DIETARY SOY PROTEIN ISOFLAVONOID EFFECTS ON THE REPRODUCTIVE TRACT OF THE NONHUMAN PRIMATE AND NEOPLASTIC HUMAN PROSTATE GLAND

By

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This project could not have been completed without the support and guidance given to me by my research advisory committee members: Drs. Michael Adams, Mark Cline, Scott Cramer, Thomas Register, and Suzy Torti. Their doors were always open to me when I needed them and they asked the kind of questions that can only stem from experience, fundamental questions that I will continue to ask myself with each new research project I begin. When I think of what it means to be a scholar, I think of them. My enthusiasm for a career in research was cultivated by my advisors, Mark Cline and Michael Adams, who continually showed me the right direction but let me hold the reins. I am fortunate to have had such patient and thoughtful mentors. I hope to serve a mentee with such care in the future. Dr. David Sidransky was kind enough to allow me to work in his laboratory to complete the epigenetic studies for this project and with the help and expertise of Mariana Brait, Leonel Maldonado, and Mohammad Hoque of the Johns Hopkins University, this project was completed. The technical assistance of Hermina Borgerink, Jean Gardin, and Debbie Golden of the Wake Forest University Primate Center was invaluable. Each of them could not have been kinder or more supportive of me. I do not think I could have been more fortunate than to have Drs. Kathryn Shelton and Kelly Ethun as my fellow graduate students. They are both such bright, funny, and motivated people. I will miss seeing them every day. I met Dr. Jennifer Cann on my first day at Wake Forest and she has become like a sister to me. Her advice and encouragement carried me through and helped me maintain a positive frame of mind. My parents have always championed my efforts with unwavering enthusiasm, whether those have been learning to ride a bicycle or striving to meet my educational and career goals. They are my cornerstone. There are not enough words to describe how much I love them or how grateful I am to them for giving me the strength to always face forward and to be myself. Lastly, I have to thank my dogs. Behind their eyes is a world I do not know, but they do their best to share it with me.
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LIST OF ABBREVIATIONS

5-AZAC- 5'-aza-2'-deoxycytidine
ββ-ER2- 8-vinylestra-1,3,5(10)-triene-3,17_-dil
16α-LE2- 3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lacton
ACTB- Beta Actin
ANCOVA- Analysis of Covariance
AR- Androgen Receptor
B- Biochanin A
BUN- Blood Urea Nitrogen
BPH- Benign Prostatic Hyperplasia
Bx- Biopsy
C-C- Case Control
CBC- Complete Blood Count
CdP- Caudal Prostate
CrP- Cranial Prostate
D- Daidzein
DES- Diethylstilbestrol
DHM- Differential Methylation Hybridization
DNA- Deoxyribonucleic Acid
DNMT- DNA Methyltranferase
DPN- 2, 3-bis(4-Hydroxyphenol)-Priopionitrile
DRE- Digital Rectal Examination
E-Equol
ECOG- Eastern Cooperative Oncology Group
ED- Enterodiol
EL- Enterolactone
ER- Estrogen Receptor
ERα- Estrogen Receptor Alpha
ERβ- Estrogen Receptor Beta
ESR2- Estrogen Receptor Beta
ETOH- Ethyl Alcohol
F- Formononetin
G- Genistein
GC-MS- Gas Chromatography-Mass Spectrometry
GSTP1- Glutathione-s-pi
GY- Glycitein
H&E- Hematoxylin and Eosin
HDAC- Histone Deacetylase
High-SI- High-Soy Isoflavonoid
HTx- Hormone Treatment
HPLC/ESI-MS/MS- High-Pressure Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry
IHC- Immunohistochemistry
ISO- Isoflavonoids
LC-ESI-MS/MS- Electrospray Ionization Liquid Chromatography Tandem Mass Spectrometry
Low-SI- Low-Soy Isoflavonoid
LuRKO- Luteinizing Hormone Knockout Mice
MANCOVA- Multivariate Analysis of Covariance
mRNA- Messenger Ribonucleic Acid
MSP- Methylation Specific PCR
NA- Not Applicable
ND- Not Done
No-SP- No Soy Protein
nM- Nanomolar
NSD- No Significant Difference
O6MGMT- O6-Methylguanine-DNA Methyltransferase
ODMA- O-Desmethylangolensin
OR- Odds Ratios
PIN- Prostatic Intraepithelial Neoplasia
PCNA- Proliferating Cell Nuclear Antigen
PCR- Polymerase Chain reaction
PrCa- Prostate Cancer
PR- Progesterone Receptor
PSA- Prostate-Specific Antigen
QMSP- Quantitative Methylation Specific PCR
RARβ2- Retinoic Acid Receptor Beta 2
RP- Radical Prostatectomy
RR- Relative Risk
Rtx- Radiation Treatment
SCID- Severe Combined Immunodeficiency
SEM- Standard Error of the Mean
SP- Soy Protein
Sx- Surgery
TSA- Trichostatin A
TR-FIA- Time Resolved Fluoroimmunoassay
WW- Watchful Waiting
ABSTRACT

Perry, Donna L.

DIETARY SOY PROTEIN ISOFLAVONOID EFFECTS ON THE NONHUMAN PRIMATE AND NEOPLASTIC HUMAN PROSTATE GLAND

Dissertation under the direction of J. Mark Cline, D.V.M., Ph.D., D.A.C.V.P., Professor, Department of Pathology, Comparative Medicine Section

Epidemiologic studies have revealed marked disparities in prostate cancer incidence throughout the world. Prostate cancer incidence is estimated to be 10 to 60 times lower in Asian men living in their country of origin when compared to African American and Caucasian American men. The precursor lesion of prostate cancer, prostatic intraepithelial neoplasia, follows a similar epidemiologic pattern, being detected earlier and with greater frequency in American men than in Asian men. Disparate socioeconomic status, diagnostic screening, body mass index, serum and tissue hormone concentrations, prostate tissue sex steroid receptor expression levels, androgen and estrogen receptor polymorphisms, and diet have all been proposed to explain these discrepancies in prostate cancer incidence in men living in Western countries when compared to men living in Asia. Lifestyle factors became a more intense research focus when epidemiologic studies revealed that prostate cancer incidence doubles in Asian American immigrants living in the United States. However, the prostate cancer incidence in Asian immigrants still remains approximately half that of Caucasian American men, suggesting that epigenetic, genetic, and lifestyle differences likely
play a role in prostate cancer development. One of the most salient lifestyle
differences between American men and Asian men is their diet. Traditional
Asian diets are rich in soy protein and lower in saturated fat than American diets.
It is important to note that prostate cancer incidence is increasing worldwide,
even in traditionally “low risk” countries. This increase in prostate cancer
incidence is associated with the “nutrition transition” or consumption of
“Westernized” diets that are more energy-dense, higher in animal protein,
saturated fat, vegetable oil, simple sugars, and lower in fiber and
phytochemicals. These diets are replacing more traditional diets rich in legumes,
fiber, and cereals world-wide. Soy, a legume, contains polyphenolic, estrogen-
like compounds termed isoflavonoids. Following the consumption of a soy-
containing meal, isoflavonoids are absorbed in the gut, and can be detected in
the serum in nanomolar concentrations. These polyphenolic compounds are
structurally similar to the endogenous sex steroid hormone estradiol (17β
estradiol) and are able to bind to endogenous estrogen receptors (ER) with
varying affinities. There are two ER receptor subtypes, ERα and ERβ. Both ER
subtypes are expressed in the prostate gland. Estrogen receptor α is
preferentially expressed in the stroma and ERβ is preferentially expressed in the
epithelium. The effects of estrogen and estrogen-like compounds are mediated
through these two receptors, although in the prostate gland they are thought to
have opposing effects. Estrogen receptor β expression is thought to have
antiproliferative and anti-inflammatory effects, while ERα expression is thought to
promote cell proliferation, squamous metaplasia, and inflammation or prostatitis.
We conducted two parallel studies to investigate the effects of soy isoflavonoid consumption on the prostate gland. The purpose of our first study was to investigate the effects of isoflavonoids in the normal prostate gland. We used the cynomolgus macaque (Macaca fascicularis) model, a species sharing 97% genetic homology with humans for this placebo-controlled dietary study. We found no significant adverse effects of isoflavonoid consumption on the prostate gland in these 91 adult male macaques receiving: 1) a soy-free, casein-lactalbumin-based diet (n=30), 2) a low-soy isoflavonoid diet, approximating 75 mg human equivalents per day (n=30), or 3) a high-soy isoflavonoid diet, approximating 150 mg human equivalents per day (n=31) for 31 months. The purpose of our second study was to investigate the epigenetic effects of short-term isoflavonoid consumption on ERβ in men with organ-localized prostate cancer. We hypothesized that ERβ DNA promoter methylation would decrease in prostate cancer with soy isoflavonoid treatment and this would result in an increase in ERβ expression. DNA promoter methylation density of a gene is inversely correlated with the gene’s transcription and subsequent protein expression. During this 4 week, phase IIb, randomized, placebo-controlled clinical trial 62 men were randomized to receive 50 grams of protein/day composed of either 1) casein-lactalbumin protein (soy-free) or 2) soy protein containing a 100 mg/day dose of isoflavonoids. This dietary treatment resulted in an unexpected significant increase in promoter methylation of the ERα gene in the neoplastic epithelium and a 50% decrease in expression of the estrogen responsive gene, progesterone receptor, but no change in ERβ DNA promoter methylation.
methylation or expression. These findings demonstrate that dietary soy isoflavonoid treatment can result in significant epigenetic changes in the promoter regions of sex steroid receptor genes in prostate cancer, alter sex steroid hormone receptor expression, and may restore expression of the two ERs to a ratio that more closely resembles that found in normal prostate epithelium.
CHAPTER 1
INTRODUCTION

Racial Disparities in the Incidence of Prostate Cancer and Prostatic Intraepithelial Neoplasia

Prostate cancer is the most common noncutaneous cancer in men and the second leading cause of cancer death in men with an 186,320 estimated new cases diagnosed in 2008 (1). African American men have the highest prostate cancer incidence, being 1.5 times more likely to develop the disease when compared to Caucasian American men (2). Prostate cancer incidence in Asian American men is one- to three fold lower than the incidence in Caucasian American men (3,4). This gap in incidence is more pronounced if the incidence rate is compared to that of Asian men living in their country of origin, where the prostate cancer incidence is 10 to 60 times lower than the incidence reported in African American and Caucasian American men living in the United States, although prostate cancer incidence is increasing yearly in all races (5-7).

Disparate socioeconomic status, diagnostic screening, serum and tissue hormone concentrations, androgen and estrogen receptor polymorphisms leading to differences in prostate tissue sex steroid receptor activity and/or expression levels, BMI, and diet have all been proposed to explain the racial discrepancies in prostate cancer incidence seen in African and Caucasian American men when compared to men of Asian descent (2,8-27). (Figure 1)

Interestingly, the incidence of a controversial precursor lesion, prostatic intraepithelial neoplasia (PIN) (28), follows a parallel epidemiologic pattern (29).
In men without concurrent prostate cancer, African American men have twice the frequency of PIN than Caucasian men of similar age (30). Multifocal to diffuse high grade PIN is also detected a decade earlier in the prostate glands of African American men (men their 20’s) (31) than in either Caucasian (Figure 2) or Asian men though this is based on a single, widely-cited, study (32). In this study Sakr et al. showed multifocal to diffuse high grade PIN was detected in Caucasian men at 0, 2, 5, and 12% in their 3rd, 4th, 5th, and 6th decade of life while in African American men multifocal to high grade PIN was detected in the 3rd, 4th, 5th, and 6th decade of life at 2, 6, 12, and 28%, although no statistics were performed on this data.

In contrast to men without concurrent prostate cancer, African American men have twice the frequency of PIN than Caucasian men of similar age (30). Multifocal to diffuse high grade PIN is also detected a decade earlier in the prostate glands of African American men (men their 20’s) (31) than in either Caucasian (Figure 2) or Asian men though this is based on a single, widely-cited, study (32). In this study Sakr et al. showed multifocal to diffuse high grade PIN was detected in Caucasian men at 0, 2, 5, and 12% in their 3rd, 4th, 5th, and 6th decade of life while in African American men multifocal to high grade PIN was detected in the 3rd, 4th, 5th, and 6th decade of life at 2, 6, 12, and 28%, although no statistics were performed on this data.

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prostate cancer, men with prostate cancer have a similar incidence of concurrent PIN regardless of race, ranging from 76 to 86% (29,31,33-35). However, it is widely recognized that African and Caucasian American men develop more clinically apparent prostate cancer and experience higher mortality than Asian men (6,22,36). In addition, African American men typically develop clinically relevant disease 5 years younger than Caucasian men (37). African American men also have higher concentrations of the prostate-specific serine protease, prostate-specific antigen (PSA), than Caucasian men, even in young adulthood (38). The recorded incidence rates of PIN appear to parallel the racial discrepancies in prostate cancer incidence. The cause of these racial discrepancies in prostate cancer incidence remains unexplained.

The Role of Androgens and Androgen Receptor in Prostate Cancer Development

Androgens are required for normal prostate growth and maintenance (39). Testosterone is the major androgen present in the circulation. Dihydrotestosterone (DHT), the most bioactive androgen, is converted from circulating testosterone by two isoenzymes, 5α reductase 1, which predominates in the skin, hair, and liver, and 5α reductase 2, which predominates in the genital skin and prostate gland (40). Androgens act as powerful mitogens in the prostate gland, stimulating prostate epithelial and stromal cell growth, differentiation, and maintenance through androgen receptor (AR) binding and transactivation (41). Castration before the onset of puberty, depriving the body of androgen production by testicular Leydig cells, prevents the prostate gland from
developing (42,43). A widely held hypothesis was that circulating and intraprostatic androgen concentrations would, by acting as powerful mitogens, parallel the racial disparities seen in PIN and prostate cancer incidence, thereby explaining the marked disparities seen in prostate cancer incidence rates by race.

Unfortunately, the role of androgens in prostate cancer development is not that clear cut. Many early studies reported serum testosterone and dihydrotestosterone concentrations or the ratio of dihydrotestosterone to testosterone to be higher in African American men than in Caucasian men and the concentrations in Asian men to be even lower than those in either African American or Caucasian American men (39,44-46). However, the majority of more recent publications have reported no significant differences in androgen concentrations in men with prostate cancer when examined by race and no significant increased risk of developing prostate cancer with higher circulating androgen concentrations (11,23,47-53). In fact, some studies have reported lower testosterone concentrations are associated with an increased incidence of prostate cancer and/or higher prostate tumor grade (54-59). Others have found no differences or association between circulating or intra-tissue androgen or estrogen concentrations with increasing prostate cancer grade (11,12).

A randomized clinical trial conducted in men designed to evaluate the effects of reductions in intraprostatic or local dihydrotestosterone production through the use of the 5α reductase 2 inhibitor, finasteride, called the Prostate Cancer Prevention Trial (60), provided little insight into the role androgens on the
racial discrepancies in prostate cancer incidence because out of the 18,882 men accrued, 92% were Caucasian (60), 4% were African American, 3% were Hispanic, and 1% were composed of other minority groups (61). However, treatment with finasteride resulted in 25% reduction in the biopsy prevalence of prostate cancer (62). Similar reductions in biopsy-detectable prostate cancer were achieved in a randomized, placebo-controlled study using dutasteride, a dual 5α reductase inhibitor (inhibits isoforms 1 and 2). Dutasteride treatment resulted in a 22% relative risk reduction of biopsy-detectable prostate cancer (63). Although the outcome of these two studies that evaluated the use of 5α reductase inhibitors on prostate cancer incidence and progression are promising and may provide insight into the role of timing and concentration of androgen exposure in prostate cancer development, their role in prostate cancer development and progression currently remains unclear.

It has also been suggested that significant differences in circulating hormone concentrations that might lead to increased prostate cancer risk may only be detectable in young men, under the age of 35 years, before the onset of andropause (44,64), or that differences in in utero exposure to circulating hormones, in the mothers of men that develop prostate cancer (65), may be the predisposing factor leading to an increased risk for prostate cancer. From an epigenetic standpoint both are intriguing possibilities.

Androgen receptor protein expression is often maintained in prostate cancer, even in the face of androgen-independent disease (66). Increased AR expression has been reported in the neoplastic prostatic epithelium of African
American men when compared to Caucasian men (24,67) although the impact on disease progression of increased or decreased AR expression levels in prostate cancer is controversial. Prostate tumors exhibiting more AR expression have been associated with more aggressive disease (68), while others have found expression levels not to be a useful prognostic indicator (69,70), and some have reported lower AR expression in neoplastic prostatic epithelium when compared to expression levels in normal prostatic epithelium (68,70). In the supporting stroma of the prostate gland, lower AR expression in prostate cancer has been associated with more aggressive disease (71).

Common androgen receptor microsatellite trinucleotide repeats include CAG, encoding polyglutamine, and GGN, encoding polyglycine, both located within the NH2-terminal (transactivation) domain of the AR protein (72). Shorter polyglutamine tracts (shorter CAG repeats) \textit{in vitro} are more transcriptionally active and in men, shorter CAG repeats have been associated with more aggressive prostate tumors and diagnosis at a younger age (72). This description of earlier an age at diagnosis with higher prostate cancer grade is consistent with the epidemiologic data of prostate cancer incidence in African American men. Indeed African American men have been reported to have the shortest polyglutamine tracts (CAG repeats) in the transactivation domain of their ARs (73), while Caucasian men have intermediate length CAG repeats. Men of Asian descent have the longest CAG repeats, or the least transcriptionally active ARs (74). More recent studies, however, have demonstrated no association
between CAG or GGN repeats and prostate cancer risk in African American men (75,76).

Short GGN repeats are associated \textit{in vitro} with hairpin formation of the mRNA transcript resulting in decreased mRNA translation of the AR transcript and presumably decreased AR protein expression in the prostate epithelium and stroma \textit{in vivo}. A recent study in 72 men with short GGN repeats found no difference in AR staining immunohistochemically, but did find shorter GGN repeats were associated with higher Gleason tumor score (higher tumor grade) (77). The true clinical significance of these AR polymorphisms in prostate cancer risk remains unresolved. Further studies in men that correlate the length of AR microsatellite trinucleotide repeats with prostate tissue outcomes, including Gleason grade, concentrations of AR mRNA, and quantitative assessment of AR protein concentrations via Western blot and/or IHC are needed to determine their clinical or prognostic significance.

Numerous AR mutations have been reported in prostate cancer (approximately 60), but the incidence of these mutations is generally reported to be low, being detected in less than 5% of prostate tumors (78). A shared feature of these mutations is either enhanced ligand response to androgen and/or ligand promiscuity. These mutated receptors gain the ability to utilize adrenal-derived androgens, estrogens, and progesterone, among other hormone or hormone-like molecules, as ligands through mutations in the region of the ligand binding pocket. However, mutations in other regions of the protein have also been reported that may enhance AR stability, coactivator interactions, transactivation,
and nuclear translocation, even in the absence of ligand binding. Unfortunately, androgen deprivation therapy appears to place selection pressure on prostate tumor cells. These androgen-deprived tumor cells have more mutations in the ligand binding domain of their ARs than those of untreated men (78). Androgen deprivation therapy may also promote the development of ARs that lack a ligand binding domain entirely, yet retain the ability to induce AR signaling (79).

While androgen hormones and their receptors are necessary for prostate gland development, it is unlikely that androgens alone lead to the development of prostate cancer. Additional research into the role of androgen hormones and the AR in prostate cancer development and progression is necessary.

Soy Protein and Isoflavonoid Consumption and Prostate Cancer Risk

Another salient difference between American men and Asian men is their diet. A traditional Asian diet is rich in soy protein (80) and lower in saturated fat than an average American or Western diet (15). North American, European, and Australian men consume 1-2 g (3-6 mg isoflavonoids) of soy protein daily while some Asian men consume an estimated 8-11 g (24-30 mg isoflavonoids) daily (80). Interestingly, prostate cancer incidence doubles in Asian American immigrants between the ages of 45-69 with consumption of a Western diet, although the prostate cancer incidence in Asian Americans remains approximately one half that seen in Caucasian American men (6,26,81,82), indicating that both genetic and dietary differences likely play a role in prostate cancer development.
Studies designed to assess an association between prostate cancer risk and consumption of soy isoflavonoids have demonstrated mixed results (83). Some studies conclude no effect of soy consumption on prostate cancer risk (16,84) while other studies demonstrate a decreased risk of prostate cancer in men that consume higher concentrations of soy (85-90). These studies vary widely in design, sample size, patient age, types of soy-containing foods included in dietary questionnaires and/or type of soy food consumed, time course, and in primary outcome (tumor of any grade detected, rise or fall in PSA value) (Table 1). However, there is compelling epidemiologic evidence for a role of soy consumption in reducing prostate cancer risk.

Soy protein contains estrogen-like compounds termed isoflavonoids. Isoflavonoids are polyphenolic compounds that are structurally similar to the endogenous sex steroid hormone, estradiol (17β estradiol), and are able to bind to estrogen receptors (ERs) with varying affinities (91,92). Isoflavonoids may have agonistic or antagonistic effects on ERs (93) depending on species, dose, cell type, ratio of estrogen receptor subtype expressed in the tissue, and endogenous hormone milieu (94,95).

**Estrogen Receptor Subtypes Expressed in the Prostate and their Proposed Effects**

Currently, there are two known nuclear ER receptors, ERα and ERβ. The prostate gland expresses both of these ER subtypes. Estrogen receptor α
**Table 1. Studies Investigating Soy Consumption in Men and Relative Risk of Prostate Cancer**

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>n</th>
<th>Age Range (yrs)</th>
<th>Study Type</th>
<th>Nationality</th>
<th>Type of Assessment</th>
<th>Food or Isoflavonoid Assessed</th>
<th>RR (95% CI)</th>
<th>P</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oishi K</td>
<td>1988</td>
<td>100 incident cases/100 BPH cases/100 hospital controls</td>
<td>50-79</td>
<td>C-C</td>
<td>Japanese</td>
<td>Dietary Questionnaire</td>
<td>Miso</td>
<td>0.64 (0.31-1.34)</td>
<td>Not reported</td>
<td>None made regarding soy intake and prostate cancer.</td>
</tr>
<tr>
<td>Severson RK</td>
<td>1989</td>
<td>174 incident cases/7999 cohort</td>
<td>Not reported</td>
<td>Cohort</td>
<td>Japanese living in Hawaii</td>
<td>Dietary Questionnaire</td>
<td>Miso, Tofu, soy sauce</td>
<td>1.24 (0.51-3.04)</td>
<td>Miso</td>
<td>P=Not reported Tofu P=0.054 Increased consumption of tofu is associated with a decreased risk of prostate cancer. Findings may be due to chance.</td>
</tr>
<tr>
<td>Jacobsen BK</td>
<td>1998</td>
<td>225 incident cases/12,395 cohort</td>
<td>Not reported</td>
<td>Cohort</td>
<td>Japanese living in Hawaii</td>
<td>Dietary Questionnaire</td>
<td>Soy milk</td>
<td>0.60 (0.30-1.10)</td>
<td>P=0.02</td>
<td>Findings suggest men with high consumption of soy milk are at reduced risk of prostate cancer.</td>
</tr>
<tr>
<td>Strom SS</td>
<td>1999</td>
<td>83 incident cases/107 controls</td>
<td>Mean: 61 cases 60.6 controls</td>
<td>C-C</td>
<td>Caucasians United States</td>
<td>Dietary Questionnaire</td>
<td>Sixteen phyto-estrogens estimated</td>
<td>G 0.71 (0.39-1.30)</td>
<td>D 0.57 (0.31-1.05)</td>
<td>F 0.99 (0.54-1.81)</td>
</tr>
<tr>
<td>Villeneuve PJ</td>
<td>1999</td>
<td>1623 incident cases/1623 controls</td>
<td>50-74</td>
<td>C-C</td>
<td>Multietnic, Canadian Provinces</td>
<td>Dietary Questionnaire</td>
<td>Soy products Tofu or soybean</td>
<td>0.80 (0.60-1.10)</td>
<td>P=0.29</td>
<td>Dietary consumption of most [soy] food was not associated with prostate cancer (including tofu).</td>
</tr>
<tr>
<td>Kolonel LN</td>
<td>2000</td>
<td>1619 incident cases/1619 controls</td>
<td>“Up to 84 yrs”</td>
<td>C-C</td>
<td>African American, Caucasian, Japanese, Chinese American or Canadian</td>
<td>Dietary Questionnaire</td>
<td>Soy foods Legumes</td>
<td>Soy foods 0.62 (0.44-0.89) Legumes excluding soy 0.68 (0.53-0.88) All legumes 0.62 (0.49-0.80)</td>
<td>Soy foods P=0.08</td>
<td>Legumes excluding soy P=0.01</td>
</tr>
<tr>
<td>Lee MM</td>
<td>2003</td>
<td>133 incident cases/265 controls</td>
<td>50-89</td>
<td>C-C</td>
<td>Chinese</td>
<td>Dietary Questionnaire</td>
<td>Soy milk, tofu, fermented beans, G, D</td>
<td>Tofu 0.58 (0.35-0.96) Soy foods 0.51 (0.28-0.95) G 0.53 (0.29-0.97) D 0.56 (0.31-1.04)</td>
<td>Tofu P=0.032</td>
<td>Soy foods P=0.061</td>
</tr>
<tr>
<td>Ozasa K</td>
<td>2003</td>
<td>52 incident cases/151 controls</td>
<td>63-74</td>
<td>Nested</td>
<td>Japanese</td>
<td>Serum</td>
<td>G, D, E</td>
<td>G 0.76 (0.52-1.12) D 0.76 (0.31-1.76) E 0.39 (0.15-0.98)</td>
<td>G P=0.54</td>
<td>D P=0.50</td>
</tr>
<tr>
<td>Nomura AMY</td>
<td>2004</td>
<td>304 incident cases/5,805 cohort</td>
<td>Not reported</td>
<td>Cohort</td>
<td>Japanese Americans in Hawaii</td>
<td>Dietary Questionnaire</td>
<td>Tofu</td>
<td>0.82 (0.54-1.23)</td>
<td>P=0.76</td>
<td>Found no discernible association between tofu intake and prostate cancer risk.</td>
</tr>
<tr>
<td>Jian L</td>
<td>2004</td>
<td>130 incident cases/274 controls</td>
<td>Mean 72.7 cases 71.4 controls</td>
<td>C-C</td>
<td>Chinese</td>
<td>Dietary Questionnaire</td>
<td>Fermented soy products</td>
<td>2.02 (1.08-3.78)</td>
<td>P=0.003</td>
<td>Consumption of preserved foods may increase the risk of prostate cancer.</td>
</tr>
<tr>
<td>Allen NE</td>
<td>2004</td>
<td>196 incident cases/18,115 controls</td>
<td>51-89</td>
<td>Nested</td>
<td>Japanese</td>
<td>Dietary Questionnaire</td>
<td>Tofu, Miso</td>
<td>All soy 0.53 (0.24-1.14) Tofu 0.47 (0.20-1.08) Natto 0.25 (0.05-1.24)</td>
<td>All soy P=0.11</td>
<td>Tofu P=0.16</td>
</tr>
<tr>
<td>Sonoda T</td>
<td>2004</td>
<td>140 incident cases/140 controls</td>
<td>59-73</td>
<td>C-C</td>
<td>Japanese</td>
<td>Dietary Questionnaire</td>
<td>Soy food, miso, natto</td>
<td>Soy foods 0.62 (0.44-0.89) Soy foods 0.51 (0.28-0.95) G 0.53 (0.29-0.97) D 0.56 (0.31-1.04)</td>
<td>Soy foods P=0.08</td>
<td>Soy foods P=0.061</td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>n</td>
<td>Age Range (yrs)</td>
<td>Study Type</td>
<td>Nationality</td>
<td>Type of Assessment</td>
<td>Food or Isoflavonoid Assessed</td>
<td>RR (95% CI)</td>
<td>P</td>
<td>Conclusion</td>
</tr>
<tr>
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</tr>
<tr>
<td>Bosetti C</td>
<td>2006</td>
<td>1294 incident cases/1451 controls</td>
<td>46-74</td>
<td>C-C</td>
<td>Italian</td>
<td>Dietary Questionnaire</td>
<td>Flavonones</td>
<td>0.96 (0.75-1.23)</td>
<td>P=0.56</td>
<td>Flavanones</td>
</tr>
<tr>
<td>Hedelin M</td>
<td>2006</td>
<td>209 incident cases/214 controls</td>
<td>66.8 cases/67.8 controls</td>
<td>C-C</td>
<td>Swedish</td>
<td>Serum Dietary Questionnaire</td>
<td>Serum EL</td>
<td>0.74 (0.41-1.32)</td>
<td>P=0.56</td>
<td>Flavanones</td>
</tr>
<tr>
<td>Heald CL</td>
<td>2007</td>
<td>433 incident cases/483 controls</td>
<td>50-74</td>
<td>C-C</td>
<td>Scottish</td>
<td>Serum Dietary Questionnaire</td>
<td>Serum G, D</td>
<td>1.01 (0.76-1.36)</td>
<td>P=0.21</td>
<td>Phytoestrogen</td>
</tr>
<tr>
<td>Kurashashi N</td>
<td>2007</td>
<td>307 incident cases/43,509 cohort</td>
<td>40-69</td>
<td>Cohort</td>
<td>Japanese</td>
<td>Dietary Questionnaire</td>
<td>Serum G, D</td>
<td>1.01 (0.76-1.36)</td>
<td>P=0.21</td>
<td>Phytoestrogen</td>
</tr>
<tr>
<td>Park S-Y</td>
<td>2008</td>
<td>4404 incident cases/4,483 controls</td>
<td>59.3-60.7</td>
<td>Cohort</td>
<td>Multiethnic</td>
<td>Dietary Questionnaire</td>
<td>Tofu, miso, vegetarian meat, G, D, GY, legumes</td>
<td>Localized PrCa G 0.27 (0.05-2.37) D 0.49 (0.24-1.00) Soy 0.01 (0.26-1.10) Advanced PrCa G 0.35 (0.35-2.05) D 0.10 (0.43-2.87) Soy 0.01 (0.27-2.00)</td>
<td>P=0.20</td>
<td>Soy intake associated with decreased risk of localized prostate cancer &amp; increased risk of advanced prostate cancer.</td>
</tr>
<tr>
<td>Li X-M</td>
<td>2008</td>
<td>28 incident cases/28 controls</td>
<td>54-62</td>
<td>C-C</td>
<td>Chinese</td>
<td>Dietary Questionnaire</td>
<td>Tofu, soy milk</td>
<td>0.29 (0.11-0.79)</td>
<td>P=0.02</td>
<td>Study suggests that consumption of soybeans would decrease the risk of prostate cancer.</td>
</tr>
<tr>
<td>Ward H</td>
<td>2008</td>
<td>193 incident cases/228 controls</td>
<td>45-75</td>
<td>C-C</td>
<td>European, not otherwise specified</td>
<td>Serum Urine</td>
<td>Serum G, D EL, ED, ODMA, GY Urine G, D, EL, ED, ODMA GY</td>
<td>Serum 1.01 (0.93-1.10) Urine 0.98 (0.90-1.08)</td>
<td>P=0.81</td>
<td>Urine P=0.72</td>
</tr>
</tbody>
</table>

The results do not support a protective effect of flavonoids on prostate cancer in this Italian population characterized by a high intake of flavonoid-containing foods.

Intake of foods rich in phytoestrogens was strongly associated with decreased risk of prostate cancer. Estimated dietary intake of total or individual lignan or isoflavonoid compounds was not associated with prostate cancer risk.

Findings support the hypothesis that serum enterolactone and soy food consumption protect against prostate cancer. An association between prostate cancer risk and isoflavone intake or serum concentrations was not found. Future retrospective studies should include subjects with greater variation of phytoestrogen and/or soy food intake and assess lignan precursors.

Soy intake associated with decreased risk of localized prostate cancer & increased risk of advanced prostate cancer.

Findings suggest that legume intake is associated with a moderate reduction in prostate cancer risk and that the isoflavones in soy products are probably not significant contributors to this effect.

Study suggests that consumption of soybeans would decrease the risk of prostate cancer.
### Table 1. Studies Investigating Soy Consumption in Men and Relative Risk of Prostate Cancer

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>n</th>
<th>Age Range (yrs)</th>
<th>Study Type</th>
<th>Nationality</th>
<th>Type of Assessment</th>
<th>Food or Isoflavonoid Assessed</th>
<th>RR (95% CI)</th>
<th>P</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kurahashi N</td>
<td>2008</td>
<td>201 incident cases/402 controls</td>
<td>40-69</td>
<td>Nested C-C</td>
<td>Japanese</td>
<td>Serum</td>
<td>Serum G, D, E, GY</td>
<td>Localized PrCa G 0.54 (0.29-1.01) D 0.63 (0.35-1.13) E 0.43 (0.23-0.82) Gy 0.78 (0.45-1.36) Advanced PrCa G 1.77 (0.42-7.41) D 1.64 (0.34-7.87) E 2.39 (0.55-10.32) GY 1.89 (0.32-11.33)</td>
<td>Localized PrCa G P=0.03 D P=0.12 E P=0.02 Gy P=0.68 Advanced PrCa G P=0.47 D P=0.48 E P=0.50 GY P=0.50</td>
<td>Isoflavones may prevent the development of prostate cancer.</td>
</tr>
<tr>
<td>Travis RC</td>
<td>2009</td>
<td>950 incident cases/1042 controls</td>
<td>43-76</td>
<td>Nested C-C</td>
<td>European, not otherwise specified</td>
<td>Serum</td>
<td>Serum G, D, E, EL, ED</td>
<td>G 0.74 (0.54-1.00) D 0.80 (0.60-1.07) E 0.99 (0.70-1.39) EL 0.77 (0.57-1.04) ED 0.88 (0.67-1.16)</td>
<td>G P=0.05 D P=0.21 E P=0.46 EL P=0.92 ED P=0.29</td>
<td>Higher concentrations of genistein may reduce prostate cancer risk.</td>
</tr>
<tr>
<td>Park S-Y</td>
<td>2009</td>
<td>288 incident cases/540 controls</td>
<td>45-75</td>
<td>Nested C-C</td>
<td>Multiethnic</td>
<td>Urine</td>
<td>Urine G, D, E, EL</td>
<td>D 0.55 (0.31-0.98) Latinos only.</td>
<td>P=0.01</td>
<td>High urinary excretion of daidzein seemed protective against prostate cancer.</td>
</tr>
<tr>
<td>Yan L*</td>
<td>2009</td>
<td>Meta-analysis</td>
<td>N/A</td>
<td>Meta-analysis</td>
<td>Data analyzed by Asian populations &amp; Western populations</td>
<td>Meta-analysis Studies: Epidemiologic (15) Isoflavones &amp; PrCa risk (9) Extracted adjusted RR &amp; OR of the highest &amp; lowest reported categories of soy intake.</td>
<td>Soy intake Nonfermented soy foods Fermented soy foods Isoflavones Asian populations Western Populations</td>
<td>Soy intake Nonfermented soy foods 0.70 (0.56-0.88) Fermented soy foods 1.02 (0.73-1.42) Isoflavones 0.88 (0.76-1.02) Asian Populations 0.52 (0.34-0.81) Western Populations 0.99 (0.85-1.16)</td>
<td>Soy intake P=0.01 Nonfermented soy foods P=0.1 Fermented soy foods P=0.92 Isoflavones P=0.09 Asian Populations P=0.01 Western Populations P=0.91</td>
<td>Results suggest that consumption of soy foods is associated with reduction in prostate cancer risk in men. Protection may be associated with the type and quantity of the soy food consumed.</td>
</tr>
</tbody>
</table>

All RR are for the most frequent or highest concentration of food assessed and/or serum concentrations analyzed. *Meta-analysis

Abbreviations: Biochanin A (B), Case Control (C-C), Daidzein (D), Enterodiol (ED), Enterolactone (EL), Equol (E), Formononetin (F), Genistein (G), Glycitein (GY), O-desmethylangolensin (ODMA), Odds Ratios (OR), Prostate Cancer (PrCa), Relative Risk (RR)
expression predominates in the stroma and ERβ expression predominates in the epithelium (96,97). The effects of estrogen or estrogenic compounds in the prostate are mediated through these two ERs but they are thought to have opposing effects. Estrogen receptor α expression and activation is thought to promote proliferation, squamous metaplasia, and inflammation or prostatitis and ERβ expression and activation is thought to have antiproliferative and anti-inflammatory effects in the prostate gland (96,98). The antiproliferative effect of ERβ has been demonstrated in cell culture studies (99,100) and in a variety of murine animal models since 1998 (101,102).

Epithelial hyperplasia of the prostate gland has been reported in ERβ knockout mice (retaining ERα expression) (100). Prins et al. provided additional insight into the role of the two ER subtypes in a study of neonatal estrogen imprinting in 2001. In this study, wild-type mice treated from days 1 through 5 of life with diethylstilbestrol (DES) (2 µg/day) exhibited expansion of the stromal compartment of the prostate gland, a continuous layer of basal cells (mice normally have a discontinuous basal cell layer lining prostatic acini), periductal fibrosis, lymphocytic prostatitis, and epithelial hyperplasia and dysplasia that increased in severity with age. These changes are often referred to as “developmental estrogenization.” Estrogen receptor α knockout mice (retaining ERβ expression) treated with the same dose of DES for 5 days exhibited none of the pathologic changes exhibited by the wild type mice. Estrogen receptor β knockout mice exhibited the same pathologic changes seen in the wild type mice: expansion of the stromal compartment, lymphocytic prostatitis, and epithelial
hyperplasia and dysplasia, in addition to a continuous basal cell layer lining prostatic acini, and periductular fibrosis. These ERβ knockout mice also exhibited decreased AR expression in the dorsolateral lobe of the prostate gland as adults when compared to ERα knockout mice. These findings indicate that ERα mediates neonatal imprinting or “developmental estrogenization” of the prostate gland (103). Unfortunately it has recently been discovered that the ERα knockout mice utilized for these studies retain a low concentration of a chimeric ERα protein that is 61kDa in length that may be capable of carrying out estrogen-dependent transactivation activities (104).

However, McPherson et al. demonstrated a similar antiproliferative role for ERβ in the prostate gland using aromatase knockout (ArKO) prostate tissue recombinants. Local estrogen production in the prostate gland is absent in the absence of stromal and epithelial aromatase. These constructs were used to determine the role of prostate-produced estrogens, or local estrogenic activity, in prostate gland development. Four constructs composed of ArKO or wild-type (wt) mouse epithelium and seminal vesicle mesenchyme (stroma) were used for this study. Two homotypic constructs: 1) wild type epithelium (wt-E) and stroma (wt-S), 2) aromatase knockout epithelium (ArKO-E) and stroma (ArKO-S), and two heterotypic constructs: 3) ArKO-S/wt-E, and 4) wt-S/ArKO-E. These tissue recombinants were grafted under the renal capsule of intact male severe combined immunodeficiency (SCID) mice for 6 weeks. After 6 weeks the homotypic ArKO constructs (aromatase-deficient epithelium and stroma) exhibited a 3 fold increase in epithelial hyperplasia that was not seen in the wt
constructs. Comparable epithelial hyperplasia was seen in the heterotypic ArKO-S/wt-E recombinants and wt-S/ArKO-E recombinants. This epithelial hyperplasia was entirely abrogated by treatment with the ERβ agonist, 8β-ER2 (8-vinylestra-1,3,5(10)-triene-3,17β-diol), making the prostate gland constructs indistinguishable from the wild-type prostate tissue recombinants. Expression of the proliferation marker, proliferating cell nuclear antigen (PCNA), was also significantly (P<0.05) reduced in the epithelium of the ArKO-S/ArKO-E (27.5±2.5 to 16.5±0.8%), ArKO-S/wt-E (28.9±2.9 to 18.9±0.8%), and wt-S/ArKO-E (31.2±3.8 to 20.5±0.4%) prostate tissue recombinants with 8β-ER2 treatment but there was no change in PCNA expression in the stroma of any prostate tissue recombinant.

In this same study, aromatase knockout mice exposed to the same ERβ agonist (8β-ER2) for 6 weeks caused attenuation of the epithelial hyperplasia that normally occurs in these knockout mice, making them indistinguishable from the prostates of wild type mice, but this effect was not quantified. No effect on the epithelial hyperplasia typically seen in the prostate gland epithelium of ArKO mice was observed when these mice were treated with the ERα agonist, 16α-LE2 (3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lacton), but ERα agonist treatment did result in an increased inflammatory response within the tissue recombinants, however, it was not quantified (105). These findings suggest that ERβ activation within the prostate gland has antiproliferative effects on the prostate gland epithelium, supporting the evidence reported previously by Prins et al. in ERβ knockout mice.
Savolainen et al. utilized luteinizing hormone receptor knockout mice (LuRKO) to elucidate the role of the two different ER subtypes in the prostate gland. Luteinizing hormone receptor knockout mice lack postnatal androgen production in the testes because they lack pituitary gland luteinizing hormone release and, as a result, regulation of the hypothalamic-pituitary-gonadal axis. These mice were given exogenous testosterone or dihydrotestosterone replacement to facilitate normal prostate gland development for 8 or 16 weeks and then a variety of hormone treatments. When these LuRKO mice were treated simultaneously with testosterone and the ERβ agonist, DPN (2, 3-bis(4-hydroxyphenol)-priopionitrile), a reduction in prostate epithelial area from 68.8% to 42.3% in the ventral prostate lobe and from 71.6% to 57.8% in the dorsal lateral prostate lobe occurred when compared to the LuRKO mice that received testosterone alone. In addition, the prostatitis score decreased from 3+ (of a possible 5+) in 4 of 8 mice treated with testosterone alone to a prostatitis score of 1+ in 1 mouse of 8 treated with testosterone and the ERβ agonist, DPN.

Treatment with the testosterone and the ERα antagonist, ICI 182,780, had no significant effect on epithelial hyperplasia in the ventral (68.8% to 62.9%) and dorsal prostate (71.6% to 70.2%) but exacerbated prostatitis from a score of 1+ to 3+ (of a possible 5+) in both prostate lobes (106). The findings using LuRKO mice again indicate that activation of the ERβ subtype has antiproliferative and anti-inflammatory effects in the prostate gland epithelium and activation of the ERα subtype has pro-inflammatory effects in the prostate gland.
The majority of publications that have investigated ERβ expression in organ-localized prostate cancer have reported decreases in expression (97,107-111), although some have reported increases (112,113). Expression of the two ER subtypes is partially controlled through DNA promoter methylation in normal and neoplastic prostate tissue (114-116).

Walton et al. evaluated expression of ERα, ERβ, and AR after co-treatment of the AR positive cell line, LNCaP, and two AR negative cell lines, DU-145 and PC3, with the DNA methyltransferase (DNMT) inhibitor, 5'-aza-2'-deoxycytidine (5-AZAC), at 8.8 µM for 72 h and the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), at 300 nM for 24 hours. Significant increases in ERβ mRNA and protein expression were seen in the LNCaP cell line alone (P<0.05) with co-treatment. Co-treatment with 5-AZAC and TSA resulted in re-expression of AR the DU-145 cell line (P<0.01). Estrogen receptor α expression increased in both LNCaP and DU-145 cell lines (P<0.05). Progesterone receptor expression paralleled that of ERα in all prostate cell lines (P<0.001) (115).

Interestingly, soy isoflavonoids have been shown in vivo and in vitro to alter gene expression through changes in DNA methylation. In vitro treatment of the LNCaP and PC3 cell lines with 20 µM of the isoflavonoid metabolite genistein for 6 days resulted in a reversal of promoter methylation of the tumor suppressor gene, retinoic acid receptor β (RARβ), and re-expression of RARβ mRNA (117). In contrast to these in vitro studies, an in vivo study in mice that measured CpG island methylation density using differential methylation hybridization arrays (DMH) demonstrated the opposite result. Increases in CpG island methylation
within the prostate gland were detected in four 8 week-old C57BL/6J mice fed
300 mg genistein/kg diet for 4 weeks when compared to 4 mice receiving a
casein-based diet alone. In the same study, 4 mice fed the same dose of
genistein (300 mg genistein/kg diet) for 2 weeks and casein for 2 weeks or visa
versa, demonstrated an intermediate DMH array intensity between the mice fed
casein alone and those fed genistein for 4 weeks. Due to the small sample size,
statistics were not performed on the intensity data gathered from these DMH
arrays (118).

In light of the epidemiologic evidence, variations in prostate cancer
incidence within different populations and ethnicities is likely due to a combined
genetic-nutrient interaction (15,19,119,120) and it is probable that ER subtype
expression in the prostate gland has an effect on prostate cancer incidence and
progression. The overall goal of this research was to investigate the effects of
dietary soy isoflavonoids on the prostate gland in two parallel studies.

Our first study was a placebo-controlled, pre-clinical trial designed to
investigate the effects long-term isoflavonoid consumption in normal prostate
gland in 91 adult male cynomolgus macaques (Macaca fascularis) (chapters 2
and 3).

We hypothesized that:

Thirty-one months of continuous dietary treatment with soy protein-derived
isoflavonoids at 75 and 150 mg human equivalents/day would have no
adverse effect on the reproductive tract (chapter 2) or expression of sex
hormone receptors (AR, ERα, ERβ, PR), the proliferation marker Ki67, or
the cyclin dependent kinase inhibitor p27KIP-1 in the prostate gland epithelium or stroma measured by IHC (chapter 3).

Specific Aims:

1. Measure serum isoflavonoid concentrations and serum sex hormones at baseline and following 31 months of isoflavonoid treatment to confirm successful delivery of soy isoflavonoids.

2. Measure body and organ weights, perform epididymal and testicular sperm counts, and assess prostate gland epithelial and stromal expression of serum sex hormones (AR, ERα, ERβ, PR), the proliferation marker Ki67, and the cyclin dependent kinase inhibitor p27KIP-1.

Our second study was a phase IIb clinical trial designed to evaluate the effect of short-term (4 week) isoflavonoid consumption at a dose of 100 mg/day on DNA promoter methylation of the ERβ gene in prostate tissue collected from 62 men with organ-localized prostate cancer (chapter 4).

We hypothesized that:

ERβ epigenetic silencing through DNA promoter methylation in neoplastic prostatic epithelial cells can be reversed by dietary soy isoflavonoids when given at physiologically achievable levels, as shown by a positive correlation between serum and/or intraprostatic isoflavonoid concentrations and ERβ expression and an inverse correlation with neoplastic cell proliferation.
Specific Aims:

1. To determine intra-prostatic tissue soy isoflavonoid concentrations and compare these concentrations to concentrations measured in serum.
   Hypothesis: Soy isoflavonoids will concentrate within prostate tissue at concentrations 4 to 13 fold above those found in serum.

2. To determine ERβ DNA promoter CpG island methylation within prostate tissue collected from men treated with soy isoflavonoids and casein lactalbumin protein.
   Hypothesis: ERβ DNA promoter CpG island methylation will be decreased within the prostate cancer of men treated with soy isoflavonoids when compared to those receiving a similar concentration of casein lactalbumin protein.

3. Correlate intraprostatic soy isoflavonoid concentrations with ERβ DNA promoter methylation data and ERβ protein expression data using immunohistochemical markers for sex steroid hormones (ERβ, ERα, AR, PR) proliferation (Ki67), and cell cycle regulatory molecules (p27KIP-1).
   Hypothesis: Soy isoflavonoids will concentration in prostate tissue resulting in a reduction in ERβ DNA promoter methylation decreasing prostate cancer epithelial cell proliferation.
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DIETARY SOY PROTEIN CONTAINING ISOFLAVONOIDS DOES NOT
ADVERSELY AFFECT THE REPRODUCTIVE TRACT OF MALE
CYNOMOLGUS MACAQUES (MACACA FASCICULARIS)

Donna L. Perry, Jennifer M. Spedick, Thomas P. McCoy, Michael R. Adams,
Adrian A. Franke, J. Mark Cline

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Stylistic variations are due to the requirements of the journal. D. Perry performed
the experiments, statistical analyses, and prepared the manuscript. J. Spedick
performed the sperm counts. T. McCoy assisted with the statistical analysis. Dr.
Adrian Franke performed the liquid chromatographic-photodiode array
electrospray MS quantitation of serum isoflavonoids. Drs. Michael Adams and J.
Mark Cline acted in an advisory and editorial capacity.
Dietary soy protein containing isoflavonoids does not adversely affect the reproductive tract of male cynomolgus macaques (*Macaca fascicularis*)\(^1\)

Donna L. Perry\(^2\), Jennifer M. Spedick, Thomas P. McCoy, Michael R. Adams, Adrian A. Franke, J. Mark Cline

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Abstract

Short term dietary studies of soy protein-derived isoflavonoids, using rodent and nonhuman primate models, have documented variable effects on the reproductive tract. Long-term effects of dietary soy and/or isoflavonoids on the reproductive tract of nonhuman primates have not been determined. The objective of this study was to assess the effects of long-term consumption of dietary soy isoflavonoids on histomorphology of the mammary glands and prostate gland, testis, and sperm counts in adult male cynomolgus macaques. Ninety-one adult male cynomolgus macaques (Macaca fascicularis) were fed diets for 3 y differing only in protein source: 1) a soy-free, casein-lactalbumin-based diet or 2) a low-soy isoflavonoid diet (~6 mg·kg\(^{-1}\)·d\(^{-1}\)) or 3) a high-soy isoflavonoid diet (~12 mg·kg\(^{-1}\)·d\(^{-1}\)). Serum isoflavonoids were measured by liquid chromatographic-photodiode array electrospray MS. Mammary gland, prostate gland, and testes were obtained at post-mortem and evaluated histopathologically and histomorphometrically. Epididymal and testicular sperm counts were performed. Serum isoflavonoid concentrations at 4 h post-feeding differed among all groups (P<0.001) and were (mean ± SEM) 67±23 (soy-free diet), 799±44 (low-soy isoflavonoid diet), and 1458±80 nmol·L\(^{-1}\) (high-soy isoflavonoid diet). Diet did not alter serum estradiol and testosterone concentrations or epididymal and testicular sperm counts. Organ weights and histologic indices did not differ among treatment groups. Mammary gland histopathologic and histomorphometric analysis revealed no abnormalities and no indication of gynecomastia. We found no evidence of an adverse effect of soy
isoflavonoids at physiologically relevant doses within the reproductive organs of adult male macaques.

**Key Words:** soy, isoflavonoid, macaque, mammary gland, prostate gland, testis

**Introduction**

Men that consume a diet rich in soy-derived protein, typically in Asian countries, have been reported to have a much lower prostate cancer incidence than men consuming a traditional Western diet (1-4). These isoflavonoid concentrations have been estimated to range from 25 to 50 mg per day (6 to 11 g soy protein) (5). This epidemiological observation has prompted many to postulate that weakly estrogenic isoflavonoids present in soy proteins may have protective, i.e, antiproliferative and pro-apoptotic effects in the prostate gland (6). Dietary studies evaluating the effects of soy-derived isoflavonoids in rodent models have reported results varying from no discernable negative effects to marked atrophy of the accessory sex glands (7-10). This variability in *in vivo* responses to dietary isoflavonoids may be due in part to differences in sensitivity of the various inbred laboratory rodent strains to these estrogen-like compounds (11, 12). *In vitro* and *in vivo* studies evaluating effects of soy-derived isoflavonoids on sperm quality have also produced conflicting results (13, 14). Several short-term pharmacokinetic studies evaluating the metabolism of soy isoflavonoids and several studies evaluating their effect on prostate-specific antigen have been performed in men. These studies have demonstrated that the isoflavonoid metabolite daidzein concentrates in prostatic fluid, although the majority of
studies demonstrate no effect on elevated or rising prostate-specific antigen (PSA) levels (15-18). To our knowledge, long-term studies evaluating the effects of continuous dietary soy isoflavonoid consumption on reproductive tissues have not yet been attempted (18-21).

The purpose of this study was to evaluate the effects of long-term dietary isoflavonoid supplementation on the morphology of the reproductive tissues in the adult male cynomolgus macaque (*Macaca fascicularis*), an animal model sharing 97% genetic identity with humans (22).

**Materials and Methods**

Ninety-one adult male cynomolgus monkeys (*Macaca fascicularis*) were imported from Indonesia (Institut Pertanian Bogor) and lived in social groups composed of 4 individuals. Effects on the cardiovascular system and behavior have been reported previously (23, 24). Age was estimated from dentition to range from 15 to 23 y at the study’s end. Briefly, all monkeys were fed a moderately atherogenic diet, designed to mimic the diet consumed by most men in Western countries, and randomized into three groups differing only by the source of dietary protein. The control group (n=30 monkeys, no-SP) was fed a soy-free and isoflavonoid-free, casein-lactalbumin based diet. The low isoflavonoid group (n=30, low-SI) was fed a mixture of alcohol-washed (isoflavonoid-depleted) and unwashed soy protein isolate containing 0.94 mg of isoflavonoids per gram of product or ~6 mg·kg⁻¹·d⁻¹, approximating a human soy isoflavonoid dose of 75 mg·d⁻¹. The high soy isoflavonoid treatment group (n=31, high-SI) was fed a soy
protein isolate containing 1.88 mg soy isoflavonoid per gram of product or ~12 mg·kg⁻¹·d⁻¹, approximating a human soy isoflavonoid dose of 150 mg·d⁻¹. These diets were continuously fed over a 31-mo treatment period. The source of the diets and a detailed description of the components in each have been described previously (23). All procedures involving monkeys were approved by the Institutional Animal Care and Use Committee of Wake Forest University School of Medicine and adhered to the National Research Council’s Guide for the Care and Use of Laboratory Animals.

**Serum Isoflavonoids**

Serum was collected under ketamine sedation (10 mg·kg⁻¹ intramuscular) by femoral vein venipuncture at 4 h after feeding. Serum isoflavonoids including genistein, dihydrogenistein, daidzein, dihydrodaidzein, glycine, O-desmethylangolensin, and equol were measured using liquid chromatographic-photodiode array electrospray MS with isotopically labeled internal standards (7, 23, 25).

**Serum Hormones**

Blood samples were collected in late morning to control for diurnal variation. Total testosterone, free testosterone, and androstenedione concentrations were determined on unextracted samples using solid phase radioimmunoassay with a commercially available kit (Coat-A-Count, Diagnostic Products). Estrogen was determined after ethyl ether extraction using a radioimmunoassay kit (DSL 4800, ultrasensitive estradiol, Diagnostics Systems Laboratory). CV for individual assays were: total testosterone 9.6%, free testosterone 6.8%, estradiol 7.9%.
Necropsy and Histomorphometry

The monkeys were humanely killed by pentobarbital overdose (30 mg·kg\(^{-1}\) intravenous) and a complete necropsy was performed immediately with collection, weighing, and 24 h fixation of all major organs in 4% paraformaldehyde. After paraffin embedding, 5 µm hematoxylin and eosin-stained sections of the testes, seminal vesicles, mammary glands, prostate, and adrenal glands were examined by a pathologist (DLP).

*Histomorphometry.* Histomorphometry was done using a video image analysis system (Sony 3-chip color CCD camera, Pentium III computer, a Scion CG-7 video capture board, and Scion image public domain software). Measurements included prostatic glandular, luminal, and stromal areas (expressed as percentages), and prostatic glandular epithelial height. Areas were measured at 200X magnification in 3 randomly chosen areas, by tracing the basement membrane and luminal epithelial border of each prostatic acinus followed by calculation of the percentage areas.

*Mammary gland.* Glandular epithelial tissue was measured at 200X magnification within 5 randomly chosen fields by tracing the basement membrane surrounding each mammary epithelial structure. Mammary gland and nipple width were measured at 100X magnification from histologic sections.

*Sperm Counts.* The number of intact sperm heads in 0.25 g of homogenized testicular and epididymal tissues were counted at using a Neubauer hemocytometer as previously described (26). Counts were repeated if
there was a >20% variation in sperm head counts among hemocytometer chambers.

Statistics

The goal of the analysis was to test the effects of soy protein/isoflavonoid treatment on organ weight, gland morphometry, sperm counts, and hormone levels. Descriptive statistics were calculated to assess normality and equality of variances. Nonparametric Kruskal-Wallis tests were performed to test for differences in isoflavonoid levels because those data had unequal variances that were not corrected by transformation. ANCOVA was used to determine effects of soy treatment on sex hormones and sperm counts. Multivariate Analysis of Covariance (MANCOVA, Wilks’ Lambda test) was used to determine effects of soy treatment on prostate gland morphometry, mammary gland morphometry, and organ weight.

Analyses were performed with and without weighting for covariates such as age, serum isoflavonoid concentrations, serum hormones at baseline and during treatment. Bonferroni corrections and weighting for covariates did not change outcomes. Analyses were performed using SAS version 9.1.3 (SAS Institute) and Statistica version 6.1 (Statsoft, 2004). Differences were considered significant at P < 0.05.
Results

Isoflavonoids

Serum isoflavonoid concentrations consistently reflected differences among groups in isoflavonoid consumption over the 31-mo treatment period (Kruskal-Wallis P < 0.001), indicating successful dietary delivery of the 2 different concentrations of soy isoflavonoids (Table 1).

Organ weights and sperm counts

Neither testicular and prostate gland weights nor testicular and epididymal sperm counts differed among the 3 groups (Table 1). The weights of other major organs, also were not affected by the diets (data not shown).

Histology and histomorphometry

Prostate gland. We did not detect differences in architecture (atrophy, hypertrophy, dysplasia) at the microscopic level within stromal and epithelial (glandular) compartments of the prostate gland among treatment groups. However, there were 5 monkeys with basal cell adenomas within the cranial lobe of the prostate gland. These basal cell adenomas were present in 1 monkey in the no-SP group, 2 monkeys in the low-SI group, and 2 monkeys in the high-SI group. Basal cell adenomas are considered incidental or background lesions in cynomolgus macaques and have been described previously (27, 28).

Histomorphometrically the percent of stromal area versus glandular (epithelial) area did not differ in the cranial and caudal prostate glands among the 3 groups (Table 2).
**Mammary gland.** We observed no evidence of gynecomastia or abnormal glandular morphology or proliferation in the monkeys with the exception of one in the high-SI treatment group, which was diagnosed with an intraductal mammary gland adenocarcinoma and was published previously (29). The incidence of mammary gland carcinoma in male cynomolgus macaques is unknown at this time, although is thought to be rare. As such, it is impossible to determine if this neoplasm is related to soy isoflavonoid treatment or within an expected incidence rate for this species of macaque. With respect to quantitative measures, the high-SI treatment group had the smallest mammary gland nipple width and this width was statistically significantly less than that of the control group (P < 0.025) (Table 2).

**Serum hormones**

Serum estradiol, testosterone, or androstenedione did not differ among the 3 groups at baseline or following the 31-mo dietary soy protein treatment period (Table 3).

**Discussion**

This 31-mo soy protein-derived isoflavonoid intervention study confirmed successful delivery isoflavonoids through dietary consumption by measurement of serum isoflavonoid concentrations. Serum hormones, organ weights, prostate and mammary gland histomorphometry, testicular and epididymal sperm counts were evaluated within a no-soy, a low-soy (~6 mg·kg⁻¹·d⁻¹ or 75 mg·person⁻¹·d⁻¹), and a high-soy isoflavonoid (12 mg·kg⁻¹·d⁻¹ or 150 mg·person⁻¹·d⁻¹) treatment
group. We found no adverse effects of dietary isoflavonoids in the organs and variables evaluated.

These findings are in contrast to our previous dietary isoflavonoid metabolite treatment study demonstrating marked accessory sex gland atrophy by Cline et al. (7) in apolipoprotein E null C57BL/6J mice. In this study mice were fed soy isoflavonoid metabolites genistein (G) and daidzein (D) in ratios of 2G:1D, 10G:1D, and 1G:10D at a dose of 120 mg·7576 kJ\(^{-1}\)·d\(^{-1}\) or 40 mg·kg\(^{-1}\)·d\(^{-1}\) from 6 to 22 wk of age. All mice receiving the isoflavonoid-enriched soy diet exhibited accessory sex gland atrophy. Spearow et al. (11) speculated that differences among murine strains in responses to isoflavonoids may be due to differences in liver and testicular sulfotransferase activity. The C57BL/6 mouse strain, used in the Cline et al. (7) study, was more sensitive to estrogenic compounds than the ICR strain of mice. In a study utilizing the ICR strain of mice fed genistein at lower concentrations of 2.5 mg·kg wt\(^{-1}\)·d\(^{-1}\) for 5 wk postweaning, no lesions within the testis, epididymis, or prostate gland occurred (30). Yet, in a different study utilizing ICR mice fed genistein at 2.5 and 5 mg·kg wt\(^{-1}\)·d\(^{-1}\) for 5 wk postweaning, there were no changes in accessory sex gland weight, but Leydig cell hyperplasia occurred histopathologically (9).

Interestingly, hyperplasia of Leydig cells, estimated as an increase in Leydig cell numbers of 74%, was seen in newborn marmosets fed a commercially available soy protein-based infant formula containing concentrations of soy isoflavonoids estimated to range from 18 to 41 mg aglycone·L\(^{-1}\). The testes of these marmosets were compared to those of their
fraternal twin fed a control diet of commercially available cow’s milk-based infant formula for 40 d (31). Upon evaluation of the co-twin marmosets at adulthood (120 to 138 wk of age), both groups proved fertile, being able to impregnate a female marmoset, although the testes of the soy-infant formula fed marmosets were 14% heavier than males fed a cow’s milk-based infant formula. The testes of the soy infant-formula-fed monkeys had larger numbers of both germ cells (Sertoli cells) and Leydig cells (interstitial cells) with no evidence of long-term adverse effect of soy isoflavonoids on reproductive function as a result of the soy isoflavonoids fed during development (32).

In a study by Svechnikov et al. (33), male Sprague-Dawley rats were fed genistein at a dose 1 g·kg⁻¹ of diet from 3 mo to 6 mo of age (21.1 mg genistein·d⁻¹ or 46.2 mg·kg⁻¹·d⁻¹ body weight). The Leydig cells of these rats were isolated, and concentrations of testosterone were determined in culture following stimulation with human chorionic gonadotropin or butyryl cAMP. Although serum concentrations of testosterone did not differ in the genistein treated group when compared to untreated controls, the Leydig cells evaluated in culture did not respond to stimulation with human chorionic gonadotropin or butyryl cAMP, which the authors attributed to reduction in mitochondrial p450 scc enzyme expression. These findings provide in vitro evidence of a negative effect of genistein administration on Leydig cell testosterone production; although assessment of testicular morphology was not performed to determine whether increases in Leydig cell number were present, as previously observed in mice and marmosets. Further studies to investigate the effects of soy isoflavonoids on
Leydig cell density and function are warranted, although we are unaware of data demonstrating reduced fertility as a result of ingestion of soy protein or isoflavonoids by nonhuman primates or humans.

In this study, no evidence of gynecomastia was seen within the low-SI and high-SI treatment groups. Fisher et al. (17) reported gynecomastia and breast tenderness in 3 of 20 men enrolled in a study evaluating the safety and pharmacokinetics of soy isoflavonoids over a 3-mo treatment period. However, these results may not apply to the general population. All 3 men were previously diagnosed with prostate cancer and were given a purified isoflavonoid extract at a high dose, i.e., 300-600 mg genistein · d⁻¹ and 150-300 mg daidzein · d⁻¹. One of these 3 men reported to have gynecomastia, was being treated with the androgen receptor antagonist, Casodex, and had symptoms of gynecomastia at baseline and before initiation of soy isoflavonoid treatment. Furthermore, these symptoms continued past discontinuation of isoflavonoid treatment. A second man also had gynecomastia at baseline, prior to the initiation of soy isoflavonoid treatment, that was attributed to use of PC-Spes, a diethylstilbestrol-contaminated herbal product with the recognized side effect of nipple tenderness and gynecomastia. This product has since been withdrawn from the marketplace. The third report of gynecomastia during the study resolved spontaneously by the subsequent follow-up visit. Giampietro et al. (34) evaluated the effects of the use of soy-protein-based formula in children ranging from 7 to 96 mo of age and found no evidence of precocious puberty, gynecomastia, or altered bone metabolism. Although the study of neonatal soy-isoflavonoid formula-fed twin
marmosets performed by Sharpe et al. (31) and Tan et al. (32), might have shed some light on this question, the mammary glands from these monkeys were not evaluated.

Taken together, these studies demonstrate that concerns over the effect of soy-protein-derived isoflavonoids on the reproductive tract in male primates are not yet supported scientifically to date (35, 36). Our study demonstrated no adverse effects of soy protein on the reproductive organs of adult male cynomolgus macaques. At this time demonstrable evidence does not exist to indicate that, when used in moderate doses, consumption of soy isoflavonoids reduces fertility or causes gynecomastia in men or nonhuman primates.

To our knowledge, this was the first study to evaluate the long-term effects of dietary soy-protein-derived isoflavonoid consumption in the adult male nonhuman primate. Future studies are necessary to explain the mechanism by which regular consumption of a diet rich in soy-derived isoflavonoids may lower the incidence of prostate cancer (1, 2). These future studies should also address the effects of other soy components commonly consumed by human beings that act alone or in combination with isoflavonoids.

Acknowledgments

We thank Hermina Borgerink, Beth Phifer, Dianna Swaim, Lisa O'Donnell, Joseph Finley, Jean Gardin of the Wake Forest University School of Medicine and Lauri Custer of the Cancer Research center of Hawaii for technical assistance.
Table 1

Serum isoflavonoid concentrations, accessory and sex gland organ weights, and testicular and epididymal sperm counts in monkeys fed No-SP, Low-SI, and High-SI diet for 31 mo

<table>
<thead>
<tr>
<th></th>
<th>No-SP</th>
<th>Low-SI</th>
<th>High-SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Isoflavonoid, nmol · L⁻¹</td>
<td>67±23ᵃ</td>
<td>799±44ᵇ</td>
<td>1458±80ᶜ</td>
</tr>
<tr>
<td>Rt Testis, g</td>
<td>18.48±0.85</td>
<td>20.36±0.64</td>
<td>21.15±0.63</td>
</tr>
<tr>
<td>Lt Testis, g</td>
<td>18.92±0.78</td>
<td>19.77±0.70</td>
<td>20.97±0.60</td>
</tr>
<tr>
<td>Prostate Gland, ² g</td>
<td>5.06±0.21</td>
<td>5.14±0.18</td>
<td>5.14±0.21</td>
</tr>
<tr>
<td>Sperm Counts, ³ nx10⁶ · g⁻¹</td>
<td>89±6.18</td>
<td>84±4.67</td>
<td>92±4.61</td>
</tr>
<tr>
<td></td>
<td>2108±222</td>
<td>2284±128</td>
<td>2471±196</td>
</tr>
</tbody>
</table>

¹Values are means ± SEM.

²Cranial and caudal prostate gland weight in total.

³Mean sperm counts in the right and left testes and epididymides were averaged.
Table 2

Prostate gland percent epithelial and stromal areas and mammary gland histomorphometry in monkeys fed No-SP, Low-SI, and High-SI diet for 31 mo

<table>
<thead>
<tr>
<th></th>
<th>No-SP</th>
<th>Low-SI</th>
<th>High-SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>31</td>
</tr>
</tbody>
</table>

**Epithelial Area**, %

<table>
<thead>
<tr>
<th></th>
<th>Cranial Prostate</th>
<th>Caudal Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-SP</td>
<td>65.27±1.73</td>
<td>31.55±1.41</td>
</tr>
<tr>
<td>Low-SI</td>
<td>63.30±1.96</td>
<td>34.04±1.07</td>
</tr>
<tr>
<td>High-SI</td>
<td>62.14±1.72</td>
<td>32.01±1.00</td>
</tr>
</tbody>
</table>

**Stromal Area**, %

<table>
<thead>
<tr>
<th></th>
<th>Cranial Prostate</th>
<th>Caudal Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-SP</td>
<td>32.90±1.17</td>
<td>50.78±1.92</td>
</tr>
<tr>
<td>Low-SI</td>
<td>32.12±1.47</td>
<td>47.41±1.14</td>
</tr>
<tr>
<td>High-SI</td>
<td>33.86±1.11</td>
<td>50.42±1.04</td>
</tr>
</tbody>
</table>

**Mammary Gland Histomorphometry**, mm

<table>
<thead>
<tr>
<th></th>
<th>Gland Width</th>
<th>Nipple Height</th>
<th>Nipple Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-SP</td>
<td>4.87±0.64</td>
<td>3.26±0.15</td>
<td>3.78±0.10</td>
</tr>
<tr>
<td>Low-SI</td>
<td>5.40±0.84</td>
<td>3.08±0.14</td>
<td>3.49±0.11</td>
</tr>
<tr>
<td>High-SI</td>
<td>6.24±0.98</td>
<td>3.10±0.11</td>
<td>3.38±0.09</td>
</tr>
</tbody>
</table>

1. Values are means ± SEM. Means in a row with superscripts without a common letter differ, P < 0.05.

2. Percentages exclude the central luminal area of the prostatic glands.
Table 3

Serum sex hormones in monkeys fed No-SP, Low-SI, and High-SI diet for 31 months. ¹

<table>
<thead>
<tr>
<th></th>
<th>No-SP</th>
<th>Low-SI</th>
<th>High-SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>Estradiol, pmol L⁻¹</td>
<td>62.37±15.57</td>
<td>57.60±11.12</td>
<td>66.56±14.90</td>
</tr>
<tr>
<td>Testosterone, nmol L⁻¹</td>
<td>11.23±1.46</td>
<td>10.89±1.25</td>
<td>12.45±1.49</td>
</tr>
<tr>
<td>Androstenedione, nmol L⁻¹</td>
<td>5.03±0.66</td>
<td>4.15±0.42</td>
<td>4.29±0.42</td>
</tr>
</tbody>
</table>

¹Values are means ± SEM.
Literature Cited


CHAPTER 3

DIETARY SOY ISOFLAVONOID EFFECTS ON EXPRESSION OF SEX STEROID HORMONE RECEPTORS, P27Kip-1, AND KI67/MIB-1 IN THE PROSTATE GLAND OF CYNOMOLGUS MACAQUES (MACACA FASCICULARIS)

Donna L. Perry, Michael R. Adams, J. Mark Cline

The following manuscript was submitted to The Journal of Nutrition, October 2009. Stylistic variations are due to the requirements of the journal. D. Perry performed the experiments, statistical analyses, and prepared the manuscript. Drs. Michael Adams and J. Mark Cline acted in an advisory and editorial capacity.
Dietary soy isoflavonoid effects on expression of sex steroid hormone receptors, p27\textsuperscript{KIP-1}, and Ki67/MIB-1 in the prostate gland of cynomolgus macaques (\textit{Macaca fascicularis})\textsuperscript{1}

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The author has no conflicts of interest.

\textsuperscript{3}The author has no conflicts of interest.

\textsuperscript{4}The author has no conflict of interest.
Abstract and Key Words:
The study objective was to assess the effects of long-term consumption of dietary soy isoflavonoids on the prostate gland using immunohistochemical markers for sex steroid receptors (estrogen receptor α and β, androgen receptor, progesterone receptor), the cyclin dependent kinase inhibitor protein p27KIP-1, and the proliferation marker Ki-67/MIB-1. Ninety-one adult male cynomolgus macaques (Macaca fascicularis) were fed diets for 31-mos differing only in protein source: 1) a soy free, casein-lactalbumin-based diet or 2) a low-soy isoflavonoid diet (approximately 6 mg·kg⁻¹·d⁻¹) or 3) a high-soy isoflavonoid diet (approximately 12 mg·kg⁻¹·d⁻¹). Estrogen receptor β was increased in the stroma of the cranial prostate lobe in animals receiving the low-soy diet when compared to those receiving the control and high-soy diets. The cyclin dependent kinase inhibitor p27KIP-1 was increased in epithelium of the cranial prostate lobe and in the stroma of the cranial and caudal prostate lobe in macaques receiving the low-soy diet when compared to those receiving the control and high-soy diet. Diet did not alter expression of the other sex steroid receptors or expression of the proliferation marker Ki-67. We found no immunohistochemical evidence of an adverse effect of dietary soy isoflavonoids given at physiologically relevant doses.
within the prostate gland of adult male macaques. Increases in p27^KIP-1 in the prostate epithelium and stroma of the low-soy diet group indicate dietary soy isoflavonoids may have antiproliferative effects in the prostate gland. **Key words:** soy, isoflavonoid, steroid receptor, p27^KIP-1, Ki-67, prostate

**Introduction**

Consumption of soy protein has been associated with significantly lower prostate cancer risk in epidemiologic studies (1-4). However, the potential of adverse reproductive system effects of soy isoflavonoid metabolites due to their estrogen receptor (ER) binding and transactivating ability (5-8) is a concern that has prompted a number of dietary studies using rodent models designed to evaluate the effects of soy-derived isoflavonoids on the male reproductive tract. These rodent studies have reported results varying from no discernable negative effect to marked atrophy of the accessory sex glands (9-12). Differences in sensitivity of the various inbred laboratory rodent strains to these estrogen-like compounds (13, 14) have been postulated to explain these inconsistent effects. *In vitro* and *in vivo* studies evaluating effects of soy-derived isoflavonoids on sperm quality in rodents have also produced conflicting results (15, 16). To address this issue in an animal model more closely related to humans, we studied the effects of continuous (31-mo) dietary soy isoflavonoid consumption on reproductive tissues in the male cynomolgus macaque (*Macaca fascicularis*). As previously reported (17), consumption of a soy protein rich diet (6 to 11 g soy protein daily) (18) had no significant adverse effect on body weight, organ weight, histomorphometry of
the mammary glands or prostate gland, and no differences in testicular or epididymal sperm count were detected. To further characterize intra-tissue effects of long-term dietary isoflavonoids on the prostate gland epithelium and supporting connective tissue stroma, we performed immunohistochemical staining and quantification for the sex steroid receptor proteins: ERα and ERβ, androgen receptor (AR), and progesterone receptor (PR), the cell cycle marker p27KIP-1, and the proliferation marker Ki-67/MIB-1.

Materials and Methods

Ninety-one adult male cynomolgus monkeys were imported from Indonesia (Institut Pertanian Bogor) and lived in social conditions composed of 4 individuals. Effects on the cardiovascular system and behavior have been reported previously (19, 20). Age was estimated from dentition to range from 15 to 23 y at the study’s end and did not differ by treatment condition. All monkeys were fed a moderately atherogenic diet, designed to mimic the diet consumed by most men in Western countries, and randomized into three groups differing only by dietary protein source. Control animals (n=30 monkeys, no-SP) were fed a soy-free and isoflavonoid-free, casein-lactalbumin based diet. Animals in the low isoflavonoid group (n=30, low-SI) were fed a mixture of alcohol-washed (isoflavonoid-depleted) and unwashed soy protein isolate containing 0.94 mg of isoflavonoids per gram of product or 6 mg·kg⁻¹·d⁻¹, approximating a human soy isoflavonoid dose of 75 mg·d⁻¹. Animals in the high soy isoflavonoid treatment group (n=31, high-SI) was fed a soy protein isolate containing 1.88 mg soy
isoflavonoid per gram of product or 12 mg·kg⁻¹·d⁻¹, approximating a human soy isoflavonoid dose of 150 mg·d⁻¹. These diets were continuously fed over a 31-mo treatment period. Diet source and a detailed description of the components in each have been described previously (19). All procedures involving monkeys were approved by the Institutional Animal Care and Use Committee of Wake Forest University School of Medicine and adhered to the National Research Council's Guide for the Care and Use of Laboratory Animals. Wake Forest University School of Medicine is accredited by the AAALAC.

**Immunohistochemistry**

Immunohistochemical staining was performed on paraformaldehyde fixed, paraffin-embedded prostate gland tissue using commercially available primary monoclonal antibodies for the following markers: ERα (NCL-ER-6F11, Novocastra, Newcastle upon Tyne, United Kingdom), ERβ (PPG5/10, Serotec, Raleigh, NC), PR (NCL-PGR, Novocastra), AR (AR27 Novocastra), Ki-67 (Ki-67/MIB-1, DAKO Carpinteria, CA), p27KIP-1 (Abcam Inc., Cambridge, MA).

Antigen retrieval was performed with citrate buffer (pH 6.0), biotinylated rabbit antimouse antibody as a linking reagent, and alkaline phosphatase-conjugated Streptavidin as the label, with a Vector Red chromogen (Vector Laboratories, Burlingame, CA) as reported previously (21).

**Immunohistochemical quantification**

Immunohistochemical staining density was quantified using a computerized grid filter overlay at 20x magnification as described previously (21). Cells of acinar structures and stroma were counted separately. The number of
immunohistochemically positively stained cells was measured as a percentage of the total numbers of cell counted (100 cells for each compartment). All measurements were made by observers blinded to treatment group.

**Statistical methods**

The goal of the analysis was to test the effects of dietary soy protein containing isoflavonoids on density and intensity of immunohistochemical staining. Descriptive statistics were calculated to assess normality and equality of variances. Density of immunohistochemical staining was analyzed by treatment group using ANOVA when distributed normally and when normality could not be achieved, Kruskal-Wallis tests were performed. All data are reported as mean ± standard error with a two tailed significance level of P<0.05. Data was analyzed using JMP (version 7.0.2, SAS Institute Inc.).

**Results**

**Sex steroid receptor immunohistochemistry**

Estrogen receptor β expression was increased in the cranial prostate lobe stroma (P<0.05) of animals receiving the low-soy diet when compared to expression in animals receiving the control diet and high-soy diet (Figure 1). Expression of ERβ did not differ within the epithelium of the cranial or caudal prostate gland lobes or the stroma of the caudal prostate gland lobe (Figures 2, 3, and 4). Expression of AR, ERα, and PR did not differ within the epithelium or stroma of the cranial or caudal prostate gland lobes in any diet condition at the 5% level (Figures 1-4).
Cyclin dependent kinase inhibitor p27\textsuperscript{KIP-1}

Epithelial cell expression of the cell cycle inhibitor protein p27\textsuperscript{KIP-1} within the caudal prostate gland lobe was increased (P<0.002) in the macaques receiving the low-soy diet when compared to those receiving the control diet and high-soy diet. Stromal cell expression of p27\textsuperscript{KIP-1} was increased in both the cranial (P<0.005) and caudal (P<0.05) prostate gland lobes in the macaques receiving the low-soy diet when compared to those receiving the control diet and the high-soy diet (Figure 5).

Proliferation marker Ki-67

Dietary soy did not alter expression of the cell proliferation marker Ki-67 in the prostate gland when compared to the control diet at the 5% level (Figure 6).

Discussion

The purpose of this study was to evaluate the effects of soy diets containing 6 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} and 12 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} of soy isoflavonoids, concentrations approximating 75 and 150 mg·person\textsuperscript{-1}·d\textsuperscript{-1}, respectively, on prostate gland epithelial and stromal expression of sex steroid hormone receptors, the cyclin dependent kinase inhibitor p27\textsuperscript{KIP-1}, and the proliferation marker Ki67/MIB-1 in an animal model that shares greater than 90% genetic homology with humans (22, 23).

All differences in expression were detected in the low-soy diet condition: ER\textbeta expression was increased in the stroma of the cranial prostate gland lobe and the cyclin dependent kinase inhibitor p27\textsuperscript{KIP-1} was increased in the epithelium of the caudal prostate gland lobe and increased in the stroma of both prostate
gland lobes. These effects resulted in an inverted U-shaped dose curve. The low and high soy diets had no significant effect on expression of AR, ERα, PR, or the proliferation marker Ki-67/MIB-1.

In the prostate gland, estrogen receptor β or ERS2, first described in 1996 (24), is expressed predominantly in the prostate epithelium while estrogen receptor α is predominantly expressed predominantly in the stroma (25, 26). Activation of these two receptors by estrogen or estrogen-like compounds is thought to have opposing effects (27). Estrogen receptor α signaling in the prostate gland promotes proliferation, squamous metaplasia, and inflammation or prostatitis. The actions of ERβ are not as well understood but activation of the receptor is thought to have antiproliferative and anti-inflammatory effects (25, 27). The antiproliferative effect of ERβ activation has been demonstrated in cell culture studies (28, 29) and in a variety of murine models since the development of the first ERβ knockout mouse in 1998 (30, 31). Early murine studies used ERα knockout mice generated with a Neo cassette inserted into exon II (32) and unfortunately it has been recently demonstrated that a chimeric ERα protein that is 61 kDa in length is retained in this knockout mouse model (33). However, the antiproliferative effects of ERβ have been demonstrated in two other genetically engineered murine models with the use of specific ERβ agonists: the aromatase enzyme knockout and the luteinizing hormone receptor knockout mouse (LuRKO).

The stromal compartment is the location of estrogen synthesis in the normal prostate gland (34). Aromatase knockout prostate tissues are estrogen
deficient. McPherson et al. embedded aromatase knockout prostate tissue recombinants under the renal capsule of intact severe combined immunodeficiency (SCID) mice to determine the role of local estrogenic activity in prostate gland development. After a period of 6 wks these aromatase deficient tissue recombinants exhibited a 3 fold increase in epithelial hyperplasia and significant increases in the proliferation marker proliferating cell nuclear antigen (PCNA). This proliferative effect within the prostate epithelium was entirely abrogated in animals receiving the ERβ agonist, 8β-ER2. Treatment of these mice with the ERα agonist, 16α-LE2, had no attenuating affect on the prostatic epithelial hyperplasia but resulted in an unquantified increase in prostatic inflammation (35). These results indicate that estrogens are required in synergy with androgens for normal prostate gland development and ERβ activation can abrogate epithelial hyperplasia in the prostate gland.

Salvoleinen et al. used LuRKO mice to demonstrate the antiproliferative and anti-inflammatory effects of ERβ signaling in the prostate gland. These mice are androgen deficient postnatally and require exogenous androgen to induce prostate gland development. Luteinizing hormone receptor knockout mice treated with testosterone to stimulate prostate gland development and treated simultaneously with the ERβ agonist, [2,3-bis(4-hydroxyphenol)-propionitrile] (DPN), exhibited a reduction in prostate epithelial area from 68.8% to 42.3% in the ventral prostate lobe and from 71.6% to 57.8% in the dorsal lateral prostate lobes when compared to LuRKO mice receiving testosterone alone. In addition, the prostatitis score decreased from 3+ (of a possible 5+) in 4 of 8 mice treated
with testosterone alone to a prostatitis score of 1+ in 1 mouse of 8 treated with testosterone and the ERβ agonist, DPN (36).

However, it is likely an oversimplification to interpret ER signaling as being exclusively attributable to ERα or ERβ homodimerization. In MCF-7 breast cancer cell lines expressing endogenous ERα and an inducible form of ERβ, re-chromatin immunoprecipitation (re-ChIP) assays have revealed the formation of ERα and ERβ heterodimers. These ERα/ERβ heterodimers decreased mRNA expression of the pS2 gene (trefoil factor 1) and increased expression of the antiapoptotic gene, BCL9, when compared to mRNA expression in the MCF-7 cell line lacking ERβ expression (37). Reductions in pS2 and progesterone receptor mRNA levels, both genes classically thought of as being regulated by ERα, were also seen with expression of ERβ in a T47D tet-off ERβ breast cancer cell line (38). Expression of ERβ also significantly reduced cell proliferation in both cell lines (37, 39).

In vivo, expression of the two ER subtypes is partially controlled by DNA promoter methylation (40, 41). Neoplastic transformation of human prostatic epithelium has been associated with decreases in ERβ expression in men with organ localized prostate cancer (26, 42-45), although some investigators report increases in ERβ expression with neoplastic transformation (46, 47). In prostate cancer cell lines, re-expression of ERβ through the use of demethylating and/or histone deacetylating agents has been associated with increased apoptosis (48). Interestingly, soy isoflavonoids have been demonstrated to demethylate nuclear receptors such as retinoic acid receptor β (RARβ) (49) and this may be the
mechanism by which ERβ expression was increased in the stroma of the cranial prostate gland lobe in these macaques.

The stromal observations in this study have relevance to benign prostatic hyperplasia (BPH), an expansion of the epithelial and stromal compartments of the prostate gland, typically most pronounced in the transition zone, although the pathogenesis of the disease is poorly understood (50). Some investigators speculate that estrogen may play a role in BPH development because men are typically afflicted with symptoms with the onset of andropause, when the ratio or relative concentrations of systemic concentrations of estrogen to testosterone increase (51, 52). The ability of soy isoflavonoids to bind to and transactivate both ER subtypes (7, 8) has raised the concern that soy consumption may also increase the risk of BPH development (53).

Two studies have measured prostate gland volume and intraprostatic isoflavonoid concentrations to determine if there is a relationship between soy consumption and BPH. Hong et al. analyzed isoflavonoid concentrations in prostate tissue from 25 men. Fifteen of these men had BPH and a prostate gland volume greater than 40 cm³ and 10 of these men had invasive bladder cancer and a prostate gland volume of 25 cm³ or less (the control group). The concentrations of the isoflavonoid metabolite genistein were 65.43 ng·mL⁻¹ in the BPH group and 86.96 ng·mL⁻¹ in the control group (54).

Brössner et al. analyzed prostate tissue isoflavonoid concentrations from 94 men; 63 with BPH and 31 with prostate cancer. The men with BPH were separated into small volume (prostate gland less than 35 cm³) or large volume
prostate gland greater than 50 cm$^3$). In men with small volume BPH, the mean genistein tissue concentrations were significantly higher (20.9 ng·g$^{-1}$ dry weight) than in men with large volume BPH (8.9 ng·g$^{-1}$ dry weight) (55).

Each study concluded that the differences in isoflavonoid concentration in BPH tissue may influence benign prostatic enlargement but did not suggest that isoflavonoids may be protective against BPH development. The several-fold higher prostate tissue concentrations of the isoflavonoid metabolite genistein in the Hong et al. study, when compared to concentrations in the Brössner et al. study, was attributed to the men in the Brössner et al. study consuming a traditional Western diet. A traditional Western diet is typically much lower in soy isoflavonoids than a traditional Asian diet (55). The prostate tissue was not analyzed immunohistochemically for sex hormone receptor expression or molecular markers of proliferation in either study.

Tsursaki et al. demonstrated that expression of ER$\beta$ is lower in the transition zone of the human prostate gland when compared to expression in the peripheral zone and speculated that this zonal difference in ER$\beta$ receptor expression may increase the risk of transition zone hyperplasia or BPH because of the accumulating evidence of the antiproliferative effects of ER$\beta$ expression in prostate tissue (56). If this speculation is correct, the mild increase in ER$\beta$ expression in the prostate stroma of these macaques in low-soy diet group would be considered a beneficial effect. A similar study evaluating the expression of ER$\alpha$, ER$\beta$, and AR in the prostate by Leav et al. did not find zonal differences in ER$\beta$ stromal expression (26). Assessment of zonal expression of these markers
was not possible in this study because the prostate in the macaque is separated into a cranial and caudal lobe and not compartmentalized into zones as is the prostate gland in humans (57, 58).

The significant increases in expression of the cyclin dependent kinase inhibitor \( p27^{KIP-1} \) in the epithelium of the caudal prostate lobe and in the stroma of both prostate lobes further supports that the low-soy isoflavonoid diet resulted in an antiproliferative effect in the prostate gland epithelium and stroma. \( p27^{KIP-1} \) binds to cyclin E/cdk2 and is one of the proteins that controls progression of the cell cycle from the G1 to S phase (59). Expression levels of \( p27^{KIP-1} \) of less than 45% in prostate cancer biopsy specimens are a negative prognostic indicator of time to biochemical recurrence in men (60). The isoflavonoid metabolite genistein has been shown to result in a dose dependent increase in \( p27^{KIP-1} \) expression prostate cancer cell lines (61, 62). In addition, we have previously demonstrated that a low-soy isoflavonoid diet (approximately 6 mg·kg\(^{-1}\)·d\(^{-1}\)) or high-soy isoflavonoid diet (approximately 12 mg·kg\(^{-1}\)·d\(^{-1}\)) did not alter the histomorphometry of either the epithelial or stromal compartment in the prostate gland in these macaques (17).

It is important to note that all macaques maintain the bacterial flora in their intestinal tract necessary to convert the isoflavonoid metabolite daidzein to the isoflavan S-equol which has a higher binding affinity for ERs than genistein and daidzein (63). Production of equol in cynomolgus macaques can be markedly reduced by altering the gut flora with oral antibiotic treatment (64). Some researchers speculate equol is the bioactive molecule responsible for the
proposed cytoprotective effects seen in habitual soy consumers that lowers prostate cancer risk (65, 66). It is estimated that 15 to 30% of Americans maintain the bacterial flora necessary to convert daidzein to equol (67) and this nonhuman primate study would most closely model those individuals.

Given what is currently known regarding ERβ signaling and cell cycle inhibitory effects of p27KIP-1 expression in the prostate gland, the increases in expression of both markers in the epithelium and stroma in the prostate gland of the macaques receiving the low-soy diet suggests that long term soy protein and isoflavonoid consumption may have an antiproliferative effect in the prostate gland.

This 31-mo dietary soy intervention study at concentrations approximating a human soy isoflavonoid dose of 75 and 150 mg·d⁻¹ demonstrated no adverse effects on the expression of sex hormone receptor, cell cycle regulatory, and proliferation marker proteins in the prostate gland of male cynomolgus macaques. Further studies to elucidate the mechanisms by which soy consumption may exert beneficial effects in the prostate gland, as reflected by the increases in ERβ and p27KIP-1 expression, seen in this dietary soy study are necessary.

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Author’s contributions to the manuscript: Donna L. Perry wrote the manuscript, conducted research, analyzed the data, and had responsibility for final content. Michael R. Adams and J. Mark Cline designed the research project and performed study oversight. All authors read and approved the final manuscript.
FIGURE 1. Stromal cranial prostate lobe sex steroid receptor expression by IHC after 31-mos dietary soy treatment. ER beta stromal staining was increased in the stroma of the low soy diet (P<0.05) condition when compared to the control and high soy diet conditions. Values are means ± SEM (n=91).

FIGURE 2. Stromal caudal prostate lobe sex steroid receptor expression by IHC after 31-mos dietary soy treatment. No significant differences were detected in the stroma among the low and high soy condition. Values are means ± SEM (n=91).
FIGURE 3. Epithelial cranial prostate lobe sex steroid receptor expression by IHC after 31-mos dietary soy treatment. No significant differences were detected in the epithelium among the low and high soy condition. Values are means ± SEM (n=91).

FIGURE 4. Epithelial caudal prostate lobe sex steroid receptor expression by IHC after 31-mos dietary soy treatment. No significant differences were detected in the epithelium among the low and high soy condition. Values are means ± SEM (n=91).
FIGURE 5. Epithelial and stromal p27 expression in the cranial and caudal prostate lobe by IHC after 31-mos dietary soy treatment. p27 expression increased in the caudal prostate lobe epithelium (P<0.002) and in the stroma of the cranial (P<0.005) and caudal (P<0.05) in the low soy condition. Values are means ± SEM (n=91).

FIGURE 6. Epithelial and stromal Ki67/MIB-1 expression in the cranial and caudal prostate lobe by IHC after 31-mos dietary soy treatment. No significant differences were detected in the stroma among the low and high soy condition. Note expression was nearly undetectable in this normal tissue. Values are means ± SEM (n=91).
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CHAPTER 4

EFFECT OF SHORT TERM DIETARY SOY ISOFLAVONOID TREATMENT ON DNA PROMOTER METHYLATION OF SIX GENES IN ORGAN-LOCALIZED PROSTATE CANCER

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Effect of short term dietary soy isoflavonoid treatment on DNA promoter methylation of six genes in organ-localized prostate cancer

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Abstract and Key Words:

Background. Consumption of a soy rich diet has been associated with decreased prostate cancer incidence in epidemiologic studies. Soy isoflavonoid metabolites bind to estrogen receptors (ERs) and have been shown to decrease DNA promoter methylation in vitro. We hypothesized that 4 weeks of dietary soy would decrease $ER\beta$ DNA promoter methylation and increase $ER\beta$ protein expression in prostate cancer.

Methods. Sixty-two men were randomized into 2 groups and given 25 g protein packets containing 1) soy-free, casein-lactalbumin protein (no-SP) or 2) soy protein (SP) and instructed to consume 2 packets (50 g protein, ~100 mg/d total isoflavonoids) daily for 4 weeks prior to radical prostatectomy. Foci of adenocarcinoma, normal tissue, and benign prostatic hyperplasia (BPH) were isolated for DNA promoter methylation analysis of $ER\beta$, $ER\alpha$, retinoic acid receptor $\beta$2 ($RAR\beta$2), glutathione-s-transferase ($GSTP1$), $O^6$-methylguanine-DNA methyltransferase ($O^6MGMT$), and $p16^{INK4a}$. Protein expression for $ER\beta$, $ER\alpha$, progesterone receptor (PR), androgen receptor (AR), the proliferation marker
Ki67/MIB-1, and the cyclin dependent kinase inhibitor p27^{kip-1} were measured immunohistochemically.

**Results.** No significant differences in $ER\beta$ DNA promoter methylation or protein expression were found in men receiving isoflavonoids. Promoter methylation of $ER\alpha$ was increased in prostate cancer in the SP group ($P<0.011$). $RAR\beta2$ and $GSTP1$ were significantly methylated in prostate cancer ($P<0.0001$) and unaffected by 4 weeks of isoflavonoid treatment. $p16^{INK4a}$ and $O^6MGMT$ were negligibly methylated in prostate cancer.

**Conclusions.** Increases in $ER\alpha$ promoter methylation in prostate cancer of men receiving isoflavonoids suggest dietary isoflavonoids may alter the $ER\alpha:ER\beta$ ratio closer to the ratio found in normal prostate.

**Key words:** androgen, estrogen, progesterone, receptor, p27^{kip-1}

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**INTRODUCTION**

In the late 1980’s Adlercreutz *et al.* demonstrated that consumption of a traditional Japanese diet leads to high serum concentrations and urinary excretion of plant-derived isoflavonoids and suggested that consumption of diets rich in these estrogen-like molecules (1) may explain the large disparity in cancer incidence between native Japanese and people living in Western countries (2,3). This nutrient hypothesis is supported by evidence from immigration studies and by the effect of the nutrition transition.

Prostate cancer is the most common noncutaneous cancer and the second leading cause of cancer death in men in the United States (4). Incidence
doubles in Asian American immigrants exposed to a Western lifestyle between the ages of 45-69 when compared to their native counterparts but still remains approximately one-half the incidence in Caucasian American men (5-8). This suggests that both genetic differences and diet likely play a role in prostate cancer development. However, prostate cancer incidence is increasing worldwide, even in traditionally “low-risk” countries. This increase in incidence is due to several proposed factors including improved screening and diagnosis and more thorough reporting (9). Interestingly, this increase also coincides with the nutrition transition or the global “Westernization” of diets (10). These diets are more energy-dense, higher in animal protein, saturated fat, vegetable oil, simple sugars, and lower in fiber and are replacing more traditional, less processed diets, rich in fiber, legumes, phytochemicals, and cereals worldwide (11-16).

For over 20 years efforts have been made to support the hypothesis that diet can affect cancer incidence and to find the mechanism and constituent or constituents within isoflavonoid-rich diets to explain their proposed benefits. Unfortunately, studies in men designed to demonstrate an association between consumption of higher concentrations of soy isoflavonoids and lower prostate cancer risk vary widely in design, sample size, patient age, time course, the soy-containing foods included in either dietary questionnaires or the type of supplement supplied, and in primary outcome (tumor grade, rise or fall in prostate-specific antigen concentrations, mortality). As a result, these studies have demonstrated mixed results (10). Some studies conclude no effect of soy consumption on prostate cancer risk (17,18) and other studies demonstrate a
decreased risk of prostate cancer in men that consume soy (19-22). The epidemiologic evidence for a role of dietary soy consumption in reducing prostate cancer risk remains compelling but uncertain (20) and warrants further investigation.

Our study was designed to evaluate a genetic-nutrient interaction through the use of a short-term dietary soy protein intervention in men with organ-localized prostate cancer. We measured DNA promoter methylation of nuclear hormone receptor genes (ERβ, ERα, RARβ2), genes involved in DNA repair (O6MGMT) and “antioxidant defense” (GSTP1), and the cyclin dependent kinase inhibitor gene, p16INK4a. Each gene has previously been demonstrated to be methylated with malignant transformation of the prostate epithelium either in vitro or in vivo with increases in promoter methylation resulting in decreases or silencing of gene expression (23-28).

MATERIALS AND METHODS

Enrollment Criteria/Patient Selection

The criteria for enrollment included histopathologically documented, organ-localized adenocarcinoma of the prostate with no evidence of metastatic disease or prior treatment for prostate cancer in a man scheduled to undergo radical prostatectomy with an Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2, that was older than 18 years of age. A computed tomography (CT) scan was evaluated for metastatic disease if the prostate-specific antigen (PSA) concentrations were greater than or equal to 20.
ng/mL at the time of enrollment. Exclusion criteria included a history of hormone treatment, concurrent dietary supplementation with soy, vitamin D, or fish oil, evidence of active nephrolithiasis or a history of hypercalcemic syndrome, and a bone scan or CT consistent with metastatic disease. Prior to study initiation men received a physical exam including a digital rectal examination (DRE) and their height, weight, pulse, blood pressure, and body temperature were recorded. Blood was collected and a complete blood count (CBC), platelet count, electrolyte panel, blood urea nitrogen (BUN) and creatinine concentrations, PSA, and liver enzymes were documented.

**Experimental Design**

This study was a double-blind, randomized, placebo-controlled trial. Participants were randomized into 2 groups to receive either placebo protein (casein lactalbumin) or soy protein for 4 weeks (30 days). Blood was collected at baseline and blood and prostate tissue were collected after 4 weeks of dietary treatment, at the time of radical prostatectomy. All participants provided informed consent and protocols and procedures were approved by the Institutional Review Board of Wake Forest University School of Medicine.

**Dietary Protein Intervention**

The placebo group (n=31, no-SP) were given 60 individual packets containing 25 mg of a soy-free and isoflavonoid-free, casein lactalbumin (milk) protein. The soy isoflavonoid group (n=31, SP) were given 60 packets containing 25 mg of an unwashed soy protein isolate containing 3.4 mg conjugated isoflavonoids weight per gram of soy protein (2.0 mg aglycone equivalents per
gram of soy protein). Each man was instructed to mix one 25 mg protein packet in his drink of choice in the morning and evening providing a daily total of 50 mg soy protein and 100 mg isoflavonoids (aglycone equivalents). All men were instructed not to consume any additional soy products during the study period and to otherwise adhere as closely as possible to their normal diet. After 4 weeks of dietary protein treatment, on the day prior to or the morning of scheduled radical prostatectomy, blood and prostate tissue were collected and a repeat CBC, electrolyte panel, BUN, creatinine, liver enzyme tests, and PSA were measured.

**Serum and Prostate Tissue Isoflavonoid Measurements**

Serum isoflavonoid measurements were performed by high-pressure liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) from serum collected at baseline and after 4 weeks dietary protein treatment, including genistein, dihydrogenistein, daidzein, dihydrodaidzein, glycistein (serum only), o-desmethylangolensin, and equol using isotopically labeled internal standards as recently described (1).

**Prostate Tissue Collection**

Immediately after radical prostatectomy, the capsular margins of the prostate gland were marked with dark ink (Davidson Marking System®, Brady Products; Bloomington, MN) and a 3 to 5 mm section of prostate tissue, transverse to the urethra, at approximately the mid-portion of the prostate was sampled for this study. This tissue was fixed in paraformaldehyde for 24 h then placed in 70% alcohol until embedded in paraffin for histologic sectioning.
prostatectomy specimen was evaluated by a board certified pathologist to confirm the biopsy diagnosis, provide Gleason tumor grade, and tumor stage (29).

Lesion Selection for DNA Promoter Methylation Analysis

Paraffin embedded prostate tissue was sectioned and placed on clean, uncharged glass slides. To map the location of adenocarcinoma, normal tissue, and BPH tissue, the first section made and every 5th serial section after the first was sectioned at 4 µ in thickness stained with H&E and examined by light microscopy to confirm the location of prostate tissue foci that were later collected for DNA promoter methylation analysis. Adenocarcinoma lesions selected were composed of >70% neoplastic epithelium. Foci were identified under a stereomicroscope by lesion type and outlined with color coded indelible ink on the coverslip of the H&E sections. The intervening serial sections were 10 µ and left unstained and paraffin embedded. These unstained sections were carefully aligned beneath H&E mapped tissue sections using a stereomicroscope and all the unmapped tissue was carefully scraped off the slide, leaving only the mapped foci for DNA promoter methylation analysis. After the unwanted tissue was removed, the mapped foci were immediately identified by color code using indelible ink on the bottom of the glass slide. These foci of paraffin embedded adenocarcinoma, normal prostate tissue, and BPH were then lifted off the glass slides with a clean blade and placed into 1.5 mL eppendorf tubes for later DNA isolation.
DNA Isolation

Paraffin was removed from the tissue by immersion in xylene for 2 h at 75°C. Tissue sections were rehydrated with decreasing concentrations of ETOH and digested in 1% sodium dodecyl sulfate and 0.5 mg/mL Proteinase K for 48 h and extracted with phenol-chloroform (2 vol/1 vol), and precipitated with ethanol as described previously (30,31).

Bisulfite Treatment

Conversion of unmethylated cytosine residues to uracil residues was performed using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA) and cleanup for quantitative methylation-specific PCR (QMSP) analysis following the manufacturer's instructions. Briefly, 2 µg genomic DNA from each sample was mixed with 85 µL Bisulfite Mix, 35 µL DNA Protect Buffer, and RNase-free water to a total pre-reaction volume of 140 µL. The thermal cycler conditions used to perform the bisulfite DNA conversion were 99°C 5 min, 60°C for 25 min, 99°C 5 min, 60°C 85 min, 99°C 5 min, 60°C 175 min, and 20°C hold. After bisulfite conversion samples were transferred to clean 1.5 mL microcentrifuge tubes. Buffer BL was added and the mixture was transferred to the Epitect spin column and centrifuged. The sample was washed and centrifuged with Buffer BW and Buffer BD. Bisulfite converted DNA was eluted from the column with Buffer EB and centrifugation and stored at -80°C until use.

Quantitative Methylation Specific PCR (QMSP)

The bisulfite-modified DNA was used as the template for fluorescence-based real-time PCR as described previously (32). The QMSP reactions were
carried out in triplicate in a final volume of 20 µL and contained 2 µL bisulfite-modified DNA; 600 nmol/L concentrations of forward and reverse primers; 200 nmol/L probe; 0.6 units platinum Taq polymerase (Invitrogen); 200 µmol/L concentrations each of dATP, dCTP, dGTP, and dTTP; and 6.7 mol/L MgCl₂. Primers and probes were designed to amplify the promoters of the 6 genes of interest and the reference gene β actin (*ACTB*). Primer and probe sequences and annealing temperatures are included in appendix A.

The thermal cycler conditions to perform the amplification were 95°C for 3 min followed by 50 cycles at 95°C for 15 secs and 60°C for 1 min. Amplification reactions were carried using a 7900 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA) in 384-well plates and analyzed by a sequence detector system (SDS 2.2.1, Applied Biosystems). Each plate included patient DNA samples, positive (in vitro methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls and multiple water blanks. Leukocyte DNA from a healthy individual was methylated in vitro with excess SssI methyltransferase (New England Biolabs, Ipswich, MA) to generate completely methylated DNA and serial dilutions (90-0.009 ng) of this DNA were used to construct a calibration curve for each plate. All samples were within the assay’s range of sensitivity and reproducibility based on amplifications of internal reference standard [threshold cycle (Cₜ) value for *ACTB* of 40]. The relative level of methylated DNA for each gene in each sample was determined as a ratio of QMSP-amplified gene to the reference gene (*ACTB*) and multiplied by 1,000 for easier tabulation (average value of triplicates of gene of interest
divided by the average value of the triplicates of $ACTB \times 1,000$). The samples were categorized as methylated or unmethylated based on the sensitivity of the assay.

**Immunohistochemistry**

Immunohistochemical (IHC) staining was performed on paraformaldehyde fixed, paraffin-embedded prostate gland tissue using commercially available primary monoclonal antibodies for the following markers: $\text{ER}_\alpha$ (NCL-ER-6F11, Novocastra, Newcastle upon Tyne, United Kingdom), $\text{ER}_\beta$ (PPG5/10, Serotec, Raleigh, NC), PR (NCL-PGR, Novocastra), AR (AR27 Novocastra), Ki-67 (Ki-67/MIB-1, DAKO Carpinteria, CA), p27$^{\text{KIP-1}}$ (Abcam Inc., Cambridge, MA), and PCNA (PC10 NCL-PCNA, Novocastra). Briefly, antigen retrieval was performed with citrate buffer (pH 6.0), biotinylated rabbit antimouse antibody as a linking reagent, and alkaline phosphatase-conjugated Streptavidin as the label, with a Vector Red chromogen (Vector Laboratories, Burlingame, CA) as reported previously (33).

**Immunohistochemical Quantification**

Immunohistochemical staining density was quantified using a computerized grid filter overlay at 20x magnification as described previously (33). Cells of acinar structures and stroma were counted separately in normal tissue, BPH, PIN, and prostate cancer. The number of immunohistochemically positively stained cells was measured as a percentage of the total numbers of
cell counted (100 cells for each compartment). All measurements were blinded to treatment group.

**Statistical Analysis**

Descriptive statistics are reported as mean ± SD. All tests conducted were two tailed with a significance level of P<0.05. The goal of the analysis was to test the effects of soy isoflavonoid treatment on DNA promoter methylation density of ERα, ERβ, GSTP1, RARβ2, O6MGMT, and p16INK4a and protein expression of ERα, ERβ, AR, PR, Ki67-MIB-1, and p27KIP-1. Analyses of promoter methylation density of ERα, ERβ, GSTP1, RARβ2, O6MGMT, and p16INK4a, protein expression of ERα, ERβ, AR, PR, Ki67-MIB-1, and p27KIP-1, and soy isoflavonoid concentration were adjusted for patient age, BMI, and within-individual correlation using SAS PROC MIXED. Patient age was used as a covariate because DNA promoter methylation of several genes including ERα, RARβ2, and GSTP1 have been reported to increase with age in normal prostate tissue and in prostate cancer (34,35). DNA promoter methylation density markers analyses tested for differences in lesion types between treatment groups, or differences in lesion type and treatment group if the interaction was nonsignificant. Analyses of soy isoflavonoid concentrations tested differences by location of sample (serum or prostate) between the no-SP and SP condition. Intraprostatic glycitein concentrations were not done. Total isoflavonoid concentrations are defined as the sum of: genistein, daidzein, equol, glycitein (serum only), and O-DMA. Immunohistochemical analyses tested for differences in treatment groups within lesion type in separate models for epithelial and
stromal measurements. All data were analyzed using SAS (SAS Institute Inc., Cary, NC).

**Patient Characteristics and Pathologic Data**

Enrollment began in December of 2003 and ended in December of 2005. Patient age ranged from 43 to 70 years. The 62 enrollees did not differ in age, body weight, height, BMI, biopsy PSA or Gleason score, prostatectomy PSA or Gleason score (Table I). Prostate tissue was collected from 58 of the 62 men enrolled. Three radical prostatectomies were cancelled following enrollment, and in one case tissue collected for the study was returned to the pathologist (RDW) for diagnostic purposes.

**RESULTS**

**Total Serum Isoflavonoid Concentrations**

After 4 weeks of dietary soy isoflavonoid treatment, total serum isoflavonoid concentrations were, as expected, significantly higher in the soy protein treatment group versus the placebo protein treatment group (P<0.0002) (Figure I).

**Total Prostate Tissue Isoflavonoid Concentrations**

After 4 weeks of dietary soy isoflavonoid treatment, total prostate tissue isoflavonoid concentrations did not differ by treatment group (P=0.69) and were not elevated above the concentrations measured in the serum (data not shown).
DNA Promoter Methylation Analysis

**Sex Steroid Receptors ERα and ERβ.** Estrogen receptor β DNA promoter methylation did not differ by treatment group with isoflavonoid treatment (P=0.52) or when analyzed outside of treatment group by lesion type (P=0.19) (Figure II). Estrogen receptor α DNA promoter methylation increased in prostate cancer with isoflavonoid treatment (P=0.01) but promoter methylation did not statistically differ in normal tissue (P=0.27) or in BPH (P=0.23) with isoflavonoid treatment or when analyzed outside of treatment group by lesion type (Figure III).

**Retinoic Acid Receptor β2.** Retinoic acid receptor β2 DNA promoter methylation was not altered by isoflavonoid treatment (P=0.79) but was highly methylated in prostate cancer when analyzed outside of treatment group by lesion type when compared to normal tissue and in BPH (P<0.0001) (Figure IV).

**Glutathione-S-Transferase pi.** As was seen in the RARβ2 DNA promoter, GSTP1 promoter methylation was not altered by isoflavonoid treatment (P=0.78) but was highly methylated in prostate cancer when analyzed outside of treatment group by lesion type when compared to normal tissue and BPH (P<0.0001) (Figure V).

**O6 Methylguanine-DNA Methyltransferase.** DNA promoter methylation of O6MGMT did not differ with isoflavonoid treatment (P=0.45) or when analyzed outside of treatment group by lesion type (P=0.13) (Figure VI).

**Cyclin Dependent kinase p16INK4a.** DNA promoter methylation of p16INK4a was detected in only a single prostate tumor within the soy treatment group. There was no DNA promoter methylation detected in the p16INK4a
promoter region measured in any other prostate tissues analyzed (data not shown).

**Immunohistochemistry**

**Sex steroid receptor immunohistochemistry.** Sex steroid receptor expression (ERβ, ERα, PR, AR) did not differ with isoflavonoid treatment in the epithelium or stroma (tables 2 and 3). There was a trend of PR receptor decrease in the prostate cancer tissues of men in the soy isoflavonoid treatment group but this did not reach statistical significance (P<0.06) (table 2).

**Proliferation marker immunohistochemistry, Ki67/MIB-1.** Expression of Ki67 did not differ with isoflavonoid treatment in the epithelium or stroma (P=0.37) (tables 2 and 3). Ki67/MIB-1 expression was statistically significantly higher in adenocarcinoma versus normal epithelium (P<0.004) when analyzed outside of treatment group by lesion type (tables 2).

**Cyclin dependent kinase inhibitor p27**

Expression of p27 did not differ with soy isoflavonoid treatment in the epithelium or stroma (tables 2 and 3).

**DISCUSSION**

The purpose of this study was to evaluate the effects of 4 weeks of dietary treatment with 50 g soy protein containing 100 mg isoflavonoids on DNA promoter methylation using methylation specific PCR and on protein expression using IHC of the sex steroid receptor ERβ in prostate cancer versus normal prostate tissue. In addition to our primary outcome we analyzed the effect of 4
weeks dietary isoflavonoid treatment on DNA promoter methylation of the sex steroid receptor $ER_\alpha$, the steroid receptor $RAR_\beta_2$, the “antioxidant gene,” $GSTP1$, the DNA repair gene, $O^6MGMT$, and the cyclin dependent kinase inhibitor gene, $p16^{INK4a}$. Protein expression of sex steroid receptors $ER_\alpha$, PR, and AR, the proliferation marker Ki67/MIB-1, and the cyclin dependent kinase inhibitor $p27^{KIP-1}$ was quantified in histologically normal prostate tissue, BPH, PIN, and prostate cancer.

Four weeks of dietary isoflavonoid treatment at 100 mg/d resulted in significantly increased serum isoflavonoid concentrations in the men receiving isoflavonoids but had no effect on $ER_\beta$, $RAR_\beta_2$, $GSTP1$, $O^6MGMT$, or $p16^{INK4a}$ DNA promoter methylation in prostate cancer, normal prostate tissue, or BPH. Similarly, protein expression of $ER_\beta$, $ER_\alpha$, AR, PR, Ki67/MIB-1, or $p27^{KIP-1}$ in the epithelium and stromal compartments did not change significantly in prostate cancer, normal tissue, or BPH with isoflavonoid treatment. Unexpectedly, DNA promoter methylation of $ER_\alpha$ was significantly increased in prostate cancer in the isoflavonoid treatment group.

The role of ER expression in the prostate gland and the cause and effects of changes in ER expression with malignant transformation have largely been unexplored. Estrogen receptor $\alpha$ is expressed predominantly in the stroma and $ER_\beta$ is expressed predominantly in the secretory epithelium of the prostate gland (36,37). Studies using transgenic ER$\alpha$ null mice suggest ER$\alpha$ signaling in the prostate gland promotes proliferation, squamous metaplasia, and prostatitis (36,38,39). Unfortunately, widely inconsistent findings in expression of both ER
subtypes in the prostate tissue of men with prostate cancer make interpretation of the role of ER expression in prostate cancer development and progression difficult.

*In vitro*, primary cultures of normal prostate epithelial cells derived from patients undergoing transrectal ultrasound-guided biopsies showed mRNA expression of the ERβ subtype, but not ERα mRNA expression. In the same study, the malignant prostate cell lines LNCaP and DU145 also expressed only the ERβ subtype while PC-3 cells expressed both ERα and ERβ mRNA. Expression of ERα in LNCaP and DU145 cells was restored in both cell lines with demethylating agents (23), suggesting that DNA methylation is one of the means by which expression of the ER subtypes are regulated within prostate epithelial cells.

In contrast, Ito et al. observed ERα and ERβ expression in LNCaP cells, and in agreement with the Lau et al. findings, ERβ mRNA expression alone in DU145 cells (40). Pasquali et al. found both ERα and ERβ mRNA and protein expression in normal prostate epithelial cells derived from men undergoing radical cystectomy for bladder cancer, but only ERα was expressed at high levels in adenocarcinoma cells collected from men with prostate cancer (41).

Studies that have compared ERα mRNA and/or protein expression in prostate cancer tissue to that in normal tissue or BPH tissue from radical prostatectomy specimens have also reported conflicting results. Increases (42-44), no change in expression (45,46), and decreases (47) in expression of ERα have been reported with malignant transformation. Hormone refractory prostate
cancers have also yielded inconsistent results in ER expression when compared to hormone sensitive prostate cancers with increased (44) and decreased (47) ERα expression reported. We are aware of no studies that have followed prostate cancer patients long enough to determine associations between ER receptor expression levels of either ER subtype and ultimate clinical outcome.

In our study, while a marked increase in ERα DNA promoter methylation was detected in the prostate cancers of isoflavonoid treated men after 4 weeks, only a slight, nonsignificant decrease in ERα protein expression was detected in the prostate cancer tissue. However, expression of PR was decreased by approximately one-half when compared to the prostate cancers of men in the no-SP treatment group, although this decrease did not reach statistical significance (P=0.06). Progesterone receptor is an ERα responsive gene (48,49) and its expression is dependent on ERα (50). The increase in DNA promoter methylation of ERα in prostate cancer would be expected to result in a decrease in ERα mRNA expression and a decrease in epithelial expression of PR.

Like ERα, PR is expressed predominantly in the stroma in normal prostate tissue (51,52) but this sex steroid receptor has been quantified much less frequently than the two ER subtypes in prostate cancer studies. In the few studies that we are aware of in which PR expression has been reported, both increases (48,53) and decreases (45,46) in mRNA and/or protein have been found with malignant transformation, although expression of PR typically parallels that of ERα in the prostate gland. Bonkhoff et al. showed that in some prostate cancers, ERα protein expression was not detectable immunohistochemically, but
PR expression in the tissue using IHC remained consistently associated with concentrations of ERα mRNA (48).

We are uncertain of the pathologic significance of the increase in ERα DNA promoter methylation and trend of decrease in PR protein expression in the prostate cancer tissue of men receiving soy isoflavonoids in this study. Cell culture studies using prostate cancer cell lines to measure the effects of isoflavonoids on DNA promoter methylation have demonstrated decreases in DNA promoter methylation with soy isoflavonoid treatment (23,25). However, in vivo studies using animal models (54,55) and one study in women (56) have demonstrated the opposite effect, an increase in DNA methylation of the genes analyzed with increasing dose of the isoflavonoid genistein.

It has been proposed by many that it may be the ratio of ERα to ERβ expression (44,57,58) that determines tissue responses to endogenous and exogenous estrogens and estrogen-like compounds in tissues that express both ER receptor subtypes, perhaps through heterodimer formation (59,60) with one of the 5 ERβ isoforms (61). Estrogen receptor β has been shown to antagonize ERα protein expression and transcriptional activity, including the induction of PR (62). In vitro studies using prostate cancer cell lines (63,64) and studies in knockout mouse models (38,65,66) suggest ERβ receptor expression has opposing effects to ERα, with ERβ expression inhibiting prostate epithelial cell proliferation and inflammation or prostatitis (36,37). The increase in DNA promoter methylation of ERα and trend toward decreases in PR expression with isoflavonoid treatment in our study suggests that 4 weeks of dietary isoflavonoid
treatment altered the ERα ERβ ratio, decreasing the transactivation effects of ERα.

We are aware of only one other study that measured the effect of soy isoflavonoids on ERα expression in men with prostate cancer. The small pilot study measured ERα expression in prostate tissue at the time of biopsy and radical prostatectomy in 11 men that received 1 of 3 different doses of soy isoflavonoids. Using IHC, decreased ERα protein expression was observed at the highest dose of isoflavonoids (224 mg/d) after 14 days of treatment. This is an isoflavonoid dose approximately twice that given to the men enrolled in our study. As in our study, no change in AR expression was detected though a less sensitive, subjective quantification method, visual scoring (0-3+), was used to evaluate expression of each receptor type. Progesterone receptor and ERβ expression were not evaluated in this small pilot study (67).

There are several plausible explanations for our finding of no significant change in ERβ DNA promoter methylation or protein expression in the prostate glands of men in this study: 1) the isoflavonoid dose was not high enough or the duration of dietary treatment was not long enough to elicit a change, 2) the average Gleason tumor grade was not high enough in the majority of prostate cancers in this study at baseline to have significant effect on ERβ DNA promoter methylation in order to effect a change with isoflavonoid treatment, 3) isoflavonoids do not reduce DNA promoter methylation in vivo, or 4) isoflavonoids do not have a significant effect on DNA promoter methylation of the ERβ gene. Nonsignificant increases in methylation of the ERβ gene in the
prostate cancer tissue of the isoflavonoid treated men were observed in this study. It is possible that dietary isoflavonoid treatment, in vivo, increases DNA promoter methylation of both ER subtypes, having an overall antiestrogenic effect through reductions in ER expression of both ER subtypes.

There was also no effect of isoflavonoid treatment at a dose of 100 mg/d on DNA promoter methylation of RARβ2 or GSTP1 in the prostate tissue analyzed in this study. The DNA promoter regions of both RARβ2 and GSTP1 genes have been shown to be highly methylated in prostate cancer previously (31,68) and both genes were highly methylated in the prostate cancer tissue when compared to normal tissue. Fang et al. showed reversal of DNA promoter methylation of the RARβ gene in LNCaP and PC3 cell lines after treatment with the isoflavonoid genistein at 20 μmol·L⁻¹ for 6 days and speculated that inhibition of DNA methyltransferase is the mechanism through which isoflavonoids caused re-expression of this gene (25). In a recent study in healthy postmenopausal women, treatment with 140 mg isoflavonoids daily had the opposite effect, significantly increasing promoter methylation of the RARβ2 gene of intraductal mammary gland epithelial cells (56).

DNA promoter methylation of the DNA repair enzyme O^6^ MGMT has been reported to be increased in prostate cancer (69), but we detected very low promoter methylation levels, a result that has been reported by Jeronimo et al. previously (70). Similarly, only a single participant in the isoflavonoid treatment group exhibited any DNA promoter methylation of the cyclin dependent kinase inhibitor p16^{INK4a}, making it impossible to assess an effect of isoflavonoid
treatment on promoter methylation of this gene. The lack of DNA promoter methylation of the $p16^{INK4a}$ gene in our localized prostate cancer samples is consistent with reports that the protein expression levels of $p16^{INK4a}$ increase with malignant transformation (71-73).

CONCLUSION

Ours is the first study to evaluate the effects dietary soy isoflavonoid treatment on DNA promoter methylation of ERs. We were unable to demonstrate any change in promoter methylation of the $ER\beta$ gene in men with organ-localized prostate cancer receiving 50 g/day soy protein and 100 mg/d isoflavonoids. DNA promoter methylation of $ER\alpha$ was significantly increased with isoflavonoid treatment. Further research into the effects of changes in expression of both ER subtypes and isoforms with malignant transformation and the possibility that dietary isoflavonoid treatment could alter ER subtype expression and restore the $ER\alpha:ER\beta$ expression ratio to a ratio that more closely reflects that of normal prostate tissue through epigenetic mechanisms are necessary.

ACKNOWLEDGMENTS

Soy products were kindly provided by The Solae Company, St. Louis, MO. We thank Michele Harmon, Libby McWilliams, Hermina Borgerink, Beth Phifer, Joseph Finley, Jean Gardin, Diana Swaim, Debbie Golden, and Lisa O’Donnell of the Wake Forest University School of Medicine and Laurie Custer of the Cancer Research Center of Hawaii for their technical assistance.
Table 1

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Figure 1

Fig. I. Serum isoflavonoid concentrations after 4 wks of dietary protein treatment. Serum isoflavonoid concentrations were significantly increased in the serum of the soy protein treated men (P<0.0002). Values are means ± SEM.
Fig. II. Estrogen receptor β DNA promoter methylation after 4 wks of dietary protein treatment. No significant differences were detected in methylation with isoflavonoid treatment. Values are means ± SEM.

Fig. III. Estrogen receptor α DNA promoter methylation after 4 wks of dietary protein treatment. ERα promoter methylation was statistically significantly increased in PrCa with isoflavonoid treatment (P=0.01). Values are means ± SEM.
Fig. IV. Retinoic acid receptor β2 DNA promoter methylation after 4 wks of dietary protein treatment. RARβ2 promoter methylation was unchanged by isoflavonoid treatment but was significantly increased in PrCa (P<0.0001) when compared to normal prostate tissue and BPH. Values are means ± SEM.

Fig. V. GSTP1 DNA promoter methylation after 4 wks of dietary protein treatment. GSTP1 promoter methylation was unchanged with isoflavonoid treatment but was significantly increased in PrCa (P<0.0001) when compared to normal prostate tissue and BPH. Values are means ± SEM.
Fig. VI. O6MGMT DNA promoter methylation after 4 wks of dietary protein treatment. No significant differences were detected in methylation with isoflavonoid treatment. Values are means ± SEM.
### Table 2. LSMEANS* of Percent Total Positively Stained Epithelial Cells (IHC)

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<th>Marker</th>
<th>Normal MEAN(SE)</th>
<th>Soy MEAN(SE)</th>
<th>BPH MEAN(SE)</th>
<th>PIN MEAN(SE)</th>
<th>Adenocarcinaoma MEAN(SE)</th>
<th>P-values**</th>
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*From models adjusted for age, BMI, and within-individual correlation
**Test of difference between Soy and Placebo within lesion type

### Table 3. LSMEANS* of Percent Total Positively Stained Stromal Cells (IHC)

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<tr>
<th>Marker</th>
<th>Normal MEAN(SE)</th>
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*From models adjusted for age, BMI, and within-individual correlation
**Test of difference between Soy and Placebo within lesion type
## Appendix A

### Appendix A. Primers and Probe Sequences and Annealing Temperatures for QMSP

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<tr>
<th>Gene</th>
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<td>GSTP1</td>
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<td>CGTCGACGCATTTCGGGGGTAGCG</td>
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<td>RARβ2</td>
<td>3p24</td>
<td>GGGATTGAATTATTTATCGGAGTTG</td>
<td>TGTCGAGAACCCCGCAGGCTCGG</td>
<td>TACCCCGACGATACCCAAAC</td>
<td>X56849</td>
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<td>p16INK4a</td>
<td>10q26</td>
<td>GTATTTTTTCGGGACGAGGCG</td>
<td>AATCCCTCGGATACCCCGACCGTTTACG</td>
<td>CGAAATATCAAACACCCCGC</td>
<td>X61657</td>
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<td>GACCGCAGACCGCAGGTAAT</td>
<td>U12818</td>
<td>150 bp</td>
<td>60</td>
</tr>
</tbody>
</table>
REFERENCES


30. Ahrendt SA, Chow JT, Xu LH, Yang SC, Eisenberger CF, Esteller M, Herman JG, Wu L, Decker PA, Jen J, Sidransky D. Molecular detection of


CHAPTER 5

INTRAPROSTATIC ISOFLAVONOID CONCENTRATIONS

One of our aims was to measure intraprostatic isoflavonoid concentrations in men receiving 100 mg/day isoflavonoids and to compare those intraprostatic isoflavonoid concentrations to concentrations achieved in the serum using HPLC/ESI-MS/MS (1). We hypothesized that prostate tissue isoflavonoid concentrations would increase 4 to 13 fold above the concentrations achieved in the serum. As mentioned in chapter 4, we found no increase in intraprostatic isoflavonoid concentrations in men receiving 100 mg/day isoflavonoids when compared to men receiving casein lactalbumin protein (P=0.64) (Figures 1 and 2, respectively).

I am uncertain why these prostate tissue samples did not demonstrate increases in isoflavonoid concentrations as has been reported by the majority of publications that have compared serum to prostate tissue isoflavonoid concentrations previously (Table 1). There are several possibilities that might explain the low prostate tissue isoflavonoid concentrations in these samples. 1) An extended duration of time between last isoflavonoid dose and tissue collection might explain the low concentrations of isoflavonoids measured in the tissue. This would seem unlikely as significant serum concentrations were detected in these men (P<0.002). 2) Degradation of isoflavonoids present in the tissue due to an extended duration of time between prostate tissue removal and snap
Figure 1. Prostate tissue isoflavonoid concentrations in men after 4 weeks of dietary soy protein treatment containing 100 mg/d isoflavonoids (SP).

Figure 2. Serum isoflavonoid concentrations in men after 4 weeks of dietary soy protein treatment containing 100 mg/d isoflavonoids (SP).
freezing of the prostate tissue samples analyzed for isoflavonoid metabolites. This is possible, although we detected no significant difference in prostate tissue isoflavonoid concentrations between the men that had open prostatectomies and robotic prostatectomies. Robotic prostatectomies often required several hours longer to complete than open prostatectomies. 3) Our isoflavonoid source and type utilized (isolated soy protein) does not concentrate in tissue.

In pursuit of answers to these questions, I chose to measure intraprostatic isoflavonoid concentrations in a subset of 45 monkeys receiving 6 and 12 mg/kg/d isoflavonoids (Figures 3 and 4, respectively) (chapters 2 and 3) because these monkeys were diet-compliant and their prostate tissue experienced less variability in handling from the time of last isoflavonoid dose to the time of necropsy dissection, removal, and sectioning for snap freezing. A subset of 15 prostate tissue samples from each diet group (a total of 45 samples) was chosen for analysis: 1) soy-free, casein-lactalbumin-based diet (no-SP) or 2) low-soy isoflavonoid diet (~6 mg·kg⁻¹·d⁻¹, low-SP) or 3) high-soy isoflavonoid diet (~12 mg·kg⁻¹·d⁻¹, high-SP). Isoflavonoids also did not concentrate in the monkey prostate gland after 31 months of treatment with dietary soy isoflavonoids (P=0.45). However, the intraprostatic isoflavonoid concentrations were similar to, but slightly higher than (low-SP, 168±147 and high-SP, 201±136 nmol/Kg) the intraprostatic isoflavonoid concentrations measured in men (72.51±74.81 nmol/Kg).

Seven published studies have measured intra-prostatic isoflavonoid concentrations in men (Table 1). Two of these studies measured isoflavonoid
Figure 3. Prostate tissue isoflavonoid concentrations in cynomolgus macaques after dietary treatment for 31 months with soy protein (SP).

Figure 4. Serum isoflavonoid concentrations in cynomolgus macaques after dietary treatment for 31 months soy protein (SP).
Table 1. Studies investigating prostate fluid or tissue isoflavonoid concentrations in men.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>n</th>
<th>Age Range (yrs)</th>
<th>Duration</th>
<th>PrCa Dxed</th>
<th>Study Type</th>
<th>Isoflavonoid Type &amp; Dose</th>
<th>Serum IF Concentrations nmol/L &amp; Method of Measurement</th>
<th>Prostate Isoflavonoid Concentrations nmol/L or nmol/Kg &amp; Method of measurement</th>
<th>Prostate Isoflavonoid Ranges (nmol/L)</th>
<th>P</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morton 1997</td>
<td></td>
<td></td>
<td>Hong Kong Chinese 31-85</td>
<td></td>
<td>NA</td>
<td>No intervention</td>
<td>Hong Kong Chinese</td>
<td>Serum IF, E 708 EL 103 ED 5.29 D 275 Portuguese E 7.10 EL 540 ED 44.65 D 18.09 British E 2.06 EL 68.04 ED 8.59 D 44.65</td>
<td>Serum IF, E 708 EL 103 ED 5.29 D 275 Portuguese E 7.10 EL 540 ED 44.65 D 18.09 British E 2.06 EL 68.04 ED 8.59 D 44.65</td>
<td>Serum IF, E 708 EL 103 ED 5.29 D 275 Portuguese E 7.10 EL 540 ED 44.65 D 18.09 British E 2.06 EL 68.04 ED 8.59 D 44.65</td>
<td>No statistics performed</td>
<td>Phytoestrogens can accumulate in prostatic fluid.</td>
</tr>
<tr>
<td>Hong 2002</td>
<td>25</td>
<td>&gt;50</td>
<td>BPH vs Control Prostates Volume &gt;40 mL and Small Volume 35-71</td>
<td></td>
<td>NA</td>
<td>No intervention</td>
<td>BPH EL 29.06 ED 5.62 D 384 G 694.37 Control Prostates ED 7.47 EL 27.28 E 56.13 D 316 G 573 GC-MS</td>
<td>BPH EL 21.76 ED 54.49 E 56.34 D 166 G 241 Control Prostates ED 16.43 EL 93.11 E 48.74 D 195 G 321 GC-MS</td>
<td>Median genistein levels greater in small volume BPH P=0.032 EL P=0.068 D P=0.34 G P=0.032</td>
<td>No provided</td>
<td>Results suggest that isoflavonoids, but not lignans, somehow influence the benign prostatic growth, and it is believed that the prostatic concentrations of genistein has the closest association among them.</td>
<td></td>
</tr>
<tr>
<td>Brössner 2004</td>
<td>63</td>
<td>46-92</td>
<td>BPH &lt;35 cm³</td>
<td></td>
<td>NA</td>
<td>Yes Observed</td>
<td>EL BPH &lt;35 cm³ 10.7 ng/g BPH &gt;50 cm³ 19.3 ng/g</td>
<td>Serum IF, EL BPH &lt;35 cm³ 10.7 ng/g BPH &gt;50 cm³ 19.3 ng/g</td>
<td>Serum IF, EL BPH &lt;35 cm³ 10.7 ng/g BPH &gt;50 cm³ 19.3 ng/g</td>
<td>Median genistein levels greater in small volume BPH P=0.052 EL P=0.068 D P=0.048 G P=0.032</td>
<td>Even in men consuming a Western diet, median genistein may influence prostate cancer and benign prostatic enlargement.</td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>n</td>
<td>Age Range (yrs)</td>
<td>Duration</td>
<td>PrCa Dxed</td>
<td>Study Type</td>
<td>Isoflavonoid Type &amp; Dose</td>
<td>Serum IF Concentrations nmol/L &amp; Method of Measurement</td>
<td>Prostate Isoflavonoid Concentrations nmol/L or nmol/Kg &amp; Method of measurement</td>
<td>Prostate Isoflavonoid Ranges (nmol/L)†</td>
<td>P</td>
<td>Conclusion</td>
</tr>
<tr>
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<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Hedlund</td>
<td>2005</td>
<td>Serum 45 Prostate 36 Low soy consumers (LS) 20 High soy Consumers (HS) 25</td>
<td>19-65</td>
<td>1 wk</td>
<td>No</td>
<td>Prospective intervention</td>
<td>“Power Dream” soy drink 42-60 mg D 43% G 53% E 44% LS 438 HS 544 G LS 409 HS 720 DHD LS 71 HS 97 O-DMA LS 35 HS 99 E LS ND HS ND HPLC EIS-MS</td>
<td>D LS 1878 HS 2779 G LS 401 HS 480 DHD LS 201 HS 541 O-DMA LS ND HS 250 E LS ND HS ND HPLC EIS-MS</td>
<td>D LS 181-6,231 HS 295-29,847 G LS 162-4,114 HS 196-5,139 DHD LS ND-3023 HS ND-10,598 O-DMA LS ND-1,182 HS ND-1,229 E LS ND-347 HS ND-9,780</td>
<td>No statistics performed on serum versus prostate fluid concentrations</td>
<td>It seems likely that high concentrations of isoflavonoids in prostate fluid are related to the ability of soy to reduce prostate cancer risk.</td>
<td></td>
</tr>
<tr>
<td>Guy</td>
<td>2008</td>
<td>16 total Untreated 4 Treated 12</td>
<td>54-77</td>
<td>3 days</td>
<td>BPH</td>
<td>Randomized double-blind placebo-controlled</td>
<td>“Evestrel” 112.5 mg D 65.7% G 65.7% E 31.7% LS 35 HS 99</td>
<td>Treated 1,450 nmol/L LC-ESI-MS/MS</td>
<td>Treated 1,050 nmol/Kg LC-ESI-MS/MS</td>
<td>Treated 300-2,300 nmol/Kg</td>
<td>Not reported</td>
<td>Isoflavone concentration in prostate remains below 5 nmol/g after high-dose supplementation.</td>
</tr>
<tr>
<td>Gardiner</td>
<td>2009</td>
<td>25 total Placebo 11 Treated 14 Available for analysis 19</td>
<td>Not reported</td>
<td>2 wks</td>
<td>Yes</td>
<td>Randomized double-blind placebo-controlled</td>
<td>“NovoSoy” 27.2 mg/tablet G 10.6 mg D 13.3 mg E 3.2 mg Total 82 mg/d (3 tablets) Placebo 2 nmol/L G 8 nmol/L</td>
<td>Treatment G 2,230 nmol/Kg D 2,380 nmol/Kg HPLC/ESI-MS/MS</td>
<td>Not reported 25th and 75th Percentiles Treatment G 520 and 3,660 nmol/Kg Treatment D 650 and 3,150 nmol/Kg</td>
<td>G P&lt;0.001 D P&lt;0.003</td>
<td>Prostate tissue is able to accumulate potentially anti-cancerous isoflavonoids.</td>
<td></td>
</tr>
</tbody>
</table>

*Plasma & prostate samples are from different groups of people
†Unless otherwise specified

Abbreviations: Biochanin A (B), Dihydrodaidzein (DHD), Daidzein (D), Electrospray Ionization Liquid Chromatography Tandem Mass Spectrometry (LC-ESI-MS/MS), Enterodiol (ED), Enterolactone (EL), Equol (E), Formononetin (F), Gas Chromatography-Mass Spectrometry (GC-MS), Genistein (G), Glycitein (GY), High-Pressure Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (HPLC/ESI-MS/MS), o-desmethylangolensin (O-DMA), Not Applicable (NA), Not Done (ND), Significant Difference (NSD), Prostate Cancer (PrCa), Time Resolved Fluorimunnoassay (TR-FIA)
concentrations in men with presumed normal prostate tissue (2,3), two in men with BPH (4,5), two in men with prostate cancer (6,7), and one study investigated BPH versus prostate cancer tissue isoflavonoid concentrations (8). Four of these studies demonstrated increased concentrations of isoflavonoids in the prostate gland tissue or fluid when compared to serum (2,3,6,7), one did not evaluate serum levels (8), one did not compare serum to prostate tissue concentrations (4), and one found no increase in the concentration of isoflavonoids in prostate tissue when compared to serum (5).

The four studies that reported increased isoflavonoid concentrations in either prostate tissue (6,7) or fluid (2,3), when compared to concentrations measured in the serum, reported wide ranges of isoflavonoid concentrations that sometimes vary by several thousand nanomoles. The highest and the widest ranging isoflavonoid concentrations that have been reported were measured in prostate fluid, not in prostate tissue. It is possible that these prostate fluid samples came from studies where men exhibited a wide variation in dietary compliance, or that there are wide variations in an individual’s ability to concentrate isoflavonoids in prostate tissue, or that the samples were contaminated with urine. Urine is the primary route of soy isoflavonoid elimination from the body (9). None of these publications have expressed concern or addressed the wide variations in isoflavonoid concentrations measured in the prostate fluid or tissue.

The ability of the prostate gland to concentrate isoflavonoids may be answered through a randomized, placebo-controlled, crossover study using diets
containing soy beans, isolated soy protein containing isoflavonoids, purified glucosides, and purified aglycones at known concentrations and ratios and delivered for a 2 to 4 week period. Collection of baseline, unfasted and fasted mid-treatment, and post-treatment salivary, serum, urine, and prostate fluid samples would likely provide invaluable information on the effect of isoflavonoid source, matrix type, and processing on the pharmacokinetics, bioavailability, and ability of the prostate tissue to concentrate isoflavonoids.
REFERENCES


We conducted two parallel studies, one in cynomolgus macaques (Macaca fascicularis) and the second in men with organ-localized prostate cancer, to investigate the effects of dietary soy isoflavonoids on the normal and neoplastic prostate gland, respectively. We demonstrated no adverse effects of soy isoflavonoids on whole body or individual organ weights, histomorphometry of the mammary glands or prostate gland, or testicular and epididymal sperm counts in 91 adult male cynomolgus macaques receiving ~6mg/kg/d and ~12 mg/kg/d isoflavonoids for 31 months, a dose approximating 75 and 150 mg/d human equivalents (1). All statistically significant differences in biomarker protein expression, measured quantitatively using immunohistochemistry, were found in the animals receiving the lower, ~6 mg/kg/d dose of soy isoflavonoids, resulting in an inverse U dose curve. Estrogen receptor β, thought to have an antiproliferative effect in the prostate gland (2), and the cyclin dependent kinase inhibitor, p27^KIP-1 (3), were increased in expression in the cranial prostate gland stroma. p27^KIP-1 was also increased in expression in the caudal prostate gland epithelium and stroma. Based on what is currently known regarding the effects of ERβ and p27^KIP-1 expression in the prostate gland, these findings suggest dietary soy isoflavonoids may exert antiproliferative effects in the prostate gland epithelium and stroma when consumed in moderate doses.
In our second study, a randomized, placebo-controlled, phase IIb clinical trial in men with organ-localized prostate cancer, we found increases in DNA promoter methylation of the sex steroid receptor ERα gene in prostate cancer after 4 weeks of dietary isoflavonoid treatment at a dose of 100 mg/d. We found only a minimal decrease in ERα protein expression in the prostate cancer tissue. However, expression of progesterone receptor (PR), a well known ERα responsive gene (4), decreased by approximately one-half in the soy isoflavonoid treated men, although this decrease did not reach statistical significant (P<0.06).

Ours is the first study to demonstrate changes in DNA promoter methylation in prostate cancer tissue of either ER subtype with a dietary isoflavonoid intervention in men. These findings suggest that changes in DNA promoter methylation are one of the mechanisms by which dietary soy isoflavonoids alter gene expression and may affect prostate cancer incidence. The overall tissue response to isoflavonoids, however, likely depends on the endogenous hormone milieu and the ratio of ER subtypes expressed in the tissue of interest (5). As stated previously (chapter 4), we are aware of no studies that have quantified ER subtype expression in prostate cancer tissue and followed patients long enough to determine associations between ER expression subtype and ultimate clinical outcome. Epigenetic changes that occur with neoplastic transformation in a variety of genes are being evaluated for use as diagnostic tools in prostate cancer (6-9). Further research into the changes in ER subtype expression that occur in the prostate gland with neoplastic
transformation and the prognostic significance of those changes, if any, need further exploration.

There have been 11 recently published intervention studies investigating the effects of isoflavonoids on prostate-specific antigen (PSA) levels in men (Table 1). Each study has utilized a different source of isoflavonoids, isoflavonoids in different ratios and forms (glycones and aglycones), and treated men for variable durations of time. The disease status of the men in these studies has spanned from healthy men with no known prostate disease to men with recurrent prostate cancer following failed treatment interventions such as radical prostatectomy or radiation treatment. The majority of these studies have demonstrated no beneficial effect of isoflavonoids.

However, the utility of PSA as diagnostic tool in organ-localized prostate cancer has become controversial (10-12). The PSA test cannot discern between clinically insignificant disease, sometimes leading to over-treatment of indolent tumors, and prostate cancers that will be lethal (13,14). In addition, having a PSA value considered by most to be within the normal range (<4 ng/mL) does not preclude the presence of a high grade tumor (15), and two large studies evaluating the efficacy of PSA screening in reducing mortality in men from prostate cancer have shown PSA screening does not reduce the mortality rate from prostate cancer (16,17). This further complicates interpretation of soy intervention studies that have used changes in PSA concentrations or doubling time/velocity as their primary outcome. Future studies investigating a
### Table 1. Studies investigating soy isoflavonoid effects on prostate-specific antigen concentrations in men.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>n</th>
<th>Age Range (yrs)</th>
<th>Duration</th>
<th>PrCa Dxed</th>
<th>Study Type</th>
<th>Isoflavonoid Type</th>
<th>Dose</th>
<th>PSA ng/mL</th>
<th>P</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adams</td>
<td>2004</td>
<td>81</td>
<td>50-80</td>
<td>12 mos</td>
<td>No</td>
<td>Randomized double-blind placebo controlled</td>
<td>G 45.6 mg D 31.7 mg GY 5.5 mg Powder</td>
<td>83 mg</td>
<td>Baseline ISO= 1.7 Post Tx ISO= 1.7 Post Tx ISO+ 2.0</td>
<td>0.94</td>
<td>No evidence that a 12 mo. 83 mg/d isoflavone treatment alters serum PSA concentration or velocity.</td>
</tr>
<tr>
<td>Dalais</td>
<td>2004</td>
<td>28</td>
<td>Wheat 60.5 Soy 61.7 Soy + Linseed 59.4</td>
<td>Yes</td>
<td>Randomized double-blind placebo-controlled</td>
<td>G, D, GY = 117mg Heat-treated soy grits Heat-treated soy grits + linseed oil</td>
<td>117 mg</td>
<td>Wheat 5.81 Soy 7.16 Soy + Linseed 6.21 Treatment Wheat 7.11 Soy 6.34 Soy + Linseed 6.99</td>
<td></td>
<td>Wheat vs. Soy Groups 0.02</td>
<td></td>
</tr>
<tr>
<td>Joniau</td>
<td>2007</td>
<td>71</td>
<td>71 at 3 mos 58 at 6 mos</td>
<td>6 mos</td>
<td>HGPIN</td>
<td>Intervention</td>
<td>ISO 100 mg Selenium 200 µg Vit. E 60 mg</td>
<td>Baseline 4.80 3 mos 3.85 6 mos 3.50</td>
<td></td>
<td>Baseline to 3 mos P= 0.0197 Baseline to 6 mos P= 0.0048 3 mos to 6 mos P=0.365 Results show a decrease in PSA level while taking a selenium, vit. E, and soy isoflavonoid supplement predicts a significantly lower risk of prostate cancer in future biopsies.</td>
<td></td>
</tr>
<tr>
<td>DeVere</td>
<td>2004</td>
<td>62</td>
<td>Failed RP 9 Failed RTx 17 HTx 14 RP + RTx 6 WW 16</td>
<td>6 mos</td>
<td>Yes</td>
<td>Nonrandomized open-label study</td>
<td>G 450 mg/d “Other aglycones” 450 mg/d G-rich extract, Soybean extract added to a mycelia culture.</td>
<td>900 mg</td>
<td>Baseline Failed RP 8.7 Failed RTx 6.6 HTx 7.0 RP + RTx 2.5 WW 8.8 Treatment Failed RP 14.2 Failed RTx 10.1 HTx 9.8 RP + RTx 4.3 WW 8.6</td>
<td></td>
<td>Random effects regression model decrease in PSA of about 5.5% per month with no overall difference after initiation of genistein extract. Genistein did not reduce PSA levels by 50% or more in 51 of 52 subjects and did not appear to be an effective treatment for prostate cancer when given alone.</td>
</tr>
<tr>
<td>Grainger</td>
<td>2007</td>
<td>41</td>
<td>Recurrent but asymptomatic PrCa</td>
<td>8 wks</td>
<td>Yes</td>
<td>Nonrandomized</td>
<td>Lycopene 25 mg G 48 mg D 24 mg</td>
<td>PSA values not reported. Baseline 12 men (30%) in slowest doubling time category, Treatment 19 men (48%) in slowest doubling time category.</td>
<td></td>
<td>P=0.08 Older men with active prostate cancer can successfully change their diet and consume tomato products and soy protein/isoflavone supplements with excellent adherence and minimal toxicity.</td>
<td></td>
</tr>
<tr>
<td>Kranse</td>
<td>2005</td>
<td>37</td>
<td>54-81</td>
<td>12 wks</td>
<td>Yes</td>
<td>Randomized, double-blind crossover</td>
<td>Verum 100 mg</td>
<td></td>
<td></td>
<td></td>
<td>Total PSA doubling times did not did not decrease during verum.</td>
</tr>
<tr>
<td>Kumar</td>
<td>2004</td>
<td>76</td>
<td>50-80</td>
<td>12 wks</td>
<td>Yes</td>
<td>Randomized double blind placebo-controlled</td>
<td>G 60 mg</td>
<td></td>
<td></td>
<td></td>
<td>Able to demonstrate total PSA was reduced in a relatively larger number of subjects consuming soy supplement compared to the placebo group, which may be a clinically significant finding.</td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>n</td>
<td>Age Range (yrs)</td>
<td>Duration</td>
<td>PrCa Dxed</td>
<td>Study Type</td>
<td>Isoflavonoid Type</td>
<td>Dose</td>
<td>PSA ng/mL</td>
<td>P</td>
<td>Conclusion</td>
</tr>
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<td>----------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Maskarinec</td>
<td>2006</td>
<td>24</td>
<td>44-69</td>
<td>3 mos</td>
<td>No</td>
<td>Randomized double-blind crossover</td>
<td>Soy foods approximated dose. Low soy grp 1 serving High soy grp 2 servings</td>
<td>0.3-10 mg low soy 9.2-69.1 mg high soy</td>
<td>Low soy 1 mo. 1.83 Low soy 3 mos. 1.95 High soy 1 mo. 2.02 High soy 3 mos. 1.74</td>
<td>P&gt;0.10</td>
<td>It is uncertain whether the small decrease in mean PSA level during the high soy diet can be translated into a potentially protective effect.</td>
</tr>
<tr>
<td>Schroder</td>
<td>2005</td>
<td>49</td>
<td>Mean 69.6</td>
<td>10 wks</td>
<td>Yes</td>
<td>Randomized double-blind placebo-controlled crossover</td>
<td>Soy 62.5 mg included in a &quot;nutritional supplement&quot; tablet</td>
<td>62.5 mg</td>
<td>Baseline 1.6 Treatment Placebo 1.5 Supplement 1.5 Slope of log transformed PSA concentrations Placebo 0.0022 Supplement 0.0009</td>
<td>Total PSA P=0.076 Slope of log transformed PSA concentrations P=0.041</td>
<td>Results showed dietary supplement significantly improved the slope of log transformed PSA concentrations when compared with placebo (P=0.041).</td>
</tr>
<tr>
<td>Urban</td>
<td>2001</td>
<td>34</td>
<td>&quot;Over 55&quot;</td>
<td>6 wks</td>
<td>No</td>
<td>Randomized double-blind placebo-controlled crossover</td>
<td>Placebo G 2.1 mg D 1.3 mg Soy G 42 mg D 27 mg</td>
<td>Placebo G 4.2 mg D 2.6 mg Soy G 84 mg D 54 mg</td>
<td>Baseline ISP- to ISP+ 9.1 ISP+ to ISP- 7.6 First period ISP- to ISP+ 8.7 ISP+ to ISP- 7.8 Second period ISP- to ISP+ 8.7 ISP+ to ISP- 8.2</td>
<td>First period 0.62 Second period 0.72</td>
<td>Isoflavone containing soy protein beverages had no statistically significant effect on serum PSA.</td>
</tr>
<tr>
<td>Vaishampayan</td>
<td>2007</td>
<td>71</td>
<td>50-91</td>
<td>6 mos</td>
<td>Yes</td>
<td>Randomized double-blind placebo-controlled crossover</td>
<td>Lycopene alone 30 mg Lycopene 30 mg + soy 40 mg</td>
<td>Lycopene alone 30 mg Lycopene 30 mg + soy 40 mg</td>
<td>Baseline Lycopene 6.1 Lycopene + Soy 6.9</td>
<td>Overall a significant rise in PSA over time (P=0.0001) Rate of PSA declined in hormone-sensitive group (P=0.015) and the hormone-refractory group (0.017)</td>
<td>Although there were no objective (partial or complete) PSA remissions in this study, a decline in the rate of PSA rise was observed in both arms of the study. Particularly, lycopene administration slowed the rate of PSA progression in both hormone-sensitive and hormone-refractory patients.</td>
</tr>
</tbody>
</table>

Abbreviations: Biochanin A (B), Dihydrodaidzein (DHD), Daidzein (D), Equol (E), Enterolactone (EL), Enterodiol (ED), Formononetin (F), Genistein (G), Glycitein (GY), Hormone Treatment (HTx), Isoflavonoids (ISO), O-desmethylangolensin (ODMA), Not Applicable (NA), Not Done (ND), No Significant Difference (NSD), Other (O), Radical Prostatectomy (RP), Radiation Treatment (RTx), Watchful Waiting (WW).
mechanism by which soy isoflavonoids may protect against the development or progression of prostate cancer need to utilize more sensitive and specific biomarkers as outcomes in addition to, or in place of, PSA. Unfortunately, a more sensitive and specific non-invasive screening test for prostate cancer is not yet commercially available. There is a great need for such a prostate cancer screening test.

Numerous studies have investigated the bioavailability of soy isoflavonoids from different sources and in different forms in both men, women, (18-34) and children (35-37). There is no consensus on how isoflavonoid source, form, and processing affects bioavailability (38). There has not yet been a controlled dietary study in an animal model that has delivered soy beans, isolated soy protein containing isoflavonoids, purified glucosides, and purified aglycones in the diet and measured isoflavonoid concentrations that result from these different sources and forms of isoflavonoids in the serum and in target tissues. Two studies recently performed in rats measured serum isoflavonoid concentrations after the delivery of 3 different isoflavonoid forms but had conflicting results.

Sepehr et al. performed a 10 day study in 90 day old Sprague-Dawley rats and delivered soy isoflavonoids from three different sources: 1) a commercially available soy supplement (Novasoy™), 2) synthetic glucosides, and 3) synthetic aglycones at the same dose (20mg/kg). Measurement of maximum peak serum isoflavonoid concentrations revealed daidzein concentrations to be 3-fold higher in rats gavaged with the synthetic daidzein glucoside and 7-fold higher in the rats
gavaged with Novasoy™ than concentrations of serum daidzein in rats gavaged with the synthetic aglycone form of daidzein (P<0.05). Serum genistein was 2-fold higher in rats gavaged with Novasoy™ than in rats receiving the synthetic glucoside or aglycone. The authors determined that the bioavailability of daidzein was greater in the glycone form based on these results. They also speculated that the increased bioavailability of genistein originating from Novosoy™ when compared to the purified glucosides and aglycones may have been due to the matrix components within the Novosoy™ product (sugars, phytosterols, etc.) protecting the genistein from degradation. While the maximum serum peak isoflavonoid concentrations were achieved from the glucoside form of isoflavonoids, the volume of distribution was significantly higher for daidzein and genistein (P<0.05) gavaged in the synthetic aglycone form than the volume of distribution achieved from either the synthetic glycone or Novosoy™ form (39).

The same authors used the same study design to test the bioavailability of different forms of isoflavonoids at the same dose (20mg/kg) but in older (20 month) Fischer 344 rats two years later (40). In this study the maximum peak serum daidzein and genistein concentrations were ~2-fold higher in the rats gavaged with Novasoy™ when compared with the rats receiving the purified isoflavonoid glucosides (P<0.05). As they reported in the previous study, the volume of distribution of daidzein was highest in the purified aglycone form (P<0.05) when compared to either the purified glucoside form or Novosoy™ form of isoflavonoids, but the volume of distribution of the aglycone form was not higher in this study for the isoflavonoid genistein. The authors speculated that in
older rats the glucoside form of isoflavonoids are poorly absorbed from the gut
and this caused the bioavailability (or maximum peak serum concentrations
measured) of genistein to be significantly higher in the aglycone gavaged rats.
Neither study measured urine or tissue isoflavonoid concentrations or differences
in plasma protein binding of the different isoflavonoid types or the degree of
isoflavonoid conjugation (sulfation or glucuronidation).

As mentioned in chapter 5, until some of these basic questions are
answered regarding what form(s) of isoflavonoids are most bioactive and
bioavailable, the answers to questions regarding soy isoflavonoid effects on a
variety of disease states, including prostate cancer and other types of cancer, will
continue to be difficult to reconcile with the epidemiologic evidence that suggests
soy consumption provides a protective effect. It is important to address some of
these basic questions regarding the bioavailability and tissue distribution of soy
isoflavonoids in order to conduct well-controlled preclinical and clinical trials that
may, in the future, resolve some of the contradictory findings reported in studies
designed to evaluate the effects of soy isoflavonoids on the incidence and
progression of prostate cancer that have been published in the literature thus far.

In this study, we assessed the effect of dietary soy isoflavonoids on DNA
promoter methylation of 6 individual genes known to be epigenetically altered in
some prostate cancers when compared to normal prostate tissue. Global
methylation studies evaluating the effect of isoflavonoid metabolites have shown
that dietary soy isoflavonoid intervention increases DNA methylation in non-
coding regions of the genome (heterochromatic regions, retrotransposable
elements) also resulting in changes in gene expression (41,42). Interestingly, global methylation studies performed on a variety of different tumor types including prostate (43,44), lung (45), colon (46), breast (47), and hematopoietic (48) neoplasms, have demonstrated that global decreases in DNA methylation or hypomethylation occurs (49) in these neoplasms. Decreases in global DNA methylation result in increased genomic instability and this is thought to contribute to carcinogenesis (50). It is possible that soy isoflavonoid consumption may decrease cancer incidence by increasing global genomic DNA methylation, resulting in increased global genomic stability. This is a hypothesis I hope to test in the future.

In summary, we demonstrated that moderate doses of dietary soy isoflavonoids have no adverse effects on the male reproductive system and increase expression of prognostically favorable biomarkers in the normal prostate gland. In the neoplastic prostate gland, short-term dietary isoflavonoid treatment resulted in increased \( ER_\alpha \) DNA promoter methylation in the malignant prostate epithelium. Further studies are needed to understand the role of both ER subtypes in the normal prostate gland, the changes in ER subtype expression that occur with malignant transformation, and the impact short-term and long-term dietary soy isoflavonoid consumption may have on the development and progression of prostate cancer.
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Manuscripts:

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Perry DL, Adams MR, Cline JM. Dietary soy isoflavonoid effects on expression of sex steroid hormone receptors, p27\(^{\text{Kip-1}}\), and Ki67/MIB-1 in the prostate gland of cynomolgus macaques (*Macaca fascicularis*). (submitted)

