

# DNA Stability and Serum Selenium Levels in a High-Risk Group for Prostate Cancer

Nishi Karunasinghe,<sup>1</sup> Jacqueline Ryan,<sup>2</sup> John Tuckey,<sup>2</sup> Jonathan Masters,<sup>2</sup> Michael Jamieson,<sup>3</sup> Larry C. Clarke, James R. Marshall,<sup>4</sup> and Lynnette R. Ferguson<sup>1</sup>

<sup>1</sup>Discipline of Nutrition, The University of Auckland, Auckland, New Zealand; <sup>2</sup>Urology Department, Auckland Hospital, Auckland, New Zealand; <sup>3</sup>Oncology Department, Waikato Hospital, Hamilton, New Zealand; and <sup>4</sup>Roswell Park Cancer Institute, Buffalo, NY

## Abstract

The essential micronutrient, selenium, is at low levels in the New Zealand diet. Selenium is a component of a number of proteins involved in the maintenance of genomic stability, and recommended daily allowances (RDA) are set on saturation levels for glutathione peroxidase (GPx), a key enzyme in surveillance against oxidative stress. It has been assumed but not proven that this level will be adequate for other key selenoenzymes. The "Negative Biopsy Trial" identifies a group of New Zealand individuals at high risk of prostate cancer, whose serum selenium levels will be monitored and who will be supplemented with a yeast-based tablet, with or without selenium, over an extended time. Access to patients on this trial provides the opportunity to ask the more generic question as to whether selenium levels in this population are adequate to maintain genomic stability. The single cell gel

electrophoresis (comet) assay was used to study DNA damage in blood leukocytes harvested from these volunteers. Average serum selenium levels before randomization was  $97.8 \pm 16.6$  ng/ml, low by international standards. For the half of the population below this mean value, lower serum selenium levels showed a statistically significant inverse relationship ( $P = 0.02$ ) with overall accumulated DNA damage. Although other interpretations cannot be excluded, the data suggest that the selenium intake in half of this population is marginal for adequate repair of DNA damage, increasing susceptibility to cancer and other degenerative diseases. It also raises the question as to whether glutathione peroxidase saturation levels are appropriate indicators of the optimal selenium levels for a given population. (Cancer Epidemiol Biomarkers Prev 2004;13(3):391–397)

## Introduction

New Zealand soils are notoriously low in selenium, and locally produced plant foods and animal products typically reflect that low level. Human dietary intakes of between 20 and 60  $\mu\text{g}/\text{day}$  of selenium have been reported in parts of New Zealand (1, 2), whereas people in high-selenium areas of the world, such as parts of China, might expect an intake of around 5000  $\mu\text{g}/\text{day}$  (3). There have been interventions to increase New Zealander's dietary selenium levels through the introduction of Australian wheat and increased use of supplemental selenium in animal foods. However, blood levels of the New Zealand population remain substantially lower than the international norm (2).

Selenium is recognized as an essential nutrient. Altogether, more than 30 selenoproteins have been identified, not all of which have had their full structure and functions completely characterized (4). Consider-

able debate has centered on the appropriate recommended daily allowance (RDA) levels for selenium (5). The current levels are based on the amount to maximize synthesis of the plasma isoform of glutathione peroxidase (GPx) as measured by the plateau of its activity. However, GPx is not the only selenoenzyme in antioxidant defense. Selenium is an example of a micronutrient for which the level necessary to prevent an obvious deficiency state may be substantially lower than the level necessary for optimal health and/or the prevention of degenerative disease (6). Ecological studies have linked low selenium status with increased risk of cancer, especially prostate cancer (7, 8), and human intervention studies have also implied that selenium supplementation may reduce prostate cancer risk (9–11). Cancer statistics indicate that the New Zealand population is among the world's highest in the incidence of many types of cancers and the fourth highest for prostate cancer among the countries with predominant Caucasian populations and similar economic development (12). The study by Clark *et al.* (11) provides preliminary evidence that there could be a benefit of selenium supplementation to levels of 200  $\mu\text{g}/\text{day}$ , more than three times the RDA, in the reduction of prostate cancer. If true, this could be of considerable importance to the New Zealand population.

Cancer develops as a multistep process, in which the accumulation of DNA damage and mutations is an

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Note: Larry C. Clarke is deceased.

Requests for reprints: Lynnette R. Ferguson, Discipline of Nutrition, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand. Phone: 64-9-373-7599 ext. 86372; Fax: 64-9-373-7502. E-mail: l.ferguson@auckland.ac.nz

integral part (13). DNA damage and mutations can arise from endogenously generated free radicals (14), or as the result of exposure to exogenous factors such as dietary carcinogens (15). Both endogenous and exogenous effects on DNA damage may be further exacerbated by the induction of the inflammatory response, which will act to promote carcinogenesis (16). On the basis of the results of animal studies, selenium has been suggested to exert anticancer effects through a number of mechanisms (17). Antioxidant action will reduce DNA damage from free radicals, modulation of xenobiotic response enzymes involved in carcinogen metabolism and clearance will reduce the impact of exogenous dietary carcinogens, and inhibition of the inflammatory response will also be beneficial. Both animal models and the results of human intervention studies suggest that selenium will protect not only against several types of cancer but also against other diseases associated with free radical damage, such as cardiovascular disease (18, 19). However, it is not clear whether some or all of these effects are operative in humans at RDA levels, or how the selenium levels that can be reached through human dietary supplementation relates to blood levels that show protective effects in animal models. Although there are human studies available to show levels of selenium necessary to saturate GPx enzymes (20), we are not aware of a substantial database showing the net effect of different selenium levels on accumulation and/or repair of oxidative and other DNA damage. This information is critical to assessing the required level of selenium in the population, and the overall effect that supplementation might achieve.

One arm of a USA-based double-blinded, placebo-controlled, phase III clinical trial is being conducted at the Auckland Hospital in New Zealand, to investigate whether selenium supplementation will reduce the likelihood of prostate cancer development in high-risk patients. The "Negative Biopsy Trial" enrolls patients with elevated prostate specific antigen (PSA) but negative biopsy for prostate cancer. Such individuals are known to have a high risk of developing prostate cancer in the succeeding 5 years (21). The single cell gel electrophoresis (comet) assay provides a sensitive means of monitoring the cumulative levels of DNA damage, and ease of repair of this damage, at the level of individual human blood lymphocytes (22). The present study reports the preliminary findings of the action of selenium on the stability of the DNA in blood leukocytes in the Auckland negative biopsy cohort. The assessments were carried out at 6 months after initiation of a 5-year trial, before unblinding of the investigations which will not take place until around 2006.

## Materials and Methods

**Cohort.** Forty-three human subjects, between the ages 50 and 75 years, were recruited from records at the Auckland Hospital Urology Clinic. Recruitment criteria were a PSA > 4, and a negative biopsy for prostate cancer, less than 80 years of age, 5-year life expectancy, no history of prior malignancy except for basal cell or squamous cell carcinoma of the skin, and not taking more than 50 µg of selenium a day as a dietary supplement. Patient characteristics are summarized in Table 1. After a run-in period of 1 month, subjects were

randomized to receive either a placebo, 200 or 400 µg of selenium/day in the form of a seleno yeast (SELENO EXCELL TABLETS A2603 LOT 1086ZR5A, A2601 LOT 1086ZR6A, and A2602 LOT 1086ZR7A, respectively), manufactured for Nutrilite, Sandy O'Day, Beach Boulevard, Buena Park, CA. This protocol will supplement selenium to 66.66% of the trial subjects by at least 200 µg. Non-fasting venous blood samples were collected into sodium heparin tubes for selenium and comet assays and serum separator tubes for PSA assay at the initial visit, at randomization, and 6 months after this point.

Serum was isolated within 2 h of collection using density gradient centrifugation, and samples frozen to -20°C before storage. Samples were analyzed using hydride generation atomic absorption spectrometry (Instrumentation Laboratories, Model 951 dual channel AAS equipped with a single slot burner head) using protocols of Hershey and Oostdyk (23). Quality control included multiple aliquots of exhaustively analyzed human plasma as external control samples. A coefficient of variation of <7% (for duplicate analysis) was the criterion of acceptance. These assays were done at the Ruakura Animal Health Laboratory (now called Gribbles Veterinary Pathology), Hamilton, New Zealand.

A further sample of serum was isolated within 2 h of collection using density gradient centrifugation, and analyzed for PSA using Abbott AxSYM MEIA total PSA method following manufacturer's protocols. The rates of change of PSA before and after supplementation were calculated as the slopes of the lines *versus* time.

**Comet Assay for DNA Damage.** The comet assay developed by Singh *et al.* (24) was used to assess the stability of DNA. Preparation of the fresh blood slides was carried out by topping a pre-agarose-coated [1 % normal melting point agarose in PBS] and dried slide with a mixture of 5 µl of blood and 75 µl of 0.5% low

**Table 1. Characteristics of the study population (n = 43)**

<i>Age at baseline level</i>	
Range	50-75
Mean ± SD	65.91 ± 6.26
<i>BMI (kg/m<sup>2</sup>)</i>	
	26.97 ± 6.95
<i>Tobacco consumption</i>	
Current smokers (%)	4.65
Previous smokers (%)	55.81
Previous smoking <20 years	16.28
Previous smoking >20 years	39.53
Average smoking years	
	16.58
0 cigarettes smoked per year (%)	45.24
<500 cigarettes smoked per year (%)	26.19
>500 to <1000 cigarettes smoked per year (%)	23.81
>1000 cigarettes smoked per year (%)	4.76
<i>Alcohol consumption</i>	
Non-alcohol users (%)	14.29
Alcohol users < once a month (%)	2.38
Alcohol users 1 day or <1 day per week (%)	28.57
Alcohol users 2-3 days per week (%)	14.29
Alcohol users 3-7 days a week (%)	40.48
<i>Previous history of skin carcinomas (%)</i>	
	9.52

melting point (LMP) agarose in PBS. The blood mixture was covered with a uniform layer of 0.5% LMP agarose in PBS. The gels were lysed at 4°C in a solution containing 1% Triton X-100, 10% DMSO, 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris (pH 10.0) for 18–20 h. The DNA was subsequently denatured for 20 min in an alkaline buffer [300 mM NaOH and 1 mM EDTA (pH > 13)] and electrophoresed for 20 min at 25 V and 300 mA in the same buffer. These assays and its variants are described more fully elsewhere (25–28).

For endonuclease III treatment, lymphocytes were isolated by centrifugation on a Ficol-based density gradient, embedded in agarose on an agarose precoated microscope slide, and lysed for 18–20 h as before. To measure DNA base damage specific to certain types of oxidative stress in addition to strand breaks, the nucleoids were incubated after lysis with endonuclease III, an endonuclease that specifically recognizes and repairs oxidized pyrimidines. The resulting nucleoids were denatured and electrophoresed for 20 min as before.

Additional blood samples were assayed for their resistance to oxidative stress, by challenging them with either hydrogen peroxide (28) or bleomycin (29) for 1 h at 0°C before assaying as above. For these challenges, 20 µl of blood were treated with 1 ml of cold 200 µM hydrogen peroxide in PBS or 1 ml of cold bleomycin (2 µg/ml) in PBS. Another 20 µl of blood were left in 1 ml of cold PBS for the control. These were left on ice for 1 h and centrifuged at 200 × g for 3 min. The supernatant was removed and the remainder was mixed with 300 µl of 0.5% LMP agarose, layered on to slides precoated with agarose, lysed, denatured, and electrophoresed as before.

The presence of breaks in the DNA allows the DNA to extend toward the anode, resulting in a comet-like image when stained with ethidium bromide and visualized under a fluorescence microscope (25). The additional breaks formed at the sites of base oxidation increase the relative amount of DNA in the tail of the comet. The yield of strand breaks indicates the antioxidant status of the cells (30). These assays provided an overall measure of DNA breakage after various types of DNA damage. For all of these assays, tail moment is calculated as Tail Length \* Tail % DNA/100 and an increase in tail moment provides evidence of an increased number of strand breaks in the nucleoid DNA. All steps for the comet slide preparation were carried out in diffused light to prevent additional DNA damage.

Comets were scored using a fluorescent microscope fitted with 100 W mercury burner from Osram, an excitation filter of 515–560 nm and a barrier filter of 590 nm, a CCD camera from Hitachi Kokusai Electric Inc., and Komet 5 Single Cell Gel Electrophoresis Analysis software from Kinetic Imaging Ltd. A total of 50 or 100 comets was scored per patient per treatment at a magnification of 250. The camera exposure time was set at 120 ms and the tail break length applied was 25 µm.

**Statistical Methods.** The comet tail moments were strongly skewed within each individual; for this reason, the data were logarithmically transformed before analysis. Because the hypothesis of interest concerned the spread of the data before and after treatment commenced (high values becoming lower, small values increasing), a Likelihood Ratio test of equal variances

was performed using Linear Mixed Models and the SAS statistical package (31). Regression analysis was performed to study the correlation of logarithmically transformed mean tail moments (geometric mean tail moments) against serum selenium levels.

## Results

The starting selenium content in serum varied from 59 to 128 µg/l (0.74–1.62 µm/l) with an average of  $97.8 \pm 16.6$  (1.24 µm/l  $\pm$  0.21). The serum selenium levels of all individuals at baseline level and randomization at 1 month showed a close correlation ( $r = 0.62$ ,  $P < 0.0001$ ). We have compared the baseline data with previously published data from Auckland, Christchurch and Otago subjects (1, 32–40) in Fig. 1. Although there were substantial differences seen between these serum selenium levels of individuals living in these three locations in 1977 (1), more recent data from these areas have significantly increased from those earlier values. However, there is still a marked difference in the distribution of serum selenium levels of individuals in these three geographic locations, apparent from Fig. 1.

Samples of leukocytes from blood taken before randomization into the trial, and also 6 months after supplementation, were assayed for DNA damage using the comet assay. We wished to understand whether increased serum selenium levels affected the amount of DNA damage detectable by the comet assay in any way. Figure 2, A and B, illustrates the relationship between the serum selenium level before randomization, and the mean geometric tail moments of leukocytes in fresh blood for subjects with less than 100 ng/ml of serum selenium (group A) and those with more than 100 ng/ml of serum selenium (group B), respectively. For group A subjects, there is a statistically significant inverse correlation between high DNA damage and high serum selenium. However, above a serum level of 100 ng/ml selenium, there is much less evidence that DNA damage is affected by serum selenium. A

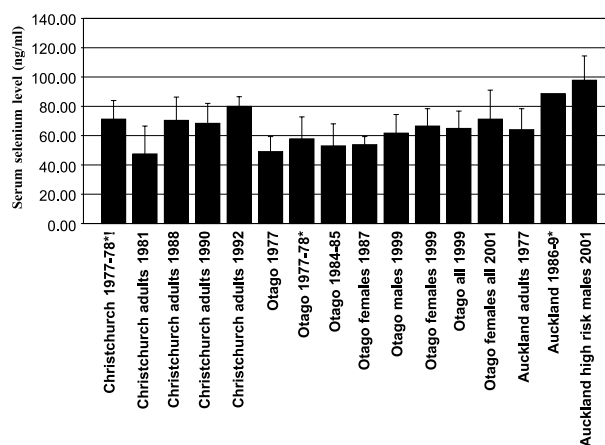


Fig. 1. Plasma/serum or whole blood (asterisk) selenium level variations recorded for New Zealand populations. Details of patient numbers and characteristics can be found in the original references 1 and 32–40.

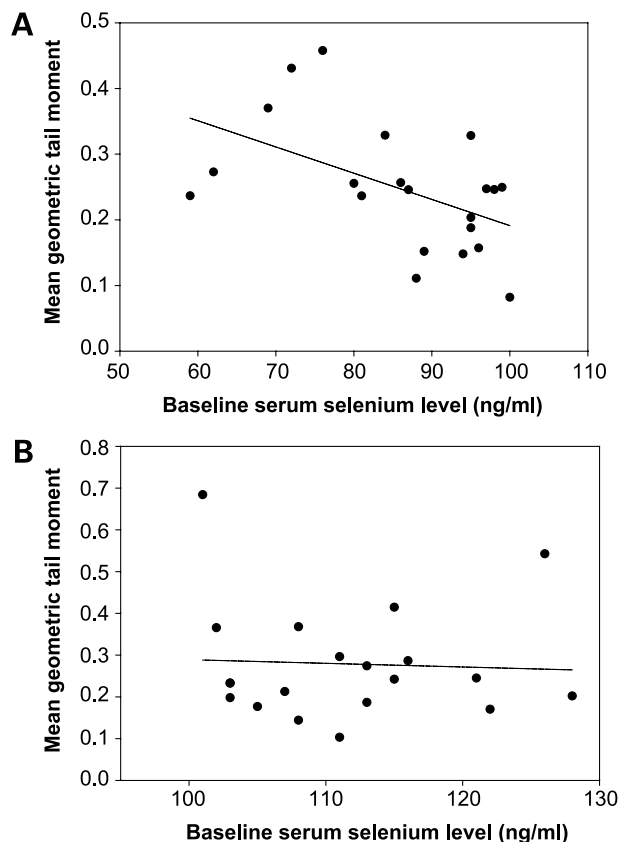


Fig. 2. Relationship between initial serum selenium levels (ng/ml) and overall DNA damage as measured by the comet assay (24). Geometric mean tail moment for each volunteer, measured at time 0, is plotted against total serum selenium in the same person as measured at the same time. **A.** Samples from individuals with initial serum selenium less than 100 ng/ml. **B.** Samples from individuals with initial serum selenium greater than 100 ng/ml. Lines, regressions calculated from the data. Correlation coefficients for these and other combinations are given in Table 2.

statistically significant relationship between detectable DNA damage in leukocytes and serum selenium was also seen in blood left in PBS as a control (Table 2). Several variations on the standard assay were also done, and the relationship between serum selenium levels and DNA damage revealed by each of these assays is also summarized in Table 2. Neither oxidized pyrimidines (detected by endonuclease III), nor accumulation of double and single strand breaks induced by a challenge with the powerful free radical-generating complexes of

bleomycin or of peroxide, showed a statistically significant relationship with higher selenium concentrations in the serum (Table 2).

There were statistically significant correlations between the mean geometric tail moments at baseline level and randomization level at 1 month for five subjects assayed for fresh blood ( $r = 0.83$ ,  $P < 0.05$ ), PBS controls ( $r = 0.97$ ,  $P < 0.01$ ), and peroxide challenge ( $r = 0.90$ ,  $P < 0.05$ ). However, there were no statistically significant correlations for the bleomycin and endonuclease III treatments between the two time points. This could either be due to reproducibility problems with these treatments or the variability among individuals. Due to this reason, the latter two treatments were not analyzed further.

The data have not been decoded at this point, so we are not presently able to compare people in different selenium supplementation groups after supplementation. However, we know that there are a number of other factors that affect the accumulation of DNA breaks, and the reparability of DNA damage. If serum selenium level has no effect on DNA damage, then those individuals who originally had high levels of damage from other factors should still show those high levels, and vice versa. After 6 months of supplementation, we know that on average, two-thirds of each group of individuals will have had selenium. Thus, we have re-examined this same group of individuals after 6 months (Fig. 3, A and B). There is no statistically significant evidence for the change in means and variance for PBS controls. However, a statistically significant narrowing of variance was observed for the peroxide challenge after 6 months although there was no statistically significant evidence for the change in means.

The rate of change of PSA was calculated for pre-trial and post-trial data (Fig. 4, A and B). There is a weak but non-statistically significant relationship between initial serum selenium and rate of change of PSA ( $P = 0.055$ ). However, this relationship was lost after 6 months of supplementation.

There were no statistically significant evidences for change in mean geometric tail moments with age, body mass index (BMI), or the previous level of exposure to cigarette smoke.

## Discussion

The most striking conclusion from this study was the indication of an association between selenium level and genomic stability. Both sets of data for untreated blood

Table 2. Correlations of leukocyte DNA damage as geometric means of tail moments with initial serum selenium level

	Initial serum selenium <100 ng/ml		Initial serum selenium >100 ng/ml	
	Correlation coefficient	Probability	Correlation coefficient	Probability
Fresh blood	-0.51	0.02 <sup>a</sup>	-0.05	0.83
Blood incubated in PBS at 0°C for 1 h	-0.47	0.03 <sup>a</sup>	+0.34	0.13
Endonuclease III sites	+0.17	0.53	-0.11	0.72
Bleomycin challenge	-0.13	0.56	+0.13	0.59
Peroxide challenge	-0.14	0.51	-0.19	0.40

<sup>a</sup>Statistically significant at  $P < 0.05$ .

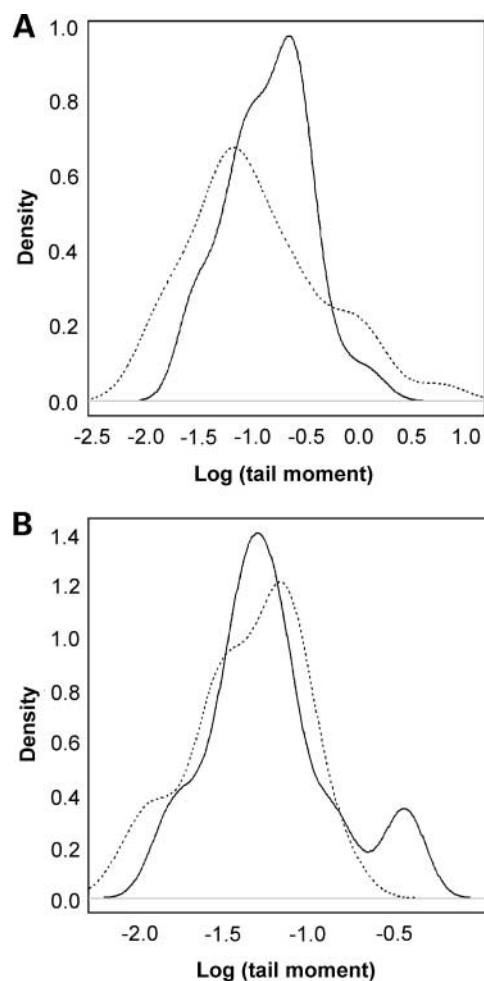


Fig. 3. Comparison of distribution of DNA strand breaks in leukocytes at 0 and 6 months of supplementation for peroxide challenge and PBS control. **A.** Peroxide: Log(geometric mean) Likelihood ratio test for equal variances Chisq, 1 *df* 6.9,  $P = 0.00861$ . Test for shift in mean  $F(df\ 1,37) = 1.04$ ,  $P = 0.315$ . **B.** PBS control: Log(geometric mean) Likelihood ratio test for equal variance Chisq, 1 *df* = 0.7,  $P = 0.402$ . Test for shift in mean  $F(df\ 1,37) = 2.86$ ,  $P = 0.099$ . Dotted line, baseline data; solid line, 6-month data.

leukocytes showed a statistically significant relationship between low serum selenium and high overall levels of DNA damage, although two other more specific types of oxidation damage appeared unaffected.

Selenium-dependent GPxs remove the products of free radicals and other reactive oxygen species (41–44), while thioredoxin reductase is a selenocysteine-containing enzyme that regulates the reduction of exposed disulfide groups (45). Both selenoproteins P and W are also thought to have antioxidant functions (46, 47), while prostatic epithelial selenoprotein has a possible redox function in protecting cells against cancer development (48). Internationally, RDAs have typically been based on the serum selenium level necessary for saturation of GPxs. There is some evidence that the physiological requirement of selenium for functional GPx mRNA is lower than the amount necessary for a fully functional GPx enzyme (49). Maximal activity of many of the other known proteins occurs at dietary selenium intakes less

than those needed for maximal GPx activity, because of the hierarchy of importance of selenoproteins in tissues (50, 51). Thus, several countries have used two-thirds the amount necessary for optimal GPx as the RDA, while others have argued against this viewpoint (5). The present data allow some comment on this controversy.

The average serum selenium level recorded in this study is within the range required for saturation of plasma GPx activity as previously reported (52) and slightly higher than the similar requirement reported by Thomson *et al.* (20). However, we note that 46.5% of the population studied was below a starting serum selenium level of 97.8 g/l (ng/ml) and therefore below the GPx saturation requirement, although generally within two-thirds of that level. It is in this group of subjects that we see the negative correlation between selenium status and DNA damage accumulation. The narrowing of the variance among peroxide-challenged group after 6 months of selenium supplement indicates that the group with higher DNA damage at baseline was receiving beneficial effects while the group that generally showed lower damage showed slightly higher damage after 6 months of supplementation.

It is recognized that the current RDA is not sufficient for maximal levels of selenoprotein P (51). It is also of

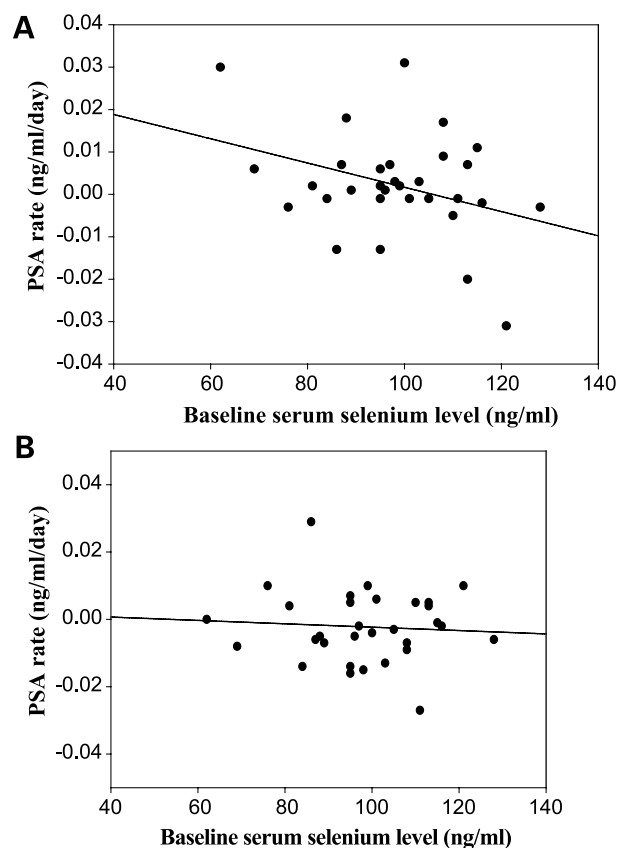


Fig. 4. Comparison between initial serum selenium levels (ng/ml) and the rate of change of PSA at time 0 (**A**) or after 6 months of supplementation (**B**). Mean values of PSA change for each volunteer, measured at time 0 (**A**) or after 6 months of supplementation (**B**), are plotted against total serum selenium in the same person as measured at time 0. Lines are regressions calculated from the data.

interest that formation of prostatic epithelial selenoprotein shows priority over that of GPx during periods of insufficient selenium intake (48). Many of the arguments on required levels have centered on the amount of selenium necessary for incorporation into selenoproteins. However, it seems that selenium may also be involved in the regulation of other gene products. Fisher *et al.* (53) reported that selenium and vitamin E deficiency differentially affected gene expression in rat liver, as detected by cDNA array technology. As well as predictable effects of selenium deficiency in leading to a down-regulation of selenium-dependent cellular GPx activity, it also led to induction of a range of genes encoding for xenobiotic metabolizing enzymes in liver, including cytochrome P450 4B1 and UDP-glucuronosyltransferase. A combined deficiency of vitamin E and selenium deficiency led to alterations in the expression level of genes encoding for proteins involved in inflammation (multispecific organic anion exporter, SPI-3 serine protease inhibitor) and acute phase response ( $\alpha$ -1 acid glycoprotein, metallothionein 1). There was also a significant down-regulation in the expression level of the antioxidant defense enzyme ( $\gamma$ -glutamylcysteine synthetase catalytic subunit). Changes in the expression of all of these genes would be likely to impact significantly on the genomic stability of the cell, and could be involved in the present data.

Because this study has still not been unblinded, we cannot yet make specific comments on effects of supplementation at different levels of selenium. However, it is of interest that for those who reported a higher DNA damage compared to the mean at the initial level showed a decrease in their tail moments for peroxide challenge after 6 months on the trial. Similarly, the rate of PSA progression has been reduced in approximately two-thirds of the group. We would note that 66% of these individuals could be expected to be on one of the selenium supplementation groups. On the other hand, the groups who reported lower DNA damage at the initial level showed an increase in their tail moments after 6 months on the trial. This indicates the importance of a critical balance of selenium required to maintain the functions at an optimal level.

The most likely interpretation of the present data is that serum selenium levels of greater than 100 ng/ml are necessary for the prevention of all types of DNA damage in human leukocytes. However, at this point, we cannot exclude other possibilities. For example, it is plausible that those individuals with higher serum selenium levels have a diet that is generally healthier, and some other factor is causal in the relationship seen. It will be 3–5 years before this study is decoded, and even then, the data may not give us all the information we need. The study is too small for high statistical significance, and higher numbers would strengthen it. It would also be useful to test effects in other members of the population who are not known to have an elevated cancer risk. The trial only considers two dose levels of selenium, and smaller increments might be appropriate. Nevertheless, with all these provisos, there is a suggestion from this study that we should be reconsidering the question of optimal levels of selenium, whether as part of whole diet or as a dietary supplement, which should be recommended for human populations.

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