites, secoisolariciresinol. enterodiol, and enterolactone. Int J Angiol 9(4): 220-225.

Puska P, Iacono JM, Nissinen A, Vartiainen E, Dougherty R, Pietinen P, Leino U, Uusitalo U, Kuusi T, Kostiainen E, Nikkari T, Seppälä E, Vapaatalo H and Huttunen J (1985). Dietary fat and blood pressure: an intervention study on the effects of a low-fat diet with two levels of polyunsaturated fat. Prev Med 14(5): 573-584.

Rickard SE and Thompson LU (1998). Chronic exposure to secoisolariciresinol diglycoside alters lignan disposition in rats. J Nutr 128(3): 615-623.

Rickard SE, Yuan YV, Chen J and Thompson LU (1999). Dose effects of flaxseed and its lignan on N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. Nutr Cancer 35(1): 50-57.

Rickard SE, Yuan YV and Thompson LU (2000). Plasma insulin-like growth factor I levels in rats are reduced by dietary supplementation of flaxseed or its lignan secoisolariciresinol diglycoside. Cancer Letters 161(1): 47-55.

Ritter JK (2000). Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chem Biol Interact 129(1-2): 171-193.

Rosner W, Hryb DJ, Khan MS, Nakhla AM and Romas NA (1999). Sex hormone-binding globulin mediates steroid hormone signal transduction at the plasma membrane. J Steroid Biochem Mol Biol 69(1-6): 481-485.

Ross D, Kepa JK, Winski SL, Beall HD, Anwar A and Siegel D (2000). NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. Chem Biol Interact 129(1-2): 77-97.

Rothman N, Steward WF, Schulte PA (1995). Incorporating biomarkers into cancer epidemiology: a matrix biomarker and study design categories. Cancer Epidemiol Biomarkers Prev 4(4): 301-311.

Saarinen NM, Wärri A, Mäkelä SI, Eckerman C, Reunanen M, Ahotupa M, Salmi SM, Franke AA, Kangas L and Santti R (2000). Hydroxymatairesinol, a novel enterolactone precursor with antitumor properties from coniferous tree (Picea abies). Nutr Cancer 36(2): 207-216.

Saarinen NM, Huovinen R, Wärri A, Mäkelä SI, Valentin-Blasini L, Needham L, Eckerman C, Collan YU and Santti R (2001). Uptake and metabolism of hydroxymatairesinol in relation to its anticarcinogenicity in DMBA-induced rat mammary carcinoma model. Nutr Cancer 41(1-2): 82-90.

Saarinen NM, Huovinen R, Wärri A, Mäkelä SI, Valentin-Blasini L, Sjöholm R, Ämmälä J, Lehtilä R, Eckerman C, Collan YU and Santti RS (2002a). Enterolactone inhibits the growth of 7,12-dimethylbenz(a)anthracene-induced mammary carcinomas in the rat. Mol Cancer Ther 1(10): 869-876.

Saarinen N (2002b). Hydroxymatairesinol from Norway spruce (*Picea abies*), a novel enterolactone precursor with anticarcinogenic properties in experimental mammary carcinoma. Institute of Biomedicine, Department of Anatomy, and Functional Foods Forum. Turku, University of Turku.

Sathyamoorthy N, Wang TT and Phang JM (1994). Stimulation of pS2 expression by diet-derived compounds. Cancer Res 54(4): 957-961.

Schottner M, Spiteller G and Gansser D (1998). Lignans interfering with 5-alpha-dihydrotestosterone binding to human sex hormone-binding globulin. J Nat Prod 61(1): 119-121.

Schulte PA and Pepera FP (1997). Transitional studies. IARC Sci Publ (142): 20-29.

Schultze-Mosgau MH, Dale IL, Gant TW, Chipman JK, Kerr DJ and Gescher A (1998). Regulation of c-fos transcription by chemopreventive isoflavonoids and lignans in MDA-MB-468 breast cancer cells. Eur J Cancer 34(9): 1425-1431.

Setchell KD and Adlercreutz H (1979). The excretion of two new phenolic compunds (Compound 180/442 and Compound 180/410) during the human menstrual cycle and in pregnancy. J Steroid Biochem 11: xv-xvi.

Setchell KD, Bull R and Adlercreutz H (1980a). Steroid excretion during the reproductive cycle and in pregnancy of the vervet monkey. J Steroid Biochem 12: 375-384.

Setchell KD, Lawson AM, Mitchell FL, Adlercreutz H, Kirk DN and Axelson M (1980b). Lignans in man and in animal species. Nature 287(5784): 740-742.

Setchell KD, Borriello SP, Gordon H, Lawson AM, Harkness R, Morgan DML, Kirk DN, Adlercreutz H, Anderson LC and Axelson M (1981). Lignan formation in man - microbial involvement and possible roles in relation to cancer. Lancet 2(8236): 4-7.

Setchell KD, Lawson AM, McLaughlin LM, Patel S, Kirk DN and Axelson M (1983). Measurement of enterolactone and enterodiol, the first mammalian lignans, using stable isotope dilution and gas chromatography mass spectrometry. Biomed Mass Spectrom 10(3): 227-235.

Setchell KD, Brown NM, Zimmer-Nechemias L, Brashear WT, Wolfe BE, Kirschner AS and Heubi JE (2002). Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am J Clin Nutr 76(2): 447-453.

Singletary KW and Gapstur SM (2001). Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms. J Am Med Assoc 286(17): 2143-2151.

Smeds A and Hakala K (2003). Liquid chromatographic-tandem mass spectrometric method for the plant lignan 7-hydroxymatairesinol and its potential metabolites in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 793(2): 297-308.

Sprando RL, Collins TF, Wiesenfeld P, Babu US, Rees C, Black T, Olejnik N and Rorie J (2000). Testing the potential of flaxseed to affect spermatogenesis: morphometry. Food Chem Toxicol 38(10): 887-892.

Stattin P, Adlercreutz H, Tenkanen L, Jellum E, Lumme S, Hallmans G, Harvei S, Teppo L, Stumpf K, Luostarinen T, Lehtinen M, Dillner J and Hakama M (2002). Circulating enterolactone and prostate cancer risk: A Nordic nested case-control study. Int J Cancer 99(1): 124-129.

Stitch SR, Toumba JK, Groen MB, Funke CW, Leemhuis J, Vink J and Woods GF (1980). Excretion, isolation and structure of a new phenolic constituent of female urine. Nature 287(5784): 738-740.

Tarpila S, Aro A, Salminen I, Tarpila A, Kleemola P, Akkila J and Adlercreutz H (2002). The effect of flaxseed supplementation in processed foods on serum fatty acids and enterolactone. Eur J Clin Nutr 56(2): 157-165.

Thompson L, Rickard S, Orcheson L and Seidl M (1996a). Flaxseed and its lignan and oil components reduce mammary tumor growth at a late stage of carcinogenesis. Carcinogenesis 17(6): 1373-1376.

Thompson LU, Seidl MM, Rickard SE, Orcheson LJ and Fong HH (1996b). Antitumorigenic effect of a mammalian lignan precursor from flaxseed. Nutr Cancer 26: 159-165.

Thompson LU, Ward WE, Tou J, Chen J, Stavro M, Li T and Goss PE (2001). Breast cancer protective effect of flaxseed lignans. Third International Conference on Natural Antioxidants and Anticarcinogens in Food, Health and Disease, Helsinki, Kuopio University Printing Office.

Toniolo P, Boffetta P, Shuker D, Rothman N, Hulka B and Pearce N (1997). Application of biomarkers in cancer epidemiology. Workshop report. IARC Sci Publ (142)1-18.

Tou JC, Chen J and Thompson LU (1998). Flaxseed and its lignan precursor, secoisolariciresinol diglycoside, affect pregnancy outcome and reproductive development in rats. J Nutr 128: 1861-1868.

Uehara M, Arai Y, Watanabe S and Adlercreutz H (2000a). Comparison of plasma and urinary phytoestrogens in Japanese and Finnish women by time-resolved fluoro-immunoassay. BioFactors 12: 217-225.

Uehara M, Lapcík O, Hampl R, Al-Maharik N, Mäkelä T, Wähälä K, Mikola H and Adlercreutz H (2000b). Rapid analysis of phytoestrogens in human urine by time-resolved fluoroimmunoassay. J Steroid Biochem Molec Biol 72(5): 273-282.

Valentin-Blasini L, Blount BC, Rogers HS and Needham LL (2000). HPLC-MS/MS method for the measurement of seven phytoestrogens in human serum and urine. J Expo Anal Environ Epidemiol 10(6 Pt 2): 799-807.

Valentin-Blasini L, Blount BC, Caudill SP and Needham LL (2003). Urinary and serum concentrations of seven phytoestrogens in a human reference population subset. J Expo Anal Environ Epidemiol 13(4): 276-282.

Wang C, Mäkelä T, Hase T, Adlercreutz H and Kurzer MS (1994). Lignans and isoflavonoids inhibit aromatase enzyme in human preadipocytes. J Steroid Biochem Molec Biol 50 (3-4): 205-212.

Wang C and Kurzer MS (1997). Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr Cancer 28(3): 236-247.

Wang C and Kurzer MS (1998a). Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr Cancer 31(2): 90-100.

Wang W, Liu LQ, Higuchi CM and Chen H (1998b). Induction of NADPH:quinone reductase by dietary phytoestrogens in colonic Colo205 cells. Biochem Pharmacol 56(2): 189-195.

Wang LQ, Meselhy MR, Li Y, Qin GW and Hattori M (2000). Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. Chem Pharm Bull 48(11): 1606-1610.

Vanharanta M, Voutilainen S, Lakka T, van der Lee M, Adlercreutz H and Salonen JT (1999). Risk of acute coronary events according to serum concentrations of enterolactone: a prospective population-based case-control study. Lancet 354(9196): 2112-2115.

Vanharanta M, Mursu J, Nurmi T, Voutilainen S, Rissanen TH, Salonen R, Adlercreutz H and Salonen JT (2002a). Phloem fortification in rye bread elevates serum enterolactone level. Eur J Clin Nutr 56(10): 952-957.

Vanharanta M, Voutilainen S, Nurmi T, Kaikkonen J, Roberts LJ, Morrow JD, Adler-creutz H and Salonen JT (2002b). Association between low serum enterolactone and increased plasma F(2)-isoprostanes, a measure of lipid peroxidation. Atherosclerosis 160(2): 465-469.

Vanharanta M, Voutilainen S, Rissanen TH, Adlercreutz H and Salonen JT (2003). Risk of cardiovascular disease-related and all-cause death according to serum concentrations of enterolactone: Kuopio Ischaemic Heart Disease Risk Factor Study. Arch Int Med 163(9): 1099-1104.

Ward WE, Jiang FO and Thompson LU (2000). Exposure to flaxseed or purified lignan during lactation influences rat mammary gland structures. Nutr Cancer 37(2): 187-192.

Ward WE, Chen J and Thompson LU (2001). Exposure to flaxseed or its purified lignan during suckling only or continuously does not alter reproductive indices in male and female offspring. J Toxicol Environ Health A 64(7): 567-577.

Velicer CM, Heckbert SR, Lampe JW, Potter JD, Robertson CA and Taplin SH (2004). Antibiotic use in relation to the risk of breast cancer. J Am Med Assoc 291(7): 827-835.

Welshons WV, Murphy CS, Koch R, Calaf G and Jordan VC (1987). Stimulation of breast cancer cells *in vitro* by environmental estrogen enterolactone and the phytoestrogen equol. Breast Cancer Res Treat 10(2): 169-175.

White E (1997). Effects of biomarker measurement error on epidemiological studies. IARC Sci Publ (142): 73-93.

Willett WC (2001). Diet and breast cancer. J Intern Med 249(5): 395-411.

Vineis P (1997). Sources of variation in biomarkers. IARC Sci Publ (142): 59-71.

Xie LH, Akao T, Hamasaki K, Deyama T and Hattori M (2003). Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and isolation of Enterococcus faecalis strain PDG-1 responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol. Chem Pharm Bull 51(5): 508-515.

Zeleniuch-Jacquotte A, Adlercreutz H, Akhmedkhanov A and Toniolo P (1998). Reliability of serum measurements of lignans and isoflavonoid phytoestrogens over a two-year period. Cancer Epidemiol Biomarkers Prev 7(10): 885-889.