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# Changes in Serum Proteomic Patterns by Presurgical $\alpha$ -Tocopherol and L-Selenomethionine Supplementation in Prostate Cancer

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## Abstract

**Background:** Evidence of the chemopreventive effects of the dietary antioxidants  $\alpha$ -tocopherol (vitamin E) and L-selenomethionine (selenium) comes from secondary analysis of two phase III clinical trials that found treatment with these antioxidants reduced the incidence of prostate cancer. To determine the effects of selenium and vitamin E in blood and prostate tissue, we undertook a preoperative feasibility study complementary to the currently ongoing Selenium and Vitamin E Cancer Prevention Trial.

**Methods:** Forty-eight patients with clinically localized prostate cancer enrolled on this 2  $\times$  2 factorial design study were randomized to take selenium, vitamin E, both, or placebo for 3 to 6 weeks before prostatectomy. Sera were collected from patients before and after dietary supplementation. Thirty-nine patients were evaluable, and 29 age-matched disease-free men served as controls. Mass profiling of lipophilic serum proteins of lower molecular weight (2-13.5 kDa) was

conducted, and mass spectra data were analyzed using custom-designed software.

**Results:** Weighted voting analyses showed a change in sera classification from cancerous to healthy for some patients with prostate cancer after dietary intervention. ANOVA analysis showed significantly different treatment effects on prediction strength changes among the four groups at a 95% confidence level. Eliminating an outlying value and performing post hoc analysis using Fisher's least significant difference method showed that effects in the group treated with the combination were significantly different from those of the other groups.

**Conclusion:** In sera from patients with prostate cancer, selenium and vitamin E combined induced statistically significant proteomic pattern changes associated with prostate cancer-free status. (Cancer Epidemiol Biomarkers Prev 2005;14(7):1697-702)

## Introduction

No reliable and sensitive method currently exists to diagnose prostate cancer or to monitor therapeutic interventions to treat it. Despite the widespread clinical use of the serum prostate-specific antigen test, it is neither sufficiently sensitive nor specific for early detection of prostate cancer, and it is not predictive of biological behavior of tumors (1-3). Because of the inherent heterogeneity of prostate tumors, it is likely that a panel of proteins could serve better than the prostate-specific antigen as "signatures" for modulation of the molecular and cellular events occurring during carcinogenesis and therapeutic intervention. In fact, studies in several different tumor types, including prostate, brain, gastrointestinal, breast, ovarian, and hepatocellular cancers, have shown that serum protein patterns could be used to differentiate cancer from controls (4-9). Proteomics, because it considers all serologic proteins, could potentially identify multiple biomarkers instead of only one. Although none of these proteins alone merits total reliance, together their profiling pattern, assisted by statistical analyses, could yield a quick and reliable

diagnosis of prostate cancer (4, 10, 11). Furthermore, this qualitative approach is able to reveal the molecular and cellular effects of therapeutic or dietary intervention in a period as short as several weeks.

Selenium and vitamin E are important intracellular antioxidants, but their function in preventing prostate cancer was initially discovered in studies intended to investigate their chemopreventive role in other cancers. Selenium's effect on prostate cancer, for example, was found during a study of its effects on skin cancer (12). In a randomized controlled study, 1,312 patients with skin cancer were given 200  $\mu$ g of selenium orally (in the form of selenized yeast) or placebo daily. The study did not find that selenium treatment protected against the development of skin cancer, but a secondary analysis of the results revealed a 63% reduction of prostate cancer in patients treated with selenium. In an update from the Nutritional Prevention of Cancer trial, investigators reported finding a continued protective effect by supplemental selenium in data through the end of the blinded treatment in 1996. Selenium reduced the overall incidence of prostate cancer by 49%, albeit the effect was limited to patients with lower baseline prostate-specific antigen and plasma selenium concentrations (<123.2 ng/mL; ref. 13).

Likewise, findings of the chemopreventive effects of vitamin E ( $\alpha$ -tocopherol) were also from secondary analysis of another study. The  $\alpha$ -tocopherol,  $\beta$ -carotene study, which evaluated 29,133 male smokers between 50 and 69 years of age from southwestern Finland, was initially designed to find vitamin E's chemopreventive effect against lung cancer as measured in incidence. Results showed no reduction in lung

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**Note:** J. Kim and P. Sun contributed equally to this work.

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cancer incidence after 5 to 8 years of dietary supplementation of  $\alpha$ -tocopherol or  $\beta$ -carotene (14); however, a secondary study of the 6-year follow-up showed a significant reduction in prostate cancer incidence among participants treated with vitamin E. Inasmuch as these studies were initially designed for lung and skin cancer, findings related to prostate cancer must be treated with caution. However, a prospective randomized clinical trial with reduction in the incidence of prostate cancer as an a priori hypothesis is still needed to evaluate the role of vitamin E and selenium as chemopreventive agents for prostate cancer.

In 2001, a phase III chemoprevention trial in prostate cancer—the Selenium and Vitamin E Cancer Prevention Trial (SELECT)—was initiated. The primary end point of SELECT is the detection of a 25% reduction in the incidence of prostate cancer for selenium or vitamin E alone with an additional 25% decrease for the combination. This multicenter, randomized, placebo-controlled, double-blind, phase III trial of supplemental selenium and vitamin E is expected to enroll >32,000 men over 7 to 15 years. Creating a complementary trial employing the identical formulations of selenium and vitamin E, we undertook a preprostatectomy placebo-controlled feasibility study to determine the biologic effects of these agents in blood and prostate tissue. Using serum samples from 39 patients evaluable for blood biomarker correlative study and 29 healthy controls, we examined whether differential serum proteomic patterns could be induced by short-term antioxidant supplementation.

## Materials and Methods

**Patients.** Forty-eight presurgical patients with clinically organ-confined prostate cancer at The University of Texas M.D. Anderson Cancer Center in Houston were enrolled in a randomized, double-blind trial of vitamin E and selenium between February 2001 and April 2002. Twenty-nine age-matched men, who consented to participate at the University of Massachusetts, served as prostate cancer-free controls, providing serum samples but receiving no treatment. The protocol and informed consent forms were approved by the respective universities' Institutional Review Boards, and an informed consent form was signed by all patients and controls before participation.

Eligibility criteria required patients to have clinical stage T1c/T2 disease, a prostate-specific antigen level <10 ng/mL within 3 months of study registration, a Gleason score  $\leq 7$ , and a prostatectomy scheduled 3 to 6 weeks after the start of treatment. Patients taking >50  $\mu$ g of selenium and/or  $\geq 300$  IU of vitamin E over 3 consecutive days within 1 month of registration were excluded. Once enrolled, patients received 400 IU of vitamin E (*all rac*  $\alpha$ -tocopheryl acetate), 200  $\mu$ g of selenium (L-selenomethionine), a combination of vitamin E and selenium, or placebo daily for 3 to 6 weeks. The formulations of selenium and vitamin E were the same as those in the ongoing SELECT. Each patient also received a multivitamin and 250 mg of vitamin C daily. Each multivitamin contained high-selenium yeast (30  $\mu$ g) and D- $\alpha$ -tocopherol (30 IU), contributing to incremental increases in supplemental selenium and vitamin E of 15% and 7.5%, respectively. Serum samples were collected from the patients before and after treatment.

**Proteomics Analysis.** The venous blood samples were collected in serum separator tubes from patients participating in the study at M.D. Anderson before and after treatment. The samples were initially allowed to clot at room temperature and then were centrifuged to separate the cellular component from the supernatant within 2 hours of collection. Aliquots of supernatant were promptly frozen and stored at  $-80^{\circ}\text{C}$  until analyzed. The samples were archived for 11 to 26

months. The samples from the 29 prostate cancer-free controls from the University of Massachusetts were processed following similar standard procedures. These control samples were archived at  $-80^{\circ}\text{C}$  for 1 to 12 months until analyzed. All samples for the training set were analyzed at the same time, and none of the samples were discarded on the basis of turbidity or hemolysis. All samples used in the experiment were thawed only once.

Lipophilic proteins/peptides were extracted from each serum sample with UltraMicroSpin C18 columns (The Nest Group, Southboro, MA) following a previously published protocol (4). In brief, sera were diluted by mixing 4  $\mu$ L crude serum of each sample with 200  $\mu$ L solution containing 5% acetonitrile (MeCN) and 1% trifluoroacetic acid, which was in turn passed through the C18 column and washed thrice with 50  $\mu$ L of the 5% MeCN/1% trifluoroacetic acid solution. The mixture was finally eluted with 30  $\mu$ L of 75% MeCN/1% trifluoroacetic acid solution. Each washing and elution cycle was done by centrifugation at  $2,500 \times g$ . Five microliters of the final eluted solution were mixed with an equal volume of 50% saturated matrix, consisting of sinapic acid in 50:50 5% MeCN/1% trifluoroacetic acid (4).

All data were obtained using the Ciphergen Protein Biosystem II with delayed extraction in conjunction with Ciphergen 2.1 software. The dried droplet method was used to crystallize 1  $\mu$ L of the matrix/sample mixture on a blank sample probe (4). An automatic protocol was designed to accomplish the data collection.

The variables for the protocol were set as follows: the mass window was set to acquire data from 0 to 20 kDa and was optimized between 3 and 10 kDa, yielding an extraction time delay of 600 ns. Each mass spectrum is the result of averaging 200 optimal shots. The intensity of the UV nitrogen laser (337 nm) was set at 240 V, the filter average was set to 0.2 times the expected peak width, and the baseline smoothing was set to 25 points before baseline correction. On the day of the experiment, external calibration was carried out with the following protein standards: [M + 1]<sup>+</sup> and [M + 2]<sup>+</sup> peaks from each standard-bovine insulin (5,733.6), bovine ubiquitin (8,564.8), and bovine cytochrome c (12,230.9). An internal calibration was completed with a combination of external protein standards and the extracted serum proteins in order to mark proteins found in each patient sample that encompassed the range of molecular weights of interest. In this way, each spectrum was internally calibrated with endogenous proteins present in each sample.

**Data Processing and Analysis.** Using Peakfit version 4.0 to perform the processing, we narrowed the mass spectra frame to capture data between 2 and 13.5 kDa, eliminating data that, from our experience, do not play a significant role in the prediction strength calculation. We then smoothed the spectra display data with a Gaussian convolute algorithm, adjusted background using a second derivative zero technique, normalized the spectra to 1.0, and zeroed the negative data. The processed mass spectra data were then exported to a text file.

The resulting text file was in turn imported into the data-processing application software MOCS,<sup>8</sup> which had been custom-designed and implemented for the weighted voting method. MOCS did all the necessary subsequent steps in the analysis automatically with the input of several variables.

First, MOCS lined up the data points from all smoothed and normalized spectra against a single mass-to-charge (*M/Z*) value array, which was calculated to the mass accuracy of the internally calibrated spectra of 150 ppm. The aligned data

<sup>8</sup> Modulized Classification System, Sun and Ho, unpublished.

were further standardized to set all intensity values between 0 and 1. The standardization was done using the equation

$$S_{\text{standardized}} = (S_x - S_{\text{min}})/(S_{\text{max}} - S_{\text{min}}),$$

where  $S_x$  is the data point that needs to be standardized,  $S_{\text{min}}$  is the lowest intensity of all spectra for the  $M/Z$  value, and  $S_{\text{max}}$  is the highest intensity of all spectra for this  $M/Z$  value. By using this standardization strategy (4), we gave all intensities equal significance.

The standardized spectra data contained the intensity of all  $M/Z$  values from 2 to 13.5 kDa. We optimized the training set data and chose the top 1,000  $M/Z$  values that had the most influence on the clustering. To determine the top 1,000  $M/Z$  values, we calculated the weighted value for each  $M/Z$ . The training set consisted of two groups: pretreatment samples from 39 men who had prostate cancer and samples from the 29 controls who did not have prostate cancer. Those  $M/Z$  values with larger distances between the means of intensity of the two groups and smaller SDs within each group were granted values of higher weight. The equation used to calculate the weighted value,  $W_{M/Z}$ , was

$$W_{M/Z} = (M_h - M_c)/(d_h + d_c),$$

where  $M_h$  is the mean of the healthy group,  $M_c$  is the mean of the group with prostate cancer,  $d_h$  is the SD of the healthy group, and  $d_c$  is the SD of the group with prostate cancer (15). This way, the  $M/Z$ s with the most significant values differentiating serum of those with prostate cancer from serum of controls have the highest weights.

The 1,000  $M/Z$ s with the highest weighted values were selected and used for comparison against the posttreatment samples in the weighted voting analysis. For each of the posttreatment samples, the training set voted on each of the 1,000 top-weighted  $M/Z$  values using the equation,

$$V_{M/Z} = W_{M/Z} \{[(S_{\text{standardized}} - M_{\text{all}})/d_{\text{all}}] - B_{M/Z}\},$$

where  $W_{M/Z}$  is the weighted value for  $M/Z$ ,  $S_{\text{standardized}}$  is the standardized intensity value of the posttreatment sample at the  $M/Z$  value,  $M_{\text{all}}$  is the mean of all training set values at this  $M/Z$ , and  $B_{M/Z}$  is the statistically derived border of this  $M/Z$  (15), defined as

$$B_{M/Z} + (M_h + M_c)/2,$$

where  $M_h$  is the mean of all samples in the healthy group and  $M_c$  is the mean of all samples in the group with prostate cancer. In this study, a positive vote indicated that at this  $M/Z$  value, the training set classified the sample into the group associated with being disease-free, whereas a negative vote classified the sample into the group associated with having prostate cancer. The resulting vote ( $V_{M/Z}$ ) for each of the 1,000  $M/Z$  values was used to calculate the sum of all positive votes ( $V_p$ ) and the sum of all negative votes ( $V_n$ ), respectively. A larger absolute value of  $V_p$  classified the sample into the group associated with being disease-free, and a larger absolute value of  $V_n$  classified the sample into the group associated with having prostate cancer. To describe the chance that the classification was accurate, we used a variable called prediction strength as a quantitative measurement (4, 15). The prediction strength is calculated with the equation

$$\text{PS} = (|V_p| - |V_n|)/(|V_p| + |V_n|).$$

The prediction strength values range from  $-1$  to  $1$ . A positive prediction strength value is associated with being disease-free,

and a negative value is associated with having prostate cancer. The closer the value is to  $0$  (the border of the two groups), the less chance that the classification is accurate; the closer it is to either  $-1$  or  $1$ , the more likely that classification is accurate. An arbitrary cutoff can be set to exclude certain samples. Setting such a cutoff increases the reliability of the prediction at the cost of lowered usability, because with this method, more samples fall into the category of nonclassifiable. In our study, no arbitrary cutoff value was applied; thus, any sample with a positive prediction strength value was classifiable as disease-free, and any sample with a negative prediction strength value was classifiable as prostate cancer.

Prediction strength values that resulted from weighted voting were calculated for the patients before and after the dietary supplementation treatment. The changes in the prediction strength values after the dietary supplementation were calculated for each sample, and the data set was analyzed using one-way ANOVA and Fisher's least significant difference method. Compared with other samples in the placebo group, sample 9 had an unusually high prediction strength value change toward the positive direction. It contributed 49.5% of the total statistical significance in the ANOVA analysis and lay outside the 95% confidence interval of the samples in the placebo group. In line with common practice, this outlier was excluded from the final analysis of the data set.

## Results

Prediction strength values determined after weighted voting analyses showed that before dietary supplementation, most serum samples from the patients with prostate cancer had negative prediction strength values, values that are associated with prostate cancer (Table 1; Fig. 1). However, after dietary supplementation, many of the serum sample values obtained from patients given the combination were positive prediction strength values, which are commonly associated with the absence of disease (Fig. 2). Generally speaking, the closer the prediction strength value to  $1$  or  $-1$ , the more likely that the classification is correct.

ANOVA analysis on the entire data set (Table 2), as well as on the set without sample 9 (Table 3), both showed significant statistical differences in prediction strength value changes among the four groups at the 0.05 level. Analysis on the data without sample 9 yielded an  $F$  ratio of 3.75 (Table 3). The probability of the result, assuming the null hypothesis (that all diet supplement treatment had the same effects on prediction strength change) is 0.020, which suggests that the effect on each arm was significantly different from the others at the 0.05 level. In the post hoc analysis of data without sample 9, the results at the 0.05 significance level using Fisher's least significant difference test suggest that the different treatment methods had significantly different effects: significant differences were found between the group receiving the combination and all other three groups (Table 4). There were no significant differences in effect among the groups which received selenium treatment, vitamin E treatment, or the placebo group (Table 4).

Furthermore, ANOVA evaluation indicated no significant differences among the groups on the basis of age ( $F$  ratio = 0.638), duration of treatment ( $F$  ratio = 1.210), pretreatment prostate-specific antigen level ( $F$  ratio = 0.630), relation to biopsy Gleason score ( $F$  ratio = 1.085), relation to prostatectomy Gleason score ( $F$  ratio = 0.144), or pretreatment performance status ( $F$  ratio = 0.256).

## Discussion

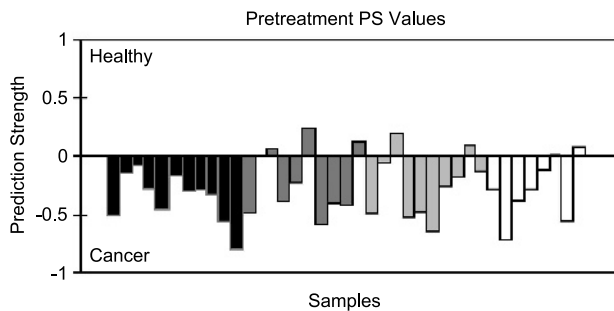
Because evidence of the chemopreventive properties of selenium and vitamin E comes from secondary analysis of

**Table 1. Prediction strength values before and after treatment**

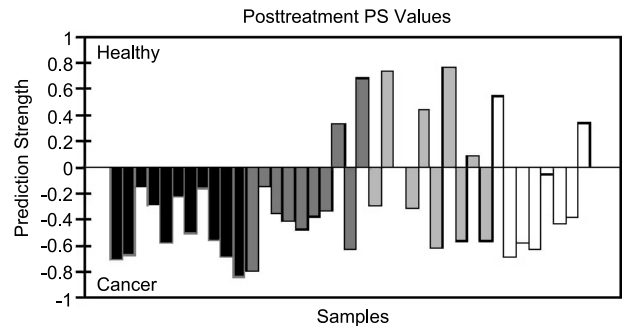
Group	Sample	Pretreatment	Posttreatment	Prediction strength change
Selenium	1	-0.506	-0.7037	-0.1977
Selenium	5	-0.135	-0.6641	-0.5291
Selenium	6	-0.086	-0.1523	-0.0663
Selenium	13	-0.271	-0.2929	-0.0219
Selenium	21	-0.439	-0.5775	-0.1385
Selenium	24	-0.168	-0.2246	-0.0566
Selenium	30	-0.300	-0.5063	-0.2063
Selenium	43	-0.283	-0.1527	0.1303
Selenium	44	-0.328	-0.5491	-0.2211
Vitamin E	4	-0.561	-0.6872	-0.1262
Vitamin E	7	-0.796	-0.8397	-0.0437
Vitamin E	14	-0.494	-0.7966	-0.3026
Vitamin E	16	-0.012	-0.1438	-0.1318
Vitamin E	20	0.061	-0.3501	-0.4111
Vitamin E	27	-0.396	-0.4090	-0.0130
Vitamin E	37	-0.221	-0.4709	-0.2499
Vitamin E	42	0.236	-0.3752	-0.6112
Vitamin E	45	-0.582	-0.3353	0.2467
Vitamin E	47	-0.408	0.3421	0.7501
Vitamin E	48	-0.418	-0.6344	-0.2164
Combination	3	0.118	0.6887	0.5707
Combination	8	-0.491	-0.2928	0.1982
Combination	12	-0.054	0.7378	0.7918
Combination	17	0.188	-0.0020	-0.1900
Combination	18	-0.527	-0.3199	0.2071
Combination	19	-0.479	0.4450	0.9240
Combination	32	-0.639	-0.6158	0.0232
Combination	33	-0.260	0.7674	1.0274
Combination	35	-0.186	-0.5728	-0.3868
Combination	40	0.089	0.0998	0.0108
Placebo	2	-0.140	-0.5650	-0.4250
Placebo	9	-0.279	0.5514	0.8304
Placebo	10	-0.717	-0.6890	0.0280
Placebo	22	-0.394	-0.5777	-0.1837
Placebo	25	-0.278	-0.6323	-0.3543
Placebo	31	-0.118	-0.0500	0.0680
Placebo	34	0.010	-0.4313	-0.4413
Placebo	38	-0.549	-0.3776	0.1714
Placebo	46	0.065	0.3482	0.2832

two phase III clinical trials, researchers conducted SELECT, a multicenter, randomized placebo-controlled, double-blind phase III trial with reduction of the incidence of prostate cancer as an end point. In order to use proteomics to characterize the biological effects of these agents in blood, we used serum samples of patients treated with the selenium and vitamin E

formulations identical to those in SELECT for 3 to 6 weeks preprostatectomy and evaluated serum samples from 29 disease-free age-matched men as controls. Because this was a study in preprostatectomy patients with prostate cancer in whom the benefits of short-term intervention of antioxidants was unknown, and because it was anticipated that recruitment of patients to a placebo-controlled study would be difficult, we decided to provide a multivitamin and extra vitamin C daily. Each tablet contained high-selenium yeast (30 µg) and D-α-tocopherol (30 IU), contributing to incremental increases in



**Figure 1.** Prediction strength values before treatment. *Left to right*, selenium (black columns), vitamin E (dark gray columns), selenium and vitamin E combination (light gray columns), and placebo (white columns). Samples with positive prediction strength values were classified as healthy and those with negative prediction strength values were classified as indicative of prostate cancer. The closer the prediction strength value was to 0, the less chance that the classification was accurate; the closer it was to either -1 or 1, the more likely that classification was accurate.



**Figure 2.** Prediction strength values after treatment. *Left to right*, selenium (black columns), vitamin E (dark gray columns), selenium and vitamin E combination (light gray columns), and placebo (white columns); see Fig. 1 for additional information.

**Table 2. ANOVA analysis**

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F ratio
Between	1.297	3	0.4322	3.016
Error	5.016	35	0.1433	
Total	6.313	38	—	

supplemental selenium and vitamin E of 15% and 7.5%, respectively. Thirty-nine of 48 patients were evaluable. Weighted voting analysis of serologic protein mass profiling data indicated a change in classification mostly in patients who had received the selenium and vitamin E combination. These patients' values changed from levels associated with disease to those associated with its absence. In post hoc analysis, the shift in serum classification, protein profiling as quantified by changes in prediction strength value, indicated that effects in the groups treated with the selenium and vitamin E combination were significantly different from those of the groups treated with selenium, vitamin E, or placebo.

The time between histologic diagnosis of prostate cancer and definitive therapy (prostatectomy) provides an opportunity to test the biological effects of chemopreventive agents with minimal side effects. The phase II preprostatectomy cancer prevention trials employing short interventions with chemopreventive agents have been widely adopted, and in this setting, proteomics provides an effective method to discover biomarkers easily measurable in such biofluids as serum. In this study, enrolling patients slated for prostatectomy, rather than disease-free men as in the SELECT, was determined best because it offered full study of the biological effects of the study agents on the prostatectomy specimen.

Our data analysis is innovative in that, by using serologic protein patterns to evaluate the patients' status, potentially many biomarkers were used and had effects on the final results. Unlike other serologic protein mass profiling studies clustering methods, which are usually aimed at differentiating only two states (with cancer and without) as diagnosis results, the use of weighted voting analysis and prediction strength values provides a way to quantitatively describe the pattern changes among multiple groups and, in turn, facilitates other statistical analysis on the results, such as ANOVA and post hoc analysis.

The lengthy and unpredictable natural history of prostate cancer makes implementing a primary chemoprevention trial logistically difficult. Prostate cancer tumor progression from microscopic to metastatic disease is poorly understood. Oxidative stress-induced genomic and mitochondrial DNA mutations have been implicated as tumor promotion factors in recent population studies (16, 17). Offering a viable option in this setting is primary prevention, especially given that prostate cancer has as long latency period (18). Although undertaking a chemoprevention trial to evaluate a drug's efficacy with cancer incidence reduction as the primary end point in prostate cancer would be both expensive and time-consuming, it is logical to identify cellular and molecular intermediate biomarkers in a particular mechanistic pathway in prostate tissue or blood, which is more easily accessible.

**Table 3. ANOVA analysis without sample 9**

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F ratio
Between	1.402	3	0.4672	3.75
Error	4.235	34	0.1246	
Total	5.637	37	—	

**Table 4. Fisher's least significant difference values without sample 9**

Group	Selenium	Vitamin E	Combination	Placebo
Selenium	—	0.781	0.0073	0.824
Vitamin E	0.781	—	0.0104	0.972
Combination	0.0073	0.0104	—	0.016
Placebo	0.0824	0.972	0.0160	—

Such intermediate biomarkers could serve as surrogate end points for a reduction in the cancer incidence in large randomized trials.

The post hoc analysis using protected Fisher's least significant difference method, which is based on the previous rejection of the null hypothesis with *F* test, showed that different dietary supplementation treatments had significantly different effects on the groups. One sample (an extreme outlier) was omitted under the assumption that results within each group are normally distributed. The clinical data of this patient was checked, and no significant difference was found between his data and data of other patients in the group. There exists the possibility that the patient may have taken alternative treatment, which could have affected his prediction strength values and skewed the range upward. Such an extreme outlying value as his will not only affect the validity of the test results, but also, by increasing the variability of each group, significantly reduce test sensitivity.

The statistical analysis of serologic protein patterns produced by the mass spectrometry method yielded a quick and direct view of the effects of dietary supplement treatment without performing actual biopsy, a procedure with findings that cannot be matched in quality by other available methods. This approach, however, did not reveal details of how the combination of selenium and vitamin E affected the mechanisms of cancer progression. Furthermore, it did not correlate changes in protein patterns to tumor grade, pretreatment prostate-specific antigen, or treatment duration. The small sample size in each category would prevent meaningful analysis.

Despite efforts to ensure that the venous blood samples in both the Massachusetts and Texas studies were collected, processed, and stored similarly, differences in the duration of storage occurred. Samples from patients with prostate cancer were archived 11 to 26 months; those from cancer-free controls were archived 1 to 12 months. Although we are unsure what, if any, bias this difference may have introduced, it should be acknowledged that differences in storage duration occurred and that they may have affected the results of the study. Having the controls at another site, although not ideal, was not expected to affect results.

Studies using protein purification in serum and tissue microarrays to examine selected proteins that are most affected by the treatment are currently under way.

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