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Effects of a Phytoestrogen Intervention and Estrogen Receptor β Genotype on Prostate Cancer Proliferation and PSA Concentrations—A Randomized Controlled Trial

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ABSTRACT

A phytoestrogen-rich diet has been suggested to reduce tumor proliferation among men with prostate cancer, and the effect may differ between men with different polymorphisms of the estrogen receptor-beta gene (ERβ). Patients with low- or intermediate-risk prostate cancer scheduled for radical prostatectomy were randomized to an intervention group ($n=71$) provided with soybeans and flaxseeds (~200 mg phytoestrogens/day) to eat until surgery (approximately 6 wk) or to a control group ($n=69$). Tumor proliferation was assessed using Ki-67 indexes, prostate-specific antigen (PSA) concentrations were analyzed in blood, and ERβ polymorphism was genotyped in all subjects. The intervention group had a 13% unit lower risk [95% confidence interval (CI): −28%, 1.8%] of a higher Ki-67 index compared to controls, but the effect was most pronounced among TT carriers of ERβ [risk difference (RD) −19%, 95% CI: −45%, 6.8%]. Subjects with genotype TC/CC had a lower risk (RD −29%, 95% CI: −46%, −1.2%) and TT genotype a higher risk (RD 25%, 95% CI: 8.7%, 42%) of increased PSA concentration, comparing the intervention group to controls. In conclusion, a phytoestrogen-rich diet may cause lower tumor proliferation and concentration of PSA in men with prostate cancer with a specific genetic upset of ERβ.

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Introduction

Prostate cancer is currently the most frequently diagnosed malignancy among men and ranks as the third leading cause of death from male-specific cancers in EU member states, according to the International Agency for Research on Cancer. Phytoestrogens, including lignans and isoflavones, are found in e.g., soybeans, rye, and seeds and have a potential protective effect against prostate cancer (1,2). In our previous study, we found a putative genetic interaction for this protective role of phytoestrogens (3). The decreased risk of prostate cancer diagnosis in men with a high intake of phytoestrogens was strongly modified by a nucleotide sequence variant (TC/CC) in the estrogen receptor-beta gene (ERβ). Phytoestrogens have a chemical structure similar to endogenous estradiol and by binding to the ERβ, they can act like a tumor suppressor (4–6). The role of ERβ in prostate cancer progression is not fully clarified; nevertheless, there is a loss of ERβ expression in prostate cancer tumors (7,8). In in vitro studies, phytoestrogens have shown proapoptotic and antiproliferative effects in prostate cancer cells, which have been both androgen-dependent and androgen-independent (9–11). Different antiproliferative effects of phytoestrogens acting through ERβ have been suggested. Examples of

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mechanisms are upregulating ERβ by reducing its promoter methylation (12,13), downregulating the androgen receptor (14,15), inhibiting nuclear translocation of the epidermal growth factor receptor, and stimulating nuclear transport of the tumor suppressor gene *PTEN* (16). Phytoestrogens may also modulate proteins involved in tumor proliferation and apoptosis (17–19).

In randomized controlled trials, phytoestrogens have been shown to reduce tumor proliferation among patients diagnosed with prostate cancer, but the scientific evidence is yet limited (20–24). A systematic review concluded that further evidence is needed from trials with adequate sample sizes and longer time intervals (25). A possible explanation for the incoherent results from studies could be that not all individuals benefit from the diet due to genetic factors, resulting in individual effects of diet on endogenous cancer protective mechanisms.

Men with prostate cancer commonly change their dietary intake or use different types of complementary or alternative medicine with non-proven scientific effects as a result of their diagnosis (26,27). In the PRODICA (impact of DIet and individual genetic factors on tumor proliferation rate in men with PROstate CAncer) trial, we provided soybeans and flaxseeds (approximately 200 mg phytoestrogens/day) to patients with prostate cancer until their surgery (28). The aim was to investigate the effect of the dietary intervention on the proliferation marker Ki-67 in tumor tissue and serum concentration of prostate-specific antigen (PSA) depending on the genotype of ERβ. Our hypotheses are:

1. In patients diagnosed with low- and intermediate-risk prostate cancer, the addition of phytoestrogen-rich foods to the diet for 6 wk, reduces prostate tumor proliferation compared to no addition of phytoestrogen-rich foods to the diet during the same period.
2. If the effect of phytoestrogens on prostate tumor proliferation exists, it is modified by the polymorphisms in the promoter region of the ERβ-gene.

Material and Methods

Study Population and Study Design

The study design has been described in detail elsewhere (28). In brief, patients diagnosed with prostate cancer cT1-cT2 [International Society of Urological Pathology (ISUP) grade <4, PSA <20] and scheduled for radical prostatectomy were recruited to the trial at the Department of Urology at Sahlgrenska University Hospital in Gothenburg, Sweden (Fig. 1). Exclusion criteria were ongoing hormone therapy, psychological or mental disorders, cognitive dysfunction, and allergy to the intervention foods. The intervention was intended to be at least 6 wk. However, for some patients, the surgery queues were shorter than 6 wk, and therefore, we decided to include all patients with at least 2 wk to scheduled surgery.

An inclusion meeting with a dietitian was scheduled with patients who agreed to participate. At the inclusion meeting, subjects filled out a questionnaire including a food frequency questionnaire (FFQ), baseline blood samples were collected, and weight and height were measured. The patients were randomized to the intervention or the control group by drawing a folded note from an envelope where half of the notes included were labeled intervention and the other half control.

To measure compliance and intake of total phytoestrogens among subjects, a 24-h dietary recall (29) by phone was performed about halfway through the study period. Endpoint blood samples were collected within 7 days of the surgery and the subjects filled out a similar questionnaire as at baseline, as close to the time of surgery as possible—ideally 1–2 days before. After the surgery, the pathology laboratory handled the prostate according to clinical routines.

Patients were recruited between February 2016 and May 2023, with the last surgery in May 2023. Ethical approval for this research was obtained by Ethical Review Board in Gothenburg, Sweden (registration numbers 410-14, T124-15; 2020-02471; 2021-03320, 2021-05878-02). The research was performed in accordance with the principles stated in the Declaration of Helsinki, and written informed consent was obtained from all included patients. The study was registered on ClinicalTrials.gov (trial registration ID: NCT02759380) on 3 May 2016 when the pilot study was finished. The study protocol underwent only minor modifications, primarily limited to administrative adjustments following the pilot study (28).

Intervention

All subjects received a brochure with dietary recommendations from the Swedish National Food Agency (30) at the inclusion meeting. Subjects were given instructions to avoid nutritional supplements, but no other dietary restrictions were given. At the inclusion meeting, subjects in the intervention group were provided with fresh frozen green soybeans, roasted yellow soybeans, and flaxseeds in amounts that were

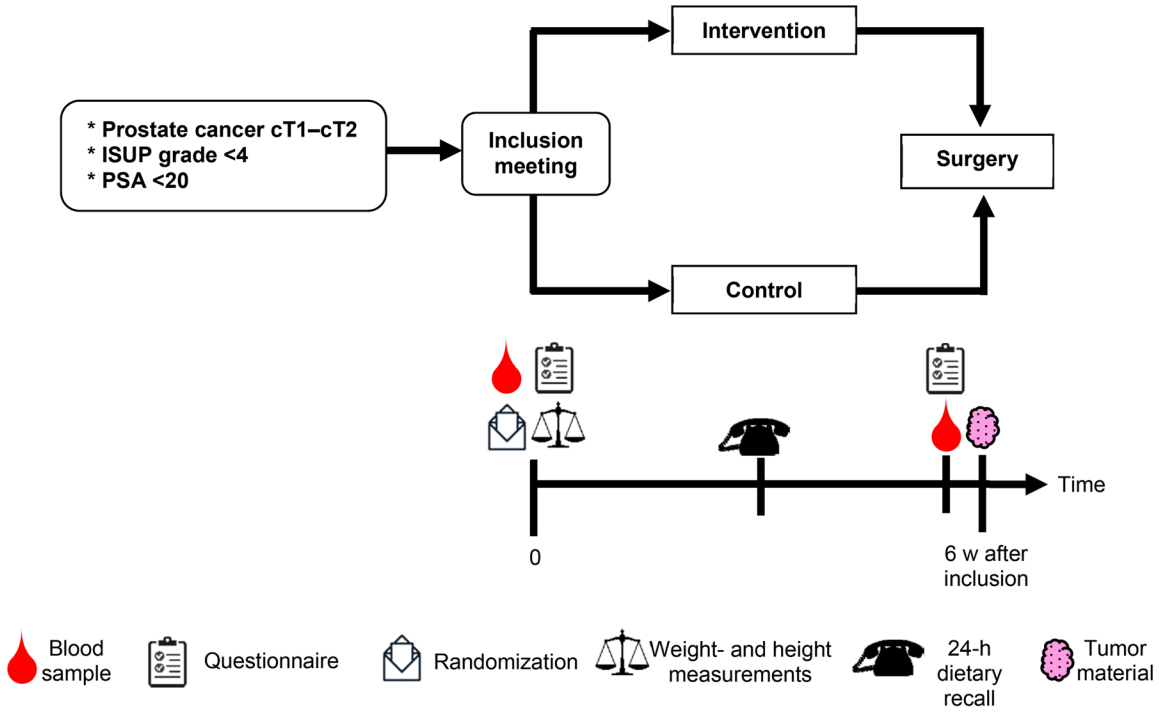

Figure 1. The study design of PRODICA. Eligible patients with prostate cancer participated in an inclusion meeting at baseline where they were randomized to an intervention or a control group, they filled out a questionnaire including a food frequency questionnaire, weight and height were measured, and blood samples were collected. Approximately halfway through the study, a 24-h dietary recall was performed with the subjects. Within 1 wk of surgery, a similar questionnaire was filled out and blood samples were collected again. After surgery, tumor material was collected according to clinical routines.

estimated to last until the surgery. They were instructed to daily eat 47 g of green soybeans, 28 g of roasted yellow soybeans, and 28 g of flaxseeds, which is estimated to provide 200 mg of phytoestrogens (100 mg isoflavones, 100 mg lignans) (31). Subjects gradually increased the intake of the intervention foods during the first 9 days according to the schedule (28). At the beginning of the study, crushed flaxseeds were used but were then replaced during the study by whole flaxseeds after 18 subjects had been included. This was due to the content of cyanogenic glycosides and the formation of hydrogen cyanide (32), explained in more detail elsewhere (28). Both the intervention and the control groups were aware of which group they were allocated to; however, the control group did not receive any information about what foods the intervention group received.

Dietary Assessments

Food Frequency Questionnaire

The FFQ has been validated against urine alkylresorcinol metabolites and 4-d estimated food records and has been described in detail elsewhere (28,33). The baseline questionnaire reflected the dietary intake during the past 3 mo, and the endpoint questionnaire reflected the time during the study intervention. Information on standard portion sizes and food composition tables were provided from The Swedish National Food Agency (34). The amounts of phytoestrogens were collected from our in-house developed database, described elsewhere (2,35). Lignans included secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, syringaresinol, medioresinol, enterodiol, and enterolactone; isoflavones included genistein, daidzein, formononetin, biochanin A, and equol; and total phytoestrogens included lignans, isoflavones, and coumestrol. After the questionnaire had been filled out, the study dietitian controlled the questionnaire for missing and improbable values (e.g., intake of food items ≥7 times per day, lower reported intakes of the intervention foods than had been reported at the 24-h dietary recall or from the remaining amounts of the intervention foods). The values were verified with the subject and were, if necessary, revised.

24-h Dietary Recall and Calculation of Compliance

The 24-h dietary recall has been described in more detail elsewhere (28). The previous day's food intake was first lined up and then a list of potentially forgotten foods was checked (28). When the 24-h dietary recall was finished, the subjects in the intervention

group were asked how much of the intervention foods they had left. The subjects also reported the remaining intervention foods at the end of the intervention period. Compliance was calculated in two ways, i.e., 1) the estimated intake of phytoestrogens based on the amount of remaining intervention foods reported at the 24-h dietary recall and the end of the intervention and 2) the total estimated intake of phytoestrogens based on the reported dietary intake at the 24-h dietary recall, calculated by using the in-house phytoestrogen database. Estimated intakes of ≥80% of the recommended intake of phytoestrogens were considered compliant for the intervention group and <80% were considered compliant for the control group.

Other Assessments

Information concerning prostate volume determined with transrectal ultrasound, ISUP grade, and concentration of total PSA that were missing at endpoint were collected from the National Prostate Cancer Register of Sweden (NPCR). NPCR is a national-wide quality register, which captures 98% of prostate cancer cases in Sweden with about 90% completeness of variables (36). The ISUP grade at the surgery collected from NPCR was compared with the pathological-anatomical information and in case of inconsistent or missing values, the pathological-anatomical information was used. Tumor volume was collected from the pathological-anatomical information. Information on the intake of Finasteride (a 5α-reductase inhibitor) and the date for the most recent prostate biopsy were collected from medical records.

Blood Samples

Procedures and analyses of the blood samples and the selection of the polymorphism of ERβ-gene have been described in detail previously (3,28). Concentrations of total and free PSA were analyzed in serum. Whole blood samples were analyzed for single nucleotide polymorphisms (SNP) in the ERβ-gene (rs2987983-13950 T/C) at Umeå University. Subjects were assigned to a genotype of either TT, TC, or CC alleles.

Tumor Material

Ki-67 was assessed with immunohistochemical detection described in detail elsewhere (28). A pathologist evaluated Ki-67 by evaluating at least 100 cells in five randomly selected areas (in total at least 500 cells) in the prostate slice with the predominant spread of the largest and dominating tumor from the formalin-fixed paraffin-embedded radical prostatectomy tissue. The Ki-67 index was calculated by the ratio of immunohistochemically positive prostate cancer nuclei divided by the total number of tumor cells evaluated × 100. The pathologist was blinded to which samples were allocated to intervention or controls.

Statistical Analysis

Statistical analysis was performed using Stata/SE version 17.0 (StataCorp LLC, College Station, TX, USA). *P* values <0.05 were considered statistically significant. The Ki-67 index was the predeclared primary outcome and the PSA concentration was the predeclared secondary outcome in the study. Data on demographics, PSA concentrations, and Ki-67 index were stratified according to the subjects' genotype of ERβ. The Mann–Whitney *U* test was used to compare the distributions between groups due to non-normally distributed data (tested with the Shapiro–Wilk test). Intention-to-treat analyses included all randomized subjects, and per-protocol analyses included subjects with ≥80% compliance. For the intervention group, we used the compliance calculated from the reported remaining intervention foods at the endpoint, and for the control group, we used the compliance calculated from the 24-h dietary recall.

If information about the most recent prostate biopsy was missing, the date of the prostate cancer diagnosis was used to calculate the time since the most recent biopsy before the surgery. PSA density was calculated by total PSA concentration divided by the prostate volume (determined with transrectal ultrasound). For the Ki-67 index, the median, mean, and maximum values were calculated from the five randomly selected areas of the tumor tissue, and in the main analysis below the median value was used.

In the primary analysis, the hypotheses were evaluated by generalized linear models providing estimates of the risk difference (RDs) and corresponding 95% confidence intervals (CIs), stratified by ERβ genotypes. The outcome of Ki-67 indexes was dichotomized according to the > median value (1) and the ≤ median value (0) of the study population. Changes in the concentrations of PSA between baseline and endpoint were dichotomized into increased (1) and unchanged or decreased (0) concentrations. The additive interaction between phytoestrogen intake and ERβ SNPs on Ki-67 and PSA concentrations was assessed in a linear odds model by the product term between the covariates representing phytoestrogen intake (coded

as intervention = 1, control = 0) and SNP genotypes (coded as TT = 0, TC/CC = 1).

Analyses were adjusted for body mass index (BMI, kg/m^2) (≤18.5, 18.5 to <25, 25 to <30, 30 to <35, ≥35), age (≥median, <median), tumor stage (T1 or TX, T2), intake of polyunsaturated fatty acids (tertiles), and the most recent prostate biopsies before surgery (>3 mo, ≤3 mo). Dietary fiber intake was not adjusted for due to the high correlation with total phytoestrogen intake. The analysis was also stratified by the reported intake of antibiotics over the last 5 yr (0 times or unknown intake, ≥1 time). In a sensitivity analysis, users of Finasteride or subjects with a long intervention period were excluded.

The power calculation has been described in detail elsewhere (28). A study sample of 118 patients provided 80% power for a two-sided test of the primary outcome of the KI-67 index, and 203 patients were needed to find an existing effect if the study sample was stratified according to the genotype of ERβ. Here, we present analyses of 154 subjects, even if we have not reached the pre-calculated power for the stratified analysis. Due to the inclusion rate being slower than expected, the data monitoring committee was consulted on whether to continue or terminate the study. The interim analysis was presented to the committee, which recommended stopping inclusion based on that the results probably do not change dramatically if the pre-calculated power will be reached. A decision was made by the research group to stop the inclusion and publish the results.

Results

Study Subjects and Characteristics

The main reasons for declining study enrollment were being occupied ($n=29$) and unwillingness to participate in the inclusion meeting in Gothenburg ($n=22$)—mostly due to long travel times (Fig. 2). Of the 154 randomized patients, 6 patients discontinued the intervention (intervention $n=4$; control $n=2$). The reasons for dropout in the intervention group were mainly related to gastrointestinal problems experienced from the intervention foods, and the subjects in the control group stated no reasons. In total, 9 subjects in the intervention group reported gastrointestinal problems related to the intervention foods, whereof 7 patients completed the participation. Besides gastrointestinal symptoms, different kinds of adverse effects were reported from 3 subjects. Based on the reported dietary intake from the 24-h dietary recall, 27% of the subjects in the intervention group and 100% of the controls were compliant. According to the reports of remaining intervention foods, 78% in the intervention group were compliant at the time of the 24-h dietary recall ($n=69$) and 76% were compliant at the endpoint ($n=67$).

The subjects had a median age of 65.5 yr (interquartile range (IQR 10), range 40–76) and the median intervention period was 47 days for both groups (intervention: IQR 32, range 7–189 days; control: IQR 28, range 8–812 days; Table 1). There were some differences in baseline characteristics between the intervention and the control groups. The intervention group had a higher proportion of cT2 tumors and a lower level of physical activity compared to the control group at baseline. At the surgery, subjects in the intervention group had a larger total tumor volume compared to the control group (Table 1).

Energy and Nutrient Intakes and Anthropometric Measurements

There were no differences in energy and nutrient intakes between the intervention and control groups at baseline (Table 2). During the intervention, the intervention group increased intake of dietary fiber, polyunsaturated fatty acids, coumestrol, isoflavones, lignans, and total phytoestrogens compared to controls, who maintained or decreased their intakes. At the endpoint, the intervention group also reported a higher intake of all these nutrients compared to the control group. The intervention group had a higher median BMI at baseline compared to controls (Table 2). However, there was no difference in weight change during the intervention between the intervention and control groups.

Effects of the Phytoestrogen Intervention on the Ki-67 Index and PSA Concentrations

The intervention group had a 13% unit decreased risk of a high Ki-67 index compared to the control group ($p=0.086$, Table 3, Fig. 3), and the effect was more pronounced among those with the TT genotype (Table 3, Supplementary Table 1). A small or opposite effects were seen in patients with genotype TC/CC. In the per-protocol analysis, the associations were statistically significant (Table 3). The median Ki-67 index was associated to genotype with a higher frequency of having a high Ki-67 in the TT group (49%) compared to 36% of those with genotype TC/CC (data not shown).

There was no statistically significant difference between the intervention and the control groups in changes in total and free/total PSA concentrations

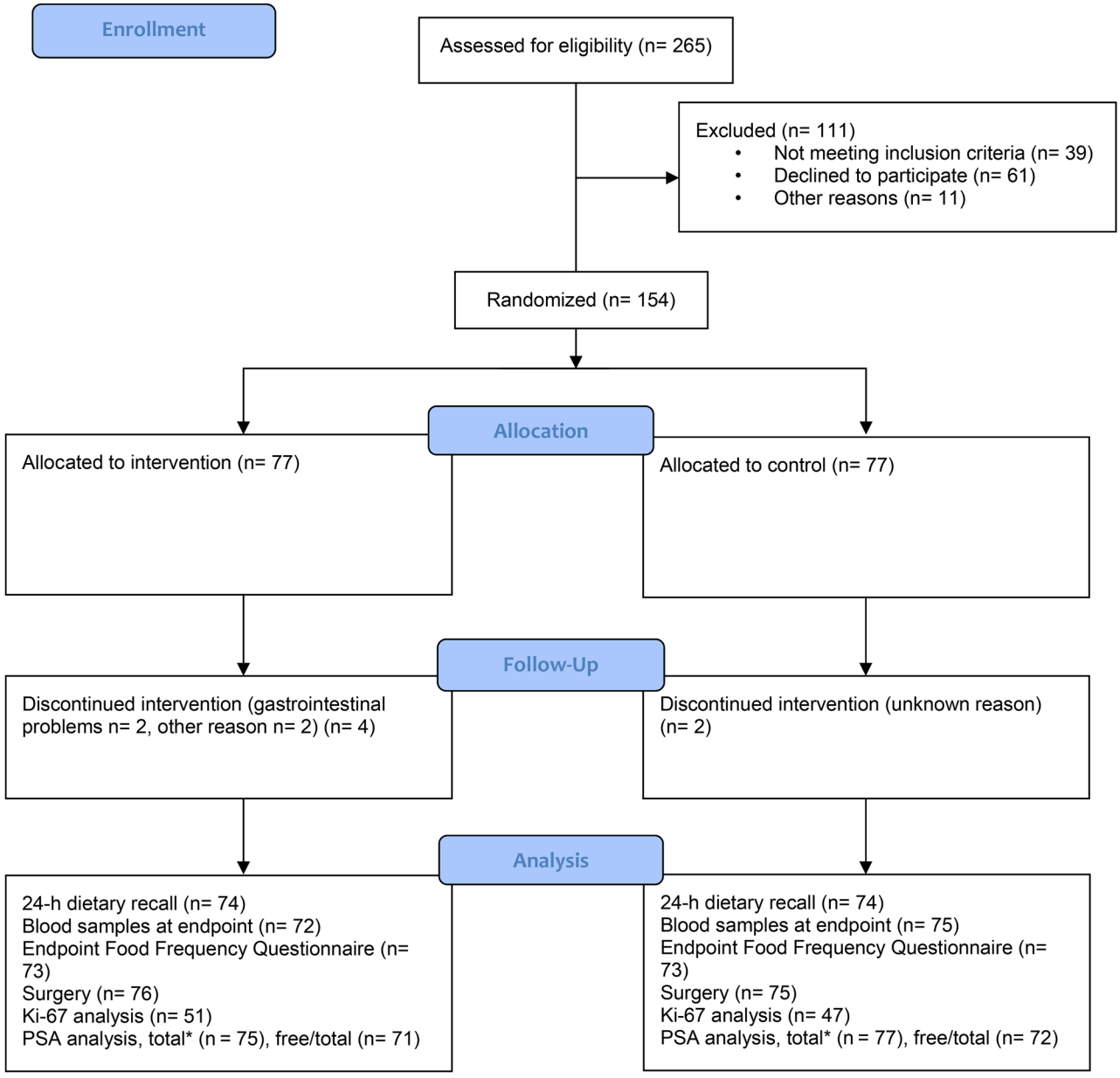

Figure 2. Flowchart of the PRODICA trial. The figure shows the number of patients that were eligible, randomized, discontinued the intervention, and included in the analyses. *Total PSA concentration that were missing at endpoint and were collected from the National Prostate Cancer Register of Sweden.

during the intervention (Table 4). However, in the stratified analysis, the results differed between subjects with different genotypes. Subjects with genotype TC/CC had a 29% decreased risk of having a rise in the total PSA concentration when eating the intervention diet compared to controls (adjusted $p = 0.001$). In contrast, subjects with genotype TT had a 25% unit higher risk of a rise in the total PSA (adjusted $p = 0.003$, Table 4). This contrasting difference in total PSA response of the intervention based on genotypes was statistically significant in an interaction analysis. The per-protocol analysis for the RDs showed similar effects for total PSA concentration (Table 4). For the analysis of free/total PSA concentration, there were no statistically significant effects on the ratio in the per-protocol analysis (Table 4).

When users of Finasteride were excluded from the analysis, the effects on the Ki-67 index became stronger ($RD_{whole\ group}$ −17%, $p = 0.030$, Table 3), while the results of PSA concentrations did not differ (data not shown). When analyses of RDs were stratified according to the reported intake of antibiotics in the last 5 yr, only users of antibiotics had a decreased risk of a higher Ki-67 index (data not shown).

Table 1. Demographics of the subjects in the PRODICA[a] trial, stratified by the genotype of estrogen receptor beta.

	Intervention ($n=75$)				Control ($n=77$)			
	Genotype TT[b] ($n=39$)		Genotype TC/CC[b] ($n=38$)		Genotype TT[b] ($n=30$)		Genotype TC/CC[b] ($n=47$)	
	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)	Range
Age, yr	64 (10)	51–76	67 (8)	43–76	67 (10)	51–75	64 (10)	40–75
Intervention period, days	47 (46)	12–189	47 (29)	7–146	44 (28)	8–812	47 (29)	14–583
The proportion of biopsies with cancer at diagnosis, %	29 (46)	8–100	25 (29)	7–100	37 (42)	8–100	21 (24)	7–100
Missing, *n* (%)	7 (18)		10 (26)		4 (13)		11 (23)	
PSA density at diagnosis, µg/l/cm^3[c]	0.14 (0.096)	0.039–0.52	0.16 (0.12)	0.069–0.55	0.17 (0.10)	0.11–0.38	0.16 (0.13)	0.078–0.69
Missing, *n* (%)	1 (3)		1 (3)		0 (0)		1 (2)	
Total tumor volume with shrinkage factor (1.3), cm^3[d]	1.8 (2.7)	0.4–9.5	2.1 (2.1)	0.6 – 16.9	1.6 (1.3)	0.3–19.1	1.5 (1.9)	0.03–4.9
Missing, *n* (%)	1 (3)		1 (3)		2 (7)		1 (2)	
Tumor stage at diagnosis	***n* (%)**		***n* (%)**		***n* (%)**		***n* (%)**	
cT1	22 (56)		23 (61)		18 (60)		34 (72)	
cT2	15 (38)		14 (37)		10 (36)		13 (28)	
cTX	1 (3)		1 (3)		2 (7)		0 (0)	
Missing	1 (3)		0 (0)		0 (0)		0 (0)	
ISUP grade at diagnosis								
1	13 (34)		15 (39)		12 (40)		22 (47)	
2	20 (53)		16 (42)		13 (43)		21 (45)	
3	5 (13)		7(18)		5 (17)		4 (9)	
Missing	1 (3)		0 (0)		0 (0)		0 (0)	
ISUP grade at the surgery								
1	12 (31)		7 (18)		5 (17)		14 (30)	
2	16 (41)		18 (47)		14 (47)		20 (43)	
3	8 (21)		12 (32)		8 (27)		9 (19)	
4–5	2 (5)		0 (0)		0 (0)		4 (9)	
Missing	1 (3)		1 (3)		3 (10)		0 (0)	
Physical activity[e]								
Low	7 (18)		10 (26)		2 (7)		5 (11)	
Moderate	20 (51)		18 (47)		18 (60)		23 (49)	
High	12 (31)		10 (26)		10 (33)		19 (40)	
Heredity for prostate cancer								
Yes	14 (36)		14 (38)		8 (27)		17 (36)	
No	9 (23)		11 (30)		6 (20)		14 (30)	
Do not know	16 (41)		12 (32)		16 (53)		16 (34)	
Antibiotic treatment during the past 12 mo								
Yes	13 (33)		8 (21)		12 (40)		12 (26)	
No	25 (64)		29 (76)		18 (60)		34 (72)	
Do not know	1 (3)		1 (3)		0 (0)		1 (2)	
Antibiotic treatment last 2–5 yr								
Yes	15 (38)		11 (29)		16 (53)		25 (53)	
No	22 (56)		20 (53)		12 (40)		17 (36)	
Do not know	2 (5)		7(18)		2 (7)		5 (11)	
Antibiotic treatment during the intervention, *n* (%)								
Yes	1 (3)		4 (11)		4 (13)		4 (9)	
No	35 (90)		33 (87)		24 (80)		42 (89)	
Missing, *n* (%)	3 (8)		1 (3)		2 (7)		1 (2)	
Intake of Finasteride during the intervention								
Yes	3 (8)		5 (13)		2 (7)		3 (6)	
No	36 (92)		33 (87)		28 (93)		44 (94)	
The most recent prostate biopsy								
≤3 mo	5 (13)		9 (24)		6 (20)		9 (19)	
>3 mo	34 (87)		29 (76)		24 (80)		38 (81)	

[a]Impact of Dlet and individual genetic factors on tumor proliferation rate in men with PROstate Cancer.
[b]Subjects were allocated to the estrogen receptor-beta genotype TT, TC, or CC.
[c]Prostate volume was determined with transrectal ultrasound.
[d]Collected from pathological-anatomical information established after the surgery.
[e]Low physical activity: 101–103, 201 p; moderate physical activity: 104, 202–203, 301–302 p; high physical activity: 204, 303–304, 401–404 p.
Activity in the daytime: sedentary (100 p); partly sedentary, sitting, and walking (200 p); mostly standing and walking (300 p), physical labor (400 p). Physical activity in evening time: sedentary (1 p), slightly strenuous activity—equal to a 30-min walk (2 p); moderately strenuous activity—equal to a bike ride of ≥30 min (3 p); sports activity (4 p).
The data were collected at baseline if not otherwise stated.
Abbreviations: ISUP, International Society of Urological Pathology.

Table 2. Energy, nutrient, and phytoestrogen intake and anthropometric measurements of the subjects in the PRODICA[a] trial.

	Intervention ($n = 73$)		Control ($n = 73$)		
	Median (IQR)	Range	Median (IQR)	Range	*P*
Energy, kcal					
Baseline	2,221 (819)	1,348–4,695	2,065 (464)	1,095–4,161	0.673
Endpoint	1,930 (669)	809–4,044	1,961 (552)	1,077–3,364	0.170
Change[b]	−65 (512)	−2,592–857	−162 (974)	−2,287–974	0.384
Carbohydrates, g					
Baseline	212 (91)	101–390	214 (77)	64–402	0.673
Endpoint	201 (72)	54–431	195 (65)	66–367	0.495
Change[b]	−3 (63)	−267–142	−16 (44)	−242–177	0.316
Protein, g					
Baseline	84 (26)	49–151	82 (25)	46–181	0.766
Endpoint	80 (19)	43–132	79 (25)	40–134	0.38
Change[b]	−3 (20)	−92–38	−5 (22)	−105–26	0.584
Fat, g					
Baseline	94 (39)	39–257	84 (26)	36–213	0.152
Endpoint	86 (37)	35–179	76 (36)	29–137	0.070
Change[b]	−6 (27)	−173–50	−7 (27)	−114–38	0.375
Fiber, g					
Baseline	22 (9)	10–45	21 (11)	9–52	0.768
Endpoint	24 (7)	12–45	20 (8)	7–44	<0.001
Change[b]	2 (6)	−14–17	−1 (5)	−18–14	<0.001
PUFA, g					
Baseline	13 (6)	6–36	12 (7)	5–39	0.352
Endpoint	14 (6)	7–32	12 (6)	5–27	0.0023
Change[b]	1 (4)	−21–11	−1 (3)	−27–9	0.0012
Fat fish, times/m					
Baseline	4 (4)	0–20	3 (3)	0–27	0.461
Endpoint	4 (4)	0–24	3 (3)	0–13	0.212
Change[b]	0 (2)	−12–19	0 (3)	−24–11	0.971
EPA + DPA + DHA, g					
Baseline	0.6 (0.5)	0.1–1.9	0.6 (0.4)	0.0–2.7	0.487
Endpoint	0.6 (0.5)	0.1–1.9	0.6 (0.4)	0.0–1.5	0.449
Change[b]	0.0 (0.4)	−1.1–1.4	0.0 (0.3)	−2.2–1.2	0.723
Coumestrol, µg					
Baseline	0.0 (0.1)	0.0–0.6	0.0 (0.1)	0.0–1.2	0.848
Endpoint	0.3 (0.3)	0.0–0.9	0.0 (0.1)	0.0–0.5	<0.001
Change[b]	0.2 (0.4)	−0.4–0.9	0.0 (0.0)	−1.0–0.4	<0.001
Lignans[c], µg					
Baseline	9,046 (15,067)	393–46,174	8,683 (12,556)	400–80,914	0.931
Endpoint	29,531 (12,071)	1,129–70,197	6,282 (9,328)	434–37,522	<0.001
Change[b]	18,491(19,617)	−9,685–57,715	−141 (6,341)	−74,464–22,247	<0.001
Isoflavones[d], µg					
Baseline	9 (89)	1–838	13 (106)	0.4–1,747	0.738
Endpoint	373 (390)	4–1,285	9 (110)	1–621	<0.001
Change[b]	297 (495)	−477–1,195	−0.1 (11)	−1,424–481	<0.001
Total phytoestrogens[e], µg					
Baseline	9,052 (14,858)	394–46,184	8,828 (12,757)	402–80,942	0.913
24-h dietary recall[f]	138,104 (42,617)	14,301–410,136	21,783 (28,735)	1,823–122,286	<0.001
Endpoint	30,073 (12,749)	1,153–70,788	6,296 (9,629)	435–37,853	<0.001
Change[b]	19,259(19,143)	−9,536–58,181	−138 (6,344)	−74,463–22,256	<0.001
BMI, kg/m^2	27.9 (4.7)	20.4–37.4	25.7 (4.0)	20.0–40.0	0.005
Weight, kg					
Baseline[g]	88 (13)	60–121	83 (15)	61–134	0.002
Endpoint	87 (14)	61–113	83 (13)	62–123	<0.001
Change[b]	0 (1)	−15–3	0 (1)	−11–7	0.895

[a]Impact of DIet and individual genetic factors on tumor proliferation rate in men with PROstate Cancer.
[b]Median difference between endpoint and baseline.
[c]Included secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, syringaresinol, medioresinol, enterodiol, and enterolactone.
[d]Included genistein, daidzein, formononetin, biochanin A, and equol.
[e]Included coumestrol, lignans, and isoflavones.
[f]Data from 74 subjects in the intervention group and 74 subjects in the control group.
[g]Data were collected from the inclusion meeting.
The data were collected from the questionnaire at baseline and endpoint except when otherwise noted. Subjects who did not fill out the questionnaire at endpoint were not included in the analyses. The Mann–Whitney *U* test was used to test the difference between groups.
Abbreviation: PUFA, Polyunsaturated Fatty Acids.

Exclusion of subjects with a long intervention period (>160 days) did not change the results for either the Ki-67 index or the PSA concentrations (data not shown).

In the Mann–Whitney *U* analysis, there were no differences in PSA concentrations, or the Ki-67 index between the intervention and the control groups, both unstratified and stratified for the ERβ genotype

Table 3. RDs with 95% CIs for the risk of higher Ki-67 index in relation to intake of phytoestrogens, stratified by estrogen receptor-beta genotype (TT or TC/CC).

			Intention-to-treat analyses				
		RD	95% CI		Adjusted RD[a]	Adjusted 95% CI[a]	*P* additive interaction[b]
Ki-67 (%)[c]	All cases ($n=98$)	−0.12	−0.31, 0.078	All cases ($n=93$)	−0.13	−0.28, 0.018	0.336
	TT ($n=51$)	−0.22	−0.49, 0.053	TT ($n=51$)	−0.19[d]	−0.45, 0.068[d]	
	TC/CC ($n=47$)	−0.027	−0.30, 0.25	TC/CC ($n=47$)	0.039[d]	−0.11, 0.19[d]	
			Per-protocol analyses				
Ki-67 (%)[c]	All cases ($n=78$)	−0.21	−0.42, −0.0033	All cases ($n=77$)	−0.16	−0.34, 0.026	0.136
	TT ($n=38$)	−0.38	−0.67, −0.090	TT ($n=37$)	−0.37	−0.65, −0.084	
	TC/CC ($n=40$)	−0.063	−0.36, 0.24	TC/CC ($n=40$)	−0.031	−0.031, −0.031	
			Users of Finasteride excluded				
Ki-67 (%)[c]	All cases ($n=91$)	−0.14	−0.34, 0.063	All cases ($n=91$)	−0.17[d]	−0.31, −0.016[d]	0.129
	TT ($n=48$)	−0.29	−0.56, −0.018	TT ($n=48$)	−0.29[e]	−0.53, −0.054[e]	
	TC/CC ($n=43$)	0.017	−0.27, 0.31	TC/CC ($n=43$)	0.062[e]	0.062, 0.062[e]	

[a]Analyses were adjusted for BMI (kg/m^2) (≤18.5, 18.5 to <25, 25 to <30, 30 to <35, ≥35), age (≥median, <median), tumor stage (T1 or TX, T2), intake of polyunsaturated fatty acids (tertiles), and the most recent prostate biopsies (>3 mo, ≤3 mo).
[b]Included the same number of subjects as the unadjusted analyses.
[c]The median value of Ki-67 was calculated from five randomly selected areas of tumor tissue.
[d]The analysis did not converge including all confounders and was therefore only adjusted for BMI, age, and tumor stage.
[e]The analysis did not converge including all confounders and was therefore only adjusted for BMI, age, tumor stage, and the most recent prostate biopsy.

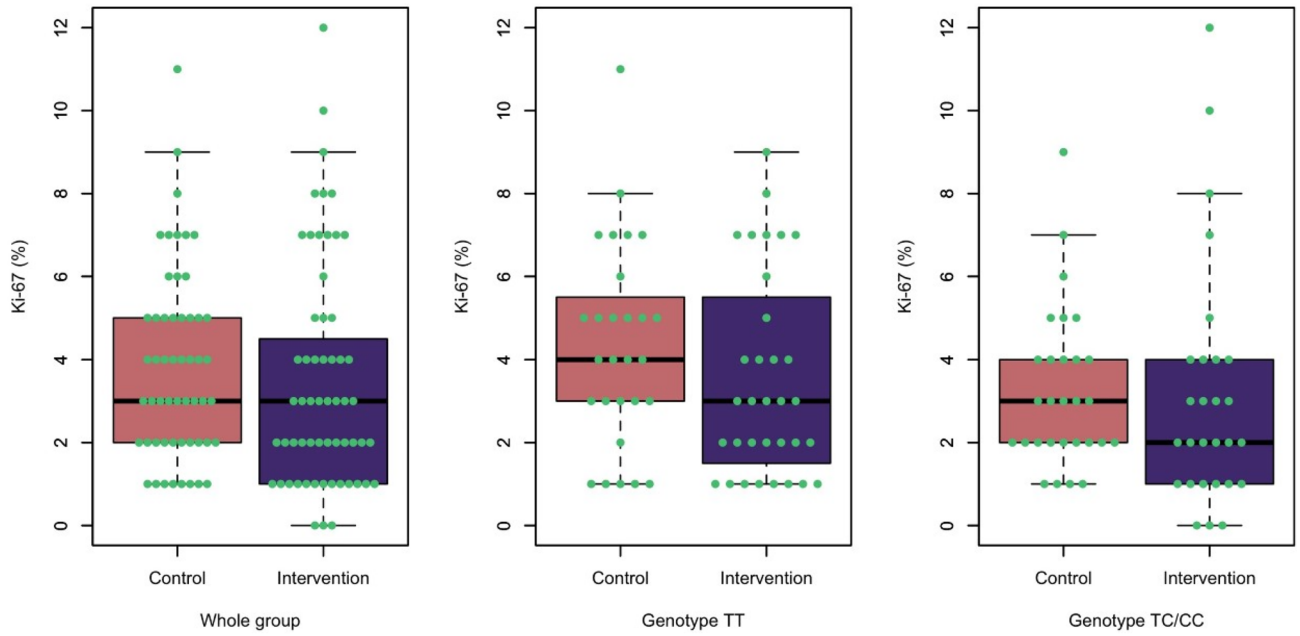

Figure 3. Tumor proliferation dotplots. Individual values of the Ki-67 index for the intervention and control groups, presented for the whole group ($n=98$, $P=0.198$) and the groups of estrogen receptor β genotype (TT $n=51$, $P=0.197$, TC/CC $n=47$, $P=0.523$). The Mann–Whitney *U* test was used to compare groups.

(Table 5, Supplementary Table 2), except for the mean and the maximum value of the Ki-67 index (Supplementary Table 3). In the control group, subjects with the TT genotype had a higher mean and maximum value of the Ki-67 index compared to the subjects with the TC/CC genotype (P_{mean} = 0.0385; P_{max} = 0.03).

Discussion

In this randomized trial in patients with prostate cancer, food items with 200 mg of phytoestrogens were provided to the men's daily diet. We found some support for the hypothesis that phytoestrogen may decrease proliferation when comparing the intervention and control groups, and the effect differed depending on the type of SNP located in the promoter region of the ERβ. We also found a genotype specific difference in the rustic PSA concentration in blood with a favorable effect of the intervention only among those with the TC/CC genotype.

The decreased risk of a higher Ki-67 index of the intervention diet was interestingly only seen in subjects with the TT genotype of ERβ. The proportion of subjects with a Ki-67 index above median value of

Table 4. RDs with 95% CIs for the risk of increasing concentrations of PSA in relation to intake of phytoestrogens, stratified by estrogen receptor-beta genotype (TT or TC/CC).

		RD	95% CI		Adjusted RD[a]	Adjusted 95% CI[a]	*P* additive interaction[b]
PSA total (µg/l)	All cases (n = 152)	0.040	−0.12, 0.20	All cases (n = 145)	−0.034	−0.18, 0.11	0.0174
	TT (n = 67)	0.25	0.022, 0.49	TT (n = 63)	0.25	0.087, 0.42	
	TC/CC (n = 85)	−0.13	−0.34, 0.085	TC/CC (n = 82)	−0.29	−0.46, −0.012	
PSA free/total (µg/l)	All cases (n = 143)	0.032	−0.12, 0.18	All cases (n = 141)	0.096	−0.025, 0.22	0.834
	TT (n = 60)	−0.0023	−0.25, 0.25	TT (n = 60)	0.071[c]	0.071, 0.071[c]	
	TC/CC (n = 83)	0.031	−0.16, 0.22	TC/CC (n = 83)	0.10[c]	−0.066, 0.27[c]	
			Per-protocol analyses				
PSA total (µg/l)	All cases (n = 125)	−0.036	−0.21, 0.14	All cases (n = 122)	−0.13	−0.30, 0.045	0.0125
	TT (n = 50)	0.23	−0.038, 0.50	TT (n = 49)	0.21	0.089, 0.33	
	TC/CC (n = 75)	−0.22	−0.44, 0.0096	TC/CC (n = 73)	−0.33	−0.50, −0.16	
PSA free/total (µg/l)	All cases (n = 121)	−0.025	−0.19, 0.14	All cases (n = 119)	0.035	−0.10, 0.17	0.477
	TT (n = 47)	0.011	−0.38, 0.16	TT (n = 47)	0.036	−0.14, 0.21	
	TC/CC (n = 74)	−0.011	−0.19, 0.21	TC/CC (n = 72)	0.041	−0.16, 0.24	

[a]Analyses were adjusted for body mass index BMI (kg/m^2) (≤18.5, 18.5 to <25, 25 to <30, 30 to <35, ≥35), age (≥median, <median), tumor stage (T1 or TX, T2), intake of polyunsaturated fatty acids (tertiles), and most recent prostate biopsies (>3 mo, ≤3 mo).
[b]Included the same number of subjects as the unadjusted analyses.
[c]The analysis did not converge including all confounders and was therefore only adjusted for BMI, age, tumor stage, and the most recent prostate biopsy.

Table 5. Serum concentrations of PSA and tumor index of Ki-67 in the subjects in the PRODICA[a] trial, stratified by estrogen receptor-beta genotype (TT or TC/CC).

	Intervention (n = 75)					Control (n = 77)					***P***[b]	
	Genotype TT (n = 37)		Genotype TC/CC (n = 38)			Genotype TT (n = 30)		Genotype TC/CC (n = 47)			TT	TC/CC
	Median (IQR)	Range	Median (IQR)	Range	*P*[c]	Median (IQR)	Range	Median (IQR)	Range	*P*[c]		
PSA total (µg/l)												
Baseline	6.8 (5.5)	1.6–21.6	6.4 (6.5)	2.3–28.9	0.352	7.4 (6.2)	1.8–24.4	6.8 (4.4)	1.9–21.1	0.119	0.365	0.292
Endpoint	6.7 (5.6)	1.3–19.9	7.1 (5.5)	2.6–27.5	0.649	8.1 (6.0)	1.4–31.6	7.0 (4.4)	2.2–19.7	0.102	0.296	0.343
Change[d]	0.1 (0.8)	−2.7–3.8	−0.3 (1.2)	−2.9 – 6.0	0.208	−0.2 (1.5)	−3.0–7.2	0.1 (1.2)	−2.5–3.1	0.638	0.686	0.175
PSA free/total (µg/l)												
Baseline	0.12 (0.050)	0.070–0.35	0.13 (0.070)	0.070–0.31	0.632	0.11 (0.070)	0.070–0.35	0.12 (0.040)	0.060–0.27	0.524	0.205	0.161
Endpoint	0.12 (0.090)	0.070–0.37	0.11 (0.070)	0.070–0.30	0.768	0.11 (0.040)	0.070–0.23	0.11 (0.050)	0.070–0.28	0.863	0.131	0.229
Change[d]	0.0 (0.030)	−0.050–0.20	−0.010 (0.020)	−0.050–0.060	0.160	0.0 (0.020)	−0.26–0.040	0.0 (0.020)	−0.13–0.050	0.413	0.872	0.526
Missing, n (%)	3 (8)		1 (3)			4 (13)		1 (2)				
	Intervention (n = 51)					Control (n = 47)					*P*[b]	
	Genotype TT (n = 28)		Genotype TC/CC (n = 23)			Genotype TT (n = 23)		Genotype TC/CC (n = 24)			TT	TC/CC
	Median (IQR)	Range	Median (IQR)	Range	*P*[c]	Median (IQR)	Range	Median (IQR)	Range	*P*[c]		
Ki-67 (%)[e]	3.0 (4.0)	1.0–9.0	2.0 (3.0)	0.0–12.0	0.611	4.0 (3.0)	1.0–11.0	3.0 (2.0)	1.0–9.0	0.104	0.197	0.523

[a]Impact of Dlet and individual genetic factors on tumor proliferation rate in men with PROstate Cancer.
[b]Difference between the intervention and control groups within the same genotype of the estrogen receptor beta.
[c]Difference between genotypes of the estrogen receptor beta within the intervention and control groups.
[d]Median difference between endpoint and baseline.
[e]The median of Ki-67 was calculated from five randomly selected areas of tumor tissue.
The Mann–Whitney U test was used to compare groups.

the study population was higher in the TT genotype group compared to the TC/CC group; also, controls in the TT genotype group had a higher mean and maximum Ki-67 index compared to the TC/CC genotype group. This might be interpreted that the TC/CC genotype is favorable compared to the TT genotype in patients with prostate cancer, but an increased intake of phytoestrogens in patients with the TC/CC genotype will bring less benefit in terms of decreased tumor proliferation. The SNP rs2987983 is located at the promoter region of the ERβ gene, associated with a small increased risk of prostate cancer (37). Also, other SNPs in the promoter region have been associated with prostate cancer risk (38). However, the biological effect is unknown. SNPs in this region may affect the binding of enhancers or repressors to regulate gene transcription. And, due to the effect on PSA concentrations in blood in the TC/CC genotype, a detection bias effect cannot be ruled out in previous epidemiological studies.

We did not find an effect of the intervention diet when we compared the values of the Ki-67 index for intervention vs. control group in the Mann–Whitney *U* analysis. This may partly be caused by the difference in several characteristics at baseline between the groups. Demark-Wahnefried et al. also found a positive effect on Ki-67 with flaxseed supplementation (20), but several other studies did not find any effect of phytoestrogen interventions on Ki-67 (24,39,40). This was potentially due to the different sizes of the study groups and thus lack of power in some of the studies. The present study shows evidence that the effect on proliferation is genotype dependent, highlighting the importance of genotyping to investigate any dietary effect on cancer aggressiveness. Even if several studies indicate that Ki-67 can be used as a predictive marker in prostate cancer (41,42), we only had values of Ki-67 at endpoint and this may not be an optimal measure of the outcome of prostate tumor proliferation. In addition, Bylund et al. (39) suggested that pretreatment prostate biopsies cause increased proliferation by the trauma caused by the biopsy needles, and a 6-wk phytoestrogen intervention may not result in a sufficiently strong reduction in the Ki-67 index.

We found opposite effects, between the two groups of ERβ genotypes, of the effect of the intervention diet on increasing total PSA concentration indicating an interaction effect of the ERβ receptor. This was in line with our hypothesis of a favorable effect of higher intake of phytoestrogens in the TC/CC genotype but contrary to the results regarding the Ki-67 index where the TT genotype had a favorable effect. Previous studies evaluating the effect of phytoestrogens on total PSA concentrations have found favorable effects (24,43) or no effects (20,44). PSA has several limitations as a prostate cancer marker, and aggressive tumors can produce less PSA per cancer compared to more indolent prostate cancers (45–47). This may be one of the reasons for the conflicting results in studies. To our knowledge, no other study has investigated the effect of phytoestrogens and the polymorphic variation in the promoter region of the ERβ gene, which may be another explanation for the varying results. Other possible reasons are underpowered studies (48), and the use of different types of phytoestrogens and thus varying bioavailability and bioactivities since they have different affinities for receptors and different pharmacodynamic properties (49).

When we excluded users of Finasteride from the analysis, the effects of decreased risk of higher Ki-67 became stronger. Finasteride is a strong inhibitor of 5-alpha-reductase and decrease dihydrotestosterone in the prostate, and our results may indicate that the intervention may be modified by other steroids in the prostate (16). Unfortunately, due to few Finasteride users, we could not investigate if the phytoestrogen intervention inhibits the effect of Finasteride on proliferation. When we stratified the RD analysis of the Ki-67 index for reported use of antibiotics, the protective effects of the intervention were the most pronounced among antibiotic users, the opposite of what we expected. Since phytoestrogens are metabolized by bacteria in the gut and the use of antibiotics affects the microbiota negatively (50,51), we expected a more pronounced effect among non-users of antibiotics. We can only speculate on the reason for this. As standard care, all patients diagnosed in Sweden will have prophylaxis with antibiotics as a single dose, and how much one dose of antibiotic will impact the microbiome in the intestine is, to our knowledge, not known. The self-reported intake of antibiotics may be incorrect due to measurement errors, or the use of antibiotics could be connected to another unknown factor.

We found that the intervention group had a larger median tumor volume at endpoint compared to the control group. However, tumor volume was not a study outcome, and the finding should, therefore, be interpreted with caution. The intervention group had a higher BMI at baseline compared to the control group, and obesity has been associated with larger tumor volumes (52).

The intervention group reported an increased intake of total phytoestrogens, isoflavones, lignans, coumestrol, dietary fiber, and polyunsaturated fatty acids at endpoint, compared to controls. All these nutrients reflect an increased intake of the intervention foods. The reported intake of total phytoestrogens at baseline was higher than in previous Swedish studies, primarily due to a significantly higher intake of lignans in our population (2,53). The higher intake of lignans in our study sample could reflect a healthier diet with a higher intake of e.g., rye and seeds (2). This is in line with previous research that several patients with prostate cancer change to a healthier diet after their disease diagnosis (26). Another plausible explanation might be that our questionnaire captured lignin intake more effectively compared to the questionnaires utilized in prior Swedish investigations.

The strengths of this study include the randomization, the sample size, the low dropout rate, and good compliance in both the intervention and the control groups. In addition to our small dropout rate, we could collect the primary outcome from subjects who had dropped out of the study. In a dietary intervention study, a concern is that the control group may

also start to eat the intervention diet. Based on the data from the 24-h dietary recall, all subjects in the control group were compliant. In the intervention group, compliance was calculated from the 24-h dietary recall, and the remaining intervention foods differed. A reason for this could have been that the subjects in the intervention group missed or ate a smaller amount of the intervention foods on the day when 24-h dietary recall was performed and then compensated for this loss on other days. Repeated 24-h dietary recalls are needed to capture habitual dietary intake (54). Therefore, we chose to use the calculation of the remaining intervention foods as a measure of compliance in the intervention group. Our previous findings on significantly higher plasma concentrations of phytoestrogens at endpoint in the intervention group compared to controls confirm that compliance was good in both groups (55). Even if some subjects in the intervention group did not reach the intended amount of phytoestrogen intake our results would be diluted, which is confirmed by the stronger effect seen in the per-protocol-analysis of the Ki-67 index.

A limitation of the present study is that we did not reach our aimed sample size, which affected the statistical power to find an effect in some of the statistical analyses. Another limitation is the change from crushed to whole flaxseeds, which we previously found to result in lower plasma concentrations of lignans (55), and probably attenuated the effects of the flaxseeds (56). In addition, the estimated amount of phytoestrogens was based on previously published biochemical analyses of foods and not specific to the food items used in this study. Thus, they may not include exactly 200 mg of phytoestrogens. Lastly, the outcomes of PSA and Ki-67 have limitations. There is a heterogeneous proliferation in the different tumors in the same patients, and the measurement of PSA in blood is highly variable from one day to another (57); this may be affected by many different aspects of the disease and changes to the microenvironment of the prostate. In addition, the clinical relevance of the PSA finding is not easily depicted due to the complexity of how PSA is produced and is leaking out into the bloodstream.

In summary, our findings suggest that a diet high in phytoestrogens may decrease tumor proliferation and total PSA concentration in men with prostate cancer with a specific genotype of ERβ. In fact, the genotype of ERβ appeared to have a greater effect than the phytoestrogen intervention, where the TC/CC genotype appeared to be the most beneficial concerning tumor proliferation. The effect of phytoestrogen on the risk for cancer progression needs further investigation before any clear recommendations can be made. Future trials could include patients during active monitoring to ensure longer duration of the intervention and stratification according to the genotype of ERβ.

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Author Contributions

RA: data curation, formal analysis, investigation, project administration, visualization, writing—original draft preparation, writing—review and editing. AJ: funding acquisition, methodology, writing—review and editing. SN: validation, writing—review and editing. RL: supervision, writing—review and editing. JS: methodology, resources, writing—review and editing. GS: conceptualization, funding acquisition, methodology, resources, supervision, writing—review and editing. MH: conceptualization, funding acquisition, methodology, project administration, supervision, writing—review and editing.

Disclosure Statement

No potential conflict of interest was reported by the author(s).

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, MH, upon reasonable request.

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