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DIETARY AND GENETIC FACTORS IN THE ETIOLOGY OF PROSTATE CANCER

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ABSTRACT

The etiology of prostate cancer is poorly understood. However, genetic factors may be more important than for many other malignancies. In addition, several studies suggest that dietary factors are of etiologic importance. In particular, dietary intake of phytoestrogens or marine fatty acids from fish may protect against prostate cancer development. Because phytoestrogens bind tightly to the estrogen receptor-beta that is involved in prostate cancer progression, we investigated whether there is a synergistic effect between phytoestrogen intake and estrogen receptor-beta (ERβ) gene polymorphisms in prostate cancer development. Furthermore, we investigated the interaction between intake of fatty fish and polymorphisms in the cyclooxygenase (COX)-2 gene, a key enzyme in fatty acid metabolism and inflammation. Finally, we examined whether the association of alcohol consumption with prostate cancer risk varies between localized and advanced cases, or between sporadic and familial cases.

We conducted a large population-based case-control study in Sweden (CAPS); in this study, we assessed dietary intake of phytoestrogens, fish consumption and alcohol drinking among 1499 cases and 1130 controls. Serum enterolactone levels were analyzed for 209 cases and 214 controls, chosen randomly. We identified four single nucleotide polymorphisms (SNPs) in the ERβ gene and five in the COX-2 gene, and genotyped these SNPs in 1314 (ERβ) or 1378 (COX-2) cases and 782 controls, respectively. Unconditional logistic regression was performed to estimate multivariate odds ratios and 95% confidence intervals. Stratified analyses, as well as both multiplicative and additive models, were used to evaluate interactions between dietary intake and SNPs on prostate cancer risk.

We found that high intake of food items rich in phytoestrogens was strongly associated with a decreased relative risk of prostate cancer, and intermediate serum levels of enterolactone were associated with a decreased relative risk. Furthermore, we found that the overall decreased risk of prostate cancer for men with a high intake of phytoestrogens was strongly modified by a promoter SNP in the ERß gene (-13950 T/C). Carriers of the variant allele had an almost 60% lower risk of prostate cancer, compared to men with low phytoestrogen intake, whereas no such association was found among men with the common genotype. Frequent consumption of fatty fish or marine fatty acids was strongly associated with a decreased relative risk of prostate cancer. The inverse association between salmon-type fish and prostate cancer was modified by a nucleotide sequence variant in the COX-2 gene (+6365 T/C). Prostate cancer cases were more likely than controls to be current or former, rather than never, drinkers. However, there was no association between recent alcohol consumption and risk of overall prostate cancer, nor advanced, sporadic, or familial prostate cancer. There was a marginal positive association between intake of any alcohol type and risk of localized disease.

In summary, our study provides strong evidence that high intake of phytoestrogens substantially reduces prostate cancer risk among men with specific polymorphic variation in the promoter region of the ERβ gene. In addition, frequent consumption of fatty fish and marine fatty acids strongly reduces the risk of prostate cancer, and this association appears to be modified by genetic variation in the COX-2 gene. Furthermore, we found no association between recent alcohol consumption and risk of overall prostate cancer, although we observed a marginal positive association with localized disease.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostatacancer är den vanligaste cancerformen bland män i västvärden och ca 10 000 män insjuknar i Sverige varje år. Andelen män som insjuknar skiljer sig mycket mellan olika länder. Varför prostatacancer är så vanligt vet vi lite om idag och de enda etablerade riskfaktorerna är ålder, etnicitet och förekomst av prostatacancer bland nära släktingar. Det finns dock ett flertal studier som tyder på att kostfaktorer är av stor betydelse.

På senare tid har intresset riktats mot en eventuell skyddande effekt av östrogenliknande föreningar i växter, s.k. fytoöstrogener. I länder som Kina och Japan är intaget av fytoöstrogener högre än i västländer och där är också förekomsten av prostatacancer lägre. Fytoöstrogener brukar delas in två grupper, lignaner och isoflavonoider. Lignaner finns framförallt i linfrö, råg, bär och olika grönsaker. Sojabönor är den största källan till isoflavonoider. Isoflavonoider kan binda till östrogen-receptorn (mottagarämne för östrogen), speciellt till östrogenreceptor beta (ERβ) och tros därmed kunna ha effekter likt östrogen. Det har visat sig i djur- och cellstudier att en minskning av ERβ i prostataceller kan öka tillväxten av cancerceller. Detta har lett till teorin att det kan finnas en interaktion mellan fytoöstrogener och ERβ som kan påverka utvecklingen av prostatacancer. Polymorfier är en benämning på vanliga små variationer i den genetiska koden (DNA) mellan individer som gör att olika individer har olika utseende, beter sig olika eller får olika sjukdomar. I befolkningen finns det även olika polymorfier av ERβ.

Resultat från en del epidemiologiska studier har visat att ett högt intag av fisk kan minska risken för prostatacancer. Man tror att en orsak till detta kan vara att framför allt fet fisk innehåller omega-3 fettsyrorna DHA och EPA. Både omega-3 och omega-6 fettsyror kan omvandlas till hormonlika ämnen s.k. prostaglandiner, vilka är involverade i immunsystemet och tillväxten av celler. Prostaglandiner som bildas från omega-6 fettsyrorna tros vara pro-inflammatoriska och kan öka tillväxten av cancerceller, medan EPA-härstammande prostaglandiner har en anti-inflammatorisk roll samt hämmar tillväxten av prostatacancer. I metabolismen av fettsyror till prostaglandiner verkar enzymet cyclooxygenase-2 (COX-2) och man har funnit att detta enzym finns i högre halter i prostatatumörer jämfört med normal prostatavävnad. I vår studie har vi tidigare funnit att män med en viss polymorfi av COX-2 genen hade en minskad risk för prostatacancer jämfört med dem som hade den vanligare genotypen.

Alkoholkonsumtion har klassats som ett cancerogent ämne och ökar risken för cancer i bland annat mag-tarmkanalen. De flesta studier på intag av alkohol och prostatacancer har dock inte visat på något samband. Några studier har sett en ökad risk för prostata-cancer vid intag av stora mängder alkohol under lång tid, medan andra sett att ett stort intag av alkohol eller rött vin minskade risken. Prostatacancer kan delas in i olika typer beroende på dess aggressivitet, avancerad cancer ger en sämre prognos medan lokaliserad cancer kan man leva med längre. Få, om några, har studerat om sambandet mellan alkohol och prostatacancer skiljer sig mellan avancerad och lokaliserad cancer.

Syftet med studie I var att studera om fytoöstrogener i kosten påverkar risken att insjukna i prostatacancer. Vidare ville vi undersöka om det finns ett samband mellan det beräknade intaget av lignaner från enkätsvar med nivåer av fytoöstrogener (enterolakton) i blod. I *studie II* studerade vi om polymorfier i ERβ påverkar effekten av fytoöstrogener på utveckling av prostatacancer. I *studie III* studerade vi om intaget av fisk och marina fettsyror har betydelse för risken att insjukna i prostatacancer samt om det finns en interaktion mellan intaget av fisk och polymorfier i COX-2 genen som påverkar risken för prostatacancer. I *studie IV* studerade vi om alkoholvanor (totalt intag samt intag av öl, vin och sprit) påverkade risken för prostatacancer samt om ett eventuellt samband skilde sig mellan avancerad och lokaliserad cancer.

Under tidsperioden januari 2001 till oktober 2002 genomförde vi en stor populations-baserad fall-kontrollstudie. Till studien inbjöds 1895 patienter med nyligen debuterad prostatacancer i åldrarna 35 till 79 år. 1684 friska kontrollpersoner identifierades genom matchning med avseende på ålder och bostadsområde. 1499 patienter och 1130 kontroller valde att delta och fick fylla i ett frågeformulär om bland annat sina matvanor, samt lämna ett blodprov. I en mindre grupp av deltagarna (209 fall och 214 kontroller) analyserades mängden av fytoöstrogenen enterolakton i blodet. Utifrån analyser av DNA gjorde vi en systematisk genomgång av polymorfier i ERβ och COX-2 generna för att hitta det mesta av den genetiska variationen i den studerade populationen. För ERβ identifierades fyra polymorfier och för COX-2 identifierades fem stycken och dessa genotypades.

I *studie I* fann vi att män som hade ett högt intag av fytoöstrogenrika livsmedel (bönor, sojabönsprodukter, linfrö, solrosfrö, bär och jordnötter) hade en 26 % lägre risk för att insjukna i prostatacancer. Vidare fann vi att män med mycket låga nivåer av enterolakton i blodet hade en ökad risk för prostatacancer. Uppmätta serumnivåer av enterolakton överensstämde inte med beräknat totalt intag av lignaner från enkäten. Det visade sig dock att sambandet påverkades av övrig kost, såsom alkohol och fett. *Studie II* visade att effekten av fytoöstrogener på prostatacancer modifierades beroende på individens genetiska uppsättning av ERβ-genen. Bland män med en variant genotyp fann vi att den skyddande effekten av fytoöstrogener ökade med högre intag av fytoöstrogener, medan vi inte fann någon skyddande effekt av fytoöstrogener bland män den vanligaste genotypen. *Studie III* visade att de som åt fet fisk (lax) en eller flera gånger per vecka hade en 43 % minskad risk för prostatacancer jämfört med dem som aldrig åt denna typ av fisk. Även män som hade ett högt intag av marina fettsyror hade en minskad risk för att insjukna i prostatacancer. Vidare fann vi en signifikant interaktion mellan intag av lax och en speciell polymorfi i COX-2 genen, men inte med någon av de fyra andra undersökta polymorfierna. I *studie IV* såg vi inget samband mellan alkoholintag och prostatacancer, vare sig för totalt alkoholintag eller för öl, vin eller sprit. Bland män med lokaliserad cancer och som drack mer än 135 g etanol/vecka fann vi en svag ökad risk jämfört med icke-drickare. Det var dock vanligare bland män med prostatacancer än bland kontroller att dricka alkohol jämfört med att vara icke-drickare.

Sammanfattningsvis, detta är den största studie som studerat intaget av fytoöstrogener och dess relation till prostatacancer i en västerländsk population. Den skyddande effekten kan bero på fytoöstogenerna i sig själva eller i kombination med andra ämnen

som finns i samma typ av livsmedel. Våra resultat bekräftar tidigare studier att fet fisk har en skyddande effekt på prostatacancer och att det är troligt att de marina fettsyrorna står för en stor del av denna effekt. Vidare bekräftar våra resultat att alkohol troligen inte är associerat med risk för prostatacancer, dock bör våra resultat angående en ökad risk för lokaliserad sjukdom studeras i andra populationer för att utreda om det föreligger ett sant samband eller om vårt fynd beror på andra orsaker. Både miljö och genetiska faktorer spelar troligen en stor roll för etiologin av prostatacancer och vår studie visar att det finns ett samspel mellan intag av kost och genetisk variation. Ytterligare studier, fram för allt i populationer med annat etniskt ursprung, behövs för att kunna utreda om skillnader i förekomsten av genetiska variationer i ERβ eller COX-2 och intaget av fytoöstrogener eller fet fisk kan förklara en del av den geografiska och etniska variationen i förekomst av prostatacancer. Slutligen vill vi rikta ett tack till alla män som har deltagit i denna studie.

LIST OF PUBLICATIONS

I. Hedelin M, Klint Å, Chang ET, Bellocco R, Johansson J-E, Andersson S-O, Heinonen S-M, Adlercreutz H, Adami H-O, Grönberg H, Augustsson Bälter K. Dietary phytoestrogen, serum enterolactone and risk of prostate cancer: the Cancer Prostate Sweden Study (Sweden). Cancer Causes and Control,17 (2);169-180, 2006

II. Hedelin M, Augustsson Bälter K, Chang ET, Bellocco R, Klint Å, Johansson J-E, Wiklund F, Thellenberg-Karlsson C, Adami H-O, Grönberg H. Dietary intake of phytoestrogens, estrogen receptor-beta polymorphisms and the risk of prostate cancer. *Submitted*

III. Hedelin M, Chang ET, Wiklund F, Bellocco R, Klint Å, Adolfsson J, Shahedi K, Xu J, Adami H-O, Grönberg H, Augustsson Bälter K. The association of frequent consumption of fatty fish with prostate cancer risk is modified by COX-2 polymorphism. *Submitted*

IV. Chang ET, Hedelin M, Adami H-O, Grönberg H, Augustsson Bälter K. Alcohol drinking and risk of localized versus advanced and sporadic versus familial prostate cancer in Sweden. Cancer Causes Control, Apr;16(3), 275-84, 2005

CONTENTS

LIST OF ABBREVIATIONS

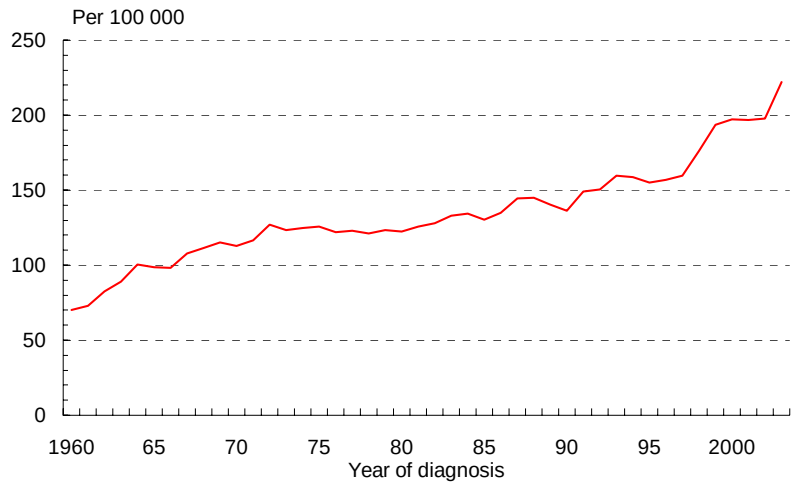
3ßAdiol	5α-androstane-3ß,17ß–diol
AA	Arachidonic acid
AR	Androgen receptor
BMI	Body mass index
CAPS	Cancer Prostate in Sweden study
CI	Confidence interval
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
DHT	5α-dihydrotestosterone
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
ERα	Estrogen receptor-alfa
ERβ	Estrogen receptor-beta
FFQ	Food frequency questionnaire
htSNP	Haplotype-tagging single nucleotide polymorphism
HWE	Hardy-Weinberg equilibrium
MAT	Matairesinol
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
PGE2	Prostaglandin E2
PSA	Prostate-specific antigen
PUFA	Polyunsaturated fatty acids
RR	Relative risk
SECO	Secoisolariciresinol
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
UTR	Untranslated region

BACKGROUND

PROSTATE CANCER

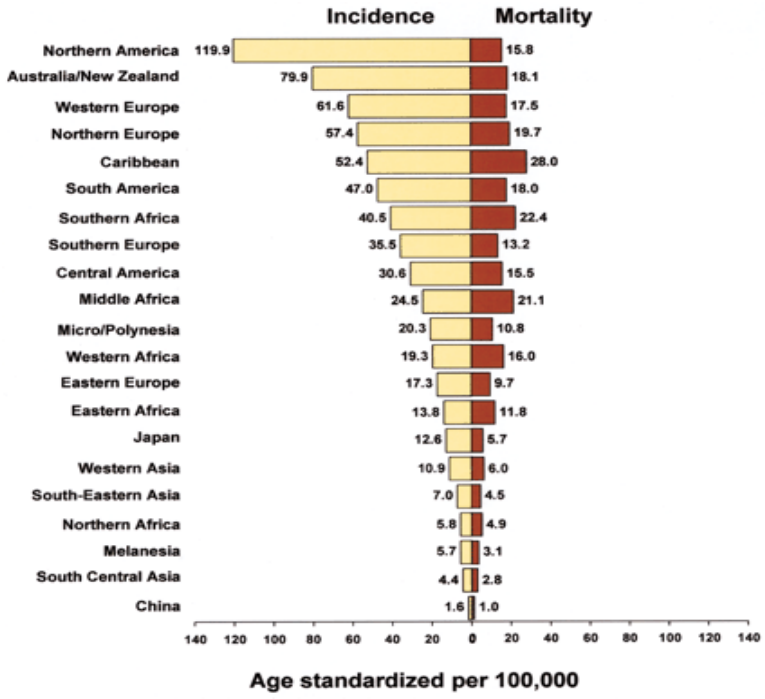
Prostate cancer is the most common cancer among men in the Western world. There were 679,000 new cases of prostate cancer worldwide in 2002, and most were diagnosed among men 65 years of age or older. In Sweden, the incidence is increasing rapidly and approximately 10,000 men are diagnosed every year (1) (Figure 1). However, the incidence rate of prostate cancer differs greatly among populations. Prostate cancer comprised of 11.7% out of all new cases of cancer in males worldwide, but 19% in developed countries and only 5.3% in developing countries (2). In Sweden, prostate cancer accounted for 36.8% of cancer diagnoses among men in 2004, and in 2003, 2,625 men died from this disease (1).

Figure 1. Incidence rate of prostate cancer in Sweden between 1960 and 2004.



Men between 30 and 40 years have been found to harbor small foci of prostate cancer (3), but most of these foci do not progress to clinically manifest prostate cancer within a normal lifetime. The incidence rates are influenced by diagnosis of latent cancers by screening asymptomatic individuals (e.g., with PSA testing), so that in countries where PSA testing is common, the ratio between the incidence rate and mortality rate may be higher than in countries were screening is less common. In Western countries, the age-adjusted survival for prostate cancer cases is significantly higher that that in developing countries. Again, much of this is a consequence of latent cancer being detected by screening procedures in developed countries, such that many cases are detected at an early stage with a better prognosis than clinically symptomatic cases (Figure 2), (2).

Figure 2. Age-standardized incidence and mortality rates for prostate cancer.



RISK FACTORS FOR PROSTATE CANCER

The increased risk of prostate cancer associated with a family history of the disease and the variation in incidence among different racial/ethnic groups support the hypothesis that genetic factors are of etiologic importance in prostate cancer. Conversely, the wide geographical variation in incidence around the world and changes in risk following migration suggest that environmental factors are also decisive (2, 4, 5).

Environmental risk factors

A large number of epidemiological studies have investigated the associations between environmental factors, especially diet, and prostate cancer. However, the findings from these studies have been inconsistent. In summary, there are several potential protective dietary factors for prostate cancer risk, including selenium, vitamin E, cruciferous vegetables, tomatoes, lycopene, legumes, fish, soy and marine fatty acids. For dietary intake of dairy products, red meat, calcium, and saturated fat and for anthropometric measures, mostly null effects or positive associations are reported (6, 7). Physical activity has been suggested to have a moderate protective effect with higher levels of activity (8, 9). Tobacco use seems not be associated with prostate cancer (10).

The prostate gland is an androgen-dependent organ, containing high amounts of the androgen receptor (AR) (11). Men who undergo castration before puberty do not develop prostate cancer (12). Like the normal prostate cells, prostate cancer cells are also generally dependent on androgens for proliferation. Based on this observation, most usual surgical and medical treatments for advanced prostate cancer aim to reduce androgen levels. With time, some cancer cells may become androgen-independent and can proliferate without androgen supply (13). However, results from epidemiological

studies regarding the possible association between testosterone and prostate cancer have yielded inconsistent results (14).

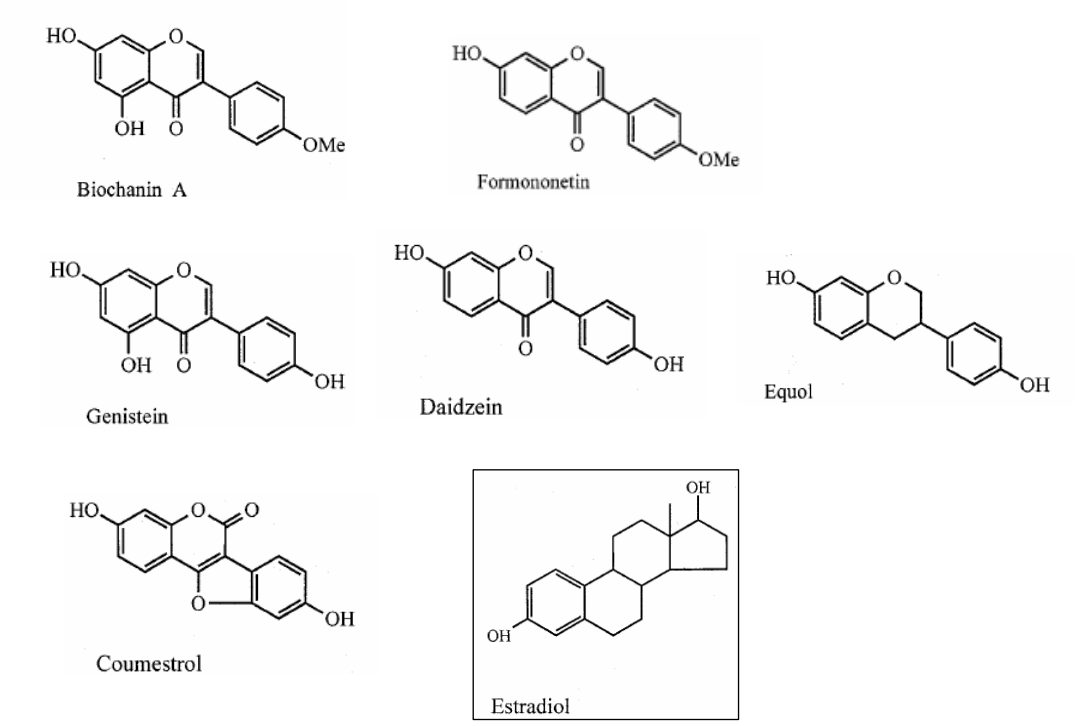
The main environmental factors of interest in this thesis are phytoestrogens, marine fatty acids and alcohol.

Phytoestrogens

Classification and metabolism

Phytoestrogens are naturally occurring hormone-like compounds found in plant foods and have a unique diphenolic structure, providing the compounds with stability (Figure 3)(15). The first reported effect of phytoestrogen intake was a potentially toxic activity, related to infertility problems in sheep fed with red clover, a rich source of phytoestrogens (16).

Figure 3. The structures of the main plant isoflavonoids and their mammalian metabolites, and of coumestrol and estradiol (15).

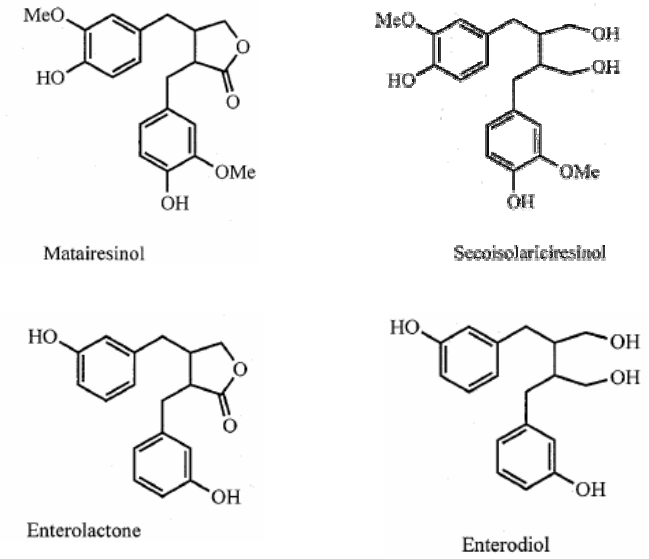


Phytoestrogens can be subdivided into coumestans, isoflavonoids, and lignans. Coumestrol (a coumestan) and the isoflavonoids genistein, daidzein, and their plant precursors biochanin A and formononetin, are mainly found in soybeans and clover. Smaller amounts have been found in other beans and in some vegetables and fruits (17). In plants, isoflavonoids occur as glycosidic conjugates but upon consumption, they are hydrolyzed by mammalian enzymes and the gut microflora to form active aglycone isoflavone compounds (15). Equols are produced from daidzein by the gut microflora, but this process seems to differ among individuals and populations (18, 19). The three most estrogenic phytoestrogens are coumestrol, genistein, and equol. Due to their structural similarity to the human hormone 17β-estradiol they bind to estrogen

receptor-beta (ERβ) with an affinity almost equal to that of the endogenous ligand (20, 21).

Compared with isoflavonoids and coumestrol, lignans have a much lower estrogenic activity (20) (Figure 4). Some of the human dietary sources of lignans are flaxseed, grain (especially rye), seeds, and berries (17). Plant lignans, such as matairesinol (MAT) and secoisolariciresinol (SECO), are converted by the mammalian gastrointestinal microflora to mammalian lignans, enterolactone and enterodiol, respectively (15). Until recently, only two plant lignan precursors for mammalian lignans were known: SECO and MAT. Lariciresinol, pinoresinol, syringaresinol and medioresinol are newly identified enterolactone precursors, found mainly in cereals. Isolariciresinol has been found in these foods as well, but seems not to be converted to enterolactone or enterodiol in the gut (22).

Figure 4. The structures of the main plant lignans and their mammalian metabolites (15).



Phytoestrogens and prostate cancer

The hypothesis that phytoestrogens protect against hormone-dependent cancers, such prostate and breast cancers, first arose from the observation that the incidence of these cancers is lower in populations, such as Japan, with a high consumption of phytoestrogens than in Western populations, where the intake of phytoestrogens is generally lower (23).

In vivo and *in vitro* experiments show protective effects of phytoestrogens on prostate cancer, and suggest that phytoestrogens may prevent cancer by a variety of mechanisms (18, 23-27):

- Through an antiestrogenic effect via the estrogen receptor
- By downregulating sex steroid receptor expression
- By reducing circulating androgen levels through increasing concentrations of sex hormone binding globulin (SHBG)

- Via negative feedback on gonadotrophin-releasing hormones and inhibition of important steroid biosynthetic enzymes (e.g., 17β- hydroysteroid dehydrogenase, 5α-reductase, aromatase)
- By inhibiting prostate cancer growth by interference with growth factors
- By increasing prostate cancer cell apoptosis
- By inhibiting angiogenesis, invasion, and metastasis
- By regulating genes involved in tumor progression
- Via antioxidant properties

Epidemiology

Table 1 summarizes epidemiological studies which have evaluated the association between intake of phytoestrogens (lignans or isoflavonoids) or soy products and risk of prostate cancer. The relative risk estimate (OR or RR) from these studies ranged from 0.3-0.95 for isoflavonoids or soy products, and from 0.66-1.20 for lignans. The results were statistically significant in 4 out of 13 studies (28-31).

Among the handful of epidemiological studies investigating isoflavonoids in Western populations, one reported an inverse association between intake of coumestrol or daidzein and prostate cancer (29), while others reported that frequent consumption of soymilk (28) or tofu (32) was associated with a reduction in risk. In one study with a multiethnic population, consumption of soy products or legumes was associated with a significant reduction in risk overall, but the associations were not significant in a subgroup of Caucasian participants (30).

Only one small study (29) investigated the association between intake of dietary lignans and prostate cancer and found no association. A few other studies have demonstrated a decreased relative risk following consumption of lignan-rich foods (30, 33-37). However, in another study no association was seen between prostate cancer risk and intake of tomatoes and fruits (30), whereas another found an increased relative risk with increasing intake of fruit and whole-grain breakfast cereals (36). Three Scandinavian nested case-control studies provided some evidence of a protective effect of serum enterolactone (38-40). In addition, the Swedish study showed that extremely low levels of enterolactone were associated with an increased risk of prostate cancer (OR for the bottom decile vs. all other deciles combined = 1.68; 95% CI: 1.03-2.74). Furthermore, two pilot studies suggested that a flaxseed-supplemented, fat-restricted diet may protect against prostate cancer (41, 42).

Table 1. Description of epidemiological studies of phytoestrogens and prostate cancer in men.

Design	Cases/ Controls,n	Race and/or study site	Exposure	Comparisons	RR/OR (95% CI)	Reference
Cohort	174/ 7999	Japanese ancestry, USA	Tofu[a]	≤1 vs. ≤5 times/week	0.35 (0.08-1.43)	Severson 1989 (32)
Cohort	225/12295	Seventh-day Adventist men, USA	Soymilk[a]	Never vs. 1 times/day	0.3 (0.10-0.90)	Jacobsen 1998 (28)
Case-control	83/107	Caucasian, USA	Genistein[a] Daidzein[a] Coumestrol[a] SECO[a] MAT[a]	Low vs high,cutoff: 29.7µg 22.8 µg 67.5 µg 482.7 µg 45.7 µg	0.71 (0.39-1.30) 0.57 (0.31-1.05) 0.48 (0.25-0.94) 1.20 (0.65-2.21) 0.89 (0.47-1.66)	Strom 1999 (29)
Case-control	1623/1623	Multiethnic, Canada	Tofu or soybean[a]	None vs. some	0.80 (0.60-1.10)	Villeneuve 1999 (43)
Case-control	90/180	Chinese, Taiwan	Soybean milk[a]	No vs. yes	0.95 (0.45-2.00)	Sung 1999 (44)
Case-control	1619/1618	Multiethnic, USA, Canada	Soy foods[a] Legumes[a]	0 vs. >39.4 g/day 2.6 vs.>51.5 g/day	0.62 (0.44-0.89) 0.68 (0.53-0.88)	Kolonel 2000 (30)
	510/501	Subgroup; Caucasian	Soy foods[a] Legumes[a]	0 vs. >39.4 g/day 2.6 vs.>51.5 g/day	0.77 (0.45-1.30) 0.97 (0.69-1.36)	
Nested case-control	794/2550	Scandinavia	Enterolactone[b]	<4.3 vs.≥15.6	1.08 (0.83-1.39)	Stattin 2002 (38)
	86/342	Subgroup; Swedish	Enterolactone, nmol/L[b]	<7.15 vs. 13.6 <7.15 vs. 25.1 <7.15 vs.≥25.1	0.66 (0.33-1.29) 0.75 (0.38-1.45) 0.87 (0.45-1.67)	
Nested case-control	214/214	Finland	Enterolactone, nmol/L[b]	<5.9 vs.24.4	0.71 (0.42-1.21)	Kilkkinen 2003 (40)
Nested case-control	265/525	Sweden	Enterolactone, nmol/L[b]	<9.4 vs. 17.6 <9.4 vs. 28.3 <9.4 vs. >28.3	0.74 (0.45-1.20) 0.95 (0.60-1.52) 1.05 (0.65-1.69)	Stattin 2004 (39)
Case-control	133/265	Chinese, China	Soy foods[a]	≤27.5 vs. 111.8 g/day	0.51 (0.28-0.95)	Lee 2003 (31)
Case-control	140/140	Japanese, Japan	Soy products[a]	≤77 vs. 187.2 g/day	0.53 (0.24-1.14)	Sonoda 2004 (45)
Cohort	304/5826	Japanese American, USA	Tofu[a]	0 vs. >240 g/day	0.82 (0.54-1.23)	Nomura 2004 (46)
Nested case-control	40/101	Japan	Genistein[b] Daidzein[b] Equol[b]	<332 vs.>765 nM <126 vs.>320 nM <1.9 vs. 60.7 nM	0.38 (0.13-1.13) 0.41 (0.15-1.11 0.34 (0.11-1.10)	Ozasa 2004 (47)

[a] assessed from dietary intake measurements
[b] measured in serum

Fish and polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) belong to two families: omega 6 (ω-6) and omega 3 (ω-3) PUFA. The ω-6 fatty acids are biosynthesized from the precursor linoleic acid, while α-linolenic acid is the parent fatty acid of the ω-3 family. Linoleic acid and α-linolenic are so-called essential fatty acids, which cannot be synthesized by animals, including humans. From these parent fatty acids, fatty acids with longer carbon chains and more double bonds can be synthesized. α-linolenic acid can to a limited extent be converted to eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Conventional dietary sources of α-linolenic acid are rapeseed oil, soy oil, dark green leafy vegetables, flaxseed, nuts and soybeans. EPA and DHA are mainly found in fatty fish, with levels that vary by the species of the fish, environmental factors and geography. The main source of ω-6 PUFAs in the typical Swedish diet is vegetable oil, such as corn oil, sunflower oil, soy oil, rapeseed oil and margarine. Arachidonic acid (AA) exists in limited levels in liver, meat and egg, but can be metabolized in human from other fatty acids in the ω-6 fatty acid family. (48)

Fish/Polyunsaturated fatty acids and prostate cancer

Increasing evidence from animal and *in vitro* studies shows that ω-3 fatty acids, especially EPA and DHA, protect against prostate cancer (49-51). In addition, recent epidemiological studies show that frequent consumption of fish is associated with a reduced relative risk of prostate cancer (50, 52, 53). Therefore, it has been suggested that high intake of fatty fish and marine fatty acids might be preventive dietary factors for prostate cancer. However, the mechanism for the potential protective effect remains unclear.

Polyunsaturated fatty acids are converted in the body to eicosanoids, which are short-lived hormone-like lipids, such as prostaglandins and thromboxanes. These compounds have several biological effects, including modulation of the inflammatory and immune responses, cell differentiation and cellular growth (51), and may be implicated in cancer development (for further reading see section: *COX-2 and prostate cancer*).

Epidemiology

Reviews of the literature on epidemiologic studies of the association between fish intake and/or fatty acids in adipose tissue, erythrocytes, serum or diet and the risk of prostate cancer (50, 52, 53) have revealed that six studies reported a significantly decreased risk of prostate cancer in association with high intake of fish, whereas seven studies showed similar but non-significant trends. However, no study found a significant positive association between dietary intake of fish, linolenic acid, or AA and prostate cancer risk, although α-linolenic acid intake was found to be associated with an increased relative risk of prostate cancer, particularly advanced prostate cancer (54), in the majority of studies. Only 3 out of 13 studies found a significantly reduced risk of prostate cancer in association with intake of EPA and DHA (50, 52, 53), and the risk of advanced prostate cancer was marginally reduced (54); none reported a positive association with prostate cancer risk. Only a few studies have evaluated association between the ratio of ω -3 to ω-6 fatty acids and prostate cancer risk, with inconsistent results (51, 54). Some smaller studies have shown that a high ω-3:ω-6 ratio in serum is inversely related to prostate cancer progression (55, 56).

Alcohol

Alcohol consumption has been suggested to be linked to various malignancies, including cancers of the pharynx, oral cavity, larynx, and esophagus, pancreas and liver (57). In addition, alcoholic beverages are classified as a human carcinogen, casually related to increased risk of cancer in the gastrointestinal tract (58). However, the role of alcohol consumptions in relation to prostate risk is unclear (57).

It has been suggested that alcohol drinking may enhance cancer development by a variety of mechanisms. These include metabolic activation of environmental carcinogens or procarcinogens by ethanol; chronic exposure to acetaldehyde, the carcinogenic major metabolite of alcohol that can produce DNA adducts; oxidative stress and damage due to increased production of reactive oxygen species (58-60); the formation of lipid peroxidation products and related DNA adducts; inhibition of DNA repair (58); and immune suppression due to heavy ethanol consumption (61, 62). On the other hand, alcohol could conceivably decrease risk of prostate cancer by lowering serum levels of androgens (63, 64), which appear to promote prostate cancer development (65), and/or by raising levels of estrogens (66, 67).

Epidemiology

The majority of epidemiologic studies suggest that alcohol drinking is not involved in the development of prostate cancer. A meta-analysis of 35 studies published prior to July 1998 determined that overall, there was no association between alcohol drinking and prostate cancer risk (57). This finding supported a previous review encompassing epidemiologic studies between 1971 and 1996, which similarly found no evidence of an association between low-to-moderate alcohol consumption and risk of prostate cancer (68). However, a minority of studies have detected an elevated risk in association with greater volume and/or longer duration of alcohol drinking (69-76), while some studies found an inverse association for heavy drinking (77), and for increasing level of red wine consumption (78) . Another recent investigation found no significant association between alcohol intake and risk of prostate cancer, but did report that risk was elevated among men who drank a large volume of alcohol at low frequency (one or two days per week), suggesting that drinking patterns rather than amounts may be associated with disease risk (79). There is no consensus as to whether the association of alcohol drinking with risk of prostate cancer varies between localized and advanced cases, or between cases with and without a family history of prostate cancer. Few, if any, previous epidemiologic studies of alcohol consumption have stratified analyses between these subgroups.

Genetic risk factors

Genetic variation in the human genome

The entire human genome contains approximately 30.000 genes and 3 billion base-pairs, and has been very consistent during the course of evolution. In comparisons between two species that have diverged from one another by millions of years, it makes little difference which individuals within each species are compared. For example, typical human and chimpanzee DNA sequences differ from one another by 1%. In contrast, when the same region of the genome is sampled from two different humans, the differences are typically less than 0.1% (80).

However, any two unrelated humans have millions of genetic differences, making them look or behave differently, or to be disparately susceptible to different diseases. Variation in the genome occurs in several ways; the smallest form of genetic variation is an exchange of one base-pair for another or a deletion/insertion of one or a few base-pairs. Mutations are variations that are rare, whereas single nucleotide polymorphisms (SNPs) refer to variations that are common in a population, with a prevalence of over 1 percent. The human genome has over 10 million polymorphisms. SNPs are often related to one another and when a mutation arises, it is generally associated with multiple other variants present on the same chromosome. Variants that are associated together are collectively known as a haplotype. For this reason, there are often strong statistical associations between polymorphisms, meaning that the presence of a particular variant at one site on a chromosome can predict or "tag" the presence of a particular variant at another site. The practical benefit of this is that only a few SNPs need to be genotyped to cover most of the variation in a region of the chromosome that are of specific interest. The recently developed International Hap Map Project has identified and genotyped more than one million SNPs in different racial/ethnic populations. Information from the project is freely available on the Internet, making it an exceptional tool for genetic studies. (80-82)

Genetic variation and risk of prostate cancer

The increased risk of prostate cancer in men with a family history of prostate cancer, and results from twin studies and family-based segregation analysis, provide strong evidence for the involvement of genetic factors in the etiology of prostate cancer (4, 5, 83). Twin studies from Sweden and the US estimated that 42% and 57%, respectively, of the risk of prostate cancer can be explained by heritable factors (5, 84). However, rare highly penetrant genes probably account for a minority of prostate cancer cases and no high-risk candidate genes have been identified (83). The remainder of the genetic influence is most likely mediated by more common genetic variants or polymorphisms. Even though the influence of polymorphisms on cancer is modest, the higher prevalence of polymorphisms than mutations in high-penetrance genes makes their overall impact on cancer substantial.

Several polymorphisms in different genes and their associations with prostate cancer risk have been investigated, although with different results (Lindström et al. *in press*). Variation in genes involved in the androgen pathway (e.g., AR, 17α-hydroxylase (CYP17) and 5α-reductase type II (SRD5A2) (Lindström et al. *in press*)) and genes

involved in the inflammation response (e.g., macrophage scavenger receptor 1 (MSR1), ribonuclease L-gene (RNASEL), macrophage inhibitory cytokine-1 (MIC-1) and interleukin-1 receptor antagonist (IL-1RN) (85-88)) have shown to be associated with prostate cancer risk. In the present thesis we focus solely on polymorphisms in the ERβ and cyclooxygenase (COX)-2 genes.

Estrogen receptor-beta (ERß)

Interest in the potential role of the estrogen receptor (ER) in prostate cancer development was heightened when the second ER, ERß, was cloned and characterized (89). ERß was found to be highly expressed in prostate epithelium, suggesting a target for a direct effect of estrogen on the prostate (90, 91).

In rodent studies, epithelial hyperplasia and insufficient differentiation in the prostate epithelium have been found in mice lacking ERβ expression (92-94). Similarly, studies on humans found that loss of ERβ expression was associated with the progression of normal prostate epithelium to prostate cancer (95-97), and that ERβ expression was inversely correlated with Gleason grade (96). Furthermore, ERβ expression has been correlated with prostate cancer prognosis (95, 96). However, other human studies found no association between ERβ and risk of prostate cancer (98). Leav et al. found that prostate carcinogenesis was characterized by loss of ERβ expression at the protein and transcript levels in high-grade dysplasias, its reappearance in grade 3 cancers, and its diminution or absence in grade 4/5 neoplasms. ERβ expression was also found in prostatic carcinoma cells metastatic to bone and lymph nodes (97), as confirmed by Lai et al (99). However, the receptor seems to play different roles at various stages in the evolution and progression of prostate cancer. This accords with the finding that ERβ was differentially expressed at successive stages of prostate cancer development (97). The precise biological function of the protective effect of ERß is unknown, but it has been suggested that ERß is involved in inhibiting invasion, proliferation and stimulating apoptosis by regulation of other steroid receptors and protein levels, interference with growth factors or regulation of checkpoints in the cell cycle (92, 100, 101).

In CAPS a weak positive association between a SNP located in the promoter region of ERβ gene (rs2987983) and prostate cancer was found (102). However, one small Japanese case-control study, including 147 cases and 266 controls, found no association between another SNP in ERβ and risk of prostate cancer (103).

Some phytoestrogens show a higher binding affinity to the ERß than to the ERα (20, 21, 104, 105) suggesting that phytoestrogens might interact with ERß in the development of prostate cancer. In paper II, we tested the hypothesis that phytoestrogens exert a protective effect on prostate cancer through interaction with specific subtypes of the ERß gene.

Cyclooxygenase (COX)-2

Cyclooxygenase (COX) is a key enzyme in eicosanoid synthesis and converts AA to prostaglandins and other eicosanoids. Two isoforms of COX have been identified. COX-1 is expressed in many tissues and cell types, and is involved in processes

including gastric acid secretion, vascular homeostasis and water reabsorption by the renal tubules. In contrast, COX-2 is inducible and involved in differentiative processes such as inflammation and ovulation (106). COX–2 is highly expressed in a number of cancers, including prostate cancer (107-109). Overexpression results in enhanced synthesis of prostaglandins, and malignant prostate tissue converts AA to prostaglandin E2 (PGE_2) at a ten-fold higher rate than benign tissue (110).

Emerging evidence that chronic inflammation is involved in the etiology of prostate cancer (88) is supported by previous findings that use of non-steroidal anti-inflammatory drugs (NSAIDs) is inversely associated with prostate cancer risk (111). It has been proposed that a protective effect of NSAIDs is mediated through inhibition of the COX enzymes. AA-derived eicosanoids favor the growth of malignant cells by increasing cell proliferation, impeding immune surveillance, inducing angiogenesis, and inhibiting apoptosis (49, 112). In contrast, ω-3 derived eicosanoids have anti-inflammatory effects and may prevent prostate cancer growth by stimulating apoptosis and up-regulating genes coding for antioxidant enzymes (49, 51).

Perhaps the most prominent mechanism of the protective effect of ω-3 fatty acids may be via their suppressive effect on the biosynthesis of AA-derived eicosanoids. This inhibition occurs at several levels: 1) high intake of ω-3 fatty acids partly replaces AA incorporation into membrane phospholipids, resulting in decreased availability of precursors for AA-derived eicosanoids; 2) ω-3 fatty acids have a higher affinity than ω-6 fatty acids for several enzymes (e.g., desaturases and elongases) in the metabolism of fatty acid conversion, and ω-3 fatty acids are therefore preferentially metabolized; and 3) marine fatty acids, namely EPA and DHA, suppress COX-2 and lipooxygenases, and compete with ω-6 fatty acids as the substrate for these enzymes (49, 51, 113).

A diet with a high ratio of ω-3 to ω-6 fatty acids results in a shift toward production of EPA-derived eicosanoids rather than AA-derived eicosanoids and, as a result, may inhibit the development of prostate cancer.

Little is previously known about the possible role sequence variants within the COX-2 gene plays in prostate cancer etiology. In the only published study to date, SNPs in the promoter region were associated with a decreased risk for prostate cancer among African Americans (114). In CAPS, we previously found a association between genetic variants of the COX-2 gene and risk of prostate cancer (115). Dietary intake of marine fatty acids from fish may be protective and COX-2 may alter the effect of these fatty acids in the development of prostate cancer. In paper III, we aimed to explore interactions between fish intake and genetic variation in the COX-2 gene.

AIMS

The specific aims of the work underlying this thesis are:

- To assess the dietary intake of phytoestrogens among Swedish men
- To validate the reported dietary intake of lignans with serum levels of enterolactone
- To investigate whether risk for prostate cancer in a Swedish population is associated with exposure to phytoestrogens, as measured by I) dietary intake of food products rich in phytoestrogens, II) dietary intake of individual phytoestrogen compounds, and III) serum enterolactone levels
- To evaluate whether there is an interaction between dietary intake of phytoestrogens and polymorphisms in the estrogen receptor-beta (ERβ) that influences the risk of prostate cancer in a Swedish population
- To investigate if there is an association between dietary intake of different fish species and fatty acids, especially marine fatty acids and the ratio of ω-3:ω-6 fatty acids, and risk of prostate cancer in a Swedish population
- To explore whether there is an interaction between fish intake and genetic variation in the COX-2 gene in the development of prostate cancer
- To assess the amount, frequency, and type of alcohol intake among Swedish men and investigate if there is an association between alcohol consumption and risk of prostate cancer overall
- To investigate whether any association between alcohol intake and prostate cancer risk varies between localized and advanced cases, or between sporadic and familial cases

MATERIALS AND METHODS

STUDY POPULATION – THE CAPS STUDY

The studies that are part of this thesis are based on the Cancer Prostate in Sweden study (CAPS). CAPS is a population-based case-control study of prostate cancer etiology. Incident prostate cancer cases were identified from 4 of the 6 regional cancer registries in Sweden, serving the northern, central, Stockholm, and southeastern health care regions, which encompass approximately 67% of Sweden's total population of about 9 million inhabitants. Together, the Swedish cancer registries record almost 100% of all incident cases in the country (116). All cases were diagnosed with pathologically or cytologically verified adenocarcinoma of the prostate (ICD-10 code: C61).
Residents of the Örebro area and northern Sweden (Västernorrland, Jämtland, Västerbotten, Norrbotten) were eligible beginning in January 1st, 2001, whereas men from Västmanland, Södermanland, Gävleborg, Dalarna, Värmland, and Uppland were eligible starting July 1st, 2001. Enrollment continued until September 30, 2002, except in Jämtland and Västerbotten counties, where recruitment ended on March 1, 2002. Participants from the northern and central health care regions, i.e., Örebro, Västmanland and Södermanland, were between 35 and 79 years of age, whereas those from the southeastern and Stockholm regions were between 35 and 65 years, in order to enrich the proportion of cases with a family history of prostate cancer, who tend to be diagnosed at an earlier age than sporadic cases. Cases were informed about the study and asked to participate via their treating physicians.

Control subjects were randomly selected from the computerized, continuously updated Swedish Population Registry, and frequency matched according to the distribution of the cases by age (within five years) and geographic residence. The controls were contacted by mail and received the same information about the study as the cases.

In total, 1895 prostate cancer cases were invited to participate. Of those, 1499 (79%) agreed to participate by completing the questionnaire and 1400 (74%) by donating a blood sample. Overall, 1352 (71%) cases both completed the questionnaire and donated blood. Of the 1684 invited controls, 1130 (67%) completed the questionnaire and 879 (52%) donated blood; 858 (51%) both completed the questionnaire and donated blood. All study participants granted informed consent at the time of enrollment in the study. The CAPS study was approved by the Ethics Committees at Karolinska Institutet and Umeå University.

Family and clinical data

All participants who reported in the initial questionnaire that they had at least one family member with prostate cancer were contacted a second time. Detailed information about family history of cancer was obtained through a second questionnaire and a subsequent telephone call. All prostate cancer cases in first, second, and, if possible, third-degree relatives were independently verified through cancer registries or medical records. Cases who had one first-degree relative with prostate cancer and controls with two first-degree relatives were defined as having familiar prostate cancer in the family. Cases without a family history of prostate cancer were

classified as sporadic cases. None of the controls fulfilled the criteria to have familiar or hereditary prostate cancer in the family.

Clinical data, including TNM (tumor, nodes, and metastasis) stage (117), tumor differentiation grade and/or Gleason score, serum prostate-specific antigen (PSA) level at diagnosis, means of diagnosis and primary treatment, were obtained from linkage to the National Prostate Cancer Registry for 95% of all patients in the study. Advanced cases were defined as those with at least one of the following criteria: T3/T4, N+, M+, Gleason score=8 to 10 or PSA level≥100 ng/ml. These criteria were chosen to identify advanced cases as those with high likelihood of dying from the disease. Localized cases were those not meeting any of the above criteria. Sixty-two cases lacked sufficient information for determining advanced or localized stage of disease. We collected self-reported data on date and type of treatment of prostate cancer. Any treatment prior to the blood draw was classified as hormone treatment, operation or radiotherapy.

EXPOSURE ASSESSMENT

All participants received a mailed information letter and an informed consent form, along with a questionnaire and a kit with 4 tubes (heparin, plasma and EDTA-treated) for blood sampling.

Individuals were instructed to donate blood at the nearest health clinic or hospital. The unprocessed samples were sent overnight by mail to the Umeå Biobank, where each sample was aliquotted into sixteen smaller tubes (plasma, serum, white and red blood corpuscles) and stored in a freezer at –80 C° until the time of analysis. Study participants were not instructed to fast before the blood draw, but health clinic personnel recorded the time when food or beverages were last consumed prior to the blood draw.

The self-administered questionnaire assessed known and potential risk factors for prostate cancer, including a dietary assessment (food frequency questionnaire (FFQ)) to measure the average intake of different food items and beverages in the last year. In addition, the participants were asked about their use of antimicrobial medications in the last year (never, 1-3 times, 4-6 times or more than 6 times). If information was missing from the mailed questionnaire, participants were contacted by phone to complete the missing information.

Assessment of food and nutrient intake

In total, the validated food frequency (118) assessed intake of 261 items, including milk and yogurt/soured milk (low, medium or high fat), low-fat cheese, high-fat cheese, cottage cheese, melted cheese, cream (12% or 40% fat), crème fraiche (17% or 34% fat), mayonnaise (32% or 80% fat), crisp bread, wheat/rye bread, wholemeal bread, butter/margarine/oil, cereals, flaxseed, wheat bran, oat bran, sunflower seed, vegetables, fruits, potatoes, rice, pasta, meat, fish, eggs, candy, ice cream, beverages (e.g., coffee, tea, soft drinks, water and alcohol) and dietary supplements (e.g., vitamins, minerals and fish oil). In order to estimate individual intake of energy and nutrients, we linked the dietary information from the questionnaire to the nutrient

database created by the Swedish National Food Administration (PC kost 2004) (48); this database lists the energy and nutrient content of 1500 food products.

Phytoestrogens

The questionnaire was specifically designed to evaluate the intake of phytoestrogen-rich food products that are commonly consumed in Sweden. In order to estimate the intake of specific phytoestrogens, we created a database for the content of genistein, daidzein, biochanin A, formononetin, coumestrol, MAT and SECO in food products. Analytical values of phytoestrogens were obtain from recently published analytical data (15, 17, 119, 120) and unpublished data (Heinonen and Adlercreutz). Most of the analyses were carried out by isotope dilution gas chromatography-mass spectrometry performed in a laboratory in Finland (15), where food products, agricultural conditions and food habits are similar to those in Sweden. For two food products (soy-based items), analysis was carried out by high-performance liquid chromatography (120, 121).

Original data on phytoestrogen concentrations were expressed for dry weights of foods and were converted by us to values for wet weight (μg/100g wet weight of a food). If analytical information about a food item was available from more than one source, the mean value was used. Of the food items in the questionnaire, 91 (35%) had phytoestrogen levels assigned directly from the original phytoestrogen database. For 65 items (25%) such as fruit soup, jam, porridge, bread and cereal products, phytoestrogen values were calculated based on information from Swedish recipes of the product or information from food manufacturers. Because of the high stability of phytoestrogens, it is unlikely that destruction occurred due to food processing, such as baking.

For the remaining 105 food items (40%), mainly those of animal and fish origin, zero values were used. From personal contact with several meat processing companies in Sweden, we found that the addition of soy to meat products, such as sausage, is not common in Sweden, and the isoflavone content of processed meat products was set to zero.

The content of the newly discovered plant lignans isolariciresinol, lariciresinol, pinoresinol, syringaresinol and medioresinol in different grain flours (Heinonen, unpublished data) was used to estimate lignan content of bread and cereal products, and was added to the database. Lariciresinol and isolariciresinol were calculated together because during the analytical work lariciresinol is converted to isolariciresinol. The extent of this conversion is not yet known. In the present study we assumed that isolariciresinol is derived from lariciresinol. The values for secoisolariciresinol include the values for anhydrosecoisolariciresinol, which also is a product of the analytical method. Based on the levels of genistein, daidzein, equol, matairesinol, enterodiol and enterolactone in raw cow milk (Adlercreutz, unpublished data), we estimated the content of these compounds in different kinds of milk products, and the information was added to the database. However, these estimations are approximate since it is unknown whether the phytoestrogen concentration is affected by dairy processing, such as fat reduction or souring of milk.

In a separate analysis, we used conversion factors to calculate the expected amount of dietary lignans to be converted to mammalian lignans (22). The rationale for this is that the fecal microflora are important for the metabolism of plant lignans to mammalian lignans (e.g., enterolactone), and different lignans are metabolized to various extents. The conversion factors were as follows: matairesinol = 0.62, secoisolariciresinol = 0.72, lariciresinol =1.01, pinoresinol =0.55, syringaresinol =0.04 and medioresinol= 0.8. The value for medioresinol was not experimentally obtained and is, therefore, an approximation. The remaining conversion factors are based on relatively few experiments and are thus quantitatively inexact. The amount of dietary lignans expected to be converted to mammalian lignans was used in the analysis of prostate cancer risk and validation of the FFQ.

Fish

Participants were asked how often, on average, they ate salmon (Salmo salar)/whitefish (Coregonus lavaretus)/char (Salvelinus alpinus) (hereafter referred to as "salmon-type fish"), Baltic herring (Clupea harengus membras)/herring (Clupea harengus)/mackerel (Scomber scombrus), cod (Gadus morhua)/saithe (Pollachius virens)/fish fingers, caviar, or shellfish (shrimp/crayfish): never, 1-3 times/month, 1-2 times/week, 3-4 times/week, 5-6 times/week, 1 time per day, 2 times per day or 3+ times per day.

Fatty acids

In order to estimate intake of fatty acids we used the Swedish National Food Administration database, which includes information about dietary content of the following fatty acids: butyric acid 4:0, lauric acid 12:0, myristic acid 14:0, palmitic acid 16:0, stearic acid 18:0, arachidic acid 20:0, palmitoleic acid 16:1, oleic or/and elaidic acid 18:1, linoleic acid 18:2, α-linolenic acid 18:3, AA 20:4, EPA 20:5, docosaapentaenoic acid 22:5 and DHA 22:6. To estimate total intake of ω-3 fatty acids, we summated intake of α-linolenic acid, EPA, docosapentaenoic acid, and DHA. To estimate total intake of ω-6 fatty acids, we combined the intake of AA and linoleic acid.

Alcohol

Participants were asked whether, one year ago, they drank alcohol at all, or whether they had stopped drinking at some age. If they currently drank alcohol, they were asked whether they drank medium beer, strong beer, wine, strong wine, or liquor, on average, never, 0-1 times per month, 2-3 times per month, 1-2 times per week, 3-4 times per week, 5-6 times per week, 1 time per day, 2 times per day, or 3 or more times per day, one year prior to the time of the questionnaire. For wine they were asked if they most often drank white or red wine. They were also asked how much, in centiliters, they drank of each type of alcohol on an average occasion.

Former drinkers were defined as individuals who had stopped drinking at least a year and a half prior to the study, in order to ensure that only men who had not been drinking for at least one full year were included. Persons who stopped drinking within the last year and half were considered current drinkers. For individuals who provided only either frequency or volume, the missing value was assumed to be the median in the study population. Seven men (six cases, one control) had missing data on both

frequency and volume of alcohol intake, and were excluded from the statistical analysis.

Frequency and volume of alcohol intake were converted into weekly intake of ethanol in grams. In Sweden, an average can (33 cl) of light beer, medium beer, or strong beer contains 6.0 g (2.3%), 9.1 g (3.5%), or 14.6g (5.6%) ethanol, respectively; a glass (15 cl) of wine or strong wine contains 14.2 g (12.0%) or 20.7 g (17.5%) ethanol, respectively; and a shot glass (4 cl) of liquor contains 12.6 g (40%) ethanol. To calculate number of drinks per week, one can of medium or strong beer, one glass of wine, and one shot glass of liquor were each considered as one drink.

Serum enterolactone

In paper I, we analyzed circulating enterolactone concentration in serum using time-resolved fluoroimmunoassay (122) with slight modifications (123), in a randomly selected subgroup comprising 221 controls and 218 cases. Laboratory personnel were blinded to the case-control status of the samples. One participant with an extreme serum value of over 600 nmol/L was excluded from the statistical analysis. Enterolactone concentration was log-transformed to normalize the distribution.

Selection of single nucleotide polymorphisms (SNPs) and genotyping

ERß

The ERβ (ESR2) gene, located on chromosome 14 q23.2, is approximately 61.2kb, including 8 exons and two untranslated first exons. We conducted a search for known SNPs in public databases (124, 125) and selected a subset of SNPs from the promoter region (15kb), introns, exons and 3´ untranslated region (UTR), covering a total length of 68.5kb. There are three SNPs in coding regions, all synonymous. At the time of selection not many SNPs in ERβ were validated and even fewer had frequency data, so the main criteria for selection were that the SNPs were evenly spread throughout the gene. We selected 37 SNPs, with a mean distance between SNPs of 1800 base-pairs. These 37 SNPs were then genotyped in 94 randomly selected control subjects from CAPS using a 5´ nuclease Taq-Man assay together with fluorescently labeled Minor Groove Binders probes (102). Five SNPs were monomorphic in our population and in 10 SNPs the assay failed, leaving 22 SNPs for further analysis. To select haplotype-tagging SNPs (htSNPs) we used the htSNP2 package for the STATA software (David Clayton, Cambridge, UK).

Four SNPs-- rs2987983 (-13950 T/C), rs1887994 (-10908 G/T), rs1256040 (11309 A/G), and rs1256062 (46385 C/T)-- were selected as htSNPs, which captured 99.6% of the haplotype variation among the 94 controls. To validate the ability of the selected htSNPs to predict common genetic variation in the ERβ gene, we downloaded all SNPs (n=89) genotyped in the CEPH population in our chosen region from the HapMap database (release #20). After exclusion of rare variants (minor allele frequency <5%, n=41) the average proportion of haplotype diversity explained by our four htSNPs was 94.5% (range 67.6 to 100%) suggesting an adequate coverage. These four SNPs were genotyped, with the same method described above, in all available samples in CAPS

(1314 cases, 782 controls with extracted DNA). All selected htSNPs were in Hardy-Weinberg equilibrium (HWE) among both cases and controls ($p > 0.05$). During the genotyping we placed two positive controls for each genotype and two negative controls on each plate. In addition 29 blind duplicates were spread among the plates and all duplicates provide concordant genotype.

COX-2

The COX-2 gene, located on chromosome 1q25.2-q25.3, is less than 8 kb in length and includes 10 exons. To achieve complete coverage of the COX-2 gene, we selected SNPs at a density of 1 SNP per kb and/or every missense mutation known. These SNPs were identified through public databases (124, 125). In total, we selected 16 SNPs from the COX-2 gene, including SNPs located within the promoter, exons, introns, and the 3′ UTR. These SNPs were genotyped in 94 randomly selected control subjects from the CAPS study.

Five of the 16 SNPs-- rs2745557 (+202 C/T), rs20432 (+3100 T/G), rs4648276 (+3935 T/C), rs5275 (+6365 T/C), and rs689470 (+8365 C/T)-- had a minor allele frequency of more than 5% in the selected controls. We genotyped these five SNPs in all available samples (1378 cases and 782 controls with extracted DNA) using the MassARRAY system (SEQUENOM, Valencia, CA) (126). All of the 5 SNPs were in HWE among cases and controls, respectively (all $p > 0.05$).

STATISTICAL METHODS

Descriptive analyses

Baseline characteristics between cases and controls were compared using the two-sided t-test for continuous, normally distributed variables and the χ^2-test for categorical variables. In addition, comparison between total energy-intake, intake of main groups of macronutrients or food items in cases and controls was tested through an age-adjusted logistic regression model.

Disease – exposure associations

Associations between exposures and risk of prostate cancer were evaluated by unconditional logistic regression to estimate the odds ratio (OR), an estimate of the incidence rate ratio, and the corresponding 95% confidence interval (CI).

Categorization of variables

Variables were analyzed in their original categorical or continuous form (from the study questionnaire) or categorized according to the following criteria: intake of antibiotics (yes or no), level of education (0 to 9 years=low, 10-12 years=medium, or 13+ years=high), smoking history (ever or never), and body mass index (<25 kg/m^2, 25 to 29.9 kg/m^2, 30 to 34.9 kg/m^2, or ≥ 35.0 kg/m^2) calculated from current weight and height, age (45-49,50-54, 55-59, 60-64, 65-69, 70-74, or 75-79 years of age), calculated from date of inclusion minus date of birth.

A variable for intake of red meat was created by summarizing intake of pork, ground meat, beef, sausage, hash, liver, liver paste and sandwich meat (g/day). Milk, yogurt/soured milk, cheese, cottage cheese, crème fraiche and cream were summarized as dairy products (g/day). A variable was created from a group of food items rich in phytoestrogens by summating the intake of flaxseed, berries, nuts, peanuts, beans, sunflower seeds and soy. These foods were chosen after we ranked food items included in the questionnaire according to the lignan or isoflavonoid content per edible portion. The food items containing the highest phytoestrogen levels were those included in the summary variable.

For paper III, a variable for ω-3: ω-6 ratio was created by dividing intake of ω-3 fatty acids by the intake of ω-6 fatty acids, and then categorizing the resulting ratio into quartiles. Intake of individual seafood items was grouped into three categories (none, 1-3 times per month, or 1 or more times per week). Total intake of salmon-type fish and herring/mackerel was grouped into three categories (none, 2 or fewer times per week, 3-4 times per week, or 5 or more times per week).

Nutrient density was obtained through dividing the estimated intake of a food item or nutrient by the total energy intake (multivariate nutrient density model) (127). Participants with extremely high or low energy intake (<2100 kJ/day or >21000 kJ/day) were excluded from the statistical analysis in papers I-III (n=16).

Categorization of exposures into quartiles was based on the distribution among controls. For each comparison of serum values or dietary intake, the lowest quartile was used as the reference category. In paper IV, never-drinkers were used as the reference group for all comparisons.

Model building

All fitted models were age-adjusted (with 5-year intervals), and potential confounders were selected based on proportional (≥10%) change in ß-coefficients and previous subject matter knowledge. In papers I-III, total energy intake (as a continuous variable) was included in the models, except in analyses of serum. All covariates included in the final models were considered to be important confounding factors for the relation between the main exposure and prostate cancer, or independent risk factors based on the above selection criteria, and are listed in the table footnotes.

We used Pearson correlation coefficient analyses to evaluate whether dietary covariates were correlated. If the correlation coefficient between two covariates in the model or between covariates and the main exposure was higher than 0.6, multicolinearity issues were considered, eventually one of the covariates were excluded from the model (128).

Linear trend tests across quartiles were performed using the quartile mean or median values as continuous variables in the model. The Hosmer-Lemeshow goodness-of-fit test was used in addition to evaluation of influential observations and residuals in assessing the fit of the model (129).

Validation (Paper I)

We used correlation analyses to evaluate whether serum enterolactone concentration can serve as a reliable biomarker of lignan intake. The distributions of serum enterolactone concentration and dietary lignan intake were normalized through log-transformation. We used the Pearson correlation coefficient (hereafter called correlation) for the log-transformed variables. Subgroup analyses were also conducted to evaluate the influence of other covariates, such as animal fat density (low intake: ≤0.0054 g/day·kJ vs. high intake: >0.0054 g/ day·kJ), vegetable fat density (low intake: ≤0.0031 g/day·kJ vs. high intake: >0.0031 g/ day·kJ), red meat (low intake: ≤97.5 g/day vs. high intake: >97.5 g/ day), and age (≤68.4 years or >68.4 years), with cutoffs for dichotomous variables set at the median among controls. We also stratified analyses by body mass index (BMI; overweight: ≥25 kg/m^2 vs. normal weight: <25 kg/m^2), smoking history (never vs. ever), education (compulsory school vs. upper secondary school /university) and alcohol intake other than red wine (none/moderate vs. high intake, with the cutoff at the median of 9.2 drinks/month among current drinkers). In addition, we fitted linear regression models predicting log-transformed enterolactone from the log-transformed dietary lignan intake or SECO/MAT intake, each of the stratification variables (one at a time), and an interaction term to evaluate if the correlation was significantly different between subgroups. The validation analyses were carried out in the control group among individuals who had not taken antibiotics during the last year (n=177).

Interaction (Paper II, III)

To explore effect measure modification by SNP status, associations between dietary intake and prostate cancer risk were stratified by genotype, using the lowest levels of dietary intake as a reference. Interactions between phytoestrogen intake and ERß SNPs on prostate cancer risk were evaluated considering both multiplicative and additive effect scales. In paper II for both approaches, quartiles of phytoestrogen intake were included as a continuous variable, and each SNP was represented by an indicator variable (variant or not). In paper III, frequencies of dietary intake were represented by two indicator variables comparing medium/high fish consumption against never consumption, and each SNP was represented by an indicator variable (variant or not). On the multiplicative scale, interaction was assessed in a logistic regression model by a likelihood-ratio test of the product terms between the covariates representing dietary intake and SNP genotypes. On the additive scale, interaction was assessed by the same product terms under a linear odds model. All interaction analyses were adjusted for age and total energy intake as described above.

In papers I-III, all analyses were performed using the STATA System software, version 8.2; in paper IV, the SAS System software, release 8.2 (SAS Institute Inc., Cary, NC, 1999-2001) was used.

RESULTS

CHARACTERISTICS OF STUDY POPULATION (STUDY I – IV)

Selected baseline characteristics of the study participants are presented in Table 2. The study population was racially and ethnically homogeneous, and most of the men were born in Sweden. We found no significant difference between cases and controls with regard to smoking history, body mass index or level of education. Recent use of antimicrobials was more common among cases than controls. Most of the cases (71%) had non-PSA-detected, clinically significant prostate cancer.

Table 2. Selected baseline characteristics of prostate cancer cases and controls with questionnaire data in the CAPS study.

Characteristics	Controls n=1130	Cases n=1499
Age, years, n (%)		
45-49	7 (1)	3 (0.2)
50-54	47 (4)	38 (3)
55-59	94 (8)	185 (12)
60-64	221 (20)	353 (24)
65-69	230 (20)	343 (23)
70-74	236 (21)	251 (17)
75-79	295 (26)	326 (22)
Mean	67.8	66.8
BMI, mean (kg/m^2)	26.3	26.2
Education, n (%)	519 (46)	
Compulsory school (0-9 years)		684 (46)
Upper secondary (10-12 years)	476 (42)	601 (40)
University (13 years or more)	128 (11)	209 (14)
Missing	7 (0,6)	5 (0,3)
Country of birth, n (%)		
Sweden	1059 (94)	1427 (95)
Other European countries	71 (6)	72 (5)
Smokers, n (%)		
Never	427 (38)	581(39)
Ever	682 (60)	899 (60)
Missing	21 (2)	19 (1)
Use of antibiotics the last year, n (%)		
No	857 (76)	802 (54)
Yes	229 (20)	637 (42)
Missing	44 (4)	60 (4)
Prostate cancer stage, n (%)*		
Localized	-	828 (55)
Advanced	-	609 (41)
Unknown	-	62 (4)
PSA detected cancer, n (%)		437 (29)

* See methods section for definition of prostate cancer stage

DIETARY INTAKE

Overall dietary intake

Dietary intake of nutrients and food groups among the study participants are presented in Table 3. We found no statistically significant differences between cases and controls with regard to intake of main groups of macronutrients and there was no difference in dietary mean intake of red meat, dairy products or animal fat. However, controls had a significantly lower mean intake of vegetable fat, adjusted for age. In addition, there was no difference in frequency of dietary intake of flaxseed, soy or beans, but controls had a significant higher intake of berries. Cases had a significantly higher energy intake than controls, adjusted for age.

Table 3. Dietary intake among prostate cancer cases and controls with questionnaire data in the CAPS study.

Dietary intake	**Controls n= 1130**	**Cases n=1499**
Total energy intake, median (kJ)	8931	9334
Proportion of energy intake (%) from:		
Fat†	33	33
Protein†	16	16
Carbohydrate†	50	50
Alcohol†	1	1
Dietary intake, mean (g/day) of:		
Red meat	82	80
Animal fat	50	49
Vegetable fat	31	34
Dairy products*	548	550
Dietary intake of flaxseed, n (column %)		
Never	968 (86)	1266 (84)
1-3 times/month	51 (4)	52 (3)
More than 1 times/week	66 (6)	101 (6)
Missing	45 (4)	80 (5)
Dietary intake of soy products, n (column %)		
Never	924 (82)	1192 (80)
1-3 times/month	135 (12)	200 (13)
More than 1 times/week	31 (3)	46 (3)
Missing	40 (3)	61 (4)
Dietary intake of beans, n (column %)		
Never	557 (49)	747 (50)
1-3 times/month	446 (40)	604 (40)
More than 1 times/week	92 (8)	95 (6)
Missing	35 (3)	53 (4)
Dietary intake of berries, n (column %)		
Never	220 (19)	241 (16)
1-3 times/month	488 (43)	742 (49)
More than 1 times/week	400 (36)	477 (32)
Missing	22 (2)	29 (3)

† Proportion of total energy intake derived from fat, protein or carbohydrates

* Milk, yogurt/soured milk, cheese, cottage cheese, crème fraise and high and low fat cream

Phytoestrogens

Among both cases and controls, the average daily intake of lignans was higher than that of isoflavonoids (Table 4). In both groups, the distributions of genistein, daidzein and SECO intake were heavily skewed towards higher values. The highest median intakes of lignans among cases and controls were seen for syringaresinol and lariciresinol. In contrast, the highest values for the expected amount of lignans to be converted to mammalian lignans were seen for medioresinol and lariciresinol.

Table 5 shows the proportional contribution of various food items to the average dietary intake of lignans, isoflavonoids and coumestrol among cases and controls. Flaxseed and rye bread contributed the most to the intake of lignans, whereas soy products were the most important dietary source of isoflavonoids.

Table 4: Daily intake of phytoestrogen (μg/day) estimated from food frequency questionnaire.

	Estimated daily phytoestrogen intake						Expected amount of dietary lignans to be converted to mammalian lignans	
	Controls			Cases			Controls	Cases
Compound	Mean	Median	Range	Mean	Median	Range	Median	Median
Genistein	205	6	0-11601	296	6	0-23067	-	-
Daidzein	133	6.5	0-7352	191	7	0-14671	-	-
Coumestrol	0.1	0.1	0-5	0.1	0.1	0-9	-	-
Formononetin	2	1	0-17	2	2	0.1-16	-	-
Biochanin A	1	0.5	0-17	1	0.6	0-16	-	-
Secoisolarici-resinol[†]	1871	125	13-104313	1923	129	8-69791	90	93
Matairesinol	49	43	4-409	49	42	4.5-271	27	26
Lariciresinol*	621	583	61-2412	626	578	60-2513	583	578
Pinoresinol*	268	253	23-1144	268	252	19-1185	139	138
Syringaresinol*	1473	1389	142-5842	1491	1373	125-6016	56	55
Medioresinol*	567	535	48-2469	571	528	38-2518	428	422
Enterolactone[#]	16	13	0-167	16	13	0-102	13	13
Enterodiol[#]	0.06	0.05	0-0.6	0.06	0.05	0-0.4	0.05	0.06
Equol[#]	1	1	0-13	1	1	0-8	-	-
Total isoflavonoids [‡]	342	15	1-18975	491	16	0-37760	-	-
Total lignans [§]	4855	3045	292-106958	4929	3044	310-73754	1392	1394

[†] Total secoisolariciresinol (sum of anhydrosecoisolariciresinol and secoisolariciresinol)
[‡] Including genistein, daidzein, formononetin, biochanin A and equol
[§] Secoisolariciresinol, matairesinol, lariciresinol, isolariciresinol, pinoresinol, syringaresinol and medioresinol
***** Lignan content is available only for bread and cereal products
[#] Mammalian lignans and equol content are available only for milk products

Table 5. Dietary sources of phytoestrogens (percent of observed total daily intake).

Food groups	SECO, MAT[†]		All lignans[‡]		Isoflavonoids[§]		Coumestrol	
	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
	Proportion of observed total daily intake (%)							
Flaxseed	90	90	36	36	0	0	0	0
Bread, rye	2.3	2.2	39	38	1.6	1.1	-	-
Berries and	1.5	1.4	0.6	0.6	0	0	-	-
Bread, wheat	1.3	1.3	15	15	-	-	-	-
Vegetables	0.9	0.9	0.4	0.4	0.3	0.2	-	-
Other cereals	0.8	0.8	6.2	6.0	<0.1	<0.1	-	-
Wine, red	0.8	0.9	0.3	0.3	0	0	-	-
Fruits	0.4	0.5	0.2	0.2	-	-	-	-
Juice	0.4	0.4	0.1	0.1	<0.1	<0.1	-	-
Nuts and peanuts	0.2	0.3	0.1	0.1	0.5	0.5	-	-
Tea	0.2	0.2	0.1	0.1	-	-	-	-
Beans and peas	0.1	0.1	<0.1	<0.1	1.5	1.0	41*	29*
Wine, white	<0.1	<0.1	<0.1	<0.1	-	-	-	-
Soy products	<0.1	<0.1	<0.1	<0.1	95	96	59	71
Milk products	<0.1	<0.1	0.3	0.3	0.5	0.3	-	-
Apples	-	-	-	-	0.3	0.2	-	-
Beer	-	-	-	-	0.1	0.1	-	-
Sunflower seed	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	-	-
Other	0.7	0.6	1.3	2.5		0.3		
Total, %	100	100	100	100	100	100	100	100

[†]Secoisolariciresinol and matairesinol
[‡]Secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, syringaresinol, medioresinol, enterodiol and enterolactone
[§] Daidzein, genistein, formononetin and biochanin A and equol
*Beans only
- Present in trace amounts or analytical data are missing

Fish and fatty acids

Dietary intake of fish and fatty acids among the study participants is presented in Table 6. There was no significant difference in dietary mean intake of all fish and seafood products combined, salmon-type fish/herring, marine fatty acids, omega-3 fatty acids or fish oil supplements between cases and controls. However, controls had a significantly lower mean intake of cod, shellfish, omega-6 fatty acids and alpha-linolenic acids, adjusted for age.

Table 6. Dietary intake of fish and fatty acids among prostate cancer cases and controls with questionnaire data in the CAPS study.

Characteristics	Controls n= 1130	Cases n=1499
Dietary intake, mean (g/day) of:		
All fish and other seafood	40	41
Salmon-type fish and herring/mackerel	22	20
Cod/saithe/fish fingers	11	13
Shellfish	5	6
Marine fatty acids*	0.6	0.6
Omega-6 fatty acids†	7.9	8.5
Omega-3 fatty acids‡	2.3	2.4
Alpha-linolenic acid	1.6	1.7
Intake of fish oil supplements, n (%)		
Never	893 (79)	1176 (78)
Ever	116 (10)	147 (10)
Missing	121(11)	176(12)

* Sum of eicosapentaenoic, docosaapentaenoic and docosahexenoic fatty acids

† Sum of arachidonic and linoleic acids

‡ Sum of alpha-linolenic, eicosapentaenoic, docosaapentaenoic and docosahexenoic acids

Alcohol

Table 7 shows the frequency and distribution of alcohol intake among cases and controls. Neither frequency nor volume of beer, wine, or liquor consumption was different between cases and controls, adjusted for age.

Table 7. Frequency and distribution of alcohol intake, in occasions per week and volume per occasion.

		Controls n=1130		Cases n=1499	
Alcohol type	Median in category	n	(%)	n	(%)
Alcohol status					
Never		145	(13)	122	(8)
Former		67	(6)	112	(8)
Current		917	(81)	1259	(84)
Frequency of total alcohol intake, occasions per week					
0.0 = non-drinkers	0.0	224	(20)	248	(17)
0.1 to 1.0	0.7	187	(17)	267	(18)
1.1 to 2.0	1.4	209	(19)	252	(17)
2.1 to 3.0	2.4	149	(13)	208	(114)
3.1 or more	5.2	361	(32)	524	(35)
Frequency of beer intake, occasions per week					

non-drinkers	0.0	224	(20)	248	(17)
0 to 1.0	0.7	386	(34)	522	(35)
1.1 to 2.0	1.1	223	(20)	314	(21)
2.1 to 3.0	2.1	149	(13)	225	(15)
3.1 or more	5.0	148	(13)	190	(13)
Volume of beer intake, centiliters per occasion (1 can≈33 cl)					
non-drinkers	0.0	224	(21)	248	(18)
0 to 32	20	162	(15)	237	(17)
33 to 40	33	261	(25)	382	(27)
41 to 50	50	287	(27)	392	(28)
51 or more	100	116	(11)	141	(10)
Frequency of wine intake, occasions per week					
non-drinkers	0.0	224	(20)	248	(17)
0 to 1.0	0.1	618	(55)	819	(55)
1.1 to 2.0	1.5	170	(15)	273	(18)
2.1 to 3.0	2.1	40	(4)	48	(3)
3.1 or more	3.6	78	(7)	111	(7)
Volume of wine intake, centiliters per occasion (1 glass≈15 cl)					
non-drinkers	0.0	224	(23)	248	(19)
0 to 15	15	210	(22)	249	(19)
16 to 30	30	292	(30)	416	(32)
31 to 37	37	138	(14)	222	(17)
38 or more	50	97	(10)	152	(12)
Frequency of liquor intake, occasions per week					
non-drinkers	0.0	224	(20)	248	(17)
0 to 1.0	0.1	724	(64)	972	(65)
1.1 to 2.0	1.5	148	(13)	220	(15)
2.1 to 3.0	---	0	(0)	0	(0)
3.1 or more	3.5	34	(3)	59	(4)
Volume of liquor intake, centiliters per occasion (1 shot glass≈4 cl)					
non-drinkers	0.0	224	(22)	248	(18)
0 to 6	5	251	(25)	347	(25)
7 to 10	10	209	(21)	329	(24)
11 to 17	15	138	(14)	193	(14)
18 or more	25	196	(19)	264	(19)

SERUM ENTEROLACTONE

The controls had significantly higher serum enterolactone concentration than cases (geometric mean = 21.4 for controls and 15.3 nmol/L for cases). The median serum enterolactone concentration was 23.9 nmol/L (5^{th} to 95^{th} percentile= 3.9 to 78.3) for controls and 21.1 nmol/L (5^{th} to 95^{th} percentile= 1.5 to 79.0) for cases. The distribution of serum enterolactone differed significantly between controls who had and had not taken antibiotics during the last year (geometric mean = 18.2 vs. 24.4 nmol/L, *P*-value=0.05), but among cases the difference was not statistically significant (geometric mean 13.8 vs. 18.5 nmol/L). There was no difference in enterolactone concentration between individuals who had fasted for at least 8 hours (geometric mean 20.0) and individuals who had eaten more recently (geometric mean 19.0).

CORRELATION BETWEEN DIETARY ESTIMATES AND SERUM MEASUREMENTS

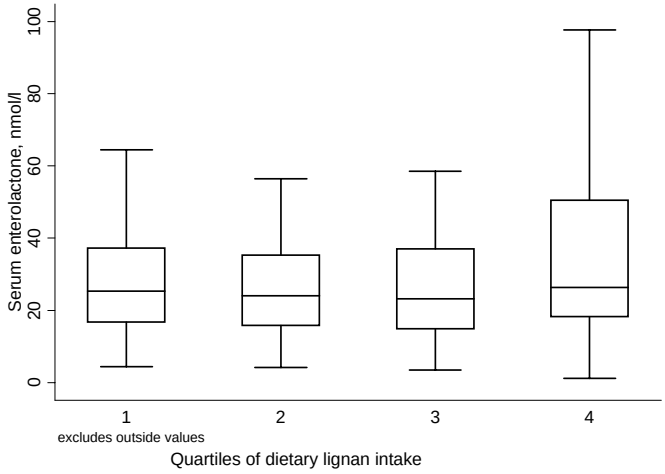
We found no significant correlation between dietary intake of total lignans and serum enterolactone level (Table 8 and Figure 5). However, there was a positive correlation between dietary intake of SECO and MAT and serum enterolactone level (correlation=0.18, *P*-value=0.02). When the data were stratified by low versus high density of animal fat intake, among those with low animal fat intake there was a significant positive correlation between serum enterolactone and dietary intake of total lignans. In contrast, there was no such correlation among those with high animal fat intake (Table 8). Serum enterolactone levels and dietary intake of total lignans were positively correlated among elderly participants, but not among younger participants (Table 8). The correlation between serum enterolactone levels and dietary lignan intake varied significantly between non- or moderate drinkers and heavy drinkers, with a significant correlation only among those who drank low amounts of alcohol (Table 8). However, there was no significant heterogeneity in the correlation between serum enterolactone and lignan intake across strata of vegetable fat or red meat intake, education level, smoking history, or body mass index. Similarly, there was no significant heterogeneity in the correlation between serum enterolactone and SECO and MAT intake across strata of any of these variables, nor were any of these factors independently associated with serum enterolactone levels.

Table 8. Correlation of dietary questionnaire measures of lignans with serum enterolactone in controls who did not use antibiotics during the last year (n=177)

Participants stratified by dietary intake of:	Correlation*	P-value
Animal fat density ≤0.0054 g/day·kJ	0.24	0.03
Animal fat density >0.0054 g/ day·kJ	-0.02	0.84
Age < 68 age	-0.11	0.31
Age > 68 age	0.24	0.03
Alcohol, high intake[#]	-0.12	0.30
Alcohol, low/moderate intake[#]	0.26	<0.01
No stratification	0.13	0.09

* Pearson correlation coefficient
[#] Cutoff at the median of 9.2 drinks/month among current drinkers

Figure 5. Distribution of serum enterolactone concentrations by quartile of lignan density of diet (µg/kJ) among controls who did not use antibiotics during the last year (n=177, 11 outliers excluded from the figure).



RISK FACTORS – DISEASE ASSOCIATIONS

Dietary phytoestrogen, serum enterolactone and prostate cancer risk (Study I)

High intake of food items rich in phytoestrogens (flaxseed, sunflower seeds, berries, peanuts, beans and soy) was associated with a monotonically decreasing overall risk of prostate cancer (Table 9). After multivariate adjustment, risk of prostate cancer was 26% lower in the highest compared to the lowest quartile of intake. The estimates were similar for advanced and localized prostate cancer (data not shown). In separate analyses of individual food items, only high intake of beans was associated with a reduced risk of prostate cancer and contributed the most to the inverse association between all phytoestrogen-rich food items and prostate cancer risk. In contrast, we found no association between dietary intake of total lignan or total isoflavonoid compounds and risk of overall, advanced or localized prostate cancer (Table 9). Similarly, there were no apparent associations with risk of prostate cancer when individual phytoestrogens were examined separately.

High serum levels of enterolactone were associated with a decreased risk of prostate cancer (Table 9). However, the trend was non-linear and J-shaped, with the strongest inverse association for intermediate levels of enterolactone-- that is, 15.3 to 23.9 nmol/L-- compared to 0 to 15.2 nmol/L. This pattern was similar after stratification of cases into those with advanced or localized disease, and did not change after multivariate adjustment for intake of antibiotics, total energy, animal fat, vegetable fat, zinc, vitamin A, protein, level of education and smoking (Table 9).

In separate analyses excluding cases who had already been treated for their prostate cancer at time of blood donation, the estimated odds ratios for the association between serum enterolactone and risk of prostate cancer did not change substantially, and we found no correlation between PSA level at the time of diagnosis among cases and enterolactone levels.

Table 9. Risk of prostate cancer in relation to serum levels of enterolactone and estimated dietary intake of food items rich in phytoestrogens, total lignans and total isoflavonoids, estimated as odds ratios (OR) with 95% confidence intervals (CIs). Exposures are categorized into quartiles, based on the distribution among controls.

	Median (interquartile range)	**Controls n**	**Cases n**	**OR[a]**	**95% CI**	**OR**	**95% CI**
Serum enterolactone concentration (nmol/L)	9.1 (0-15.2)	55	91	1.0	(reference)	1.0[b]	(reference)
	20.1 (15.3-23.9)	52	20	0.24	0.13-0.45	0.28[b]	0.15- 0.55
	29.9 (24.0-37.7)	55	46	0.54	0.32-0.92	0.63[b]	0.35-1.14
	55.4 (37.8-169.9)	52	51	0.61	0.36-1.03	0.74[b]	0.41-1.32
	P-value for linear trend			*0.33*		*0.82*	
Dietary intake of food items rich in phytoestrogen, g/day·MJ [1]	0.7 (0-1.18)	270	388	1.0	(reference)	1.0[c]	(reference)
	1.9 (1.19-2.61)	270	386	0.95	0.76-1.18	0.97[c]	0.76-1.22
	3.4 (2.62-4.70)	269	333	0.85	0.68-1.06	0.82[c]	0.64-1.04
	7.4 (4.71-47.0)	272	324	0.77	0.62-0.97	0.74[c]	0.57-0.95
	P-value for linear trend			*0.02*		*0.01*	
Dietary intake of lignans, µg/day·MJ [2]	87 (19-113)	273	371	1.0	(reference)	1.0[f]	(reference)
	134 (114-155)	270	373	0.99	0.79-1.23	0.95[f]	0.75-1.21
	179 (156-212)	273	364	0.98	0.79-1.22	0.94[f]	0.73-1.21
	296 (213-6600)	265	323	0.88	0.70-1.10	0.85[f]	0.65-1.12
	P-value for linear trend			*0.4*		*0.3*	
Dietary intake of isoflavonoids, µg/day·MJ [3]	0.8 (0.085-1.0)	272	328	1.0	(reference)	1.0[g]	(reference)
	1.3 (1.1-1.6)	268	352	1.02	0.82-1.28	1.04[g]	0.82-1.33
	1.9 (1.7-2.5)	274	367	1.09	0.87-1.36	1.05[g]	0.82-1.35
	113 (2.6-3750)	267	384	1.05	0.84-1.31	0.99[g]	0.77-1.28
	P-value for linear trend			*0.9*		*0.68*	

Adjusted for:

[a] age (in 5-year categories) for serum analysis, age and total energy intake for analysis of dietary intake

[b] age, intake of antibiotics, zinc, animal fat, vegetable fat, vitamin A and protein during the last year, level of education

[c] age, intake of antibiotics , zinc, animal fat, total energy intake, alcohol, vegetable fat, red meat, vegetables, fruit, tocopherol during the last year

[f] age, intake of antibiotics, zinc, animal fat, total energy intake, alcohol, vegetable fat, carbohydrates, isoflavonoids during the last year

[g] age, intake of antibiotics, zinc, animal fat, total energy intake, alcohol, vegetable fat, red meat during the last year

[1] Sum of flaxseed, sunflower seeds, berries, peanuts, beans and soy.

[2] Sum of matairesinol, secoisolariciresinol, lariciresinol, pinoresinol, syringaresinol and medioresinol multiplied by conversion factors and dietary enterolactone, enterodiol, (see Methods)

[3] Sum of genistein daidzein formononetin and biochanin_a

Dietary intake of fish and prostate cancer risk (Study III)

Estimates of prostate cancer risk by level of fish consumption are shown in Table 10. High intake of salmon-type fish was associated with a significantly decreased relative risk of prostate cancer. After multivariate adjustment, risk of prostate cancer was 43% lower among men who ate salmon-type fish once or more per week, compared with men who never ate salmon-type fish. Intake of herring and mackerel alone was not associated with risk of prostate cancer, but the combined intake of herring/mackerel and salmon-type fish was significantly associated with a 64% lower risk of prostate cancer for men who ate at least 5 servings of fatty fish per week. In contrast, intake of white fish (cod, saithe, fish fingers) or shellfish was significantly associated with an increased risk of prostate cancer. After multivariate adjustment, risk of prostate cancer was 45% higher for men who ate white fish once or more per week, and 81% higher for men who ate shellfish once or more per week, compared with men who never ate white fish or shellfish, respectively. There was no association between prostate cancer and total intake of fish and seafood products: the OR comparing the highest to the lowest quartile of intake was 1.07 (95% CI: 0.85-1.35). Additional adjustment for intake of fish oil supplements did not change the estimates for any of the associations. We repeated all analyses separately for cases with localized or advanced prostate cancer, and the estimates were similar across disease stages.

Dietary intake of long-chain fatty acids and prostate cancer risk (Study III)

The relative risk of prostate cancer by level of fatty acids intake is shown in Table 11. After multivariate adjustment, intake of ω-6 fatty acids was significantly associated with a 36% increased relative risk of prostate cancer in the highest compared to the lowest quartile of intake. We found no association between total intake of ω-3 fatty acids and prostate cancer risk. However, there was a statistically significant trend toward higher risk with increasing intake of α-linolenic acid. In contrast, high intake of marine fatty acids (EPA and DHA) was associated with a significantly decreased relative risk of prostate cancer; the risk was reduced by 30% in the highest compared to the lowest quartile of intake. The ratio of ω-3 to ω-6 fatty acids was associated with a significantly decreased relative risk of prostate cancer: subjects in the highest compared with the lowest quartile of ω-3:ω-6 consumption experienced a 29% lower risk. The association was even more pronounced for the ratio of EPA and DHA to ω-6 fatty acids, with a risk reduction of 34% in the highest compared with the lowest quartile of intake (Table 11). Additional adjustment for intake of fish oil supplements did not change the estimates for any of the associations. We repeated all analyses separately for cases with localized or advanced prostate cancer, and the estimates were similar across disease stages.

Table 10. Relative risk of prostate cancer in association with dietary intake of fish, estimated as odds ratios (OR) with 95% confidence intervals (CIs).

Dietary intake of fish and fatty acids	Frequency	Controls n	Cases n	OR*	95% CI	OR	95% CI
Herring/mackerel	never	169	219	1.00	(reference)	1.00†	(reference)
	1-3 per month	691	921	1.02	0.82-1.28	1.00†	0.79-1.27
	=>1 per week	223	288	0.96	0.73-1.26	1.00†	0.73-1.36
	P-value for linear trend			*0.70*		*1.00*	
Salmon-type fish	never	174	277	1.00	(reference)	1.00‡	(reference)
	1-3 per month	688	903	0.82	0.66-1.02	0.72‡	0.57-0.90
	=>1 per week	222	249	0.65	0.50-0.85	0.57‡	0.43-0.76
	P-value for linear trend			*<0.01*		*<0.01*	
Cod/saithe/fish fingers	never	237	203	1.00	(reference)	1.00§	(reference)
	1-3 per month	603	8555	1.58	1.27-1.96	1.41§	1.12-1.76
	=>1 per week	248	379	1.64	1.28-2.11	1.45§	1.12-1.88
	P-value for linear trend			*<0.01*		*<0.01*	
Shellfish	never	450	450	1.00	(reference)	1.00‖	(reference)
	1-3 per month	547	864	1.55	1.30-1.84	1.57‖	1.30-1.88
	=>1 times per	69	123	1.64	1.18-2.27	1.81‖	1.28-2.56
	P-value for linear trend			*<0.01*		*<0.01*	
Salmon-type fish and herring/mackerel	never	54	97	1.00	(reference)	1.00**	(reference)
	<=2 per week	927	1221	0.74	0.53-1.02	0.64**	0.45-0.92
	3-4 per week	85	106	0.64	0.42-0.99	0.57**	0.35-0.90
	>5 per week	29	22	0.39	0.20-0.75	0.36**	0.18-0.72
	P-value for linear trend			*<0.01*		*<0.01*	

Adjusted for:
* age (in 5-year categories) and total energy intake
† age (in 5-year categories), total energy intake, dietary intake of alcohol, food items rich in phytoestrogens, vitamin C, zinc, tocopherol, carbohydrates, saturated fat, selenium, seafood, salmon-type fish and cod
‡ age (in 5-year categories), total energy intake, dietary intake of alcohol, food items rich in phytoestrogens, vitamin C, fat other than ω-3, ω-6, EPA or DHA, seafood, cod and herring
§ age (in 5-year categories), total energy intake and dietary intake of seafood, salmon-type fish and herring.
‖ age (in 5-year categories), total energy intake and dietary intake of food items rich in phytoestrogens, vitamin C, selenium, cod, salmon-type fish and herring
** age (in 5-year categories), total energy intake, dietary intake of alcohol, food items rich in phytoestrogens, vitamin C, tocopherol, seafood and cod.

Table 11. Relative risk of prostate cancer in association with estimated dietary intake of fatty acids, estimated as odds ratios (OR) with 95% confidence intervals (CIs).

Fatty acid	Median (interquartile range)	Controls n	Cases n	OR*	95% CI	OR	95% CI
Omega-6 fatty acids[1] g/day·MJ	0.66(0.29-0.73)	281	367	1.00	(reference)	1.00†	(reference)
	0.78(0.74-0.82)	281	319	0.88	0.71-1.11	0.93†	0.72-1.19
	0.88(0.83-0.93)	281	358	0.94	0.76-1.18	1.03†	0.79-1.35
	1.05(0.94-3.05)	281	445	1.16	0.93-1.47	1.36†	1.01-1.84
	P-value for linear trend			*0.13*		*0.03*	
Omega-3 fatty acids[2] g/day·MJ	0.18(0.07-0.19)	281	364	1.00	(reference)	1.00‡	(reference)
	0.22(0.20-0.23)	281	391	1.08	0.87-1.35	1.18‡	0.91-1.52
	0.26(0.24-0.28)	281	373	1.02	0.82-1.27	1.20‡	0.88-1.63
	0.33(0.29-1.4)	281	361	0.99	0.79-1.23	1.25‡	0.88-1.78
	P-value for linear trend			*0.78*		*0.27*	
Alpha-linolenic acid g/day·MJ	0.12(0.05-0.13)	281	359	1.00	(reference)	1.00§	(reference)
	0.15(0.14-0.16)	281	334	0.93	0.74-1.16	0.98§	0.77-1.26
	0.18(0.17-0.19)	281	393	1.07	0.86-1.34	1.22§	0.93-1.61
	0.23(0.20-0.60)	281	403	1.06	0.85-1.32	1.35§	0.99-1.84
	P-value for linear trend			*0.37*		*0.03*	
Sum of EPA and DHA[3], g/day·MJ	0.03(0-0.038)	277	398	1.00	(reference)	1.00‖	(reference)
	0.05(0.039-0.053)	281	409	1.03	0.83-1.28	0.98‖	0.77-1.24
	0.06(0.054-0.077)	280	369	0.97	0.77-1.21	0.91‖	0.70-1.18
	0.11(0.078-1.08)	279	308	0.80	0.64-1.00	0.70‖	0.51-0.97
	P-value for linear trend			*0.06*		*0.05*	
Ratio of omega-3:omega-6 fatty acids	0.22(0.12-0.25)	281	441	1.00	(reference)	1.00**	(reference)
	0.27(0.26-0.28)	281	390	0.89	0.72-1.10	0.89**	0.72-1.12
	0.30(0.29-0.32)	281	360	0.83	0.67-1.03	0.83**	0.66-1.05
	0.37(0.32-1.39)	281	298	0.71	0.56-0.88	0.71**	0.55-0.92
	P-value for linear trend			*<0.01*		*<0.01*	
Ratio of EPA+DHA[3]:omega-6 fatty acids	0.03(0-0.04)	281	449	1.00	(reference)	1.00**	(reference)
	0.05(0.05-0.06)	281	390	0.87	0.70-1.09	0.84**	0.68-1.05
	0.08(0.07-0.09)	281	355	0.81	0.65-1.01	0.77**	0.62-0.97
	0.13(0.10-1.0)	281	295	0.69	0.55-0.87	0.66**	0.51-0.84
	P-value for linear trend			*<0.01*		*<0.01*	

Adjusted for:

* age (in 5-year categories) and total energy intake

† age (in 5-year categories), total energy intake and dietary intake of food items rich in phytoestrogens, dietary intake of fat other than ω-6 fatty acids, red meat, dairy products, zinc, tocopherol, vitamin D and carbohydrates

‡ age (in 5-year categories), total energy intake and dietary intake of food items rich in phytoestrogens, vitamin C, saturated fat, fruit, vegetables, red meat, dairy products, zinc, tocopherol, vitamin D, carbohydrates and fiber

§ age (in 5-year categories), total energy intake and dietary intake of food items rich in phytoestrogens, vitamin C, saturated fat, red meat, dairy products, zinc, tocopherol, vitamin D, carbohydrates, fiber and alcohol

‖ age (in 5-year categories), total energy intake and dietary intake of food items rich in phytoestrogens, vitamin C, saturated fat, fruit, vegetables, red meat, dairy products, zinc, tocopherol, vitamin D, carbohydrates, fiber, alcohol, selenium, beta-carotene and levels of education

** age (in 5-year categories), total energy intake and dietary intake of fat other than ω-3and ω-6 fatty acids, and vitamin D

[1] Sum of arachidonic and linoleic acids

[2] Sum of alpha-linolenic, eicosapentaenoic, docosaapentaenoic and docosahexenoic acids

[3] Sum of eicosapentaenoic acid (EPA), docosahexenoic acid (DHA) and docosaapentaenoic acid

Alcohol consumption and prostate cancer risk (Study IV)

Table 12 shows the estimated associations between current or former *versus* never drinking, as well as alcohol intake in terms of grams of ethanol consumed per week, and risk of prostate cancer. After adjusting for age, cases were significantly more likely than controls to be current or former drinkers, compared to never-drinkers. The relative risk was somewhat higher for former drinkers than for current drinkers, and persisted after additional adjustment for other factors (se table footnotes). However, cases and controls were similar in their average recent intake of total alcohol, beer, wine, and liquor (Table 12). Compared to non-drinkers, there was no difference in risk of prostate cancer among drinkers of any amount of any type of alcohol, adjusting for age and intake of other alcohol types. Although there was a slight suggestion of a positive association between total alcohol intake and risk of prostate cancer, all ORs were statistically non-significant, and there was no trend in increasing risk with greater consumption of alcohol.

When localized and advanced prostate cancer cases were examined separately, former drinkers were at higher risk of both localized and advanced prostate cancer than never drinkers, and current drinkers had a higher risk of localized disease only (Table 12). Although not statistically significant, there was marked heterogeneity in the association of total alcohol intake with risk of localized (*n*=634 cases) versus advanced (*n*=804 cases) prostate cancer, with a positive association between total alcohol consumption and localized disease only. Furthermore, all individual types of alcohol were at least marginally positively associated with risk of localized but not advanced prostate cancer. However, there was no evidence of a dose-response trend in either disease group.

Cases with and without a family history of prostate cancer in at least one first- or second-degree relative were evaluated separately, in order to determine whether the association with alcohol intake differed between sporadic and familial prostate cancer. Whereas current and former drinkers were more likely than never drinkers to develop sporadic prostate cancer, they showed no significant differences in risk of familial prostate cancer. As with overall prostate cancer, there was no association between intake of total alcohol, beer, wine, or liquor with risk of either sporadic or familial disease, and there was no heterogeneity in any association with alcohol intake between the two groups. The estimates were minimally affected by further adjustment for additional variables.

Table 12. Odds ratios (OR) and 95% confidence intervals (CI) for associations between alcohol intake and risk of overall, localized and advanced prostate cancer.

	All cases				Localized disease *N*=634 cases		Advanced disease *N*=804 cases		
Alcohol type	OR*	(95% CI)	OR**	(95% CI)	OR *	(95% CI)	OR *	(95% CI)	$P^{\dagger}$
Alcohol status									
Never	1.0	---	1.0	---	1.0	---	1.0	---	
Former	1.9	(1.3, 2.8)	2.1	(1.4, 3.3)	1.7	(1.0, 3.0)	1.7	(1.1, 2.5)	
Current	1.5	(1.1, 1.9)	1.6	(1.2, 2.1)	1.8	(1.2, 2.6)	1.1	(0.8, 1.5)	
Ethanol from total alcohol, grams per week									
0.0	1.0	---	1.0	---	1.0	---	1.0	---	
0.1 to 45.0	1.1	(0.8, 1.3)	1.1	(0.8, 1.4)	1.5	(1.1, 2.1)	0.8	(0.6, 1.0)	
45.1 to 90.0	1.2	(0.9, 1.5)	1.2	(0.9, 1.5)	1.4	(1.0, 2.0)	0.9	(0.7, 1.2)	
90.1 to 135.0	1.3	(1.0, 1.7)	1.3	(0.9, 1.7)	1.4	(1.0, 2.1)	1.1	(0.8, 1.5)	
135.1 or more	1.2	(0.9, 1.5)	1.3	(1.0, 1.7)	1.4	(1.0, 2.0)	0.9	(0.7, 1.2)	
	P for trend: .12		*P* for trend: .06		*P* for trend: .34		*P* for trend: .50		.78
Ethanol from beer, grams per week									
0.0	1.0	---	1.0	---	1.0	---	1.0	---	
0.1 to 15.0	1.1	(0.8, 1.4)	1.1	(0.8, 1.4)	1.5	(1.1, 2.0)	0.8	(0.6, 1.0)	
15.1 to 30.0	1.1	(0.8, 1.4)	1.0	(0.7, 1.3)	1.3	(0.9, 1.9)	0.9	(0.6, 1.2)	
30.1 to 60.0	1.2	(0.9, 1.6)	1.3	(0.9, 1.7)	1.5	(1.0, 2.1)	1.0	(0.7, 1.3)	
60.1 or more	1.2	(0.9, 1.6)	1.2	(0.9, 1.6)	1.5	(1.0, 2.2)	0.9	(0.7, 1.2)	
	P for trend: .22		*P* for trend: .21		*P* for trend: .27		*P* for trend: .58		.63
Ethanol from wine, grams per week									
0.0	1.0	---	1.0	---	1.0	---	1.0	---	
0.1 to 15.0	1.0	(0.8, 1.3)	1.0	(0.8, 1.3)	1.4	(1.0, 1.9)	0.8	(0.6, 1.0)	
15.1 to 30.0	1.2	(0.9, 1.6)	1.2	(0.8, 1.6)	1.5	(1.0, 2.2)	0.8	(0.6, 1.2)	
30.1 to 60.0	1.2	(0.9, 1.7)	1.2	(0.9, 1.7)	1.7	(1.1, 2.5)	0.9	(0.6, 1.2)	
60.1 or more	1.0	(0.8, 1.4)	1.0	(0.7, 1.4)	1.4	(0.9, 2.1)	0.7	(0.5, 1.1)	
	P for trend: .72		*P* for trend: .96		*P* for trend: .51		*P* for trend: .42		.31
Ethanol from liquor, grams per week									
0.0	1.0	---	1.0	---	1.0	---	1.0	---	
0.1 to 15.0	1.1	(0.9, 1.3)	1.0	(0.8, 1.3)	1.4	(1.0. 2.0)	0.8	(0.6, 1.0)	
15.1 to 30.0	1.1	(0.8, 1.4)	1.1	(0.8, 1.5)	1.3	(0.9, 1.9)	0.9	(0.7, 1.3)	
30.1 to 60.0	1.0	(0.7, 1.4)	1.1	(0.8, 1.6)	1.3	(0.8, 2.0)	0.8	(0.6, 1.2)	
60.1 or more	1.1	(0.8, 1.6)	1.2	(0.8, 1.7)	1.3	(0.8, 2.0)	1.0	(0.7, 1.4)	
	P for trend: .68		*P* for trend: .44		*P* for trend: .75		*P* for trend: .82		.43

Never-drinkers were used as the reference group for all comparisons, cases (n=122), controls (n=145).

* Odds ratio adjusted for age (5-year categories) and intake of other alcohol types

** Odds ratio adjusted for age (5-year categories), smoking history (ever, never), current body mass index, family history of prostate cancer, and intake of other alcohol types, dairy products, red meat, and fruits and vegetables

† *P*- value for test of heterogeneity

GENE - ENVIRONMENTAL - INTERACTION

Interactions between phytoestrogens and ERß promoter region polymorphisms (Study II)

We first explored each of the four identified SNPs in ERβ in relation to increasing total phytoestrogen intake. For each of the SNPs, we performed analyses separately for subjects homozygous for the wild type allele and those who were heterozygous or homozygous for the variant allele (TC/CC carriers). Among subjects with homozygous wild type alleles in the ERβ promoter region (-13950 T/C), phytoestrogen intake was not associated with prostate cancer risk (Table 13). In contrast, a monotonically decreasing risk with increasing levels of phytoestrogen intake was found among subjects who were heterozygous or homozygous for the variant allele. The interaction was statistically significant on both a multiplicative and an additive scale. For none of the other three SNPs (rs1887994 (-10908 G/T), rs1256040 (11309 A/G) and rs1256062 (46385 C/T)) did we find a significant interaction between genotype and phytoestrogen intake. As a corollary, all further analyses were confined to the promoter region SNP.

High intake of food items rich in phytoestrogens was associated with a decreasing overall risk of prostate cancer (Table 9). Subjects in the highest compared with the lowest risk quartile of phytoestrogen consumption experienced a statistically significant 26% decreased risk of prostate cancer. When the analysis was stratified by allele of the SNP (-13950 T/C), the relative risk of prostate cancer for TC/CC carriers decreased even further with increasing intake of phytoestrogens (Table 13). Following multivariate adjustment, the risk of prostate cancer among TC/CC carriers in the highest quartile was 57% lower than in the lowest quartile (95% CI: 33-73%), whereas we found no significant association with phytoestrogen intake among the wild type TT allele carriers.

For isoflavonoids, we found no overall association with risk of prostate cancer (Table 9). However, when we stratified by nucleotide sequence in the SNP (-13950 T/C), risk decreased monotonically with increasing intake of isoflavonoids among TC/CC carriers (Table 14), whereas no reduction in risk was seen for TT carriers. Similar results were seen for intake of coumestrol: among men who were TC/CC carriers, risk of prostate cancer was 43% lower among men with high intake compared with men with no intake of coumestrol (95% CI: 16-62%) (Table 15). In contrast, there was no association between dietary lignan intake and risk of prostate cancer, even among TC/CC carriers.

We repeated all analyses separately for case patients with localized or advanced prostate cancer, and found no evidence of heterogeneity by disease stage.

Table 13. Odds ratios (OR) with 95% confidence intervals (CIs) for the risk of prostate cancer in relation to intake of food items rich in phytoestrogens, stratified by estrogen receptor-beta alleles (rs2987983-13950 T/C). Exposures are categorized based on quartiles among all controls.

Nucleotide sequence	Frequency	Intake of foods rich in phytoestrogen, g/day·MJ * Median (interquartile range)	Controls n	Cases n	OR†	95% CI	OR‡	95% CI
TT	58%	0.8 (0-1.18)	108	155	1.00	(reference)	1.00	(reference)
		1.9 (1.19-2.60)	101	168	1.10	0.78-1.56	1.14	0.78-1.65
		3.4 (2.63-4.70)	104	152	1.04	0.74-1.47	0.98	0.67-1.44
		7.4 (4.71-34.5)	112	142	0.85	0.60-1.20	0.82	0.55-1.23
		P-value for linear trend			*0.34*		*0.27*	
TC/CC	42%	0.7 (0-1.18)	62	166	1.00	(reference)	1.0	(reference)
		1.9 (1.19-2.60)	86	161	0.70	0.47-1.04	0.69	0.45-1.05
		3.3 (2.63-4.70)	73	124	0.62	0.41-0.94	0.56	0.36-0.88
		7.3 (4.73-47.0)	91	122	0.48	0.32-0.72	0.43	0.27-0.67
		P-value for linear trend			*<0.001*		*<0.001*	
P-values for interaction, multiplicative =0.04, additive=0.04								

* Sum of flaxseed, sunflower seeds, berries, peanuts, beans and soy
† adjusted for age (in 5-year categories) and total energy intake
‡ adjusted for age and intake of antibiotics, zinc, animal fat, total energy intake, alcohol, vegetable fat, red meat, vegetables, fruit, and tocopherol during the last year

Table 14. Odds ratios (OR) with 95% confidence intervals (CIs) for the risk of prostate cancer in relation to intake of isoflavonoids, stratified by estrogen receptor-beta alleles (rs2987983-13950 T/C). Exposures are categorized based on quartiles among all controls.

Nucleotide sequence	Dietary intake of isoflavonoids, µg/day·MJ * Median (interquartile range)	Controls n	Cases n	OR†	95% CI	OR‡	95% CI
TT	0.8 (0.085-1.0)	109	128	1.00	(reference)	1.00	(reference)
	1.3 (1.1-1.6)	109	159	1.20	0.85-1.70	1.26	0.86-1.84
	1.9 (1.7-2.5)	111	154	1.18	0.83-1.68	1.20	0.81-1.78
	116 (2.6-1874)	96	176	1.40	0.98-2.00	1.47	0.98-2.20
	P-value for linear trend			*0.09*		*0.10*	
TC/CC	0.8 (0.14-1.0)	69	133	1.00	(reference)	1.00	(reference)
	1.3 (1.1-1.6)	80	147	0.93	0.69-1.40	0.87	0.57-1.34
	1.9 (1.7-2.5)	86	154	0.89	0.60-1.32	0.79	0.51-1.22
	116 (2.6-3750)	77	139	0.81	0.54-1.22	0.63	0.39-1.00
	P-value for linear trend			*0.30*		*0.05*	
P-values for interaction, multiplicative=0.09, additive=0.09							

* Sum of genistein daidzein formononetin and biochanin_a
† adjusted for age (in 5-year categories) and total energy intake
‡ adjusted for age and intake of antibiotics, zinc, animal fat, total energy intake, alcohol, vegetable fat, and red meat during the last year

Table 15. Odds ratios (OR) with 95% confidence intervals (CIs) for the risk of prostate cancer in relation to 3 levels of intake of coumestrol, stratified by estrogen receptor-beta alleles (rs2987983-13950 T/C).

Nucleotide sequence	Dietary intake of coumestrol, ng/day·MJ All cases	Controls n	Cases n	OR*	95% CI	OR†	95% CI
All cases	None	535	692	1.00	(reference)	1.00	(reference)
	<=9.7	276	395	1.06	0.87-1.28	1.03	0.84-1.26
	>9.7	270	344	0.91	0.75-1.10	0.85	0.69-1.05
	P-value for linear trend			*0.42*		*0.20*	
TT	None	213	284	1.00	(reference)	1.00	(reference)
	<=9.7	107	173	1.22	0.90-1.64	1.17	0.85-1.61
	>9.7	105	160	1.05	0.79-1.43	1.03	0.74-1.43
	P-value for linear trend			*0.58*		*0.72*	
TC/CC	None	136	288	1.00	(reference)	1.00	(reference)
	<=9.7	99	158	0.74	0.53-1.03	0.75	0.53-1.07
	>9.7	77	127	0.71	0.47-1.00	0.57	0.38-0.84
	P-value for linear trend			*0.04*		*0.003*	

P-values for interaction, multiplicative=0.64, additive=0.70

* adjusted for age (in 5-year categories) and total energy intake
† adjusted for age, intake of antibiotics, zinc, animal fat, total energy intake, alcohol, vegetable fat, red meat during the last year

Interactions between intake of salmon-type fish and COX-2 polymorphisms (Study III)

We explored each of the five identified SNPs in the COX-2 gene in relation to increasing levels of salmon-type fish intake. For each of the SNPs, we performed analyses separately for subjects homozygous for the wild type allele and those who were heterozygous or homozygous for the variant allele. The interaction between salmon-type fish intake and SNP 5275 (+6365 T/C) was significant on both the multiplicative and the additive scales. We did not find any significant interactions between genotype and intake of salmon-type fish for any of the other 4 SNPs examined (rs20432 (+3100 T/G), rs4648276 (+3935 T/C), rs2745557 (+202 C/T), and rs689470 (+8365 C/T)).

Among subjects who were heterozygous or homozygous for the variant allele (C) of the SNP 5275 (+6365 T/C), high intake of salmon-type fish was associated with a significantly decreased relative risk of prostate cancer (Table 16). Following multivariate adjustment, risk of prostate cancer was 72% lower among men who ate salmon-type fish once or more per week, compared with men who never ate salmon-type fish, whereas we found no significant association with salmon-type fish intake among subjects homozygous for the wild type allele (T).

Table 16. Odds ratios (OR) with 95% confidence intervals (CIs) for the relative risk of prostate cancer in relation to intake of salmon-type fish, stratified by cyclooxygenase-2 alleles (rs5275 +6365T/C).

Nucleotide sequence	Dietary intake of salmon-type fish	Controls n	Cases n	OR*	95% CI	OR†	95% CI
TT	never	50	85	1.0	(reference)	1.0	(reference)
	1-3 per month	189	341	1.09	0.74-1.63	1.01	0.66-1.54
	=>1 per week	49	96	1.14	0.69-1.89	1.10	0.64-1.89
	P-value for linear trend			*0.6*		*0.74*	
TC&CC	never	45	149	1.0	(reference)	1.0	(reference)
	1-3 per month	287	426	0.47	0.33-0.68	0.38	0.26-0.56
	=>1 per week	100	116	0.36	0.23-0.55	0.28	0.18-0.45
	P-value for linear trend			*<0.01*		*<0.01*	
	P-value for interaction, multiplicative=<0.01, additive=<0.01						

* Adjusted for age (in 5-year categories) and total energy intake
† Adjusted for age (in 5-year categories), total energy intake, dietary intake of alcohol, food items rich in phytoestrogens, vitamin C, fat other than ω-3, ω-6, EPA or DHA, seafood, cod and herring

GENERAL DISCUSSION

METHODOLOGY

Before discussing the scientific results of the four studies, we will first consider the underlying study methodology. Epidemiological studies can be affected by random error, which affects precision, and by systematic error, which affects validity. Precision improves with increasing study size, whereas systematic errors are independent of the study size. Validity of a study is defined as the absence of systematic error, and can be diminished by appropriate study design and assessment methods or, to some extent, be adjusted for in statistical analysis. Bias, another term of systematic error, is classified as selection bias, information bias, or confounding (130).

Precision

One of the strengths of our study is its large size, which reduced variability in the estimates of association, and was particularly necessary for examination of rare exposures. Even though the intake of phytoestrogens was low in our study population, the range was sufficient to rank subjects according to their intake. When high intake was compared to low intake in relation to risk of prostate cancer, the confidence interval for the estimate was relatively narrow, indicating that the precision of our study was high. The different genotypes we studied were common in our study population and gave us the opportunity to achieve estimates with relatively good precision. However, chance findings and, conversely, failure to detect true associations can never be completely ruled out.

Validity

Selection bias is if the association between exposure and disease differs between those who participate and those who did not participate in the study. This type of error can arise from how subjects are selected for inclusion in a study or from factors that influence study participation. We used a population-based design in order to reduce the amount of potential selection bias. However, the relatively low participation rate, especially for blood donation, among eligible controls could introduce selection bias. While it is implausible that specific genotypes would influence individuals' willingness to join the study, participants and non-participants might differ in their dietary habits. Such bias should, however, have the same influence on all strata of genotypes. The participation rate for the questionnaire only (79% for cases and 67% for controls) was higher than that for both questionnaire and blood donation (71% for cases and 51% for controls). Therefore, we compared characteristics for participants who completed the questionnaire and donated blood with those who only answered the questionnaire. Among both cases and controls, baseline characteristics (e.g., age, body mass index, level of education, and smoking status), as well as dietary intake of fish and phytoestrogens, did not differ significantly between those who did and did not donate a blood specimen. However, controls who did not provide blood had a lower intake of macronutrients (protein, fat and carbohydrates) and total energy than controls who did provide blood. This difference could have occurred if controls who did not donate blood were less motivated to complete the questionnaire fully, which would have led to less intake of food overall. However, if controls who did not donate blood truly

consumed lower amounts of fish or food containing phytoestrogens, then we would have underestimated the true protective effect of salmon-type fish or phytoestrogens intake on prostate cancer risk. The association between alcohol intake and prostate cancer risk could have been inflated, if eligible controls who drank heavily were less likely to participate than light or non-drinkers.

If aggressiveness of the disease leads to a more rapid death and is also related to the exposure, the observed associations may be affected by survival bias, a form of selection bias. However, we used a rapid case ascertainment method in the study and the proportion of deaths among the non-participating cases was relatively low (8%). Therefore, bias due to non-participation of cases was unlikely to have a major impact on results of this study.

Misclassification of exposure

Information bias can occur whenever there are errors in the information collected about or from study subjects. In epidemiological studies, this often refers to misclassification of exposure or disease. If the misclassification of exposure is systematically different between cases and controls, then it is differential and the error can either exaggerate or underestimate an effect. If the misclassification is non-differential, i.e., the same for cases and controls, the misclassification usually dilutes the association between the exposure and disease, given that the exposure is dichotomous.

Differential misclassification would arise if cancer patients recalled their dietary history differently than controls because of their disease. This type of error is referred to *recall bias,* and it is one of the potential limitations of case-control studies where the exposure information is collected after the disease has occurred. However, information about a special diet (e.g., fish, food items rich in phytoestrogens, or different fatty acids) and their possible role in prostate cancer is almost non-existent among the general population in Sweden. Hence, it is unlikely that cases would alter their recall of past food intake due to awareness of the importance of these food items. However, heavy alcohol drinking could be perceived as a generally unhealthy behavior, leading to some over-reporting among cases who attributed their disease partly to alcohol intake. This could account for the apparent positive association of current and former drinking with prostate cancer risk, and could have obscured any true inverse association with alcohol consumption. On the other hand, some under-reporting is also possible among men who did not want to admit alcohol abuse. If such under-reporting occurred non-differentially between cases and controls, then it would likely have led to a dilution of the observed association.

Validity in dietary assessments

Measurement of dietary intake using traditional nutritional tools (e.g., FFQ, interview, 24-hour recall or weighed food records) has several limitations that influence validity. Both FFQs and interviews are dependent on the participant's memory, whereas 24-hour recall and weighed food records only measure the dietary intake for a short period of time. Our choice to use a FFQ was based on its being the most practical, economical and well-validated method available for a large-scale study. In addition, our FFQ was previously validated against weighed food records, for which each participant weighed

and recorded all foods consumed during four separate weeks, 3 to 4 months apart. The Pearson correlation coefficients between the estimates derived from the questionnaire and the weighed food records generally ranged between $r = 0.2$ and $r = 0.6$ (e.g., $r =$ 0.2-0.4 for fish, $r = 0.6$ for oranges, $r = 0.2$ for broccoli, $r = 0.4$ for cabbage, $r = 0.5$-0.6 for dairy products; $P \leq 0.05$ for all correlations) (118). The validity of alcohol intake estimates in a similar questionnaire was previously evaluated in a Swedish study and responses to a one-time food frequency questionnaire were compared to fourteen 24-hour recall interviews. The Spearman correlation coefficient between the questionnaire-based and interview-based estimates was r=0.8 for total ethanol intake (M. Messerer et al., submitted). Although these correlations were statistically significant, it is unclear whether weighed food records or FFQs are a more accurate measure of average food intake over time, nor whether correlation between these metrics is expected to be high or low. Furthermore, individuals willing to weigh and record all foods consumed for a week may not be representative of other study populations.

We asked participants to report their food consumption during the last year prior to the time of the questionnaire. Because dietary patterns tend to be reasonably well correlated from year to year (127), we implicitly assumed that reported habits were generally representative of adulthood behaviour and we did not ask about earlier patterns, which would have been more difficult to recall. However, diet at one point in time may not be representative of a lifetime or even adulthood diet for many people. In addition, we were unable to account for timing of food consumption, changes in dietary habits over time, or cumulative dietary intake, some or all of which may affect the association between diet intake and prostate cancer risk. Furthermore, some degree of misclassification of dietary intake due to measurement error associated with the FFQ is unavoidable. Because the same questionnaire was completed by cases and controls, most of such misclassification was likely non-differential and would generally have biased our results predictably toward a null effect-- possibly resulting in our inability to detect some true associations.

To date, there are no comparable studies estimating dietary intake of phytoestrogens in a Swedish population to corroborate our measurements. However, in Finland, where the dietary habits are similar to those in Sweden, estimated intake of phytoestrogens is comparable with that observed in our study (120, 131). The mean intake of SECO in our study was higher than that reported among men in Finland (120, 131), but lower than among women in Germany, USA and the Netherlands (132-134). Some of the discrepancies are probably due to the laboratory method used, since various analytical methods produce different absolute amounts of measured levels of phytoestrogens. In addition, some variation was likely due to international differences in dietary habits. For example, the intake of isoflavonoids in our study was low, as expected, because the intake of soy products is low in Sweden. The intake of isoflavonoids in our study and that reported in other Western countries is much lower than the intake reported in Asian populations (31, 133). The main sources of lignans in our study-- flaxseed, rye bread, wheat bread, cereals and berries-- were similar to those in Finland, but differed slightly from dietary sources in USA and Germany (131, 132, 134).

Other reasons for the inconsistencies in results among studies of phytoestrogen intake could be differences in assessment methods for evaluating dietary intake, the ability of the questionnaire to assess relevant food items for phytoestrogen intake, and/or the phytoestrogen content in food, which may depend on factors such as agricultural conditions. Furthermore, phytoestrogen databases used in various studies differ in calculation methods for levels of phytoestrogens in food, chosen reference values of phytoestrogens, analytical methods, and origin of analyzed food items. We used information about phytoestrogen content in foods analyzed by a uniform method in a single laboratory (15). Also, no previous study has taken into account the newly discovered lignans, which likely explains why the total lignan intake in our study was higher than that in others.

One of our aims in paper I was to validate the reported dietary intake of lignans with serum levels of enterolactone. Dietary intake of total lignans was not correlated with serum enterolactone overall, although we found a significant positive correlation among participants with low intake of animal fat and among elderly participants. High fat intake has been reported to decrease serum enterolactone levels (135), and older subjects may have higher serum enterolactone values than younger subjects (136). We found a positive correlation between intake of SECO and MAT and serum enterolactone, a finding that agrees with results from a Finnish study (131).

Wide interindividual variation in serum enterolactone concentration has been seen in other studies. In studies where determinants of serum enterolactone were investigated, only up to 22% of the variation in serum enterolactone could be explained by diet, demographic characteristics (age, sex and BMI) and bowel movements (136-138). This indicates that there are individual differences in the metabolism of enterolactone that are unknown and account for the majority of interindividual variation in serum enterolactone concentration. The biotransformation of plant lignans into mammalian lignans depends on the bacterial microflora in the gut (22, 139, 140), an observation supported by the fact that use of antimicrobials drastically lowers serum and urinary levels of enterolactone (141). The lack of a correlation between dietary intake of total lignans and serum levels in our study may be explained by a number of factors, such as the inability of our questionnaire to cover all sources of lignans in the Swedish diet, or an incomplete database of lignans that does not cover all sources of enterolactone precursors. Finally, the variation in metabolism of dietary lignans to mammalian phytoestrogens may be so complex, and dependent on individual differences in gut microflora and fat intake or other determinants, that the measurement of intake using traditional nutritional tools may not be adequate to capture the association between lignans and disease risk.

In a similar validation study, dietary intake of isoflavonoids and lignans measured by FFQ was compared to urinary phytoestrogens. Dietary intake of isoflavonoids correlated well with urinary levels, whereas estimated lignan intake did not (142). Some other studies identified a significant correlation between dietary intake and serum or urinary levels of isoflavonoids (143, 144). Since we did not analyze serum levels of isoflavonoids or coumestrol, we were not able to validate the estimated dietary intake in our study. However, the FFQ may be a better tool to measure intake of isoflavonoids and coumestrol than intake of lignans. In the typical Swedish diet, major sources of

lignan precursors are spread over more food items than sources of isoflavonoids and coumestrol, and are therefore more difficult to capture. In addition, lignans in plants are largely present in the form of SECO and MAT, whereas in humans, enterolactone and enterodiol are the primary forms. Some food items, e.g. flaxseed and rye bran (15), are known to produce high levels of enterolactone, and not all persons may be aware of eating flaxseed or rye bran because it can be hidden in the diet, for example, in bread. In contrast, it is more likely that a person is aware of having consumed a large amount of beans or soy products, which are rich sources of isoflavonoids and coumestrol. This discrepancy could further reduce the accuracy of our measurement of lignan intake, relative to that of isoflavonoid and coumestrol intake, using the FFQ.

Selection of SNPs and genotyping

The aim in the selection of htSNPs in ERβ and COX-2 genes was to find as few htSNPs as possible that could describe the most haplotype diversity in our population. At the time of selection, not many SNPs in ERβ and COX-2 were validated and even fewer had frequency data, so the main criterion for selection was that the SNPs were evenly spread throughout the gene. However, since then, the HapMap database has been updated and we were able to validate the ability of the selected htSNPs to predict common genetic variation in the genes. We found that the average proportion of haplotype diversity explained by our four chosen htSNPs in the ERβ gene was 94.5% (range: 67.6 to 100%), suggesting an adequate coverage. However, for the COX-2 gene only sparse (5 SNPs) genetic information is available from the Hap Map Project, therefore we are not able to evaluate the coverage achieved by our genotyped SNPs.

To evaluate whether the SNPs genotyping methods were valid, both positive and negative controls were used on each plate. Blind duplicates were spread among the plates and all duplicates provided concordant genotype. The success rate—that is, the proportion of successfully genotyped SNPs among all assays attempted—was adequate in our study (mostly over 95%). In addition, Hardy-Weinberg equilibrium was used to test if the genotype frequencies were different from those expected in the population, and we found that all selected htSNPs were in HWE among both cases and controls.

Modelling of gene-environment interactions

The conceptualization of biological interaction in causation of disease has been the subject of lively debate (145), with different causal models leading to different measures of association. We applied both multiplicative and additive effect scales, and observed significant statistical interaction between genotype (of ERβ or COX-2, respectively) and dietary intake (of phytoestrogens or salmon-type fish, respectively) under both models. In addition, we used stratified analyses to examine heterogeneity in associations with genotypes across categories of dietary intake. Although conclusive biological interpretation of the observed interactions cannot be established, our findings provide a basis both for improved detection of the effects of phytoestrogen or fish intake, and possibly for targeting of interventions.

Misclassification of disease

The Swedish cancer registries record almost 100% of all incident cases, and all adenocarcinomas of the prostate included in this study were verified by biopsies or cytological methods. Because there is limited PSA testing in our study population, our results pertain mainly to non-PSA-detected, clinically significant prostate cancer. However, prostate tumors progress slowly compared with many other types of cancer, and many men with prostate cancer may never develop clinical symptoms. As a result, any observed association between an exposure and risk of prostate cancer may not be clinically important. Because of the high incidence of prostate cancer in the general population, some men in the control group are likely to develop prostate cancer after enrollment. From the date of inclusion of study participants (January 1st, 2001) to February 1st, 2005, 26 controls were diagnosed with prostate cancer. Controls with a prostate cancer diagnosis at the time for analysis were excluded (n=13). Nevertheless, any misclassification of disease status in this study is likely unrelated to recalled dietary history.

Confounding

A simple definition of confounding is a mixing of the effect of an exposure on disease with a third (confounding) factor, leading to a bias; in other words, in the situation of confounding, the exposed and unexposed groups are not comparable. A confounding factor must be associated with the disease and the exposure under study, but is not an intermediate factor in the casual chain between the exposure and the disease. The confounding factor can be associated with the disease either as a cause or as a proxy for a cause, but not as an effect of the disease. Confounding can be controlled for in the study design or, given adequate information on the confounding factor(s), in the statistical analysis.

There are only a few well established risk factors for prostate cancer. Age is one of the strongest risk factors. In the design of our study, cases and controls were frequency matched by age; as a methodological consequence, all analyses were adjusted for age. Racial/ethnic origin is also a known risk factor and may also be related to our exposures under study. However, our study population was racially and ethnically homogenous, which reduces the risk of confounding by unmeasured genetic and environmental factors.

There are several proposed risk factors, especially related to diet, for prostate cancer, Intake of different food items and nutrients is often correlated, and identification of confounding factors can be difficult. We used directed acyclic graphs (146) and previous subject matter knowledge to describe confounding of our exposure-disease associations. Among identified confounding factors, those with proportional (≥10%) change in ß-coefficients were included in the final models.

Intake of most nutrients tends to be positively correlated with energy intake, and total energy intake itself is generally influenced by body size, metabolic efficiency and physical activity (127). Even relatively small changes in caloric intake cannot be made unless changes in weight or physical activity also occur. In the absence of such changes, therefore, most alterations in absolute nutrient intake must be accomplished

by changing the composition of the diet rather than the total amount of food. Consequently, nutrient intake in relation to total energy intake (i.e., nutrient density), rather than absolute nutrient intake, may be of more interest in association with disease risk. If energy intake is also related to the disease it may confound the risk estimates, and controls in our study had a lower intake of total calories. To adjust for the confounding effect of energy intake we used the multivariate nutrient density model (127), in which nutrient densities are obtained by dividing the estimated dietary intake of nutrients by the total energy intake. However, using nutrient density can have serious pitfalls if energy intake itself is related to the disease. Because the nutrient density variable contains the inverse of energy intake as a component, nutrient density tends to be associated with the disease in the opposite direction from that of energy intake, even if the absolute nutrient has no association with the disease. The abovementioned model accounts for this by holding total energy intake constant; thus, this method is an "isocaloric" analysis and does control for confounding by energy intake. The coefficient for calories will generally be interpretable as representing the effect of calories in the usual biologic sense because nutrient densities are not inherently part of or highly correlated with total energy intake. The observed association between energy intake and prostate cancer in our study might be interpreted as biased. If controls were less motivated to complete the questionnaire fully, this would have led to lower calculated intake of food overall, leading to differential misclassification of energy intake among controls. If this were true, it would still be appropriate to use nutrient densities in the analysis, since this approach provides an estimation of the composition of the reported diet.

Low BMI, high education, frequent physical activity and never-smoking status are all factors that can be proxies for a general healthy lifestyle, and any or all of these factors may influence prostate cancer risk. If a healthy lifestyle is related to dietary intake phytoestrogens, marine fatty acids or alcohol, as well as to risk of prostate cancer, then these factors would be potential confounders. However, none of these factors has been reported to be a strong risk factor for prostate cancer. In our study, BMI, education, and smoking were not independent risk factors for prostate cancer and did not change the estimates substantially when included in the models. We were not able to account for physical activity; however, we adjusted for total energy intake, thereby removing some of any possible confounding effect of physical activity, which is correlated with energy intake. Since there is no strong evidence of an association between physical activity and prostate cancer risk, our inability to control for physical activity presumably did not substantially change our findings. Nevertheless, although we controlled for different dietary and other factors, there remains the possibility of residual confounding due to imperfect measurement of dietary habits or other unmeasured factors.

INTERPRETATION AND IMPLICATIONS

Study I and Study II

In paper I, we found that high intake of food items rich in phytoestrogens was strongly associated with a decreased risk of both localized and advanced prostate cancer. High intake of beans was the strongest determinant of the inverse association between all phytoestrogen-rich food items and prostate cancer risk. In contrast, estimated dietary

intake of total or individual lignans or isoflavonoids was not associated with prostate cancer risk overall. However, in paper II, we found that the overall decreased risk of prostate cancer for men with a high intake of phytoestrogens, coumestrol, or isoflavonoids was strongly modified by a nucleotide sequence variant in the ERß gene, and was seemingly confined to men who were C-allele carries of a SNP located in the promoter region (-13950 T/C). C-allele carriers with high phytoestrogen intake had an almost 60% lower risk of prostate cancer, compared to C-allele carriers with low phytoestrogen intake, whereas no such association was found among men with the wild type TT genotype. We applied both multiplicative and additive effect scales, and observed significant statistical interaction under both models. This suggests that phytoestrogens and ERβ interact synergistically in a fraction of the population to reduce prostate cancer risk (147), although the precise biology of the interaction is not known. Although at present there are also no functional data showing that the promoter SNP (-13950 T/C) affects the expression of ERß in the prostate, our findings suggest that the (-13950 T/C) SNP, or some other genetic variant(s) in strong linkage disequilibrium with this SNP, modifies the protective effect of phytoestrogens on prostate cancer.

With regard to potential carcinogenic mechanisms, phytoestrogens may be involved in the endocrine control of prostate cell growth by influencing the balance between AR and ERß. Testosterone and its metabolite 5α-dihydrotestosterone (DHT) cause proliferation of prostate epithelial cells through binding to the AR. In contrast, by binding to ERß, 5α-androstane-3ß,17ß–diol (3ßAdiol), a metabolite of DHT, represses the expression of AR and thereby inhibits androgen-driven proliferation while promoting cell differentiation (148, 149). Since some phytoestrogens bind strongly to ERß, the effect of these compounds on prostate epithelia may be the same as that of 3ßAdiol. Experimental studies have shown that physiological concentrations of genistein downregulate AR expression by binding to ERß (26, 150, 151).

If TC carriers of the ERβ promoter region SNP (-13950 C/T) have a higher expression of ERß and relatively low levels of endogenous ligands, then phytoestrogens in the prostate might act as a substitute for the natural ligand, and confer the protective effect that normally comes from binding to ERß. Polymorphisms in the ERß gene might also entail structural changes in the receptor that increase its binding affinity for phytoestrogens.

No previous studies have investigated the interaction between phytoestrogens and polymorphic variation in the ERβ gene in influencing the risk of prostate cancer. However, in a related study, isoflavonoids were found to be negatively correlated with plasma estradiol levels only among women with a certain type of polymorphism in the estrogen receptor-alpha (ESR1) *Pvu* II gene, suggesting that isoflavonoid intake may interact with this gene in the development of breast cancer (152).

In the absence of related epidemiologic evidence, our findings accord with the known biologic properties of the three main groups of phytoestrogens. That is, isoflavonoids and coumestrol bind to ERβ with an affinity almost equal to that of an endogenous ligand, 17β-estradiol (20, 21), although a higher concentration of isoflavonoids than of 17β-estradiol is required to induce ERβ transcription and stimulation of cell growth

(104). Compared with isoflavonoids and coumestrol, lignans have a much lower binding affinity for ERβ (20), and they were unassociated with prostate cancer risk in our study population.

However, lignans may prevent prostate cancer development by mechanisms independent of ERß. Our results from the serum analysis support the hypothesis that lignans protect against prostate cancer, although the relationship between serum enterolactone and prostate cancer risk appears to be non-linear. In contrast, our analysis of estimated dietary lignan compounds does not support such a relationship, perhaps in part because of the limitations of our questionnaire in assessing intake of lignans, as discussed above.

Similar to two smaller Swedish studies (38, 39), we found the strongest inverse association with the second quartile of serum enterolactone. This J-shaped risk function – found also in studies of testicular cancer (153) and breast cancer (154) – is difficult to explain. It could be that high enterolactone levels cause alterations in hormone balance or other factors that influence both lignan metabolism and prostate cancer risk.

None of the three published epidemiological studies of enterolactone and prostate cancer (38-40) took antibiotic use or intake of other food/nutrients into consideration. In our study, the use of antimicrobials was more common among cases than controls, most likely in part because patients are often treated with antimicrobials when biopsies are taken or as treatment for urinary tract infection. The controls who took antibiotics within the last year had significantly lower serum enterolactone levels than those who did not, strongly supporting the possibility that antibiotic use decreases enterolactone levels (141). Therefore, we adjusted for antibiotic use in our analysis.

Serum enterolactone was measured after the onset of disease in cases. If the disease itself or its treatment can alter the metabolism of lignans to enterolactone, this could explain the inverse association between serum enterolactone and prostate cancer risk, as well as the lack of correlation between lignan intake and serum enterolactone in our study. However, our observations of no differences in the results between localized and advanced disease, even after the exclusion of treated cases, and of no correlation between PSA and enterolactone levels, suggest that neither the disease process nor the treatment of prostate cancer influenced enterolactone levels in our study. A single measurement of enterolactone may not reflect levels over a long time period. However, repeated measurements of serum enterolactone one year apart in another study showed an intra-class correlation of 0.6, suggesting that enterolactone is reasonably stable over time within individuals (155). We had relatively few observations on serum enterolactone, and we cannot rule out that our results are due to chance alone.

Endogenous steroid hormones and phytoestrogens share in many ways the same metabolism (15). Dietary factors influence the metabolism of both endogenous hormones and phytoestrogens (140, 156, 157). As a corollary, an inverse association between phytoestrogens and prostate cancer risk may be due to the effects of phytoestrogens themselves, or could be due to other beneficial properties of foods that contain phytoestrogens, which may act in synergy with other compounds to exert their overall effects. In addition, the dose, duration, and timing of exposure to

phytoestrogens through life are probably important for the effects of phytoestrogens on prostate cancer development (15).

The effects of phytoestrogens are pleiotropic in nature, and different types of phytoestrogens (including both lignans and isoflavonoids) vary in their effects on prostate cancer (18, 23, 26). If the effects of isoflavonoids are dependent on specific genotypes, then part of the inconsistency in results from other studies of isoflavonoids and prostate cancer may be explained by differences in the distribution of ERβ gene polymorphisms across the studied populations. On the other hand, the effect of lignans on prostate cancer may be influenced by other factors independent of genetic variation in ERβ (158).

Study III

We found that frequent consumption of fatty fish was strongly associated with a decreased relative risk of prostate cancer, whereas intake of lean fish and shellfish was associated with an increased risk. These results are further supported by our findings that high intake of marine fatty acids was associated with a significant reduction of prostate cancer risk. Moreover, a high ratio between intake of marine fatty acids and ω-6 fatty acids was strongly associated with a decreased prostate cancer risk, supporting our hypothesis that the fatty acids EPA and DHA are involved in the etiology of prostate cancer.

There are several possible explanations for the null findings in other studies. Almost all of them looked at total intake of fish and did not differentiate among species of fish; such misclassification might entail underestimation of any protective effect of fatty fish. The intake of marine fatty acids in some study populations may have been too low to show a potential protective effect, and/or the range of exposure may have been too narrow, limiting the ability to detect an association with prostate cancer.

There is some evidence of a stronger inverse association between fish intake and prostate cancer from studies conducted in countries with a high *per capita* intake of marine fatty acids, an indicator of high intake of fatty fish, compared to results from studies conducted in countries with low *per capita* intake (53). These findings are supported by another Swedish study that found a strong negative association between fish intake and prostate cancer (159). Furthermore, the intake of fatty fish is relatively high in Sweden compared with other countries (160), and the intake of EPA and DHA in our study population in particular was relatively high compared with intake in non-Swedish Western study populations (161, 162)

The ratio of ω-3/ ω-6 fatty acids might be more important than the absolute intake of ω-3 fatty acids in inhibiting the development of several diseases, including cancer and various inflammatory and autoimmune diseases (51). The ratio of ω-3/ω-6 fatty acids in Western diets is lower than that in Far Eastern countries, where the incidence of prostate cancer is also lower (51). In accordance with other studies, we found that the ω-3 fatty acid α-linolenic acid was associated with an increased risk of prostate cancer.

Hence, the ratio of marine:ω-6 fatty acids may be a better measure of beneficial dietary fat intake than the ratio of ω-3:ω-6 fatty acids.

Our finding that intake of lean fish and shellfish was associated with an increased risk of prostate cancer is difficult to explain. However, the lack of an inverse association may be explained in part by the much lower levels of EPA and DHA in these types of seafood than in salmon-type fish and herring/mackerel (163), or by the fact that fish fingers contain a relatively low proportion of fish meat. In addition, seafood from open seas contains varying levels of contaminants (e.g., methyl-mercury, organochlorine compounds , polychlorinated biphenyls , and dioxins), whereas salmon-type fish consumed in Sweden is generally farm-raised and contains a lower level of contaminants (10). There is, however, no conclusive evidence that these compounds are associated with prostate cancer risk (164, 165).

In our study, the inverse association between high intake of salmon-type fish and risk of prostate cancer was modified by a nucleotide sequence variant in the COX-2 gene, and was seemingly confined to men who were C-allele carries of the SNP rs5275 (+6365 T/C). High salmon-type fish consumers with the C-allele had a 72% lower risk of prostate cancer. However, no such association between intake of salmon-type fish intake and prostate cancer was found among men with the more common TT genotype. This suggests that the protective effect of fish is modified by variation in the COX-2 gene.

To our knowledge, no other epidemiological studies have evaluated the interaction between intake of fish and polymorphisms in the COX-2 gene in the etiology of prostate cancer. However, fish intake modified the association between COX-2 genotypes and colorectal adenoma in a case-control study (166). In a small intervention study, COX-2 expression was decreased among men with untreated prostate cancer consuming a low-fat diet supplemented with fish oil (167). Genetic variation in the COX-2 gene may interact with fish consumption by influencing the synthesis and/or metabolism of eicosanoids, and could enhance the anti-inflammatory effect of marine fatty acids on prostate cancer risk. However, the precise mechanism of the effects of an interaction between COX-2 genetic variation and fish intake on prostate cancer development remains unclear.

Study IV

Our study suggests that recent intake of total alcohol, beer, wine, and liquor is not associated with risk of prostate cancer. This lack of association was observed for advanced but not localized disease, and for both sporadic and familial prostate cancer.

Despite the lack of association with any amount of total alcohol intake, we observed that both current and former drinkers had a significantly higher risk of prostate cancer than never-drinkers. However, because actual alcohol, beer, wine, and liquor consumption showed no relationship with prostate cancer risk, the apparent positive association of ever versus never drinking with risk of prostate cancer may be due to other factors correlated with drinking habits. Alternatively, it is possible that only

distant past alcohol intake is associated with risk of prostate cancer, leading to a positive association with ever drinking but not with recent intake.

Differences in associations between localized and advanced prostate cancer could arise if the timing of the effects of alcohol were limited to early disease development, such that any association with late-stage disease would be attenuated over time. Alternatively, alcohol could promote a clinically less aggressive form of prostate cancer. It could also be that disease symptoms altered drinking habits even a year before diagnosis in advanced cases, leading them to report patterns that were unrepresentative of previous intake, whereas habits of men who went on to develop localized prostate cancer were unaffected by symptoms.

Our findings contradict a handful of studies that detected a positive association between alcohol intake and risk of all prostate cancer (69-76). As with the very modest relative risks that we detected in association with localized disease, the excess risk in these studies has generally been low or moderate, with only a couple of studies (74, 75) reporting relative risks above 2.0 for long-term and/or heavy drinkers, compared to non-drinkers. Our data also conflict with a report of an inverse association between alcohol drinking and prostate cancer risk in a large prospective cohort study (77), although the apparent protective effect in this investigation was detected mainly among a small number of men who drank heavily in the distant past. Overall, our findings confirm the majority of studies, which show no association with alcohol consumption with prostate cancer risk (57, 68).

Inconsistency among prior studies may be explained by several aspects, including variation in study design, study population, exposure assessment, and prevalence of environmental and/or genetic co-factors. Volume, frequency, and duration of drinking have not always been evaluated separately in prior studies. The timing of alcohol exposure being addressed (e.g., current or ten years ago) has varied among study questionnaires, and different types of alcoholic beverages have often not been assessed individually. In addition, given that we observed a positive association only with risk of localized prostate cancer, variation in the proportions of localized and advanced cases among previous study populations may explain discrepant results.

In summary, our results are consistent with the preponderance of evidence suggesting that alcohol drinking is unrelated to risk of prostate cancer. Furthermore, we observed that alcohol intake is not associated with risk of advanced, sporadic, or familial prostate cancer, although it is marginally associated with risk of localized disease. Although previous studies have extensively examined the association between alcohol intake and prostate cancer, no others, to our knowledge, have stratified between localized and advanced or sporadic and familial cases.

CONCLUSIONS

From the results of this case-control study on prostate cancer among Swedish men, our conclusions are as follows:

- Dietary intake of lignans and isoflavonoids ranges widely among Swedish men, and the distribution of intake is heavily skewed towards higher values. The intake of isoflavonoids in this population is relatively low, whereas the intake of lignans is comparable with that observed in studies from Finland. Flaxseed and rye bread contribute the most to the overall intake of lignans, whereas soy products are the most important dietary source of isoflavonoids in this population.

- Dietary intake of total lignans is not correlated with serum enterolactone level overall; the correlation appears to be influenced by other dietary factors.

- High intake of food items rich in phytoestrogens is strongly associated with a decreased relative risk of both localized and advanced prostate cancer.

- Estimated dietary intake of total or individual lignans or isoflavonoids is not associated with prostate cancer risk overall.

- Serum enterolactone levels are inversely, but non-linearly, associated with risk of prostate cancer.

- The inverse association between high intake of foods rich in phytoestrogens and risk of prostate cancer is modified by a nucleotide sequence variant in the ERβ gene.

- High intake of fatty fish and marine fatty acids is strongly associated with a decreased relative risk of prostate cancer.

- High intake of lean fish and shellfish is associated with an increased risk of prostate cancer.

- A high ratio between intake of marine fatty acids and ω-6 fatty acids is strongly associated with a decreased relative risk of prostate cancer.

- The inverse association between high intake of fatty fish and risk of prostate cancer is modified by a nucleotide sequence variant in the COX-2 gene.

- Recent intake of total alcohol, beer, wine, and liquor, respectively, is not associated with risk of overall prostate cancer, nor advanced, sporadic, or familial prostate cancer. There is a marginal positive association between intake of any alcoholic type of drink and risk of localized prostate cancer.

FUTURE RESEARCH

This is the first study in a Western population that examines the association between risk of prostate cancer and exposure to phytoestrogens using three different exposure measurements. Our results support the hypothesis that high overall intake of foods rich in phytoestrogen compounds lowers the risk of prostate cancer. However, further prospective epidemiological studies using improved food databases and experimental studies are needed to identify the specific compounds that provide the putative protective effect, and to determine precisely how the complex metabolism of phytoestrogens may interact with other mechanisms to prevent cancer. This may also enlighten us about the apparently non-linear association between serum levels of enterolactone and prostate cancer.

Studies in other human populations, especially of different races or ethnicities, are needed to determine whether differences in the distribution of ERβ or COX-2 polymorphisms and intake of phytoestrogens and fish, respectively, can explain some of the geographical and racial/ethnic variations in prostate cancer risk. Further, experimental studies are needed to evaluate the biological function of the studied SNPs or other genetic variants in strong linkage disequilibrium with these SNPs.

Other studies, especially with prospective exposure data, can help reveal whether our findings of; lean fish, shellfish and α-linolenic acid being associated with an increased relative risk of prostate cancer, and the heterogeneity of the association with alcohol between localized and advanced prostate cancer, are due to bias, chance or a true biological difference.

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