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MODULATION OF ESTROGEN SIGNALLING BY ENTEROLACTONE AND ITS DIETARY SOURCES

by

Pauliina Damdimopoulou

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From the Department of Cell Biology and Anatomy, Institute of Biomedicine, & Functional Foods Forum, University of Turku, Turku, Finland, and Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden

Supervised by

Professor Sari Mäkelä, M.D., Ph.D.
Functional Foods Forum and
Department of Biochemistry and Food Chemistry
University of Turku
Turku, Finland

Co-supervisor

Ingemar Pongratz, Ph.D.
Department of Biosciences and Nutrition
Karolinska Institutet
Huddinge, Sweden

Reviewed by

Docent Marina Heinonen, Ph.D.
Department of Applied Chemistry and Microbiology
University of Helsinki
Helsinki, Finland

Professor Barbara Demeneix, Ph.D.
Evolution des Régulations Endocriniennes
Centre National de la Recherche Scientifique
Paris, France

Dissertation opponent

Professor Lilian Thompson, Ph.D.
Department of Nutritional Sciences
University of Toronto
Toronto, Ontario, Canada

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“Why not.”

-Tassos

To Tassos

ABSTRACT

Pauliina Damdimopoulou. Modulation of estrogen signalling by enterolactone and its dietary sources

University of Turku, Department of Cell Biology and Anatomy, Institute of Biomedicine, & Functional Foods Forum, 20014 University of Turku, Finland, and Karolinska Institutet, Department of Biosciences and Nutrition, SE-14157 Huddinge, Sweden

Enterolactone (ENL) is a gut microbe metabolite of dietary plant lignans present in the human diet. Plant lignans are found in cereals, vegetables, fruits, berries, and beverages derived therefrom. Flaxseed is one of the richest dietary sources of plant lignans though cereal products are generally the main source of lignans in human diet. ENL is considered the bioactive form of lignans and has been suggested to reduce breast cancer risk through modulation of estrogen signalling. However, the data supporting this hypothesis are scarce. ENL stimulates breast cancer cell proliferation *in vitro* but reduces growth of estrogen-dependent mammary carcinomas *in vivo*. The interaction between ENL and estrogen receptors (ERs), the key regulators of breast cancer growth, has remained poorly understood.

In this work, modulation of estrogen signalling by ENL and its dietary sources was studied by using a combination of cell culture and animal models. *In vitro*, ENL transactivates both ERs with preference for ERα, and the binding affinity of ENL towards ERα is enhanced in cell culture conditions, suggesting metabolic activation of ENL. In ovariectomized estrogen reporter mice, ENL significantly induces reporter gene expression in the uterus and vagina, but not in bone and the mammary gland. It is concluded that ENL is a selective ER modulator (SERM) with an ER subtype, tissue, and cell type -selective activity. Administration of flaxseed in the diet, giving rise to high serum concentration of ENL, is, however, devoid of estrogenic activity in immature rat uterus and mammary gland, and in ovariectomized reporter mice. Instead of being estrogenic, some of the tested cereal products reduced 17β-estradiol (E_2) -induced reporter gene activity and uterine growth. In particular, rye bran in the diet attenuated E_2-induced estrogen signalling. These results suggest that both ENL and its dietary sources can modulate estrogen signalling. Furthermore, the results indicate that estimation of biological activity of food items is difficult based on studies conducted with pure compounds only.

Keywords: cereal products, dietary lignans, enterolactone, estrogen receptor, estrogen reporter mouse, flaxseed, phytoestrogens

TIIVISTELMÄ

Pauliina Damdimopoulou. Enterolaktonin ja sen ravinnon lähteiden vaikutukset estrogeenivasteisiin

Turun yliopisto, Solubiologian ja anatomian osasto, Biolääketieteen laitos, & Funktionaalisten elintarvikkeiden kehittämiskeskus, 20014 Turun yliopisto, ja Karoliininen instituutti, Biotieteiden ja ravitsemustieteen laitos, SE-14157 Huddinge, Ruotsi

Enterolaktoni (ENL) on suolistomikrobien tuottama aineenvaihduntatuote, jonka esiasteita ovat ravinnon kasvilignaanit. Kasvilignaaneja on runsaasti viljoissa, vihanneksissa, hedelmissä ja marjoissa sekä niistä jalostetuissa tuotteissa. Pellavansiemenessä on erittäin korkea lignaanipitoisuus, mutta viljatuotteet ovat ihmisravinnon määrällisesti merkittävimpiä kasvilignaanilähteitä. ENL:a pidetään kasvilignaanien biologisesti aktiivisena muotona, ja on esitetty, että ENL vähentää rintasyöpäriskiä muokkaamalla estrogeenivasteita. ENL:n vaikutuksista estrogeenivasteisiin tiedetään kuitenkin hyvin vähän. Solumalleissa ENL lisää rintasyöpäsolujen kasvua, mutta *in vivo* -koemalleissa ENL vähentää estrogeeniriippuvaisten maitorauhassyöpien kasvua. Estrogeenien kasvua stimuloivat vaikutukset välittyvät estrogeenireseptoreiden kautta, ja nämä reseptorit ovat keskeisessä asemassa rintasyövän kehittymisessä. ENL:n vuorovaikutus estrogeenireseptoreiden kanssa tunnetaan heikosti.

Tässä työssä tutkittiin ENL:n estrogeenivaikutuksia sekä solu- että eläinmalleissa. Solumalleissa ENL aktivoi estrogeenivasteisen raportoijageenin ilmenemistä molempien estrogeenireseptorialatyyppien, ERα:n ja ERβ:n, kautta, mutta ensisijaisesti ERα-välitteisesti. ENL:n sitoutumisvoimakkuus ERα:a kohtaan kasvoi soluviljelyolosuhteissa, mikä viittaa ENL:n aktivoitumiseen soluissa mahdollisesti aineenvaihdunnan seurauksena. Kun ENL:a annettiin estrogeeniraportoijahiirille, raportoijageeni aktivoitui kohdussa ja vaginassa mutta ei luussa tai maitorauhasessa. Nämä tulokset viittaavat selektiiviseen ER-modulointiin eli siihen, että ENL toimisi ns. SERM-yhdisteenä. Kun estrogeeniraportoijahiirille annettiin pellavaa rehun seassa, seerumin ENL-pitoisuus nousi merkittävästi, mutta raportoijageeni ei kuitenkaan aktivoitunut hiirien kudoksissa, eikä pellava ravinnossa myöskään vaikuttanut estrogeenin kaltaisesti nuorien rottien kohdun painoon tai maitorauhasen kasvuun. Sen sijaan viljatuotteet vähensivät 17β-estradiolin (E_2) aiheuttamia vasteita raportoijahiirissä. Erityisesti ravintoon lisätty ruislese vähensi E_2:lla aiheutettua raportoijageenivastetta ja kohdun kasvua. Tulokset osoittavat, että ENL ja sen ravinnon lähteet voivat muokata estrogeenivasteita. Tulokset osoittavat myös, että ravinnon biologisia vaikutuksia ei voida kovin helposti arvioida puhdasainetutkimusten perusteella.

Avainsanat: enterolaktoni, estrogeeniraportoijahiiri estrogeenireseptorit, kasviestrogeenit, kasvilignaanit, pellavansiemen, viljavalmisteet,

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ABBREVIATIONS

4OHT	4-hydroxy-tamoxifen
αERKO	estrogen receptor α knock-out
AF-1/AF-2	activation function 1/activation function 2
ALP	alkaline phosphatase
AP-1	activation protein 1
βERKO	estrogen receptor β knock-out
BMI	body mass index
BTM	basal transcription machinery
C_6C_3	propylbenzene
CEAD	coulometric electrode array detector
CPS	counts per second
DAB	diaminobenzidine
DAPI	4'-6-diamidino-2-phenylindole
DBD	DNA binding domain
DES	diethylstilbestrol
DEX	dexamethasone
DMBA	dimethylbenz[a]anthracene
DMSO	dimethylsulfoxide
E_2	17β-estradiol
EGF	epidermanl growth factor
END	enterodiol
ENL	enterolactone
EP	17β-estradiol dipropionate
ERα	estrogen receptor α
ERβ	estrogen receptor β
ERE	estrogen responsive element
EtOH	ethanol
FBS	foetal bovine serum
FRAP	fluorescence recovery after photo bleaching
GC	gas chromatography
GEN	genistein
GF-AAS	grafite furnace atomic absorption spectrometry
GFP	green fluorescent protein
GOPTS	(3-glycidyloxylpropyl)trimethylsiloxan
H3/5/12	helix 3/5/12
H&E	hematoxylin&eosin
HMR	hydroxymatairesinol
HPLC	high pressure liquid chromatography
ICI	ICI 182,780
IGF-1	insulin like growth factor 1
IUPAC	International Union of Pure and Applied Chemistry
LAR	lariciresinol
LBD	ligand binding domain

LC	liquid chromatography
MAPK	mitogen activated protein kinase
MED	medioresinol
MeOH	methanol
MNU	methylnitrosourea
MR	matairesinol
NR	nuclear receptor
OECD	Organization for Economic Co-operation and Development
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PgR	progesterone receptor
PINO	pinoresinol
PKA/PKC	protein kinase A/C
PND	post natal day
PTWI	provisional tolerable weekly intake
RIfS	reflectometric interference spectroscopy
RLU	relative light unit
SDG	secoisolariciresinol diglucoside
SECO	secoisolariciresinol
SERM	selective estrogen receptor modulator
SES	sesamin
Sp-1	specificity protein 1
SYR	syringaresinol
TEB	terminal end bud
Tk	thymidine kinase
TR-FIA	time resolved fluoroimmunoassay

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the roman numerals I-III. Some unpublished data is also included.

(I) **Penttinen P**, Jaehrling J, Damdimopoulos AE, Inzunza J, Lemmen JG, van der Saag P, Pettersson K, Gauglitz G, Mäkelä S, Pongratz I. Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. Endocrinology 2007, 148(10):4875-4886.

(II) Khan G*, **Penttinen P***, Cabanes A, Foxworth A, Chezek A, Mastropole K, Yu B, Smeds A, Halttunen T, Good C, Mäkelä S, Hilakivi-Clarke L. Maternal flaxseed diet during pregnancy or lactation increases female rat offspring's susceptibility to carcinogen-induced mammary tumorigenesis. Reproductive Toxicology 2007, 23(3):397-406.

*The authors contributed equally to this work.

(III) **Penttinen-Damdimopoulou P**, Power K, Hurmerinta T, Nurmi T, van der Saag P, Mäkelä S. Rye bran, wheat bran, flaxseed, and soy in the diet modulate estradiol-induced responses in estrogen reporter mice. (Manuscript)

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1 INTRODUCTION

Incidence of cancer varies between countries (AICR, 2007). Immigrants moving from a low cancer incidence region to a higher incidence region soon adapt to the risk level of the new region, demonstrating the importance of environmental factors, such as diet, in defining the risk of cancer (Kolonel *et al.*, 2004). Dietary phytoestrogens have been suggested to lower the risk of breast cancer. For example, the lower risk of breast cancer among Asian women compared to western women might depend on greater consumption of soy isoflavones in Asia (Adlercreutz, 2002; Kolonel *et al.*, 2004; Wu *et al.*, 2008). In western countries, similar associations have been made between consumption of high-fibre low-fat diets and the risk of breast cancer (Gerber, 1998; Adlercreutz, 2002). As fibre-rich food products are good sources of plant lignans, putative phytoestrogens, it has been proposed that lignans partially mediate the beneficial effects of fibre on human health (Adlercreutz, 1984; Adlercreutz, 2002; Adlercreutz, 2007).

Lignans are polyphenolic compounds widely distributed in higher plants. Over 500 different lignan structures have been identified, and these plant secondary metabolites probably help plants to combat insects and fungi (Ayres & Loike, 1990; Gang *et al.*, 1999). Lignans are characterized by a structure consisting of two propylbenzene (C_6C_3) units (Figure 1) with a varying degree of aromatic substitution (Moss, 2000). In most edible plants, lignans consisting of C8-C8' linked C_6C_3 units are encountered as glucosides bound to the fibre fraction (Ayres & Loike, 1990). When dietary plant lignans, such as secoisolariciresinol (SECO), matairesinol (MR), pinoresinol (PINO), and lariciresinol (LAR), are ingested, they are metabolized by gut microbiota into enterolignans (also known as mammalian lignans) (Axelson *et al.*, 1982; Borriello *et al.*, 1985; Heinonen *et al.*, 2001). Enterolignans enterodiol (END) and enterolactone (ENL) are absorbed, together with plant lignans, and can be quantified in various human body fluids (Bannwart *et al.*, 1989; Adlercreutz *et al.*, 1995; Morton *et al.*, 1997a; Boccardo *et al.*, 2003; Nurmi *et al.*, 2003; Smeds *et al.*, 2006). Epidemiological studies demonstrating an inverse correlation between serum concentration of ENL and the risk of breast cancer have supported the hypothesis of dietary lignans as anticarcinogenic substances (Boccardo *et al.*, 2004; Olsen *et al.*, 2004; Piller *et al.*, 2006a; Piller *et al.*, 2006b).

The phenolic chemical structure (Figure 2) and initial observation of cyclic excretion of ENL in women led to classification of lignans as phytoestrogens (Setchell *et al.*, 1980; Stitch *et al.*, 1980; Setchell *et al.*, 1981). Subsequent studies have demonstrated that the affinity of ENL towards the mediators of estrogenicity, estrogen receptors (ERs), is low (Mueller *et al.*, 2004). Moreover, ENL does not promote uterine growth in rodents (Setchell *et al.*, 1981; Waters & Knowler, 1982; Saarinen *et al.*, 2002a), suggesting a lack of estrogenic activity. In experimental mammary carcinoma studies a reduction of estrogen-dependent tumour growth has consistently been shown with ENL, plant lignans, and dietary sources of lignans (Thompson, 2003; Saarinen *et al.*, 2007), an observation that may imply antiestrogenic activity of lignans

and ENL. In contrast to these observations are reports showing ENL-induced proliferation of estrogen-dependent cell lines and induction of estrogen regulated genes *in vitro* (Welshons *et al.*, 1987; Mueller *et al.*, 2004; Cosentino *et al.*, 2007; Saarinen *et al.*, 2007; Dip *et al.*, 2008) At present, the nature of the interaction between ENL and ERs remains a matter of controversy.

The two ERs, ERα and ERβ, are ligand-inducible transcription factors that mediate the physiological effects of female sex steroid 17β-estradiol (E_2). According to the classical model of signal transduction through ERs, the inactive receptors are activated by estrogens, recruit co-regulators, and modulate target gene expression through binding to gene promoters containing estrogen responsive elements (EREs) (Nilsson *et al.*, 2001; Gronemeyer *et al.*, 2004). In addition to endogenous estrogens, many environmental compounds can bind to and modulate the activity of ERs (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998). Moreover, many ligands displaying selective binding to either ERα or ERβ have been identified in nature, and also intentionally synthesized by man (Kuiper *et al.*, 1998; Swaby *et al.*, 2007). Selective ER modulators (SERMs) display estrogen agonist activity in certain tissue types while being inactive or antiestrogenic in others (Shang & Brown, 2002; Swaby *et al.*, 2007). Phytoestrogens have been suggested to be "natural" SERM-like compounds (Setchell, 2001).

Current literature concerning the estrogenic activity of ENL may suggest the selective regulation of estrogen signalling. Thus, the aim of this study was to investigate selective and tissue -specific regulation of ER signalling by ENL. Estrogen reporter gene models were employed *in vitro* and *in vivo* to evaluate the effects of ENL on ER signalling. Furthermore, as exposure to ENL in humans occurs through consumption of diets containing ENL precursors (plant lignans), common dietary sources of lignans were also studied in parallel with purified ENL *in vivo*.

2 REVIEW OF THE LITERATURE

2.1 Lignans in plants

2.1.1 Classification, distribution, and function

The name lignan was originally proposed by Haworth in 1936 to describe naturally occurring compounds formed of two β-β' linked cinnamic acid residues (Haworth, 1936; Ayres & Loike, 1990; Moss, 2000). According to the International Union of Pure and Applied Chemistry (IUPAC) lignans are natural plant products derived from cinnamic acid residues; however, for naming purposes lignans should be considered as the union of two C_6C_3 units (Moss, 2000). Hundreds of structures belonging to the lignan family have been recognized in nature (Ayres & Loike, 1990). The major classes of lignans are the C8-C8' (or β-β') coupled lignans, referred to as lignans, and structures where the coupling between the subunits occurs by bonding other than C8-C8', referred to as neolignans (Ayres & Loike, 1990; Moss, 2000). Further, if the two C_6C_3 residues are linked by an oxygen (ether) bridge, the compounds are called oxyneolignan, the union of three residues is called sesquineolignans, and of four residues dineolignans (Moss, 2000). The structure of cinnamic acid, as well as of a C_6C_3 unit with IUPAC recommended numbering, and examples of a lignan and neolignans skeletons, are shown in Figure 1. It is important to notice that although the C_6C_3 units are the basis for lignan nomenclature, no lignans with unsubstituted aromatic rings have been encountered in nature (Ayres & Loike, 1990).

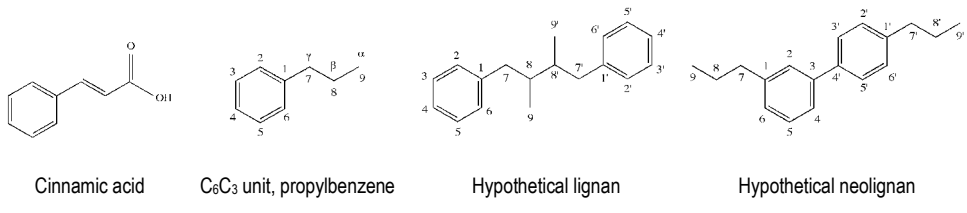


Figure 1. Structural makeup of lignans. Lignans are defined as natural products derived from two cinnamic acid residues, but for naming purposes the structures are considered as the union of C_6C_3 (propylbenzene) units. The IUPAC recommended numbering is shown in the structures. The unions of two C8-C8' coupled C_6C_3 units are called lignans, and structures where coupling between the two subunits occurs by bonding other than C8-C8' are referred to as neolignans.

Lignans are widespread in the plant kingdom. They are ubiquitously distributed in higher plants and are encountered in all parts of plants (roots, rhizomes, stem, leaves, fruits) (Ayres & Loike, 1990). Large amounts of unconjugated lignans are found in coniferous trees, where the concentration in branches, heartwood and knots may be up to 10 % of dry weight (Ekman, 1976; Ekman, 1979). In edible plants, the highest lignan concentrations are found in flaxseed, where the concentration may reach 0.3 % on wet weight basis (Milder *et al.*, 2005a; Thompson *et al.*, 2006). In contrast to trees,

lignans rarely occur unconjugated in the human diet. Instead, in most edible plants, lignans are bound as glucosides to the fibre fraction.

The function of lignans and neolignans in plants is most likely in the self-defence system, where these molecules serve as fungicides and insecticides (Ayres & Loike, 1990; Gang *et al.*, 1999). The concentration of lignans increases upon fungal attack (Ayres & Loike, 1990; Gang *et al.*, 1999), suggesting that lignans are phytoalexins.

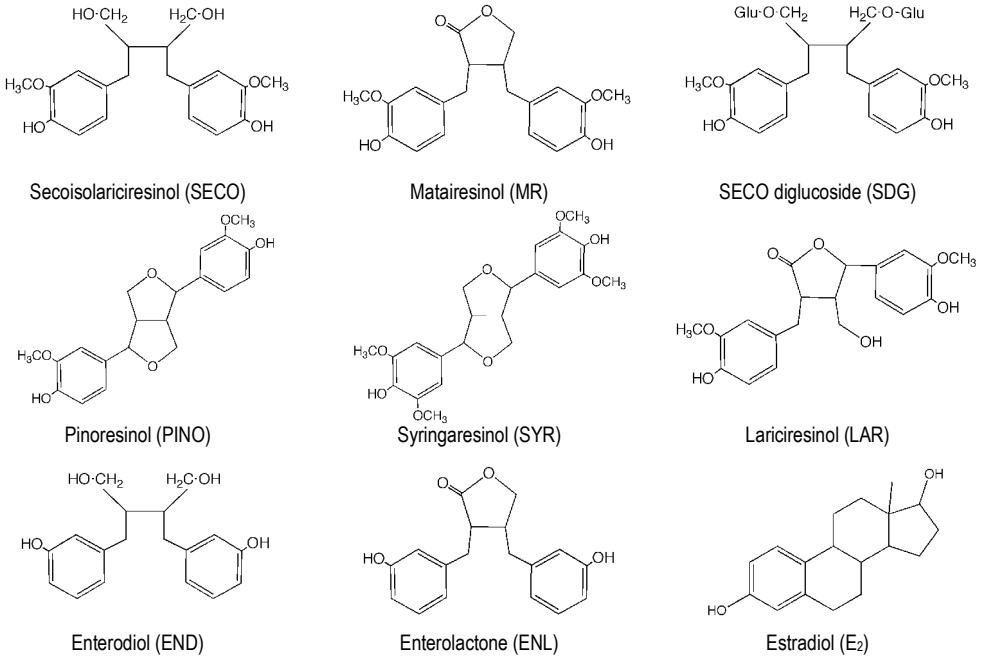


Figure 2. Examples of lignans. The structures of common dietary plant lignans SECO, MR, SDG, PINO, SYR, and LAR, and the gut microbe metabolites of plant lignans, enterolignans END and ENL, are depicted. The structure of the female sex steroid E_2 is shown for comparison.

2.1.2 Dietary lignans

2.1.2.1 Concentration of lignans in the human diet

In edible plants lignans are bound to fibre, thus, lignans are an integral part of a healthy fibre-rich human diet. The most common dietary lignans are C8-C8' (or β-β') type lignans (Figure 2). For a long time, SECO and MR were regarded as the principal plant lignans present in the human diet (Axelson *et al.*, 1982; Borriello *et al.*, 1985; Horn-Ross *et al.*, 2000a; Valsta *et al.*, 2003). This view has been recently revised after identification of several other plant lignans in many common food items (Milder *et al.*, 2005a; Thompson *et al.*, 2006; Smeds *et al.*, 2007a). LAR, PINO, syringaresinol (SYR), hydroxymatairesinol (HMR), medioresinol (MED), and sesamin (SES) are some of the recently identified dietary plant lignans (Figure 2) (Penalvo *et al.*, 2004;

Penalvo *et al.*, 2005a; Milder *et al.*, 2005a; Thompson *et al.*, 2006; Smeds *et al.*, 2007a; Penalvo *et al.*, 2008).

During the past decade, concentration of SECO and MR has been analysed in a wide range of dietary plants (seeds, oilseeds, nuts, cereals, legumes, fruits, vegetables, berries) and plant-derived beverages (Mazur *et al.*, 1996; Mazur *et al.*, 1998; Mazur & Adlercreutz, 2000; Mazur *et al.*, 2000). After the relative recent discovery of the additional plant lignans in the human diet, a more comprehensive picture of lignan concentrations in diet has been obtained through studies including up to 24 different plant lignans into the analyses (Penalvo *et al.*, 2004; Penalvo *et al.*, 2005a; Milder *et al.*, 2005a; Thompson *et al.*, 2006; Smeds *et al.*, 2007a; Penalvo *et al.*, 2008). These studies have indisputably demonstrated that in addition to not being the only plant lignans in the human diet, SECO and MR are often present in smaller quantities than the more recently discovered dietary plant lignans.

Several databases containing lignan concentrations in food items have been constructed and most of them contain information on SECO and MR only (Pillow *et al.*, 1999; Horn-Ross *et al.*, 2000a; Kiely *et al.*, 2003; Valsta *et al.*, 2003; Blitz *et al.*, 2007). For example, the Finnish National Food Composition Database (Fineli) was expanded in 2003 to contain concentrations of SECO and MR in 180 foods and over 1000 composite food items, based on published concentration data in the literature (Valsta *et al.*, 2003). Two databases containing concentration information on four plant lignans have been created. In the Netherlands, the content of SECO, MR, PINO, and LAR in 83 commonly consumed solid foods and 26 beverages was analyzed (Milder *et al.*, 2005a). The richest sources of lignans in this study were flaxseed (301 mg/100g) and sesame seeds (29 mg/100g), followed by bread (0.02-12 mg 100g), *Brassica* vegetables (0.2-2 mg/100g), and fruits (0-0.5 mg/100g) (Milder *et al.*, 2005a). In beverages, total plant lignan concentration varied from 0 μg/100ml in cola to 90 μg/100ml in South African red wine (Milder *et al.*, 2005a). In Canada, SECO, MR, PINO, and LAR content in 121 food items was analyzed and compiled into a database (Thompson *et al.*, 2006). Similar to the Dutch study, the richest sources of lignans were flaxseed (379 mg/100g) and sesame seeds (8 mg/100g), followed by bread (0.004-7 mg/100g), vegetables (*Brassica* 0.08-0.09 mg/100g), and fruits (0.002-0.4 mg/100g) (Thompson *et al.*, 2006). In beverages, total lignan concentration varied from traces in milk to 40 μg/100g in red wine (Thompson *et al.*, 2006). In both studies, most analyzed foods contained more PINO and LAR than SECO and MR. Consequently, many food items, such as tomato, broccoli, and strawberry, are far better sources of lignans than previously thought (Table 1). Concentrations of SECO, MR, PINO, and LAR from several independent studies, in selected food items, are presented in Table 1.

Table 1. Concentration of plant lignans in some selected food items (μg/100g fresh weight).

Food item	SECO	MR	PINO	LAR	Reference*
Seeds					
Flaxseed	294,210	553	3,324	3,041	Milder 2005
	375,322	153	730	2,808	Thompson 2006
	165,759	529	871	1,780	Smeds 2007
	323,670	5,202	2,460	3,670	Penalvo 2005
Sesame seed	66	481	29,331	9,470	Milder 2005
	7	123	6,815	1,052	Thompson 2006
	240	1,137	47,136	13,060	Smeds 2007
Cereals and cereal products					
Multi-grain bread	6,163	19	377	185	Milder 2005
	4,770	1	4	10	Thompson 2006
Rye bread	13	14	172	122	Milder 2005
	122	0	9	11	Thompson 2006
Refined wheat bread	17	0	28	38	Milder 2005
	1	0	1	2	Thompson 2006
Rye	462	729	1,547	1,503	Smeds 2007
	38	27	381	324	Penalvo 2005
Wheat	868	410	138	672	Smeds 2007
	35	3	37	62	Penalvo 2005
Legumes					
Soy beans	79	2	89	100	Thompson 2006
	334	2	446	287	Penalvo 2004
	67	0	33	81	Penalvo 2008
Vegetables					
Broccoli	38	0	315	972	Milder 2005
	6	0	6	82	Thompson 2006
Garlic	50	0	200	286	Milder 2005
	42	5	482	54	Thompson 2006
	55	0	45	84	Penalvo 2008
Onion	18	0	0	19	Milder 2005
	22	9	1	0	Thompson 2006
Carrot	93	0	19	60	Milder 2005
	4	0	0	2	Thompson 2006
Cucumber	8	0	1	59	Milder 2005
	2	0	1	1	Penalvo 2005
	41	0	0	65	Penalvo 2008
Tomato	2	0	14	42	Milder 2005
	1	0	2	6	Thompson 2006
	1	0	5	11	Penalvo 2005
	3	0	12	11	Penalvo 2008
Potato	2	0	0	17	Milder 2005
	0	0	0	1	Thompson 2006
Fruits and berries					
Peach	27	0	186	80	Milder 2005
	14	2	37	10	Thompson 2006
	11	0	83	38	Penalvo 2008
Apple	0	0	0	1	Milder 2005
	0	0	1	1	Thompson 2006
	0	3	0	55	Penalvo 2008
Banana	0	0	0	0	Milder 2005
	1	0	0	1	Thompson 2006
	0	0	0	17	Penalvo 2008
Orange	5	2	24	47	Milder 2005
	3	2	3	9	Thompson 2006
	11	0	9	19	Penalvo 2005
Strawberry	5	0	212	117	Milder 2005
	51	0	21	33	Penalvo 2008

*References: Milder 2005, (Milder *et al.*, 2005a); Penalvo 2004, (Penalvo *et al.*, 2004a); Penalvo 2005, (Penalvo *et al.*, 2005a); Penalvo 2008, (Penalvo *et al.*, 2008); Smeds 2007, (Smeds *et al.*, 2007a); Thompson 2006, (Thompson *et al.*, 2006)

2.1.2.2 Exposure to dietary lignans through diet

Exposure to dietary lignans has been evaluated in different populations using food frequency questionnaires and lignan databases. As concentrations of lignans in food items vary in different databases (Table 1) and food frequency questionnaires are subjected to recall bias, the lignan exposure calculations are estimations only. Lignan exposure through dietary intake has been estimated in Finland (Valsta *et al.*, 2003; Kilkkinen *et al.*, 2003a), the Netherlands (Keinan-Boker *et al.*, 2002; Milder *et al.*, 2005b; Milder *et al.*, 2007), the USA (Horn-Ross *et al.*, 2000b; de Kleijn *et al.*, 2001), and the UK (Bhakta *et al.*, 2006) (Table 2). Among elderly American women, the average daily intake of SECO and MR was estimated as 0.2 mg, and coffee and orange juice were the primary sources of lignans (Horn-Ross *et al.*, 2000b). Another American study found 3-fold higher intake of SECO and MR in elderly women, 0.6 mg/d, and in this study the main sources of lignans were fruits, bread, cereals, rice, grains, and berries (de Kleijn *et al.*, 2001). In Europe, three studies have evaluated the dietary intake of lignans. Dutch elderly women consumed considerable amounts of SECO and MR, 1.1 mg/d (Keinan-Boker *et al.*, 2002), compared to their American peers (Horn-Ross *et al.*, 2000b; de Kleijn *et al.*, 2001). Grain products, vegetables, fruits, and beverages were identified as the most important sources in this study (Keinan-Boker *et al.*, 2002). In a random population in Finland, the average daily intake of SECO and MR was 0.4 mg/d (Valsta *et al.*, 2003). The intake was higher for women (0.6 mg/d) than for men (0.3 mg/d), and the main sources of lignans were seeds, cereals (rye in particular), berries, fruits, and vegetables (Valsta *et al.*, 2003). Another Finnish study, using the same database as Valsta *et al.*, found less than 0.2 mg/d intakes for both women and men, with the most important sources being fruits, berries, and cereals (Kilkkinen *et al.*, 2003a). The intake of SECO and MR was similar (<0.2 mg/d) in South Asian women and British women living in the UK, and the main source in both groups was bread (Bhakta *et al.*, 2006). As a summary, the intake of SECO and MR in Europe and USA is < 1 mg/d, and the most important sources are cereal products, beverages, fruits, vegetables, and berries.

The intake of four different plant lignans, SECO, MR, PINO, and LAR, has been estimated in two studies. The average intake of these lignans among a representative subset of Dutch adults was 1.2 mg/day, and the intake did not differ between men and women (Milder *et al.*, 2005b). Beverages contributed 37 % to the intake, vegetables 24 %, nuts and seeds 14 %, bread 9 %, and fruits 7 % (Milder *et al.*, 2005b). In another study by Milder *et al.*, the intake of the four plant lignans among Dutch endoscopy patients was determined as 1.0 mg/d (geometric mean) (Milder *et al.*, 2007). PINO and LAR accounted for 75-80 % of the lignan intake in these two studies, demonstrating the predominant occurrence of these two lignans in the human diet, compared to SECO and MR. The results of Milder *et al.* are in line with most earlier intake estimates, if the results from the studies focusing on SECO and MR only are regarded as 20-25 % of the "total" intake (Table 2). Overall, the average daily intake of plant lignans is most probably > 1 mg/d in most western populations if all four dietary plant lignans are included in the calculations. It remains to be studied how many other kinds of plant

lignans occur in common human diets, and how much they contribute to daily lignan exposure.

Table 2. Lignan intake (mg/d) in general populations

Study population (n)	Food intake assessment	Source of lignan concentrations	Lignans included	Intake (Average±SD)	Reference
Elderly American W (n=447)	FFQ	Analysis of 112 food items	SECO MR	0.2	(Horn-Ross *et al.*, 2000b)
Postmp American W (n=964)	FFQ over the past year	Literature	SECO MR	0.6±0.4	(de Kleijn *et al.*, 2001)
Dutch elderly W (n=17,140)	FFQ over the past year	Literature	SECO MR	1.1	(Keinan-Boker *et al.*, 2002)
Random Finnish population (n=2,862)	24-hour recall	Literature and analysis of food items	SECO MR	W 0.6±1.7 M 0.3±9.3	(Valsta *et al.*, 2003)
Random Finnish population (n=2,852)	24-hour recall	Database developed earlier (Valsta 2003)	SECO MR	W 0.15±0.0 M 0.17±0.0	(Kilkkinen *et al.*, 2003a)
Representative subset of Dutch adults (n=4,661)	2-day recall	Analysis of 109 food items	SECO MR PINO LAR	1.2±2.1	(Milder *et al.*, 2005b)
South Asian W living in England (n=221) and British W (n=49)	24-hour recall	Literature	SECO MR	Asian 0.1±0.1 British 0.2±0.1	(Bhakta *et al.*, 2006)
Dutch endoscopy patients (n=637)	FFQ over the past year	Database developed earlier (Milder 2005b)	SECO MR PINO LAR	1.0*	(Milder *et al.*, 2007)

FFQ, food frequency questionnaire; M, men; postmp, postmenopausal; W, women
*Geomeric mean

2.2 Lignans in humans

2.2.1 Metabolism of lignans

2.2.1.1 Gut microbes and metabolism of dietary plant lignans to enterolignans

Nearly three decades ago, two research groups independently reported the presence of a phenolic compound of unknown origin in human urine (Setchell *et al.*, 1980; Stitch *et al.*, 1980). The compound was identified as a lignan but was suggested to originate from the ovaries due to a cyclic excretion pattern in females (Setchell *et al.*, 1980; Stitch *et al.*, 1980). However, it was soon discovered that this compound, ENL, was excreted in equal amounts by ovariectomized and intact rats, while ENL was not detected in the urine of germ-free rats (Axelson & Setchell, 1981). Further, administration of antibiotics to humans decreased the urinary excretion of ENL to a negligible level (Setchell *et al.*, 1981). In subsequent work, dietary SECO and MR were recognized as precursors of ENL, and gut microbes as the essential link between dietary lignans and enterolignans (Axelson *et al.*, 1982; Borriello *et al.*, 1985; Bowey *et al.*, 2003). To date, the reasons behind the cyclic excretion pattern of ENL in women are not understood. However, gut microbes have been suggested to play a role in this phenomenon. The fluctuating levels of estrogens in bile reaching the gut during the menstrual cycle perhaps affects the activity and/or number of colonic microbes involved in lignan metabolism (Adlercreutz, 2007).

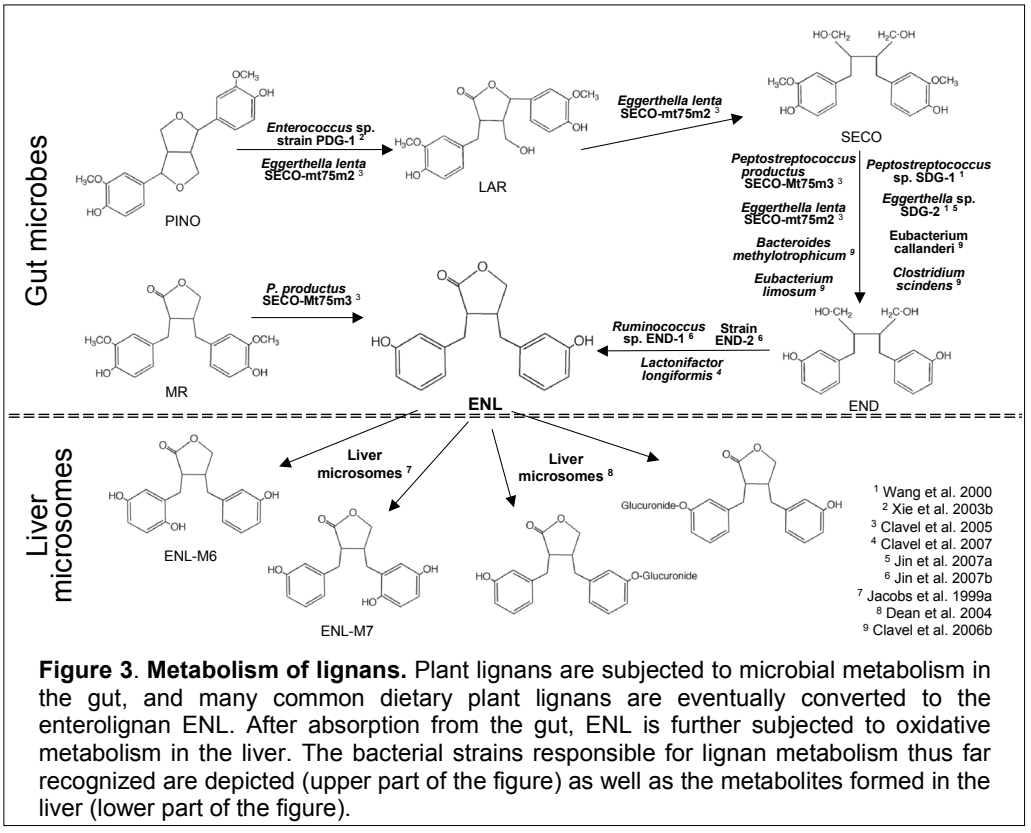


Figure 3. **Metabolism of lignans.** Plant lignans are subjected to microbial metabolism in the gut, and many common dietary plant lignans are eventually converted to the enterolignan ENL. After absorption from the gut, ENL is further subjected to oxidative metabolism in the liver. The bacterial strains responsible for lignan metabolism thus far recognized are depicted (upper part of the figure) as well as the metabolites formed in the liver (lower part of the figure).

Several dietary plant lignans are subjected to metabolism in the gut. For example, LAR, PINO, SYR, HMR, SES, and arctigenin are converted to ENL (Borriello *et al.*, 1985; Heinonen *et al.*, 2001; Saarinen *et al.*, 2002b; Xie *et al.*, 2003a; Xie *et al.*, 2003b; Smeds *et al.*, 2004; Penalvo *et al.*, 2005b). SECO is first metabolized to END and then to ENL, while MR is directly converted to ENL (Figure 3) (Axelson *et al.*, 1982; Borriello *et al.*, 1985). PINO is first metabolized to LAR, which is further converted to SECO, and finally converts to END and ENL (Figure 3) (Heinonen *et al.*, 2001; Xie *et al.*, 2003b). SECO can also be metabolised to ENL without intermediate formation of END (Xie *et al.*, 2003b). The efficiency of the metabolic conversions varies. Evaluated by *in vitro* fermentation with human faecal microflora, the conversion rates of eight different plant lignans varied from 0 % for cycloLAR to 101 % for LAR (Heinonen *et al.*, 2001). The conversion rates for MR, SECO, PINO-diglucoside, SYR-diglucoside, and HMR were 62 %, 72 %, 55 %, 4 %, and 15 %, respectively (Heinonen *et al.*, 2001). In addition to cycloLAR, nortrachelogenin is a plant lignan that is not metabolized to enterolignans (Saarinen *et al.*, 2005). Both enterolignans and plant lignans are detected in human serum and urine (Bannwart *et al.*, 1989; Adlercreutz *et al.*, 1995; Nurmi *et al.*, 2003; Smeds *et al.*, 2006), suggesting that both types of lignans are absorbed from the gut.

The end metabolite of plant lignans (of those that can be metabolized to enterolignans) in the gut is ENL, judged from *in vitro* fermentation studies with human faecal microbiota (Borriello *et al.*, 1985; Xie *et al.*, 2003a; Xie *et al.*, 2003b). The work to identify bacterial species responsible for lignan metabolism in the gut has been intense, and several bacterial strains have recently been characterized (Wang *et al.*, 2000; Xie *et al.*, 2003a; Xie *et al.*, 2003b; Clavel *et al.*, 2005; Clavel *et al.*, 2006a; Clavel *et al.*, 2006b; Clavel *et al.*, 2007; Possemiers *et al.*, 2007; Jin *et al.*, 2007a; Jin *et al.*, 2007b; Jin *et al.*, 2007c). The current understanding of the lignan metabolism by gut microbiota is illustrated in Figure 3 (upper part). It is important to realize that each depicted conversion step involves multiple intermediate metabolites (Wang *et al.*, 2000; Xie *et al.*, 2003b; Clavel *et al.*, 2006a), which are not presented in Figure 3. Several general observations about the lignan metabolism in the gut have been made. The microbes involved in lignan metabolism encompass several functionally distant species of bacteria, many of which are part of dominant human gut microbiota (Clavel *et al.*, 2006b). However, individual variation in the composition of gut microbiota may lead to differences in lignan metabolism. In particular, the conversion of END to ENL seems to be a feature of subdominant species in the gut, while conversion of plant lignans to END occurs more readily (Clavel *et al.*, 2005; Possemiers *et al.*, 2007). Interestingly, women have a higher number of bacteria capable of END → ENL conversion than men (Clavel *et al.*, 2005). Oral antibiotic treatments destroy microbes involved in enterolignan formation and full recovery of the gut microbiota may take over a year (Setchell *et al.*, 1981; Kilkkinen *et al.*, 2002).

2.2.1.2 Oxidative metabolism of ENL

ENL has been suggested to undergo oxidative metabolism in liver microsomes. When ENL was incubated with Aroclor-induced rat liver microsomes *in vitro*, altogether six aliphatic and six aromatic monohydroxylation products were detected, while two aromatic and five aliphatic products were formed in incubations with non-induced human liver microsomes (Jacobs *et al.*, 1999). *In vivo* in rats, six aromatic and five aliphatic monohydroxylated ENL derivatives were found in bile and urine after treatment with ENL or flaxseed (Niemeyer *et al.*, 2000). In human urine, only traces of two *in vitro* identified aromatic hydroxylation products of ENL were detected after flaxseed supplementation (Jacobs *et al.*, 1999). However, four additional aromatic hydroxylation products, that were not detected in *in vitro* incubations, were discovered in the same samples (Jacobs *et al.*, 1999). It is not clear whether all these six aromatic hydroxylation products found in human urine are truly formed by liver microsomes, as some of them have also been described as intermediate metabolites in lignan metabolism by gut microbes (Jacobs *et al.*, 1999; Xie *et al.*, 2003a). Moreover, the authors later speculated on the authenticity of their results, as monohydroxylated derivatives of ENL can be formed as artefacts during the sample extraction procedure (Niemeyer *et al.*, 2003). Hence, to date, it is not clear whether the oxidative metabolism of enterolignans occurs *in vivo*. In Figure 3 (lower part) the structures of the aromatic hydroxylation products of ENL found both in human liver microsomal incubations *in vitro* and in human urine are presented.

2.2.1.3 Sulfatation and glucuronidation of ENL

In biological fluids, only a small fraction of ENL occurs in free unconjugated form. The major form of ENL in human serum and urine is monoglucuronide (Figure 3 lower part) (Adlercreutz *et al.*, 1993; Adlercreutz *et al.*, 1995). In human urine, 85-99 % of ENL is found as glucuronides and 1-5 % as sulphates (Axelson & Setchell, 1980; Adlercreutz *et al.*, 1995). In serum, 70-80 % of ENL is conjugated to glucuronic acid (Adlercreutz *et al.*, 1993). In rats, a similar pattern of conjugation, with glucuronidation predominating, is found (Axelson & Setchell, 1981). It has been suggested that the conjugation of ENL takes place in the liver, and that the conjugates are excreted in bile and reabsorbed from the gut, *i.e.* ENL undergoes enterohepatic circulation (Axelson & Setchell, 1981).

Several cell lines can conjugate ENL *in vitro*. When incubated with rhesus monkey liver microsomes, or human and monkey hepatocytes, ENL is rapidly glucuronidated (Dean *et al.*, 2004). In contrast, the human hepatoma HepG2 cell line rapidly converts ENL to sulphates (83 %) and to a lesser extent to glucuronides (13 %) (Adlercreutz *et al.*, 1992). Similarly, the human breast cancer MCF-7 cell line conjugates ENL to sulphates (91 %) (Mousavi & Adlercreutz, 1992). Interestingly, the human colon epithelial cell lines HT29 and CaCo-2 can also convert ENL to glucuronides and sulphates, suggesting that conjugation of lignans could take place already upon absorption from the gut (Jansen *et al.*, 2005).

2.2.2 Lignans in human body fluids

2.2.2.1 Factors affecting concentration of ENL in serum

The obvious determinants of serum ENL concentration are dietary intake of ENL precursors and the health of gut microbiota. Several additional factors affecting the circulating level of ENL have been recognized. The association between dietary habits, demographic factors, and serum ENL has been assessed in healthy individuals in Finland, the USA, Denmark, and in a large cross-European population consisting of individuals from 16 different regions (Kilkkinen *et al.*, 2001; Horner *et al.*, 2002; Kilkkinen *et al.*, 2002; Kilkkinen *et al.*, 2003a; Johnsen *et al.*, 2004; Peeters *et al.*, 2007). In these studies, intake of grains and cereals, vegetables, caffeine/coffee, fibre, fruits, berries, and alcohol have been positively associated with serum ENL concentration (Kilkkinen *et al.*, 2001; Horner *et al.*, 2002; Kilkkinen *et al.*, 2003a; Johnsen *et al.*, 2004). The food items of importance differ between the studies, and might reflect different consumption patterns in the different countries. Constipation is often reported as a positive determinant of serum ENL concentration, and it probably reflects the longer fermentation time of plant lignans in the gut (Kilkkinen *et al.*, 2001; Johnsen *et al.*, 2004). Current smokers and subjects with high or low body mass index (BMI) tend to have less ENL in circulation than their non-smoker and normal weight peers (Kilkkinen *et al.*, 2001; Horner *et al.*, 2002; Johnsen *et al.*, 2004). Other suggested determinants of serum ENL concentration are age and years of education (positive correlation), gender (women have higher serum ENL) and intake of fat (high

intake reduces serum ENL) (Kilkkinen *et al.*, 2001; Horner *et al.*, 2002; Kilkkinen *et al.*, 2002; Kilkkinen *et al.*, 2003a; Johnsen *et al.*, 2004; Peeters *et al.*, 2007). Interestingly, the large cross-European study encompassing nine countries and 16 regions found region to be the most significant determinant of serum ENL concentration (Peeters *et al.*, 2007). The highest concentration of ENL was found in Danish people, and the lowest in Southern Europeans (Peeters *et al.*, 2007). Use of oral antibiotics is a separate important determinant of serum ENL concentration. The concentration of ENL decreases after use of antibiotics, and complete restoration of ENL production may take up to 16 months (Kilkkinen *et al.*, 2002).

Individual variation in serum ENL concentration is remarkable: the concentration varies even within the same day, and fasting before sampling does not reduce the variation (Stumpf & Adlercreutz, 2003; Hausner *et al.*, 2004; Sonestedt *et al.*, 2007). It has been estimated that three separate serum samples are needed for a reliable estimation of an individual's serum ENL concentration (Stumpf & Adlercreutz, 2003). Perhaps due to the use of one sample only, the recognized determinants of serum ENL concentration explain only a fraction of the variation in ENL concentration in different populations. In Finnish men, 3 % of the variation was explained with constipation and consumption of whole grain products, fruits and berries (Kilkkinen *et al.*, 2001). In Finnish women, 14 % of the variation was explained with BMI, smoking, age, constipation, and consumption of vegetables (Kilkkinen *et al.*, 2001). In American men and women, age, gender, BMI, fibre, caffeine, and alcohol explained 22 % of the serum ENL concentration variation, while fibre alone explained 13 % (Horner *et al.*, 2002). In Danish postmenopausal women, 16 % of the variation in serum ENL concentration was explained by consumption of whole grain, leafy vegetables, coffee, and cabbage, and by BMI, bowel activity, and smoking (Johnsen *et al.*, 2004). A factor that has not been considered in any of these studies is the bacterial strains that convert END to ENL (Figure 3). As these species are subdominant in the gut (Clavel *et al.*, 2005; Possemiers *et al.*, 2007), their number and activity could greatly influence serum ENL concentration.

Determinants of serum ENL concentration have also been evaluated in epidemiological studies primarily designed to investigate the association between ENL and risk of cancer. In Dutch endoscopy patients, consumption of fruits, nuts, seeds, and wine were positively associated while consumption of beer negatively associated with the concentration of ENL in serum (Milder *et al.*, 2007). A model adjusted for lignan intake, age, weight, smoking status, and bowel activity explained 13 % of serum ENL concentration (Milder *et al.*, 2007). The effects of dietary fibre, smoking, BMI, age, and oral antibiotics on serum ENL concentration have been detected in several independent epidemiological studies investigating breast, prostate and colon cancer (Pietinen *et al.*, 2001; Hulten *et al.*, 2002; Kilkkinen *et al.*, 2003b; Grace *et al.*, 2004; Kilkkinen *et al.*, 2004; Stattin *et al.*, 2004; Hedelin *et al.*, 2006; Kuijsten *et al.*, 2006; Zeleniuch-Jacquotte *et al.*, 2006; Kuijsten *et al.*, 2008). Hence, it seems that the determinants of serum ENL concentration are similar in healthy general populations and in cancer patients and controls.

2.2.2.2 Concentration of ENL in human serum

The concentration of ENL in human body fluids is heavily skewed towards higher values. While the general population has a lower nanomolar concentration of ENL in serum, certain individuals may have 1000-fold higher concentrations. Description of serum ENL distribution in a population is, therefore, best achieved by reporting median and range of concentrations instead of traditional average and standard deviation. Approximately 20 % of published ENL studies to date deal with serum ENL concentration, and one third of these studies report the concentration as median and range. Few additional studies report range but no median. All these studies are summarized in Tables 3-5, grouped as general population studies (Table 3), epidemiological studies (Table 4) and supplementation studies (Table 5). In most of these studies the lowest measured concentration of ENL has been below 1 nM (Tables 3-5), while the highest reported values range from 40 nM to 1.3 μM in general populations (Table 3), from 40 nM to 2.5 μM in epidemiological studies (Table 4), and from 10 nM to 0.8 μM after dietary supplementations (Table 5). Although the range of observations varies in these studies, the median concentration in all studies has been approximately 10-50 nM, reflecting the typical low nanomolar concentration in the majority of subjects (Table 3-5).

In supplementation studies, the serum concentration of ENL before and after ingestion of berries or cereal products (Table 5) does not differ remarkably from concentrations measured in general populations (Table 3). However, flaxseed supplementation results in considerably higher serum ENL concentration than typically observed in general populations. Twenty-four hours after ingestion of a single dose of flaxseed (a cake containing 15 g flaxseed) the serum ENL concentration in healthy men was 37-147 nM (Morton *et al.*, 1997b). After a 2-week supplementation period with flaxseed (25 g/day) the serum ENL in postmenopausal Australian women was 140-820 nM (Morton *et al.*, 1994) and a 10-day supplementation with ground flaxseed (0.3 g/kg body weight) yielded 120-540 nM concentrations in Dutch men and women (Kuijsten *et al.*, 2005). Clearly, consumption of flaxseed differs significantly from consumption of other sources of lignans, such as cereals and berries, in terms of achieved serum lignan concentrations. This is also expected, when the exceptionally high concentration of lignans in flaxseed is considered; flaxseed contains approximately 100-fold more ENL precursors than common cereals rye and wheat, and 1000-fold more than *e.g.* strawberries (Table 1). The whole average daily intake of lignans in different populations (Table 2) could be obtained from 0.3 g flaxseed. The heavily skewed distribution of serum ENL concentrations might at least partly depend on flaxseed consumers.

The methodology used in serum lignan quantification has been diverse. Traditional chromatographic methods [gas (GC), liquid (LC), and high pressure liquid (HPLC) chromatography] allow the quantification of several different lignans simultaneously, but require expertise in analytical chemistry and special equipment. A time resolved fluoroimmunoassay (TR-FIA) kit for the measurement of ENL in biological fluids was introduced in 1998 (Adlercreutz *et al.*, 1998). This kit allows rapid and simple

quantification of ENL, even in laboratories that are not specialised in analytical chemistry. However, it was recently suspected that the antibody used in the kit only recognizes one of the two ENL stereoisomers, and may therefore give too low results (Adlercreutz, 2007). A meta-analysis of the ENL concentrations reported in general population studies (Table 3) suggests that this might be the case. When TR-FIA is compared against the chromatographic methods (GC, LC, HPLC), the TR-FIA studies tend to find a smaller range of ENL concentrations (Mann-Whitney *U*-test, $p<0.05$). It is unclear whether the choice of analytical method, perhaps leading to smaller range of detected concentrations, could affect the results obtained, for instance, in epidemiological studies.

Table 3. Serum/plasma ENL concentration (nM) in general populations.

Subjects	Method	N	Median	Range	Mean±SD	Reference
Finnish pre and postmp W	GC-MS	Omnivorous W, 14 Vegetarian W, 14	27.3 50.5	10.4-74.1 17.9-1,078.2	33.3±19.8 252.6±370.1	(Adlercreutz *et al.*, 1993)
Portuguese, Hong Kong, and British M	GC-MS	Portuguese M, 50 Hong Kong M, 53 UK M, 36	nr nr nr	n.d.-55.6 0.4-365.4 0.2-41.2	13.1 20.8 13.1	(Morton *et al.*, 1997a)
American pre and postmp W	GC-MS	Visit 1, 60 Visit 2, 60 Visit 3, 60	22.5 22.7 18.5	0.2-561 0.3-655 0-463	nr nr nr	(Zeleniuch-Jacquotte *et al.*, 1998)
Japanese and Finnish W	TR-FIA	Japanese W, 111 Finnish W, 87	7.7 20.7	0.8-85.5 3.1-103.5	13.3±15.6 25.0 ±16.6	(Uehara *et al.*, 2000)
Finnish M and W	TR-FIA	W, 1,212 M, 1,168	16.6 13.8	0-182.6 0-95.6	20.5 17.3	(Kilkkinen *et al.*, 2001)
American M and W	TR-FIA	W+M 193	nr	0.6-155.3	nr	(Horner *et al.*, 2002)
Japanese M and W, British M and W	GC-MS	Japanese W, 125 Japanese M, 102 British W, 133 British M, 43	10.8 10.8 15.8 18.8	0-129 0-323 0.3-102 1.5-145	22.7±31.3 32.6±58.7 18.7±16.4 24.4±24.5	(Morton *et al.*, 2002)
Mildly hypercholesterolemic Finnish M	TR-FIA	100	nr	1.1-70.8	16.6	(Vanharanta *et al.*, 2002)
British M and W	LC-MS	300	14.4	nr-1,301	nr	(Grace *et al.*, 2003)
American M and W	HPLC-MS/MS	208	nr	nd-375	12	(Valentin-Blasini *et al.*, 2003)
British M	LC-MS/MS	267	nr	nd-162.1	nr	(Low *et al.*, 2005a)
British postmp W	LC-MS/MS	109	nr	nd-177	nr	(Low *et al.*, 2005b)
Norwegian postmp W	TR-FIA	616	16.7	0.3-176.9	nr	(Stuedal *et al.*, 2005)
Finnish M and W	HPLC-MS/MS	W, 5 M, 5	55.0 22.3	16.8-110.0 10.0-85.8	56.1±38.7 31.6±30.8	(Smeds *et al.*, 2006)
Swedish postmp W	TR-FIA	Non-fasting, 21 Fasting, 21	18 22	1.3-82 0.5-65	nr nr	(Sonestedt *et al.*, 2007)

M, men; nd, not detected; nr, not reported; postmp, postmenopausal; premp, premenopausal; W, women

Table 4. Serum/plasma ENL concentration (nM) in epidemiological studies

Subjects	Method	N	Median	Range	Mean±SD	Rerefenre
Swedish premp and postmp BC cases and ctrls	TR-FIA	248 cases 492 ctrls	nr	0-143.5 (for all)	26.8 / 19.3 22.9 / 20.4 (two cohorts)	(Hulten *et al.*, 2002)
Finnish M with coronary events	TR-FIA	1,889	nr	0.2-205.1	17.1±14.0	(Vanharanta *et al.*, 2003)
Italian W with palpable cysts	TR-FIA	191	17	1-140	20.4±1.3	(Boccardo *et al.*, 2003)
Italian W with breast cysts	TR-FIA	18 cases 365 ctrls 383 cases+ ctrls	8.5 16.0 16	nr nr 1-140	14.7±4.3 19.8±1.0 19.6±0.1*	(Boccardo *et al.*, 2004)
British premp and postmp BC cases and ctrls	LC-MS/MS	284 cases + ctrls	nr	nd-1,301	nr	(Grace *et al.*, 2004)
Finnish type I diabetes patients	TR-FIA	Normalbuminuric F, 50 Normalbuminuric M, 50 Microalbuminuric F, 50 Microalbuminuric M, 50 Macroalbuminuric F, 50 Macroalbuminuric M, 50 ESRD F, 50 ESRD M, 50	9.8 13.7 10.7 17.8 26.8 39.2 44.9 37.3	1-56 0.6-84 0.7-44 0.4-185 0.5-900 0.6-471 0.9-2,537 0.9-2,170	13.4±11.3 17.5±15.4 14.8±11.6 20.8±26.6 73.6±167.5 62.2±79.0 180.9±397.5 206±400.3	(von Hertzen *et al.*, 2004)
Danish postmp BC cases and ctrls	TR-FIA	857 cases-controls	27.2	0-455	38.0±40.5	(Johnsen *et al.*, 2004)
Finnish BC cases and ctrls	TR-FIA	206 cases 215 ctrls 421 cases + ctrls	nr nr nr	nr nr 0.6-155.2	25.2±22.2 24.0±21.3 nr	(Kilkkinen *et al.*, 2004)
Danish postmp BC cases and ctrls	TR-FIA	381 cases	nr	0.1-454.6	nr	(Olsen *et al.*, 2004)
Swedish PC cases and ctrls	TR-FIA	218 cases 221 ctrls 439 cases + ctrls	21.1 23.9 nr	nr nr 0-169.9 Outlier >600	nr nr nr	(Hedelin *et al.*, 2006)
Finnish M smoker coronary event cases and ctrls	GC-MS	340 cases 420 ctrls 760 cases + ctrls	nr 13.0 nr	nr nr 0.2-106.1	17.8±19.8 18.1±17.5 nr	(Kilkkinen *et al.*, 2006)
German premp BC cases and ctrls	TR-FIA	220 cases 237 ctrls	6.3 9.7	0-225.7 0.1-65.7	11.6±nr 12.2±nr	(Piller *et al.*, 2006a)
Scottish PC cases and ctrls	GC-MS	249 cases 205 ctrls	11.8 16.2	nd-330.3 nd-537.6	nr nr	(Heald *et al.*, 2007)
Dutch pre/perimp and postmp BC cases and ctrls	LC-MS/MS	87 pre/perimp cases 296 postmp cases 87 pre/perimp ctrls 296 postmp ctrls	10.0 9.9 10.3 10.0	0.5-123.3 0-193.7 0.3-125.9 0-174.3	nr nr nr nr	(Verheus *et al.*, 2007)

BC, breast cancer; Ctrl, control; ESRD, end-stage renal disease; M, men; nd, not detected; nr, not reported; PC, prostate cancer; perimp, perimenopausal; postmp, postmenopausal; premp, premenopausal; W, women

* SEM

Table 5. Serum/plasma ENL concentration (nM) in supplementation studies.

Subjects	Supplement	Method	N	Time point	Median	Range	Mean±SD	Rerefence
Postmp Australian W	Flaxseed 25 g/d for 2 wks	GC-MS	23	Pooled	nr	140-818	394	(Morton *et al.*, 1994)
Western M	Cake with 15 g flaxseed and 15 g soy flour	GC-MS	4	0h 24h	15.8 114.0	6.7-24.1 36.5-147.2	15.6±9.3 102.9±47.8	(Morton *et al.*, 1997b)
Finnish M and W	Strawberries, 500 g	TR-FIA	7	0h 24h	10.6 16.5	1.7-22.4 2.4-50.0	10.3±6.7 20.6±16.3	(Mazur *et al.*, 2000)
Finnish M and W	Recommendation to eat fruits, berries and vegetables, 12 wks	TR-FIA	85	0 wks 12 wks	12.2 19.5	0.9-85.2 (overall)	nr nr	(Stumpf *et al.*, 2000)
Over-weight Americans	Refined or whole grain diet (100% controlled), 6 wks	TR-FIA	11	Refined Whole grain	8.4 17.8	0-35.9 0.7-46.1	11.1±11.9 19.2±15.6	(Jacobs *et al.*, 2002)
Swedish PC patients	Rye or wheat bread, 3 wks	TR-FIA	10 8	Rye, BL 3wks Wh., BL 3wks	nr nr nr nr	nr 9.9-159.8 nr nr	32.8±20.9 70.3±53.4 28.0±23.0 20.7±12.5	(Bylund *et al.*, 2003)
Dutch M and W	Flaxseed (whole, crushed or ground) 0.3 g/kg body weight, 10 d	LC-MS/MS	12	BL Whole Crushed Ground	nr nr nr nr	nr 29-262 22-277 122-539	9.5±1.1* 65±16* 85±17* 167±25*	(Kuijsten *et al.*, 2005)
Finnish postmp W	Rye or wheat bread, 8 wks	TR-FIA	39	BL Rye Wheat	nr nr nr	0.1-74.7 4.4-105 0.7-39.4	32.8±4.8* 53.5±10.0* 22.7±4.0*	(Linko *et al.*, 2005)°
Finnish M and W	Sesame seed, 50 g	HPLC-CEAD	4	1h	4.3	nd-13.7	5.6±5.9	(Penalvo *et al.*, 2004c)
Finnish M and W	Sesame seed, 50 g	HPLC-CEAD	4	0h 24h	nr nr	0.6-9.9 nr	4.3±2.5* 567±324*	(Penalvo *et al.*, 2005b)
Finnish M and W	Whole grain wheat and rye crisp bread, 1 wk	TR-FIA	8W 7M	BL, W Wh., W Rye, W BL, M Wh., M Rye, M	nr nr nr nr nr nr	7.2-18.7 5.8-41.3 1.3-38.7 0.8-10.9 0.7-21.4 1.4-35.8	12.9±1.1 16.2±3.0 22.8±3.1 6.1±1.0 5.9±1.8 18.0±3.2	(Linko-Parvinen *et al.*, 2007)

BL, baseline; M, men; nr, not reported; PC, prostate cancer; postmp, postmenopausal; W, women; wks, weeks

* SEM; ° outliers excluded from the study

2.2.2.3 Concentration of ENL in other human body fluids

Only few studies have addressed the question of possible bioaccumulation of ENL in tissues (Table 6). Boccardo *et al.* (Boccardo *et al.*, 2003) measured ENL concentration in breast cyst aspirate, and Morton *et al.* in prostatic fluid (Morton *et al.*, 1997a). Both studies concluded that ENL may accumulate in tissues, since the tissue fluid levels were higher than corresponding serum concentrations in nearly all subjects (Morton *et al.*, 1997a; Boccardo *et al.*, 2003). An additional study has measured ENL in early amniotic fluid, and accordingly found a comparatively high level of ENL (Engel *et al.*, 2006). The serum concentrations in pregnant mothers were not measured in this study and hence no conclusions on possible accumulation in amniotic fluid can be drawn. Although the reported concentrations in amniotic fluid appear high, they could merely reflect a generally higher ENL concentration in pregnant women. Urinary excretion of ENL is reported to increase in early pregnancy (Setchell *et al.*, 1980; Stitch *et al.*, 1980).

Table 6. ENL concentration (nM) in other body fluids

Subjects	Sample	Method	N	Median	Range	Mean±SD	Reference
British, Chinese, Portuguese and Hong Kong M	Prostatic fluid	GC-MS	British, 17 Chinese, 15 Portuguese, 22 Hong Kong, 20	nr nr nr nr	nd-523.0 1.0-459.3 nd-2,095.2 nd-590.0	68.1 110.3 543.1 103.9	(Morton *et al.*, 1997a)
Italian W	Breast cyst fluid	TR-FIA	258	63	0-872.0	107.5±9.9	(Boccardo *et al.*, 2003)
Pregnant W	Amniotic fluid (week 20 of pregnancy)	LC-ECD	21	321.5	39.6-375.5	nr	(Engel *et al.*, 2006)

ECD, electrochemical detection; M, men; nd, not detected; nr, not reported; W, women

2.3 Effects of ENL and its dietary sources on breast and endometrium in humans

2.3.1 The hypothesis about ENL and breast cancer

When ENL was originally detected in human urine, several observations led to the hypothesis that ENL was of ovarian origin: the excretion in women was cyclic, high in early pregnancy, absent in newborn, and low or absent in men (Setchell *et al.*, 1980; Stitch *et al.*, 1980). When the structure of ENL was elucidated, and ENL was recognized as a lignan, excitement about possible connections to cancer arose (Setchell *et al.*, 1981), since podophyllotoxin, another member of the lignan family, had been successfully developed into anticancer drugs etoposide and tenoposide (Ayres & Loike, 1990). Furthermore, the presence of phenolic rings in the structure of ENL together with the initial ideas of ovarian origin led to a postulation of estrogenic properties, and subsequently possible modulation of the risk of estrogen-dependent cancer. Soon after, Adlercreutz *et al.* reported that urinary excretion of ENL was lower among breast cancer patients than healthy control women (Adlercreutz *et al.*, 1982). Since then, the linkage between lignans (ENL in particular) and hormone-dependent

cancer (breast cancer in particular) has been intensively studied. To date, no consensus on the role of lignans in cancer has been obtained.

2.3.2 Epidemiological evidence on the effects of ENL on breast cancer risk

2.3.2.1 Lignan intake and breast cancer

In prospective settings, an inverse correlation between lignan intake and breast cancer has been detected among French postmenopausal women (Touillaud *et al.*, 2007), but no correlation has been found among French premenopausal (Touillaud *et al.*, 2006), Dutch pre- and postmenopausal (Keinan-Boker *et al.*, 2004), or American pre- and postmenopausal (Horn-Ross *et al.*, 2002) women. In case-control studies, a protective effect for lignan intake on premenopausal breast cancer has been observed in American and German women (McCann *et al.*, 2002; Linseisen *et al.*, 2004; McCann *et al.*, 2004; McCann *et al.*, 2006; Piller *et al.*, 2006b). In one of the studies, the protective effect was limited to ER-negative breast cancer (McCann *et al.*, 2006), and in two of the studies the effect was limited to carriers of the A2A2 allele of CYP17 gene (McCann *et al.*, 2002; Piller *et al.*, 2006b). In three studies, conducted in the USA and Britain, no connection between lignan intake and premenopausal breast cancer was found (Horn-Ross *et al.*, 2001; dos Santos Silva *et al.*, 2004; Fink *et al.*, 2007). The evidence pointing to postmenopausal breast cancer risk reduction by lignan intake is scarce. Only one study suggests risk reduction among American women (Fink *et al.*, 2007), while in five studies no effects of lignan intake on postmenopausal breast cancer risk have been observed (Horn-Ross *et al.*, 2001; McCann *et al.*, 2002; dos Santos Silva *et al.*, 2004; McCann *et al.*, 2004; McCann *et al.*, 2006).

Other reports in the literature describing the effects of lignan intake on breast include an Australian study that found an association between higher lignan intake and more favourable breast cancer prognosis (Ha *et al.*, 2006). In this study, women recently diagnosed with invasive breast cancer filled dietary questionnaires before entering surgery. Intake of lignans was significantly associated with reduced risk of lymphovascular invasion and reduced tumour size, and increased incidence of progesterone receptor (PgR) positive cancer (Ha *et al.*, 2006). In Canada, the association between lignan intake during adolescent and breast cancer risk later in life was studied (Thanos *et al.*, 2006). In this case-control study, women diagnosed with breast cancer, and matched controls, completed a questionnaire concerning their dietary habits during adolescence (between 10-15 years of age). The risk of breast cancer later in life correlated inversely with the intake of lignans during adolescence (Thanos *et al.*, 2006).

2.3.2.2 Serum ENL and breast cancer

In prospective settings, no association between serum ENL concentration and breast cancer risk has been seen in British pre- and postmenopausal (Grace *et al.*, 2004), Finnish pre- and postmenopausal (Kilkkinen *et al.*, 2004), American postmenopausal (Zeleniuch-Jacquotte *et al.*, 2004), or Dutch pre- and postmenopausal (Verheus *et al.*,

2007) women. However, an inverse correlation between serum ENL and risk of breast cancer was found in Italian pre- and postmenopausal women with palpable cysts (Boccardo *et al.*, 2004), and in Danish postmenopausal women (Olsen *et al.*, 2004) in prospective settings. The protective effect in the latter study was limited to ER-negative breast cancer (Olsen *et al.*, 2004). Serum ENL concentration has also been linked to increased risk of breast cancer. In three separate cohorts in northern Sweden, women in the highest and lowest percentiles of serum ENL concentration (40-140 nM and 0-6 nM, respectively) were at higher risk of breast cancer than women with intermediary concentrations (10-27 nM) (Hulten *et al.*, 2002). Another study conducted in the USA also indicated increased risk of premenopausal breast cancer in women in the highest quintile (>24 nM) of serum ENL concentrations versus women in the lowest quintile (<5 nM) (Zeleniuch-Jacquotte *et al.*, 2004). The results are puzzling, as the concentrations suggested to increase the risk in these two studies, >24-40 nM, are comparable to those reported to protect from breast cancer in the studies of Olsen *et al.* and Boccardo *et al.* (Boccardo *et al.*, 2004; Olsen *et al.*, 2004). In case-control studies, a significant inverse correlation between serum ENL and breast cancer risk has been observed in pre- and postmenopausal Finnish women (Pietinen *et al.*, 2001) and premenopausal German women (Piller *et al.*, 2006a). In German premenopausal women, the protective effect of ENL was strongest among CYP17 A2A2 allele carriers (Piller *et al.*, 2006b).

In one study, the association between circulating ENL and normal breast tissue morphology was investigated. Plasma concentration of ENL correlated with breast mammographic density in postmenopausal Norwegian women: women in the highest quartile had 2 % denser mammograms than women in the lowest quartile of ENL concentrations (Stuedal *et al.*, 2005).

2.3.3 Epidemiological evidence on the effects of ENL on endometrium

Two epidemiological studies on lignans and endometrial cancer have been conducted thus far, and as in the case of breast cancer, with conflicting results. In prospective settings, circulating ENL was not associated with the risk of endometrial cancer in American, Swedish or Italian pre- and postmenopausal women (Zeleniuch-Jacquotte *et al.*, 2006). Even though BMI was the strongest predictor of endometrial cancer, and BMI was inversely correlated with ENL in cases, the effect of ENL on endometrial cancer was not significant (Zeleniuch-Jacquotte *et al.*, 2006). In a case-control study, consumption of lignans, SECO in particular, was associated with reduced risk of endometrial carcinoma in American pre- and postmenopausal women (Horn-Ross *et al.*, 2003). In this study, the highest risk for endometrial cancer was observed among obese postmenopausal women with the lowest consumption of lignans (Horn-Ross *et al.*, 2003).

Altogether, the epidemiological evidence on breast and endometrial cancer risk modulation by lignans is confusing. Case-control studies tend to find a connection between lignans and cancer risk reduction more easily than prospective studies. This could depend on lifestyle changes upon receiving cancer diagnosis, or perhaps on an

altered metabolism of lignans in cancer patients. Another possible explanation for the confusing results could be the timing of lignan exposure. It has been proposed that exposure to estrogenic compounds *in utero* through maternal diet and prepubertally during adolescence has an impact on the risk of breast cancer later in life, with exposure during adolescence having a protective effect (Hilakivi-Clarke *et al.*, 2001; De Assis & Hilakivi-Clarke, 2006). This hypothesis is supported by the study by Thanos *et al.*, where decreased breast cancer risk was observed among women who consumed more lignans at the age of 10-15 years (Thanos *et al.*, 2006). Some of the studies also suggest that certain gene alleles could affect the cancer risk modulation by ENL and lignans (McCann *et al.*, 2002; Piller *et al.*, 2006b). It is possible that the protective effects of lignans are stronger in individuals with certain alleles of *e.g.* cytochrome P450 genes.

2.4 Effects of ENL and its dietary sources on estrogen target tissues *in vivo*

2.4.1 Assays of estrogen action in rodents

2.4.1.1 Uterotrophic test

The rodent uterotrophic test is based on the marked morphological changes that occur in the uterus in response to estrogens. Many variations of the test are used, differing in the route of exposure, time of exposure, and age and hormonal status of the animals. However, the basic principle of the test is the same: estrogenic compounds increase the weight of the uterus while antiestrogenic compounds antagonize estrogen-induced uterine growth. The Organization for Economic Co-operation and Development (OECD) has recently validated the uterotrophic test as a short-term screening test for estrogenic properties (OECD Test No. 440: Uterotrophic Bioassay in Rodents, adopted 16 Oct, 2007). In this guideline, the preferred model organism is immature rat due to the intact hypothalamus-pituitary-gonadal axis, which is not present when adult ovariectomized animals are used. According to the guideline, immature rats should be dosed (*s.c.* or *p.o.*) prepubertally [between post natal days (PND) 18 and 25] with the tested compound on three subsequent days and the weight of the uterus should be recorded 24 hours after the last dose.

The uterine weight increase in response to E_2 is a result of a complex cascade of events intended to prepare the uterus for embryo implantation (Wang & Dey, 2006). In response to E_2 stimulation, the expression of hundreds of genes changes within hours after exposure (Hewitt *et al.*, 2003; Moggs *et al.*, 2004). Likewise, the first morphological changes occur within hours: stromal oedema is typically observed 4-6 hours post E_2 exposure and proliferation approximately 24-36 hours post E_2 administration (Hewitt *et al.*, 2003; Moggs *et al.*, 2004). Hence, in the early phase of uterotrophic response, the uterine weight increase is due to water imbibition, and in the later phase to cell proliferation. It has been suggested that the proliferation response in the uterus is mediated by the non-classical estrogen signalling pathway, while water imbibition would be induced by the classical pathway, *i.e.* through ERE-controlled genes (O'Brien *et al.*, 2006). Studies with ERα knock-out mice (αERKO) have

demonstrated that the estrogen-induced uterine growth is strictly ERα-dependent (Lubahn *et al.*, 1993; Hewitt *et al.*, 2003). However, growth factors, such as epidermal growth factor (EGF) and insulin like growth factor 1 (IGF-1), can also induce uterine growth (Curtis *et al.*, 1996; Klotz *et al.*, 2002). Moreover, although growth factors fail to increase uterine weight in the absence of ERα, they can induce expression of estrogen responsive genes in αERKO uteri (Hewitt *et al.*, 2005), suggesting that growth factor and ER pathways interact in multiple ways in uterotrophic response. Other signalling pathways have also been indicated in uterotrophic response. For instance, canonical Wnt signalling is required for E_2-induced uterine growth (Hou *et al.*, 2004). Altogether, the uterine growth response seems to result from a complex interplay between multiple separate signalling pathways, orchestrated by ERα.

2.4.1.2 Experimental models on estrogen action in the mammary gland

Estrogenic activity of compounds in the mammary gland of rodents can be studied during the development of the gland and in mammary carcinogenesis. Mammary gland development occurs mainly after birth. Until puberty, the growth of the mammary gland is isometric, *i.e.* it grows with the same pace as the body, and does not depend on estrogens (Mallepell *et al.*, 2006). However, at puberty the growth of the mammary epithelium becomes dependent on estrogens. E_2 induces ductal elongation by stimulating epithelial growth into the surrounding mammary fat pad at terminal end buds (TEBs), located at the tip of the prolonging ducts. This allometric growth is strictly dependent on epithelial ERα, as the mammary glands of αERKO mice fail to develop beyond the rudimentary structures present prior to puberty (Mallepell *et al.*, 2006). The TEB structures are highly proliferative, hormone sensitive, home stem cells, and are the targets of malignant transformations in carcinogen-induced mammary tumour models in rodents. Exposure to estrogens prepubertally would enhance the maturation of the mammary gland, and thereby confer resistance to carcinoma later in life (De Assis & Hilakivi-Clarke, 2006). The maturation of the mammary gland can be followed at different developmental stages by measuring the size of the mammary epithelium and quantifying the number of the TEB structures.

The mammary gland is most sensitive to breast cancer initiative events during the pubertal growth phase (Cardiff & Wellings, 1999). Although life time exposure to estrogens is a well known risk factor for breast cancer, the mechanisms of estrogen involvement in breast cancer induction and promotion are not well understood (Russo & Russo, 1998). In humans, breast cancers are classified based on their estrogen dependency into ER positive and ER negative carcinomas. Compounds suggested to act on breast cancer through ER-mediated mechanisms, such as phytoestrogens, would be expected to affect the estrogen-dependent ER positive breast cancers. In mouse models, progressed mammary carcinomas are estrogen-independent, but in rats most are estrogen-dependent (Nandi *et al.*, 1995). Hence, estrogen-dependent mammary carcinomas are better mimicked in rat models. A widely used estrogen-dependent mammary carcinoma model is the DMBA [dimethylbenz(a)anthracene] -induced mammary tumour model in rats (Welsch, 1985). In this model, treatment of intact female rats with a single dose of DMBA at the age of 50 days induces mammary

tumours arising from the TEBs. The timing of exposure is important, demonstrating the vulnerability of the mammary gland at the time when the highly proliferative TEBs are present. Although DMBA-induced tumours are estrogen-dependent and mostly adenocarcinomas, thereby resembling human breast cancer, they are also prolactin-dependent and rarely metastasize, in which they differ from human breast cancer (Welsch, 1985). In order to better model human cancer, immunodeficient mice xenografted with human breast cancer cells have been used. Although these models allow the study of human derived cancer cells *in vivo*, the properties of the common *in vitro* cultured cells may differ from cancer cells in primary breast tumours. Furthermore, the gene expression profiles of breast cancer cells grown in culture or in immunodeficient mice display only a minor overlap after treatment with estrogen, even when the same cell line is used, indicating the pivotal role of cellular environment in the behaviour of breast cancer cells in experimental models (Harvell *et al.*, 2006). In the DMBA model and in mice xenografted with estrogen-responsive cancer cells, estrogenic compounds promote tumour growth while estrogen antagonists inhibit the growth.

2.4.1.3 Estrogen reporter mice

Along with the creation of ER knock-out mice, it became evident that estrogen activity is essential in many tissues outside the female reproductive system (Harris, 2007; Zhao *et al.*, 2008). The rodent uterotrophic test and mammary gland models, albeit well characterized and widely used, are limited in their restricted tissue selection. The lack of well recognized, feasible end points of estrogen exposure in non-reproductive tissues is perhaps one of the reasons why assays of estrogenicity mainly rely on the uterus and mammary gland. To overcome this apparent limitation, transgenic estrogen reporter mice have been created (Ciana *et al.*, 2001; Nagel *et al.*, 2001; Toda *et al.*, 2004; Lemmen *et al.*, 2004a). The genome of these mice is modified with an insertion of estrogen-inducible transgene. In tissues where components required for estrogen activity are present, such as ER and necessary co-factors, estrogenic compounds are expected to stimulate the expression of the reporter gene. The four separate estrogen reporter mouse models created to date rely on similar core promoter sequence, the consensus ERE-sequence, but on different reporter genes. In the mouse model of Nagel *et al.*, a 3xERE-thymidine kinase (tk) promoter drives the expression of β-galactosidase (Nagel *et al.*, 2001), the mouse model of Ciana *et al*. expresses Luciferase under the control of a 2xERE-tk promoter (Ciana *et al.*, 2001), the mouse model of Toda *et al*. bears 4xERE-hsp68 driven green fluorescent protein (GFP) (Toda *et al.*, 2004), and the mouse model of Lemmen *et al.* expresses Luciferase from a 3xERE-TATA promoter (Lemmen *et al.*, 2004a). The characterization of these mouse lines has indicated that estrogens can affect ERE-mediated transcription in nearly all tissue types (Ciana *et al.*, 2001; Nagel *et al.*, 2001; Toda *et al.*, 2004; Lemmen *et al.*, 2004a). Further, the reporter gene can be induced by environmental contaminants (bisphenol A), synthetic drugs [diethylstilbestrol (DES), tamoxifen] and dietary phytoestrogens [genistein (GEN)] (Nagel *et al.*, 2001; Lemmen *et al.*, 2004a; Lemmen *et al.*, 2004b; Montani *et al.*, 2008). In addition to providing information about the effects of compounds on ER-mediated transactivation *in vivo* in different tissues, the

reporter mice also offer the possibility to measure traditional end points of estrogen exposure, such as uterine growth. Indeed, by combining the reporter gene approach to traditional end points of estrogen exposure, the role of ER signalling in the particular response can be evaluated. Thus far, lignans have not been studied in reporter mouse models.

2.4.2 Effects of purified lignans on the uterus

Soon after its discovery, ENL was tested for uterotrophic properties. When given to immature mice, ENL (15 μg–20 mg/kg *s.c.*) did not promote uterine weight 24 hours post injection (Setchell *et al.*, 1981). Similarly, ENL (0.3-30 μg/rat, ≈ 10 μg–1 mg/kg *s.c.*) did not significantly increase uterine RNA synthesis up to 24 hours post injection in immature rats (Waters & Knowler, 1982). However, when ENL was administered 22 hours prior to treatment with E_2, it significantly reduced E_2-induced RNA synthesis. This phenomenon was only seen when ENL was administered 22 hours before the treatment with E_2, not if the compounds were co-administered or if ENL was injected 12 hours before E_2 (Waters & Knowler, 1982). The lack of estrogenicity of ENL in uterotrophic tests was confirmed 20 years later with immature rats. No effects on uterine weight were observed, when ENL, or its precursors HMR, SECO and MR (50 mg/kg *p.o.*), were gavaged to immature rats for seven days (Saarinen *et al.*, 2000; Saarinen *et al.*, 2002a).

In long-term studies, such as the mammary carcinoma studies, uterine weight at sacrifice is often recorded. ENL precursors SDG, HMR, and LAR did not affect the uterine weight in DMBA-induced rats (Thompson *et al.*, 1996a; Rickard *et al.*, 1999; Saarinen *et al.*, 2000; Saarinen *et al.*, 2001; Saarinen *et al.*, 2008). LAR did not affect uterine weight in ovariectomized immunodeficient mice in the presence of E_2 either (Saarinen *et al.*, 2008). When SDG was administered to pregnant rats, the uterine weight of female offspring was transiently increased on PND21, but when exposure occurred during lactation, SDG did not alter uterine weight (Tou *et al.*, 1998; Ward *et al.*, 2001). In ovariectomized immunodeficient mice bearing MCF-7 xenografts in the absence of E_2, ENL (10 mg/kg *s.c.*) administration for 22 weeks did not affect the uterine weight (Power *et al.*, 2006b). However, ENL significantly reduced uterine weight after a 7-week daily administration regimen (10 mg/kg *p.o.*) in non-ovariectomized DMBA rats (Saarinen *et al.*, 2002a).

Estrogens are involved in the development and growth of endometrial carcinomas. Although several endometrial carcinoma models in rodents exist (Vollmer, 2003), they have not yet been applied to lignan studies, with one exception. The effects of HMR consumption on endometrial carcinoma were investigated in Donryu rats, which develop endometrial adenocarcinomas spontaneously with age (Vollmer, 2003). To accelerate the carcinoma formation, Donryu rats were induced with N-ethyl-N'-nitro-N-nitrosoguanidine, and subsequently treated with HMR in their diet (200 and 600 ppm, resulting in ≈10 and 30 mg/kg/d exposure) for a year (Katsuda *et al.*, 2004). While HMR did not significantly affect the incidence of proliferative lesions of the endometrium, the incidence of adenocarcinomas was reduced (Katsuda *et al.*, 2004).

2.4.3 Effects of dietary lignan sources on the uterus

Dietary lignan sources have not been studied in conventional uterotrophic test settings. However, the uterine weight after exposure to a flaxseed diet has been recorded in mammary carcinoma studies. Flaxseed has not significantly influenced the weight of the uterus in any of the performed studies (Rickard *et al.*, 1999; Chen *et al.*, 2007a; Chen *et al.*, 2007b). The effects of developmental exposure to flaxseed have also been studied. When pregnant rats were administered a 10 % flaxseed diet throughout pregnancy and lactation, the uterine weight of the dams and their female offspring was increased on the post delivery day/PND 21 (Tou *et al.*, 1998). However, the effect in the offspring was transient, and the differences were no longer significant on PND50 and PND132 (Tou *et al.*, 1998). When rat pups were exposed to 10 % flaxseed during lactation only or during lactation and adolescence, no differences in uterine weight were detected on PND21, PND50, or PND132 (Ward *et al.*, 2001).

2.4.4 Effects of purified lignans on mammary gland maturation and carcinogenesis

2.4.4.1 Purified dietary ENL precursors and the mammary gland

The most studied purified dietary lignan *in vivo* is flaxseed SDG. When rats were exposed to SDG (1.5 mg/d *p.o.*) through pregnant dams *in utero* and through milk during lactation, the number of TEBs was reduced on PND50 (Tou & Thompson, 1999). In a subsequent study, SDG was given in the diet to rat pups only during lactation or during lactation and adolescence, and in both setups, the number of TEBs was reduced on PND50 (Ward *et al.*, 2000). When rats exposed to SDG during lactation were examined on PND21 and PND50, proliferation in TEBs was increased on PND21 and the number of TEBs decreased on PND 50, suggesting that SDG enhances mammary gland maturation (Tan *et al.*, 2004). As enhanced mammary gland maturation has been suggested to protect against mammary carcinoma, the authors tested the effect of lactational SDG exposure on DMBA-induced mammary carcinoma development. Tumour incidence, size, multiplicity, and total tumour load were reduced in the SDG-exposed rats compared to the vehicle-exposed controls, suggesting that enhanced mammary gland maturation after lactational exposure to SDG reduces susceptibility to mammary carcinomas later in life (Chen *et al.*, 2003).

Administration of SDG to adult rats post carcinogen induction also inhibits the growth of mammary carcinomas. Daily administration of SDG (1.5 mg/day *p.o.*) at the initiation or promotion phase of DMBA-induced mammary carcinogenesis reduced tumour growth (Thompson *et al.*, 1996a; Thompson *et al.*, 1996b), and in methylnitrosourea (MNU) -induced mammary carcinoma model SDG (0.7 mg/d or 1.4 mg/d *p.o.*) reduced the invasiveness and bettered the grade of tumours, but had less clear effect on tumour volume and multiplicity (Rickard *et al.*, 1999). Similar to SDG, other dietary ENL precursors also inhibit carcinoma growth in the DMBA model. HMR given by gavage *p.o.* after DMBA induction, or mixed in the diet before the induction, inhibits tumour growth (Saarinen *et al.*, 2000; Saarinen *et al.*, 2001). SES, an ENL precursor present in sesame seed, reduces the number of tumours when

administered in the diet to DMBA-induced rats (Hirose *et al.*, 1992), and LAR reduces the growth of mammary gland tumours when administered *p.o.* to DMBA-induced rats or in the diet to immunodeficient mice bearing MCF-7 xenografts (Saarinen *et al.*, 2008).

2.4.4.2 Purified ENL and mammary cancer

Studies conducted with different ENL precursors suggest that ENL could be the mediator of the anticarcinogenic properties of dietary plant lignans. The effects of ENL on mammary carcinoma have been investigated in three different studies. When DMBA-induced rats were treated with ENL (10 mg/kg *p.o.*) daily for seven weeks, starting after tumours were established, the tumour growth was significantly reduced (Saarinen *et al.*, 2002a). The effect was more pronounced on tumours formed during the 7-week treatment period, and was also seen as a reduced proportion of growing and increased proportion of non-growing tumours (Saarinen *et al.*, 2002a). In the second study, ovariectomized immunodeficient mice were xenografted with the human breast cancer MCF-7 cells and an E_2 pellet was implanted (Power *et al.*, 2006a). When the tumours were established, the pellets were removed and mice were treated with daily injections of ENL (10 mg/kg *s.c.*) for 22 weeks (Power *et al.*, 2006a). ENL treatment did not support the growth of the tumours (Power *et al.*, 2006a). In the third study, the effect of ENL on E_2-supported tumour growth in MCF-7-xenografted immunodeficient mice was studied (Bergman Jungestrom *et al.*, 2007). Ovariectomized, E_2-pelleted mice with established tumours were treated with daily injections of ENL (15 mg/kg *s.c.*) for five weeks. The treatment significantly reduced E_2-supported tumour growth (Bergman Jungestrom *et al.*, 2007).

2.4.5 Effects of dietary lignan sources on mammary gland maturation and carcinogenesis

By far the most studied dietary lignan source in experimental models is flaxseed. The first implications of the effects of flaxseed on the mammary gland were indicated by Serraino & Thompson (1991) who reported that rats treated with flaxseed diet (5 %, 10 %) had less proliferating cells and nuclear aberrations in TEBs 24 hours post DMBA administration (Serraino & Thompson, 1991). Later, both 5 % and 10 % flaxseed diets were shown to reduce the number of TEBs on PND50 when rats were exposed *in utero* and during lactation, or continuously from gestation on until PND50 (Tou & Thompson, 1999). In subsequent studies, rats were exposed to 10 % flaxseed diet during lactation only or during lactation and adolescence, and the number of TEBs was again reduced in both setups on PND50 (Ward *et al.*, 2000). The effect of 10 % flaxseed diet during lactation was further dissected by studying the mammary gland morphology on PND21 in addition to PND50. Consistent with the theory of advanced maturation of mammary gland, the flaxseed diet increased the number of TEBs on PND21 and decreased their number on PND50 (Tan *et al.*, 2004). When the rats exposed to flaxseed during lactation were induced with DMBA, the tumour growth, incidence, and multiplicity were reduced, suggesting that developmental flaxseed exposure may reduce susceptibility to mammary carcinomas by enhancing the

maturation of the mammary gland (Chen *et al.*, 2003). However, in another study, the incidence and multiplicity of DMBA-induced tumours were increased when rats were exposed to 15 % defatted flaxseed *in utero* (Yu *et al.*, 2006). In the same study, mammary gland morphology was evaluated, and no changes in the number of TEBs were observed on PND21 or PND50 (Yu *et al.*, 2006). These results suggest that exposure *in utero* only differs from exposure during gestation and lactation or lactation only.

Flaxseed reduces tumour growth even in adult carcinogen-induced rats. A flaxseed diet (5 %) given at an early or late stage of carcinogenesis inhibits the growth of tumours in the DMBA model (Serraino & Thompson, 1992; Thompson *et al.*, 1996b). In the MNU-induced carcinoma model a 5 % flaxseed diet did not alter tumour volume, multiplicity or incidence, but reduced tumour grade and invasiveness (Rickard *et al.*, 1999). The results obtained with flaxseed diets in immunodeficient mice bearing MCF-7 xenografts are in line with the observations in the DMBA studies. When established MCF-7 tumours are maintained with E_2 pellets, 10 % flaxseed significantly reduces tumour growth (Chen *et al.*, 2004; Bergman Jungestrom *et al.*, 2007; Chen *et al.*, 2007b), and in the absence of E_2, flaxseed does not support growth of tumours (Chen *et al.*, 2004; Saarinen *et al.*, 2006; Chen *et al.*, 2007a).

Collectively, *in vivo* studies suggest that ENL, plant lignans, and dietary sources of lignans are anticarcinogenic and do not possess estrogenic activity in established estrogen-dependent mammary tumours. The inhibition of estrogen-dependent carcinoma growth could imply antiestrogenicity. However, developmental studies with SDG and flaxseed suggest that lignans may enhance mammary gland maturation when exposure occurs prepubertally, and have uterotrophic effects when exposure occurs *in utero*, both of which could be indications of estrogenic activity. Moreover, increased susceptibility to mammary carcinogenesis has been reported after *in utero* exposure to flaxseed, which according to some hypotheses could indicate estrogenic activity (De Assis & Hilakivi-Clarke, 2006). Hence, *in vivo* studies do not provide a clear ER-related mechanism for the activity of ENL and lignans. The effects of purified ENL on the uterus and mammary gland are summarized in Table 7.

Table 7. Effects of purified ENL on the uterus and mammary gland *in vivo*

Target tissue	End point	Result	Interpretation from ER point of view	References
Uterus	Growth	• No effect on uterine weight in a 24 h assay in immature mice (15 μg-20 mg/kg, *s.c.*) • No effect on uterine RNA synthesis in a 24 h assay in immature rats (10 μg-1 mg/kg *s.c.*) • Reduction of E_2-induced RNA synthesis in immature rats when administered 22 h before E_2 • No effect on uterine weight in a 7-day assay in immature rat (50 mg/kg *p.o.*) • Reduction of uterine weight in a 7-week DMBA study in adult intact rats (10 mg/kg *p.o.*) • No effect on uterine weight in a 22-week xenograft study in the absence of E_2 in immunodeficient mice (10 mg/kg *s.c.* 22 weeks)	No estrogenic activity Antiestrogenic activity	(Setchell *et al.*, 1981) (Waters & Knowler, 1982) (Saarinen *et al.*, 2002a) (Power *et al.*, 2006b)
	Carcinoma	Not determined	-	-
Mammary gland	Development	Not determined	-	-
	Carcinoma	• Inhibition of tumour growth in DMBA-induced rats (10 mg/kg *p.o.* 7 weeks) • No promotion of tumour growth in immunodeficient mice bearing MCF-7 xenografts in the absence of E_2 (10 mg/kg *s.c.* 22 weeks) • Inhibition of tumour growth in immunodeficient mice bearing MCF-7 xenografts in the presence of E_2 (15 mg/kg *s.c.* 5 weeks)	No estrogenic activity Antiestrogenic activity	(Saarinen *et al.*, 2002a) (Power *et al.*, 2006a) (Bergman Jungestrom *et al.*, 2007)

2.5 Interaction between ENL and the estrogen receptors

2.5.1 Estrogen receptors

ERs are ligand-inducible transcription factors that modulate gene expression in response to female sex steroids, estrogens. ERs belong to a super family of evolutionally conserved nuclear receptors (NRs), which in humans is composed of 48 known receptor proteins (Laudet, 1997; Nilsson *et al.*, 2001; Gronemeyer *et al.*, 2004). Together with receptors for androgens, progesterones, thyroid hormone, mineralocorticoids and glucocorticoids, ERs belong to the steroid/thyroid hormone receptor subfamily within the NR superfamily. Half of the NRs, including ERs, regulate gene expression in response to a ligand, while the other half do not have a recognized ligand and are called orphan receptors (Nilsson *et al.*, 2001; Gronemeyer *et al.*, 2004). Thus far, two distinct ERs have been characterized, namely ERα (Walter *et al.*, 1985; Greene *et al.*, 1986) and ERβ (Kuiper *et al.*, 1996) (official nomenclature NR3A1 and NR3A2, respectively) (Laudet *et al.*, 1999). The full length human ERα is a 595 amino acids long protein, while the full length ERβ is somewhat shorter, 530 amino acids (Moore *et al.*, 1998). Several alternative splice variants of both receptors have been discovered in normal and cancer tissue but the significance of most of these variants in estrogen signalling remains unclear (Herynk & Fuqua, 2004).

2.5.1.1 Structure of the ERs

The ER domain arrangement is characteristic to NRs (Laudet, 1997; Nilsson *et al.*, 2001; Gronemeyer *et al.*, 2004). The N-terminal A/B-domain harbours ligand independent activation function 1 (AF-1) and an interaction surface for co-regulators,

the central C-domain mediates DNA binding through two zinc finger structures, the D-domain functions as a flexible hinge and contains nuclear localization signal, and the C-terminal E/F-domain is responsible for ligand binding, harbours ligand dependent AF-2, and another interaction surface for co-regulators (Figure 4). The two ERs display 47 % overall sequence homology (Enmark *et al.*, 1997). The highest degree of sequence conservation between the two receptors is observed in the DNA binding domain (DBD) (97 %) and the lowest in the N-terminal A/B-domain (18 %) (Enmark *et al.*, 1997). The low conservation of the A/B-domain is reflected by substantial differences in the activity of AF-1 between the two receptors (McInerney *et al.*, 1998; Webb *et al.*, 1999). The ligand binding domains (LBDs) of ERα and ERβ are 60 % homologous by sequence, and crystal structures have revealed that the two LBDs, formed of 12 α-helices (Figure 4), are very similar in terms of 3D structure (Brzozowski *et al.*, 1997; Pike *et al.*, 1999). However, the ligand binding cavities differ between ERα and ERβ by two amino acid residues, and the ligand binding pocket of ERβ is subsequently 20 % smaller than that of ERα (Pike *et al.*, 1999).

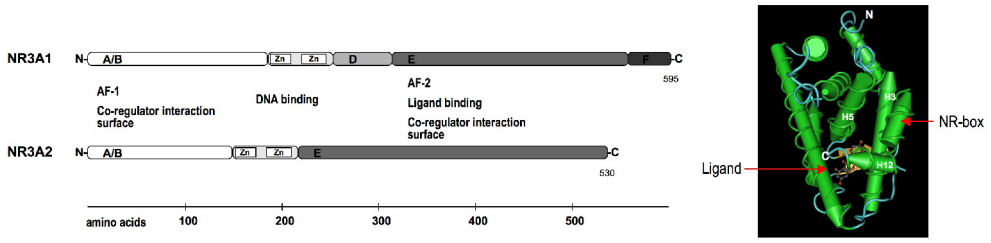


Figure 4. Structure of the ERs. Schematic presentation of the domain arrangement of the full length human ERα and ERβ (to the left) and crystal structure of the ERα LBD (to the right). The N-terminal A/B-domain harbours ligand independent activation function AF-1 and a co-regulator interaction surface, the central C-domain mediates DNA binding through two zinc fingers, and the C-terminal E/F-domain contains the LBD, ligand dependent AF-2, and a co-regulator interaction surface. The ER LBD is formed of 12 α-helices. When an agonist binds to the LBD, helix 12 (H12) covers the opening of the ligand binding cavity and forms a co-regulator interaction surface together with H3 and H5. Co-regulators with an LxxLL-motif (NR-box) can bind to the surface formed. The presented domain arrangement is retrieved from The Expert Protein Analysis System server of the Swiss Institute of Bioinformatics (www.expasy.org), and the crystal structure from the National Center for Biotechnology Information public protein structure database (www.ncbi.nlm.nih.gov).

2.5.1.2 Activation of the ERs

In the absence of ligands, ERs are maintained in a latent, high ligand binding affinity conformation by co-chaperone proteins (Pratt & Toft, 1997). Upon ligand binding, a major conformational change occurs in the receptor leading to dissociation of the co-chaperone molecules, and exposure of co-regulator interaction surfaces. The ligand-activated ERs either homo- or heterodimerize and bind to ERE-elements on DNA. The classical ERE consists of inverted repeats, separated by 3 nucleotides, of pentanucleotides resembling the consensus sequence GGTCA (GGTCAnnnTGACC). Many variations of the consensus sequence occur (Gruber *et al.*, 2004). Moreover, ERs

can bind to other promoter sequences indirectly through tethering to other transcription factors, such as Activating Protein 1 (AP-1) and Specificity protein 1 (Sp-1) (Carroll *et al.*, 2006). Once the ligand-activated ER dimer has bound to DNA, it recruits co-factors and communicates with the basal transcription machinery (BTM), and ultimately regulates the transcription of the target gene (Figure 5) (Metivier *et al.*, 2006). The protein complexes that form on responsive promoters are dynamic and possess several different activities, such as histone acetyl transferase, histone deacetylase, histone and DNA methyl transferase, helicase, and ubiquitin ligase activity, all required for appropriate target gene transcription (Metivier *et al.*, 2006; Metivier *et al.*, 2008). For example, approximately 30 proteins have been recognized in the complex that forms on the human pS2 promoter upon E_2 stimulation (Metivier *et al.*, 2003). Through the various co-regulator proteins ERs regulate the transcription of the target gene, leading to increased or decreased expression. The transcriptional cycle is completed by clearance of the promoter through proteosomal degradation of ER (Reid *et al.*, 2003; Metivier *et al.*, 2006). Ligands are not always needed for ER activity: other cellular signalling pathways can modulate ER through phosphorylation. For example, growth factor signalling and ER signalling are coupled through mitogen activated protein kinase (MAPK). Further, general cellular regulators of phosphorylation, such as protein kinases A and C (PKA, PKC), can modulate ER activity (Nilsson *et al.*, 2001). A simplified overview of classical ER signalling pathway is presented in Figure 5.

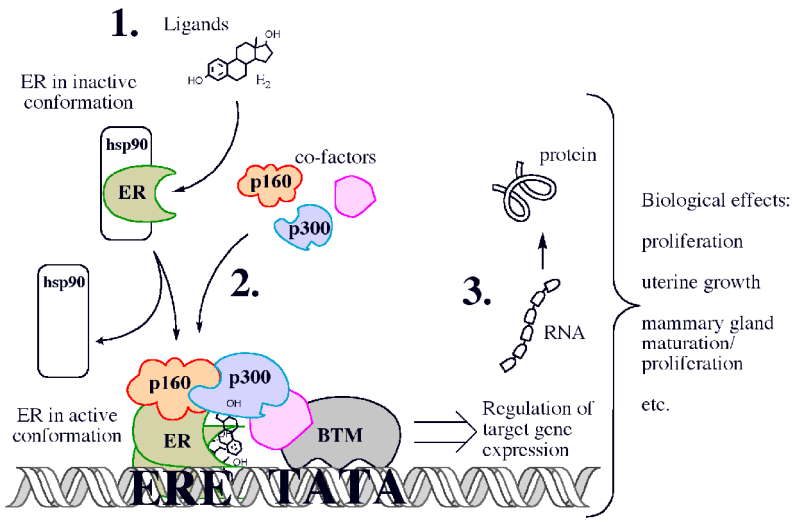


Figure 5. Schematic presentation of classical ER-mediated signalling. 1. In the absence of a ligand, ER is kept in a latent high ligand binding affinity conformation by co-chaperones, such as heat shock protein 90 (hsp90). 2. Upon ligand binding, the co-chaperone complex dissociates and the receptor dimerizes. A co-activator binding surface is exposed and co-regulators, such as p160 and p300, can associate with the receptor on enhancer elements (EREs) of target genes on DNA. The co-regulators bridge the ER to the BTM. 3. Target gene transcription is initiated (or activity is repressed).

2.5.1.3 Distinct in vivo *functions of ERα and ERβ*

The two ERs are widely expressed, yet have specific individual expression patterns. ERα is expressed in the female reproductive tract, mammary gland, bone, liver, kidney, cardiovascular system and adipose tissue, while ERβ is expressed in the urogenital tract, mammary gland, bone, cardiovascular system, lung, and gastrointestinal tract (Enmark *et al.*, 1997; Kuiper *et al.*, 1997). Even when present in the same tissue, expression is often limited to different cell types. For instance, in the ovary, ERβ is expressed in the granulosa cells and ERα in the theca cells (Saunders *et al.*, 2000). Although estrogens and their receptors are indispensable for normal female reproductive function, they also play a role in a multitude of targets outside the reproductive system, including the central nervous system, skeletal system, cardiovascular system, immune system, and male reproduction (Couse & Korach, 1999; Harris, 2007; Zhao *et al.*, 2008). The different physiological functions of the two ERs are illustrated by the isoform-specific knock-out mice, αERKO and βERKO (Couse & Korach, 1999; Hewitt *et al.*, 2005; Harris, 2007). Neither of the receptors is required for embryonic development; however, the postnatal phenotypes or αERKO and βERKO mice clearly indicate that the ERs are required for normal development and homeostasis (Lubahn *et al.*, 1993; Krege *et al.*, 1998; Dupont *et al.*, 2000). The αERKO females are infertile, have hypoplastic uteri that do not respond to estrogenic stimulus, polycystic ovaries characterized by absence of *corpora lutea*, rudimentary mammary gland structures, and altered mating behaviour (Lubahn *et al.*, 1993; Dupont *et al.*, 2000; Hewitt *et al.*, 2003). The reproductive phenotype of βERKO females is milder; the βERKO females are subfertile or infertile, have rare or none *corpora lutea* in their ovaries, and display normal mating behaviour (Krege *et al.*, 1998; Dupont *et al.*, 2000). The gross morphology of the uterus and mammary gland of βERKO mice is normal (Krege *et al.*, 1998; Dupont *et al.*, 2000; Hewitt *et al.*, 2003); however, βERKO uteri show exaggerated response to E_2 and the mammary gland becomes abnormal upon aging (Gustafsson & Warner, 2000; Wada-Hiraike *et al.*, 2006). As a summary, ERα seems indispensable in mediating estrogen activity in classical target tissues, *i.e.* in the female reproductive tract and mammary gland. ERβ, on the other hand, seems to be a mediator of estrogen signalling in non-classical target tissues, such as the colon, prostate and central nervous system. For example, βERKO mice develop hypertension as they age, have malignant changes in colon and prostate epithelium, and display increased anxiety (Krege *et al.*, 1998; Krezel *et al.*, 2001; Zhu *et al.*, 2002; Imamov *et al.*, 2004; Imwalle *et al.*, 2005; Wada-Hiraike *et al.*, 2006).

2.5.1.4 Selective regulation of ER activity

ERs are promiscuous in their spectrum of ligand binding capacities. In addition to estrogens, a multitude of other compounds, including drugs such as DES, heavy metals such as cadmium, phytoestrogens such as GEN, and industrial chemicals such as bisphenol A, can bind and regulate the activity of ERs (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998; Stoica *et al.*, 2000). The binding affinities of xenoestrogens for the two ERs differ, leading to preferential activation of one isoform over the other (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998). Moreover, the binding of structurally diverse ligands

generates different conformational changes in the LBD, which subsequently affects interaction with co-regulatory proteins and transcriptional activity of the receptor (Brzozowski *et al.*, 1997; Paige *et al.*, 1999; Roelens *et al.*, 2006). Binding of an agonist to ER LBD leads to a conformation where helix 12 (H12) moves across the ligand binding cavity surface, closing the ligand binding pocket and forming an interaction surface for co-regulators together with H3 and H5 (Figure 4) (Brzozowski *et al.*, 1997; Paige *et al.*, 1999). AF-2 interacting co-regulators possessing a conserved LxxLL-motif (NR-box), such as the p160 family coactivators SRC1-3 and TRAP220, can interact with this surface (Figure 4) (McDonnell & Norris, 2002). In case an antagonist binds to ER, the LBD H12 does not relocate to the typical agonist position, the co-regulator interaction surface is not properly formed, and the receptor interacts with co-repressors like N-CoR and SMRT instead of co-activators. More complexity to the co-regulator action is provided, for instance, by co-regulators such as RIP140 and SHP, that bind to the same surface as the LxxLL containing co-activators, but instead of promoting they repress transcription and thereby function as anti-co-activators (Heldring *et al.*, 2007).

In many cases, ERα and ERβ signal in opposite ways in response to the same ligand. For example, E_2 activates transcription through ERα on AP-1 promoters, while transcription is repressed when ERβ mediates the signal (Paech *et al.*, 1997). In cell culture ERα mediates proliferation, while ERβ induces apoptosis in response to E_2 (Helguero *et al.*, 2005). Furthermore, ERα and ERβ might have different target genes. It was recently demonstrated that although the two receptors bind a set of the same elements on DNA, both also have their unique, distinct binding regions in genomic DNA (Liu *et al.*, 2008).

Altogether, the differing ligand binding affinities of ERα and ERβ, the crucial role of the LBD conformation in dictating subsequent activity, different co-activator pools available in cells, and the different biological roles of the two receptors pave the way for selective modulation of estrogen signalling. The concept of selective ER modulation (SERM activity) was originally derived from the biological activity of tamoxifen, a non-steroidal drug that has been used in the treatment and prevention of ERα-positive breast cancer since the 1970s (Jordan, 2003). Tamoxifen is an antiestrogen in the breast but has estrogen-like properties in bone and uterus. Concern about tamoxifen safety has been evoked by observations of higher risk for endometrial carcinoma, blood clots, and resistance of the tumours to treatment in long-term use (1998; Cuzick *et al.*, 2002). Tamoxifen has become a model compound in SERM studies, and it has considerably increased the understanding of ER function. The conformation of tamoxifen-bound ERα does not allow the binding of LxxLL-motif co-activators (Brzozowski *et al.*, 1997), and accordingly, the receptor recruits co-repressors N-CoR and SMRT to estrogen-responsive promoters in MCF-7 cells (Shang *et al.*, 2000; Liu & Bagchi, 2004). However, in the human endometrial cancer Ishikawa cells tamoxifen-bound ERα induces the expression of estrogen-responsive genes c-myc and IGF-1 by recruiting co-activator SRC-1 to the promoters (Shang & Brown, 2002). This differential activity of tamoxifen between endometrial and breast cell lines

seems to be dependent on the relative expression levels of the co-regulators SRC-1, N-CoR, and SMRT (Shang & Brown, 2002; Keeton & Brown, 2005). The relative concentrations of co-activators in the different cells is regarded as one of the explanations for tissue-specific SERM activity. The existence of yet unidentified SERM specific co-regulators has also been recently suggested (Heldring *et al.*, 2004; Kong *et al.*, 2005). Interestingly, dietary phytoestrogens have been proposed to be natural SERM-like compounds based on their selectivity towards ER isoforms. For example, GEN, an isoflavone derived from soy, preferentially interacts with ERβ (Kuiper *et al.*, 1998; An *et al.*, 2001; Manas *et al.*, 2004; Damdimopoulos *et al.*, 2008). However, the details of the mechanisms of phytoestrogen-triggered SERM activity, as well as the significance of such activity to humans consuming phytoestrogens, remain to be investigated.

2.5.2 Estrogenicity of ENL

2.5.2.1 Receptor binding affinity of ENL

Ever since the discovery of ENL, interaction with the ERs has been one of the suggested mechanisms of action of this compound (Setchell *et al.*, 1981). However, the affinity of ENL towards ERs is low. In a competitive ligand binding assay utilizing human ERs, the IC_{50} values of ENL for ERα and ERβ were 6.7 μM and 39 μM, respectively (Mueller *et al.*, 2004). These values are clearly higher than those measured in human body fluids under any reported circumstances (Tables 3-6), and would suggest that ENL is a weak ligand for the ERs at best.

2.5.2.2 Transactivation of the ERs by ENL

Only few endogenous genes have been studied after ENL exposure. In MCF-7 cells and in a primary culture of rat uterine cells, 10 μM ENL stimulated the expression of PgR, and in a primary culture of rat pituitary cells the same concentration of ENL induced prolactin synthesis (Welshons *et al.*, 1987). Measured with Northern blot, ENL increased the expression of pS2 at a slightly lower concentration (1 μM) in MCF-7 cells (Sathyamoorthy *et al.*, 1994).

Three studies have assessed the estrogenicity of ENL in reporter gene assays. In Ishikawa cells stably transfected with either ERα or ERβ, the potencies of different phytoestrogens were tested on two different promoter constructs. ENL was a weak transactivator of ERα and ERβ both on the consensus ERE promoter and on the complement C3 promoter (Mueller *et al.*, 2004). Compared to DES, the relative potency of ENL on the ERE promoter was 0.01 %, and on the C3 promoter even smaller (Mueller *et al.*, 2004). In the same study, the authors reported that ENL was a partial antagonist in the Ishikawa cells, reducing DES-induced activity by 20-40 % (Mueller *et al.*, 2004). In the human embryonic kidney HEK293 cell line, ENL was also a weak ER agonist in an ER transactivation assay (Saarinen *et al.*, 2000). In this study, the reporter gene consisted of one copy of the consensus ERE fused to the alkaline phosphatase (ALP) reporter gene. Activation through ERα and ERβ was

reported to require over 1 μM concentration of ENL, and no antagonistic effects were observed when cells were co-treated with E_2 and ENL (Saarinen *et al.*, 2000). In a recent study, ENL was shown to activate expression of C3-promoter driven Luciferase in MCF-7 cells through ERα, starting at 1 μM concentration, but not through ERβ (Carreau *et al.*, 2008). In the same study, ENL antagonized E_2-induced ERβ transactivation but had no effect on E_2-induced signalling through ERα (Carreau *et al.*, 2008).

ER transactivation by ENL has thus far only been studied on classical ERE-driven genes; hence it is unclear whether ENL can regulate the expression of non-classical, *e.g.* AP-1 regulated genes. Recently, the genome-wide effects of ENL and E_2 on gene expression were compared in MCF-7 cells. When the cells were treated with 1 μM ENL, the expression of 96 genes was significantly altered (5-fold difference, $p<0.01$). These genes were similarly affected by E_2, indicating that ENL and E_2 have overlapping target genes in MCF-7 cells (Dip *et al.*, 2008). The induction of only a subset of E_2–responsive genes in MCF-7 by ENL in the study of Dip *et al.* implies target gene selectivity. Promoter studies of the genes regulated by ENL could reveal elements required for the activity of ENL bound ER.

2.5.2.3 Effects of ENL on proliferation of estrogen-dependent cell lines

Several independent studies have shown that ENL stimulates the growth of estrogen-dependent cell lines. The cells most often used in the context of ENL-stimulated growth are human breast cancer MFC-7 and T-47D cells that express ERα endogenously. Welshons *et al.* were the first to show cell growth stimulation in response to ENL treatment: both MCF-7 and T-47D proliferation (measured as DNA content) increased after treatment with 1 μM and 10 μM ENL, respectively, and the effect was blocked with the antiestrogen tamoxifen (Welshons *et al.*, 1987). Inhibition of growth was observed with 100 μM ENL in both cell lines (Welshons *et al.*, 1987). Subsequently, Mousavi & Adlercreutz showed that 0.5–10 μM ENL stimulated the growth of MCF-7 cells (measured as DNA content), while a higher concentration (50 μM) inhibited the growth (Mousavi & Adlercreutz, 1992). In the same study, the authors reported that E_2 and ENL negated each other's proliferative effects in co-treatments (Mousavi & Adlercreutz, 1992). In yet another study, 1 μM ENL stimulated MCF-7 growth to the same extent as 0.1 nM E_2 (Sathyamoorthy *et al.*, 1994). Wang & Kurzer showed MCF-7 proliferation (DNA synthesis) with 10-50 μM ENL and inhibition of growth at higher concentrations (Wang & Kurzer, 1997). Later, the same authors reported that the ENL-induced MCF-7 growth was negated with tamoxifen, and that 10 μM ENL enhanced the growth stimulatory effects of 10 pM E_2 (Wang & Kurzer, 1998). When proliferation was measured as the number of viable cells instead of DNA concentration, 10 μM ENL significantly increased MCF-7 proliferation, and the effect was blocked by antiestrogens tamoxifen and ICI 182,780 (ICI) (Saarinen *et al.*, 2007). ENL had no effects on E_2-induced proliferation in co-treatments (Saarinen *et al.*, 2007). When proliferation was measured as cell cycle progression, differing results with ENL in MCF-7 were obtained. The fraction of cells in the S-phase was significantly increased already with pikomolar (pM) concentrations of ENL (Cosentino

et al., 2007). As in the proliferation studies, the effect was blocked with tamoxifen, suggesting an ER-mediated mechanism (Cosentino *et al.*, 2007). Additionally, treatment with 1 μM ENL significantly increased the antiapoptotic protein Bcl-2 and reduced the proapoptotic protein Bax levels, indicating proliferative effects. However, ENL was not an efficient cell cycle inducer, as the maximal effect remained at 25 % of that of E_2 (Cosentino *et al.*, 2007).

The results from the *in vitro* studies assessing ENL-ER interaction are summarized in Table 8. Clearly, proliferation assays and gene expression profiling suggest that ENL possesses estrogenic properties. However, the discrepancy between ligand binding assays, demonstrating very low affinity for the ERs, and the proliferation assays, showing significant changes at concentrations as low as the pikomolar range, remains obscure. Additionally, the *in vivo* studies with ENL suggest antiestrogenic activity (Table 7), which has clearly not been observed in the *in vitro* studies.

Table 8. Summary of ENL – ER interaction *in vitro*

Event	Assays	Result	Interpretation	References
ER binding	Ligand binding assay (fluorescein labelled E_2, human ERs)	• IC_{50} values for ERα and ERβ 6.7 μM and 39 μM, respectively	ENL is a poor ER ligand	(Mueller *et al.*, 2004)
ER transactivation	Induction of endogenous genes Gene expression profiling	• Increase in PgR and prolactin protein levels with 10 μM ENL in MCF-7 and primary culture of rat pituitary and uterine cells • Increase in pS2 mRNA level with 1 μM ENL in MCF-7 • Ninety-six E_2-regualted genes significantly regulated by 1 μM ENL in MCF-7	ENL has estrogen agonist effects on gene expression at μM concentrations ENL is an ER transactivator at μM concentrations	(Welshons *et al.*, 1987) (Sathyamoorthy *et al.*, 1994) (Dip *et al.*, 2008)
	Reporter gene assays	• Activation of 3xERE-Luciferase, C3-Luciferase, and ERE-ALP with >1 μM ENL in Ishikawa, HEK293 and MCF-7		(Saarinen *et al.*, 2000) (Mueller *et al.*, 2004) (Carreau *et al.*, 2008)
Proliferation	Proliferation of estrogen dependent cell lines	• Increased proliferation (DNA synthesis) in MCF-7 with 0.5-50 μM ENL • Increased number of viable MCF-7 cells with 10 μM ENL • Increased fraction of MCF-7 in the S-phase with 0.1 pM ENL • Increased proliferation (DNA synthesis) in T-47D with 1 μM ENL • Inhibition of proliferation (DNA synthesis) in MCF-7 with >50 μM ENL • Inhibition of E_2-induced proliferation in MCF-7 • Enhancement of E_2-induced proliferation in MCF-7 • No effect on E_2-induced proliferation in MCF-7	ENL has estrogen agonist effects on cell growth and proliferation at 0.1 pM - 50 μM concentrations High concentration of ENL (>50 μM) inhibits cell growth and proliferation No consistent results of antiestrogenic properties in proliferation assays	(Mousavi & Adlercreutz, 1992) (Sathyamoorthy *et al.*, 1994) (Wang & Kurzer, 1997) (Wang & Kurzer, 1998) (Cosentino *et al.*, 2007) (Saarinen *et al.*, 2007)

3 AIMS OF THE STUDY

At present, there is data suggesting that ENL may be an ER agonist *in vitro* (Table 8). However, *in vivo* models propose antiestrogenic activity for ENL (Table 7). Furthermore, in epidemiological studies, contradictory results have been presented about the involvement of ENL in breast cancer risk regulation. Given the complexity of ER-mediated signalling, it is possible that these discrepancies are due to the SERM-like activity of ENL. The aims of this study were to assess the SERM-like properties of ENL with reporter gene models *in vitro* and *in vivo,* and to compare the activity of purified ENL to the ingestion of dietary ENL precursors in order to evaluate the possibilities of predicting the effects of complex diets based on pure compound studies.

More specifically, the aims of this study were as follows:

1. To study the modulation of estrogen signalling by ENL

   - by using estrogen reporter gene assays *in vitro*
   - by assessing the ER binding properties of ENL in different binding assays
   - by studying tissue-specific estrogen-inducible reporter gene activation in ovariectomized 3xERE-Luciferase mice

2. To study the modulation of estrogen signalling by dietary sources of ENL

   - by testing the effects of flaxseed and rye bran on the uterus and mammary gland in rats
   - by studying the effects of flaxseed, rye bran, and wheat bran on the expression of the estrogen-inducible reporter gene and on uterine weight in ovariectomized 3xERE-Luciferase mice in the presence and absence of E_2

4 MATERIALS AND METHODS

4.1 *In vitro* assays (Study I)

4.1.1 Ligands and hormones

Racemic ENL was obtained from VTT (Technical Research Centre of Finland, Helsinki, Finland), E_2, 4-hydroxy-tamoxifen (4OHT) and dexamethasone (DEX) were purchased from Sigma (St. Louis, MO, USA), and DES from Acros Organics (Geel, Belgium). ICI was obtained from Astra Zeneca (Södertälje, Sweden). Ethanol (EtOH) (for ENL, E_2, ICI, and DEX) and dimethylsulfoxide (DMSO) (for 4OHT) were used as solvents.

4.1.2 Transactivation assays

The plasmids used in this study (pSG5-ERα, pSG5-ERβ, 3xERE-TATA-Luciferase, pCMV-β-Galactosidase, Gal4-ERα-LBD, Gal4-ERβ-LBD, 5xGal4-TATA-Luciferase, and p-EGFP-ERα) were kind gifts from Dr. Ingemar Pongratz and Dr. Anastasios Damdimopoulos. Stable 3xERE-Luciferase mouse mammary epithelial (HC11-ERE) cells and human cervix adenocarcinoma (HeLa) cells were routinely maintained in Dulbecco's RPMI 1640 medium supplemented with 10 % foetal bovine serum (FBS), 0.05 mg/ml gentamycin, 0.01 mg/ml insulin, and 0.01 μg/ml EGF, and in Dulbecco's DMEM with 10 % FBS, 2 mM L-glutamine, and 50 U/ml penicillin/streptomycin, respectively. Medium, penicillin/streptomycin, gentamycin, and FBS were purchased from Gibco (Paisley, Scotland), and insulin and EGF from Sigma (St. Louis, MO, USA). For reporter gene assays, cells were seeded 40 000 per well in 12-well plates, and used for transfection (HeLa), or for treatment (HC11-ERE), the following day. HeLa cells were transfected with 5 ng/well of pSG5-ERα or pSG5-ERβ expression vectors together with 200 ng 3xERE-TATA-Luciferase reporter gene, and 50 ng pCMV-β-Galactosidase, or with 10 ng/well of Gal4-ERα-LBD or Gal4-ERβ-LBD together with 100 ng 5xGal4-TATA-Luciferase and 50 ng pCMV-β-Galactosidase. Transfections were performed in medium without phenol red with Lipofectamine reagents (InVitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Ligands were added to cells four hours after transfection in fresh white medium containing 5 % charcoal-dextran stripped FBS (HyClone, Logan, Utah, USA) and antibiotics. All treatment mediums contained 0.1 % EtOH. For assays where 4OHT was used, a control containing 0.1 % DMSO was also used. Luciferase activity was measured after 48 hours' treatment using BioThema's Luciferase assay kit (Haninge, Sweden) and β-Galactosidase expression with Tropix's Galacto-Light Plus kit (PE Biosystems, Bedford, MA, USA) according to the manufacturer's instructions. Produced light was recorded with a Lucy 3 Microplate Luminometer (Anthos Labtech Instruments, Salzburg, Austria). Luciferase activity was normalized against β-Galactosidase expression (HeLa) or protein concentration measured with BioRad's Protein Assay (Hercules, CA, USA) using bovine serum albumin as standards (HC11-ERE). All the treatments were performed in triplicate in one assay, and each assay was performed three times.

4.1.3 Receptor binding assays

The radioligand displacement assay was performed at KaroBio (Huddinge, Sweden) following published methods (Barkhem *et al.*, 1998). The Reflectometric Interference Spectroscopy (RIfS) -based ER binding assay was performed at the University of Tübingen by Mr. Jan Jaehrling. For the RIfS-assay, cell culture supernatant from a routine transfection assay was collected and buffered with HEPES to pH 7.0. RIfS-transducer chips of 1 mm D 263 glass with layers of 10 nm Nb_2O_5 and 330 nm SiO_2 (Unaxis Balzers AG, Balzers, Liechtenstein) were first cleaned for functionalization in 1 M NaOH for 2 min, washed with tap water, and cleaned and mechanically dried with KIMTECH tissues (Kimberly-Clark, Reigate, UK). Then the transducer chips were treated with freshly prepared Piranha solution [mixture of 30% hydrogen peroxide and concentrated sulphuric acid at a ratio of 2:3 (v/v)] for 30 minutes in an ultrasonic bath. After rinsing with Milli-Q water and drying in a nitrogen stream, the surface was immediately activated for polymer functionalization by incubation with (3-glycidyloxylpropyl)trimethylsiloxan (GOPTS) (Fluka Chemie GmbH, Buchs, Switzerland) for 1 hour. The surface was subsequently cleaned with water-free acetone and dried in a nitrogen stream. RIfS transducers were modified with two layers of polymer [diaminopolyethylene glycol (mean molecular weight 2 kDa) (Rapp Polymere, Tübingen, Germany)] as previously described (Birkert *et al.*, 2000) and by subsequent immobilization of aminodextran (100 kDa) (Innovent, Jena, Germany) to reduce the non-specific binding of sample components to the sensor, and to provide binding sites for the DES-derivative. Carboxyl groups were introduced by letting the sensor react with glutaric anhydride (Sigma-Aldrich, Deisenhofen, Germany) at 70°C for 2 hours (Piehler *et al.*, 1996). DES was modified in a two-step synthesis to introduce a spacer termed by a primary amino group. This DES-derivative was covalently bound to the sensor using standard peptide chemistry to provide specific binding sites for ER. Before assembly into the flow cell, the transducers were rinsed with Milli-Q water and thoroughly dried in a nitrogen stream. Eighteen microlitres of each sample was pre-incubated with the same volume of an ERα solution (PanVera, Inc., Madison, WI, USA) (final concentration 200nM) in HEPES pH 7.4 for 90 minutes at room temperature and then stored on ice until measurement. The sample was guided to the sensor using a syringe driven flow system (Hamilton, Bonaduz, Switzerland) optimized for small sample volumes. The duration of a single measurement was 865 seconds. As a reference, medium (DMEM with EtOH, no incubation with cells) without pre-incubation with ERα was used to check for non-specific binding of the sample to the sensor. The resulting signal was used to correct the other binding curves to obtain only specific ERα binding signals. Maximum binding was determined and inhibition factors (%) calculated as:

$$Inhibition(\%) = \frac{N - S}{N - P} * 100$$

where N is the signal of the blank sample (medium containing EtOH, negative control) which was set to 0% inhibition (full binding), and P the signal of medium containing E_2 which was set to 100% inhibition (media not incubated with cells), and S the ER binding signal of the sample measured.

4.1.4 Receptor mobility and sub-nuclear localization assays

For the Fluorescence Recovery After Photobleaching (FRAP) assay, HeLa cells were seeded in 35 mm plates and transfected with 250 ng p-EGFP-ERα using Lipofectamine reagents (InVitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, cells were treated overnight with the ligands and used for FRAP assay using Leica's laser scanning confocal microscope (Heidelberg, Germany). For imaging and bleaching, the GFP tags were excited using the 488 nm laser line of an Ar-Kr laser and the emitted light was captured between 500 and 550 nm. From each plate 10 cells were selected, and an area less than 10 % of the nucleus was point-bleached for 4 seconds with full laser power. In order to avoid artefacts, cells expressing high levels of GFP were excluded. Images were recorded after the bleaching every 0.5 second for a total of 20 seconds. Fluorescence recovery curves were created for the bleached point, and for an unbleached area in all nuclei. The general drop in fluorescence observed due to the bleaching was monitored with the help of background quantification, and it was used to normalize the fluorescence recovery curves. The assay was repeated three times.

For the sub-nuclear localization assay, HeLa cells were grown on glass cover slips, treated with poly-L-lysin (Sigma, St. Louis, MO, USA), in 6-well plates, and transfected as described above. Following overnight treatment with ligands, the cells were fixed with 3.7 % paraformaldehyde, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions, and glass plates were mounted to object glasses with FluorSave Reagent (Calbiochem, Darmstadt, Germany). The results were viewed with Leica's AS MDW multi dimensional imaging workstation (Wetzlar, Germany) equipped with CoolSNAP HQ CCD camera (Roper Scientific, Duluth, GA, USA). The filter used for excitation of the GFP tag was 495 nm (bandwidth 12) and emission was collected at 530 nm (bandwidth 30). For DAPI a 420 nm (bandwidth 30) filter was used for excitation and emission was collected at 465 nm (bandwidth 20). Z-stacks of the cells' nuclei were created by capturing images at 0.3 μm intervals throughout the nuclei. Image restoration of the z-stacks was achieved by the 3D blind deconvolution procedure using the AutoQuant program (Troy, NY, USA).

4.2 *In vivo* studies (Studies I, II, III)

The rodent studies were performed at animal facilities of the University of Turku, Karolinska Institutet (Huddinge, Sweden) and Georgetown University (Washington DC, USA). The study protocols were approved by local animal authorities [licence numbers 1444/04, 1592/04 (University of Turku), S-109-07 (Karolinska Institutet), and 06-096 (Georgetown University)]. In all studies, animals were housed under standard conditions of the respective animal departments with free access to feed and tap water throughout the experiments.

4.2.1 Experimental animals (Studies I, II, III)

In Study I, 40 homozygous 3xERE-Luciferase reporter mouse females (Lemmen *et al.*, 2004a) were produced at the animal department of Karolinska Institutet through homozygote mating. In Study II, female Sprague-Dawley rats were obtained from Charles River Laboratories on day 7 of gestation, and immature female rats were produced at the University of Turku animal department. In Study III, homozygous 3xERE-Luciferase reporter males (Lemmen *et al.*, 2004a) were mated with wild type C57Bl females (Harlan, Horst, the Netherlands) at the animal department of the University of Turku, and heterozygous female offspring were used in the studies.

4.2.2 Diets (Studies I, II, III)

4.2.2.1 Composition of experimental diets (Studies I, II, III)

In all rodent studies, semisynthetic basal diets were used in order to minimize exposure to plant fibre and soy protein, sources of lignans and isoflavones, commonly used in open formula rodent chows. In Study I, C1000 basal diet (Altromin, Lage, Germany) was used. In Study II, in experiments focusing on developmental exposure, AIN93G basal diet (Harlan Teklad, Madison, WI, USA) supplemented with 0 %, 5 %, or 10 % flaxseed was used. The supplemented diets were corrected for the main nutrients to correspond to the basal diet. In the immature rat uterotrophic assay C1000 basal diet (Altromin, Lage, Germany) and basal diet supplemented with 5 % defatted flaxseed flour or 5 % rye bran were used. In Study III, AIN93M basal diet (SDS, Whitham, UK), or basal diets supplemented with rye bran (11 %), wheat bran (9.9 %), flaxseed (14 %), or soy granules (22 %) were used. The supplemented diets were corrected for the main nutrients to correspond to the composition of the basal diet. Flaxseed used in Study II was obtained from BulkFoods (http://www.bulkfoods.com) and defatted flaxseed flour from MTT Agrifood Finland (Jokioinen, Finland). Crushed flaxseed, rye bran (Studies II and III), wheat bran, and soy granules used in Study III were bought from a local supermarket in Turku, Finland.

The compositions of the basal diets, as reported by the manufacturers, were the following: 54 g/kg sunflower oil, 173 g/kg casein, 31 g/kg cellulose, 110 g/kg sucrose, and 456 g/kg cornstarch in the C1000 diet; 70 g/kg corn oil, 200 g/kg casein, 50 g/kg cellulose, 100 g/kg sucrose, 132 g/kg maltodextrin, and 398 g/kg cornstarch in the AIN93G diet; and 40 g/kg rape seed oil, 140 g/kg casein, 50 g/kg cellulose, 100 g/kg sucrose, 156 g/kg maltodextrin, and 466 g/kg cornstarch in the AIN93M diet.

4.2.2.2 Analysis of lignans, isoflavones, and cadmium in diets (Studies II, III)

The concentrations of isoflavones (GEN, daidzein, genistin, daidzin, glycitein) and lignans (MR, SECO, PINO, LAR, SYR) in the experimental diets used in Study III were analysed by Dr. Tarja Nurmi at the University of Kuopio. Both isoflavones and plant lignans were determined in all diets. Isoflavones were analysed as described earlier (Penalvo *et al.*, 2004b), and plant lignans with a modification of the method previously published by Penalvo *et al.* (Penalvo *et al.*, 2005a). Briefly, samples were

hydrolysed as described earlier, but instead of solid phase extraction, samples were extracted twice with diethyl ether. Combined extracts were evaporated and samples were purified with QAE-Sephadex A-25 in acetate form. Instead of GC-MS applied in the original method, samples were analysed with the HPLC coupled to coulometric electrode array detector (CEAD) using the chromatographic conditions described earlier for urinary lignans (Nurmi *et al.*, 2003). Recovery of the analytes was determined with a standard mixture treated like the samples. Recovery values ranged from 57% (MR) to 100 % (PINO), which were comparable to earlier published values (Penalvo *et al.*, 2005a).

The concentration of cadmium was analysed in the diets used in Study II and in the cereals, soy, and flaxseed used in Study III using graphite furnace atomic absorption spectrometry (GF-AAS) (Solaar M6 Dual Zeeman AAS Spectrometer, Thermo Electron Spectroscopy Ltd., Cambridge, England). The samples (0.3 g) were digested in duplicates into ultrapure NHO_3 (Fluka Chemie Gmbh, Buchs, Schwitzerland) and H_2O_2 (J.T. Baker, Deventer, Holland) (5/2 vol/vol) in closed vessels using a microwave oven. In the analysis, palladium (0,5 g/l) was used as a matrix modifier, and the concentration of cadmium in the samples was determined with the standard addition method. Commercial reference material (lyophilized poplar leaves) handled in a similar manner was used as a quality control, and samples with only HNO_3 and H_2O_2 were used as blanks.

4.2.3 Optimization of the *in vivo* imaging protocol

An *in vivo* imaging protocol was set up and optimized for Study III. Imaging was conducted with Xenogen IVIS50 Imaging System equipped with an XGI-8 Gas Anaesthesia System (Caliper Life Sciences, Runcorn, UK). The two optimized parameters were 1) the dose of the Luciferase substrate D-Luciferin and 2) the time between the administration of D-Luciferin and imaging. In the literature, a commonly used dose of D-Luciferin in estrogen reporter mice is 100-200 mg/kg and 10-15 minutes is the usual delay between substrate administration and imaging (Ciana *et al.*, 2003; Lemmen *et al.*, 2004a; Lemmen *et al.*, 2004b). Adult intact male mice (n=8) were injected with 1 mg/kg (*i.p.*) E_2-dipropionate (EP) (Sigma, St. Louis, MO, USA), and imaged 15 hours post injection. Four animals received 100 mg/kg D-Luciferin (Synchem, Felsberg/Altenburg, Germany) and four 25 mg/kg (*s.c.*). Images were taken at 0, 3, 6, 9, 12, 15 minutes after D-Luciferin injection. Additionally, images were taken at approximately 45 minutes, 2 hours, and 12 hours post D-Luciferin administration to determine the persistence of the substrate in mice. Based on the pilot study, 25 mg/kg and 3 minutes were chosen as suitable parameters to be used in future studies.

4.2.4 Experimental protocols (Studies I, II, III)

4.2.4.1 Effects of ENL on reporter gene activity in 3xERE-Luciferase mice (Study I)

Forty adult female reporter mice were ovariectomized and allowed to recover for two weeks at the animal department of Karolinska Institutet (Huddinge, Sweden). During the recovery period, mice consumed a semisynthetic basal diet. Mice were randomized into five treatment groups (n=8 in each). Two groups were injected *i.p.* with 1 mg/kg EP (Sigma, St. Louis, MO, USA) and two groups with 10 mg/kg ENL (VTT, Helsinki, Finland). One group received vehicle only (rape seed oil). Mammary glands, uteri, vaginas, and bones (*tibia*) were collected 12 and 24 hours post injection for Luciferase analysis, and control tissues were collected at 12 hours only. Tissues were snap frozen in liquid nitrogen and stored at -86 °C. One uterine horn from each animal was collected in formalin and stored in EtOH.

4.2.4.2 Effects of dietary lignan sources on rat uterus and mammary gland (Study II)

The effects of flaxseed and rye bran on immature rat uterus and mammary gland were tested at the animal department of the University of Turku. Immature female pups were weaned on PND19 and randomised to four treatment groups (n=8 in each). On weaning, rats in the positive and negative control groups received a semisynthetic basal diet while rats in the two other groups received the basal diet supplemented with 5 % rye bran or 5 % defatted flaxseed flour. Positive control rats received a daily injection of E_2 (1 mg/kg *s.c.*) (Sigma, St. Louis, MO, USA), while other rats received vehicle only (rape seed oil). The treatment period was continued for one week (PND19-25), and weight gain and food consumption were measured daily. On PND26, rats were sacrificed by carbon dioxide suffocation and neck dislocation. The serum was collected and stored at -20 °C for lignan analysis. The uteri were carefully blotted, weighed, collected in formalin, and stored in EtOH. The liver and kidneys were weighed and stored at -20 °C for cadmium analysis. The abdominal mammary glands were dissected and processed into whole mounts.

The effect of developmental flaxseed exposure on mammary gland morphology and tumorigenesis was tested by Professor Leena Hilakivi-Clarke *et al.* at the animal department of the Georgetown University (Washington DC, USA). Pregnant rats received a semisynthetic basal diet (n=21) or the basal diet supplemented with 0 %, 5 %, or 10 % flaxseed (n=7 in each) from gestational day 7 on. The rats were kept on the flaxseed diets until they gave birth, and at that point switched to the basal diet. Two days after the offspring were born, all female pups were pooled and randomly assigned to be housed with a dam that was fed the same diet during pregnancy as the pups. Each dam had a total of 10 female pups. The pups were weaned on PND25. The dams kept on basal diet throughout pregnancy were divided to three groups of three lactating dams each on post-delivery day 5, and fed either 0 %, 5 %, or 10 % flaxseed diets. Dams and pups were kept on these diets until post-delivery day 25, when the pups were weaned. In both exposure setups, mammary glands were dissected from 8-week old pups and processed into whole mounts and paraffin sections (n=4-5 in each group).

The susceptibility to mammary tumorigenesis was evaluated by gavaging 50-day old rats, exposed to flaxseed *in utero* or during lactation, with 10 mg DMBA (n=22-25 in each group). Tumour development was followed by palpations once a week.

4.2.4.3 Effects of dietary lignan sources on reporter gene activity in 3xERE-Luciferase mice (Study III)

One hundred adult female reporter mice were ovariectomized and allowed to recover for two weeks at the animal department of the University of Turku. During the recovery period, mice consumed a semisynthetic basal diet. After the recovery period, mice were randomized to five dietary groups: basal diet (n=23), soy diet (n=19), flax diet (n=19), rye diet (n=19) and wheat diet (n=20). Samples were collected 40 hours (acute exposure) and 14 days (sub-chronic exposure) post diet change (n=6-7 in each group). The remaining mice were injected with E_2 (50 μg/kg *i.p.*) (Sigma, St. Louis, MO, USA), except for four animals in the basal diet group that received vehicle only (10% DMSO in rape seed oil). Induction of the reporter gene was followed with the optimized *in vivo* imaging protocol described above (chapter 4.2.3). Mice were imaged at 4, 8, 12, 16, 22 and 28 hours (±10 minutes) post ligand injection. Additionally, a background image was taken from all mice prior to the E_2 injection. An area over the upper abdomen, where strongest response to E_2 treatment was observed, was quantified with LivingImage 4.09A Carbon software (Xenogen, Lake Oswego, Oregon, USA). Additionally, the time point of maximal induction was determined for each mouse. In each setting (acute, sub-chronic, and post E_2), tissues [brain, pituitary gland, mammary gland, bone (*tibia*), muscle (*quadriceps*), liver, uterus, and vagina] were collected for Luciferase analysis and serum was drawn for lignan and isoflavone analysis. In addition, the weight of the uterus, liver, and kidneys were recorded at sacrifice. The tissues were snap frozen in liquid nitrogen and stored at -86 °C, and the serum was stored at -20 °C. One uterine horn from each mouse was collected in formalin and stored in EtOH. Food consumption and body weight gain were recorded once a week throughout the experiment.

4.2.5 *Ex vivo* analyses (Studies I, II, III)

4.2.5.1 Luciferase activity in tissue samples (Studies I, III)

Frozen tissue samples were homogenized under liquid nitrogen, dissolved in sample buffer (25 mM Tris acetate pH 7.8, 1.5 mM ethylenediaminetetraacetic acid, 10 % glycerol, 1 % triton X-100) containing freshly added 2 mM dithiothreitol and Complete Mini proteinase inhibitor tablets (Roche Diagnostics GmbH, Penzberg, Germany) and centrifuged. Supernatant was used for Luciferase activity measurement using BioThema's Luciferase assay kit (Haninge, Sweden) according to the manufacturer's instructions. Luminescence was recorded with a Victor^2 Multilabel Counter (PerkinElmer, Turku, Finland) and results were corrected for protein concentration in the samples, measured with BioRad's Protein Assay (Hercules, CA, USA) using bovine serum albumin standards. Background noise originating from the Victor^2 Multilabel Counter detector was measured by reading each plate once before the

luminescence reaction was started. Average background noise was subtracted from the sample readings before the results were corrected for protein concentration. Hence, the results were calculated as:

$$RLU = \frac{CPS(sample) - CPS(noise)}{C(protein)}$$

where RLU stands for relative light units, CPS for counts per second, and C for concentration. Negative values were considered as no detectable activity and assigned a value of 0.

4.2.5.2 Morphology of mammary gland and uterus (Studies I, II, III)

In Study II, abdominal mammary glands were collected and processed into whole mounts for morphological evaluation. The whole mounts were evaluated for the amount and size of TEBs; total number of TEBs was calculated and the size of 10 TEBs per mammary gland was measured with ImageJ (http://rsb.info.nih.gov/ij/) from photographs taken with an Olympus DP70 Digital Camera System. In studies I, II and III, a piece of uterine horn was collected. The uterine horns were embedded in paraffin and processed into hematoxylin&eosin (H&E) stained tissue sections. Sections were viewed under an Olympus BX51 Microscope, and images were taken with an Olympus DP70 Digital Camera System. In all studies, sections were evaluated visually for markers of estrogenicity (epithelial thickening, mitotic figures, stromal oedema, and immune cell infiltration). Additionally, stromal oedema was scored in Study I from non-existent to profound, and epithelial height was measured in Study III with ImageJ (http://rsb.info.nih.gov/ij/).

4.2.5.3 Immunohistochemical stainings (Studies I, II)

In Study I, expression of Luciferase, CyclinD1 and Ki67 was monitored with immunohistochemical stainings. Uterine horns were processed into 5 μm thick tissue sections, deparaffinized in UltraClear (J.T. Baker, Deventer, Holland) and EtOH, and antigens were retrieved with microwave oven treatment in citric acid buffer pH 6.0. Endogenous peroxidase activity was blocked with H_2O_2. The primary antibodies were polyclonal rabbit anti-Luciferase (Sigma, St. Louis, MO, USA), monoclonal rabbit anti-CyclinD1 (NeoMarkers, Fremont, CA, USA), and polyclonal rat anti-Ki67 (DAKO, Glostrup, Denmark). Prior to the addition of anti-Ki67 antibodies the sections were blocked for 1 hour in 10 % rabbit serum. No blocking was used for Luciferase and CyclinD1 stainings. After overnight incubation with the primary antibody solutions at +4 °C, the slides were washed with phosphate buffered saline (PBS) and secondary antibodies were added. For the Luciferase and CyclinD1 stainings DakoCytomation's rabbit EnVision System-HRP kit was used for secondary antibodies, and for the Ki67 staining peroxidase conjugated rabbit anti-rat antibodies (Rockland, Gilbertsville, PA, USA) were used. The antibody complexes were visualized with diaminobenzidine (DAB) (rabbit EnVision System-HRP, DakoCytomation, Carpinteria, CA, USA). The background was stained with Mayer's hematoxylin, sections were dehydrated with EtOH and UltraClear, and mounted with

UltraKitt (J.T. Baker, Deventer, Holland). The Luciferase stainings were quantified by scoring the response to four categories based on the extent of the staining. CyclinD1 and Ki67 stainings were quantified by calculating the number of the positive cells in stroma and on luminal and glandular epithelia. In Study II, expression of proliferating cell nuclear antigen (PCNA), ERα and ERβ in mammary gland sections was determined. The primary antibodies were polyclonal goat anti-PCNA, polyclonal rabbit anti-ERα and polyclonal rabbit anti-ERβ (all from Vector Laboratories, Burlingame, CA, USA). Stainings were quantified by counting the positive cells (PCNA and ERβ) or by scoring (ERα). The percentage of apoptotic nuclei was determined by counting number of positive cells after staining with the *in situ* apoptosis ApopTag Peroxidase detection kit (Serological Corporation, Toronto, ON, Canada).

4.2.5.4 Serum lignan and isoflavone analyses (Studies II, III)

Serum was collected for lignan analysis in Study II from immature rats, and for lignan and isoflavone analysis in Study III from adult mice. Serum samples from Study II were analyzed by Dr. Annika Smeds at Åbo Akademi. The analysis was performed as described previously for rat urine and human serum (Smeds *et al.*, 2004; Smeds *et al.*, 2006). Briefly, 100 µl of serum samples were enzymatically hydrolyzed overnight at +37 °C with filtered *H. pomatia*, after which internal standards, d_6-ENL and d_6-MR, were added. Quality controls and standard samples containing ENL, END and SECO were prepared from pure reference compounds. After solid phase extraction at pH 4.0 with Oasis HLB Extraction Cartridges (Waters, Milford, USA) samples were evaporated to dryness under N_2-flow, reconstituted in methanol (MeOH)/0.1% acetic acid 20/80 (v/v) and analyzed with HPLC-MS/MS as described (Smeds *et al.*, 2006). Serum samples from Study III were analyzed by Dr. Tarja Nurmi at the University of Kuopio. Three serum samples per group from acute and sub-chronic time points were selected for the analysis. Due to the limited volume of serum samples, lignans were analysed only from mice on the wheat, rye and flax diets, and isoflavones only from mice on the soy diet. Both lignans and isoflavones were analysed from the control mouse serum. The samples were prepared for the analyses as described earlier (Nurmi & Adlercreutz, 1999; Penalvo *et al.*, 2004c) with slight modifications. Briefly, 200 µl of serum samples were enzymatically hydrolysed overnight at +37 °C with four volumes of 0.2 M sodium acetate buffer pH 5 containing 0.2 U/ml of β-glucuronidase and 2 U/ml of sulphatase. Cool samples were extracted twice with 4 mL of diethyl ether and combined extracts were evaporated under N_2-flow. Isoflavones and their metabolites were measured from samples dissolved in the original volume of 70 % MeOH. Samples from the control groups were first dissolved in the original volume of MeOH. Then half of the volume was taken for further purification carried out prior to lignan analysis, and the other half was evaporated under N_2-flow again, and finally dissolved in the half of the original volume of 70 % MeOH for isoflavone analysis. Samples for lignan analysis were purified with QAE-Sephadex A-25 in acetate form as described earlier by Penalvo *et al.* (Penalvo *et al.*, 2004c). Quality controls and standard samples containing daidzain, equol, *O*-desmethylangolensin (synthesized by Professor Hase *et al.*, Laboratory of Organic Chemistry, University of Helsinki), GEN and glycitein (Nutrilab, Giessen, Netherlands), or SECO, MR, LAR, PINO, SYR

(ArboNova, Turku, Finland), END and ENL (VTT Technical Research Centre, Espoo, Finland) were prepared from pure reference compounds. Analyses were performed with HPLC-CEAD. The chromatographic conditions for isoflavones and their metabolites (Penalvo *et al.*, 2004c) and for lignans and their metabolites (Nurmi *et al.*, 2003) have been described previously.

4.2.5.5 Measurement of cadmium in tissues (Study II)

In Study II, uptake of cadmium from the diets was determined by measuring the concentration in the liver and kidneys, two major sites of cadmium storage in the body. One whole kidney and a piece of liver (0.3 g) were digested and analyzed as described above (4.2.2.2) by Dr. Teemu Halttunen at the University of Turku. Instead of poplar leaves, dog fish muscle was used as a quality control.

4.2.6 Statistical analysis (Studies I, II, III)

Normally distributed data (organ weights, body weights, food consumption, cadmium exposure, cadmium concentration in organs, number and area of TEBs, ERα, ERβ and PCNA staining, tumour multiplicity) were analysed with one-way or two-way ANOVA followed by Fisher LSD (Study II) or Tukey's (Study III) *post hoc* tests. *In vivo* imaging data (Study III) was Log-transformed and analysed with two-way ANOVA and Tukey's test. Unparametrical data (Luciferase activity in tissue homogenates, lignan concentration in serum, expression of Ki67 and CyclinD1, tumour latency) were analysed with Kruskall-Wallis ANOVA followed by pair-wise comparisons of treatment groups to the control group with Mann-Whitney *U*-test or Dunn's test, and p-values were corrected for multiple comparisons with Bonferroni correction (Studies I-III). The difference in the time point of maximal Luciferase induction was analyzed with the Chi Square test (Study III). Results were considered statistically significant if $p<0.05$.

5 RESULTS

5.1 *In vitro* assays (Study I)

5.1.1 Transactivation of ERs

The effect of ENL on ER transcriptional activation was tested in two cell lines using the 3xERE-Luciferase reporter gene. In stably transfected HC11-ERE cells, expressing ERα and ERβ endogenously, ENL dose-dependently increased the expression of Luciferase, starting at 1 μM concentration (Study I). In HeLa cells, transiently transfected with either ERα or ERβ and the 3xERE-Luciferase reporter gene, ENL dose-dependently activated reporter gene expression through both ER isoforms (Figure 6). Interestingly, activation through ERα occurred at a lower concentration (100 nM) than through ERβ (1 μM) (Figure 6), suggesting that ENL is selective towards ERα. Increased Luciferase expression was only observed in the presence of transfected ERs, and the induced activity was repressed to basal level with antiestrogens 4OHT and ICI, suggesting an ER-mediated mechanism (Figure 6). The role of LBD in ENL induced ER transactivation was studied with ER-LBD-Gal4 constructs, and a similar response to the full length receptors was obtained (Study I). These assays suggest that ENL activates ERs by directly binding to the LBD of the receptors.

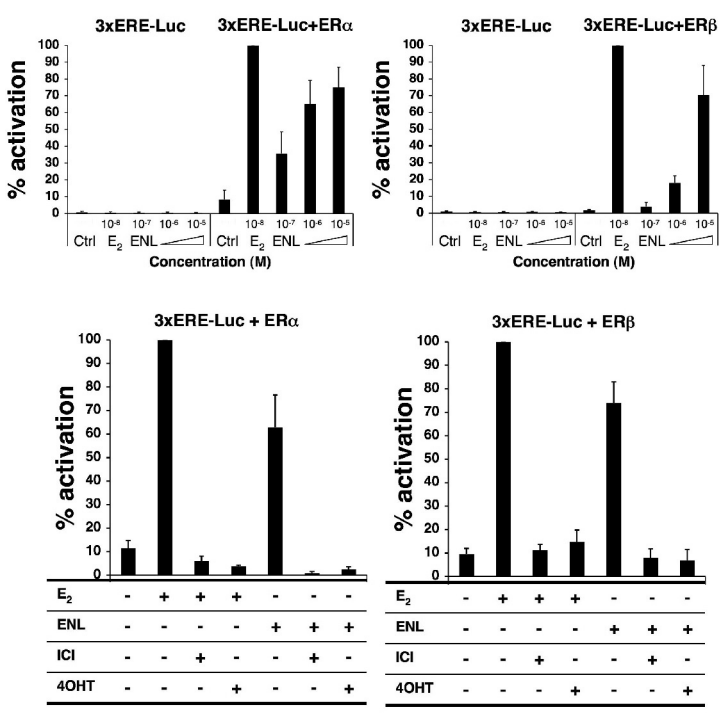


Figure 6. Transactivation of ERs in transiently transfected HeLa cells. HeLa were transfected with expression vectors for either ERα or ERβ together with the 3xERE-Luciferase (3xERE-Luc) reporter gene, and treated with increasing concentrations of ENL. Dose-dependent activation occurred through both receptors; however, activation through ERα was achieved at a lower concentration (100 nM) than through ERβ (1 μM) (upper panel). The Luciferase induction was blocked by the antiestrogens 4OHT and ICI (lower panel).

5.1.2 Ligand binding assays

The affinity of ENL towards ERα and ERβ was tested in a conventional ligand binding assay based on displacement of radiolabelled E_2 from ERs. The affinity of ENL towards both receptors was poor [IC_{50}(ERα)=130 μM, IC_{50}(ERβ)=99 μM] (Study I). The disagreement between ER transactivation data (Figure 6) and the binding affinity data suggests that the cell context is needed for the estrogenic activity of ENL. To measure this, a novel ligand binding assay was set up. In the RIfS-based assay, the binding affinity of crude cell culture medium towards ERα was measured after incubation with HeLa or after similar incubation in the absence of cells (Figure 7). As controls, medium without ligands, medium with E_2, and medium with DEX were used. The medium without ligands and medium containing DEX did not display binding affinity towards ERα (Figure 7). While the presence of HeLa did not alter the ERα binding activity of medium containing E_2, the ERα binding activity of medium containing ENL was enhanced by incubation with HeLa (Figure 7). These results suggest that ENL gains higher ERα binding activity in cells, perhaps due to conversion to higher binding activity compound(s).

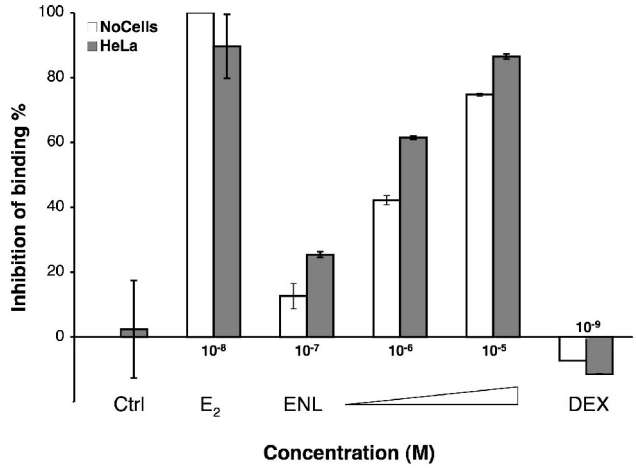


Figure 7. The binding affinity of cell culture medium towards ERα. Cell culture media incubated for 48 hours in the presence or absence of HeLa were tested for ERα binding in a RIfS-based binding assay. While the binding affinity of medium containing E_2 was not altered by the presence of HeLa, the affinity of medium containing ENL was increased by the incubation with HeLa. Cell culture medium with no added ligands (Ctrl) or medium containing dexamethasone (DEX), a ligand for the glucocorticoid receptor, were used as controls.

5.1.3 ERα mobility and sub-nuclear localization

The binding assays suggest that the binding affinity of ENL towards ERα is greater in a cell-based environment compared to cell-free systems. In order to further confirm interaction between ERα and ENL in a cell-based environment, receptor mobility and translocation assays were performed. In the presence of ligands, the mobility of ERα is slowed down and the receptor translocates into specific speckle-like structures in the cell nucleus (Htun *et al.*, 1999; Stenoien *et al.*, 2001; Damdimopoulos *et al.*, 2008). HeLa were transfected with ERα-GFP and treated overnight with ENL. Antiestrogen ICI was used as a control, since it is reported to irreversibly immobilize ERα (Stenoien *et al.*, 2001; Damdimopoulos *et al.*, 2008). ENL treatment slowed down the mobility of ERα-GFP to the same extent as E_2, assessed with a FRAP-assay (Figure 8 upper

panel). In line with this, ERα-GFP was re-organized into speckle-like structures within the nucleus after treatment with ENL and E_2 (Figure 8 lower panel). These results add further proof to a direct interaction between ENL and ERα.

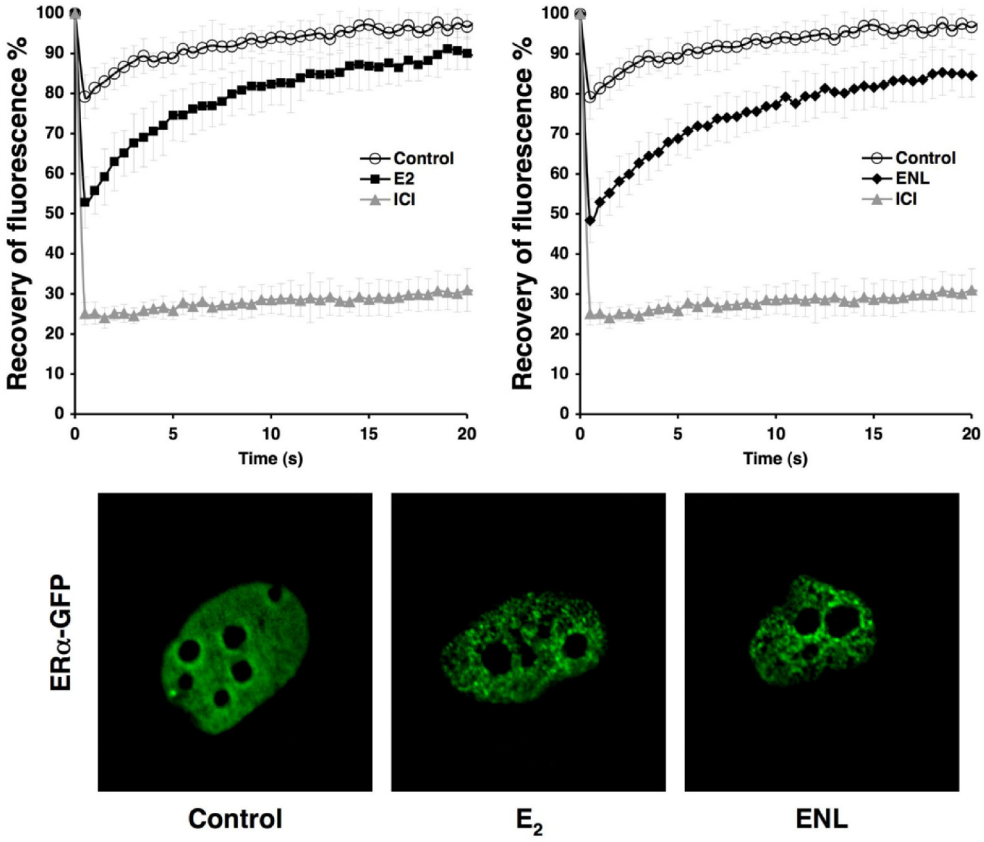


Figure 8. ERα-GFP mobility and sub-nuclear localization. HeLa cells were transfected with ERα-GFP and treated overnight with ENL, E_2, ICI, or vehicle only (control). Mobility of the receptor was slowed down to the same extent with ENL and E_2 (upper panel). The localization of ERα-GFP in the nucleus was homogenous in the absence of a ligand (control) and speckle-like after treatment with E_2 and ENL (lower panel).

5.2 *In vivo* assays (Studies I, II, III)

5.2.1 Effects of ENL in 3xERE-Luciferase reporter mice (Study I)

5.2.1.1 Activation of reporter gene

To study ER-mediated transactivation by ENL *in vivo*, adult ovariectomized reporter mice were administered a single dose of ENL (10 mg/kg) or EP (1 mg/kg) and tissues were sampled 12 and 24 hours later. Reporter gene response was measured in the abdominal mammary gland, uterus, vagina, and bone (*tibia*), and compared to the

response in untreated mice. Significant activation of the reporter gene in all collected tissues at both measured time points was observed after treatment with EP (Figure 9). In contrast, ENL induced significant Luciferase expression in the uterus and vagina but had no effect on the mammary gland and bone (Figure 9). The response evoked by ENL at 12 hours in the uterus was comparable to EP, suggesting that ENL might possess significant estrogenic activity in this tissue type (Figure 9). The reporter gene response in the uterus was further confirmed with immunohistochemical staining of Luciferase (Study I).

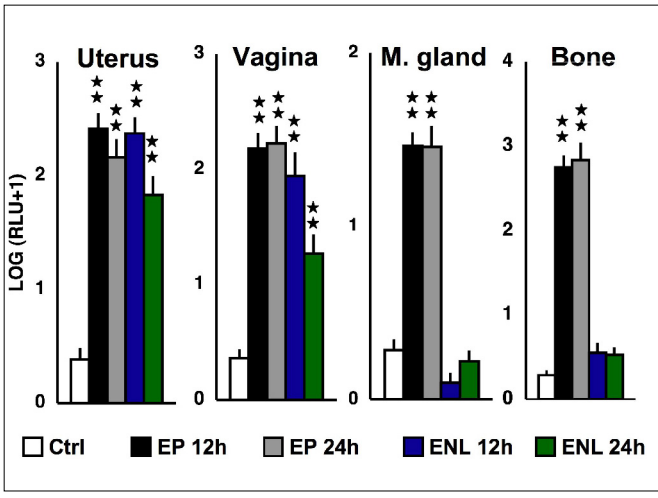


Figure 9. Luciferase response in selected tissues. The effects of ENL (10 mg/kg) and EP (1 mg/kg) on the Luciferase expression in the uterus, vagina, mammary gland and bone in ovariectomized 3xERE-Luciferase reporter mice 12 and 24 hours post exposure. ENL induced Luciferase expression in the uterus and vagina but not in the mammary gland or bone. The bars are the average of Log transformed activity (RLU) +SEM. The stars indicate significant difference to the control (Ctrl) mice evaluated with Kruskall-Wallis ANOVA and Mann-Whitney *U*-test. (**) p<0.01.

5.2.1.2 Uterine morphology

Due to the strong reporter gene response in the uterus after ENL treatment, the uteri were examined further for indications of estrogen exposure. Stromal oedema, mitotic figures, and epithelial height were monitored and graded visually on H&E-stained tissue sections. Control uteri showed no signs of oedema, the luminal epithelium was flat, and mitotic figures were absent (Study I). Treatment with EP resulted in classical estrogenic effects: at 12 hours stromal oedema was profound in 75 % of the uteri and moderate in the remainder of the samples, and at 24 hours the oedema was accompanied by mitotic figures on the luminal and glandular epithelium and the epithelial height was increased (Study I). A modest response was observed in the ENL treated mice. After 12 hours, 29 % of the animals displayed moderate stromal oedema and the rest of the samples showed no signs of oedema (Study I). At 24 hours, no oedema, mitotic figures, or epithelial thickening were detected in the ENL group, and the uteri resembled those of the control group (Study I). These results suggest that ENL can promote endogenous estrogen responses in the mouse uterus. However, compared to EP, these morphological responses are modest, short lived, and limited to stromal oedema.

5.2.1.3 Expression of CyclinD1 and Ki67

The effect of ENL on the uterus was further characterized with immunohistochemical stainings for two estrogen responsive genes, CyclinD1 (a direct target gene) and Ki67 (a proliferation marker gene). The stainings were quantified by calculating the number of positive nuclei on the luminal epithelium, glandular epithelium and in the stroma. Treatment with EP significantly increased the amount of CyclinD1 positive cells at both time points on the luminal epithelium and in the stroma, and after 24 hours on the glandular epithelium (Study I). In line with this, EP treatment significantly increased Ki67 staining at 24 hours post treatment on the luminal epithelium and in the stroma, but had no effects on the glands (Study I). The effect of ENL on CyclinD1 expression was very similar to EP, the only difference being on the luminal epithelium, where ENL only had effects 24 hours post treatment (Study I). Surprisingly, the effect of ENL on Ki67 staining was very different from EP. While EP increased Ki67 expression on the luminal epithelium and in the stroma, ENL significantly induced expression of Ki67 only on the glandular epithelium (Study I). These results indicate the ENL can induce expression of endogenous estrogen marker genes in mouse uterus. Furthermore, the activity of ENL is cell type -specific, as induction of Ki67 only occurs on the glandular epithelium. Additionally, these data indicate that the reporter gene is a sensitive marker of estrogen activity, as it also reflects changes in endogenous gene expression.

5.2.2 Effects of dietary sources of ENL on the uterus and mammary gland in rats (Study II)

5.2.2.1 Immature rat uterotrophic test

The estrogenic responses evoked by ENL in reporter mouse uteri suggest estrogen agonist activity for ENL in this organ. Normally, ENL is not consumed as a pure compound but rather in the form of precursors in the diet. The effect of two dietary lignan sources, flaxseed and rye bran, were therefore tested in an immature rat uterus bioassay. While rye is an important source of lignans in the human diet, flaxseed is the richest known source of lignans (Valsta *et al.*, 2003; Thompson *et al.*, 2006). In addition to lignans, flaxseed contains cadmium (Angelova *et al.*, 2004; Hocking & McLaughlin, 2006), a heavy metal with reported estrogen effects on the uterus and mammary gland (Johnson *et al.*, 2003). Thus, flaxseed is a source of a mixture of compounds that are suggested promote estrogen effects *in vivo* based on pure compound studies (Johnson *et al.*, 2003). Immature female rats were kept on a basal diet supplemented with 5 % defatted flaxseed or 5 % rye bran for seven days (PND18-PND26). Control mice were kept on a basal diet, and half of the control animals received a daily injection of E_2. The morphology of the uterus and mammary gland was assessed on PND27. Food consumption and body weight gain did not differ between the experimental groups (Study II). The absolute weights of the liver and kidneys were not altered by the treatments; however, the relative weight of kidneys was higher in the E_2 group compared to control (Study II). E_2 treatment (1 mg/kg/d) resulted in typical changes in the morphology of the uterus and mammary gland; uteri

were heavier, showed pronounced thickening of the epithelia and infiltration of immune cells, and the mammary glands had fewer but larger TEBs than the control (Figure 10, Study II). Flax and rye diets did not affect the weight of the uterus, nor did the gross morphology of the uteri and mammary glands in the flax and rye groups differ from mice on the basal diet (Figure 10, Study II). The concentrations of cadmium in the flax, rye, and basal diets were 45.6, 8.2, and 6.7 µg/kg, respectively. The calculated average exposure to cadmium through diet was 42.4, 7.7, and 6.0 µg/kg/week in the flax, rye and basal diet groups, respectively (Study II). In accordance with the dietary exposure, cadmium was significantly accumulated in the liver and kidneys of the flax group rats, while no accumulation was observed in the rye group (Figure 10). Serum lignans (SECO, END and ENL) were significantly elevated only in the flax group (Figure 10, Study II). Thus, despite significant exposure to a mixture of putative ER modulators (cadmium and ENL), the rats in the flax group did not display any typical morphological changes in the uterus and mammary gland.

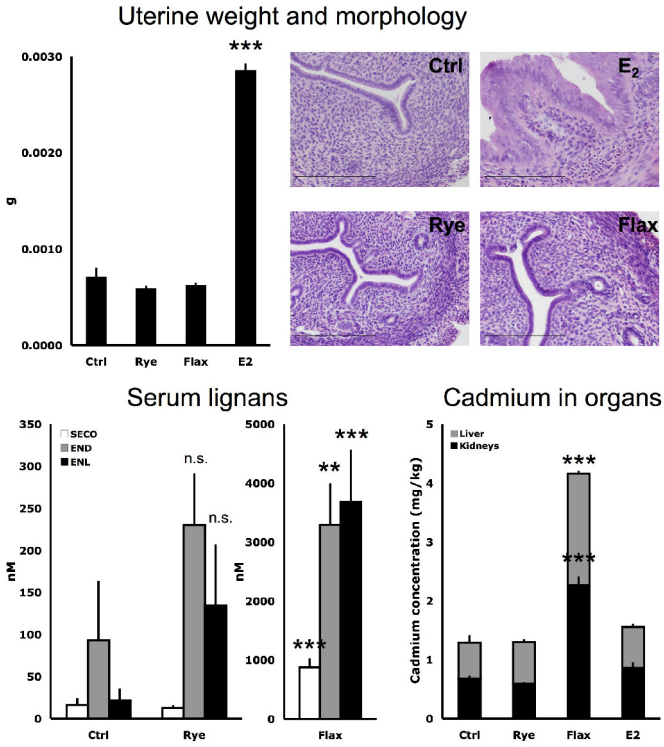


Figure 10. The effects of rye and flax exposure in immature rats. Effects of the test diets on the uterine weight and morphology (scale bars are 200 µm), serum lignans (observe the different scales in the diagrams), and on accumulation of cadmium in tissues in immature female rats after a 7-day exposure. After treatment with E_2, uterine weight was significantly increased and typical morphological changes in uterine histology were observed. Uterine weight and morphology were not affected by the flax and rye diets. The flax diet significantly increased serum lignan concentration and accumulation of cadmium in the liver and kidneys while the other diets did not. The bars are average + SEM. The stars indicate statistical difference to the control (Ctrl), evaluated with Kruskall-Wallis ANOVA and Mann-Whitney *U*-test (serum lignans) or one-way ANOVA and Fisher LSD test (cadmium in tissues). (**) $p<0.01$, (***) $p<0.001$, n.s. not significant.

5.2.2.2 Susceptibility to mammary carcinoma

The effect of developmental exposure to flaxseed on susceptibility to DMBA-induced mammary carcinomas was studied by exposing rats to a 5 % and 10 % flax diet *in utero* or postnatally (Study II). Developmental exposure to the 10 % flax diet shortened tumour latency and increased tumour multiplicity (Study II). Tumour latency was also shortened by the 5 % flax diet *in utero*, but no differences in tumour multiplicity or latency were observed when exposure occurred postnatally (Study II). The flax diets did not alter the number of TEBs or apoptosis and proliferation (PCNA staining) in the mammary gland at the age of 8 weeks, nor did the diets alter tumour incidence after DMBA induction (Study II). Developmental exposure to 10 % flax diet decreased ERβ positive staining in the mammary gland (Study II), and exposure *in utero* also increased ERα staining, evaluated at the age of 8 weeks (Study II). *In utero* exposure to 5 % flaxseed did not affect ER staining, but postnatal exposure decreased the level or both receptors at the age of 8 weeks (Study II).

Collectively, the results from the uterus bioassay suggest that dietary defatted flaxseed and rye bran do not possess estrogen activity that could be observed as increased uterine weight or altered mammary gland development, despite the fact that the rats in the flax group were significantly exposed to a mixture of putative dietary estrogen agonists. However, flaxseed in the diet may alter development of estrogen-dependent tumours if exposure occurs at early life stages.

5.2.3 Optimization of the *in vivo* imaging protocol

A colony of 3xERE-Luciferase mice was established at the University of Turku, and a protocol for *in vivo* imaging was set up. The optimized parameters were 1) the dose of Luciferase substrate D-Luciferin, and 2) the time between dosing of D-Luciferase and imaging. Eight intact adult reporter mouse males were treated with 1 mg/kg EP, and imaged 15 hours post injection. Mice received either 25 or 100 mg/kg D-Luciferin, and images were taken 0, 3, 6, 9, 12, 15, 45, 120, and 720 minutes post D-Luciferin administration. A robust signal was obtained in both 25 mg/kg and 100 mg/kg groups already 3 minutes post substrate administration, and the signal remained stable between 3-15 minutes (Figure 11, left side). In the higher dose group, the signal remained higher than in the lower dose group at all measured time points, and did not return to baseline even 12 hours post administration of D-Luciferin (Figure 11, right side). In fact, surprisingly, the signal slightly increased between 2 and 12 hours in the 100 mg/kg group (Figure 11 right side). In the lower dose group, the signal did not return to the baseline by 12 hours either, but the luminescence had stabilised to a low level already 2 hours post injection. Based on these results, 25 mg/kg was chosen as a suitable dose of D-Luciferin, and 3 minutes post D-Luciferin injection as an appropriate time point for imaging. These parameters improve animal welfare by minimizing the time mice are anaesthetized, and reduce the cost of the experiments by lowering the need for D-Luciferin.

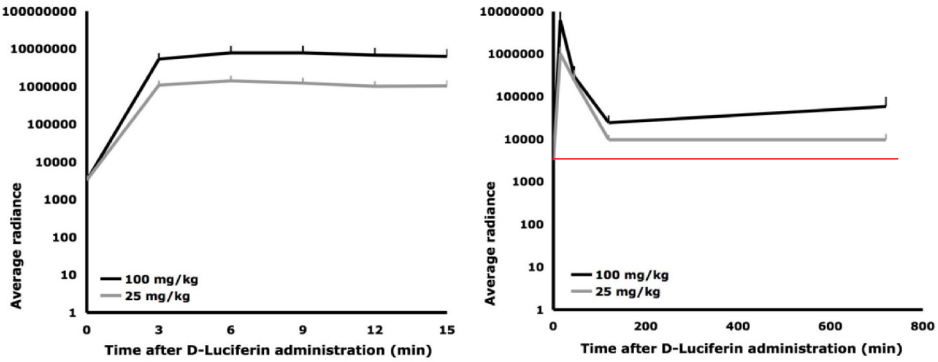


Figure 11. The optimization of the *in vivo* imaging protocol. Intact males were treated with 1 mg/kg EP and imaged 15 hours later. The mice were injected with 25 mg/kg or 100 mg/kg D-Luciferin, and images were taken 0-15 min post injection (diagram on the left). The decay of the signal was followed with images taken at 45, 120 and 720 minutes after the D-Luciferin injection (diagram to the right). The red line indicates the baseline (luminescence at 0 minutes).

5.2.4 Effects of dietary sources of ENL in 3xERE-Luciferase reporter mice (Study III)

5.2.4.1 Activation of reporter gene in acute and sub-chronic exposure

Despite the high circulating level of lignans and significant accumulation of cadmium in the tissues, no uterine or mammary gland effects were observed in immature rats on the flax diet. However, developmental exposure to flaxseed increased susceptibility to mammary carcinoma, suggesting some activity for flaxseed *in vivo*. As estrogen signalling is not limited to the uterus and mammary gland, the effects of flaxseed could be detected in some other tissue type. In Study I, the reporter gene activity accurately reflected the effects of ENL on endogenous estrogen target genes in reporter mouse uteri, and seems therefore a reliable marker of subtle estrogen action in tissues. To study whether dietary sources of ENL could affect ER-mediated transcription *in vivo*, ovariectomized female reporter mice were administered diets containing either flaxseed (14 %), rye bran (11 %), wheat bran (9.9 %) or soy granules (22 %). Wheat bran was included in the study due to a recent report suggesting that wheat is a good source of a wide variety of lignans (Smeds *et al.*, 2007a). Soy, on the other hand, is a rich source of isoflavones such as GEN, which has documented estrogen effects *in vivo* in estrogen reporter mice (Montani *et al.*, 2008). All food items used were purchased from a normal supermarket and were hence meant for human use. The prepared rodent diets were analysed for lignans and isoflavones. Flax, rye and wheat diets contained the expected concentration of lignans (53, 0.3 and 0.2 mg/100g, respectively) (Table 1). Although soy beans contain lignans, the concentration of lignans in the soy diet (0.4 mg/100 g) was higher than anticipated based on reported concentrations in the literature (Table 1). The soy diet contained 20 mg/100g isoflavones, which is in accordance with published values for the soy bean (Thompson *et al.*, 2006). As expected, flaxseed used in the flax diet contained substantially more cadmium (95.2 μg/kg) than rye bran (2.0 μg/kg), wheat bran (6.4 μg/kg) and soy granules (8.7 μg/kg).

Mice were exposed to the experimental diets for 40 hours or two weeks. Food consumption in the experimental groups was initially higher than in the basal diet group but did not differ at other time points (Study III). The consumption of the flax diet remained non-significantly higher than the consumption of other diets at all time points, and only mice on the flax diet significantly gained weight during the 2-week experimental period (Study III). The relative weights of liver and kidneys were not altered by the diets (Study III). Calculated average exposure to lignans in rye, wheat and soy group was 0.4, 0.3, and 0.6 mg/kg/d, while mice in the flax group were exposed to approximately 100 mg/kg/d (Study III). As the average exposure to lignans in humans is approximately 0.02 mg/kg /d for a 60 kg person (Table 2), the exposure to lignans in rye, wheat, soy, and in particular in flax mice, was higher than typically observed in human populations. Likewise, the exposure to isoflavones in the soy group (30 mg/kg/d) (Study III) was higher than reported for Asian people (Wakai *et al.*, 1999). The intake of cadmium from flax, rye, wheat, and soy in diet was approximately 105, 1, 7, and 10 μg/kg/week, respectively.

The exposure to lignans and isoflavones was confirmed with serum analysis of lignans, isoflavones and their metabolites. Control mice had a low level of enterolignans in serum (median concentrations 23 nM END and 4 nM ENL), and the concentrations were not significantly altered by rye or wheat consumption (Study III). The concentrations of ENL in these groups reflected well the lower nanomolar range reported in human populations (Tables 3-4). Consumption of flax diet significantly increased the concentration of END (median 4.7 μM) and ENL (median 340 nM) (Study III), and similar concentrations of ENL have also been reported in flaxseed supplementation studies in humans (Morton *et al.*, 1994; Kuijsten *et al.*, 2005). No isoflavones were detected in the serum of mice on the basal diet (Study III). The median concentration of isoflavones in the serum of soy mice was 1.7 μM (Study III), which can also be considered attainable in humans (Adlercreutz *et al.*, 1993).

Luciferase response was measured in the uterus, vagina, mammary gland, bone (*tibia*), brain, pituitary gland, liver, and muscle (*quadriceps*). After acute exposure, Luciferase expression was significantly increased in the mammary gland, pituitary gland, and liver by soy diet (Figure 12). None of the other diets affected the reporter gene at acute exposure, nor did any diet induce Luciferase after sub-chronic exposure (Figure 12, Study III). As soy is reported to exert estrogenic effects on bone (Power *et al.*, 2007), the *femur* and *lumbar vertebra* were also analysed from the mice in the soy group. Soy did not induce Luciferase expression in these bone types either (Study III).

5.2.4.2 Modulation of E_2-induced reporter gene activity by dietary sources of ENL

Dietary sources of ENL did not increase reporter gene activity in any of the collected tissues, despite high exposure to lignans and confirmed significant circulating levels of ENL in the serum of the flax mice (Study III). In order to assess possible antiestrogenic activity of dietary sources of ENL, ovariectomized reporter mice were maintained on the flax, rye, wheat, and soy diets for two weeks and challenged with E_2. Control mice were maintained on the basal diet and injected with E_2 (positive control) or vehicle

only (negative control). Luciferase induction was followed in real time with the optimized imaging protocol. Positive control mice exhibited significant Luciferase induction at 4-12 hours post E_2 administration, and the time of maximal activity was recorded at 8 hours (Figure 13, Study III). Experimental diets had dramatic effects on E_2-induced Luciferase activity. Rye diet attenuated E_2-induced Luciferase expression to an extent at which the measured signal did not significantly differ from the negative control mice (Figure 13, Study III). Further, wheat and soy diets reduced the E_2-induced signal so that significant induction was only measured at 8 hours post E_2 injection. The time point of maximal Luciferase induction was not altered by rye, wheat, or soy diet (Figure 13, Study III). The flax diet did not reduce E_2-induced Luciferase expression, but significantly delayed the time point of peak activity from 8 hours to 10 hours post injection (Figure 13, Study III). When Luciferase expression was measured in different tissues *ex vivo*, increased activity compared to negative control was measured in the uterus, mammary gland, pituitary, muscle, and bone of the E_2-treated mice (Figure 13, Study III). Luciferase activity was not significantly different from the negative control in the vagina, brain, and liver (Figure 13, Study III). There were no significant differences in E_2-induced reporter gene response between the different dietary groups (Figure 13).

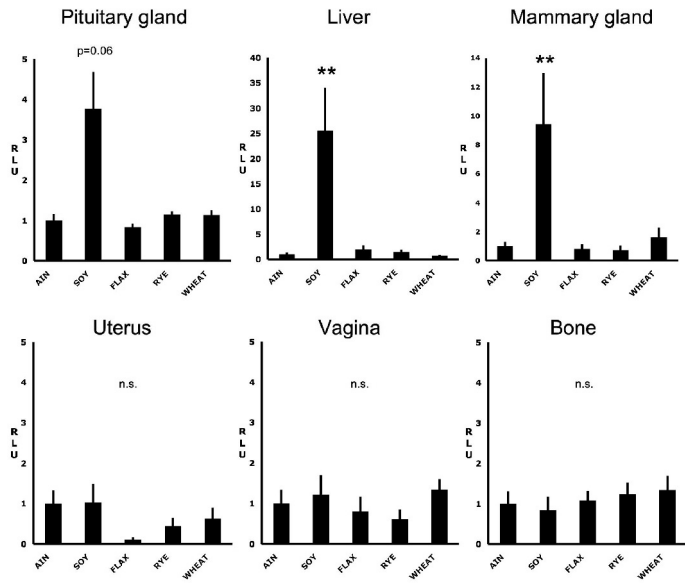
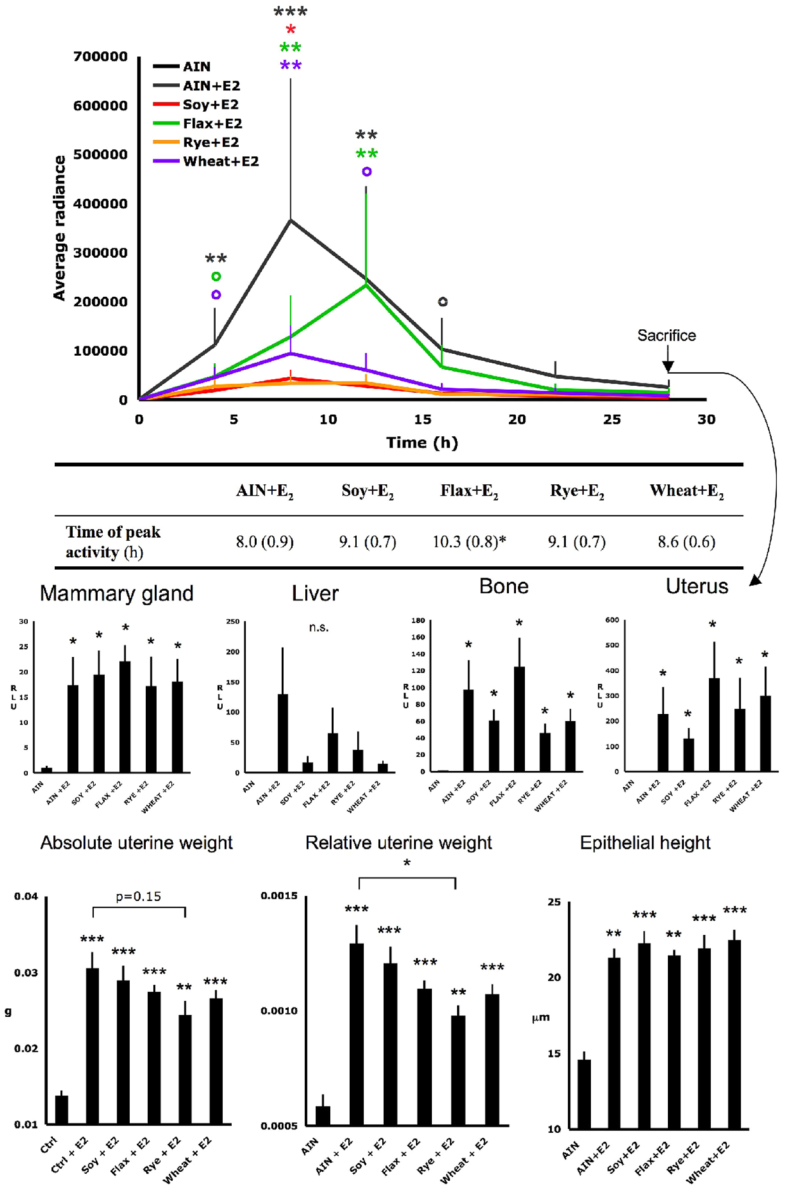


Figure 12. Luciferase response to dietary lignan sources in selected tissues. Effect of soy, flax, rye and wheat on Luciferase expression in the pituitary gland, liver, mammary gland, uterus, vagina, and bone in ovariectomized 3xERE-Luciferase reporter mice in acute exposure. The soy diet increased Luciferase expression in the pituitary gland, liver, and mammary gland. The other diets had no effects on the reporter gene. The bars are average + SEM. The response in the basal diet group (AIN) is set to one. The stars indicate statistical difference to the control (AIN) evaluated with Kruskall-Wallis ANOVA and Mann-Whitney *U*-test. (**) $p<0.01$, n.s. not significant.

5.2.4.3 Effect of dietary sources of ENL on uterus

Estrogenic effects of the dietary sources of ENL were also evaluated with traditional end points in the uterus. Uterine weight was recorded, morphology evaluated visually on H&E-stained sections, and epithelial height measured. In acute exposure, relative uterine weight was reduced by the wheat diet; however, no changes in absolute weights were observed (Study III). After sub-chronic exposure, no changes in uterine weights were observed (Study III). After exposure to E_2, the weight of the uterus was significantly increased in all E_2-treated groups compared to negative control (Figure 13). Interestingly, rye diet significantly reduced E_2-induced uterine weight compared to the positive control group, confirming an antiestrogenic role for rye in estrogen signalling (Figure 13). Estrogenic markers (edema, mitotic figures, epithelial thickening) were evaluated from H&E-stained tissue sections visually, and no differences in the gross morphology were observed between the groups (Study III). To assess possible subtle effects of the diets, thickness of the luminal epithelium was measured from the sections. None of the diets altered the thickness in acute or sub-chronic exposure. The epithelia were significantly thicker in all E_2-treated mice compared to the negative control (Figure 13). The diets did not significantly affect E_2-induced thickening compared to the positive control group (Figure 13, Study III).

Collectively, the results from the reporter mouse studies with dietary ENL sources suggest that the dietary intake of lignans does not possess similar estrogenic activity as ENL given as a pure compound in the reporter mice. Interestingly, all the dietary sources of lignans that were tested modulated the reporter gene response induced by E_2. This suggests that dietary sources of ENL can modulate ER-mediated transcription *in vivo*.



	AIN+E_2	Soy+E_2	Flax+E_2	Rye+E_2	Wheat+E_2
Time of peak activity (h)	8.0 (0.9)	9.1 (0.7)	10.3 (0.8)*	9.1 (0.7)	8.6 (0.6)

Figure 13. Modulation of estrogen response by the soy, flax, rye, and wheat diets in reporter mice. Effects of the diets on the E_2-induced Luciferase expression, uterine weight and epithelial thickness in ovariectomized 3xERE-Luciferase reporter mice. Rye, wheat, and soy in diet attenuated E_2-induced Luciferase expression when measured in real time by *in vivo* imaging (diagram on the top). The time of peak Luciferase activity was delayed by the flax diet (table in the middle). Experimental diets did not affect E_2-induced Luciferase expression (RLU) at the tissue level (mammary gland, liver, bone, and uterus). Rye significantly reduced relative uterine growth after E_2 injection. The diets did not affect E_2-induced epithelial height. The stars indicate statistical difference to the control (AIN, or AIN+E_2 where indicated) evaluated with two-way ANOVA and Tukey's test on Log transformed data (*in vivo* imaging), Chi Square test (time of peak activity), Kruskall-Wallis ANOVA and Mann-Whitney *U*-test (Luciferase activity in tissues), and one-way ANOVA and Tukey's test (uterine weigth and epithelial height). (°) p<0.1, (*) p<0.05, (**) p<0.01, (***) p<0.001, n.s. not significant.

6 DISCUSSION

6.1 ENL preferentially activates ERα

Transactivation of ERα and ERβ by ENL was assessed with estrogen responsive 3xERE-Luciferase reporter gene *in vitro*. Reporter gene activity was dose-dependently increased by ENL in HC11-ERE cells expressing both ERs endogenously, and transient transfection assays in HeLa cells demonstrated that the activation is mediated by both receptors; however, preferentially by ERα (Study I). Furthermore, the ENL-induced ER activation is mediated by the LBD of the receptors; LBD-Gal4 constructs were sufficient for ENL induced reporter gene activation, and activation of full length receptors was blocked by antiestrogens (Study I). The concentration sufficient to activate ERα, 100 nM, is often measured in the serum of humans in their habitual diets (Table 3), and even exceeded in supplementation studies with flaxseed (Table 5). The concentration sufficient to activate ERβ, 1 μM, is not typically observed in general populations (Table 3). The micromolar concentration of ENL has been reported in old Finnish vegetarians, British men and women, and in end-stage renal disease patients (Adlercreutz *et al.*, 1993; Grace *et al.*, 2003; Grace *et al.*, 2004; von Hertzen *et al.*, 2004). Hence, it is more likely that ENL would act primarily through ERα in general populations. Three other studies have assessed the ER isoform specificity of ENL. In the studies of Saarinen *et al*. and Mueller *et al.*, no clear selectivity was observed. As the ENL-induced ER transactivation in both these studies was poor altogether, it might have been difficult to detect isoform specificity (Saarinen *et al.*, 2000; Mueller *et al.*, 2004). A recent study, Carreau *et al.* reports similar ERα selectivity as observed in Study I. ENL induced complement C3 promoter -driven reporter gene expression through ERα at 1-10 μM concentrations in MCF-7 cells, while no activation occurred through ERβ at the same concentrations (Carreau *et al.*, 2008). The higher concentration required for ERα activation in the study of Carreau *et al.* compared to Study I might depend on the reporter gene construct, as ENL is a weaker ER agonist on the C3 promoter than on the consensus ERE promoter (Mueller *et al.*, 2004). The results concerning ERβ transactivation in the study of Carreau *et al.* are hard to interpret, as MCF-7 endogenously express ERα, and ERβ is suggested to be a dominant negative regulator of ERα (Pettersson *et al.*, 2000). HeLa cells used in Study I do not express ERs endogenously, and offer, therefore, a more reliable system for determining ER isoform -specific transactivation. These results also highlight the role of the reporter gene construct and the cell line in determining the magnitude of the response to SERM-like compounds.

6.2 Tissue, cell type, and gene -selective activity of ENL

The *in vitro* transactivation assays suggest that ENL preferentially acts through ERα (Study I). The effects of ENL on ER-mediated transcriptional activation were further studied *in vivo* in 3xERE-Luciferase reporter mice. ERα has been implicated as an important mediator of estrogen activity in female reproductive tissues and in bone (Lubahn *et al.*, 1993; Dupont *et al.*, 2000; Mallepell *et al.*, 2006; Harris, 2007). In the reporter mice, ENL selectively activated reporter gene expression in the uterus and

vagina, but not in the mammary gland and bone (Study I). The significant effects in the uterus and vagina strengthen the idea of ENL as an ERα activator. However, the lack of ENL activity in the mammary gland and bone suggests that the ERα expression pattern is not the only determinant of ENL-induced ER-mediated responses *in vivo*. The effects of ENL on estrogen-sensitive end points in the uterus, mammary gland and bone have been studied previously, and none of the studies has reported any significant estrogen agonist activity for ENL in these tissues (Setchell *et al.*, 1981; Waters & Knowler, 1982; Saarinen *et al.*, 2002a; Power *et al.*, 2006a; Power *et al.*, 2006b; Bergman Jungestrom *et al.*, 2007). Lack of traditional uterotrophic response after ENL treatment was also confirmed in Study I; hence, ERE-driven reporter gene activation in the uterus does not necessarily reflect typical proliferative growth response.

Although ENL did not promote typical proliferative growth response in the uterus, modest stromal oedema was present in 29 % of the ENL-treated animals at 12 hours post treatment. In the mouse uterus, the different morphological changes that occur in response to estrogens are induced by distinct sets of genes activated or repressed in a carefully controlled timely manner (Hewitt *et al.*, 2003; Moggs *et al.*, 2004). The ENL-induced partial estrogen response in the uterus might be due to activation of only a subset of the estrogen responsive genes. For instance, the ENL bound ER could be selective towards classical ERE-regulated genes, and fail to activate non-classical Sp-1 and AP-1 regulated estrogen responsive genes. Studies with the NERKI mice, which lack the classical ERE-signalling, have shown that the uterine epithelial proliferation is controlled through the non-classical (AP-1, Sp-1) signalling pathways (O'Brien *et al.*, 2006). The uterine stroma does not respond to estrogen treatment in these mice, indicating the ERE-dependency of *e.g.* stromal oedema. Immunohistochemical stainings in Study I revealed that both EP and ENL induced CyclinD1, a cyclin that initiates the mammalian cell cycle, in the uteri. CyclinD1 activation by estrogens is absent in the NERKI mice. The NERKI mouse studies collectively suggest that the stromal oedema, as well as CyclinD1 induction, are ERE-mediated responses in the mouse uterus (O'Brien *et al.*, 2006). Therefore, the responses seen in Study I after ENL treatment (*i.e.* stromal oedema and CyclinD1 induction) could be a result of selective activation of ERE-regulated genes by ENL-ER. Activation of AP-1 or Sp1 –driven genes by ENL should be studied in the future to test this hypothesis. Overall, the hypothesis of target gene -selective action of ENL is supported by the study of Dip *et al.* where ENL was shown to regulate only a subset of E_2-responsive genes in MCF-7 cells (Dip *et al.*, 2008). The mechanisms of the selective activity of ENL remain to be addressed in future studies. Intriguingly, EP treatment increased Ki67 expression in the luminal epithelium and in the stroma, while ENL increased the expression only in the uterine glands. This suggests that the activity of ENL within an organ is cell type -specific, and that target gene selectivity is not the only determinant of ENL activity *in vivo*.

6.3 Metabolism as a determinant of the estrogen-like activity of ENL

Reports by others have in general demonstrated weaker estrogenic activity for ENL *in vitro* (Table 8) than the findings in Study I. For instance, ENL displayed substantially lower estrogenic activity in Ishikawa cells transfected with a similar 3xERE-Luc reporter construct as used in Study I (Mueller *et al.*, 2004). Both Ishikawa and HeLa cells are human endometrial adenocarcinoma cell lines lacking endogenous ER expression. Interestingly, Ishikawa cells do not metabolize xenobiotics (Mueller *et al.*, 2004). It is possible that metabolic activity in the cell is required for estrogenic activity at low concentrations of ENL. This hypothesis was tested in a RIfS-based binding assay, where the ERα binding activity of cell culture medium can be measured. The results from the RIfS assay showed that the ERα binding activity of medium containing ENL is enhanced by incubation with HeLa cells. This suggests that ENL might be converted to more estrogenic product(s) in HeLa cells. Cellular metabolism could also explain why the estrogen-like activity of ENL has been lower in reports by others, as HeLa cells have not previously been used in ENL studies. The metabolism hypothesis would also explain the low ER binding affinity of ENL in traditional cell free binding assays. However, other mechanisms could also explain the low binding affinity to ER and yet activation of ER signalling. For example, increased kinase activity could lead to activation of ERs by phosphorylation. The effects of ENL on kinase activity have been tested in an ER negative human breast cancer cell line MDA-MB-468. ENL did not affect EGF receptor tyrosine kinase activity or PKC activity in these cells (Schultze-Mosgau *et al.*, 1998). In another study, ENL did not alter the expression of biomarkers of MAPK signalling pathway in MCF-7 tumours *in vivo* in immunodeficient mice (Power & Thompson, 2007). These studies suggest that indirect activation of ERs through induction of kinase activity is not a likely mechanism of action of ENL. Thus, the cellular metabolism of ENL into more estrogenic compound(s) remains a plausible explanation for the differences between ENL's ER binding affinity and ER transactivation capacity.

Little is known about the metabolic fate of ENL in cell systems. *In vitro,* HepG2, MCF-7, and different human colon epithelial cells conjugate ENL to sulphate and glucuronic acid (Figure 3) (Adlercreutz *et al.*, 1992; Mousavi & Adlercreutz, 1992; Jansen *et al.*, 2005). As Phase II metabolism, conjugation to glucuronic acid or sulphate, reduces the bioavailability of compounds, it hardly represents a mechanism of ENL activation. Rat, pig, rhesus monkey, and human liver microsomes can convert ENL into a variety of aliphatic and aromatic hydroxylated derivatives *in vitro* (Figure 3) (Jacobs & Metzler, 1999; Niemeyer & Metzler, 2002; Dean *et al.*, 2004). Of the several identified metabolites only two have been tested for estrogenicity: 6OH-ENL was an even weaker inducer of 3xERE-Luciferase reporter gene than ENL in Ishikawa cells (Mueller *et al.*, 2004), and a 3,4-catechol metabolite of ENL was found to be antiestrogenic in an MCF-7 proliferation assay (Xie *et al.*, 2003a). Thus, these two ENL metabolites are probably not responsible for the increased estrogenicity of ENL-medium incubated with HeLa cells. The identity of the putative ENL metabolites in the HeLa culture medium remains to be determined in future studies.

Several of the hydroxylated ENL metabolites (Jacobs & Metzler, 1999; Niemeyer & Metzler, 2002; Dean *et al.*, 2004) are also found in human urine and in rat bile and urine (Jacobs *et al.*, 1999; Niemeyer *et al.*, 2000), suggesting that oxidative metabolism of ENL might also occur *in vivo*. It is not clear whether the suggested metabolism of ENL occurs solely in liver microsomes, or whether other cell types could also metabolize ENL. The possible requirement for metabolic activation, possibly occurring at target cell level, could limit the estrogenicity of ENL to certain tissue or cell types. This hypothesis is supported by the observations of ENL activity on uterine epithelium in reporter mice. Luminal and glandular epithelium have distinct molecular signatures and roles in the mouse uterus (Niklaus & Pollard, 2006). Interestingly, the expression of genes involved in metabolism is concentrated on the glandular epithelium (Niklaus & Pollard, 2006). In Study I, ENL induced Ki67 expression specifically in the uterine glands and not in the luminal epithelium. This suggests that metabolism might indeed dictate ENL's bioactivity *in vivo*. Furthermore, the effects exerted by ENL on the mouse uterus appear to occur on a different time scale compared to EP. Where EP induced responses 12 and 24 hours post administration, the effects of ENL were more limited in duration. For example, the reporter gene response and stromal oedema were strongest at 12 hours post ENL treatment, and started diminishing thereafter (Study I). The lack of long-term effects after ENL treatment could also be due to metabolism. The parental compound and its possible estrogenic metabolite(s) might be further converted to inactive break-down products, which can no longer sustain the estrogenic response in the target cells.

6.4 Activity of purified ENL as a predictor of diet-induced effects

The studies with purified ENL provided valuable information on the mechanisms of action of this compound (Study I). However, although a recent study reported occurrence of ENL in some food items such as peanuts and almonds (Smeds *et al.*, 2007b), the main exposure to ENL in humans occurs through intake of dietary plant lignans (ENL precursors). To test whether dietary sources of ENL have effects on the uterus and mammary gland, two different models were used. In Study II, the effects of defatted flaxseed and rye bran on the growth of the uterus and maturation of the mammary gland were studied in a 7-day immature rat uterus bioassay. In Study III, the effects of crushed flaxseed, rye bran, wheat bran and soy granules on 3xERE-Luciferase activation in various tissues in ovariectomized female mice were studied after acute and sub-chronic exposure. In both studies, a significant increase in serum ENL concentration was achieved with the flax diets, and both studies failed to detect the putative estrogenic activity of the flax diet in the measured parameters. Rye and wheat diets did not significantly increase serum ENL concentration in mice, nor did they promote estrogen agonist activity in the studied tissues. It is unclear why ENL, when derived through dietary intake of plant lignans, does not possess comparable estrogenic effects to purified ENL administered as such in reporter mice. One possible explanation could be reduction of biological activity through glucuronidation. The majority of ENL is found as glucuronides in serum and bile, and the conjugation may take place already in the colon epithelium upon absorption from the gut (Axelson & Setchell, 1981; Adlercreutz *et al.*, 1993; Jansen *et al.*, 2005). When ENL is injected as

a pure compound, as was done in Study I, it might be more available to tissues before conjugation and excretion.

In contrast to the tested dietary lignan sources, the soy diet, a known source of estrogenic isoflavonoids such as GEN, induced reporter gene activity in the mammary gland, pituitary gland and liver, indicating that the reporter mouse model is responsive to diet-derived compounds. Induction of estrogen reporter gene by GEN in the liver has been described previously in intact males (Montani *et al.*, 2008). The reporter gene induction in mammary gland is in accordance with other reports on the stimulatory effects of soy and GEN on the mouse and human mammary gland (Hsieh *et al.*, 1998; McMichael-Phillips *et al.*, 1998; Saarinen *et al.*, 2006; Power *et al.*, 2006a). The role of soy and GEN in breast cancer is a matter of controversy (Messina, 2008). However, Study III indicates that dietary soy can activate ER-signalling in the mammary gland when endogenous estrogen production is low, and suggests that further studies are needed to assure the safety of soy consumption in humans.

6.5 Mixture effects and exposure time point as confounding factors

When exposure to ENL occurs through consumption of a lignan-rich diet, other possibly bioactive compounds are inevitably consumed simultaneously. For example, plant lignans and END are absorbed from the gut together with ENL. Indeed, in Studies II and III, ENL was accompanied by a high concentration of SECO and END in the serum of the rodents consuming flax diets. Perhaps the mixture of SECO, END and ENL has a different effect on ER-signalling from ENL alone. Purified SDG (glucoside of SECO) enhances mammary gland maturation in immature rats and reduces estrogen dependent tumour growth in DMBA rats, suggesting that SECO could have biological activity *in vivo* (Thompson *et al.*, 1996a; Thompson *et al.*, 1996b; Tou & Thompson, 1999; Ward *et al*., 2000; Chen *et al*., 2003; Tan *et al.*, 2004). However, as SECO is converted to END and ENL *in vivo*, it is difficult to determine the effects of different lignans in these models. Studies delivering SECO or END as an injection instead of oral gavage could help to address the role of individual lignans in the biological responses.

In addition to being a rich source of lignans, flaxseed accumulates cadmium (Angelova *et al.*, 2004; Hocking & McLaughlin, 2006). In Study II, the concentration of cadmium in the flax diet (46 μg/kg) was 7-fold higher than in the rye and basal diets and in Study III nearly 10-fold higher (95 μg/kg) than in other diets. Cadmium transactivates ERα *in vitro* at nanomolar concentration and induces estrogen effects in mammary gland and uterus at low doses (5 μg/kg) *in vivo* (Stoica *et al.*, 2000; Johnson *et al.*, 2003). The current provisional tolerable weekly intake (PTWI) for cadmium is 7 μg/kg/week, set by the World Health Organization. In the immature rat uterine growth test, the rats on a flax diet were exposed to 42 μg/kg cadmium during the 7-day experimental period, clearly exceeding the PTWI value for humans. In line with this, cadmium significantly accumulated in the liver and kidneys of the rats in the flax group (Study II). Based on pure compound studies, both ENL (Study I) and cadmium (Johnson *et al.*, 2003) would be expected to have estrogenic activity in rodent uterus.

However, no such effects were observed in Studies II and III when the exposure occurred through dietary intake instead of pure compounds. Either the mixture of lignans and cadmium is devoid of any estrogenicity in biological systems, or the measured parameters were not appropriate end points for the effects of such mixtures.

Time point of exposure to estrogenic compounds has been suggested to play an important role in defining the effect on subsequent breast cancer risk. Exposure prepubertally would lead to enhanced mammary gland maturation and decreased susceptibility to carcinoma later in life, while exposure *in utero* is suggested to increase the risk (De Assis & Hilakivi-Clarke, 2006). In Study II, exposure to flax diet *in utero* or postnatally during lactation did not affect the maturation of mammary gland in rats or decrease susceptibility to DMBA-induced mammary carcinomas (Study II). On the contrary, *in utero* exposure to flax diet was associated with increased susceptibility to mammary carcinomas. Although the gross morphology of the mammary gland was not altered by the flax diets, the ERα/ERβ ratio was increased in the epithelial structures (Study II). ERα is believed to mediate the proliferative actions of estrogens while ERβ has been suggested to inhibit ERα-mediated transcription and functions as a tumour suppressor (Dickson & Lippman, 1987; Pettersson *et al.*, 2000; Zhao *et al.*, 2003; Helguero *et al.*, 2005; Matthews *et al.*, 2006). Hence, the increased ERα/ERβ ratio in the mammary gland after exposure to a flax diet might explain the increased susceptibility to DMBA tumours. Increased susceptibility to mammary carcinoma after developmental exposure to a flax diet has been previously recorded in one study (Yu *et al.*, 2006). However, majority of developmental flaxseed exposure studies have reported the protective effects of flax diets on the mammary gland (Tou & Thompson, 1999; Ward *et al.*, 2000; Chen *et al.*, 2003; Tan *et al.*, 2004). The source of flaxseed most probably is different in the various studies. The concentration of cadmium in flaxseed varies depending on growth location (Angelova *et al.*, 2004; Hocking & McLaughlin, 2006). Perhaps differing concentrations of cadmium in the flax diets in the different experiments could explain some of the contradictory results. The role of cadmium in the biological activity of flaxseed at different developmental stages should be studied in the future by comparing flaxseed cultivars with different cadmium accumulation rates.

6.6 Antiestrogenicity of dietary lignan sources

Most studies conducted with flaxseed and mammary carcinoma report reduction of estrogen-dependent tumour growth (Serraino & Thompson, 1992; Thompson *et al.*, 1996b; Rickard *et al.*, 1999; Chen *et al.*, 2003; Chen *et al.*, 2004; Bergman Jungestrom *et al.*, 2007; Chen *et al.*, 2007b). The mechanisms of the anticarcinogenic effects of flaxseed are not known but one plausible explanation could be attenuation of estrogen signalling. In order to characterize the possible antiestrogenic activities of dietary lignan sources, ovariectomized female reporter mice were maintained on flax, rye, wheat, and soy diets for two weeks and treated with a single dose of E_2 (Study III). Somewhat surprisingly, rye, wheat, and soy in the diet reduced E_2-induced reporter gene activity, suggesting antiestrogenic activity for these diets, while the flax diet had no apparent effect on the activity (Study III). Diets high in fibre and low in fat are

suggested to lower the risk of breast cancer by modifying enterohepatic circulation of estrogens (Gerber, 1998; Forman, 2007). According to the "fibre hypothesis", dietary fibre binds estrogens in the gut, thereby lowering the amount of estrogens in the enterohepatic circulation and subsequently in serum. *In vitro*, wheat bran, rye, and soy bean bind E_2 (Arts *et al.*, 1991). However, absorption of E_2 from enterohepatic circulation by the experimental diets in Study III most likely plays only a minor role in the tissue responses, as a single dose of E_2 was administered by *i.p.* injection. In addition, no antiestrogenic effects of flax diet were observed although also flaxseed binds E_2 *in vitro* (Arts *et al.*, 1991). An extension of the fibre hypothesis suggests that the co-passengers of dietary fibre, such as dietary lignans, are partly responsible for the reduction in breast cancer risk (Adlercreutz, 2007). In Study III, the only diet giving rise to considerable concentration of lignans in serum (flax diet) did not reduce estrogen signalling (Study III). This suggests that lignans possibly do not play a central role in the estrogen antagonist effects observed in Study III, and the presence of other possibly bioactive compounds should be considered. For example, rye bran and wheat bran are good sources of alkylresorcinols, phenolic lipids with poorly known physiological functions (Ross *et al.*, 2003). Alkylresorsinols possess a phenolic ring, which is considered as an important structural determinant for ER binding (Brzozowski *et al.*, 1997). However, the role of alkylresorsinols in estrogen signalling has not been studied thus far. Furthermore, alkylresorsinols are specific for rye and wheat and thus hardly play a role in the antiestrogenic effect of soy. Instead, soy could modulate estrogen signalling by acting as a mixed agonist/antagonist. For example, in immature mice soy in the diet promotes uterine growth in the absence of hormonal treatment, but reduces the growth in the presence of DES (Mäkelä *et al.*, 1995).

The antagonistic effects of the experimental diets on E_2-induced Luciferase expression were not seen in any of the *ex vivo* analyzed tissues. This could be explained by the time point of tissue sampling. In Study III, the reporter gene response was primarily measured with *in vivo* imaging and the tissues were collected only after the kinetics of the responses had been determined, *i.e.* 28 hours post E_2 administration. The activity of E_2 peaks at 6-8 hours in reporter mice (Ciana *et al.*, 2001; Ciana *et al.*, 2003; Lemmen *et al.*, 2004a), and this time point could perhaps represent a more suitable time for studying the antagonistic effects of diets at the tissue level. Although 28 hours post E_2 exposure might have been a late time point for reporter gene studies, it is an ideal time for measuring uterine growth response (Moggs *et al.*, 2004). Indeed, the antiestrogenic effect of the rye diet was confirmed by a smaller uterotrophic response in mice consuming the rye diet (Study III). This suggests a role for rye consumption in endogenous estrogen signalling and might have important implications in terms of the regulation of estrogen stimulus in tissues. Study III does not provide mechanistic explanations for the consistent down-regulation of E_2-induced signalling in the rye group. One possible explanation could be short chain fatty acids. An interesting feature of rye consumption is production of short chain fatty acids, such as butyrate, in the gut. For example, consumption of rye bread increases butyrate concentration in the circulation of pigs (Bach Knudsen *et al.*, 2005). Intriguingly, butyrate is a histone deacetylase inhibitor that can affect transcription by preventing

deacetylation of histone tails (Dashwood *et al.*, 2006). Histone deacetylase inhibitors are currently being evaluated for treatment of breast cancer, as they induce cell cycle arrest in breast cancer cells (Dashwood *et al.*, 2006; De los Santos *et al.*, 2007). *In vitro*, butyrate inhibits E_2-induced expression of ERE-containing target genes as well as proliferation of MCF-7 cells (De los Santos *et al.*, 2007). Perhaps butyrate production from the rye diet explains the inhibition of E_2-induced reporter gene expression and reduced uterine growth in Study III. The antiestrogenic activity of rye suggests intriguing possibilities for reducing estrogen stimulus in tissues, and subsequently possibly the risk of estrogen-dependent diseases such as breast cancer. However, it remains to be studied whether rye consumption could also affect estrogen signalling in humans.

7 SUMMARY AND CONCLUSIONS

ENL is a gut microbe metabolite of plant lignans present in human diets. ENL has been suggested to lower the risk of breast cancer, and in animal models significant anticarcinogenicity of ENL, plant lignans, and dietary lignan sources has been documented. Many lines of evidence imply the modulation of estrogen signalling by ENL; however, no convincing mechanistic evidence on such activity has been reported previously. In this study, modulation of estrogen signalling through ER-mediated mechanisms was extensively studied using cell culture and rodent models. Collectively the results indicate that it is very challenging to predict the biological activity of dietary compounds if only pure compound studies are employed.

More precisely, the main findings of this work are:

1. **ENL-induced estrogen effects are preferentially mediated through ERα.** The transactivation assays demonstrate that activation of ERα occurs readily at concentrations attainable in general populations, while concentrations needed to activate ERβ are seldom achieved in human populations. Furthermore, in line with the *in vitro* results, ENL activated the estrogen reporter gene in the uterus and vagina, typical ERα tissues, *in vivo* in reporter mice.

2. **ENL might give rise to more estrogenic compounds in cell culture.** A novel binding assay set up in this study indicates that the ERα binding affinity of ENL is augmented in cell culture conditions. The putative estrogenic metabolites should be identified in future studies, and their involvement in human breast cancer risk modulation should be assessed.

3. **Estrogenic activity of ENL is tissue, cell type, and gene -specific.** In the reporter mice, ENL selectively regulated the ERE-driven reporter gene in the uterus and vagina but not in bone and the mammary gland. In the uterus, the effect of ENL on endogenous genes was concentrated on the epithelium of the glands, and the partial uterotrophic response suggests that not all estrogen responsive genes are activated by ENL in this organ. The mechanisms of the SERM-like activity of ENL should be addressed in future studies by studying co-factor specificity of ENL-bound ER and activity of this complex on different types of estrogen responsive promoters.

4. **Results obtained with purified ENL do not predict the biological activity of the high circulating level of ENL obtained through plant lignan -rich diets in animal models**. Several different dietary sources of ENL were tested for their estrogenic effects in immature rats or in reporter mice, and none of the diets induced signs of estrogenicity, despite high serum concentration of ENL in flaxseed fed animals. Detoxification systems, such as Phase II metabolism, could reduce the bioavailability of ENL *in vivo*. Reporter mouse studies with oral administration of ENL should give an answer to this question.

5. **Developmental exposure to flaxseed increases susceptibility to mammary carcinoma in rats.** This observation is in contrast to many other reports on the same topic, and may reflect differences in the composition of flaxseed used in the different studies. Cadmium is a heavy metal with proposed estrogenic effects at low concentrations. It accumulates in flaxseed and the concentration varies depending on growth location. The role of cadmium in the increased susceptibility to mammary carcinoma after developmental exposure to flaxseed should be addressed in future studies with the help of flaxseed varieties with high and low cadmium content.

6. **Direct E_2-induced ER-transactivation, measured as reporter gene activation, can be modulated *in vivo* with diet.** All experimental diets modulated the E_2-response. However, the most dramatic effect was observed with the rye bran diet. The rye diet effectively attenuated E_2-induced Luciferase activity and significantly reduced uterotrophic response. The role of dietary lignans in this activity is not clear, and other possible bioactive compounds derived from rye, such as alkylresorsinols and butyrate, should be considered in future work in addition to lignans.

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