

## A Combination of Micronutrients Is Beneficial in Reducing the Incidence of Prostate Cancer and Increasing Survival in the *Lady* Transgenic Model

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### Abstract

We have previously shown that administration of a combination of micronutrients (selenium, vitamin E, and lycopene) inhibits prostate cancer (PCa) development in the *Lady* transgenic model. In the present study, we examine timing of initiation of micronutrients, and the effect of micronutrient combinations, on PCa development in *Lady* transgenic model.

Transgenic males were randomized to either a control diet; control diet supplemented with human equivalent doses of vitamin E, selenium, and lycopene (E+S+L); or control diet supplemented with vitamin E and selenium (E+S). In separate experiments, the combination of E+S+L was initiated at varying time points (4, 8, 20, and 36 weeks of age).

A combination of E+S+L resulted in a significant reduction in PCa and liver metastasis when intervention was commenced within 8 weeks of age ( $P < 0.0001$ ). Immunohistochemical analysis revealed a strong correlation between disease-free state with up-regulation of the prognostic marker p27<sup>Kip1</sup> ( $P < 0.0001$ ) and decreased expression of proliferating cell nuclear antigen and significantly increased apoptotic index ( $P < 0.0001$ ). On the contrary, a combination of E+S was not effective in preventing PCa, with a high proportion (84.6%) of animals developing PCa and a small proportion (11.5%) developing high-grade PIN.

Early commencement of micronutrients (E+S+L) is beneficial in reducing PCa. Lycopene is an essential component of the combination and effective (when used with E+S) for PCa prevention. These observations provide support for their chemopreventive effect and some clues about their mechanism of action. These key findings will be complementary to the outcome from the Selenium and Vitamin E Chemoprevention Trial.

Recent data have established that the risk of prostate cancer (PCa) can be reduced by preventative measures (1). Substantial preclinical and epidemiologic studies have identified several micronutrients as potential PCa prevention agents (2–4). This in particular includes selenium, vitamin E, and lycopene. These molecules have a wide range of *in vitro* antitumor properties, but their actual benefit in preventing PCa has not been firmly established.

Our studies of the *in vitro* use of selenium, vitamin E, and lycopene in human PCa cell lines (5–7) and more impor-

tantly in a transgenic mouse model (8) have shown the powerful effect of these micronutrients in inhibiting PCa development. Dietary micronutrients protect against cellular oxidative damage by neutralizing oxygen free radicals (9). We have previously shown that a combination of these micronutrients induces cell cycle arrest with up-regulation of cell cycle regulatory protein p27<sup>Kip1</sup> (5–7). Selenium, an essential micronutrient, has been identified as a chemopreventive agent in various cancers, including the prostate. There exists an inverse association between selenium intake, tissue levels, and the incidence of prostate as well as other human cancers (10, 11). Vitamin E, the major intracellular antioxidant, has a wide range of anticancer properties (12, 13). Lycopene, a naturally occurring carotenoid, is a potent antioxidant and free radical scavenger (14, 15). Several studies have shown an inverse relationship between dietary intake of lycopene-rich food and risk of PCa (16–18).

Many individuals take vitamins, minerals, and other macronutrients or micronutrients with the hope of preventing chronic diseases such as cancer. The data supporting these agents are usually derived from cross-sectional epidemiologic data and *in vitro* or *in vivo* animal studies. Large-scale randomized trials of these agents for disease

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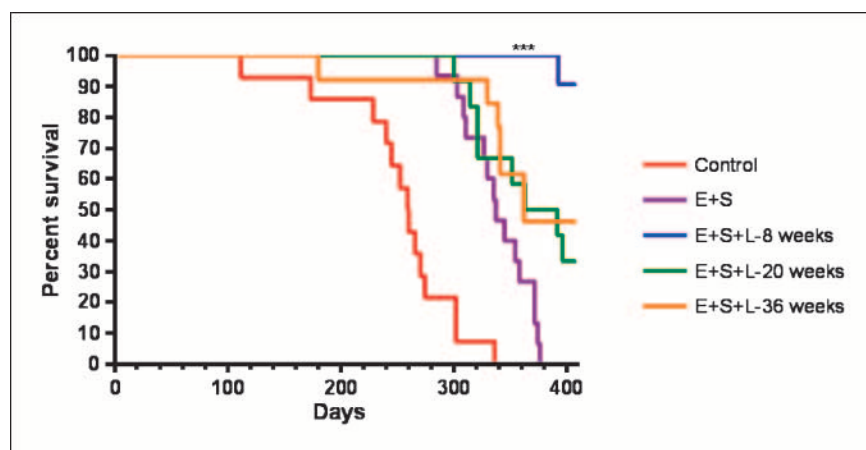
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**Fig. 1.** Increased survival of animals with micronutrient supplementation. Animals that were placed on a diet supplemented with either combination (E+S or E+S+L at 4, 8, 20, and 36 wk of age) showed a significant increase in survival ( $P < 0.0001$ , log rank) compared with the controls (\*, 8 versus 20; \*\*\*, E+S+L versus E+S; \*\*\*, control versus all groups).

prevention are rare. Prior non-a priori end point phase III studies as well as *in vitro* cell line data suggest that vitamin E and selenium (E+S) could potentially prevent PCa. Long-term clinical trials have shown a 30% to 70% reduction in the incidence of PCa following daily administration of micronutrients such as selenium (10, 19, 20). However, in all such studies, PCa reduction was not an a priori hypothesis; hence, these findings were hypothesis generating rather than testing proof of efficacy of the compounds. The NIH sponsored the Selenium and Vitamin E Chemoprevention Trial (SELECT), which assessed E+S as primary prevention for PCa (21, 22). This study is intended to provide definitive evidence of the benefit (or lack thereof) of these micronutrients in PCa prevention by 2012. However, it is not designed to provide information about the importance of timing of micronutrient administration, dose, combinations of micronutrients beyond E+S, or the genetic and molecular mechanisms by which these micronutrients achieve their effect. A recently published interim futility analysis (23) led to the cessation of this trial. It is our belief that strong preclinical data must exist to justify randomized trials. Although the failure of SELECT is disappointing, it remains plausible that other micronutrient combinations may have beneficial effect. Moreover, earlier administration of even E+S may be beneficial for a slowly progressive disease such as PCa, which actually begins when men are in their fourth decade of life. In this study, we have examined in an *in vivo* system a variety of micronutrient combinations and effect of timing of micronutrient administration.

The *Lady* transgenic PCa model has been used in our previous studies and was chosen to investigate these current issues. This *Lady* transgenic model is a less aggressive version of the original transgenic adenocarcinoma of the mouse prostate (TRAMP) and spontaneously develops metastatic PCa and mimics the progressive forms of human disease in many ways. The TRAMP model uses the SV40 early region that expresses both the small and large T antigens, whereas the *Lady* transgenic (LPB-Tag) uses only the large T antigen (24, 25). These mice develop precursor lesions [prostatic intraepithelial neoplasia (PIN)] followed by localized and eventually metastatic PCa, affording an opportunity to study many events in PCa progression. In addition, these animals predictably de-

velop invasive carcinoma with glandular differentiation (adenocarcinoma) as well as neuroendocrine PCa that commonly metastasizes to the liver (25).

Variation in micronutrient intake influences outcome in many ways, but among the most obvious is the association between micronutrient combination and tumor outcome. The present study was undertaken (a) to determine whether the inhibition of PCa seen with the combination of vitamin E, selenium, and lycopene (E+S+L) can be achieved with E+S alone and (b) to address the effect of timing of initiation of micronutrients on the prevention of PCa. These observations will be compatible with a mechanism involving micronutrient combination and intervention times on host tumor incidence and subsequently signaling pathways in neoplastic tissue.

## Materials and Methods

### Transgenic mice

The female *Lady* (12T-10) transgenic mice (25) developed on a pure background were obtained from Dr. Robert J. Matusik (Vanderbilt Prostate Center, Vanderbilt University Medical Center, Nashville, TN). Breeders were fed standard rodent diet obtained from Purina Mills Test Diet. After weaning of the animals by 4 wk of age, the gender of the offspring was determined, males were separated, and a tail biopsy was collected from each mouse. Tail DNA was used for determination of transgene incorporation by PCR. Transgenic animals were then randomized to the various groups. Two distinctive studies were carried out. (a) To investigate if the preventative effect of micronutrients was mediated by inhibiting cancer initiation or progression, transgenic males were randomized to one of the following groups: control diet (started on diet at 4 wk of age) and control diet supplemented with a combination of E+S+L were started at either 4, 8, 20, or 36 wk of age. (b) To examine the micronutrients that were essentially contributing to this reduced incidence, transgenic males were randomized at 4 wk of age to one of the following diets: control diet, control diet with a combination of E+S, and control diet supplemented with a combination of E+S+L. Transgenic males, included in experiments, were placed on formulated diets until the end of the treatment period (58 wk of age) or based on clinical condition when essential.

The standard rodent diet, which is commonly used for chemoprevention studies in animals, was used as the "control diet" and the base diet for preparation of the micronutrient-supplemented diets. The micronutrients added were based on our previous studies (8)

and were in proportion to the human equivalent of (per day) 800 IU vitamin E ( $\alpha$ -tocopherol succinate), 200  $\mu$ g selenium (seleno-DL-methionine), and 50 mg lycopene (based on a 70 kg male standardization). The micronutrient supplements were provided to Purina Test Mills for inclusion in the diets. The nutritional content of the standard diet was as follows: protein, 20.3%; fat, 25.2%; and carbohydrate, 54.5%. The diet was prepared at approximately 4- to 6-mo intervals, a period short enough to avoid depletion of vitamins by air oxidation. The diets were nonirradiated, stored at 4°C at all times, stable for 6 mo (assayed by the company), and free of phytoestrogens. This diet purchased from Purina Test Mills is a defined diet prepared according to good laboratory practice guidelines, with or without the supplements.

Throughout the study, animals were weighed every other week. Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the Sunnybrook Health Sciences Centre and the Canadian Council on Animal Care.

### Preparation and analysis of blood and tissues

Animals were sacrificed at the termination of the experimental period (58 wk of age) or based on clinical condition. All animals were examined at necropsy for gross organ abnormalities, kidney, liver, spleen, bladder, seminal vesicle, and prostate. Tissues collected at necropsy were routinely fixed in 10% (v/v) buffered formalin. Five-micrometer sections were cut from paraffin-embedded tissues, mounted, stained with H&E, and processed for histopathologic evaluation.

### Immunohistochemistry

Expression of p27<sup>Kip1</sup> and proliferating cell nuclear antigen (PCNA) was determined immunohistochemically. Paraffin sections of tissue blocks were deparaffinized, rehydrated, and boiled in citrate buffer (pH 7.0). Sections were blocked with 0.3% hydrogen peroxide in methanol followed by normal serum and incubated overnight at 4°C with the primary antibody [anti-p27<sup>Kip1</sup> rabbit polyclonal antibody (Santa Cruz Biotechnology) diluted 1:100 (200  $\mu$ g/mL) in PBS; PCNA rabbit polyclonal antibody (Santa Cruz Biotechnology) diluted 1:50 (200  $\mu$ g/mL) in PBS; and anti-SV40 Tag (AB-2) monoclonal antibody (Oncogene Research) diluted 1:100 (200  $\mu$ g/mL) in PBS]. Slides

were then reacted with biotin-labeled anti-rabbit IgG/anti-mouse IgG and incubated with preformed avidin-biotin peroxidase complex (Vector Laboratories). Metal-enhanced diaminobenzidine substrate (Vector Laboratories) was added and sections were counterstained with hematoxylin, dehydrated, and mounted.

### Detection of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

A modified terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique was used to detect apoptotic cells in paraffin sections with the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection System (Chemicon). Briefly, after deparaffinization, sections were boiled in 2× SSC (citrate) buffer (pH 6.0), and the endogenous peroxidase activity was blocked. Digoxigenin-labeled nucleotides were introduced by terminal deoxynucleotidyl transferase and stained using antidigoxigenin-peroxidase. Peroxidase activity on sections was detected by immersion in freshly mixed diaminobenzidine substrate and samples were counterstained with 0.5% methyl green. The number of positively stained cells per high-power field was counted in eight random fields and averaged, and the apoptotic index was calculated as described below.

### Immunoblotting

Prostate/tumor tissues from animals in each group were cut into 1-mm pieces and homogenized separately in ice-cold RadioImmuno Precipitation Assay lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol/L EDTA] supplemented with a mixture of protease and phosphatase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 0.02 mg/mL each of aprotinin, leupeptin, and pepstatin; Sigma Chemical Co.). Protein amounts were quantitated by Bradford analysis. Protein (40  $\mu$ g) suspended in lysis buffer was loaded in lanes of SDS-containing polyacrylamide gels, electrophoresed, and transferred to membranes (Immobilon transfer membrane, Millipore). Blots were blocked for 1 h with blocking buffer (5% nonfat dry milk in PBS containing 0.2% Tween 20) followed by sequential incubation with primary and secondary antibodies. Antibody-protein complexes were visualized by electrochemiluminescence. Densitometry was done using the Molecular Dynamics Imaging System and ImageQuant software.

**Table 1.** Effect of micronutrients on the incidence and histologic grade of the prostate tumors

Histologic grading	Control (n = 12), % (n)	E+S (n = 26), % (n)	E+S+L age of animals at intervention (wk), % (n)			
			4 (n = 10)	8 (n = 13)	20 (n = 10)	36 (n = 11)
No tumor	8.3 (1)	3.9 (1)	90 (9)*	76.9 (10)†	—	—
Prostate tumor						
HGPIIN	16.7 (2)	11.5 (3)	—	7.7 (1)	70 (7)	45.5 (5)
Adenocarcinoma	0 (0)	19.2 (5)	10 (1)	7.7 (1)	10 (1)	27.3 (3)
Undifferentiated	75 (9)	65.4 (17)	—	7.7 (1)	20 (2)	27.2 (3)

NOTE: Tumors that showed glandular differentiation were classified as adenocarcinoma. Tumors that were composed of sheets of round to spindle cells arranged in sheets were considered undifferentiated. Within this group, some of the cells showed features such as nuclear molding and scant cytoplasm, suggestive of neuroendocrine differentiation. Hyperplasia and low-grade PIN were reported under the category of no tumor. Tumor was subdivided into HGPIIN, adenocarcinoma, and undifferentiated carcinoma. Intervention when commenced within the first 8 wk of age resulted in a highly significant reduction in the incidence of PCa [90% ( $P < 0.0001$ ) and 76.9% ( $P < 0.0001$ ) reduction at 4 and 8 wk of age]. The supplementation of E+S had no major effect on reducing the incidence of PCa, with a high proportion of animals in the E+S group displaying PCa (84.6%) and a small proportion displaying HGPIIN (11.5%). This percentage was essentially comparable with the control animals that had no micronutrient intervention.

\*Significantly different from control ( $P < 0.0001$ ).

†Significantly different from control ( $P < 0.0001$ ).

**Table 2.** Effect of micronutrients on the incidence of liver metastasis

Histologic grading	Control (n = 12), % (n)	E+S (n = 26), % (n)	E+S+L age of animals at intervention (wk), % (n)			
			4 (n = 10)	8 (n = 13)	20 (n = 10)	36 (n = 11)
No metastasis	25 (3)	34.6 (9)	90 (9)*	84.6 (11) <sup>†</sup>	70 (7) <sup>‡</sup>	45.5 (5)
Metastatic undifferentiated	75 (9)	65.4 (17)	10 (1)	15.4 (2)	30 (3)	54.5 (6)

NOTE: Histologically, liver tissue was classified as normal or those that showed metastatic undifferentiated carcinomas. The incidence of liver metastasis was significantly lower if micronutrient intervention was commenced early on in the life of the animals [10% ( $P < 0.0001$ ) and 15.4% ( $P < 0.0001$ ) at 4 and 8 wk of age]. There was reduction in the frequency of liver metastasis in the animals supplemented with E+S, although this was not significant.

\*Significantly different from control ( $P < 0.0001$ ).

<sup>†</sup>Significantly different from control ( $P < 0.0001$ ).

<sup>‡</sup>Significantly different from control ( $P < 0.05$ ).

to quantitate the relative amounts of protein detected on Western blots. The following primary antibodies were used:  $\beta$ -actin mouse monoclonal antibody (Sigma) at 1:10,000 dilution and anti-SV40 Tag (AB-2) monoclonal antibody (Oncogene Research) diluted 1:100 (200  $\mu$ g/mL) in PBS. Horseradish peroxidase-labeled anti-mouse IgG (1:5,000 dilution) served as the secondary antibody.

#### Immunohistochemical evaluation and scoring—proliferation and apoptotic indices

Immunohistochemically stained slides were evaluated by two independent individuals in a blinded fashion without knowledge of the outcome or other immunohistochemical results. Paraffin sections from prostate tissue of all animals from the different groups were included in the study. Each histologic section was screened and assessed for the percentage of tumor nuclei displaying staining. When homogeneous distribution of the immunoreactivity was observed, sections were evaluated with a 40 $\times$  objective. In heterogeneous distribution of the immunoreactivity, the entire tissue section was screened using a 40 $\times$  objective to arrive at the estimated degree of positivity. Staining intensities were recorded by counting eight randomly selected fields of ~150 cells each. The proliferative and apoptotic index (%) was determined by counting the number of positively stained nuclei within the prostate tissue of *Lady* transgenic mice  $\times 100$  / total number of cells.

Samples were considered positive for p27 and PCNA and counted if nuclear staining was observed in 10% of tumor cells. For p27, the presence of cytoplasmic staining was not recorded as the staining was predominantly nuclear. In no case was there a change in category (positive/negative result) or a difference >5%. Images were captured using a 20 $\times$  objective with  $\times 200$  total magnification using an Olympus BX51 microscope with UPlanSApo objectives and Olympus Q-Colour-5 camera with Q capture Pro software.

#### Statistical method

Tumor development and the disease-free data were plotted for the various groups and the  $P$  value was calculated. Fishers' exact test was used to compare incidences of PCa in different groups. The significance of differences for immunohistochemical analysis was calculated using the Student's  $t$  test. Results were considered significant at the 5% critical level ( $P < 0.05$ ). All calculations were done using Statistical Analysis System (version 9.1.3 for Windows; SAS Institute) and S-PLUS (version 6.2 for Windows; MathSoft) statistical software packages.

## Results

#### Effect of micronutrients on body weight

Animal weights were measured biweekly. Differences in body weight between the groups were not apparent at any

time during the study weeks after commencement of the diets. All animals placed in the various groups were observed to have had normal weight gain. Daily supplementation with micronutrients through the diet was well tolerated with no evidence of micronutrient-related toxicity (data not shown).

#### Effect of tumor growth and survival

Kaplan-Meier analysis was done to compare survival of transgenic mice on micronutrient-enriched and control diets (Fig. 1). Log-rank test showed a survival advantage with any of the micronutrient combination. At 58 weeks of age (study end point), we observed that there was a significant increase in the survival proportion of the animals in all of the intervention groups compared with the controls. Animals that were placed on a diet supplemented with either combination (E+S or E+S+L) or initiated micronutrients (E+S+L) at 8, 20, and 36 weeks of age showed a significant increase in median survival ( $P < 0.0001$ , log rank) compared with the controls (Fig. 1). Animals in a control group could not be continued on the diet beyond 48 weeks of age due to tumor burden. All mortality cases were primarily of PCa-related causes and included a very small proportion (control, 2 animals; E+S, 3 animals; E+S+L 20 weeks, 2 animals; and E+S+L 36 weeks, 2 animals). To examine the effect of the diets on mortality specifically from PCa, we did survival analysis excluding those mice that died of non-PCa-related causes.

#### Early commencement of micronutrients reduced the incidence of PCa and liver metastasis

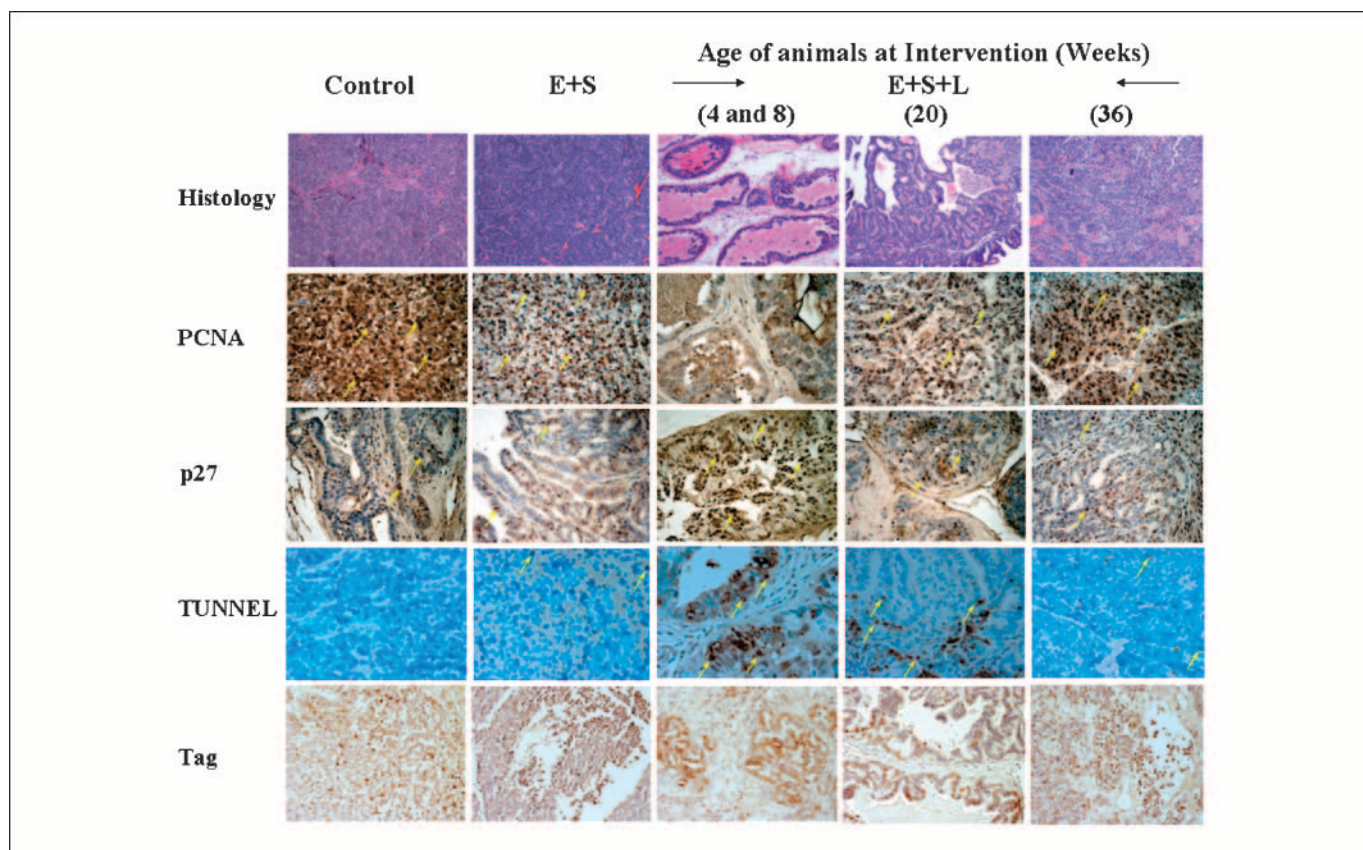
Dietary micronutrients (E+S+L) significantly reduced the incidence of PCa in *Lady* transgenic mice. It was noted that 75% of the animals that provided a control diet had developed PCa and 16.7% high-grade PIN (HGPIN). On the contrary, only a very small proportion (10%) of the animals on the E+S+L (started on the supplemental diet at 4 weeks of age) developed tumors. The remaining 90% had normal, benign prostates ( $P < 0.0001$ ). Animals were also initiated on E+S+L at different times beginning at 4, 8, 20, or 36 weeks of age. Supplementation was continued until the end of the treatment period (maximum of 58 weeks of age). Intervention with a combination of E+S+L when commenced within 8 weeks of age resulted in a highly significant reduction in the incidence of PCa (76.9% had normal prostate;  $P < 0.0001$  at 8 weeks of age; Table 1).

None of the animals that commenced on micronutrients at 20 and 36 weeks of age had normal prostates. However, commencement of micronutrients at 20 weeks of age resulted in a significant number (70%;  $P < 0.0001$ ) of animals with HGPIN with a substantial incidence (30%) of PCa. Interestingly, animals that started on the supplemented diet at 36 weeks of age had higher PCas (54.5%) and an equally high number presenting with HGPIN (45.5%). It was very evident that delaying intervention times beyond 8 weeks of age resulted in a significantly higher proportion of animals developing HGPIN at 20 weeks of age and with further delay in intervention to 36 weeks of age resulted in increase in the number of cancers relatively no animals bearing normal prostate (Table 1).

HGPIN, a known precursor of invasive carcinoma, is defined as atypical ductal or acinar epithelium with nuclear enlargement, hyperchromatism, and chromatin clumping, confined to the duct or acinus without evidence of stromal invasion. In the animal model, PIN may form large masses

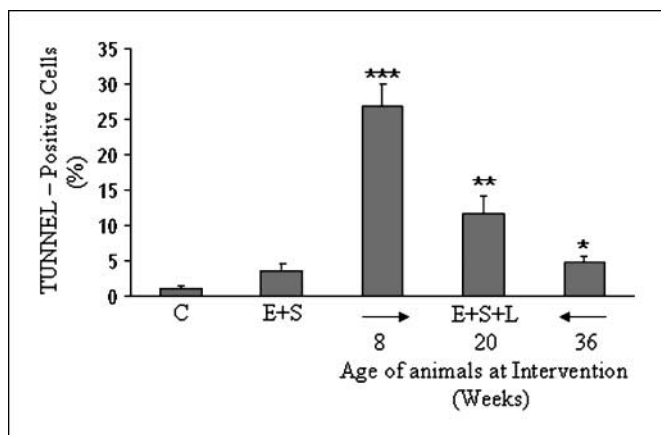
that may be grossly evident, but microscopically, the masses have regular rounded borders. Invasive carcinoma has a growth pattern that in contrast to PIN exhibits destructive local invasion. Tumors may show glandular differentiation (adenocarcinoma), squamous differentiation (squamous cell carcinoma), or neuroendocrine differentiation (neuroendocrine carcinoma). Tumors lacking morphologic features of specific differentiation are classified as undifferentiated carcinoma.

In our study, tumors that showed glandular differentiation were classified as adenocarcinoma. Tumors that were composed of sheets of round to spindle cells arranged in sheets were considered undifferentiated. Within this group, some of the cells showed features such as nuclear molding and scant cytoplasm, suggestive of neuroendocrine differentiation. Hyperplasia and low-grade PIN were reported under the category of "no tumor." Tumors were subdivided into HGPIN, adenocarcinoma, and undifferentiated carcinoma (Table 1).



**Fig. 2.** Histopathology and immunohistochemical analysis of animals supplemented different micronutrient combinations and at varying intervention times. Prostate tissue from animals on the various micronutrient combinations that were collected at necropsy was fixed in 10% formalin, processed, and embedded in paraffin, cut at 5  $\mu$ m of thickness, and stained with H&E. Animals that commenced on E+S+L within the first 8 wk of age had normal prostate. Animals on E+S+L diet had normal prostate compared with the animals on E+S or standard diet. Staining intensities were recorded by counting eight randomly selected fields of ~150 cells each. The proliferative and apoptotic index (%) was determined by counting the number of positively stained nuclei within the prostate tissue of *Lady* transgenic mice  $\times 100$  / total number of cells. Staining intensities are expressed as percentages. Images were captured using a 20 $\times$  objective with  $\times 200$  total magnification.  $P$  value expressed calculated as that significantly different from control. Histology: control, undifferentiated; E+S, undifferentiated; E+S+L (4 and 8 wk of age), normal; E+S+L at 20 wk of age, HGPIN; E+S+L at 36 wk of age, adenocarcinoma. PCNA: staining intensity: control,  $52 \pm 7.2\%$ ; E+S,  $43.8 \pm 7.2$  ( $P < 0.05$ ); E+S+L intervention at 4 and 8 wk of age,  $25.2 \pm 2.5\%$  ( $P < 0.0001$ ); E+S+L intervention at 20 wk of age,  $46.2 \pm 8.9\%$  ( $P < 0.001$ ); E+S+L intervention at 36 wk of age,  $31 \pm 3.7\%$  ( $P < 0.05$ ). p27<sup>Kip1</sup>: staining intensity: control,  $<10\%$ ; E+S,  $<10\%$ ; E+S+L intervention at 4 and 8 wk of age,  $46.2 \pm 8.9\%$  ( $P < 0.001$ ); E+S+L intervention at 20 wk of age,  $<10\%$ ; E+S+L intervention at 36 wk of age,  $<10\%$ . TUNEL: apoptotic index: control,  $1 \pm 0.5$ ; E+S,  $3.6 \pm 0.85\%$ ; E+S+L intervention at 4 and 8 wk of age,  $26.8 \pm 3.1\%$  ( $P < 0.0001$ ); E+S+L intervention at 20 wk of age,  $11.8 \pm 2.4\%$  ( $P < 0.001$ ); E+S+L intervention at 36 wk of age,  $4.8 \pm 0.9\%$  ( $P < 0.05$ ). Tag: staining intensity: control,  $49.3 \pm 2.9\%$ ; E+S,  $47.3 \pm 3.5\%$ ; E+S+L intervention at 4 and 8 wk of age,  $30.8 \pm 2.3\%$  ( $P < 0.05$ ); E+S+L intervention at 20 wk of age,  $37.6 \pm 2.1\%$  ( $P < 0.05$ ); E+S+L intervention at 36 wk of age,  $43.6 \pm 3.8\%$ .

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**Fig. 3.** Graphical representation of the percentage of apoptosis induced by micronutrients as determined by immunohistochemistry. The apoptotic index (%) was calculated in the various groups by immunohistochemical analysis. The apoptotic index (%) was determined by counting the number of positively stained nuclei within the prostate tissue of *Lady* transgenic mice  $\times 100$  / total number of cells. Control,  $1 \pm 0.5$ ; E+S,  $3.6 \pm 0.85$ ; E+S+L intervention at 4 and 8 wk of age,  $26.8 \pm 3.1$  ( $P < 0.0001$ ); E+S+L intervention at 20 wk of age,  $11.8 \pm 2.4$  ( $P < 0.001$ ); E+S+L intervention at 36 wk of age,  $4.8 \pm 0.9$  ( $P < 0.05$ ).

However, all animals in this group showed improved survival compared with the controls.

At necropsy, *Lady* transgenic animals on a control diet showed a 75% incidence in liver metastasis by the end of the intervention period (Table 2). The incidence of liver metastasis was significantly lower if micronutrient intervention was commenced early on in the life of the animals [10% ( $P < 0.0001$ ) and 15.4% ( $P < 0.0001$ ) at 4 and 8 weeks of age]. Along with the reduction in the incidence of PCa with micronutrient supplementation, there was also a significantly lower incidence of liver metastasis, although micronutrients were commenced at 20 weeks of age (30% at 20 weeks of age;  $P < 0.05$ ) compared with controls (75%). Histologically, liver tissue from 20- and 36-week-old animals showed metastatic undifferentiated carcinomas similar to that from control animals, whereas animals commenced on micronutrients from 4 and 8 weeks of age had histologically normal livers (data not shown).

#### **Administration of micronutrients at an early age reduced the expression of proliferative marker (PCNA) and increased the extent of apoptosis in the prostate of *Lady* transgenic mice**

Histologic analysis revealed control tumors to be mostly undifferentiated. Animals started on the E+S+L-supplemented diet at 4 and 8 weeks of age had normal benign prostates compared with HGPIN and adenocarcinoma/undifferentiated tumors at 20 and 36 weeks of age (Fig. 2). Micronutrient consumption resulted in a significant reduction in proliferation of prostate tissue with reduced expression of proliferative cell nuclear antigen (Fig. 2). Delayed intervention (later than 8 weeks of age) was associated with increased expression of PCNA comparable with that of the untreated control. Scoring of prostate tissue for antigenic staining revealed that  $52 \pm 7.2\%$  of nuclei in the control tissue stained positive for PCNA. Tissue from animals that commenced at 4 and 8 weeks of age had  $<10\%$  ( $P < 0.0001$ ) of positively stained nuclei, escalating sig-

nificantly with delayed intervention [ $25.2 \pm 2.5\%$  ( $P < 0.05$ ) and  $31 \pm 3.7\%$  ( $P < 0.05$ ) at 20 and 36 weeks of age, respectively; Fig. 2].

Another important observation of our study was a marked induction of apoptosis in the prostate with micronutrient treatment. The present study has shown that when a decrease in proliferation was observed with the administration of micronutrients, there was an associated increase in the apoptotic index. A combination of E+S+L initiated at an early age selectively induces apoptosis of cells ( $26.8 \pm 3.1\%$  at 4 and 8 weeks versus  $1 \pm 0.5\%$  in the control;  $P < 0.0001$ ; Figs. 2 and 3). Delaying the time of intervention beyond 8 weeks resulted not only in a significant proportion of cells expressing PCNA but also fewer apoptotic cells, although not highly significant yet showing the potency of the combination [ $11.8 \pm 2.4\%$  ( $P < 0.001$ ) and  $4.8 \pm 0.9\%$  ( $P < 0.05$ ) at 20 and 36 weeks of age, respectively; Figs. 2 and 3].

#### **Intervention with micronutrients early on in life altered cell cycle regulation with up-regulation of prognostic tissue marker p27<sup>Kip1</sup>**

Immunohistochemistry done on formalin-fixed paraffin-embedded tissue showed a strong correlation between disease-free state and increased levels of p27<sup>Kip1</sup>. A uniformly intense immunoreactivity for p27<sup>Kip1</sup> was seen in the early intervention groups (Fig. 2). Staining for p27 was predominantly nuclear. The staining intensity in the tissue of E+S+L-treated animals at 4 and 8 weeks of age was  $46.2 \pm 8.9\%$  compared with  $<10\%$  staining in the untreated controls (graded by two blinded individuals;  $P < 0.001$ ). In the delayed intervention groups, there was a significant decline in the levels of expression of p27 ( $<10\%$  in the 20 and 36 weeks of age).

#### **Lycopene is an essential component of the PCa prevention combination**

The supplementation of E+S had no major effect on reducing the incidence of PCa, with a high proportion of animals in the E+S group displaying PCa (84.6%), a small proportion developing HGPIN (11.5%), and 3.9% that were normal (Table 1). This percentage was essentially comparable with the control animals that had no micronutrient intervention, wherein 75% of the animals developed PCa and 16.7% with HGPIN (Fig. 2). However, there were a small proportion of tumors in the E+S-treated group (19.2%) that were classified as adenocarcinoma, 65.4% as undifferentiated, and the remaining 11.5% as HGPIN (Table 1; Fig. 2). It is to be noted that animals on a E+S-supplemented diet showed a significant reduction in the wet weight of the tumors obtained at necropsy ( $1.62 \pm 0.62$  g versus  $3.35 \pm 0.57$  g;  $P < 0.001$ ; Fig. 4). Histologic analysis revealed a reduction in the frequency of liver metastasis in animals on a E+S diet, although this was not significant (Table 2).

#### **Administration of E+S had no significant effect in the expression of proliferative marker (PCNA), apoptotic index, or cell cycle marker, p27<sup>Kip1</sup>**

Although the administration of E+S had no significant reduction in the incidence of PCa, there was a significant reduction in tumor wet weight accompanied by an overall survival benefit. Nuclear staining for PCNA was scored at  $52 \pm 7.2\%$  in the control tissue,  $43.8 \pm 7.2\%$  in the E+S group ( $P < 0.05$ ), and

<10% of the E+S+L group ( $P < 0.0001$ ; Fig. 2). The staining intensity for p27<sup>Kip1</sup> in the animals placed on E+S was comparable with the untreated control with a <10% staining, compared with E+S+L that exhibited a highly significant staining of  $46.2 \pm 8.9\%$  ( $P < 0.001$ ; Fig. 2). A combination of E+S caused no significant effect in apoptosis. The apoptotic index was  $3.6 \pm 0.85\%$  with E+S treatment compared with  $26.8 \pm 3.1\%$  ( $P < 0.0001$ ) with E+S+L treatment (Figs. 2 and 3).

### Expression of large T antigen in prostate of *Lady* transgenic mice

In view of the fact that prostate tumorigenesis in the transgenic mice is driven by the expression of SV40 T antigen specifically in prostate epithelial cells, it is always desirable to find the effect of a given chemopreventive/antitumor agent on the expression levels of SV40 T antigen. Hence, we analyzed the expression of Tag in prostate of mice by immunohistochemical staining on paraffin sections and Western blot on tissue lysates. As visualized in Fig. 2 (immunohistochemical staining), by counting the number of positively stained nuclei, we observed that Tag oncoprotein was expressed in the prostates of all *Lady* transgenic mice treated with and without micronutrients in the different groups, but with some degree of variability. The percentages of Tag-positive cells were the following: control,  $49.3 \pm 2.9\%$ ; E+S,  $47.3 \pm 3.5\%$ ; E+S+L intervention at 4 and 8 weeks of age,  $30.8 \pm 2.3\%$ ; E+S+L intervention at 20 weeks of age,  $37.6 \pm 2.1\%$ ; and E+S+L intervention at 36 weeks of age,  $43.6 \pm 3.8\%$  (Figs. 2 and 5A).

Results from Western blot analysis of the prostate/tumor tissue and subsequent densitometric analysis correlated with the immunohistochemical analysis (Fig. 5B). When the appearance of cancer was inhibited, with more normal cells, we observed slightly lower levels of Tag expression. This is not surprising because, depending on early stage before transformation or at late stages, there are different cell populations present, resulting in different levels of expression of Tag. Hence, changes in Tag expression maybe due to a mixture of cell types rather than an actual change in gene expression per cell. This is suggestive of the fact that the mechanism of a combination of micronutrient action against PCa is not related to

altered or down-regulation of the transgene expression but rather to direct suppression of carcinogenesis.

### Discussion

Epidemiologic and clinical evidence suggests that micronutrients may be of value for the prevention and control of PCa (3, 26, 27). Using the *Lady* transgenic model, we show a definite association between a micronutrient-supplemented diet, disease-free state, and increased survival benefit. This was particularly evident with a combination of E+S+L that is in line with our previously reported *in vitro* synergy between micronutrients (7). This is with specific bearing to their antioxidant action and growth-inhibitory effect on PCa cells (7, 14, 27, 28), as well as their additive effects *in vitro* on androgen receptor target gene expression and oxidative stress reduction in prostate tumors (29).

In our study of varying the time of initiation of micronutrients (E+S+L), we observed that the percentage of animals with normal prostate decreased significantly with increasing age at which supplementation was instituted. The percentage of animals with normal prostate was 90% and 76.9% when E+S+L was given at the beginning of weaning (4 and 8 weeks of age) and practically no animal detected with normal prostate when commenced at 20 and 36 week of age (Table 1). Although initiation was commenced at 36 weeks of weaning, 54.5% of the animals had cancer, of which 27.3% were adenocarcinoma, 27.2% were undifferentiated, and the remaining 45.5% had HGPIN. It is to be noted that a small proportion (10%) of animals in the E+S+L group that were commenced on the micronutrients at 4 weeks of age developed PCa and were classified as adenocarcinomas. In this group that was classified as adenocarcinoma, the prostate tissue that was sampled was replaced by tumor with no residual benign glands to evaluate for HGPIN. It is likely that were the animals sacrificed at an earlier date, HGPIN would have been detected.

Development and progression of PCa is associated with the loss of normal epithelial morphology, along with concomitant acquisition of invasive, metastatic, and ultimately fatal properties (30, 31). *Lady* transgenic animals go through different stages of tumor development from PIN to neoplasia that mimic the development of human PCa. These unique features of the *Lady* transgenic model in an age-specific manner provide opportunities to conduct studies in cancer prevention and therapy at various stages of disease progression. It was interesting to note that when intervention times were delayed beyond 8 weeks of age, there were still a significant proportion of animals developing HGPIN (70% and 45.5% at 20 and 36 weeks of age). This reemphasizes the importance of micronutrient supplementation at the time of development of microfoci so as to prevent both cancer as well as HGPIN. However, a micronutrient-supplemented diet started at 36 weeks of age still had an influence on tumor differentiation and survival. This study raises the compelling question of the effect of lycopene alone in future investigations. We speculate that the micronutrients act in synergy to exhibit the maximal effect and lycopene would be required in combination with E+S to obtain this desired effect.

Among the carotenoids,  $\beta$ -carotene and lycopene have been studied with respect to their possible roles in preventing PCa (3, 4, 14, 27, 32–34). The results of several epidemiologic stud-

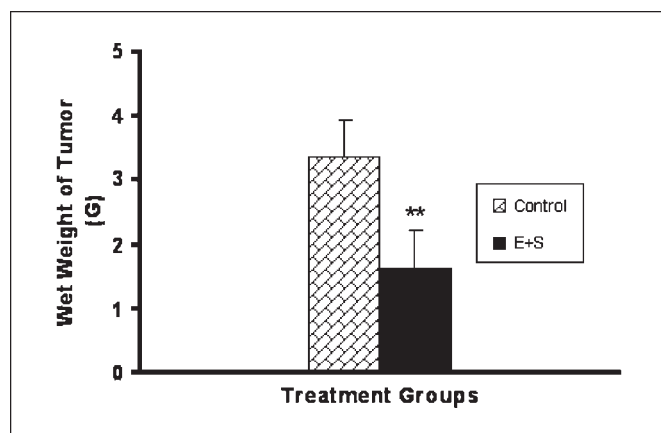
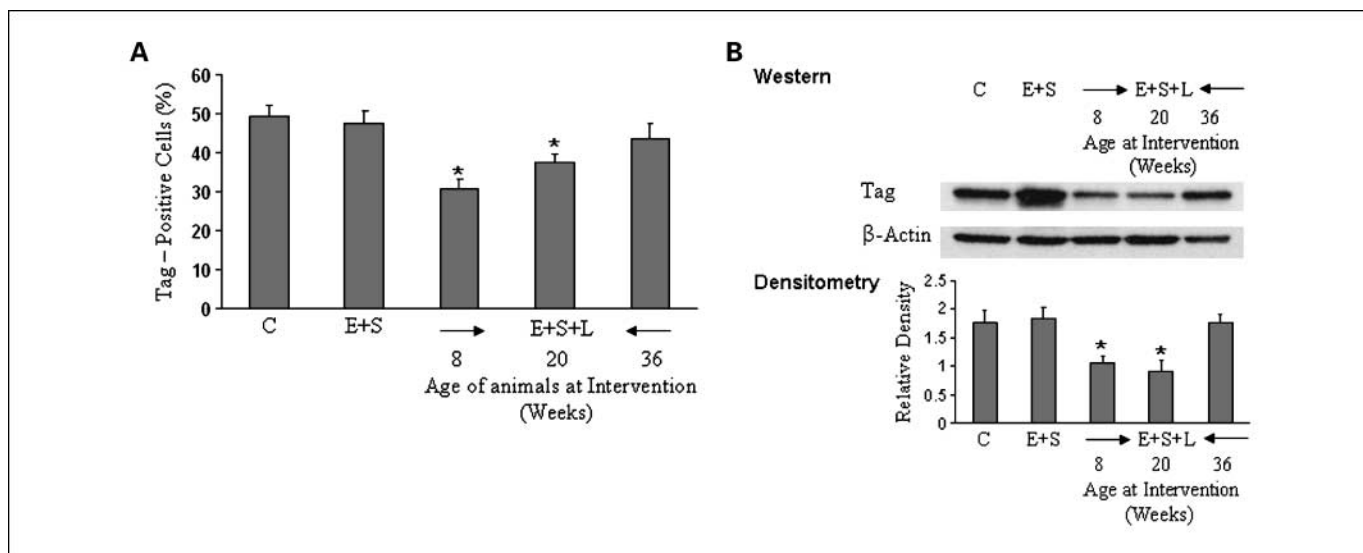


Fig. 4. Wet weight of the prostate tumors obtained at necropsy. Animals on a E+S-supplemented diet showed a significant reduction in the wet weight of the tumors obtained at necropsy. Tumor weights in the E+S group weighed  $1.62 \pm 0.62$  g versus  $3.35 \pm 0.57$  g in the controls ( $P < 0.001$ ).



**Fig. 5.** A, graphical representation of the percentage of Tag-positive epithelial cells as determined by immunohistochemical analysis. There was expression of T antigen in the prostate tissue of all animals in the various groups in varying intensities, irrespective of supplementation with micronutrients. Staining intensity: control,  $49.3 \pm 2.9\%$ ; E+S,  $47.3 \pm 3.5\%$ ; E+S+L intervention at 4 and 8 wk of age,  $30.8 \pm 2.3\%$  ( $P < 0.05$ ); E+S+L intervention at 20 wk of age,  $37.6 \pm 2.1\%$  ( $P < 0.05$ ); E+S+L intervention at 36 wk of age,  $43.6 \pm 3.8\%$ . B, quantitation of T antigen in the various groups ascertained by Western blot analysis and densitometry: T antigen was detected on immunoblots by electrochemiluminescence from protein obtained from prostate tissue/tumor lysates.  $\beta$ -Actin was used as an internal control for protein loading and transfer. One representative sample is depicted for each group. Densitometric analysis was done (Tag band intensities adjusted with  $\beta$ -actin) using the Molecular Dynamics Imaging System and ImageQuant software to quantitate the relative amounts of protein detected on Western blots.

ies investigating the relationship between  $\beta$ -carotene intake and risk of PCA have been inconsistent (35, 36). Until recently, only *in vitro* data (37, 38) have supported the anticancer role played by lycopene; however, their efficacy *in vivo* has been convincing to a much lesser extent. There have been few studies *in vivo* that have shown a chemoprevention effect of lycopene either alone or in combination with certain selected micronutrients. This could be explained by the fact that, apart from optimizing the dose and form of lycopene, its combination with other micronutrients or phytochemicals might prove to be more effective in inhibiting PCA *in vivo*. A high intake of tomato-based products resulting in a considerable exposure to lycopene has been correlated with a reduced PCA risk (39, 40). Lycopene is present in the human prostate at significant concentrations (41), a finding that rendered lycopene as an attractive candidate for primary PCA chemoprevention.

Vitamin E and selenium have been studied for their growth-inhibitory effect in different model systems. Previous studies have shown the beneficial effect with reduced mortality from PCAs among subjects receiving  $\alpha$ -tocopherol in the Alpha-Tocopherol Beta-Carotene trial (42). We have previously published that vitamin E inhibits the growth of PCA cells *in vitro* mediated by alteration in cell cycle regulatory molecules, specifically p27 (5). In a second study, we have shown that a combination of E+S was shown to potentiate or act in synergy, thereby enhancing the proportion of cancer prevention (7). It is possible that vitamin E is effective only under certain situations and in selected model systems. Although a combination of E+S was not effective as that of E+S+L in impeding prostate tumor growth, it was still beneficial in reducing tumor burden as well as increasing overall survival rates.

Our observations provide support for the concept that intervention with E+S+L initiated at an early age was beneficial in reducing the PCA incidence and increasing disease-free survival.

In addition, we observed a marked reduction in the levels of expression of the proliferative marker (i.e., PCNA). Increased proliferation of PCA cells ultimately results in tumor invasion and metastasis, leading to significant mortality in humans (43). Unfortunately, >60% of the newly diagnosed cases of PCA develop metastatic forms of the disease (44). In the present study, a combination of E+S+L at an early age was found to be effective in completely abolishing distant site metastases and cellular proliferation as shown by the proliferation markers. PCNA serves as a requisite auxiliary marker for increased proliferation for DNA polymerase  $\delta$ -driven DNA synthesis and is cell cycle regulated (45). As shown in Fig. 2, administration of micronutrients markedly suppressed proliferation and PCNA protein expression in the prostates of *Lady* transgenic mice. Results reveal that  $52 \pm 7.2\%$  of nuclei in the control tissue that were stained positive for PCNA, compared with <10% in the prostate tissue from E+S+L-treated animals, commenced at 4 and 8 weeks of age ( $P < 0.0001$ ).

We have further evidence that these micronutrients mediate their effect through p27<sup>Kip1</sup>, an important marker implicated in the prognosis of several cancers (46–48). Our results are in accordance with previously reported data that frequent loss of cyclin-dependent kinase inhibitor p27 in PCA is correlated with advancing biological aggressiveness, implicating deregulation in tumor progression. We show that supplementation with E+S+L augmented expression of p27<sup>Kip1</sup> similar to that seen in normal tissue. Although there are several mechanisms by which the individual micronutrients function, a combination of E+S+L increased the cell cycle inhibitor p27. Many of these antioxidants, including lycopene, hamper cancer cell proliferation, thus indicating that they possess the therapeutic capability (49, 50). Their capacity to inhibit tumorigenesis, both at the initiation stage (as antioxidants) and the progression stages (as cancer cell proliferation inhibitors), may have



secured their initial success in the chemoprevention studies (51). We suggest a similar possibility that the observed ability of the E+S+L diet to decrease rate of prostate tumorigenesis may be related to a combination of both the antioxidant potential and the inhibitory activity against PCa cell proliferation.

Another important observation of our study was a marked induction of apoptosis in the prostate by micronutrient treatment. In recent years, apoptosis has gained much attention as a preferential way of eliminating the unwanted cancerous cells (44, 52–56). At present, only a few agents are known to possess the potential for selective elimination of cancer cells (57). Studies from our laboratory have shown that a combination of E+S+L initiated at an early age selectively induces significant apoptosis of cancerous cells without affecting the normal cells. Similar observation has been reported from many laboratories worldwide (53–56). Our results show that micronutrients administered to *Lady* transgenic mice results in massive apoptosis of neoplastic prostatic cells and further suggest that the combination of micronutrients could be an effective agent for a preferential elimination of cancerous and precancerous cells via a programmed cell death. Based on our data, we believe that the observed inhibition of PCa tumorigenesis and subsequent metastasis through the use of micronutrients (E+S+L) is caused by decreased proliferation with increased apoptosis.

Tag oncoprotein was expressed in varying levels in the prostates of *Lady* transgenic mice treated with and without micronutrients. If you inhibit the appearance of cancer, then likely you will have more normal cells that do not express Tag. The problem still is that, depending on when we look at the tissue, early before transformation or at late stages, there will be different cell populations present. Early stage will have normal, whereas when PIN appears, there is still some normal-looking cells, and late when neuroendocrine cancer occurs most cells are involved. Therefore, changes in Tag expression maybe due to a mixture of cell types rather than an actual change in gene expression per cell (58, 59). This is suggestive of the fact that the mechanism of a combination of micronutrient action against PCa is not related to altered or down-regulation of the transgene expression but rather due to direct suppression of carcinogenesis.

Lippman et al. (23) has most recently published secondary analyses of the randomized controlled trial “SELECT” where selenium or vitamin E, alone or in combination at the doses and formulations used, did not prevent PCa in this population of relatively healthy men. These were men 50 years or older (African-American men) or 55 years or older (all other men), with a serum prostate-specific antigen level of 4 ng/mL or less and a digital rectal examination not suspicious for PCa. These findings are timely as we have analogous findings using our *in vivo* transgenic model system that a combination of E+S was not effective in reducing the incidence of PCa. Interestingly, we have also reported in our present study that the addition of lycopene to the combination caused a significant effect in reduction in tumor incidence as well as increasing survival of the animals. We have also shown that the observed beneficial effect was pronounced when the supplementation was initiated at an early age, at the time of microfoci development.

We are currently assessing the mechanism by which the addition of lycopene to the combination of E+S may have inhibited

tumor growth. We speculate that micronutrients can influence a variety of biological processes and through mechanisms dependent and/or independent of their antioxidant functions. They may lower oxidative stress and affect hormone and growth factor signaling, cell communication and apoptosis, or, as seen in this situation, a cell cycle arrest. It has been documented in literature that reactive oxygen species is associated with carcinogenesis and tumor progression in prostate and several other tissues. This can be the basis of oxidative damage if not trapped efficiently in the tissue (51). During aging, the prostate tissue gradually loses the antioxidant capability (60), making the prostatic epithelium highly sensitive to oxidative stress-induced mutation, leading to the neoplastic transformations (61). There are a few topical publications that show an additional benefit of combining dietary chemopreventive agents (62) or micronutrients, particularly vitamin E or selenium (8), for reduction of PCa growth (63). Combination of dietary agents and their resultant interaction are probably due to the synergistic effect of compounds against the cancer cell proliferation, in addition to their probable antioxidant activity of the individual compounds (51). On the other hand, cooperative interaction between these micronutrients may result from a different mechanism of action or a direct effect of nutrients on each other. However, antioxidant activity is the only prevailing suggested individual mechanism of action for lycopene, selenium, vitamin E, and other chemoprevention agents that are currently being tested in clinical trials for PCa.

When the events in the progression of a normal cell to a malignant lesion are considered, many targets can be visualized that can serve as windows of opportunity for chemoprevention of cancer. These opportunities involve both inhibition and induction of pathway(s) by chemopreventive agents. Hence, it is crucial to identify a “cocktail approach” that relies on a combination of agents, which individually are effective against distinct targets. These agents have a unique function and distribution. In addition, as seen in our present study, although specific agent played a definitive role in cancer prevention, the synergistic/additive effects of another agent working through similar or different mechanism(s) enhance the efficacy in a positive manner.

Insufficient clinical evidence exists to warrant recommending drastic dietary changes to patients to reduce their risk of PCa. However, despite the plethora of confounding factors present in clinical studies to assess these effects and conditions, the sum total of published data including our own remains compelling with regard to the potential for the micronutrients in PCa prevention. Our compelling evidence presented in this article motivates further work for large-scale phase III/IV trials that are eventually needed for proof of clinical benefit with regard to initiating micronutrients and the benefit of selected cocktails.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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