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Role of lignan sources in tumour formation in multiple intestinal neoplasia mice

ACADEMIC DISSERTATION

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To my family

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Oikarinen Seija I, Role of lignan sources in tumour formation in multiple intestinal neoplasia mice [dissertation]. Helsinki, University of Helsinki. 2005.

Abstract

Plants lignans are ubiquitous compounds in plants, and some of them, such as matairesinol and secoisolariciresinol, are converted into the mammalian lignans enterolactone and enterodiol by gut microflora. Lignans have been suggested to decrease risks of hormone-related cancers in man and to retard tumorigenesis in animal models of cancer. Diet has a major impact on the incidence of colorectal cancer, and lignans have been proposed to prevent this form of cancer. Multiple intestinal neoplasia (Min) mice, which have an inherited mutation in the *adenomatous polyposis coli* gene, and thus, a spontaneous formation of intestinal adenomas, serve as a useful experimental model in studies of diet and colon cancer.

The aims of this study were to elucidate the role of lignan sources and lignan metabolism in adenoma formation in Min mice, and to see if there is gender or genotype based differences in these parameters. To study this, the mice were fed lignan-rich foods, such as flaxseed and rye bran, and the pure lignans 7-hydroxymatairesinol, matairesinol and secoisolariciresinol in aglycone forms. Furthermore, the role of other dietary components in flaxseed and rye bran (besides lignans) in tumour formation, and the effects of diets on levels of such cell-signalling parameters as β-catenin, cyclooxygenase-2 and protein kinase C-ζ were also elucidated.

The results showed that flaxseed and rye diets providing such lignans as secoisolariciresinol diglycoside and syringaresinol showed no clear anticarcinogenic effects in Min mice. Studies with the three pure lignans yielded conflicting results; the crude extract of 7-hydroxymatairesinol was chemopreventive, pure matairesinol had tumour growth-favouring effects, and pure secoisolariciresinol had a neutral effect. The exact mechanism of action of these lignans was unclear, but it did not appear to depend on enterolactone formation. We found also that neither plasma enterolactone nor the intestinal enterodiol, enterolactone or secoisolariciresinol had

chemopreventive effects on adenoma formation in male and female Min mice. Furthermore, we found gender-based differences in the gut enterolactone metabolism and the number of colon tumours. The results suggested also that other dietary components in flaxseed and rye bran, e.g. plant oils and fibre, modulated intestinal adenoma formation in this mouse model. A diet rich in α-linolenic acid may directly or by changing in the ratio of n-6 to n-3 fatty acids of the intestinal mucosa inhibit colon tumorigenesis. Fibres may have either some additive chemopreventive or slightly tumour-growth promotive effects. Fractionation of rye bran did not increase its anticarcinogenic properties. Subcellular localizations of β-catenin and protein kinase C-ζ in the adenoma and mucosa tissue might be pivotal in inhibiting adenoma formation. A low level of nuclear β-catenin in the adenomas of 7-hydroxymatairesinol-fed mice and a high level of protein kinase C-ζ in the mucosal membranes of flaxseed-fed mice were associated with a low tumour number and size in this animal model. The amount of COX-2 was shown to increase during tumour growth, but the experimental diets did not affect the level of this enzyme in the adenomas. Thus role of COX-2 in tumour progression remained unclear.

List of original publications

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

I Oikarinen S, Heinonen S-M, Nurmi T, Adlercreutz H, Mutanen M. No effect on adenoma formation in Min mice after moderate amount of flaxseed. Eur J Nutr 2005;44:273-280.

II Oikarinen SI, Pajari A-M, Salminen I, Heinonen S-M, Adlercreutz H, Mutanen M. Effects of a flaxseed mixture and plant oils rich in alpha-linolenic acid on the adenoma formation in multiple intestinal neoplasia mice. Br J Nutr 2005, in press.

III Oikarinen S, Heinonen S, Karppinen S, Mättö J, Adlercreutz H, Poutanen K, Mutanen M. Plasma enterolactone or intestinal *Bifidobacterium* levels do not explain adenoma formation in multiple intestinal neoplasia (Min) mice fed with two different types of rye-bran fractions. Br J Nutr 2003;90:119-125.

IV Oikarinen SI, Pajari A-M, Mutanen M. Chemopreventive activity of crude hydroxymatairesinol (HMR) extract in Apc^{Min} mice. Cancer Letters 2000;161:253-258.

V Pajari A-M, Smeds AI, Oikarinen SI, Eklund PC, Sjöholm RE, Mutanen M. The plant lignans matairesinol and secoisolariciresinol administered to Min mice do not protect against intestinal tumor formation. Cancer Letters 2005, in press.

These publications have been reproduced with the kind permission of their copyright holders. Some unpublished data are also presented.

Abbreviations

AC	aberrant crypt
ACF	aberrant crypt foci
AIN	American Institute of Nutrition
AOM	azoxymethane
APC/Apc	adenomatous polyposis coli
CEAD	coulometric electrode array detector
CFU	colony-forming unit
COX	cyclooxygenase
DMBA	dimethylbenz(a)anthracene:
END	enterodiol
ENL	enterolactone
ER	oestrogen receptor
GC-MS	gas chromatography-mass spectrometry
HENL	7-hydroxyenterolactone
HMR	7-hydroxymatairesinol
HPLC	high-performance liquid chromatography
HPLC-MS/MS	high-performance liquid chromatography-tandem mass spectrometry
IC_{50}	an inhibitor concentration blocking 50% of V_{max}
IGF	insulin-like growth factor
KRAS	proto-oncogene coding a signalling protein
LAR	lariciresinol
MAT	matairesinol
MIN	multiple intestinal neoplasia
MLH1, MSH2, MSH6	mismatch repair genes
MNU	*N*-methyl-*N*-nitrosourea
PGE_2	prostaglandin E_2
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PIN	pinoresinol
PKC	protein kinase C
PUFA	polyunsaturated fatty acid
SECO	secoisolariciresinol
SD	standard deviation
SDG	secoisolaricaresinol diglycoside
SMAD2/4	genes coding signalling proteins downstream of transforming factor β
SYR	syringaresinol
TR-FIA	time-resolved fluoroimmunoassay

1 Introduction

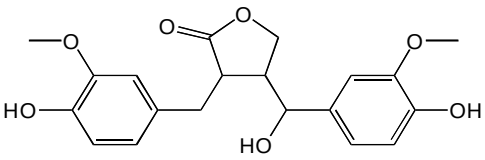
1.1 Lignans

1.1.1 Origin and structure

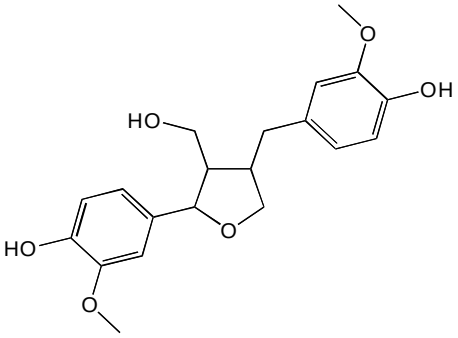
Plants contain a large group of non-nutrient (poly)phenolic compounds which are synthesized to protect plants from photosynthetic stress, reactive oxygen radicals, wounds and attackers such as microbes and herbivores. Plant (poly)phenols are derived from phenylalanine units, and they have in common at least one aromatic ring structure with hydroxyl group(s). Lignans are a group of plant (poly)phenols. Lignans are related to lignin, a structural cell wall component of vascular plants. They are both derived from monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols (C_6C_3) *via* their respective pathways (Davin & Lewis 2000). They are characterized by the coupling of two C_6C_3 units by a bond between positions C8 and C8' (Moss 2000). Lignans are ubiquitous in plant roots, stems, leaves, seeds and fruits, and they have been suggested to be involved in plant defence. Several hundred different lignan structures have been identified in various plants (Ayres & Loike 1990). The lignans included here, i.e. 7-hydroxymatairesinol, matairesinol and secoisolariciresinol, lariciresinol, pinoresinol and syringaresinol, and two mammalian lignans enterodiol and enterolactone, which are formed in the mammalian body, are shown in **Figure 1**.

1.1.2 Food sources and dietary intake

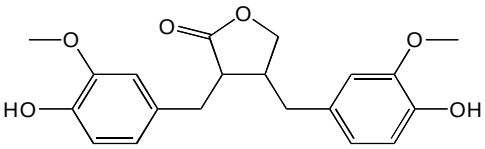
The most commonly analysed lignans in edible plants are secoisolariciresinol and matairesinol (Mazur *et al.* 1996, Mazur 1998). The highest amount of lignans, mainly secoisolariciresinol diglycoside, was found in defatted flaxseed meal, which contains up to 600-700 mg of secoisolariciresinol per 100 g dry weight (Mazur & Adlercreutz 1998). However, in other foods, *e.g.* cereals, vegetables and fruits and berries, the levels of secoisolariciresinol and matairesinol are generally low. These lignans are also found in such beverages as tea, coffee and wine. Although lignans are ubiquitous in foods, the intake of secoisolariciresinol and matairesinol has been estimated to be barely 1 mg/day in Western countries, varying greatly between individuals (Strom *et al.* 1999, Horn-Ross *et al.* 2000*a*, 2002*a*, de Kleijn *et al.* 2001, Keinan-Boker *et al.*



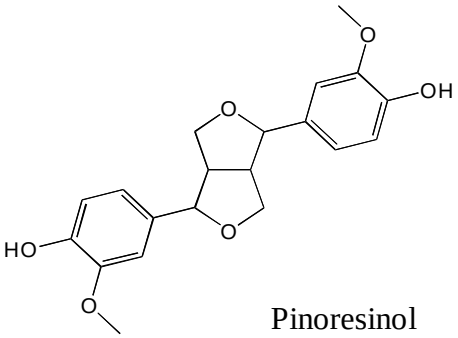
7-Hydroxymatairesinol
(HMR)



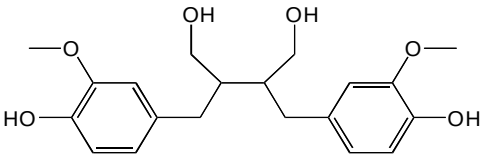
Lariciresinol
(LAR)



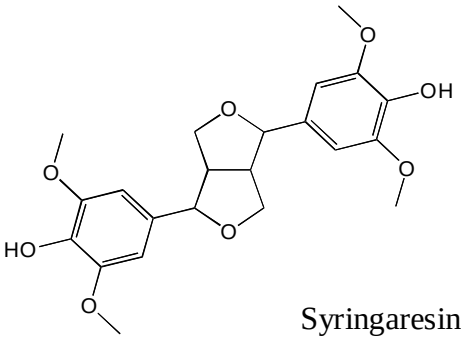
Matairesinol
(MAT)



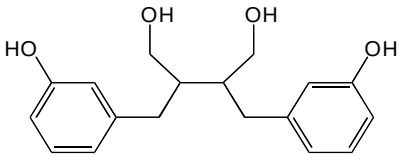
Pinoresinol
(PIN)



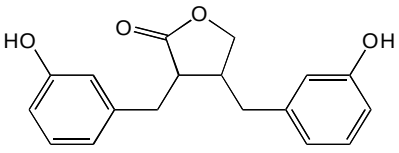
Secoisolariciresinol
(SECO)



Syringaresinol
(SYR)



Enterodiol
(END)



Enterolactone
(ENL)

Figure 1. Structures of dietary lignans from plants and the mammalian lignans enterodiol and enterolactone (diagrams kindly provided by Professor K. Wähälä's research group, Department of Organic Chemistry, University of Helsinki).

2002, Valsta *et al.* 2003, Linseisen *et al.* 2004) (**Table 1a**). Milder *et al.* (2005*a*) have recently reported that the intake of four plant lignans secoisolariciresinol, matairesinol, lariciresinol and pinoresinol was 1.2 mg/day in Dutch adults (**Table 1b**).

1.1.3 Metabolism in human subjects and animals

In foods, lignans occur as glycosidic conjugates, which are hydrolysed by bacterial β-glucosidases in the gut and then released as aglycones. Gut microflora can metabolize plant lignans further into mammalian lignans (Axelson & Setchell 1981, Borriello *et al.* 1985). The importance of microflora in the metabolism of lignans has been demonstrated both in germ-free animals (Axelson & Setchell 1981) and in humans by the administration of antibiotics, which prevented the production of mammalian lignans (Setchell *et al.* 1981). Enterodiol and enterolactone, two mammalian lignans discovered in human urine already in the 1980s (Setchell *et al.* 1980, Stitch *et al.*1980), are the metabolites of the plant lignans secoisolariciresinol and matairesinol, respectively (Borriello *et al.* 1985). Enterodiol can be further oxidized to enterolactone. Other plant lignans are also precursors of mammalian lignans. *In vitro* and *in vivo* experiments have revealed the plant precursors of the mammalian lignans enterodiol and enterolactone to be secoisolariciresinol, matairesinol, lariciresinol, cyclolariciresinol (isolariciresinol), pinoresinol, syringaresinol, 7-hydroxymatairesinol, arctigenin and its glycoside arctiin (Saarinen *et al.* 2000, 2002*b*, Heinonen *et al.* 2001, Wang 2002, Xie *et al.* 2003) (**Figure 2**). In addition, 7-hydroxyenterolactone and enterofuran have been identified as mammalian lignan metabolites in human urine (Adlercreutz 1995, Liggins *et al.* 2000). An *in vitro* study showed that 7-hydroxyenterolactone was a metabolite of 7-hydroxymatairesinol, and enterofuran a metabolite of lariciresinol, pinoresinol and secoisolariciresinol (Heinonen *et al.* 2001). Some differences in proportions of different lignan metabolites emerge when comparing results from *in vitro* and *in vivo* experiments. For example, 7-hydroxymatairesinol was converted to both 7-hydroxyenterolactone and enterolactone in an *in vitro* incubation with faecal flora (Heinonen *et al.* 2001), whereas orally administered 7-hydroxymatairesinol was mainly metabolized enterolactone in rats (Saarinen *et al.* 2000).

Table 1a. Daily intakes (μg/d) of plant lignans matairesinol and secoisolariciresinol in selected Western Countries.

References	Population	*n*	Matairesinol	Secoisolariciresinol	Main lignan sources
Strom *et al.* 1999	Caucasian men in USA	107	**46** (3, 1 082)	**483** (9, 139 927)[1]	Black tea, flaxseed bread, cranberry products
Horn-Ross *et al.* 2000*a*	Elderly African-American, Latina and Caucasian women in California	447	**36**	**139**[2]	Orange juice, coffee
de Kleijn *et al.* 2001	Postmenopausal Caucasian women in USA	964	**19** (12-28)	**560** (399-778)[3]	Fruits (no citrus), breads, cereals, rice and grains, berries
Horn-Ross *et al.* 2002*a*	California Teachers Study cohort	111 526	**23** (12-33)	**85** (48-121)[4]	-
Keinan-Boker *et al.* 2002	Middle-aged and elderly Dutch women	17 140	**73** (43-106)	**988** (676-1 285)[3]	Grain products, fruit, red and white wines
Valsta *et al.* 2003	Finnish men and women	2 862	**38** ± 18	**396** ± 1571[5]	Seed, cereals, fruits, berries and vegetables
Linseisen *et al.* 2004	Premenopausal German women	666	**29** (20-39)	**529** (274-1 280)[3]	Nuts and seeds, coffee, bread, onion/garlic, wine

[1-5] Values are [1] median (minimum, maximum); [2] mean; [3] median (25^{th}-75^{th}) percentiles; [4] mean (20^{th}-80^{th}) percentiles; [5] mean ± SD.

Table 1b. Daily intakes (μg/d) of plant lignans in a Dutch population.

References	Population	*n*	Matairesinol	Secoisolariciresinol	Lariciresinol	Pinoresinol	Main lignan sources
Milder *et al.* 2005*a*	Dutch men and women	4660	**11** (2, 22)	**292** (93, 277)	**535** (238, 897)	**403** (108, 764)[1]	Beverages, vegetables, nuts and seeds, bread and fruits

[1] Values are mean (10^{th}-90^{th}) percentiles.

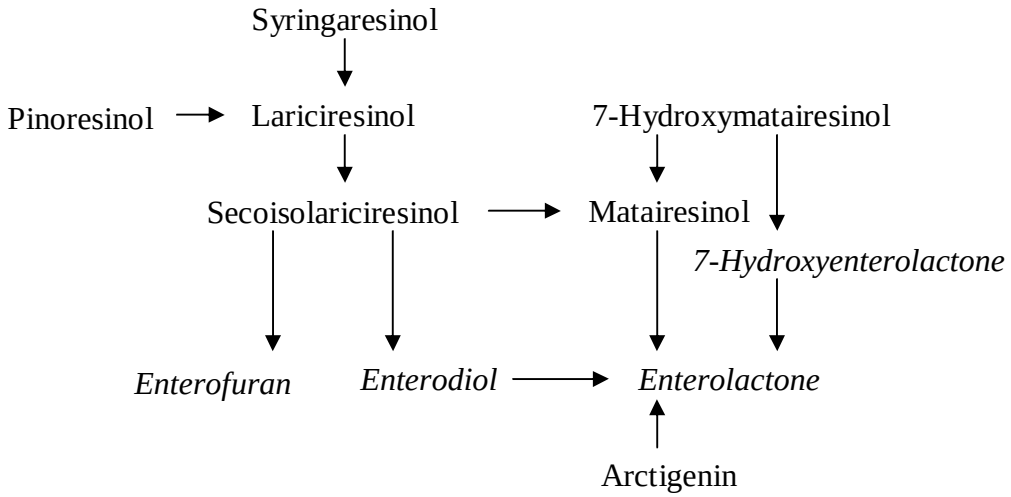


Figure 2. Proposed metabolic pathways of plant lignans to the mammalian lignans enterofuran, enterodiol, enterolactone and 7-hydroxyenterolactone (modified from Heinonen *et al.* 2001, Saarinen *et al.* 2000, 2002*b*, Xie *et al.* 2003).

Absorption of lignans occurs as aglycones. Wood-originated lignans are already in unconjugated form (aglycones) and thus can be absorbed in the upper parts of the small intestine. Lignans can be reconjugated in the intestinal epithelium during absorption or in the liver by UDP-glucuronosyltransferases and sulphotransferases (Dean *et al.* 2004, Jansen *et al.* 2005). Additional metabolism beyond glucuronidation or sulphation may also occur in the liver; enterolactone and enterodiol with extra hydroxyl groups have been identified in human urine after flaxseed ingestion (Jacobs *et al.* 1999). Oxidative metabolism has been suggested to be a means of disposing of lignans from the mammalian body (Wang 2002). Plasma lignans circulate either as glucuronide and sulphate conjugates or as free forms. Lignans are excreted in the urine and *via* bile into faeces. Enterolactone and enterodiol are excreted in the urine mainly as glucuronides, sulphatases, and to a minor extent as free aglycones. Conjugated lignans, which are excreted through bile, undergo enterohepatic recycling (i.e. lignans are re-excreted *via* the bile duct into the intestinal tract, deconjugated by the bacterial β-glucuronidases and sulphatases, and reabsorbed by the intestinal cells) or they are flushed away in faeces in free form. Most of the lignans are eventually excreted *via* faeces and urine in mammalian organisms. Rickard and Thompson (1998) reported that after ingestion of ^{3}H-secoisolariciresinol diglycoside, over 50% of the lignans were excreted in faeces and 30% were present in urine. In tissues, the

greatest concentrations were in those tissues involved in lignan metabolism, *e.g.* intestinal, hepatic, and renal tissues and in blood. Moreover, some lignans were found in the uterus. The presence of lignans in human semen, saliva, breast aspirate or cyst fluid and prostate fluid has also been reported (Adlercreutz 1995).

In humans, average levels of enterolactone in serum/plasma are usually less than 30 nmol/l (Stumpf *et al.* 2000*a*, Kilkkinen *et al.* 2001, 2003*a*, Pietinen *et al.* 2001, Stattin *et al.* 2002). However, inter-individual variation is large, and in individuals consuming lignan-containing foods, *e.g.* whole-grain products, fruit, vegetables and berries, serum/plasma enterolactone levels may be considerably over 100 nmol/l (Adlercreutz *et al.* 1982, Kilkkinen *et al.* 2001, Johnsen *et al.* 2004). Interestingly, lignan-rich food consumption and health-related variables (*e.g.* smoking) usually explain only part of this inter-individual variation. The gut microflora has an important role in lignan metabolism, and the use of antimicrobials decreases enterolactone concentrations in urine and serum (Setchell *et al.* 1981, Kilkkinen *et al.* 2002). Stumpf & Adlercreutz (2003) and Hausner *et al.* (2004) reported large intra-individual within-day and day-to-day variations in serum enterolactone concentrations, and authors suggested that a single measurement of enterolactone is insufficient to estimate the basal enterolactone level in human subjects.

1.1.4 Biological activities

Currently of interest to researchers of the nutritional and medical sciences are lignans and other (poly)phenolic compounds due to their antioxidative, anti-inflammatory and anticarcinogenic activities and possible effects on human health (Liu 2004, Arts & Hollman 2005). A large body of *in vitro* and *in vivo* data has previously been reviewed by Adlercreutz (1998), Thompson (1998) and Saarinen (2002). Only the (*in vivo*) lignan studies considered most significant with regard to cancer prevention are referred to here.

Mammalian lignans enterolactone and enterodiol have some structural similarity to endogenous oestrogens and therefore are included in the family of phytoestrogens (i.e. oestrogenic compounds of plant origin). Mammalian lignans have been reported to possess low oestrogenic activity, e.g. they can stimulate proliferation and DNA/protein synthesis of breast cancer cell lines at 1-10 μM concentrations

(Welshons *et al.* 1987, Sathyamoorthy *et al.* 1994, Wang & Kurzer 1997, 1998, Mousavi & Adlercreutz 1992). High enterolactone and enterodiol concentrations (IC_{50} values above 10 μM) can also inhibit the growth of ER-positive and ER-negative breast cancer cells (Wang & Kurzer 1997, Schulze-Mosgau *et al.* 1998). Weak estrogens, such as lignans, have been postulated to have oestrogenic or even antiestrogenic effects depending on the level of endogenous oestrogens (Mousavi & Adlercreutz 1992, Adlercreutz 1998). The action of lignans is attributed to their proposed ability to bind to oestrogen receptors (ER)α and β. Compared with 17β-estradiol, enterolactone has been shown to bind with a low affinity to human ERα and β and to act as partial agonist and antagonist (Mueller *et al.* 2004). The interplay of lignans and ER α/β might result in competition with endogenous oestrogens, thus modulating the biological activity of oestrogens in target tissues. Furthermore, enterolactone has been proposed to affect sex hormone production *in vitro* by inhibiting the action of steroid-metabolizing enzymes such as aromatase (Adlercreutz *et al.* 1993, Wang *et al.* 1994), an enzyme converting testosterone and androstenedione to 17β-oestradiol and oestrone, respectively. Lignans have also been reported to have the ability to enhance the synthesis of sex hormone binding globulin (SHBG) *in vitro* (Adlercreutz *et al.* 1992), and to possess binding affinities to SHBG, thus modulating the binding of sex hormones (Schöttner & Spiteller 1998). An ER-independent pathway may be involved as well. Anticancer effects of secoisolariciresinol diglycoside, enterolactone, enterodiol and 7-hydroxymatairesinol may, in part, be associated with their antioxidant capacities, as observed *in vitro* assays (Prasad 1997, Kitts *et al.* 1999, Saarinen *et al.* 2000). In most studies, plant and mammalian lignan concentrations were at micromolar levels, which can be over one hundred/thousand-fold the concentrations generally found in the plasma of human subjects.

Although the action of lignans action has been postulated to occur *via* ERs, conclusive evidence for oestrogen-like activity *in vivo* has not been found. Early studies with enterolactone showed no clear oestrogenic effects on uterine weight or uterine RNA synthesis in female mice and rats (Setchell *et al.* 1981, Waters & Knowler 1982). In the latter study, enterolactone inhibited oestrogen-stimulated RNA synthesis in the rat uterine only when administered 22 hours before oestradiol. Saarinen *et al.* (2000,

2002*a*, 2005) subsequently reported that enterolactone, 7-hydroxymatairesinol, matairesinol and secoisolariciresinol (50 mg/kg bw) had no oestrogenic/antioestrogenic activity or aromatase-inhibiting capacity in immature rats, as judged by uterine growth. Nor did administration of 7-hydroxymatairesinol (50 mg/kg bw or 0.15% diet) cause oestrogenic or antiandrogenic effects in adult male rats, as judged by male accessory sex gland and testis weights (Saarinen *et al.* 2000, 2005). Furthermore, enterodiol and enterolactone at concentrations < 1 μM did not show any activation of a reporter gene *via* α- and β- type oestrogen receptors (Saarinen *et al.* 2000). In these studies, the mechanism of the action of lignans in DMBA-induced mammary tumours remained partly unknown. Several studies with young and adult rats have shown that flaxseed supplementation, pure secoisolariciresinol diglycoside and nortrachelogenin may cause some oestrogen-like effects in rats, but the effect is dependent on the life stage (Orcheson *et al.* 1998, Tou *et al.* 1998, 1999, Ward *et al.* 2000, 2001, Saarinen *et al.* 2005).

Human studies have demonstrated non-consistent effects of flaxseed on endogenous sex hormone production and metabolism. Shultz *et al.* (1993) reported no change in plasma total or free testosterone levels or SHBG level in men consuming 13.5 grams of flaxseed per day for six weeks. No change in the serum sex hormone or SHBG levels, or progesterone/17β-estradiol ratio was found after flaxseed (10 g), wheat bran (28 g) and flaxseed plus wheat bran (10 g + 28 g) feeding in premenopausal women (Frische *et al.* 2003). Three studies with postmenopausal women reported that consumption of flaxseed changed sex hormone levels in the urine or serum (Haggans *et al.* 1999, Hutchins *et al.* 2001, Brooks *et al.* 2004). Urinary excretion of oestrogen metabolites were significantly increased after consumption of ground flaxseed (10-25 g per day) (Haggans *et al.* 1999, Brooks *et al.* 2004). Hutchins *et al.* (2001) reported reduced serum concentrations of 17β-oestradiol and oestrone sulphate, and an increased serum concentration of prolactin after flaxseed (5-10 g per day). However, no change in the serum levels of other sex hormones or SHBG were found in this study. Thus, short-term experiments with flaxseed have yielded no conclusive evidence for oestrogenic action of lignans. Similarly, no clear changes in the metabolism of sex hormones or SHBG, which has suggested to be in part responsible for lowering the risk of hormone-dependent cancers, were detected.

1.2 Lignans and cancer

1.2.1 Human studies

During the last two decades, the health effects of lignans in humans have received much attention. In the early 1980s, results of a descriptive study showed that the urinary excretion of enterolactone was lower in breast cancer patients than in omnivorous and vegetarian controls (Adlercreutz *et al.* 1982). Later, the incidence of so-called Western diseases, including breast, prostate and even colon cancer, was proposed to be explained by decreased intake of lignans and isoflavones (Adlercreutz 1990). There are three main ways to study the effect of lignans on the risk of various cancers in humans. In the first approach, urinary or plasma enterolactone levels have been used as indicators of lignan consumption and metabolism. The drawback of this approach is that enterolactone may reflect only short-term intake of plant lignans and may be influenced by the use of antibiotics (Kilkkinen *et al.* 2002) or individual variation in metabolism. In the second approach, an estimated intake of plant lignans has been used as a marker. This approach may provide a good estimation of long-term exposure to dietary lignans. So far, only matairesinol and secoisolariciresinol have been included into food databases (Pillow *et al.* 1999, Horn-Ross *et al.* 2000*b*, Valsta *et al.* 2003) which may lead to the underestimation of plant lignan intake (Milder *et al.* 2005*b*). The third approach, an indirect analytical method using an *in vitro* fermentation of lignan-containing foods with human faecal microflora (Thompson *et al.* 1991), has been used to estimate the intake of mammalian lignans and risk of breast cancer. Observational studies on the relationship between lignans and the risk of various cancers in human subjects are summarized in **Table 2**.

Table 2. Observational studies on the association between lignans and risk of different cancers in human subjects.

References	No. of cases	No. of controls	Age (years)	Medium	Lignan	Adjusted OR/RR (95% CI)[1]	*P* for trend	Association
Breast cancer								
Ingram *et al.* 1997	144	144	30-84	Urine	END	0.73 (0.33-1.64)	0.288	None
					ENL	0.36 (0.15-0.86)	0.013	Negative
					MAT	2.18 (0.83-5.76)	0.308	None
Pietinen *et al.* 2001	194	208	25-75	Serum	ENL	0.38 (0.18-0.77)	0.03	Negative
			Premenopausal		ENL	0.42 (0.10-1.77)	0.18	None
			Postmenopausal		ENL	0.50 (0.19-1.28)	0.10	None
Dai *et al.* 2002	250	250	25-64	Urine	END	0.43 (0.26-0.71)	<0.01	Negative
					ENL	0.42 (0.25-0.69)	<0.01	Negative
					END + ENL	0.40 (0.24-0.64)	<0.01	Negative
Dai *et al*. 2003	117	117	Postmenopausal	Urine	END + ENL	0.50 (0.23-1.10	0.09	None
Boccardo *et al.* 2004	18	383	25-79	Serum	ENL	0.36 (0.14-0.93)[2]	-	Negative
den Torkelaar *et al.* 2001	88	268	50-64	Urine	ENL	1.43 (0.79-2.59)[3]	0.25	None
Hultén *et al.* 2002	248	492	51, 58 (means)	Plasma	ENL	1.1 (0.7-1.7)	-	None
Kilkkinen *et al.* 2004	206	215	25-75	Serum	ENL	1.30 (0.73-2.31)	0.48	None
Olsen *et el.* 2004	381	381	Postmenopausal	Plasma	ENL	0.93 (0.86-1.01)	0.09	None
Zeleniuch-Jacquotte *et al.*	189	189	Premenopausal	Serum	ENL	1.6 (0.7-3.4)	0.13	None
2004	228	228	Postmenopausal		ENL	1.0 (0.5-2.1)	0.95	None
McCann *et al.* 2002	301	316	Premenopausal	Diet	END + ENL	0.49 (0.32-0.73)[4]	-	Negative
	439	494	Postmenopausal		END + ENL	0.72 (0.51-1.02)[4]	-	None

McCann *et al.* 2004	315	593	Premenopausal	Diet	MAT + SECO	0.66 (0.44-0.98)	-	Negative
	807	1 443	Postmenopausal		MAT + SECO	0.93 (0.71-1.22)	-	None
Linseisen *et al.* 2004	278	666	Premenopausal	Diet	MAT	0.58 (0.37-0.94)	0.025	Negative
					SECO	1.12 (0.73-1.73)	0.648	None
					MAT + SECO	1.1 (0.72-1.70)	0.837	None
					END	0.61 (0.39-0.98)[4]	0.034	Negative
					ENL	0.57 (0.35-0.92)[4]	0.008	Negative
					END + ENL	0.61 (0.39- 0.98)[4]	0.034	Negative
Horn-Ross *et al.* 2001	1 272	1 610	35-79	Diet	MAT	1.1 (0.89-1.5)	-	None
					SECO	1.3 (1.0-1.6)	-	None
					MAT + SECO	1.3 (1.0-1.6)	-	None
Horn-Ross *et al.* 2002*a*	568	111 526	21-103	Diet	MAT	1.1 (0.8-1.4)	0.2	None
					SECO	1.2 (0.9-1.6)		None
Keinan-Boker *et al.* 2004	280	15 555	49-70	Diet	END + ENL	0.7 (0.5-1.1) [4]	0.06	None
Prostate and testicular cancers								
Stattin *et al.* 2002	794	2 550	Middle-aged & old	Serum	ENL	1.08 (0.83-1.39)	-	None
Kilkkinen *et al.* 2003*b*	214	214	50-69	Serum	ENL	0.71 (0.42-1.21)	0.37	None
Stattin *et al.* 2004	265	525	60 (median)	Plasma	ENL	1.22 (0.80, 1.86)	0.21	None
Strom *et al.* 1999	83	107	61 (mean)	Diet	MAT	0.89 (0.47-1.66)	-	None
					SECO	1.20 (0.65-2.21)	-	None
Walcott *et al.* 2002	159	136	18-55	Diet	MAT + SECO	0.96 (0.11-8.09)	0.27	None
					END + ENL	0.73 (0.21-2.56)	0.09	None

Table 2. *Continues*

References	No. of cases	No. of controls	Age (years)	Medium	Lignan	Adjusted OR/RR (95% CI)[1]	*P* for trend	Association
Colorectal cancer								
Lundin 2001	117	232	?	Plasma	ENL	0.99 (0.52-1.88)	-	None
Endometrial cancer								
Horn-Ross *et al.* 2003	500	470	35-79	Diet	MAT	1.6 (0.99-2.4)	0.07	None
					SECO	0.63 (0.40-0.98)	0.009	Negative
					MAT +SECO	0.68 (0.44-1.1)	0.03	Negative
Ovarian cancer								
McCann *et al.* 2003	124	696	40-85	Diet	MAT + SECO	0.43 (0.21-0.85)	-	Negative
Thyroid cancer								
Horn-Ross *et al.* 2002*b*	817	793	20-74	Diet	MAT	0.72 (0.46-1.1)	0.49	None
					SECO	0.56 (0.35-0.89)	0.009	Negative
					MAT + SECO	0.68 (0.43-1.1)	0.07	None
Premalignant lesions of the cervix								
Hernandez *et al.* 2004	112	183	>18	Plasma	END	2.7 (1.1-6.3)	0.01	Positive
					ENL	2.4 (1.0-5.8)	0.06	None

[1] Adjusted odds ratio/relative risk (95% confidence intervals), highest group compared with lowest group
[2] Serum enterolactone ≤ 8 nmol/l compared with serum enterolactone > 8 nmol/l
[3] Crude odds ratio.
[4] Lignan intakes expressed as the sum of enterolactone and enterodiol production *in vitro* from foods.

Three case-control studies have reported an inverse association between urinary/serum enterolactone and risk of **breast cancer** (Ingram *et al.* 1997, Pietinen *et al.* 2001, Dai *et al.* 2002). A later study by Dai *et al.* (2003) showed only a tendency for a reduced risk of breast cancer with increasing urinary excretion of mammalian lignans. They reported, however, a stronger inverse association between urinary isoflavonoids/lignans and breast cancer risk among women with a high body mass index or waist:hip ratio. The authors suggested that these overweight women, who often have a high level of oestrogens and insulin and a low SHBG level and 2-hydroxyestrone:16α-hydroxylation ratio, may benefit from high intake of phytoestrogens such as isoflavonoids and lignans. Recently, a follow-up study has shown that a high serum enterolactone concentration has a strong protective effect on breast cancer risk in women with palpable cysts (Boccardo *et al.* 2004). By contrast, in one prospective case-control study with postmenopausal Dutch women, higher urinary enterolactone excretion was associated with a non-significant increase in risk of breast cancer (den Tonkelaar *et al.* 2001). Serum/plasma enterolactone level was not associated with risk of breast cancer in four case-control studies nested within cohorts in Sweden, Finland, Denmark and USA (Hulten *et al.* 2002, Kilkkinen *et al.* 2004, Olsen 2004, Zeleniuch-Jacquotte *et al.* 2004). In one case-control and in two cohort studies, no associations between intake of lignans and risk of breast cancer were found (Horn-Ross *et al.* 2001, 2002*a*, Keinan-Boker *et al.* 2004). In three case-control studies, the intake of mammalian lignans and matairesinol was, however, inversely associated with the risk of breast cancer in premenopausal women (McCann *et al.* 2002, 2004, Linseisen *et al.* 2004). McCann *et al.* (2002) reported also that premenopausal women with at least one A2 allele of the *cytochrome P450c17α (CYP17)* gene benefit most from a lignan-rich diet (enterolactone: OR 0.12, 95% CI 0.03-0.50). The authors suggested that lignans might provide the greatest protective effect among women who have high endogenous hormone levels and perhaps a higher risk for breast cancer. An intervention study by Thompson *et al.* (2000) with breast cancer patients showed that dietary flaxseed (25 g/d) reduced the Ki67 labelling index (a proliferation marker) in tumours, suggesting an anticancer effect of flaxseed.

Studies examining the association between lignans and **prostate cancer** risk are sparse. Serum enterolactone concentration was not found to be protective against

prostate cancer in three nested case-control studies (Stattin *et al.* 2002, 2004, Kilkkinen *et al.* 2003*b*). Similarly, intake of lignans was not associated with risk of prostate or testicular cancer (Strom *et al.* 1999, Walcott *et al.* 2002). An intervention study with prostate cancer patients showed that the consumption of a low-fat (20% of energy), flaxseed-supplemented (30 g/d) diet affected several biomarkers associated with prostatic neoplasia (Demark-Wahnefried *et al.* 2001). The mean proliferation index was lower, and the apoptotic index higher in the prostate tumours of diet-treated patients compared with controls. Significant decreases were also found in the levels of total serum testosterone, free androgen, and total cholesterol in diet-treated patients compared with baseline measurements. The authors suggested that the low-fat, flaxseed-supplemented diet might have an influence *via* a hormonal mechanism (lignan production) on prostate cancer biomarkers. However, possible effects of fat restriction and n-3 fatty acids were not discussed. In an intervention study, Bylund *et al.* (2003) reported that when rye-bran or wheat-bran bread was fed to prostate cancer patients, the apoptotic index was increased in biopsies of prostate tumours in the rye-bran bread group but not in the wheat-bran bread group. However, plasma enterolactone levels were not associated with changes in the apoptotic index, indicating that other components of rye bran were involved.

The relationship between lignans and other types of cancers has also been investigated. No association was found between plasma enterolactone level and risk of **colorectal cancer** or risk of cancers of the colon and rectum separately in one unpublished study (Lundin 2001). Horn-Ross *et al.* (2002*b*, 2003), however, reported reduced risks of **thyroid** and **endometrial cancers** with a high dietary intake of secoisolariciresinol. When the subgroups of women were studied further, the authors found a significantly reduced risk of endometrial cancer with increasing plant lignan intake among postmenopausal women (OR = 0.57, 95% CI 0.34-0.97, P_{trend} = 0.02). However, among premenopausal women, no such association was not observed (OR = 0.77, 95% CI 0.26-2.3, P_{trend} = 0.42). In line with these two studies, an inverse association was detected between the intake of plant lignans and the risk of **ovarian cancer** (McCann *et al.* 2003). In a recent study, Hernandez *et al.* (2004) reported a positive association between plasma enterodiol levels and **premalignant lesions of the cervix**.

In summary, observational studies have yielded contradictory results on the relationship between lignans and risk of different cancers. Out of 16 case-control and cohort studies, eight showed an inverse association between lignans and breast cancer risk, and eight found no clear evidence of such an association. All five studies of lignans and risk of prostate/testicular cancers showed null results. Among five studies of lignans and risks of colorectal, endometrial, ovarian and thyroid cancers, or risk of premalignant lesions of the cervix, one study was null, three studies showed low risks and one study showed an elevated risk. The results of these studies are, however, only preliminary, and requiring confirmation by future research. It is also noteworthy that in some studies the association between lignans and risks of cancers was dependent on menopausal status or *cytochrome P450c17α (CYP17)* allele status. This may indicate that certain subgroups of women benefit more by increasing lignan intake. This suggestion requires verification in future studies.

1.2.2 Animal studies

The effects of lignan-rich foods or pure lignan isolates on breast, prostate and colon cancers in experimental animals are summarized in **Table 3**. In most studies, flaxseed has been used as a model substance to test the effect on lignans in carcinogenesis. Flaxseed and defatted flaxseed at a level of 2.5 - 10% level have been shown to reduce risks for **mammary cancer** in dimethylbenz(a)anthracene (DMBA)-treated rats during initiation and the early and late promotion stages of the cancer process (Thompson *et al.* 1996*a*, *b*, Thompson 1998). Similarly, purified lignan secoisolaricaresinol diglycoside at 1.5 mg/d inhibited the development of new tumours in this rat model (Thompson *et al.* 1996*a*, *b*). Ground flaxseed, rich in lignans and n-3 fatty acids, has been shown to decrease the growth rate and metastasis of oestrogen receptor (ER)-negative human breast cancer cells in nude mice (Chen *et al.* 2002, Dabrosin *et al.* 2002). In contrast to these studies, 2.5-5% ground flaxseed or secoisolaricaresinol diglycoside at 0.7-1.4 mg/d gave inconsistent results in *N*-methyl-*N*-nitrosourea-treated rats: low secoisolaricaresinol diglycoside level promoted, high secoisolaricaresinol diglycoside level inhibited and flaxseed feeding had no effect on tumour multiplicity (Rickard *et al.* 1999). Authors concluded that discrepancies between this and previous studies on flaxseed might be related to differences in experimental design, e.g. the use and dose of a different carcinogen and a basal diet.

Table 3. Effects of lignans or lignan-rich diets on breast, prostate and colon cancers in experimental animals.

References	Model	Lignan intake or experimental diet	Effect (compared with controls)
Breast cancer			
Thompson *et al.* 1996*a*	DMBA-treated rats (late promotion stage) [1]	SDG 2 200 nmol per day [2], 1.82% flaxseed oil [3], flaxseed 2.5% and 5%	All experimental diets reduced the volume of established tumours by at least 50%. SDG intake reduced the volume of new tumours.
Thompson *et al.* 1996*b*	DMBA-treated rats (early promotion stage) [1]	SDG 1.5 mg per day [2]	SDG intake reduced the number of tumours per tumour-bearing rat by 37%, and the number of tumours per total number of rats by 46%.
Rickard *et al.* 1999	MNU-treated rats (early promotion stage) [1]	SDG 0.7 and 1.4 mg per day, 2.5% and 5% ground flaxseed	No effect of flaxseed diets on tumour size, multiplicity or incidence was observed. Low SDG level promoted and high SDG level inhibited tumour multiplicity. All treatment groups showed decreased tumour invasiveness and grade.
Chen *et al.* 2002	ER-negative human breast cancer cells in nude mice (late promotion stage) [1]	Ground flaxseed 10%	Flaxseed diet decreased tumour growth rate, total incidence of metastasis (by 45%), proliferation and expression of insulin-like growth factor 1 and epidermal growth factor receptor in tumours.
Dabrosin *et al.*2002	ER-negative human breast cancer cells in nude mice (late promotion stage) [1]	Ground flaxseed 10%	Flaxseed diet decreased tumour growth rate, incidence of metastasis in other organs and extracellular levels of vascular endothelial growth factor.
Hirose *et al.* 2000	PhIP-treated rats (initiation and promotion stages) [1]	Arctiin 0.2% and 0.02%	Arctiin given in post-initiation period decreased the multiplicity (but not the incidence) of tumours.
Kitamura *et al.* 2003	PhIP-treated rats (initiation and	Trachelogenin 0.2% and 0.02%	Trachelogenin did not decrease tumour incidence or inhibit

	promotion stages) [1]		the growth of mammary tumours.
Saarinen *et al.* 2000	DMBA-treated rats (late promotion stage) [1]	HMR extract 15 mg per kg bw per day	HMR extract reduced the total tumour volume and increased the proportion of regressing and non-growing tumours.
Saarinen *et al.* 2001	DMBA-treated rats (initiation and late promotion stage) [1]	HMR extract 4.7 mg per kg bw (pure HMR 87.6 mg per kg diet)	HMR intake when started one week before the DMBA treatment decreased tumour growth rate and total tumour volume at the end of the feeding period by 22%.
Saarinen *et al.* 2002*a*	DMBA-treated rats (late promotion stage) [1]	ENL 1 and 10 mg per kg bw	ENL at 10 mg per kg reduced the total tumour volume, and the total volume of new tumours established during the experiment.
Saarinen *et al.* 2005	DMBA-treated rats (late promotion stage) [1]	Nortrachelogenin 15 mg per kg bw	Nortrachelogenin did not inhibit the growth of mammary tumours.
Prostate cancer			
Landström *et al.* 1998	Antrogen-sensitive prostate tumour implants in rats	Defatted soy flour 33%, rye bran 33%, rye- endosperm 33%, heat-treated rye bran 33%	Soy flour and rye-bran intake delayed tumour growth at early stages.
Bylund *et al.* 2000	Antrogen sensitive human tumour cells in nude mice	Rye-bran 30%	Rye-bran intake inhibited tumour growth and lowered prostate-specific antigen secretion.
Zeng *et al.* 2004	SV40T antigen transgenic rats	Arctiin 0.004%, 0.02% and 0.1%	No significant effect of arctiin on neoplastic lesions of the prostate was observed
Bylund *et al.* 2005	LNCaP human prostate cancer xenografts in athymic mice (early promotion stage) [1]	HMR 0.15% and 0.30%	HMR intake mice decreased tumour growth and tumour cell proliferation index (0.30% HMR), and increased apoptotic index.

Table 3. *Continues*

References	Model	Lignan intake or experimental diet	Effect (compared with controls)
Colon cancer			
Serraino & Thompson 1992	AOM-treated rats (early promotion stage) [1]	Flaxseed flour (ground flaxseed) 5% and 10%, defatted flaxseed meal 5% and 10%	All experimental diets reduced the number of ACs by 41-53% and ACF by 48-57% in the descending (distal) colon.
Jenab & Thompson 1996	AOM-treated rats (early promotion stage) [1]	SDG 1.5 mg per day [3], flaxseed 2.5% and 5%, defatted flaxseed 2.5% and 5%	SDG intake reduced the total number of ACs by 29% in the distal colon. All experimental diets reduced the number of AC per focus (AC multiplicity) in the distal colon.
Davies *et al.* 1999	AOM-treated rats (initiation stage) [1]	Rye bran 30%	Rye-bran diet decreased the number of tumours in the colon.
Mutanen *et al.* 2000	Min mice	Rye bran 10%	Rye-bran group had a tendency to gain lower amounts of adenomas in the small intestine than the non-fibre group.
van Kranen *et al.* 2003	Min mice	Rye bran 30%, flaxseed 5%	No protective effect of rye bran or flaxseed on intestinal adenoma formation was observed.
Hirose *et al.* 2000	PhIP-treated rats (initiation and promotion stages) [1]	Arctiin 0.2% and 0.02%	Arctiin given during the post-initiation period decreased the total number of ACF

Abbreviation are explained on page 8.
[1] Animals put on experimental diets before the carcinogen treatment/cancer cell injection (initiation stage), shortly after the carcinogen treatment/cancer cell injection (early promotion stage) or several weeks after the carcinogen treatment (late promotion stage).
[2] Lignan dose is equivalent to that present in 15 g of 5% flaxseed diet.
[3] Fatty acid dose is equivalent to that present in 15 g of 5% flaxseed diet.

The antitumour effect of various flaxseed-containing diets appeared not to be dose-dependent, and a higher level of flaxseed did not provide extra protection against tumorigenesis. The exact mechanism of lignan action during different stages of mammary tumorigenesis was not elucidated, although the possible mechanism of lignan protection was supposed to be associated to the factors reviewed in the section entitled "Biological activities" (page 14). In addition, antitumour effects of flaxseed were linked with reduced expressions of insulin-like growth factor-1, epidermal growth factor receptor and vascular endothelial growth factor in ER-negative tumours (Chen *et al.* 2002, Dabrosin *et al.* 2002). Flaxseed also contains high quantities of α-linolenic acid (18:3n-3), which may inhibit the cancer process. Thompson *et al.* (1996*a*) reported that flaxseed oil-supplemented diet (equal to a level of 5% flaxseed) inhibited tumour growth when mammary tumours were already established but had no effect on new tumour development. With regard to pure lignan isolates, arctiin, 7-hydroxymatairesinol and enterolactone have been shown to inhibit mammary carcinogenesis in carcinogen-treated rats (Hirose *et al.* 2000, Saarinen *et al.* 2000, 2001, 2002*a*) but tracheloside or nortrachelogenin administrations had not such effects in carcinogen-treated rats (Kitamura *et al.* 2003, Saarinen *et al.* 2005).

The effect of lignans on **prostate cancer** is unclear. Landström *et al.* (1998) suggested that phytoestrogens (isoflavonoids of soy and lignans of rye bran) might be responsible for delayed prostate tumour growth in rats. In a subsequent study, consumption of a rye-bran diet (30%) delayed prostate cancer growth in nude mice, but the effect was not related to enterolactone production (Bylund *et al.* 2000). Two studies with pure lignans showed conflicting results. Arctiin from *Arctium lappa* (burdock) seeds failed to prevent prostate cancer in SV40T antigen transgenic rats (Zeng *et al.* 2004) whereas 7-hydroxymatairesinol inhibited tumour growth of the LNCaP human prostate cancer xenografts in athymic mice (Bylund *et al.* 2005).

The impact of lignans on **colon carcinogenesis** has been studied mainly with carcinogen-treated rats, with formation of early precancerous lesion (aberrant crypt foci, ACF) or tumour in the colon being used as an end-point (Serrano & Thompson 1992, Jenab & Thompson 1996, Davies *et al.* 1999, Hirose *et al.* 2000). Flaxseed and defatted flaxseed meal (at a level of 5% or 10%) decreased the number of aberrant

crypts (AC) and ACF in the descending (distal) colon of carcinogen-treated rats by 41-53% and 48-57%, respectively (Serraino & Thompson 1992). However, the effect of flaxseed was not dose-dependent. Nor was it related to the urinary excretion of mammalian lignans or the presence of α-linolenic acid in the diets. In a subsequent study, where carcinogen-treated rats were fed either a control diet or a diet supplemented with flaxseed and defatted flaxseed meal (at level of 2.5% or 5%) or pure secoisolariciresinol diglycoside, the number of AC per focus (AC multiplicity) was significantly reduced in the distal colon of flaxseed and secoisolaricaresinol diglycoside groups (Jenab & Thompson 1996). The protective effect was partly due to secoisolaricaresinol diglycoside, and there was a negative association between total urinary lignan excretion and AC multiplicity. The oil component of flaxseed was not considered an effective agent in this study. In other study, a 30% rye-bran diet decreased the number of colon tumours in carcinogen-treated rats, and the authors suggested that the rye-bran lignans were responsible for this effect (Davies *et al.* 1999). Similarly, Mutanen *et al.* (2000) reported that multiple intestinal neoplasia (Min) mice fed rye bran had the lowest adenoma number (26.4 ± 12.1, mean ± SD) and incidence of colon adenomas (33%) of all experimental groups, although these values were not significant when compared with the non-fibre group (34.6 ± 7 and 71%). However, lignans were not measured in this study, and their role in tumour prevention remained unclear. Later, van Kranen *et al.* (2003) reported that flaxseed or rye-bran consumption did not explain adenoma formation in Min mice. Only one study has evaluated the role of pure lignan isolate in colon carcinogenesis (Hirose *et al.* 2000). When pure lignan arctiin from *Arctium lappa* (burdock) seeds were fed to carcinogen-treated rats, the number of ACF per colon was significantly decreased in the group fed 0.02% arctiin during the initiation period, and in groups fed either 0.02% or 0.2% arctiin during the post-initiation period.

Yan *et al.* (1998) and Li *et al.* (1999) have reported on the effects of flaxseed and pure secoisolaricaresinol diglycoside on **experimental metastasis** of melanoma cells in mice. In both studies, B16BL6 murine melanoma cells were injected into C57BL/6 mice, which resulted in the formation of metastatic lung tumours. Flaxseed at levels of 5% and 10% reduced tumour area, tumour volume and the median number of tumours by 54% and 63%, respectively (Yan *et al.* 1998). In the second study,

secoisolaricaresinol diglycoside at 147 and 293 μmol/kg reduced tumour area and tumour volume. However, only the highest secoisolaricaresinol diglycoside level (equivalent to a 10% flaxseed diet) reduced the median number of tumours by 53% (Li *et al.* 1999). This indicates that flaxseed *per se* is more potent in preventing metastasis than an equivalent amount of pure secoisolaricaresinol diglycoside.

In summary, experimental studies of lignans or lignan sources and mammary and colon carcinogenesis showed both protective and neutral effects. Secoisolaricaresinol diglycoside from flaxseed, arctiin, 7-hydroxymatairesinol and enterolactone have been the most potent anticarcinogenic compounds in animal studies. However, the role of the oil component of flaxseed in tumour prevention remains obscure, and may be underestimated. The effect of lignans or sources of lignans on prostate carcinogenesis in animals and humans seem not to be convincing. Only one study showed that 7-hydroxymatairesinol had anticancer properties on a prostate cancer model. The potential antitumourigenic effects of lignans or lignan-rich foods may involve both ER-mediated and ER-independent mechanisms.

1.3 Colon carcinogenesis

1.3.1 Colorectal cancer

Colorectal cancer is the third most common cancer (9.4%) worldwide after lung and breast cancers. It ranks fourth in mortality (7.9%), after lung, stomach and liver cancers (Stewart & Kleihues 2003). Nearly 950 000 new colorectal cancer cases are diagnosed yearly, with over 490 000 deaths. It affects men and women almost equally, with about 498 000 new cases in men and 446 000 in women per year. Incidence rates of this cancer are highest in North America, Europe, Australia and Japan, and lowest in Africa and Asia. In Finland, three leading cancers in 2003 were lung, prostate and colorectal cancer in men with incidence rates of 95, 34 and 26 per 100 000, respectively. Women's leading cancers in 2003 were breast, colorectal and corpus uteri cancers with incidence rates of 84, 19 and 14 per 100 000, respectively (Finnish Cancer Registry, http:// www.cancerregistry.fi/eng/statistics/ updated 11.7.2005).

Colorectal cancer mainly occurs sporadically and is affected by both genetic and environmental factors (Weitz *et al.* 2005). Ethnic, migrant and twin studies show that environmental factors, including diet, are the strongest contributors to cancer risk, accounting for over 70% of colorectal cancers (Willet 2002, Stewart & Kleihues 2003). Diets high in vegetables and fruits, and perhaps fibre, and low in fat and red meat have been suggested to be associated with a decreased risk of colorectal cancer (Potter 1999). In addition, the use of nonsteroidal anti-inflammatory drugs or hormone replacement therapy has consistently been associated with a reduced risk of colorectal cancer (Grodstein *et al.* 1999, Writing Group for the Women's Health Initiative Investigators 2002, Hawk *et al.* 2004). Physical activity also appears to confer some protection (Potter 1999).

Colorectal cancer is inherited in approximately 5-10% of cases. The two most common inherited forms are familial adenomatous polyposis (FAP) and Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (de la Chapelle 2004). FAP patients have hundreds to thousands of adenomas throughout the colon and rectum. Germline mutations in the *adenomatous polyposis coli* (*APC*) gene underlie FAP (Nishisho *et al.* 1991), and the penetrance of this syndrome is nearly 100%. Lynch patients are prone not only to colorectal cancer but also to cancer of other organs, such as the endometrium, ovaries, stomach and small intestine, which hinders diagnosis of the syndrome (Lynch & de la Chapelle 2003, Lynch *et al.* 2004). Germline mutations in the mismatch repair genes, including *MLH1*, *MSH2* and *MSH6*, cause Lynch syndrome, and the syndromes' penetrance is approximately 80% for colorectal cancer. Microsatellite instability was described as a hallmark of Lynch syndrome (Aaltonen *et al.* 1993).

Most cancer-causing mutations occur in somatic cells, and sporadic colorectal carcinomas arise from a multistage process in which epithelial cells harbour multiple genetic changes in tumour-suppressor genes and proto-oncogenes (Fearon & Vogelstein 1990, Kinzler & Vogelstein 1996). Some of the genetic changes underlying intestinal tumour progression are summarized in **Figure 3**. The adenoma-carcinoma sequence is widely accepted to be one of the main pathways, and the *APC* tumour-suppressor gene to be one of the first genes mutated during intestinal neoplasia (Powell *et al.* 1992). The *APC* gene is mutated in 80% of sporadic colon

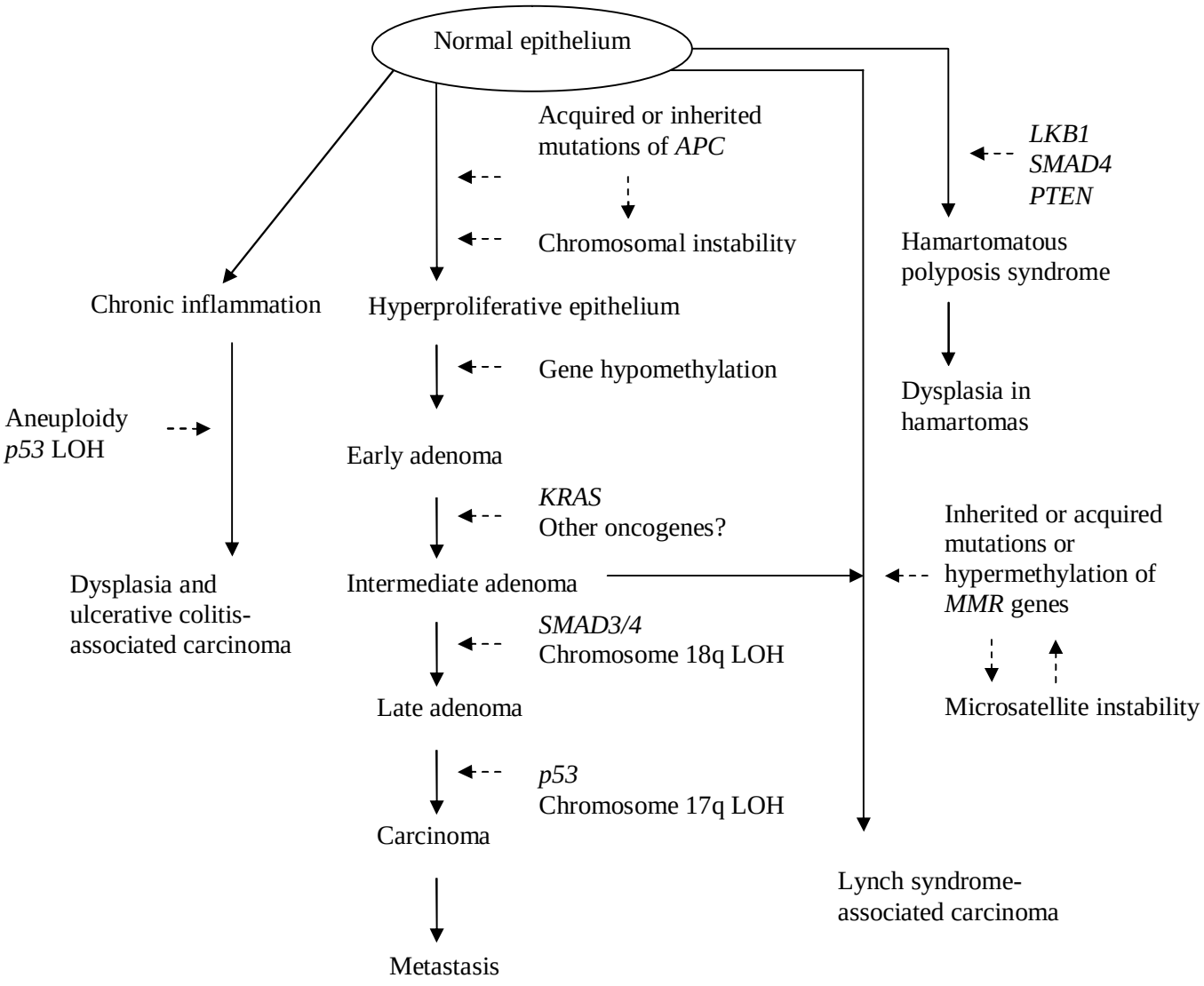


Figure 3. Colorectal carcinomas arise from a multistage process (black arrows) in which epithelial cells harbour multiple genetic changes in tumour-suppressor genes and proto-oncogenes (dashed arrows) (modified from Fearon & Vogelstein 1990, Potter 1999 and Fodde *et al.* 2001). The majority of sporadic tumours are suggested to develop *via* an *APC*-dependent pathway. *APC*, adenomatous polyposis coli; *KRAS*, a proto-oncogene coding a signalling protein; *LKB1*, a serine/threonine kinase gene; LOH, loss of heterozygosity (in cells that carry a mutated allele of a tumour-suppressor gene, the gene becomes fully inactivated when the cell loses a large part of the chromosome carrying the wild-type allele); *MMR* genes, mismatch repair genes; *p53*, a gene coding a multifunction tumour-suppressor protein; *PTEN*, a tumour-suppressor gene coding a protein that attenuates signals originating at tyrosine kinase receptors, e.g. insulin-like growth factor 1 receptor; *SMAD3/4*, genes coding signaling proteins downstream of transforming factor β.

cancers and in all patients with FAP, and is followed by mutation or loss of other tumour-suppressor genes (*p53* and *SMAD2/4*) and mutation of the *KRAS* proto-oncogene. In addition to a mutation in one allele, inactivation of tumour-suppressor genes requires losses of a part of the chromosome carrying the wild-type allele (loss of heterozygosity, LOH). The *APC*-related pathway of transformation is typical of distal (left) colorectal cancers, whereas proximal (right) colorectal cancers more often possess microsatellite instability and defects in mismatch repair genes. A differential pattern of gene expression between the proximal and distal colon may, in part, cause the tissues' susceptibilities to certain pathways of tumorigenesis (Glebov *et al.* 2003).

The APC protein is a huge multifunction protein which contains 2 843 amino acids and regions that interact with several proteins, including axin/conductin, β-catenin, tubulin and microtubule-associated protein EB1 (Fodde *et al.* 2001). Thus, APC is involved in cellular adhesion, migration, signalling and proliferation. Interested readers are referred to an extensive review of the various functions of APC and their roles in colon cancer (Näthke 2004). One well-studied role of APC is in a Wnt signalling. In the cytosol, the APC protein together with glycogen synthesis kinase 3 beta (GSK3β) and axin/conductin post-transcriptionally regulate the level of β-catenin protein (Behrens *et al.* 1998, Hart *et al.* 1998). This complex phosphorylates β-catenin, thus targeting it for ubiquitination and destruction by proteasomes. In the presence of Wnt signalling, a dishevelled (Dsh) protein inhibits APC/GSK3β/axin activity so that more β-catenin is present in cells. In cancer, a dysfunction in the APC protein causes, among other things chromosomal instability in epithelial cells and improper regulation of cellular β-catenin pools (Henderson 2000, Fodde *et al.* 2001). Mutations in the *APC* gene lead to the accumulation of hypophosphorylated β-catenin protein in the cytosol and later in the nucleus, where β-catenin together with transcription factors (T cell factor/lymphoid enhancer-binding factor) (Behrens *et al.* 1996, Korinek *et al.* 1997) constitutively activate the expression of target genes such as *C-myc* and *cyclin-D1* (He *et al.* 1998, Tetsu & McCormick 1999). In addition, genes coding for matrix metalloproteinase matrilysin, peroxisome proliferator-activated receptor δ (*PPARδ*), components of the AP-1 transcription complex (*c-jun* and *fra-1*), urokinase-type plasminogen activator receptor (*uPAR*), zonula occludens- (ZO-1) and vascular endothelial growth factor (VEGF) have been identified as target

genes for the β-catenin/Tcf complex (Crawford *et al.* 1999, He *et al.* 1999, Mann *et al.* 1999, Easwaran *et al.* 2003). The role of β-catenin in intestinal tumorigenesis is further supported by β-catenin mutations with a stable phenotype being found in colorectal tumours lacking *APC* mutations (Morin *et al.* 1997, Sparks *et al.* 1998). β-Catenin binds also to E-cadherin and α-catenin in the adherent junction complexes, which form homophilic interactions between neighbouring cells and anchor to actin filaments (Cowin 1994). Thus, β-catenin has an important role in maintaining cell-cell contacts.

1.3.2 $Apc^{Min/+}$ (Min) mice as a model of colon carcinogenesis

Colon carcinogenesis can be studied either by inducing ACF or tumour formation with chemical carcinogens or by using mouse models with defects in the *Apc* gene (Green & Hudson 2005). One such mouse model for human FAP is multiple intestinal neoplasia (Min) or $Apc^{Min/+}$ mice, which was generated by random chemical carcinogenesis of C57BL/6J mice (Moser *et al.* 1990). Some of the progeny had a phenotype characterized by multiple adenoma formation in the small intestine and colon, and therefore, the gene defect was named multiple intestinal neoplasia. Most of the intestinal adenomas (range 30-100) were in the small intestine, especially in the distal part, and only a few adenomas were found in the colon. The lifespan of Min mice was around 120 days, and the cause of death was severe chronic anaemia and/or intestinal obstruction. Breeding studies showed that tumour development was dependent on a single dominant mutant allele, and the Min character was transmitted to 50% of progeny. The germline mutation was later mapped to mouse chromosome 18, and it appeared to be a transversion point mutation that alters nucleotide 2549 from a T to an A in the *Apc* allele (Su *et al.* 1992). This converts codon 850 from one encoding a leucine to a stop codon, which in turn causes truncated Apc protein formation. Heterozygous Min mice had one wild-type and one mutated *Apc* allele in all somatic and gemline cells. Mice homozygous for the Min mutation died at the latest on day seven of gestation (Moser *et al.* 1995). Intestinal adenomas in Min and in other *Apc*-deficient mice were homozygous for the *Apc* defect, and loss of the chromosome carrying the wild-type *Apc* allele preceded intestinal tumour development (Oshima *et al.* 1995). Inactivation of the normal *APC* allele has also been shown in tumours of FAP patients (Levy *et al.* 1994, Lamlum *et al.* 1999).

Mutations in the *APC* gene predispose people to colorectal cancer, whereas in Min mice, due to their short life span, most of the tumours are benign adenomas, or sometimes adenocarcinomas, but never metastatic tumours. Mutations in *KRAS* and *p53* genes have not been found in Min mice.

Other genes can modify tumour multiplicity in Min mice. Dietrich *et al.* (1993) reported that tumour number in Min mice was strongly affected by genetic background. A *modifier of Min1* (*Mom1*) locus accounts for about 50% of genetic variation in tumour number in two mouse strain backcrosses. A candidate for *Mom1* is the gene for a secretory phospholipase A2 (*sPLA$_2$*). PLA$_2$s represent a family of proteins which cleave membrane phospholipids at the sn-2 position, releasing free fatty acids, including arachidonic acid (Bilger *et al.* 1996). Cyclooxygenase-2 (COX-2) enzyme converts arachidonic acid to prostaglandins such as PGE$_2$. A large body of data supports the important role of COX-2 in colonic neoplasia (Gupta & Dubois 2001). COX-2 expression is elevated in human adenomas and adenocarcinomas and in adenomas of Min mice (Ebenhard *et al.* 1994, Williams *et al.* 1996). Moreover, in an *Apc* mutated background, mice null for COX-2 had suppressed polyp formation (Oshima *et al.* 1996). The risk of colorectal adenoma in human subjects has recently been reported to be modified by single nucleotide polymorphisms in genes related to the arachidonic acid pathway such as secretory and cytosolic PLA$_2$ and COX-2 (Siezen *et al.* 2005). Taken together, these studies have shown the relevance of cellular fatty acid metabolism in colonic neoplasia.

Hormonal factors also seem to play a role in the progression of colorectal cancer in humans since hormone-replacement therapy in postmenopausal women decreases the risk of colorectal cancer (Grodstein *et al.* 1999, Writing Group for the Women's Health Initiative Investigators 2002, Hawk *et al.* 2004). The protective mechanism is unclear, but a decrease in secondary bile acid concentration by oestrogen or a direct effect of oestrogen on epithelial cells has been suggested (McMichael & Potter 1980). A more recent hypothesis involves ERβ, which was cloned in 1996 (Kuiper *et al.* 1996) and found also to be present in the intestinal mucosa of the colon (Foley *et al.* 2000). Endogenous oestrogen was also demonstrated to protect against *Apc*-induced tumour formation, and this protection was associated with a relative increase in ERβ

and a decrease in ERα in intestinal tissues (Weyant *et al.* 2001). Later the same study group showed that treatment of ovaryectomized Min mice with 17β-estradiol and the phytooestrogen coumestrol, but not the soy isoflavone genistein, resulted in a significant reduction in tumour number (Javid *et al.* 2005). The data reported thus far suggest that both endogenous oestrogen and functional ERβ may protect against colon cancer. The proposed actions of lignans in colon tumorigenesis might be direct (inside the intestinal lumen) or indirect (systemic *via* blood), possibly being mediated though the ER. However, ER-independent mechanisms may also be involved.

In summary, the genetic background (defect in *Apc* gene) and phenotype (formation of multiple intestinal adenomas) are similar to Min mice and human FAP patients, with the exception that in Min mice most of the adenomas are found in the small intestine. Despite this, Min mice are considered a suitable model for studying development of intestinal neoplasia. Precancerous lesions (adenomatous polyps and ACF) are also considered one of the strongest end-point markers for dietary chemoprevention studies in animals and in human subjects (Rafter *et al*. 2004).

2 Aims of the study

Based on epidemiological findings and experimental studies, lignans or sources of lignans, such as flaxseed and rye bran, might have a role in colon cancer prevention. The objective of this study was to investigate the effects of plant and mammalian lignans on adenoma formation in Min mice, which serve a model for colon carcinogenesis. Mice were fed diets supplemented with lignan-containing foods, e.g. flaxseed (I, II), rye-bran and its extracts (III), and pure wood-originated lignans 7-hydroxymatairesinol (IV), matairesinol and secoisolariciresinol (V). The formation of the mammalian lignans enterolactone and enterodiol was analysed from intestinal contents and plasma (I-III, V) to determine whether the effect of lignans on adenoma formation in Min mice is direct or systemic. These studies were designed to answer the following questions:

1. Do lignan-rich diets have an effect on intestinal adenoma formation in Min mice?

2. Is there an association between plasma enterolactone level or intestinal lignans and adenoma formation?

3. Are there gender or genotypebased differences in lignan metabolism and adenoma formation?

4. Are there some dietary components in flaxseed and rye bran besides lignans which might be related to adenoma formation?

5. Does a lignan-rich diet affect cell-signalling parameters, such as β-catenin, cyclooxygenase-2 and protein kinase C, in the intestinal tissues of Min mice?

3 Study designs and methods

This section gives a brief overview of study designs and methods. Detailed descriptions of materials and methods can be found in the original publications (I-V).

3.1 Diets and study designs

All diets were semi-synthetic AIN-93G-based (Reeves *et al.* 1993) high-fat diets. The fat concentration (20 g/100 g) and fat composition of the experimental diets were designed to approximate those in a typical Western-type diet; the diet provided intakes of saturated, monosaturated and polysaturated fatty acids in an approximate ratio of 3:2:1. The bran/fibre- supplemented diets were prepared by diluting non-fibre with the addition of a bran/fibre source, and take into account the protein, carbohydrates and fat provided by brans. All diets were similar with respect to protein (20%), carbohydrates (40%) and fat (40%) on an energy basis (kJ), excluding the energy values contained in dietary fibre and derived from fermentation. Lignan levels of the experimental diets and estimated lignan intakes in Studies I-V are summarized in **Table 4**.

Flaxseed is the richest source of lignans in food, and it has been used as a model substance in experimental studies of the relationship between lignans and cancer. **Studies I and II** were designed to test the effect of flaxseed lignans, mainly secoisolariciresinol diglycoside, on adenoma formation in Min mice. Both intestinal lignan levels and plasma enterolactone levels were analysed. Male and female Min mice and wild-type mice were used in Study I to determine whether conversion plant lignans differ between the sexes, and whether a genotype-based difference exists in lignan metabolism between Min and wild-type mice. In Study II, the flaxseed mixture diet was composed such that the level of defatted flaxseed was five times higher than in Study I. Furthermore, to elucidate the role of the oil component of the flaxseed mixture in adenoma formation, a lignan-free diet with an oil composition similar to the flaxseed-supplemented diet was made. In Study II, the intestinal lignan level, the plasma enterolactone level, the fatty acid composition of the colon mucosa and cell

Table 4. Lignan levels of experimental diets, and estimated lignan intakes in Studies I-V.

Study	Diets	Lignan level/kg diet		Estimated lignan intake/day bw kg in aglycone form[3]	
		μmol	mg	μmol	mg
I	Non-fibre (control)	<0.23	<0.1	-	-
	Defatted flaxseed 0.5%	73	26	7	2.5
II	Non-fibre (control)	nd[1]	nd[1]	-	-
	Flaxseed mixture 15%	380[2]	140[2]	38	13
	Oil mixture 9%	nd[1]	nd[1]	-	-
III	Non-fibre (control)	0.4	0.1	-	-
	Rye bran 10%	15.4	5.5	1.4	0.5
	Soluble extract 7.9%	20.5	7.3	2	0.7
	Insoluble extract 7.9%	7.5	2.7	<1	<0.26
IV	Inulin 2.5% (control)	nd[1]	nd[1]	-	-
	Inulin 2.5% + Rye bran 10%	nd[1]	nd[1]	-	-
	Inulin 2.5% + 7-hydroxy-matairesinol extract 0.02%[4]	260	94	25	9
V	Non-fibre (control)	nd	nd	-	-
	Matairesinol 0.02%	560	200	50	20
	Secoisolaricirecinol 0.02%	560	200	50	20

[1] nd, not determined.
[2] Calculations were based on the lignan level of defatted flaxseed (5 mg/g) in Study I.
[3] Food consumption per mouse was estimated to be 2.4 g per day.
[4] Extract contained 47% 7-hydroxymatairesinol.

signalling parameters, such as β-catenin, cyclooxygenase-2 and protein kinase C-ζ, were analysed.

Study III evaluated whether two rye-bran fractions, i.e. the soluble extract and the insoluble fraction, result in distinct bifidogenic effects or enterolactone production in Min mice, and whether these parameters are associated with intestinal tumorigenesis in this animal model. The fractions of rye bran were prepared on the basis of water solubility, which resulted in different fibre compositions and *in vitro* fermentation properties of the two rye fractions. The spectrum of rye-bran lignans differs from that of flaxseed, with the main lignan in rye bran being syringaresinol, although pinoresinol, lariciresinol, secoisolarisiresinol and matairesinol are also found. Processing of rye bran did not affect relative proportions of various lignans, but total lignan concentrations of rye fractions were changed. The soluble extract had the highest and the insoluble fraction the lowest lignan concentration. In Study III, the mice were fed the experimental diets twice, and the two experiments were referred to

as Experiments 1 and 2. The adenoma and *Bifidobacterium* results were presented as pooled from Experiments 1 and 2, and lignan results from Experiment 2 alone.

Methods for isolating wood-originated lignans in large quantities from branch knots of Norway spruce (*Picea abies*) are available (Ekman 1976). The major lignan isolated from branch knots is 7-hydroxymatairesinol, which represents up to 60% of all isolated lignans. 7-Hydroxymatairesinol can be used as a starting material for chemical synthesis to produce other pure lignans, e.g. matairesinol and secoisolariciresinol. **Studies IV and V** were carried out to test the effects of pure lignans 7-hydroxymatairesinol (IV), matairesinol and secoisolariciresinol (V) on adenoma formation in Min mice. Subcellular expression of β-catenin was analysed in the adenoma and mucosa tissues in Study IV.

3.2 Sources of lignans

Defatted flaxseed came from Elixi Oil Oy (Somero, Finland), and flaxseed (Linobene®) and oil mixtures from HK-Ruokatalo (Vantaa, Finland). Dr. Sirpa Karppinen (VTT, Espoo, Finland) prepared and analysed the insoluble fraction and the soluble extract of rye bran as described in Study III. Rye bran was purchased from Melia (Raisio, Finland).

The isolation and composition of the (–)-7-hydroxymatairesinol extract from Norway spruce (*Picea abies*) was similar to that described by Saarinen *et al.* 2000. The isolate was a gift from Hormos Medical Ltd. (Turku, Finland), and it contained 47% 7-hydroxymatairesinol, 17% other lignans (liovil, secoisolariciresinol, conidendric acid, matairesinol, lignan A, conidendrin and lariciresinol) and 36% polar, non-volatile, high molecular weight organic components. 7-Hydroxymatairesinol was a mixture of two diastereomers, (–)-7-hydroxymatairesinol (major isomer) and (–)-7-*allo*-hydroxymatairesinol (minor isomer) (Mattinen *et al.* 1998).

(–)-Matairesinol was synthesized from 7-hydroxymatairesinol isolated from Norway spruce according to Eklund *et al.* (2003). (–)-Secoisolariciresinol was synthesized from matairesinol by reduction with $LiAlH_4$ essentially as described by Eklund *et al.*

(2002). Purities of the synthesized compounds were over 95%. Associate Professor Rainer Sjöholm's research group (Åbo University, Finland) provided both matairesinol and secoisolariciresinol for diet experiments.

3.3 Experimental animals

The Laboratory Animal Ethics Committee of the University of Helsinki approved the study protocols of all experiments (I-V). Male C57BL/6J- *Apc*$^{Min/+}$ (Min) mice (5-6 weeks of age) for Studies III (Experiment 1) and IV were obtained from Jackson Laboratory, ME, USA. The Min pedigree was maintained at the Laboratory Animal Centre, University of Helsinki, by mating wild-type C57BL/6J females with Min males originally obtained from Jackson Laboratory. Offspring were genotyped after weaning by using Promega's Wizard® Genomic DNA Purification Kit followed by allele-specific polymerase chain reaction (Dietrich *et al.* 1993). Male and female Min mice aged 5-6 weeks were used for Studies I-III (Experiment 2) and Study V, and their wild-type siblings were also used in Study I. Mice were assigned randomly to the experimental diets and housed in plastic cages, with a controlled room temperature (20-22^{o}C) and a 12-h light-dark cycle. They had free access to the experimental diets and tap water. The body weights of the animals were recorded weekly. If mice had a rapid decrease in weight or a prolapse of the rectum, they were excluded from the experiment.

3.4 Analytical methods

3.4.1 Tumour scoring and sample collection

At the end of the feeding periods, mice were killed by carbon dioxide asphyxiation at the age of 11-12 weeks (I, III, IV) or 15 weeks (II, V). A blood sample was collected from the abdominal aorta and centrifuged at 6000 x *g* for 1 min, after which the plasma was stored at -70^{o}C for enterolactone analyses. At autopsy, the uterus was excised and weighed (V). The small intestine and colon were removed and cut open longitudinally, and the contents of the small intestine and caecum were collected and stored at -70^{o}C for lignan analysis (I, II) and *Bifidobacterium* analysis (III). The intestinal tissues were washed with ice-cold saline, and scoring of adenomas was done as described by Mutanen *et al.* (2000). Briefly, the small intestine and colon + caecum

were rinsed after they were spread flat, mucosal surface up, on a microscope slide. The number, diameter and location of adenomas were determined with a dissection microscope with a magnification of 67 x by two observers blind to the dietary treatment. The minimum detection limit of the adenoma diameter was 0.3 mm. Adenoma tissue was clipped off, and the small intestinal mucosa was scraped. Tissues were snap-frozen in liquid nitrogen and stored at -70^{o}C for further analysis.

3.4.2 β-Catenin, COX-2 and PKC protein analysis by Western blot

Subcellular localization of β-catenin and PKC is considered to be an important determinant of their function. The mucosa and adenoma tissues of the small intestine were therefore fractionated into nuclear, cytosolic and membranous fractions as described by Pajari *et al.* (1998, 2003) for Western blot analysis. Normalized amounts of the fractionated proteins and controls for inter-assay variation were resolved by sodium dodecyl sulphate – polyacryl amide gel electrophoresis, and blotted onto membranes capable of binding proteins. Signals of specific proteins were visualized by using primary and secondary antibodies as described in Studies II and IV. Blots were scanned and analysed, and results in duplicate were expressed as sample band intensity (optical density of the band multiplied by the band area) divided by control band intensity.

3.4.3 Lignan analysis

In Studies I-III, Professor Herman Adlercreutz's group (University of Helsinki, Finland) carried out lignan analysis of tissue samples, brans and diets. Plasma enterolactone was analysed by using time-resolved fluoroimmunoassay (Adlercreutz *et al.* 1998, Stumpf *et al.* 2000*b*), and lignans (enterodiol, enterolactone, and secoisolariciresinol) of the small intestine and caecal contents were measured by using HPLC with coulometric electrode array detector (Mazur *et al.* 1996, Nurmi & Adlercreutz 1999). Lignans (lariciresinol, matairesinol, pinoresinol, secoisolariciresinol and syringaresinol) of brans and diets were analysed by using gas chromatography-mass spectrometry (Mazur *et al.* 1996, modified by Nurmi, Heinonen and Adlercreutz, unpublished results). In Study V, the plasma lignans (cyclolariciresinol, enterodiol, enterolactone, 7-hydroxyenterolactone, 7-hydroxymatairesinol (both diastereomers), lariciresinol, matairesinol and

secoisolariciresinol) were analysed by Associate Professor Rainer Sjöholm's group (Åbo University, Finland) by using HPLC-tandem mass spectrometry. Due to the analysis procedure, the value of each lignan represents the sum of all conjugated and unconjugated forms of this particular lignan.

3.4.4 Bifidobacterium analysis

Dr. Jaana Mättö (VTT Biotechnology, Finland) carried out *Bifidobacterium* analysis as described in Study III. Bifidobacteria were enumerated from the samples by culturing on bifidobacteria-selective agar and identified by colony and microscopic morphology and simple phenotypic tests. The detection limit was 10^4 colony-forming units/g wet weight.

3.4.5 Fatty acid analysis

In Study II, Irma Salminen (National Public Health Institute, Finland) carried out fatty acid analysis of the colonic mucosa tissue by using gas chromatography. Values for fatty acids are expressed as a percentage of total fatty acids.

3.5 Statistical analysis

Data were analysed either with the non-parametric Kruskal-Wallis test followed by the Mann-Whitney U-test (IV) or with the Mann-Whitney U-test alone to compare experimental groups with the control group, and genders within a diet group (I-III,V). Associations between variables were analysed with the non-parametric Spearman's correlation test (I, II). Colon adenoma number was evaluated with by χ^2 test, and two-way analysis of variance (general linear model) was used to test normally distributed plasma enterolactone data for diet x gender and diet x genotype interactions in Study I. Distributions of two variables were analysed with the Wilcoxon signed-ranks test (II). Statistical analyses were performed with SPSS software, version 6.1-10.0 (SPSS Inc., Chicago, IL) or Stat View software, version 5.0.1 (SAS Institute Inc., Cary, NC). Differences were considered significant at $P < 0.05$.

4 Results and discussion

4.1 General observations

In our experience, weight gain of a Min mouse during the feeding period is an indicator of a mouse's health, and a decrease in weight at the end of the feeding period is usually associated with a high tumour load. The weight gain and final body weights did not differ between dietary groups in Studies I, II (females) and III-V. In Study II the Min males fed the flaxseed diet had a significantly higher body weight at the end of the 10-week feeding period than control males ($P < 0.05$). Visual evaluation of mice during the feeding period revealed that 50% of animals in the 7-hydroxymatairesinol group had some shedding of hair, which might indicate that the level of 7-hydroxymatairesinol extract in the diet or its composition had some unwanted effects. When matairesinol and secoisolariciresinol were fed to mice (Study V), no such effect on fur was observed.

4.2 Adenoma number and size

The first aim of this project was to determine whether lignan-rich diets affect intestinal tumour formation in Min mice. The adenoma results from all studies are summarized in **Table 5**. The end-points were the number and size of tumours in the small intestine and colon, and the incidence of colon adenomas (the number of tumour-bearing animals). The range of adenoma number and adenoma distribution between the small intestine and the colon here were similar to those found in other studies (Moser *et al.* 1990, Corpet & Pierre 2003).

Table 5. Effects of experimental diets on adenoma number and size of multiple intestinal neoplasia (Min) mice in Studies I-V.

			Small intestine		Colon and caecum			
Study	Diets	*n*	Number[1]	Size (mm)[1]	Incidence	(%)[2]	Number[1]	Size (mm)[1]
I	Non-fibre	12 ♂	57 ± 30	1.0 ± 0.2	7/12	(58)	1.2 ± 1.1	2.7 ± 0.7
		13 ♀	57 ± 27	0.9 ± 0.1	8/13	(62)	0.7 ± 0.6	2.0 ± 0.6
	Defatted flaxseed	17 ♂	46 ± 17	1.0 ± 0.2	9/17	(53)	0.8 ± 0.9	2.5 ± 0.7
		16 ♀	56 ± 27	0.9 ± 0.1	5/16	(31)	0.4 ± 0.6	2.4 ± 0.7
II	Non-fibre	14 (total)	54 (31, 86)	1.2 (0.9, 1.3)				
		8 ♂			4/8	(50)	0.5 (0, 3)	2.9 (2.6, 3.1)
		6 ♀			0/6	(0)	-	-
	Flaxseed mixture	11 (total)	37 (13, 81) *	0.9 (0.8, 1.2) *				
		6 ♂			3/6	(50)	0.5 (0, 2)	3.5 (3.3, 3.8) *
		5 ♀			1/5	(20)	0 (0, 2)	3.3 (3.3, 3.3)
	Oil mixture	11 (total)	42 (15, 90) [§]	1.0 (0.7, 1.4) *				
		6 ♂			2/6	(33)	0.5 (0, 3)	3.0 (2.8, 3.3)
		5 ♀			2/5	(40)	0 (0, 2)	1.7 (1.1, 2.3)
III [3]	Non-fibre	21 ♂&♀	31 ± 11	1.2 ± 0.2	7/21	(33)	0.5 ± 0.9	2.7 ± 0.8
	Rye bran	23 ♂&♀	42 ± 25	1.2 ± 0.2	15/23 *	(65)	1.3 ± 1.5 *	2.7 ± 0.8
	Soluble extract	23 ♂&♀	44 ± 23 [‡]	1.4 ± 0.2	16/23 *	(70)	1.0 ± 1.0 *	2.8 ± 1.0
	Insoluble extract	12 ♂	38 ± 14	1.3 ± 0.2	8/12	(67)	0.9 ± 0.9	2.9 ± 1.1
IV	Inulin	8 ♂	40 ± 9	1.2 ± 0.1	6/8	(75)	1.3 ± 1.1	1.0 ± 0.7
	Inulin + Rye bran	7 ♂	36 ± 7	1.2 ± 0.2	4/7	(57)	0.9 ± 0.9	1.2 ± 1.2
	Inulin + 7-hydroxymatairesinol extract	8 ♂	27± 11 *	1.1 ± 0.2	6/8	(75)	1.3 ± 1.1	1.1 ± 0.8
V	Non-fibre	26 ♂&♀	51 ± 27	1.2 ± 0.2	13/26	(50)	0.5 ± 0.1	3.0 ± 0.8
	Matairesinol	11 ♂&♀	67 ± 25 [†]	1.4 ± 0.2 *	4/11	(36)	0.4 ± 0.5	2.9 ± 1.1
	Secoisolariciresinol	13 ♂&♀	54± 28	1.2 ± 0.2	3/13	(23)	0.4 ± 0.9	2.4 ± 0.8

[1] Values are mean ± SD or median (min, max). * Significantly different ($P < 0.05$) compared with the control group; [§] P = 0.095; [‡] $P = 0.063$; [†] $P = 0.052$.
[2] The number of animals (%) bearing tumours in the colon and caecum.
[3] Data of the pooled results of experiments 1 and 2 are presented in Study III. See the original publication for further details.

In Studies I and II, Min mice were fed diets containing either low or high levels of flaxseed, providing mainly secoisolariciresinol diglycoside at levels of 73 and 380 μmol per kg diet, respectively. A diet with a low amount of flaxseed (Study I) had no effect on adenoma number and size in the small intestine. Nor was there any change between diet groups in adenoma number, size, incidence or distribution in the colon, although the flaxseed-fed males and females had a non-significant tendency for a decreased adenoma number in the colon. In the subsequent flaxseed study (II), the flaxseed mixture rich in lignans and α-linolenic acid decreased the number of adenomas in the small intestine by 31% ($P < 0.05$). The adenoma size of the small intestine was also decreased in the flaxseed mixture group and in the oil group (both $P < 0.05$). The males of the flaxseed mixture group had an increased adenoma size in the colon ($P < 0.05$). Otherwise, the incidence, number and size of the colon adenomas were not different between control and treatment groups.

In Study III, diets supplemented with rye bran and rye fractions contained mainly syringaresinol plus other lignans at levels between 7 and 21 μmol per kg diet. The pooled results of Experiments 1 and 2 showed that the soluble extract increased (albeit not significantly) the number of adenomas in the small intestine ($P = 0.063$). The major differences between the experimental groups were found in the distal small intestine, where most of the adenomas were situated (see detailed results in the original publication). The rye-bran and the soluble-extract groups ($P = 0.032$-0.037) had significantly more colon adenomas than the non-fibre control group. The size of the colon adenomas was not significantly altered between the experimental groups. The colon adenoma number of the rye-bran group might be a chance result since no difference in colon adenoma number was found between the rye-bran (10%) and non-fibre control groups in our previous study (Mutanen *et al.* 2000). The effect of rye bran on adenoma formation was also neutral in Study IV. In this study, the mice fed the diet supplemented with inulin + rye bran had a similar adenoma number and size to mice fed the inulin (control) diet.

Crude 7-hydroxymatairesinol extract, and pure matairesinol and secoisolariciresinol preparations were used in Studies IV and V, providing lignans at levels of 260 and 560 μmol per kg diet, respectively. The diet supplemented with the 7-hydroxymatairesinol extract decreased the number of adenomas in the small intestine by 33% ($P < 0.05$) compared with the control group. The matairesinol-fed mice had an increased adenoma size ($P < 0.05$) in the small intestine, whereas secoisolariciresinol had no effect on adenoma formation in the small intestine. The colon adenoma parameters did not differ between groups in Studies IV and V.

Do lignan-rich diets have an effect on intestinal adenoma formation in Min mice when adenoma number and size are the end-points? The results of Studies I-V do not give an unequivocal answer to this question. Defatted flaxseed at moderate levels was not protective against intestinal adenoma formation in Study I. In the subsequent flaxseed study, the oil diet inhibited intestinal adenoma growth nearly as effectively ($P < 0.05$) as the flaxseed mixture diet, suggesting that the oil component was mainly responsible for the antitumour effect of the flaxseed diet and not the lignans *per se*. In Study III, the soluble extract, which was partly hydrolysed and easily fermentable, contained a large amount of mammalian lignan precursors, supported the growth of *Bifidobacterium* and promoted adenoma growth in Min mice. Adenoma sizes of the non-fibre (lignan-free) and rye-bran groups were, however, smaller than that of the soluble-extract group. The effects of these rye preparations on adenoma formation thus appeared not to be mediated through lignans. In Studies IV and V, pure lignan isolates were used, and therefore, changes in tumour formation in Min mice could be related directly to lignans. The effect of 0.02% secoisolariciresinol was neutral with regard to adenoma formation in the small intestine and colon. However, it is interesting that both flaxseed (diet providing secoisolariciresinol diglycoside) and secoisolariciresinol groups had a tendency for decreased adenoma number and incidence in the colon in Studies I and V. At present, we do not have an explanation for this observation, nor is it easy to confirm since a low colonic adenoma number is not an ideal end-point marker in Min mice.

The chemical structures of 7-hydroxymatairesinol and matairesinol differ only by one hydroxyl group. However, discordant effects on adenoma formation in Min mice were found after 7-hydroxymatairesinol and matairesinol feedings. The diet supplemented

with the 7-hydroxymatairesinol extract inhibited and that with matairesinol promoted tumour formation in the small intestine. This controversy may be due to different experimental designs, including composition of experimental diets, or it may partly reflect true differences in effects of these lignans. The control diet in Study I was 2.5% inulin, which was used to promote adenoma formation, and not the non-fibre diet used in Studies II-V. Differences in the chemical structure of pure compounds might also be highly important with regard to lignan metabolism and chemoprevention. Saarinen *et al.* (2002*b*) reported that the urinary excretion of enterolactone in rats was greater in the matairesinol group than in the 7-hydroxymatairesinol group after a single oral dose of aglycone lignans (25 mg/kg per kg body weight). The authors suggested that the presence of the hydroxyl group at carbon atom number 7 in the 7-hydroxymatairesinol molecule slowed down the formation of enterolactone, resulting in a higher level of 7-hydroxymatairesinol inside the gut lumen. Saarinen *et al.* (2005) reported also that nortrachelogenin which resembles 7-hydroxymatairesinol, but has a hydroxyl group at C-8 instead of C-7, was not enterolactone precursor *in vivo*. Furthermore, 7-hydroxymatairesinol but not nortrachelogenin has been shown to inhibit the growth of mammary tumours in carcinogen-treated rats (Saarinen *et al.* 2000, 2001). In our study, the 7-hydroxymatairesinol extract also contained other lignans, which might together affect adenoma formation.

Due to the limited availability of purified lignans, only a few studies have attempted to elucidate the role of lignans in animal models for colon cancer. Jenab and Thompson (1996) and Hirose *et al.* (2000) have shown protective effects of secoisolariciresinol and arctiin on early precancerous lesions in carcinogen-treated rats. Recently, van Kranen *et al.* (2003) reported a study where Min mice were fed 5% flaxseed or 30% rye-bran diets, which provided flaxseed and rye-bran lignans of 140 mg and 0.6 mg per kg diet, respectively. The authors reported that neither 5% flaxseed nor 30% rye-bran diets were able to decrease intestinal adenoma formation; in fact, female Min mice fed a 30% rye-bran diet had an increased number of small intestinal adenomas.

In conclusion, the current experimental evidence regarding the role of lignans in colon neoplasia is limited and shows conflicting results. Our findings as well as those of van Kranen *et al.* (2003) do not support the assumption that flaxseed and rye diets providing such lignans as secoisolariciresinol or syringaresinol would exert an anticarcinogenic

effect in Min mice. Secoisolariciresinol administered as a pure compound at a level 0.02% (w/w) also failed to show a chemopreventive effect. The effects of matairesinol and 7-hydroxymatairesinol on adenoma formation in Min mice were conflicting and should be clarified in further studies.

4.3 Mammalian lignan levels in the plasma

Our results showed that plasma enterolactone or enterodiol levels did not explain adenoma formation in Min mice. We can not state that a high mammalian lignan level is necessary for colon cancer chemoprevention, and the oil diet without lignans was protective against adenoma growth in this mice model. Our findings are in line with a study of human subjects, in which plasma enterolactone level was not found to be associated with colorectal cancer risk (Lundin 2001). However, experimental and human studies have shown that the role of plasma enterolactone in other types of cancer, e.g. mammary cancer, may be more prominent. The plasma lignan results are therefore discussed in an attempt to shed light on lignan metabolism *in vivo*.

The plasma enterolactone levels of Min mice fed the experimental diets are summarized in **Figure 4**. The order of enterolactone production between dietary groups was as follows: matairesinol group (median 203 nmol/l) > secoisolariciresinol group (median 85 nmol/l) ≈ flaxseed mixture group (median 97 nmol/l) > 0.5% flaxseed group (median 47 nmol/l) > 10% rye-bran group (median 32 nmol/l) > 7.9% soluble-extract group (median 15 nmol/l) > non-fibre control and oil groups (median < 10 nmol/l). All lignan-supplemented groups had significantly higher levels of plasma enterolactone($P < 0.05$) than the control groups. Flaxseed and rapeseed oils in the oil diet were not sources of plant lignans, and thus, intestinal or plasma lignan levels of the oil group did not differ from the control group. Overall, rye-diet-fed mice were lower enterolactone producers than flaxseed- or wood-lignan-fed mice. Rye bran is a source of other plant lignans, such as lariciresinol, pinoresinol and syringaresinol, in addition to matairesinol and secoisolariciresinol, which are the two lignans known to metabolize to enterolactone and enterodiol *in vivo*. The *in vitro* conversion of lariciresinol, pinoresinol and syringaresinol to both enterodiol and enterolactone has been reported (Heinonen *et al.* 2001).

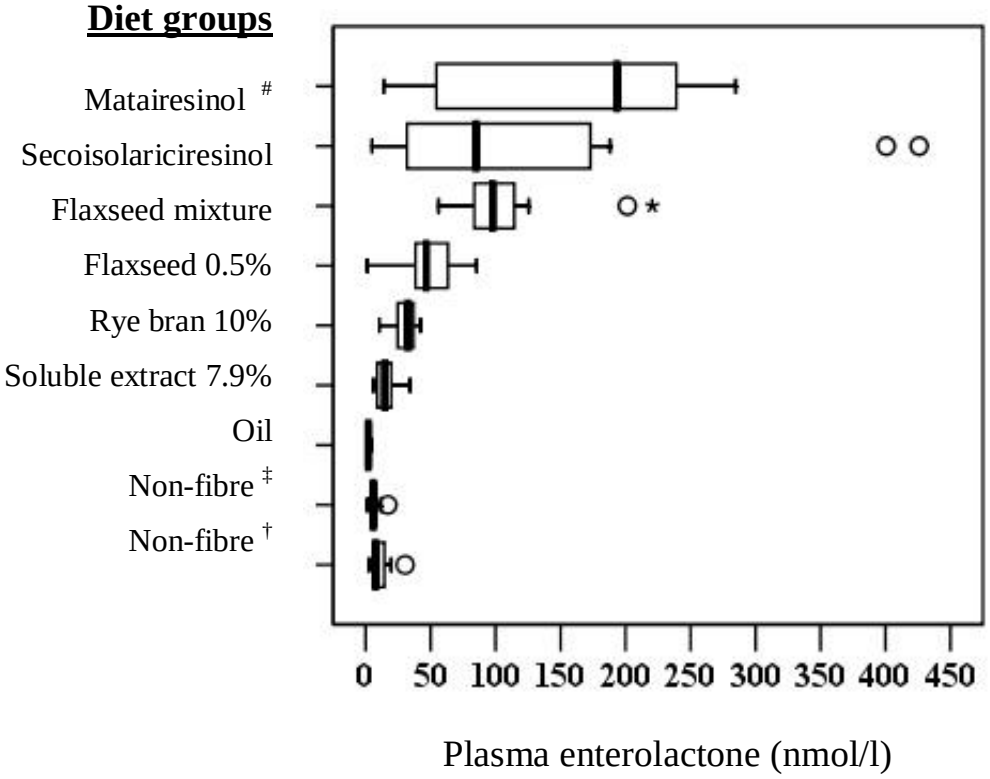


Figure 4. Plasma enterolactone levels (nmol/l) of multiple intestinal neoplasia (Min) mice fed the experimental diets in Studies I-III and V. For each group, the box represents the interquartile range, which contains 50% of values. The whiskers (vertical lines) extend from the box to the highest and lowest value. Horizontal lines across the boxes indicate medians. Outliers (○) are cases with values between 1.5 and 3 box-lengths from the upper or lower edge of the box, and extremes (*) are values more than 3 box-lengths from the upper or lower edge of the box. # An extreme enterolactone value (975 nmol/l) in the matairesinol group was excluded from the figure to make box-plots more understandable. ‡ The box represents the pooled plasma enterolactone values of all non-fibre control groups analysed by time-resolved radioimmunoassay. † The box represents those plasma enterolactone values analysed by high-performance liquid chromatography-tandem mass spectrometry.

A large inter-individual variation in plasma enterolactone was found within the dietary groups. This variation was largest in the matairesinol and secoisolariciresinol groups. The reason for this is unclear. Similarly, a large inter-individual variation in serum lignan levels has been found in experimental animals and in human subjects consuming their habitual diets (Kilkkinen *et al.* 2001, Saarinen *et al.* 2001, Johnsen *et al.* 2004).

Because no reference was available in the literature, we also examined whether gender or genotype differences are present in lignan metabolism in mice. We found that plasma enterolactone did not differ between wild type and Min mice in Study I or between genders in Studies II-V with the exception of secoisolariciresinol-fed mice: the plasma enterolactone level was lower in males (49 nmol, $n = 6$) than in females (224 nmol, $n = 6$, $P = 0.010$) fed the same diet. This result may, however, be a chance result.

In Study V, nine different lignans were analysed in plasma of the Min mice. The plasma matairesinol, secoisolariciresinol, enterodiol and enterolactone levels of mice fed the experimental diets are summarized in **Figure 5**. Levels of 7-hydroxymatairesinol (both diastereomers), 7-hydroxyenterolactone, lariciresinol and cyclolariciresinol were near or below detection limits in most mice, and therefore, the data are not shown. Both matairesinol and secoisolariciresinol were efficiently absorbed. This is in line with other studies (Saarinen *et al.* 2001, 2002*b*, Smeds *et al.* 2004) reporting that the plant lignan 7-hydroxyenterolactone can be found in the serum of 7-hydroxyenterolactone-fed rats, and that both a single oral dose and a ten-day administration of secoisolariciresinol and matairesinol results in considerable excretion of secoisolariciresinol and matairesinol in urine. The levels of plant lignans in the plasma of matairesinol- and secoisolariciresinol-fed mice were surprisingly high (medians over 200 nmol/l), which presents the possibility that plant lignans, at least in the case of matairesinol, have an independent effect on tumorigenesis.

The plasma enterolactone levels did not directly reflect lignan levels in diets. Although a fivefold difference was present in the dietary levels of defatted flaxseed between the two flaxseed studies, only a twofold difference was found in plasma enterolactone levels. The level of flaxseed in the second study may have exceeded the capacity of microflora to metabolize lignans. Similarly, the results of the rye study indicated that the plasma enterolactone levels did not directly reflect the analysed lignan contents of

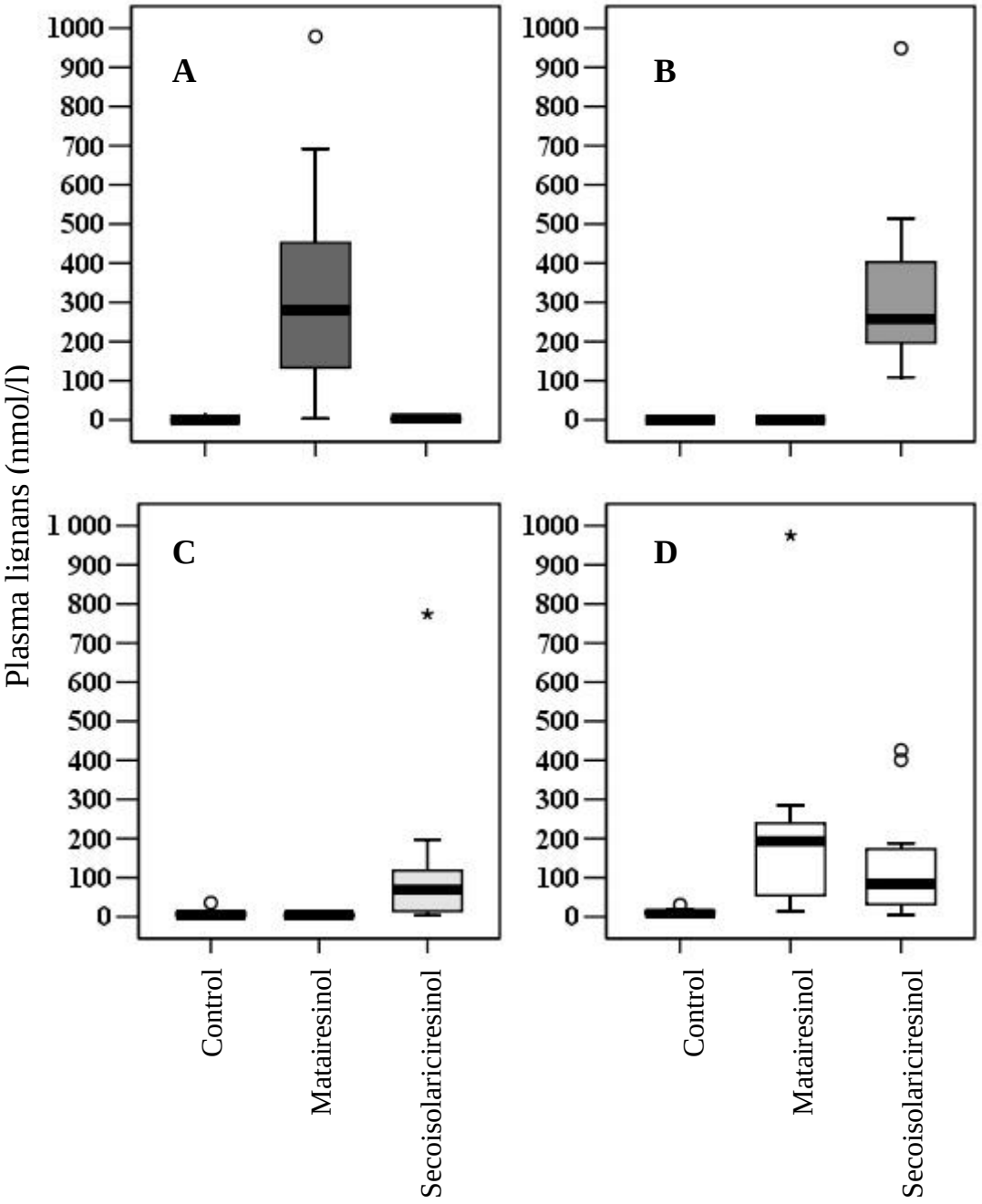


Figure 5. Plasma matairesinol (A), secoisolariciresinol (B), enterodiol (C) and enterolactone (D) levels (nmol/l) of male and females multiple intestinal neoplasia (Min) mice fed the experimental diets in Study V. For each group, the box represents the interquartile range, which contains 50% of values. The whiskers (vertical lines) extend from the box to the highest and lowest value. Horizontal lines indicate medians across the boxes. Outliers (○) are cases with values between 1.5 and 3 box-lengths from the upper or lower edge of the box, and extremes (*) are values more than 3 box-lengths from the upper or lower edge of the box.

the rye diets. Based on the lignan analysis, the soluble-extract diet contained more lignans than the rye-bran diet. Mean plasma enterolactone was, however, highest in the rye-bran group (mean 30 nmol/l), and not the soluble-extract group (16 nmol/l). The ray-bran matrix may be more resistant to analytical hydrolysis than the soluble fraction, but may be degraded extensively inside the colon, thus freeing bound lignan for metabolism and absorption. In addition, slowly fermentable rye bran may be more susceptible to enterolactone formation than the fermentable soluble extract. Intestinal microflora adaptation to the experimental diets could also lead to differences in plasma enterolactone levels.

Rickard *et al.* (2000) have reported that flaxseed and/or secoisolariciresinol reduced plasma concentration of insulin-like growth factor (IGF)-1 in rats treated with or without *N*-methyl-*N*-nitrosourea carcinogen. The authors suggested that the anticancer effect of flaxseed and lignans may be related, in part, to the reduction of plasma IGF-1 level, with the major target organ for lignan action suggested to be the liver, which also regulates the synthesis of IGFs and IGF-binding proteins. Interested readers are referred to Pollak *et al.* (2004), who recently reviewed the role of the IGF-1 signalling pathway in cellular proliferation, survival and apoptosis. To test this hypothesis between lignans and plasma IGF-1 levels, we analysed the plasma IGF-1 levels of mice fed control, flaxseed and oil diets in Study II. Plasma samples were treated and IGF-1 levels were analysed by radioimmunoassay according to manufacturer's instructions (rat IGF-1 RIA, Diagnostic Systems Laboratories, Inc., Webster, Tx). We found no significant differences in plasma IGF-1 levels between the dietary groups. The plasma IGF-1 levels (ng/ml) were 498 ± 108, 557 ± 74 and 520 ± 111 (mean ± SD) for the control, flaxseed and oil groups, respectively (unpublished results). That the plasma IGF-1 levels of the flaxseed and oil groups appeared to be slightly elevated compared with the control group, but these values did not reach statistical significance ($P = 0.189$ and $P = 0.584$ for flaxseed and oil groups, respectively). Thus, our preliminary result with IGF-1 does not support the suggestion that plasma IGF-1 has a role in prevention of adenomas in Min mice.

4.4 Mammalian lignan production inside the intestinal lumen

Mammalian lignan formation or secoisolariciresinol level did not explain adenoma number or size in Min mice, although substantial levels of lignans were present in the lumen. Interestingly, the Min males fed the flaxseed diets had a higher level of intestinal enterolactone than the Min females fed the same diet. In addition, wild-type mice fed the flaxseed diet had a gender difference in caecal enterolactone levels. The reason for this is unclear, and no comparable studies exist in the literature. Bacterial bioconversion of the plant lignan secoisolariciresinol to the mammalian lignans or enterohepatic circulation of enterolactone might be different between male and female mice. Further study of lignan metabolism is thus warranted. We also found that Min genotype or phenotype by some unknown mechanism influences intestinal lignan metabolism. Min mice had less intestinal enterodiol and enterolactone than their wild-type littermates. However, the responses to diets were similar in both genotypes. One explanation for these results may be that bleeding from the intestinal adenomas changes levels or populations of microorganisms or bacterial enzyme activities, thus modifying dietary lignan metabolism. We also found that plasma enterolactone levels do not reflect gender or genotype differences in gut enterolactone.

4.5 Effects of other dietary components in flaxseed and rye fractions on adenoma formation in Min mice

4.5.1 Fatty acids

The type of fat has been suggested to affect colonic neoplasia in experimental animals and human subjects (Hansen Petrik *et al.* 2000, Davidson *et al.* 2004, Roynette *et al.* 2004). Particularly, the roles of n-3 and n-6 polyunsaturated fatty acids (PUFAs) on colon tumorigenesis have been studied extensively. In general, n-3 PUFAs (from fish oil) have been proposed to act as tumour preventive agents in colon cancer experiments. The proposed underlying mechanisms by which these fatty acids might exert their effect on colonic neoplasia include alteration of membrane phospholipid turnover and prostaglandin synthesis (Rao *et al.* 1996), downregulation of protein kinase C βII activity (Murray *et al.* 2002) and anti-apoptotic Blc-2 protein level (Hong *et al.* 2003) and increased apoptosis of colonic cells (Davidson *et al.* 2004).

The role of another n-3 PUFA, namely α-linolenic acid (18:3n-3), in chemoprevention was evaluated in Study II. The control diet contained (g/kg diet) 24 g of linoleic acid, whereas the values of α-linolenic acid were 37-39 g in both flaxseed and oil diets. Both flaxseed and oil diets similarly changed the colonic fatty acid profile, a marker for fatty acid intake (see Table 4 in Study II). Changes in proportions of PUFAs also led to changes in the ratio of n-6 to n-3 PUFAs, which were 1.6:1 ($P < 0.001$) and 1.7:1 ($P < 0.001$) for the flaxseed and oil groups, respectively, and 4.4:1 for the control group. Thus, the effect of oil may be due to a direct effect of α-linolenic acid or changes in the ratio of n-6 to n-3 fatty acids of the intestinal mucosa. α-Linolenic acid has been reported to decrease tumour or ACF formation in some but not all studies of colon cancer (Narisawa *et al.* 1994, Onogi *et al.* 1996, Hansen Petrik *et al.* 2000, Dwivedi *et al.* 2005). Furthermore, the oil component of flaxseed was considered less important than lignans as an effective agent by Serraino and Thompson (1992) and Jenab and Thompson (1996).

COXs, the key enzymes in regulating the synthesis of such prostaglandins as prostaglandin E_2 (PGE_2), have been suggested to be responsible for cancer-promoting effects in humans and animals. Wang *et al.* (2005) recently demonstrated that PGE_2 can enhance intestinal proliferation and COX-2 expression in adenomas of Min mice through a positive feedback loop. Furthermore, the authors reported that activation of a Ras-mitogen-activated protein kinase (MAPK) signalling cascade was required for PGE to induce COX-2 expression. Hansen Petrik *et al.* (2000) have similarly shown that the antitumorigenic effect of n-3 eicosapentaenoic acid (EPA) on adenoma formation in Min mice was related in part to alterations in prostaglandin biosynthesis and a decreased level of PGE_2 in the intestinal tissue. However, the synthesis of prostaglandins is also dependent on n-6 and n-3 chain PUFAs such that n-3 long-chain PUFAs can displace arachidonic acid, and eicosanoids derived from n-3 long-chain PUFAs oppose the action of those derived from n-6 fatty acids. Unfortunately, because we did not measure the PGE_2 level in intestinal tissues due to the lack of intestinal samples, the role of PGE_2 in Study II remains obscure.

Increased COX-2 expression favours colonic neoplasia, and non-steroidal anti-inflammatory drugs acting as COX inhibitors play an important role in cancer prevention (Gupta & Dubois 2001). In Study II, we analysed COX-2 expression in adenomas to determine whether this could explain the inhibition of adenoma growth found in the flaxseed and oil groups relative to the control group. We found that the COX-2 protein level was not dependent on diets, although a positive correlation was present between COX-2 level and adenoma growth (r = 0.536, P = 0.001, n = 33). Unfortunately, we did not measure COX-2 activity, which might also be modulated by the experimental diets. In another study, the administration of 1,4-phenylene *bis*(methylene) selenocyanate decreased adenoma number in Min mice, and also lowered COX-2 activity in adenomas, while leaving the level of COX-2 protein unchanged (Rao *et al.* 2000).

Boolbol *et al.* (1996) and Mahmoud *et al.* (1997) reported that enterocyte apoptosis and migration upwards to the apex of the villus were decreased by 27-47% and 25%, respectively, and cell proliferation was decreased by 45% in the normal-appearing mucosa of Min mice as compared with wild-type mice. Tumour prevention by plant phenolics, caffeic acid phenethyl ester and curcumin, have later been shown to be associated with increased levels of enterocyte apoptosis and proliferation (Mahmoud *et al.* 2000). Recently, Davidson *et al.* (2004) reported that fish oil (n-3 PUFA) feeding increased the level of apoptosis in the upper part of colonic crypts compared with corn oil (n-6 PUFA) and olive oil (n-9 monounsaturated fatty acids) treatments. Furthermore, microarray analysis of colonocyte expression profiles showed that fish oil up-regulated genes involved in apoptosis and differentiation. Thus, changes at the cellular level of apoptosis may be the key in cancer prevention. To determine whether the experimental groups in Study II would experience a change in the level of enterocyte apoptosis, we analysed the number of cytoplasmic histone-associated DNA fragments by an assay based on a quantitative sandwich-enzyme immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics GmbH, Mannheim, Germany). In this assay, the histone-associated DNA fragments indicate DNA degradation of apoptotic cells. The measuring of apoptosis was carried out according to manufacturer's instructions, and the absorbance values of control (1.0 ± 0.9, mean ± SD, n = 6), flaxseed (1.1 ± 1.0, n = 6) and oil (1.5 ± 0.5, n = 6) groups were standardized to the corresponding values obtained from wild-type mice fed the control diet (1.2 ± 1.0, n =

6) (unpublished results). When the relative level of enterocyte apoptosis was set to 100% for wild-type mice, the respective values for control-, flaxseed-, and oil-diet-fed Min mice were 83% (P = 0.873), 97% (P = 1.000) and 130% (P = 0.263). These preliminary results showed that the flaxseed and oil groups had a non-significant tendency for an increased level of apoptosis in the intestinal mucosa, which might be associated with lowered tumour load and smaller tumour size in these experimental groups.

In summary, the results suggest that the oil component of flaxseed and rapeseed oils has a chemopreventive effect on adenoma formation, especially with regard to adenoma growth in Min mice. The exact mechanism for prevention remains unclear, but it may be related to α-linolenic acid and perhaps diet-induced changes in cellular apoptosis. The results of this experiment warrant further investigation.

4.5.2 Fibre

In Study II, the flaxseed diet also contained wheat fibre, which consists mainly of cellulose and hemicellulose isolated from wheat bran. Several studies have shown that wheat bran is protective against colon cancer in carcinogen-treated rats (McIntry *et al.* 1993, Zoran *et al.* 1997, Jenab & Thompson 1998). In Min mice, 5-10% wheat bran or cellulose supplementation inhibited adenoma formation in the small intestine but not in the colon compared with a high-fat, non-fibre diet (Yu *et al.* 2001). *Apc*$^{\Delta 716}$ mice fed a low-risk diet (20% wheat bran and 5% fat) were observed to have less polyps in the small intestine and the colon than their peers receiving a high-risk diet (2·5% wheat bran and 20% fat) (Hioki *et al.* 1997). However, this study did not separate the effects of fat and fibre on adenoma formation. In our previous study, we found that adenoma number in the small intestine and colon of a high-fat wheat bran (10% w/w) group or a low-fat AIN-93G (contains 5% cellulose) group was not different from that of a high-fat non-fibre group (Mutanen *et al.* 2000). Therefore, while the effect of wheat fibre in Study II is somewhat unclear, wheat fibre may confer some protective effect against adenoma formation.

We conducted Study III to determine whether fractionation of rye bran increases its anticarcinogenic properties, which might be related to bifidogenicity and lignans. We

found that the rye fraction, refer to here as the soluble extract, actually promoted adenoma growth in Min mice, whereas rye bran and insoluble-fraction had neutral effects. All rye diets had equal levels of fibre (4%), and therefore, the amount of rye fibre as such does not explain adenoma growth. The fractionation of rye bran was based on water solubility, and it was highly fermentable *in vitro* (Karppinen *et al.* 2001). Although the fermentability of the soluble-extract was not proven to be the reason for the enhanced growth pattern of adenomas, our other studies with highly fermentable inulin fibre, which increased adenoma number and size in Min mice, support this interpretation (Mutanen *et al.* 2000, Pajari *et al.* 2003). The results of our studies with Min mice consistent with earlier research which has shown that soluble fibres, e.g. oat bran, pectin and guar gum, increase the number and incidence of colonic tumours in carcinogen-treated rats (Jacobs & Lupton 1986, McIntry *et al.* 1993, Zoran *et al.* 1997).

The role of fibres in adenoma formation in Min mice is sometimes questioned since most of the adenoma formation occurs in the small intestine, whereas the colon is the main site for bacterial fermentation of fibres. It has, however, been shown that gut fermentation (*via* SCFA and small enteropeptides) increases epithelial cell proliferation in both the large and the small intestine (Goodlad *et al.* 1995, Sakata 1995). The presence of adenomas, mainly in the distal small intestine, may also affect the flow of digesta, which in turn could influence colonization of microbes and microbe metabolism in this area.

Fibre stimulates bacterial growth, increasing faecal bulk, and may cause alterations in colonic microflora, e.g. changes in bacterial species and enzyme activities (Kim 2000). Attempts to increase such bacterial groups as *Bifidobacterium* and *Lactobacillus* have been considered beneficial to the host's health (Salminen *et al.* 1998). The aim of Study III was to determine whether bifidobacteria level had any effect on adenoma formation in Min mice fed different rye preparations. The results revealed that processing of rye bran into two fractions did not affect bifidogenicity of the substrate. A considerable number (10^9 cfu/g) of *Bifidobacterium* were found in mice fed the standard chow prior to the feeding experiments. Removal of fibre in the non-fibre diet decreased the level of *Bifidobacterium* to below detection limits (less than 10^4 cfu/g). However, 6-7 weeks of feeding with three different rye diets sustained *Bifidobacterium* at similar levels (10^8-10^9 cfu/g). The results clearly show that water solubility does not affect bifidogenicity

of rye bran; even the insoluble fraction sustained bifidobacteria growth, as has been demonstrated for soluble preparations *in vitro* (Crittenden *et al*. 2002). The results further show that the non-fibre group had a concomitant decrease in intestinal *Bifidobacterium* level during the feeding period, yet did not have an increased number of adenomas in the colon. Thus, growth of bifidobacteria in Min mice does not appear to regulate colon adenoma formation. Pierre *et al.* (1997), on the other hand, showed that short-chain fructo-oligosaccharides (sc-FOSs), used as a prebiotic substrate for *Bifidobacterium*, decreased the number of colon adenomas. The authors concluded, however, that T cells related to immunosurveillance, and not bifidogenicity, participate in the mechanism of colon tumour initiation in Min mice fed sc-FOSs (Pierre *et al.* 1999). The role of prebiotics and/or probiotic bacteria in colon carcinogenesis has also been studied by using carcinogen-treated rats (Arimochi *et al.* 1997, Challa *et al.* 1997, Onoue *et al.* 1997, Reddy *et al.* 1997, Singh *et al.* 1997, Rowland *et al.* 1998, Femia *et al.* 2002). The results of these studies are, however, inconsistent, indicating how strong the influence of background diet, bacteria strain or prebiotic source can be.

In summary, the type of fibre may modulate adenoma formation in Min mice, although the exact mechanism is unclear. Wheat fibre consisting mainly of cellulose may have some additive chemopreventive effect, whereas highly fermentable fibres may enhance intestinal tumour formation in Min mice. *Bifidobacterium* level does not appear to explain adenoma formation in this animal model.

4.6 Effect of diets on signal transduction parameters in Min mice

4.6.1 β-Catenin

The improper regulation of cellular β-catenin pools is one of the driving forces in *Apc*-induced colonic neoplasia (Henderson 2000). Diet has been shown to affect β-catenin localization in cellular compartments in Min mice; a decreased tumour number was associated with reduced β-catenin staining as well as redistribution of β-catenin to a more “normal” localization mainly at the intracellular junctions (McEntee *et al.* 1999, Schmelz *et al.* 2001, Pajari *et al.* 2003, Misikangas *et al.* 2005). Thus, to find the reason for the decreased number of adenomas in mice fed flaxseed (Study II) and 7-hydroxymatairesinol (Study IV), we analysed the subcellular localization of β-catenin

in the mucosa and adenoma tissues. The levels of cellular β-catenin are summarized in **Table 6**.

The only significant diet effect on subcellular β-catenin levels was observed in 7-hydroxymatairesinol-fed mice in which the nuclear β-catenin level in the adenoma tissue (0.41 ± 0.25, relative units) was decreased significantly compared with the control group (3.15 ± 2.90, relative units, $P = 0.003$). Thus, the nuclear β-catenin levels in the adenoma and in the surrounding mucosa tissues did not differ significantly from each other in the 7-hydroxymatairesinol group (Table 6). In other groups, the nuclear β-catenin level was significantly elevated in adenoma tissue compared with the surrounding mucosa tissue (Table 6). This indicates the unsuccessful post-transcriptional regulation of β-catenin in adenomas due to the loss of the wild-type *Apc*-gene. Interestingly, we found that cytosolic β-catenin in adenomas was significantly increased compared with the same fraction in mucosa tissue in Study IV, but not in Study II (Table 6). A plausible explanation for this can be found in study designs. In Study IV, all diets contained 2.5% inulin as a non-lignan fibre source, which served as a promoter of adenoma formation (Mutanen *et al.* 2000, Pajari *et al.* 2003). Pajari *et al.* (2003) demonstrated that the promotion of tumour development was significantly accompanied by the accumulation of cytosolic β-catenin in adenoma tissue.

Table 6. Differential expression of β-catenin in the mucosa and adenoma tissues of mice fed the experimental diets in Studies II and IV. [1]

Fraction	Study II			Study IV		
	Non-fibre	Flaxseed	Oil	Inulin	Inulin/ Rye bran	Inulin/HMR
Cytosol	ns	ns	ns	Ad > Muc	Ad > Muc	Ad > Muc
Membrane	ns	Muc > Ad	Muc > Ad	ns	ns	ns
Nuclear	Ad > Muc	Ad > Muc	Ad > Muc	Ad > Muc	Ad > Muc	ns

[1] Abbreviations are as follows:
ns, non-significant difference between the mucosa and adenoma tissues in β-catenin expression.
Ad>Muc, β-catenin expression in the adenoma tissue significantly higher than that of the mucosa tissue: $P < 0.05$ (Wilcoxon signed-ranks test).
Muc>Ad, β-catenin expression in the mucosa tissue significantly higher than that of the adenoma tissue: $P < 0.05$ (Wilcoxon signed-ranks test).

A reduced association between membranous E-cadherin and β-catenin has been found in Min mice as compared with their wild-type littermates (Carothers *et al.* 2001). Misikangas *et al.* (2005) reported that at five weeks of age Min mice had less membranous E-cadherin and β-catenin in the mucosa than their wild-type littermates. Inulin feeding, which increased the adenoma size, reduced the levels of E-cadherin and β-catenin in the mucosal membranes in both Min and wild-type mice compared with control groups (Misikangas *et al.* 2005). In Study II, there were no differences between diet groups in membranous β-catenin measured in the adenoma tissue. However, in the normal-appearing mucosa, membrane β-catenin levels were slightly elevated in the flaxseed group relative to the control group (median 1.8 vs. 0.8, $P = 0.09$) (Figure 3 in Study II). This might indicate a protective effect of defatted flaxseed, wheat fibre, flaxseed and rapeseed oils on mucosal integrity, and thus, on intestinal adenoma formation.

4.6.2 Protein kinase C-ζ

Protein kinase C (PKC) isozymes are involved in diverse biological processes, including cellular proliferation, differentiation and apoptosis as well as malignant transformation (Black 2001). Human and animal studies have shown that PKC-βII is up-regulated, and PKC-α, -βI, -δ and -ζ are down-regulated in colon tumourigenesis (Kahl-Reiner *et al.* 1994; Wali *et al.* 1995; Klein *et al.* 2000, Murray *et al.* 2002). Klein et al. (2000) reported a reduced protein expression of PKC-α, -βI, and -ζ in adenoma tissue compared with adjacent mucosal tissue of Min mice by using an immunohistochemistry method. Recently, atypical PKCs (ζ, and λ/τ) have been shown to co-localize with Par-proteins at cell-cell contacts in epithelial cells, thus establishing cell polarity, and also to phosphorylate GSK-3β and subsequently induce association of Apc with microtubules (Etienne-Manneville & Hall, 2003*a*,*b*). We chose PKC-ζ of all PKC isozymes for our studies because its localization in cell fractions can be modulated by diet (Pajari *et al.* 2000), it is down-regulated during the cancer process (Klein *et al.* 2000), it mediates the effect of various anticarcinogenic substances (Roy *et al.* 1995, Wali *et al.* 1995, 1996), and it has been shown to interact with the Apc pathway (Etienne-Manneville & Hall 2003*b*).

By Western blot analysis, we found that PKC-ζ expression (sum of 70-kDa and 75-kDa bands) was lower in the membranes of adenoma tissue compared with the same fraction in the normal-appearing mucosa without a major reduction in PKC-ζ levels in tissues (Table 5 in Study II). This is in line with a study where the majority of PKC-δ and PKC-ζ were localized in the cytosolic fraction of AOM-induced tumours but not in the normal mucosa (Davies & Johnson 2002). We also found that membrane PKC-ζ expression in the mucosa tissue was inversely associated with the adenoma number in the same area in Min mice (r = -0.460, P = 0.008, n = 32). Our results suggest that membrane-associated PKC-ζ protects against cancer, although the mechanism is still unknown. An *in vitro* experiment with Caco-2 cell has demonstrated that EGF-induced PKC-ζ translocation to membranes is necessary for stabilization of the microtubule skeleton and cell barrier function against oxidative injury (Banan *et al.* 2002). Furthermore, through interaction with atypical PKC, the mammalian Par-proteins regulate the formation of epithelial cell-cell junctions, which are essential for the establishment of epithelial cell polarity (Etienne-Manneville & Hall 2003*b*). In normal murine intestinal epithelium, PKC-ζ was detected mainly in the post-mitotic cells of the upper crypt and surface mucosa, and in the membrane/cytoskeletal compartments (Saxon *et al.* 1994; Verstovsek *et al.* 1998). Therefore, the presence of PKC-ζ in the membrane fraction of epithelial cells seems to be involved in the normal differentiation and maturation processes.

In summary, the subcellular localization of β-catenin and PKC-ζ may exert an effect on colonic neoplasia. PKC-ζ results showed that the diet might affect subcellular localization of cell signalling molecules also in the normal-appearing mucosa. However, the mechanism of action is complex and far from clear. The relevance of subcellular localization of β-catenin and PKC-ζ in adenoma formation in Min mice requires confirmation in future studies.

4.7 Other results

No published data are available regarding gender differences in adenoma number in Min mice (Moser *et al.* 1990). In Study I, however, we found that Min males had twice as many adenomas in the colon (0.90 ± 0.94, mean ± SD, n = 29) as Min females (0.45 ± 0.57, n = 29, P = 0.048). No gender difference was present in adenoma number in the small intestine in the control and flaxseed groups. The gender difference in the colon adenoma number in Min mice was unexpected, and no references were found in the literature. To study this phenomena further, the adenoma data of all non-fibre control-diet-fed male and female mice were collected in Studies I-V, and in studies conducted by Pajari *et al.* (2003) and Päivärinta *et al.* (unpublished data). Altogether, 44 males and 44 females had been fed with the non-fibre diet until the age of 12-15 weeks. A pooled data analysis supported the finding that colon adenoma number was significantly higher in Min males than in Min females (0.8 ± 1.0, n = 44 vs. 0.4 ± 0.7, n = 44, P = 0.034, Mann-Whitney U-test). The adenoma number in the small intestine, by contrast, did not differ between the males and females fed the non-fibre diet (54 ± 23 vs. 55 ± 27, P = 0.940). The observation of a gender difference in colon adenoma number will suggest that the underlying mechanism may be hormone-related. Indeed, studies of Weyant *et al*. (2001) and Javid *et al.* (2005) have been shown that 17β-estradiol can modulate intestinal tumour formation in ovariectomized Min mice. Our result and the adenoma number in the colon being low question the suitability of using colon adenoma number as an end-point marker for cancer prevention studies in Min mice. Research on a better end-point marker for the colon is needed.

5 Summary and conclusions

The effects of lignan-rich foods and pure lignans on intestinal tumorigenesis were investigated using multiple intestinal neoplasia (Min) mice. We used flaxseed and rye bran as lignan sources with different lignan profiles. Secoisolariciresinol is the primary lignan in flaxseed, whereas mainly syringaresinol is found in rye bran and rye fractions. Availability of pure lignan isolates, e.g. 7-hydroxymatairesinol from spruce, and synthesis of other lignans, e.g. matairesinol and secoisolariciresinol, by using 7-hydroxymatairesinol as a starting material enabled us to also evaluate the effect of pure lignans on colon tumorigenesis. The main findings of Studies I-V may be summarized as follows:

1. Flaxseed and rye diets providing such lignans as secoisolariciresinol diglycoside and syringaresinol (I-III) as well as pure secoisolariciresinol supplementation (V) showed no anticarcinogenic effects in Min mice. The crude extract of 7-hydroxymatairesinol had chemopreventive effects (IV), while pure matairesinol had tumour-promoting effects in this mouse model (V). The underlying mechanisms by which these lignans exerts opposite effects on tumorigenesis might, in part, be related to the direct actions of these compounds.

2. Supplementation of the diet with lignan-rich components of flaxseed and rye-bran or lignan preparations increased the levels of plant and mammalian lignans in both the small intestine and the colon of Min mice, and also increased plasma enterolactone to levels of 100 nmol/l or even higher. Studies with pure lignans showed that enterolactone was the main plasma metabolite in matairesinol-fed mice, whereas both enterolactone and enterodiol were found in the plasma of secoisolariciresinol-fed mice. Substantial amounts of the lignan precursors matairesinol and secoisolariciresinol were also present in plasma (I-III, V). We found also that gender and genotype affect enterolactone metabolism inside the gut lumen. Furthermore, plasma enterolactone levels did not directly correlate with lignan levels found in the gut (I, II). However, neither plasma enterolactone nor the intestinal enterodiol, enterolactone or secoisolariciresinol levels were related to adenoma formation in the Min mouse model (I-II, V).

3. The lignan sources (flaxseed and rye bran) also provide other dietary components, such as fat and fibre, which may affect tumorigenesis. The action of lignans should therefore not be stressed over other possible effective agents. The oil part of the flaxseed mixture (rich in α-linolenic acid) was mainly responsible for inhibition of intestinal tumorigenesis, particularly with regard to adenoma growth (II). α-Linolenic acid may directly or by changing in the ratio of n-6 to n-3 fatty acids of the intestinal mucosa inhibit colon tumorigenesis. The type of fibre may also modulate adenoma formation in Min mice, although the exact mechanism is unclear. Wheat fibre may have had some additive chemopreventive effect (II). Fractionation of rye bran did not increase its anticarcinogenic properties (III). If the fractionation of rye bran was based on water solubility, and the rye fraction contained rapidly fermentable fibres, it was slightly harmful in Min mice. Rye bran and fractions also sustained the growth of *Bifidobacterium*. However, the level of this bacterium in the gut did not regulate adenoma formation in this mice model.

4. Subcellular localization of cell-signalling proteins β-catenin and PKC-ζ in the mucosa tissue might be pivotal in inhibiting adenoma formation (IV,V). A low level of nuclear β-catenin in the adenomas of 7-hydroxymatairesinol-fed mice and a high level of PKC-ζ in the mucosal membrane of flaxseed-fed mice was associated with a low tumour number and size in this animal model. The amount of COX-2 was shown to increase during tumour growth, but the experimental diets did not affect the level of this enzyme in the adenomas. Thus role of COX-2 in tumour progression remained unclear

5. The colon adenoma number was affected by gender such that male Min mice had twice the number of adenomas in the colon as female Min mice. This questions the suitability of using colon adenoma number as an end-point marker.

Our knowledge of the relationship between lignans and the risks of various cancers in human subjects and experimental animals has grown greatly during the past few years. Although the results with this mouse model indicate that some lignans (e.g. enterolactone, enterodiol and secoisolariciresinol) do not have an apparent chemopreventive effect on colon carcinogenesis, these studies raised some new questions which warrant further studies. Small differences in the chemical structure of

pure lignans might be important with regard to lignan metabolism and chemoprevention. Thus more detailed information is needed on lignans actions *in vivo*. The oil component of the flaxseed mixture appeared to have a chemopreventive effect on adenoma growth in Min mice. The effect of this α-linolenic acid-rich oil component on colon tumourigenesis and possible mechanistic pathways (e.g. PKC-ζ and apoptosis) should be verified and researched further. Exploiting such techniques as immunohistochemistry may open new possibilities for examining the effects of dietary components on subcellular localization of cell-signalling proteins, which seemed to be pivotal for adenoma progression. The gender difference in lignan metabolism was unexpected, and the reason for it remains unclear. The exact effects of gender on lignan metabolism and perhaps on colon adenoma number are worthy of further studies.

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