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Phytoestrogens and prostate cancer

Experimental, clinical, and epidemiological studies

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Till Zackarias

Nothing will benefit human health and increase the chances for survival of life on Earth as much as the evolution to a vegetarian diet.

Albert Einstein

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ABSTRACT

PHYTOESTROGENS AND PROSTATE CANCER

Experimental, clinical, and epidemiological studies

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Dietary factors may affect development and progression of prostate cancer. Experimental and epidemiological studies have suggested an effect of phytoestrogens on prostate cancer and lignans are the predominant phytoestrogen in a Western diet.
The effects of a diet rich in phytoestrogens, in particular lignans, as compared to a control diet, were assessed in several prostate cancer models.
In paper I, 70 athymic nude mice with transplanted subcutaneous LNCaP tumours, an androgen sensitive human prostate cancer cell line, were fed one out of six phytoestrogen rich diets or a control diet after tumour injection. The rye diet, with high lignan content, decreased tumour take and growth, decreased secretion of prostate specific antigen and increased apoptosis. Addition of fat to the rye diet decreased the beneficial effects.
In paper II, transgenic mice designed to develop prostate cancer (TRAMP) were fed rye bran or a control diet from the age of four weeks. Rye bran decreased prostate epithelial cell volume by 20%, and increased cell apoptosis by 31% as compared to the control diet.
In paper III, we examined the effects of 7-hydroxymatairesinol (HMR), a purified lignan, in nude mice with subcutaneous LNCaP tumours in two different concentrations as compared to a control diet. Mice on the HMR diets had a reduced tumour take rate, lower total tumour volume, increased proportion of non-growing tumours, and increased apoptosis as compared to the control diet.
In Paper IV, a three week intervention study exploring the effects of rye bran bread vs. a control diet in men with prostate cancer was performed. The men in the rye group had increased levels of plasma enterolactone and in biopsies from the prostate after the intervention an increase in apoptosis was observed in comparison with biopsies obtained before the intervention.
In paper V, we examined the association between plasma levels of enterolactone, and risk of prostate cancer in a nested case control study. In the Northern Sweden Health and Disease Cohort, enterolactone concentrations were measured in plasma obtained at a mean time of 5 years before diagnosis from 265 cases of prostate cancer, and from 525 matched controls. We found no significant association between plasma enterolactone and risk of prostate cancer. Men with very low enterolactone levels (bottom decile) however, had significantly higher risk of prostate cancer.
Phytoestrogen rich diet including soy, rye bran, substances purified from rye, and a purified lignan (HMR) all inhibited prostate tumour growth. However, it cannot be concluded that the effects observed were due solely to lignans as other components in rye grain such as tannins, phytic acid, ferulic acid, vitamins and minerals may have contributed to the beneficial effects. Thus, additional studies are needed to further elucidate the effects of phytoestrogens on prostate cancer development and progression.

Key words: prostatic neoplasm, phytoestrogens, lignans, enterolactone, rye, 7-hydroxymatairesinol, nude mice, TRAMP mice, nested case control study, LNCaP

ABBREVIATIONS

BrdU	Bromodeoxyuridine
DLP	Dorsolateral prostate lob
End	Enterodiol
Enl	Enterolactone
GC-MS	Gas chromatography with mass spectrometric detection
HPLC	Reversed phase high performance liquid chromatography
HMR	Hydroxymatairesinol
IGF	Insulin-like growth factor
IGFBP	Insulin growth factor binding protein
ISEL	*In situ* end labelled
LC-MS	Liquid chromatography with mass spectrometric detection
LI	Labelling index
MAb	Monoclonal antibody
Mat	Matairesinol
PB	Probasin promoter
PCa	Prostate cancer
PCR	Polymerase chain reaction
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
Seco	Secoisolariciresinol
SHBG	Sex hormone binding globulin
SV 40	Simian vacuolating virus No. 40
Tag	T antigen
tPa	Tissue plasminogen activator
TRAMP	Transgenic adenocarcinoma in mouse prostate
TR-FIA	Time-resolved fluoroimmunoassay
VP	Ventral prostate lob

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which will be referred to by their Roman numerals:

I Annika Bylund, Jie-Xian Zhang, Anders Bergh, Jan-Erik Damber, Anders Widmark, Anders Johansson, Herman Adlercreutz, Per Åman, Martin J Shepherd, and Göran Hallmans. Rye Bran and Soy Protein Delay Growth and Increase Apoptosis of Human LNCaP Prostate Adenocarcinoma in Nude Mice. *Prostate 2000; 42(4): 304-14*.

II Pernilla Wikström, Annika Bylund, Jie-Xian Zhang, Göran Hallmans, Pär Stattin, and Anders Bergh. Rye Bran Diet Increases Epithelial Cell Apoptosis and Decreases Epithelial Cell Volume in TRAMP (transgenic adenocarcinoma of the mouse prostate) Tumors. *Nutr Cancer 2005;53(1): 111-6.*

III Annika Bylund, Niina Saarinen, Jie-Xian Zhang, Anders Bergh, Anders Widmark, Anders Johansson, Eva Lundin, Herman Adlercreutz, Göran Hallmans, Pär Stattin, and Sari Mäkelä. Anticancer Effects of a Plant Lignan 7-Hydroxymatairesinol on a Prostate Cancer Model *In Vivo*. *Exp Biol Med (Maywood) 2005; 230(3): 217-23*.

IV A Bylund, E Lundin, J-X Zhang, A Nordin, R Kaaks, U-H Stenman, P Åman, H Adlercreutz, T Nilsson, G Hallmans, A Bergh, and P Stattin. Randomised controlled short-term intervention pilot study on rye bran bread in prostate cancer. *Eur J Cancer Prev 2003; 12(5): 407-15.*

V Pär Stattin, Annika Bylund, Carine Biessy, Rudolf Kaaks, Göran Hallmans, Herman Adlercreutz. Prospective study of plasma enterolactone and prostate cancer risk (Sweden). *Cancer Causes Control 2004; 15(10): 1095-102.*

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INTRODUCTION

Geographic differences in PCa

In North America and Europe, prostate cancer (PCa) is the most common cancer among men, and it is much more frequent than in southeast Asia, where the incidence of PCa is low [1]. In contrast, the incidence of latent, clinically insignificant prostate cancer is equally high world-wide, but the mortality from PCa is much higher in Western countries than in southeast Asia (Fig 1) [1]. Furthermore, there is an increased risk of PCa in men migrating from low to high incidence countries [2], indicating that life-style including diet may be an important etiological factor in PCa. Traditional Japanese and Chinese diets are rich in foods containing phytoestrogen compounds, whereas a Western diet contains little of these phytochemicals (Fig 2). Plasma and urinary levels of phytoestrogens are much higher in areas where cancer incidence is low in comparison with areas of high cancer incidence. In Scandinavia, the incidence of PCa is lower in the north eastern parts of Finland than other parts of Finland. In northeastern Finland, compared to other regions in Finland, people eat large amounts of lignan rich bread [3]. These geographical differences suggest that environmental factors – such as diet and in particular phytoestrogens – may play a role in the etiology of PCa.

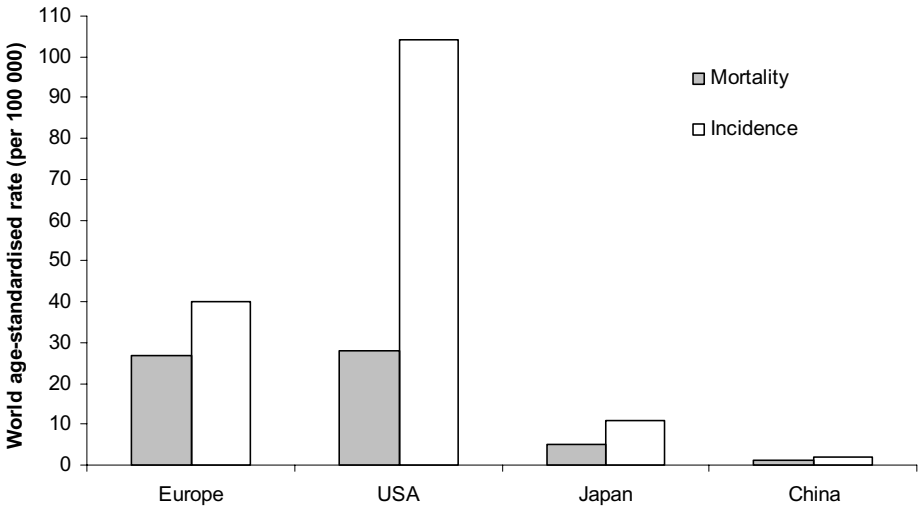

Figure 1. World age-standardized mortality and incidence rates (cases per 100 000) of prostate cancer in Europe, USA, Japan and China (data compiled from Ferlay JP, et al. *GLOBOCAN 2000: Cancer incidence, mortality, and prevalence worldwide*. 2001, Lyon, France: IARC Press).

Definition of a phytoestrogen

The definition of a phytoestrogen is "any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous oestrogens and structurally are similar to mammalian oestrogen 17 β-oestradiol (E2)"[4, 5]. As oestrogen agonists and antagonists, phytoestrogens have also been classified as selective oestrogen receptor modulators (SERMs) [6].

Lignan structure and chemistry

Phytoestrogens consist of three main groups of plant compounds namely, the lignans, isoflavonoids and coumestans (Fig 3). All three groups are characterized by a diphenolic ring, making them structurally similar to endogenous oestrogens. Members of the lignan group of phytoestrogens possess a 2,3-substituted di-1,4-benzylbutane structure, formed from the dimerization of two cinnamic acid residues [7]. Precise measurement of the phytoestrogen content in food stuffs has been difficult because the analytical methods have been crude. Until recently, most of the available information on dietary phytoestrogen concentrations has been related to the isoflavone aglucones. Data on the concentrations of lignans in food is more limited. The most widely used techniques for measurement of phytoestrogens are reversed phase high performance liquid chromatography with coulometric detection (HPLC), gas chromatography with mass spectrometric detection (GC-MS), liquid chromatography with mass spectrometric detection (LC-MS) and time-resolved fluoroimmunoassay (TR-FIA). Each of these techniques has advantages and disadvantages reviewed by [8, 9].

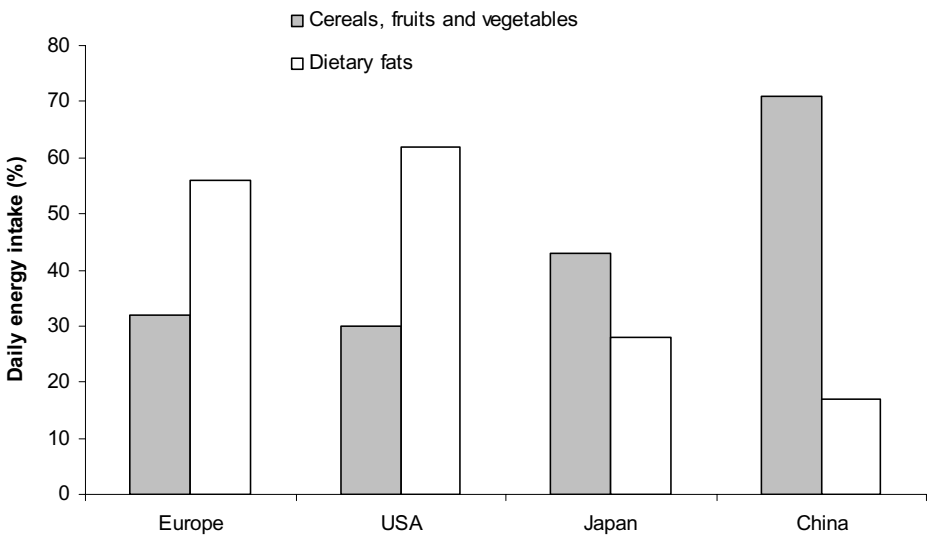

Figure 2. Dietary sources of daily energy intake, expressed as percentage, for Europe, USA, Japan and China. Dietary fats include eatable fats, added fats, diary products and meats. Adapted from Potter, J., *Patterns of diet*, in *Food, Nutrition and the Prevention of Cancer: a global perspective* 1997, American Institute for Cancer Research: Washington, DC. p. 22-34.

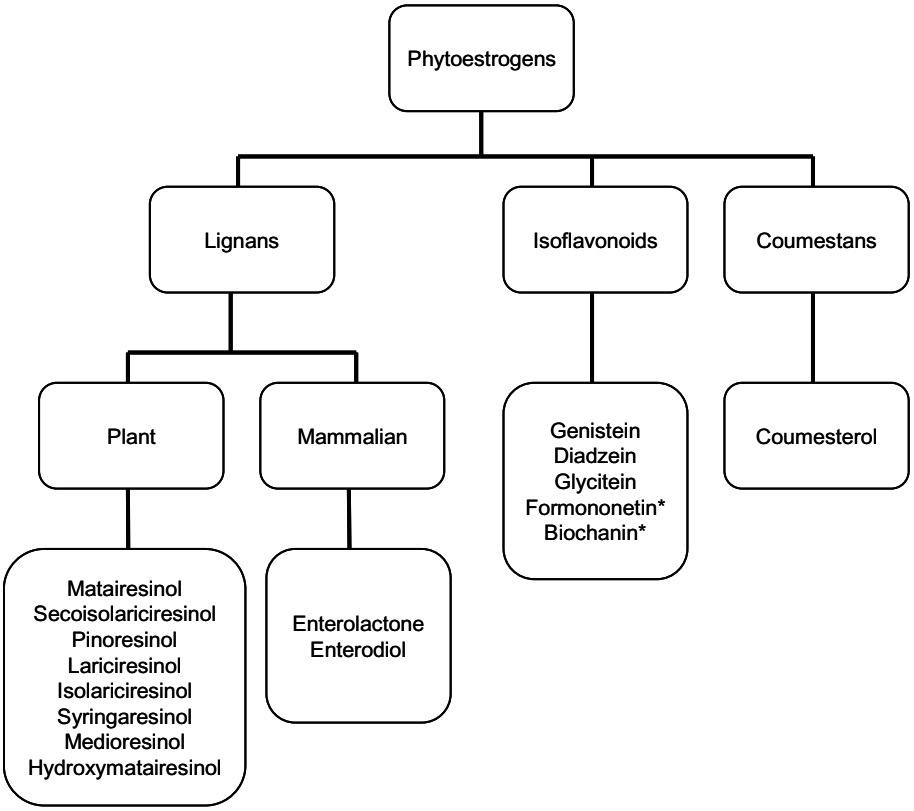

Figure 3. Classes of phytoestrogens * Biochanin A and Formononetin are precursors to the genistein and daidzein, respectively.

Effects of phytoestrogens

The significance of the structural similarity of the lignans and isoflavones to endogenous oestrogens and possible effects on cancer prevention was first suggested in the early 1980s in publications by Setchell and Adlercreutz [10]. Since then, phytoestrogens have been implicated in cancer prevention, in particular in sex-hormone dependent cancers e.g. breast and prostate cancer, through effects on synthesis, metabolism and biological activity of sex hormones, intracellular enzymes, protein synthesis, growth promoting hormones. Phytoestrogens have also been demonstrated to influence cell adhesion, malignant cell proliferation, cell differentiation, apoptosis, angiogenesis, and to act as an antioxidant [11-14], in studies mainly performed in breast cancer models.

In general, most phytoestrogens are relatively weak oestrogens, and require much higher concentrations than oestradiol to produce an equivalent biological response. Oestrogenic activity of phytoestrogens are approximately 100-1000 fold weaker than that of 17β-oestradiol [15-17], but may be present in the body in concentrations 100-fold higher than endogenous oestrogens [18-20].

Oestrogens and prostate cancer

Oestrogen compounds have been used

to treat prostate cancer. Oestrogens achieve castration by feedback inhibition on the hypothalamic-pituitary axis and reduce luteinising hormone (LH) release from the pituitary gland and subsequently reduce testicular production of testosterone. Oestrogens are derived from androgens in the male, in peripheral tissues by the aromatase enzyme. Oestrogens in the male modulate the level of free androgens in the plasma by promoting the concentration of sex hormone-binding globulin (SHBG) and by influencing the hypothalamus to produce less testosterone. Thus, oestrogens moderate the influence of androgens in prostate development.

Oestrogen receptors (ERs)

Two main isoforms of ER have been identified, namely the ERα and the ERβ [21]. Phytoestrogens possess phenolic groups spaced at a similar distance to those in the oestradiol structure. The chemical structure of phytoestrogens determines their affinity, selectivity and efficacy of their binding to oestrogen receptors (ERs) predominantly located in the nucleus of the cell. Oestradiol binds with equal affinity to both ERα and ERß [22]. However, the phytoestrogens show greater selectivity towards binding to ERβ [22]. There is a growing interest in the presence of ERs in prostatic tissues and the potential benefits of oestrogen/ phytoestrogen therapy in PCa is summarized in [23-25].

Dietary phytoestrogen intake in Western and Eastern hemisphere

The intake of phytoestrogens is 10- to 40-fold higher in Asian diets than in Western diets because of the higher intake of soy products in Asia [26, 27]. Due to the large variability in phytoestrogen concentrations in plants and foods it is difficult to provide direct comparisons between different exposure groups. Crudely, a rank order may be determined of daily isoflavone intake: an average European diet contains about 1 mg/day [28], vegetarian diet approximately 8 mg/day [28] soy formula fed infants have roughly an intake of 40 mg/day [29] and subjects on a traditional Japanese diet have an intake of about 25-50 mg/day [30, 31].

Dietary sources of lignans

Lignans are present in the woody portions of plants, the seed coat of seeds, and the bran layer in grains. Lignans occur in most cereals, fruit and vegetables, and are more widespread in different plant foods than isoflavones. Table 1 lists the quantity of selected

Table 1. Lignan content in different food plant products (µg/100g dry weight of food source).

Food	Sec	Mat
Flaxseed	369 900	1087
Rye bread	47	65
Soy beans	13-273	Tr
Strawberry	1500	78
Lingonberry	1510	0
Broccoli	414	23
Red wine	686-1280	17-22
Green tea	1794-2887	195-277

Abbreviations are as follows:
Tr, trace; Sec, Secoisolariciresinol Mat, matairesinol.
All values are from: Mazur, W., *Phytoestrogen content in foods.* Baillieres Clin Endocrinol Metab, 1998. 12(4): p. 729-42.

lignans in several common foods and drinks that have a high lignan content [32-34]. Flaxseed (linseed) is one of the richest natural source of lignans [32]. To date, approximately 200 food items have been analyzed for plant lignan content or the ability of food to produce enterolactone and enterodiol [32-37]. Food composition databases with calculated content of lignan precursors and mammalian lignans have recently been developed to allow for estimates of consumption levels in humans from food frequency formularies [36-39]. Secoisolariciresinol represents the most common component of plant lignans. The principal dietary lignans are lariciresinol, isolariciresinol, matairesinol glycoside, matairesinol, secoisolariciresinol, secoisolariciresinol diglycoside, syringaresinol, medioresinol and hydroxylmatairesinol (HMR) [10, 40, 41]. The form in which the lignans occur in foods is unknown but it has been suggested they are present as long-chain polymers [35]. For this reason, isolation of these compounds from plants and foods requires chemical treatment after which they are in the form of aglucones or glycosides [35]. Much of the research on phytoestrogens has focused on examining the concentration and biological activity of the isoflavones, coumestans, and prenylated flavonoids and to a lesser extent lignans. Although other phytoestrogens have been identified there are limited data on their biological properties and their concentrations in plants and foods. In general, commercial food processing reduces phytoestrogen concentrations [42]. Cooking reduced phytoestrogen concentrations and altered the chemical form of phytoestrogens present in food-stuffs. However, baking or frying did not change the total phytoestrogen content of food-stuffs [42].

Lignan concentrations in urine, serum etc

Mean concentrations of plasma lignans in men are in the range of 7- 33 nmol/l in various populations [43, 44]. Lignans have also been detected in other biological specimens as prostatic fluid [45], semen [46], prostate tissue [47] and faeces [48]. One of the determining factors of serum and urinary lignan levels is the plant lignan content in the diet. Supplementation with flaxseed, a rich source of mammalian lignans causes a dose-dependent response in serum [49, 50] and urinary [50-52] lignan concentrations. Several small supplementation studies with different compound and lignan intake caused up to 250-fold increased lignan concentration in urine [50] and 10-fold in serum (paper IV). Lignan rich foods [53-56], but also a vegetarian diet cause an increase in serum [57, 58] and urinary lignan levels [59]. Serum enterolactone concentrations have been negatively associated with intake of fat [60]. Lignan levels tend to be higher in persons with low and normal body mass index [61, 62], and in older people [63]. The short term reliability coefficient of serum lignan measurements has been reported to be 0.77 in samples collected once a week for a month [64], and 0.79 in samples collected daily for a week [64]. The long term reliability coefficient, in samples collected three times over a time period of two years was lower; 0.55 [65].

Pharmacokinetics, metabolism and bioavailability

The absorption, distribution, metabolism and excretion of phytoestrogens have not been fully elucidated in human

adults. Most of the current knowledge concerns the isoflavones daidzein and genistein and to a lesser extent, the lignans enterolactone and enterodiol. There is a considerable inter-individual variation in the metabolism and bio-availability of ingested phytoestrogens, which can be attributed, at least in part, to differences in gut micro-flora. In turn, the micro-flora may be influenced by factors such as use of antibiotics, bowel disease, gut motility, gastric pH, mucin secretion, bile secretion, stress, diet and intestinal transit time [63, 66-69]. In humans complex enzymatic metabolic conversions of ingested lignans and isoflavonoids occur in the gastrointestinal tract, resulting in the formation of heterocyclic phenols with a close similarity in structure to oestrogens. Plant lignans are not oestrogenic in themselves, but are converted to the enterolignans enterolactone and enterodiol by the gut micro-flora [10]. The most studied plant lignans, matairesinol and secoisolariciresinol occur as glycosidic conjugates [70, 71]. Matairesinol is converted to enterolactone through demethylation and dehydroxylation. Secoisolariciresinol is transformed to enterodiol, and can be further oxidized to enterolactone. Like endogenous oestrogens, lignans are located in the enterohepatic circulation and are excreted mainly in urine but also to some extent in faeces [72, 73]. After ingestion, the major serum peak level of the isoflavones occurs at 4-6 hours and given its half-life of between 4 and 8 hours, nearly all of the isoflavones are excreted after 24 hours [74]. Furthermore the elimination half-life of enterolactone 12.6 hours predicts that steady state will be reached after 2 days (4 x 12.6 h) [75]. Studies in ileostomy patients [72], and in subjects administered antibiotics [70] have confirmed that conversion from plant to mammalian lignans depends on the gut flora. Detection of plant lignans in the urine, indicates that they may also be absorbed from the gastrointestinal tract in unchanged form [76, 77]. The cause may be insufficient bacterial capacity or precursor overload. 7-hydroxymatairesinol (HMR), a structural "cousin" of matairesinol and the most abundant single component of spruce (*Picea abies*) lignans, was metabolized mainly to enterolactone in rats [78]. Two isomers of HMR have been identified in human urine together with 7-hydroxyenterolactone, the metabolite of HMR [79].

In vitro studies

Lignans

Evidence for an antiproliferative effect of lignans in *in vitro* models of prostate cancer has been reported in three studies [80-82]. The effect of enterolactone and enterodiol was investigated on the growth of LNCaP, PC-3 and DU-145 prostate cancer cell lines. Over a dose range of 10-100 μM, enterolactone inhibited the growth of these cell lines, whereas enterodiol only inhibited the LNCaP and PC-3 cells. Enterolactone was more potent (IC_{50} = 57 μM) than enterodiol (IC_{50} = 100 μM) [80]. The lignans inhibited the cell cycle of PC-3 human prostate cancer cells [81]. Furthermore enterolactone, enterodiol and matairesinol, a plant lignan, inhibited the growth of LNCaP, PC-3 and DU-145 cell lines [82].

Animal studies

Lignans

In a previous study from our group, subcutaneous transplanted Dunning

R3327 tumours in Copenhagen rats had a decreased growth when the rats were fed rye bran and soy diets compared to tumours in the control group [83, 84]. In another study in the same animal model, rats were fed one of six isocaloric diets for 18 weeks. The rye bran diet from the first experiment remained unchanged. The control diet was supplemented with cellulose, other diets were control diet with 33% enzyme-treated rye bran, 33% rye bran plus 10% soy flour and 3,3 % flaxseed diet. Only the rye bran diet significantly inhibited tumour growth. Addition of soy flour and enzymatic treatment of rye bran abolished the beneficial effect of rye bran, and the flaxseed diet had no effect on tumour growth. In contrast, Lin *et al*, found a significant decrease in tumour volume and proliferation and an increase in apoptosis in a study of a 5% (by weight) flaxseed-supplemented diet fed to male TRAMP mice compared to control mice [85]. Table 2 provides a summary of the current, PCa animal research, into the anticancer effects of lignans.

Isoflavonoids

Isoflavonoids reduced prostate tumour incidence, in chemically-induced [86, 87], spontaneous [88], transplantable [89], and transgenic [90], PCa animal models. Intake of isoflavonoids inhibited metastasis [89] and tumour growth [91], in transplanted tumours in animal models and prolonged the latency period in

Table 2. Summary of the in vivo studies on the effects of lignans on prostate cancer.

Model	Duration	Intervention	Findings	Reference
BALB/cABom mice (n=70)	9 wk	Rye and soy based diets	↓Tumour take ↓Tumour volume ↓ PSA levels ↑ Apoptosis	Paper I
BALB/cABom mice (n=36)	9 wk	0,15% or 0,3% HMR diets	↓Tumour take ↓Tumour volume ↑ Apoptosis	Paper III
TRAMP mice (n = 58)	20 wk	Rye diet	↓Tumour volume ↓Gleason grade ↑ Apoptosis	Paper II
TRAMP mice (n = 135)	20, 30 wk	5% flaxseed based diet	↓Tumour volume ↓Gleason grade ↑ Apoptosis	Lin *et al* 2002
Dunning R3327 rat (n =125)	24 wk	33 % soy flour diet 33 % rye bran diet 33 % heat treated rye bran diet 33% rye endosperm diet	↓Tumour volume ↑ Apoptosis	Landström *et al* 1998

development of spontaneous prostate tumour in rats [88]. Furthermore, isoflavonoids increased apoptosis, reduced cell proliferation, insulin-like growth factor-I, microvessel density, levels of testosterone and testes weights in rats fed a soy diet [91]. Genistein reduced expression of osteopontin, down-regulated EGF and IGF-1 receptor improved survival, in a TRAMP model [92, 93]. Genistein also decreased the development of poorly differentiated tumours and suppressed poorly differentiated prostate cancer and metastasis in an androgen-independent TRAMP model [94]. Phytoestrogens down-regulated the epidermal growth factor (EGF) pathway and inhibited the expression of tyrosine phosphorylated proteins in the dorsolateral prostate of rats [95]. Phytoestrogens also decreased testosterone levels and prostate weight without altering LH or prostate 5alpha-reductase levels in rats [96]. A low-fat diet containing soy protein and isoflavones decreased the growth rate and final weight of human LNCaP prostate tumours grown in severe-combined immunodeficient (SCID) mice [97]. *In utero* exposure to soy beans from the maternal diet, delayed the development of prostatic intraepithelial neoplasia (PIN), in male mice oestrogenised with DES shortly after birth [98]. Mice fed genistein from weaning until 28-30 days of age significantly reduced the number of mice with poorly differentiated prostate cancer [90]. Dietary exposure of rats to genistein from either conception until day 70 postpartum or from day 56 to day 70 post partum resulted in a dose-dependent down-regulation of androgen receptor and ERα and ERβ mRNA in the prostate[99]. The experiments in these studies all point to an inhibitory effect of isoflavonoids on prostate cancer development and progression. However, the concentrations used in these experiments were very high compared with dietary exposure in humans in Western populations.

Human studies

Observational studies on phytoestrogens and prostate cancer

A summary of studies of the anti-cancer effects of lignans in men is presented in Table 3. Using data from 42 countries in an ecological study, a significant inverse association was observed between consumption of soy products and prostate cancer mortality, with an effect size per kilocalorie at least four times as large as that of any other dietary factor investigated [100]. Investigations of isoflavonoids have been performed in cohort and case-control studies based mostly on food frequency questionnaires. These studies have yielded inconsistent results. Two cohort studies [101, 102] and three case-control studies [103-105] conducted in the West reported an inverse association between high isoflavonoid intake and prostate cancer risk. In contrast, in one other cohort study [106] and two case control studies [107, 108] no association was found between high isoflavonoids intake and prostate cancer risk. One cohort study [109] and two case-control studies [110, 111] conducted in Asia found an association between high soy intake and reduction in risk. Serum genistein, daidzein, and equol reduced prostate cancer risk dose-dependently in a nested case-control study among Japanese men [112]. In contrast, one cohort [113] and two case-control studies [114, 115] failed

Table 3. Summary of the human studies on the effects of lignans on prostate cancer.

Model	Duration	Study design	Findings	Reference
Human (n=18)	3 wk	295 g/day rye bran diet	↑ Apoptosis ↑ Serum plasma enl levels	Paper IV
Human (n=265)		Nested case-control	No relation between plasma enl level and PCa risk	Paper V
Human (n=25)	34 days	Flaxseed, 30g/d fat-restricted	↓ Tot serum cholesterol ↓ Tot testosterone ↓ Proliferation ↓ Free androgen index ↑ Apoptosis	Demark-Wahnefried et al. 2001
Human (n=794)	3-24 y	Nested case-control	No relation between plasma enl level and PCa risk	Stattin et al. 2002
Human (n=29)	27,4 ± 3,6 days	20 g linseed diet+ 50g soy bread 50g soy bread diet wheat bread diet	↓ total PSA ↓ free to total PSA ratio	Dalais et al 2004
Human (n=209)		Case control	↑ plasma enl level ↓ PCa risk (J=shaped)	Hedelin et al 2006
Human (n=136)	10 yr	Dietary questionnaire (105; items)	No relation between diet of vegetables and fruit and PCa risk	Tseng et al 2004
Human (n=214)	6 yr	Nested case-control	No relation between serum enl concentration/ lignan consumption and PCa risk	Kilkinnen et al 2003

to show an association between dietary intake of soy and prostate cancer risk.

Three Scandinavian studies have examined the association between serum levels of enterolactone and prostate cancer risk. In a Finnish nested case-control study of 214 men diagnosed with prostate cancer no association was observed between serum enterolactone levels and prostate cancer risk [116]. In a larger pooled nested case-control study of 794 incident prostate cancer cases and 2550 controls, no association between prediagnostic circulating enterolactone levels and prostate cancer risk was observed [44]. In a more recent Swedish case-control study of 1499 cases and 1130 controls [117] high dietary intake of lignans was associated with a 15 % reduction in risk and high serum levels of enterolactone were also associated with a slight decrease in risk [117].

Interventional studies

Demark-Wahnefried *et al*, performed a study in 25 men scheduled for prostatectomy [118]. The men consumed a low-fat (20 %) diet supplemented with 30g/day of flaxseed, for an average of 34 days before surgery. Men on this diet had decreased serum levels of total and free testosterone, total serum cholesterol and the level of proliferation and apoptosis in the tumour was affected as compared to those of historic cases (matched for age, race, PSA and Gleason score). Dalais *et al,* performed a study with a similar design in which men were given a diet supplemented with a mixture of 20g flaxseed and 50g soy, or 50g of soy grit or a control diet for a month [119]. Men in the intervention arm with 50g of soy had decreased serum PSA levels, and increased free androgen levels but no effect was demonstrated in the other intervention group. Rannikko *et al*, also performed a study with a similar study design in which the men were given 240 mg of clover phytoestrogens or placebo daily for 2 weeks [120]. Phytoestrogen treatment increased serum LH and a non-significant decline in serum testosterone was noted. Finally, in a randomised, crossover study in men with consumption of a soy beverage, no effect on PSA levels or the proto-oncogene p105erbB-2 expression was demonstrated [121].

AIMS OF THE STUDY

The general aim of this thesis was to investigate the effects of phyto-estrogens on prostate cancer, in experimental, clinical and epidemiological studies.

Specific aims were to investigate:

- if food rich in lignans, isoflavones, or extracts of rye, inhibits prostate cancer growth
- the effect of 7-hydroxymatairesinol, a purified lignan, on prostate cancer growth
- the short-term cellular and metabolic effects of high rye bran intake in men with prostate cancer
- the association between circulating concentrations of enterolactone, a lignan, and risk of prostate cancer

MATERIAL AND METHODS

The LNCaP cancer cell line

The LNCaP human prostate adenocarcinoma cell line was used in paper I and III [122]. LNCaP cells are androgen receptor (AR) positive, androgen sensitive, and express AR and ER. The AR has a single point mutation that permits it to bind to nonandrogenic sex steroids (oestrogens and progestins). LNCaP cells have a low anchorage potential and a doubling time of approximately 60 hours. Both cultures and tumours produce PAP (Prostatic acid phosphatase) and prostate-specific antigen (PSA) [122, 123]. Cultures used for the experiments were in exponential growth phase, with a doubling time of 16 hours. Each mouse was given subcutaneous inoculations of 0.05 ml Matrigel (Serva, Lab Kemi) and1μg/ml of basic fibroblast growth factor (bFGF) on each side of the back followed by inoculation of 4x 10^6 tumour cells into the same area.

Animal models

Athymic mice of the Balb/cABom strain, age 6-8 weeks, (Bomholtgård Breeding and Research Center Ltd, Denmark) so called nude mice were used in paper I and III. Xenografts acceptance is the basis for the widespread use of the nude mice as hosts for transplanted human tumours. The main characteristics, of the BALB/c nude mouse are the absence of the thymus and the absence of hair. The mice are grossly deficient in peripheral T cells, although the number of lymphocytes bearing T-cell markers (CD4, CD8), increases steadily with age, and they have a normal complement of B-lymphocytes, almost entirely composed of B-lymphocytes. A relatively normal IgM response to thymus-independent antigen is seen. Lymphokine activated killer cells (LAK) and natural killer cells (NK) are more frequent in nude mice than in normal mice [124]. Macrophage function is enhanced [125] and the number of mast-cells is normal [126].There is little difference in intestinal microbial flora between athymic and normal mice, but, nude mice have a more diverse gastric microbial flora [127]. In paper II, the C57BL/6 TRAMP mouse, a transgenic mouse model was used. The model makes use of a rat probasin promoter that regulates the prostate-specific expression of the SV40 T-antigen [128]. This model have many similarities to human prostate cancer, including an epithelial origin, a step-wise progression from PIN to cancer and further to metastasis [129, 130], and TRAMP mice have an intact immune system.

Diets

In paper I, seven different diets were used. A control diet (CC) was made from the basic components of corn starch, sucrose, low-fat milk powder (Semper AB, Sweden), corn oil and lard. Cellulose was used to adjust the fiber content and energy density in the diet. Diets supplemented with rye bran (RB) and rye bran extracted with ethyl acetate (EXRB) were prepared with the same basic components as in the CC diet plus rye bran (donated by Nordmill's AB, Uppsala, Sweden). A high fat rye bran diet (HFRB) was made with the same components as the RB diet but with high energy density and 40% energy derived from fat. A soy protein diet (SCC) had the same basic

components as the CC diet but the protein source was from soy instead of milk.

In paper II, a control diet (CC) was compared to a (CC) diet supplemented with rye bran (RB).

The major ingredients of the diets in paper III were cornstarch, sucrose, low fat milk protein cellulose, (a mixture of 50% Dicalcel 2 and 50% Dicalcel 4) corn oil, lard, and flaxseed oil. Cellulose was used to adjust the fibre content and energy density in the diet. Diets with 0.15g HMR per 100g of diet (HMR 0.15 diet) and 0.30g HMR per 100g of diet (HMR 0.30 diet) were made with the same components as the semi purified control diet (CLS). HMR extracts were isolated from Norway spruce (*Picea abies*).

In paper IV soft and crisp rye and wheat bread with a very high content of fibre were developed for the study, with the aim of a similar appearance for the rye and wheat bread. In order to have a fibre level in the wheat bread similar to that of the rye bread, a fibre rich wheat product was used (Vitacel WF 600, Rettenmaier & Söhne, Ellwagen-Holzmühle, Germany). Vitacel is a cellulose rich product with a dietary fibre content of 96 % (mainly glucose residues from cellulose). The Vitacel contained only traces of lignans, 21.6 μg secoisolariciresinol/100g dry matter and very small amounts of matairesinol. The rye crisp bread contained whole kernel rye flour 500g, rye bran 500g, fat 60g, and salt 12g as main ingredients and the corresponding wheat crisp bread contained white wheat flour 81g, Vitacel 190g, sugar 60g, dry malt 10g and salt 12g. The soft rye bread contained white wheat flour 600g, rye bran 350g, (B3-fin, Nordmill's, Uppsala, Sweden), bakers yeast 60g, fat 15g, sugar 15g and salt 15g, and the corresponding soft wheat bread white wheat flour 300g, Vitacel 100g, bakers yeast 2g, fat 7g, salt 7g and sugar 7.5g. The crisp bread was baked at several occasions at Wasabröd AB (Filipstad, Sweden) and the soft bread at Cerealia R&D (Järna, Sweden) and Ceralia AB, Gimobageriet (Umeå, Sweden) and was kept frozen at -20ºC.

Metabolic Studies

In paper I and III, metabolic studies were carried out two weeks after tumour cell injection, before the tumours were palpable. In the metabolic study, five animals in paper I and twelve animals in paper III (six mice per cage) from each dietary group were maintained in metabolic cages for three days. Body weight, food and water intake were recorded. During three days urine and fecal samples were collected and stored at -30° C.

Biochemical assays

Isotope dilution gas liquid chromatography-mass spectrometry was used to analyze the isoflavonoids and lignans in the food in paper I, II and IV [33]. Isotope dilution gas liquid chromatography-mass spectrometry in the selected ion-monitoring mode, was used to analyse phytoestrogen in urine in paper 1 and IV and oestrogens in urine in paper IV [131]. In paper III and V, the lignan concentrations in urine and plasma respectively, were measured by using a time-resolved fluor immunoassay (TR-FIA) [132]. In paper IV, the concentrations of insulin-like growth factor-1 (IGF-I), insulin growth factor binding protein's (IGFBP's), C-peptide, sex steroids, PSA and fibrinolytical factors in plasma were measured with radio immunoassays. Tissue plas-

minogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and tPA-PAI-1 complex were measured by ELISA. In paper I, PSA was analyzed using Tandem-R PSA kit (Hybritech Europe S.A., Belgium).

Immunohistochemistry

We examined the following tissues: in paper I and III, tumour specimens, in paper II lung tissue seminal vesicles, prostate lobes, and bladder, and in paper IV, 18 gauge core biopsies from the prostate tumour before and after dietary intervention. The tissue specimens were fixed in phosphate-buffered formalin, dehydrated, and embedded in paraffin. In paper II, III and IV, four μm slides were stained with haematoxylin-eosin HE) for evaluation of morphology and in paper III-IV number of apoptotic cells. In paper II, III and IV, terminal deoxynucleotidyl-transferase-mediated uridine triphosphate end-labelling (TUNEL) preparation was used for detection of apoptotic cells [44]. In paper I, paraffin sections were *in situ* end labelled (ISEL) to detect apoptotic cells [133]. For Ki-67 immunohisto-chemistry in paper II, III and IV, we used an anti-Ki67 antibody (MIB1, Immunotech SA, Marseille, France) as described before [134]. To label p27-expressing cells in paper IV, an anti-p27 antibody (BD Transduction laboratories, KY, USA) was applied, using a method previously described [134]. In paper I, detection of bromodeoxyuridine (BrdU) incorporation, which is an index of the number of cells in the S-phase of the cell cycle in tumour tissue, was used. Animals were administered an i.p. dose of 50 mg BrdU/kg body weight (Bromodeoxy-uridine 10 mg/ml, Sigma) one hour before sacrifice. Five-μm-thick sections were immunostained with a monoclonal anti-body against BrdU (DAKO, Älvsjö, Sweden) using biotinylated horse anti-mouse IgG and a peroxidase-labeled ABC (avidin biotin complex) reagent (Vector Laboratories, Burlingame, CA). In paper II, tail DNA was isolated from all mice, and transgenic animals were identified via PCR based screening, as previously described [128, 129]. Lung sections were stained with an antibody against SV40 Tag as described earlier [128, 129], in search for micrometastasis.

Morphology and Morphometry

Apoptotic index (AI) and proliferative index (PI) were investigated in non-necrotic tumour areas in paper I and III. To obtain labeling indexes, a number of tumour epithelial cells were evaluated for immunoreactivity and the proportion of labeled cells determined.

In paper I, the amount of necrosis in the tumours, defined as confluent areas of ISEL-positive cells and with a general appearance of necrosis, was assessed by a point counting stereological method [135, 136] using a 121 point eye piece graticule mounted in the eye-piece of a light microscope at 40x magnification. The number of grid inter-sections falling over viable or necrotic tumour tissue was counted and the percentage of the necrotic and viable tumour tissue was calculated. The total volume of viable tumour tissue was obtained by multiplying the percentage of viable tissue with total tumour volume.

In paper IV, all biopsies, in each patient and at each time point, containing tumour tissue were evaluated. If the total number of tumour cells in a biopsy set

was less than 350 cells, the data from that patient was not used. The apoptotic index was assessed by quantifying TUNEL staining and the proportion of morphologically defined apoptotic cells in HE stained sections.

In paper II, the different lobes of each prostate were used for histological analysis and pathological grading [137]. The volume density of epithelial cells, glandular lumina and tissue stroma in the ventral and dorsolateral prostate lobes was measured using the stereological method previously described. The number of grid-intersections falling on each tissue compartment was counted in several random sections covering the whole prostate lobe at 100x magnification.

Human studies

Inclusion criteria and recruitment of clinical cases

For the study described in paper IV, we recruited 23 men with conservatively treated prostate cancer. The men were identified through the Primary prostate cancer register at the Oncological Centre, Umeå University hospital. Inclusion criteria were histologically verified prostate cancer conservatively treated, local stage T2-T3, serum PSA< 50 ng/mL, age under 80 years, no metastasis on bone scan at the time of diagnosis, and no current or past castration therapy. Exclusion criteria were other tumours (except skin tumours) gastrointestinal disease, vegetarian diet, diabetes mellitus, and symptomatic progression of the prostate tumour. Tumour stage was evaluated according to the classification of the Union International Contre le Cancer (UICC, 1992) and tumour differentiation was evaluated according to the World Health Organization classification (Mostofi *et al.*, 1980).

Case ascertainment and control selection in nested case control study

In paper V, we used data from the Västerbotten intervention project (VIP) and the Northern Sweden part of the WHO study for Monitoring of trends and cardiovascular disease study (MONICA) which, are part of the Northern Sweden Health and Disease Cohort. Incident cases of PCa in these two cohorts were identified by linkage with the Regional cancer registry, using a nation-wide individual identification number as identity link. If several samples were available for the same case subject, the first sample was chosen. The linkage identified 265 available cases of prostate cancer. Two controls were randomly selected within sets of subjects from the same cohorts including all members alive and free of cancer at the time of diagnosis of the case, matching the index case on age (± 6 months) and date (± 2 months) of the blood sampling.

Ethical considerations

The Animal Ethics Committee of Umeå University approved the studies in papers I, II and III. The Research Ethical Committee of Umeå University Hospital approved the studies in papers IV and V. Informed consent was obtained from all men in paper IV.

Statistical methods

Comparisons between groups were made using the Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U-test in paper I, II and III. In paper IV, comparisons between analyses obtained at the same time point in the two

groups were made using the Mann-Whitney test. The Chi-square test was used to test the tumour take rate between dietary groups in paper I and III and in paper II to test differences in tumour differentiation. Spearman's correlation coefficient was used to correlate the data from selected variables in paper I and to examine the cross-sectional relationships between different analyses in paper IV. A Wilcoxon signed ranks test was used for analysis of changes between two time points in each respective group in paper IV. The Statistical Package for the Social Science (SPSS for Windows, Release 7.5 10.0 and 11.0.0, SPSS Inc.) was used. In paper V, odds ratios (ORs) for disease were calculated by conditional logistic regression for quartile levels of enterolactone in univariate and multivariate models. Cut-off points were determined on variable distributions of cases and controls combined. Confidence intervals (95%) were computed using the standard errors of the pertinent regression coefficients, assuming a normal probability distribution for the estimated coefficients. The logistic regression analyses were performed using the "PHREG" procedure for proportional hazards regression of the Statistical Analysis System (SAS).

RESULTS AND COMMENTS

Paper I

LNCaP cells were transplanted subcutaneously in nude mice. The animals consumed one out of seven study diets. Tumour take, tumour growth and PSA secretion were studied during 9 weeks. The study demonstrated that rye bran, ethyl acetate extraction from rye bran and soy protein diets inhibited the tumour growth. The most prominent effect was observed in the rye group. The tumours that became palpable in the rye group were smaller compared to those in controls (median tumour weight 0.12g ***vs.*** 0.33g) and secreted lower amounts of PSA (median 7.4 ng/ml ***vs.*** 32.0 ng/ml). Soy treatment was slightly less effective and did not prevent tumour take to the same extent as rye, although the tumours that developed were small and secreted only low amounts of PSA. The apoptotic index was higher in the rye group compared to control (mean 1.47 % ***vs.*** 0.42 %). The tumour take rate was significantly reduced in rye and ethyl acetate extract of rye compared to control (30%, 30% respective 75%) percentage of tumour cell injected sites that developed to palpable tumours. Addition of fat to the rye diet decreased the beneficial effects on tumour take and PSA secretion. Tumours were less necrotic in the soy diet group than those in the rye diet group. When tumours in the different treatment groups became palpable their growth rates were almost similar, with a mean doubling time of 8.3 days. The level of urinary secretion of enterolactone in the different treatment groups did not correlate to the anti-tumour effect.

The largest effect of rye, ethyl acetat extract of rye and soy diets occurred during the early phase of tumour growth and the effect on an established palpable tumour seemed to be more limited. The rye diet, ethyl acetat extract of rye and soy diets decreased tumour take and growth, decreased secretion of prostate specific antigen and increased apoptosis. Addition of fat abolished the beneficial effects of rye.

Paper II

In this study we examined the effects of rye diet in C57Bl/6 TRAMP mice. The experiment was started at four weeks of age i.e. before the appearance of high grade PIN or cancer. The effect on the prostate was studied at 24 weeks of age when all animals in the control group had developed cancer [138-140]. Efficacy was evaluated by measurements of primary tumour size and differentiation. Rye-fed TRAMP mice had compared to controls a lower prostate weight corrected for total body weight (1.4 mg/g ***vs.***1.9 mg/g body weight). Furthermore we saw a decrease in prostate epithelial volume density in rye group compared to control group (40 % ***vs.*** 50%). This is an effect of rye bran on tumour growth as the proportion of epithelial cells increases as tumours de-differentiates. We also noticed a somewhat less advanced histological grade in the rye group, with 8/13 (62 %) animals with moderate differentiated tumours in the rye group compared to 11/15 (73%) animals in the control group. Furthermore, the apoptosis index in the dorsolateral prostate was significantly higher in the rye-fed animals compared to the controls (2.1 % ***vs.*** 1.6 %). At the end of the study, mice on rye diet had a higher

body weight compared to controls. In the rye animals, no micrometastasis in the lungs were demonstrated, whereas micrometastasis was found in 13% of the control animals.

The limited response in TRAMP mice is probably related to the strong oncogenic pressure present in these animals with an SV40 Tag-induced disturbance in p53- and Rb-regulated cell functions.

Rye diet delayed tumour progression in TRAMP mice, with lower weight of prostate lobes, decreased epithelial cell volume and a tendency towards less advanced histological grades and increased apoptotic index.

Paper III

In this study we examined the effect of 7-hydroxymatairesinol (HMR) on LNCaP tumours in nude mice. This is the first *in vivo* study on prostate cancer to use a purified lignan. HMR was chosen as it is available in sufficient quantities from Norway spruce (*Picea abies*), and is structurally similar to matairesinol. The animals consumed two different concentrations, 0.15g and 0.30g HMR per 100g of diet compared to a control diet. Animals in HMR 0.15 group had a reduced tumour take rate (54% ***vs.*** 67%), lower total tumour weight (95mg ***vs.*** 237mg), increased percentage of non-growing tumours (46% ***vs.*** 13%), higher tumour cell apoptotic index (0.68% ***vs.*** 0.15%), compared to control animals. This study, demonstrate that a purified lignan HMR inhibits early tumour growth and delay progression in LNCaP tumours in nude mice.

Paper IV

Next, we studied the effect of a short-term intervention with rye bread in men with prostate cancer. The diets consisted of rye bread (n = 10) or a control wheat (n = 8) crisp and soft bread. The rye group consumed an average of 622 kcal (242 nmol matairesinol and 256 nmol secoisolariciresinol) daily. The control group consumed 671 kcal (10 nmol matairesinol and 50 nmol secoisolariciresinol) daily. Blood samples, ultrasound-guided core biopsies of the prostate, and urine samples were obtained before and immediately after the intervention. Plasma enterolactone levels increased in rye group from baseline (mean 32.8 nmol/L to 70.3 nmol/L) after the intervention but not in the control group (mean 28.0 nmol/L to 20.7 nmol/L). However, the difference in interindividual plasma enterolactone levels was more than ten-fold (range 9.9-159 nmol/L) despite a small variation in rye bread intake. Plasma levels of enterolactone were not linearly correlated with the amount of rye bread consumed, nor to the apoptotic index in tumour biopsies, suggesting that factors in rye, other than lignans, may be involved. We observed a significant decrease in body weight and plasma levels of C-peptide in both groups and an increase in the levels of IGFBP-1 in the rye group compared to the control group. In contrast, no changes were observed in PSA, sex hormones, insulin-like growth factor 1(IGF-1), the endothelial fibrinolytical system or levels of excreted oestrogens in urine. In the rye group apoptotic index increased significantly from (2.1 ‰ to 5.9 ‰), whereas among controls no significant increase was observed (2.2 ‰ to 3.1 ‰). An increase in cell proliferation in the second set of biopsies compared to the first set was observed. This increase in proliferation

could be due to a persistent inflammation and tissue healing response, which include a surge in growth factors, induced by the first biopsy session performed seven weeks earlier [141]. This suggests that if post treatment biopsies are used to evaluate short-term therapeutic effects of substances that inhibit cell proliferation, the persistent effect of pre-treatment biopsies have to be taken into account.

The men in the rye group had increased levels plasma enterolactone, the predominant lignan in humans and in biopsies from the prostate after the intervention an increase in apoptosis was observed in comparison with biopsies obtained before the intervention. The variation of plasma enterolactone levels was more than ten-fold, and not correlated with the amount of rye consumed nor with the apoptotic index.

This study suggests that a high rye bread intake can induce an increase in tumour cell apoptosis in prostate cancer patients.

Paper V

In this paper we studied the association between plasma enterolactone and risk of prostate cancer. In the Northern Sweden Health and Disease Cohort, enterolactone concentrations were measured by time-resolved fluoroimmunoassay (TR-FIA) in plasma taken from 265 men who were diagnosed with prostate cancer at a mean time of 5 years after blood collection, and in plasma from 525 control men, matched for age and date of blood collection. There was no significant association between quartiles of plasma enterolactone and risk of prostate cancer. Odds ratios for prostate cancer, estimated by conditional logistic regression for increasing concentrations of enterolactone in quartiles were 1.00 (referent), 0.81 (95% confidence interval 0.52-1.27), 1.03 (0.67-1.58), and 1.22 (0.80-1.86). Adjustments for body mass index (BMI), smoking status and stratification for age, lag time (the time between recruitment and diagnosis), storage time and tumour characteristics did not materially alter risk estimates. Men with very low enterolactone levels, however, had significantly higher risk of prostate cancer, odds ratio for bottom deciles versus all other deciles was 1.68 (1.03-2.74). Overall, this study did not support the hypothesis that high circulating enterolactone concentrations are protective against prostate cancer development.

As a conclusion, our results support the hypothesis that lignans act as anticarcinogenic agents, and our results suggest that the favorable effects of fiber-rich diets may, at least partly, be related to lignans.

DISCUSSION

General methodological considerations

A major limitation to the interpretation of experimental research on phyto-estrogens is that many studies do not report doses on a body weight basis (mg of compound/kg bw/day) [85, 90, 98, 138]. Therefore, comparisons between studies are difficult. Table 4 shows the consumed doses of matairesinol on a body weight basis (mg of matairesinol /kg bw/day) compiled from paper I, III and IV. In general, concentrations of lignans have been much higher in animal compared to human interventions. There was a ten-fold difference of the lignan matairesinol in the diet between the human experiment with rye bread in paper IV and the animal experiment with rye diet in paper I and 1500-3000 fold difference of the lignan content between the human experiment in paper IV and the animal experiment with hydroxylmatai-resinol diet in paper III.

In paper V, no association between plasma enterolactone and prostate cancer risk was demonstrated, in accordance with two earlier, prospective studies [44, 116]. Plasma enterolactone levels in our study corresponded to those in previous studies [69, 116]. Plasma lignan concentrations are relatively good biomarkers of dietary lignan exposure. Lignan concentrations in plasma reach steady state after two days, when consumed 2-3 times a day [75, 142]. Thus, the three weeks of supple-mentation with rye bran in paper IV must be considered sufficient, assuming plasma enterolactone to be the active component in tumour inhibition. Plasma entero-lactone in the rye bread group was much higher compared to controls (mean 70.3 nmol/L ***vs.*** 20.7 nmol/L) after the inter-vention.

Putative anti-cancer mechanisms of phytoestrogens

In paper I and III animals given rye, ethylacetat extract of rye, soy and HMR had lower tumour take rate, smaller total tumour volume, increased proportion of non-growing tumours (in paper III), and higher apoptotic index in tumours when compared to animals receiving control diet. When LNCaP cells are injected together with matrigel, a preparation

Table 4. Consumed doses of a specified lignan on a body weight bases (mg of Matairesinol /kg bw/day) compiled from paper 1, III and IV.

Model	Duration	Intervention	Consumption mg/kg/day	
BALB/cABom mice LNCaP	9wk	Rye bran diet	0,06	Paper I
BALB/cABom mice	9wk	HMR diet 0,15%	13,44	Paper III
LNCaP		HMR diet 0,30%	25,75	Paper III
Human	3 wk	Rye bread diet 295 g/day	0,008	Paper IV

containing extra cellular matrix proteins and growth factors, they form highly vascularised tumours in nude mice [143, 144]. Angiogenesis is essential for the growth and metastases of solid tumours [145] and local angiogenesis is necessary for tumours to grow larger than 1-2 mm^3 [145]. Consequently, tumour take and early growth in the LNCaP model is dependent on stimulation from extra cellular matrix components and local angiogenesis. Our results indicate that rye, ethylacetat extract of rye, 7-hydroxy-matairesinol and soy slow growth of LNCaP tumours, partly by inhibiting formation of new blood vessels. Angiogenesis balance may be critical in early tumour development [146]. Lignans may be more important in the early stage of tumour development by modulating angiogenic/antiangiogenic factors. Our observations also suggest that plant diets influence cell death in prostate tumours. Important steps in tumour progression such as the transitions from latent to clinical and from hormone sensitive to hormone insensitive prostate cancer are related to decreases in tumour cell apoptosis rather than to increases in cell proliferation in rat [147], as in human prostate cancer [148]. The response in the TRAMP model in paper II was not of the same magnitude as in the LNCaP experiments in paper I and III. This was possibly due to the strong oncogenic pressure present in these animals with SV40 Tag-induced disturbances in p53 and Rb regulated cell functions [129, 130]. Flaxseed diet produced a stronger reduction in prostate weight in TRAMP mice than rye, which suggests that the effect of rye bran is rather weak [85]. In contrast, TRAMP mice treated with genistein, the effect was rather similar to what we observed for rye [90]. In paper IV we found a significant increase in the apoptotic index in men with prostate cancer after a three week period of high intake of rye bread compared with the control group. Substances inducing apoptosis, one of our major endpoints in paper I-IV, can be expected to be effective already after a short time. For example, the effects on apoptosis from castration therapy have been observed already three days after androgen withdrawal [149].

Our observation of an increased apoptotic index in prostate biopsies in men on a rye diet is in accordance with a study on flaxseed supplementation before prostatectomy [118]. However, our observations must be interpreted with caution due to the small number of participants, the limited volume of tissue available for analysis from each participant and time point, tumour heterogeneity and the relatively large range in apoptotic index. Furthermore, it is possible that other dietary factors such as dietary fat, fruits, vegetables, and fibre and a variety of nutritive and non-nutritive dietary components, not specifically included in the intervention diet in paper IV, could exert a protective effect. Increased tumour cell apoptosis was the principal morphological response observed after treatment with rye bran, soy, ethylacetate extract of rye in experimental studies in all these tumour models.

Enterolactone levels in relation to tumour inhibition

In paper I and III there was no clear cut relation between tumour inhibition and urinary enterolactone excretion in the LNCaP animal model. A more than tenfold inter-individual variation in the

plasma concentration of enterolactone was seen among men in the rye group in paper IV despite a uniform intake. This high interindividual variation in both urinary excretion and plasma concentrations of enterolignans has also been demonstrated in other studies [50, 51, 66, 75, 142, 150]. In a study similar to ours, healthy volunteers consumed wholemeal rye bread and white wheat bread for 4 weeks and daily urinary enterolactone excretion increased significantly during the rye-bread period compared with the wheat-bread period and was 5- and 10-fold higher respectively in comparison with the amount of the two plant lignan precursors (secoisolariciresinol and matairesinol) measured in the rye bread [54]. However if it can be used to evaluate the effects of lignans are questionable. First-lignan level in prostatic fluid was much higher (10^2-10^3) than in plasma [45, 151]. Prostatic enterolactone tissue levels was (median 17.4 ng/g dry weight), with a wide interindividual variation [152]. Plasma lignan concentrations were rather low in our studies as [paper V] in other epidemiological studies, median enterolactone (15 nmol/L). Conversely, supplementation of diet with flaxseed or rye increased the serum enterolactone concentration up to more than 100nM, [paper IV][153], showing that high serum levels of lignans in humans are achievable. In paper V, plasma enterolactone is inversely related to obesity and smoking, and supports that enterolactone is a marker of a healthy life-style including high intake of whole grain cereal, fruits, and vegetables.

In summary, there are uncertainties that need to be clarified in order to elucidate the effect of hormones on prostate cancer risk [154] e.g. the relevance of hormone/ phytoestrogen measurements in a single blood sample in middle age and the relevance of circulating levels of hormones *vis a vis* intraprostatic levels.

Role of intestinal micro-flora in lignan metabolism

The levels of circulating enterolactone depend on the amount of ingested plant lignans but also on the efficacy of biotransformation and absorption of the ingested lignans [69]. A high consumption of fat lowers the biotransformation and absorption of plant lignans, presumably by changing the micro-flora of the gut [58, 69]. Previous studies found that equol production was inversely related to fat consumption [66, 155], and directly related to vegetable fibre consumption [66, 155, 156]. Adlercreutz *et al* [157] have previously shown that there are differences in lignan and isoflavone excretion among groups consuming very different diets (*e.g.*, macrobiotic, vegetarian, and omnivorous diets).

The effects of the immune deficiency of athymic mice on gastrointestinal microflora and their ability to form mammalian lignans from plant lignan precursors, is poorly understood. In paper I and III, enterolactone was detected in the urine of rye and HMR-treated nude mice, demonstrating that these mice were able to convert plant lignans to mammalian lignans. However, the proportion of the daily HMR dose recovered as urinary enterolactone was much lower in the male nude mice (6% and 3% in HMR 1.5 and HMR 3.0 groups, respectively) than in another study, with female rats (26%) [158]. This may be due to species, gender or differences in duration of lignan administration. Further, we can not

exclude the possibility that the differences in enterolactone excretions are associated with differences in gut flora, and consequently, the capacity to form enterolactone from the plant lignan precursors. Brown *et al* showed that there was very little difference between the intestinal flora between nude and normal mice. The nude mice had a more diverse microbial flora in their gut [127]. Paper IV included a washout period of four weeks between first biopsy and its accompanying antibiotic prophylaxis and the intervention period. In a Finnish study, serum enterolactone concentrations were significantly lower in subjects who had used oral antibiotics up to 12–16 months before serum sampling than in non-users. The effect on enterolactone levels indicate that the gut micro-flora is crucial in the metabolism and uptake of lignans [68]. Possibly, the antibiotic given in conjunction with the first biopsy in paper IV affected enterolactone plasma levels.

Energy/fat importance for tumour outcome

Energy intake has considerable effect on development of experimental prostate tumours [159]. Fat content influenced tumour growth in the LNCaP model [160], in ACI/Seg rats [161] and Lobound-Wistar rats [162], without increasing cell proliferation [161]. Some epidemiological studies suggest that high fat consumption in humans is related to prostate cancer death [48, 49], but there was no relation between intake of fat and prostate cancer risk in another cohort study [163]. In order to control for energy content, our control diets had the same energy content as the intervention diets. In paper I, the addition of fat to a rye diet decreased the beneficial effects on tumour take and PSA secretion. Fat also had an impact on urinary excretion of enterolactone. Biotransformation and absorption of enterolactone is decreased by a high fat intake [62, 69], which is a determinant of body weight. Thus high fat intake might explain our observation of an inverse relationship of enterolactone with BMI in paper V.

Prostate specific antigen (PSA)

PSA is a biomarker for prostate cancer and serum PSA concentrations are linearly related to prognosis and progression of prostate cancer [164-166]. Thus, an effective intervention would be expected to have an impact on PSA levels. In our study in paper I, PSA and tumour weight correlated. Rye, ethylacetat extract of rye and soy diet reduced plasma PSA levels compared with controls in nude mice. In our study in paper IV, no changes in total PSA or in the ratio of free/total PSA were demonstrated. Tumours in our study were quite advanced and thus may be unlikely to be affected by dietary changes.

In studies similar in design to paper IV, PSA decreased [119, 167, 168] [169] or the PSA velocity decreased [170] [171] or no effect was observed [172]. These studies were made with isoflavonoids and the study duration was longer than our study.

Sex steroids, SHBG, FSH, and LH and tumour inhibition

The sex hormone concentrations were not altered during intervention with rye bread in paper IV carried out in men with prostate cancer, a non-significant decrease was observed for testosterone, but no essential changes were seen for plasma levels of oestradiol, SHBG, FSH, LH, and

no change occurred in the urinary excretion of oestrogen metabolites in urine. In accordance a isoflavone intervention with similar design as ours did not affect oestradiol, testosterone, FSH, and LH concentrations or sperm volume, count, motility, morphology or testicular volume [173]. However, in a study with phytoestrogen supplementation for two weeks, up regulated mean serum LH/T ratio significantly [120].

Although it is established that sex steroid hormones, particularly androgens, are essential for the growth, development, and maintenance of healthy prostate epithelium, and to the progression of prostate cancer, epidemiologic studies have thus far failed to show that high levels of circulating androgens increase the risk of developing prostate cancer. Most prospective epidemiologic studies found no evidence that serum levels of endogenous sex hormones and their binding protein (SHBG) are associated with prostate cancer risk [174]. Interestingly, in several recent prospective studies, the risk of prostate cancer was *reduced* in men with higher levels of testosterone [175-178]. As in our results in paper IV these results indicate that short-term exposure to phytoestrogens during adulthood does not appear to affect androgen concentrations. Hormonal systems are homeostatic and mild exposure during adulthood to oestrogenic compounds may not have a biological consequence.

Enterolactone levels and smoking status

Overall, lignan levels are higher in subjects with normal and low BMI (61, 62) however, enterolactone levels were low in subjects with very low BMI (62). Smokers have lower enterolactone levels compared to former smokers and non-smokers (60, 62) [paper V], even though smokers have a lower BMI than non-smokers. Smokers consume less fruits, berries, vegetables and whole-grain products than non-smokers (179, 180). Speculatively, enterolactone may be consumed when eliminating free radicals due to oxidative stress induced by smoking.

IMPLICATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

Experimental studies in rodents show that diets supplemented with lignans or isoflavones inhibit the development of prostate cancer. However, the concentrations used are very high compared with human dietary exposure. The metabolism of isoflavones, lignans and other phytoestrogens in humans is poorly understood, and the plasma concentrations of biologically active or inactive forms of phytoestrogens have not been fully determined. Further analysis of the concentrations of phytoestrogens in food items is necessary as a basis for further studies and development of biomarkers for phytoestrogen exposure. Further information on tissue distribution of phytoestrogens is required to determine tissue specific exposures. Such studies would enable improved evaluation of systemic exposures and establish a physiological dose range in humans. The ability of phytoestrogens to interact with oestrogen receptors has been extensively studied. More research is required on phytoestrogens potential to produce biological effects by alternative oestrogenic and non-oestrogenic mechanisms. Larger prospective studies including analysis of intake and circulating levels of a number of phytoestrogens are necessary to elucidate the relationship between dietary phytoestrogens and the development of prostate cancer in humans.

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