Phase II Study of Pomegranate Juice for Men with Rising Prostate-Specific Antigen following Surgery or Radiation for Prostate Cancer

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Abstract

Purpose: Phytochemicals in plants may have cancer preventive benefits through antioxidation and via gene-nutrient interactions. We sought to determine the effects of pomegranate juice (a major source of antioxidants) consumption on prostate-specific antigen (PSA) progression in men with a rising PSA following primary therapy.

Experimental Design: A phase II, Simon two-stage clinical trial for men with rising PSA after surgery or radiotherapy was conducted. Eligible patients had a detectable PSA >0.2 and <5 ng/mL and Gleason score ≤7. Patients were treated with 8 ounces of pomegranate juice daily (Wonderful variety, 570 mg total polyphenol gallic acid equivalents) until disease progression. Clinical end points included safety and effect on serum PSA, serum-induced proliferation and apoptosis of LNCaP cells, serum lipid peroxidation, and serum nitric oxide levels.

Results: The study was fully accrued after efficacy criteria were met. There were no serious adverse events reported and the treatment was well tolerated. Mean PSA doubling time significantly increased with treatment from a mean of 15 months at baseline to 54 months posttreatment ($P < 0.001$). *In vitro* assays comparing pretreatment and posttreatment patient serum on the growth of LNCaP showed a 12% decrease in cell proliferation and a 17% increase in apoptosis ($P = 0.0048$ and 0.0004, respectively), a 23% increase in serum nitric oxide ($P = 0.0085$), and significant ($P < 0.02$) reductions in oxidative state and sensitivity to oxidation of serum lipids after versus before pomegranate juice consumption.

Conclusions: We report the first clinical trial of pomegranate juice in patients with prostate cancer. The statistically significant prolongation of PSA doubling time, coupled with corresponding laboratory effects on prostate cancer *in vitro* cell proliferation and apoptosis as well as oxidative stress, warrant further testing in a placebo-controlled study.

Adenocarcinoma of the prostate is currently the most common malignancy in men in the United States comprising 29% of all cancers. This year an estimated 232,090 men will be newly diagnosed with prostate cancer (1). There has been a trend toward improved survival in prostate cancer over the past several years. Prostate cancer 5-year survival rates have increased from 67% for the period of 1974 to 1976 to 92% for the period of 1989 to 1995 (2). However, prostate cancer remains the second most common cause of cancer death in men in the United States, accounting for 11% of all cancer deaths. This year an estimated 30,350 men will die of prostate cancer (1).

Primary management of prostate cancer for the majority of patients consists of either radical surgery or radiation therapy. Although this is adequate for permanent disease control in many patients, a significant number of patients relapse and ultimately develop metastatic disease. Radical prostatectomy is currently the most commonly used therapy for curative intent (3). However, approximately one third of prostate cancer patients with clinically confined cancer that are treated with radical prostatectomy will develop a biochemical recurrence (4, 5). Pound et al. (6) reviewed 1,997 patients that underwent radical prostatectomy for clinically localized prostate cancer and determined that 15% of patients had biochemical recurrence. Thirty-four percent of patients with biochemical recurrence developed distant metastases with only 15 years of total follow-up, median time to development of metastases was 8 years from the time of initial prostate-specific antigen (PSA)

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elevation, and median time to death from the development of metastases was 5 years (6).

There are limited treatment options for patients who have undergone primary therapy with curative intent and who have progressive elevation of their PSA without documented evidence of metastatic disease. Early initiation of hormonal ablation is associated with significant morbidity and effect on quality of life, including fatigue, hot flashes, loss of libido, decreased muscle mass, and osteoporosis with long-term use. Strategies to delay clinical prostate cancer progression and prolong the interval from treatment failure to hormonal ablation would be of paramount importance. A combination of epidemiologic and basic science evidence strongly suggests that diet and plant-derived phytochemicals may play an important role in prostate cancer prevention or treatment.

Multiple genetic and epigenetic factors have been implicated in the oncogenesis of prostate cancer. However, the molecular mechanism underlying the disease is not well understood. African American men have the highest rate of prostate cancer in the world, whereas Japanese and Chinese native to their countries who consume a low-fat and high-fiber diet with high consumption of phytochemicals that include soy products and green tea have the lowest rate (7). Epidemiologic studies suggest that a reduced risk of cancer is associated with the consumption of a phytochemical-rich diet that includes fruits and vegetables [dietary aspects of both prostate cancer prevention and treatment are well reviewed by Chan et al. (8)]. Fresh and processed fruits and food products contain high levels of a diverse range of phytochemicals of which polyphenols, including hydrolyzable tannins (ellagitannins and gallotannins) and condensed tannins (proanthocyanidins), and anthocyanins and other flavonoids make up a large proportion (9). Several phytochemicals have been proposed as potential chemoprevention agents based on animal and laboratory evidence of antitumor effects. Suggested mechanisms of anticancer effects of polyphenols include the inhibition of cancer cell growth by interfering with growth factor receptor signaling and cell cycle progression, promotion of cellular differentiation, modulation of phosphodiesterase/cyclooxygenase pathways, inhibition of kinases involved in cell signaling, and inhibition of inflammation (10–12).

The pomegranate (*Punica granatum* L.) fruit has been used for centuries in ancient cultures for its medicinal purposes (13). Pomegranate fruits are widely consumed fresh and in beverage forms as juice and wines (14). Commercial pomegranate juice shows potent antioxidant (15) and antiatherosclerotic (16) properties attributed to its high content of polyphenols, including ellagic acid in its free and bound forms (as ellagitannins and ellagic acid glycosides), gallotannins, and anthocyanins (cyanidin, delphinidin, and pelargonidin glycosides) and other flavonoids (quercetin, kaempferol, and luteolin glycosides; ref. 14). The most abundant of these polyphenols is punicalagin, an ellagitannin implicated as the bioactive constituent responsible for >50% of the potent antioxidant activity of the juice (14). Punicalagin is abundant in the fruit husk and, during processing, is extracted into pomegranate juice in significant quantities reaching levels of >2 g/L juice (14). Ellagic acid and tannins have been shown previously to exhibit *in vitro* and *in vivo* anticarcinogenic properties, such as induction of cell cycle arrest and apoptosis, as well as the inhibition of tumor formation and growth in animals (17). Recently, there have been several reports on the antiproliferative, apoptotic, angiogenic, and inhibition of nuclear factor-κB (NF-κB) activity and xenograft growth by pomegranate polyphenols (18–22). Although these data on pomegranate polyphenols and NF-κB are quite preliminary and many mechanistic questions remain to be elucidated, it is consistent with a much larger body of literature that supports the concept that plant polyphenols, including red wine resveratrol (23), green tea catechins (24), and soy isoflavones (25), are capable of decreasing proliferation and stimulating apoptosis via inhibition of NF-κB activity. Finally, pomegranate phytochemicals inhibited the *in vitro* proliferation of three prostate cancer cell lines, LNCaP, PC3, and DU145, and showed *in vivo* inhibition of xenograft growth in athymic mice (20).

To study the possible therapeutic effects of pomegranate juice on prostate cancer, a single-center, open-label, phase II clinical trial was done.

Materials and Methods

Clinical trial design. An open-label, single-arm clinical trial was done at the Clark Urologic Center, David Geffen School of Medicine, University of California at Los Angeles. A 2-year, single-center, phase II, Simon two-stage clinical trial for men with rising PSA after surgery or radiotherapy was designed and done. Eligible patients had a detectable PSA >0.2 and <5 ng/mL that was documented as rising, enough pretreatment PSA time points to calculate a baseline PSA doubling time (PSADT), no hormonal therapy before entering the study, no evidence of metastatic disease, and Gleason score ≤7.

Serial PSA measurements before study entry determined a baseline PSADT. Each patient had a minimum of three pretreatment PSA values measured over a minimum of 6 months before study entry. Patients were treated with 8 ounces of pomegranate juice by mouth daily (Wonderful variety, equivalent to 570 mg total polyphenol gallic acid equivalents daily) until meeting disease progression end points. Patients were followed in 3-month intervals for serum PSA, and blood and urine were collected for laboratory studies. Clinical end points included safety, effect on serum PSA, effect on serum hormone levels (testosterone, estradiol, sex hormone-binding globulin, dehydroepiandrosterone, insulin-like growth factor, and androstenedione), and exploratory laboratory studies. These exploratory studies included markers for treatment compliance (serum and urinary polyphenol/ellagic acid levels), markers of serum antioxidant effect (serum nitrous oxide levels), and *in vitro* assays that measure the effect of patients' serum on the LNCaP growth and apoptosis (26). For these laboratory studies, each patient during treatment was compared with baseline. Samples were collected, processed, aliquoted, labeled with anonymized study ID numbers, and batch stored at −80°C. For each assay, aliquoted samples were thawed and run simultaneously to ensure standardization of conditions across samples. For each assay, pretreatment and posttreatment samples were randomly assayed by laboratory technicians who were blinded to the corresponding clinical data. The primary end point for this study was effect on PSA variables, such as change in doubling time, and the secondary end points included safety and modulation of biomarkers. PSADT before treatment was compared with the slope of this curve after treatment, because a significant change in this slope may potentially correlate with delay in disease progression. An objective response was defined as a decrease of ≥50% in highest measured serum PSA. Progressive disease was defined as either a >100% increase in PSA (with a minimum value of 1.0 ng/mL) compared with the best response observed (nadir) or any documentation of metastatic or recurrent disease. Patients with stable disease did not qualify as objective response or progressive disease.

The study was conducted according to the Declaration of Helsinki and its amendments. The study protocol was approved by the University of California at Los Angeles Medical Institutional Review Board and the Institutional Scientific Peer Review Committee of the Jonsson Comprehensive Cancer Center. All patients gave written informed consent before participation.

The study sponsors played no role in the study design, collection, analysis, or interpretation of data or in the writing of this report.

Calculation of PSADT. The linear spline method with a knot at $t = 0$ (baseline) was used to estimate the PSADT before and after baseline for each subject. The model is $y = \alpha + \beta t + \lambda u(t)$, where $y = \log(\text{PSA})$ and $u(t) = 0$ if $t < 0$; otherwise, $u(t) = t$. PSADT is estimated: before treatment, DT1 = log 2 / β; after baseline, DT2 = log 2 / ($\beta + \lambda$).

Sample size and statistical considerations. The study was designed as a two-stage optimal flexible design (27), phase II trial with the following assumptions: the ineffectiveness cutoff was chosen equal to 5%; the targeted desirable response rate cutoff equal to 20%. Hence, the hypotheses of interest were H0: $r0.05$ against HA: $r0.20$, where r is the response rate. The α error rate (probability of accepting an ineffective treatment, a false-positive outcome) was set to 5%. The β error rate (probability of rejecting an effective treatment, a false-negative outcome) was set to 10%. The study is designed to measure whether HA-H0 = 0.15 with the following properties: if the true response rate is 5%, the treatment is rejected with a very high probability ($>1 - \alpha$), whereas if the true response rate is 20%, the treatment is rejected with a very low probability ($<\beta$). In the first stage, 24 evaluable patients are treated. If >1 patient achieved objective response or stable disease, additional 22 patients would be treated, up to a total of 46. At the end of the second stage, if <4 patients achieved objective response, the treatment would be declared ineffective. Otherwise, the study is considered to have achieved its targeted desirable response rate (20%) and the treatment is deemed promising for further testing in a phase III randomized study.

Pomegranate juice processing. Pomegranate juice was provided by the Pom Wonderful Company (Los Angeles, CA). Pomegranates were handpicked, washed, chilled to 4°C, and stored in tanks. The fruit was then crushed, squeezed, and treated enzymatically with pectinase to yield the juice and byproducts, which included the inner and outer peels and the seeds. Flavonoids constitute 40% (anthocyanins, catechins, and phenols) of total polyphenols in pomegranate juice (28). The pomegranate juice was filtered, pasteurized, concentrated, and stored at −20°C until use.

Treatment dose. Suggested clinical dosing of pomegranate juice was based on its measured antioxidant effect in dose-response studies in healthy human males at the following content daily: 0 (baseline), 3, 6, 8, 12, and 15 ounces (equal to 90, 180, 240, 360, and 450 mL/d, respectively). The results of these studies (not published) show that consumption of pomegranate juice by healthy male subjects exhibits gradual increased antioxidant capacities on using increasing dosages (0-15 ounces daily for 1 week). Based on these results, the recommended clinical use of 6 to 8 ounces (180-240 mL) of pomegranate juice daily was suggested as the optimal dosage, with significant clinical antioxidant effect and with no significant effect on serum triglycerides and glucose.

Cell culture. Androgen-dependent LNCaP prostate tumor cells were obtained from American Type Culture Collection (Manassas, VA). The cells were grown in 75-cm^2 flasks (Falcon Primaria, Bedford, MA) in RPMI 1640 without phenol red, supplemented with 10% fetal bovine serum, 200 IU penicillin, 200 mg/mL streptomycin, and 4 nmol/L L-glutamine (Omega Scientific, Inc., Tarzana, CA). The cultures were maintained at 37°C and supplemented with 5% CO_2 in a humidified incubator. Cells were passaged routinely at 80% confluence, and fresh medium was replaced every third day. Cells used in experiments were not passaged >10 times.

In vitro *proliferation assay.* The effect on the *in vitro* proliferation of LNCaP by the exchange of culture medium supplemented with 10% fetal bovine serum with medium supplemented with subject serum was determined as described previously (26).

In vitro *apoptosis assay.* Experiments were carried out in 96-well tissue culture plates at a density of 10×10^3 cells per well. Cells were prepared and plated for 24 hours as mentioned above. Using the Cell Death Detection ELISA Plus (Roche Applied Science, Indianapolis, IN), apoptosis was measured at the end of 2 days of treatment with 10% fetal bovine serum or 10% experimental sera. This apoptosis assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones and allows the specific determination of mononucleosomes and oligonucleosomes in the cytoplasm fraction of lysates. Background using incubation buffer and ABTS solution was subtracted from the absorbance measurements (405-490 nm). The results are expressed in microunits of mononucleosomes and oligonucleosomes. Cell culture and apoptosis assay were run in triplicates.

Serum lipid peroxidation. Oxidative stress both in the basal state and in an induced oxidative stress system [with the free radicals generator 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH)] was determined by measurement of serum lipid peroxidation in the patient's serum at baseline and after consumption of pomegranate juice. Serum samples were diluted 1:4 (v/v) with PBS and incubated for 2 hours at 37°C without (basal state oxidative status) or with AAPH (70 mmol/L; AAPH-induced oxidation). The extent of lipid peroxidation was measured using 100 μL of the above solutions by the lipid peroxide test, which analyzes lipid peroxide formation by their capacity to convert iodide to iodine, as measured spectrophotometrically at 365 nm (29).

The quantification of lipid peroxides, the major initial reaction products of lipid peroxidation, serves as a direct and valuable index of the oxidative status of biosystems. AAPH is a water-soluble azo-compound that is used extensively as a free radical generator in the characterization of antioxidants. Decomposition of AAPH produces molecular nitrogen and two carbon radicals. The carbon radicals may combine to produce stable products or react with molecular oxygen to give peroxyl radicals.

Evaluation of plasma nitric oxide. Concentrations of nitrite (NO_2^-) and nitrate (NO_3^-) were determined as an indirect measurement of nitric oxide (NO) production. Plasma nitrite and nitrate represent the two stable oxidation products of NO in solution, and determination of both anions represents an accurate quantitative measure of NO produced. A sensitive chemiluminescence detection procedure was used to measure plasma nitrite and nitrate. In short, plasma nitrite and nitrate (NO_x) levels were measured by the classic Griess method. Total nitrite was measured at 540 nm absorbance by reaction with Griess reagent (sulfanilamide and naphthalene-ethylene diamine dihydrochloride). Amounts of plasma nitrite were estimated by a standard curve obtained from enzymatic conversion of $NaNO_3$ to nitrite.

Determination of ellagic acid and ellagic acid metabolites by liquid chromatography-electrospray ionization/mass spectrometry. Plasma from the centrifugation of collected whole blood and aliquoted urine [1.3 mL urine with 30 μL ascorbic acid/EDTA solution (20% ascorbic acid, 0.1% EDTA, NaH_2PO_4 0.04 mmol/L (pH 3.6-final pH 2))] were collected at each study time point and stored immediately at −80°C. The high-performance liquid chromatography system and conditions for liquid chromatography-electrospray ionization/mass spectrometry are as described previously (30).

Results

The study was fully accrued to 48 participants over a period of 13 months in two stages after efficacy criteria were met. Two patients withdrew participation before their first evaluation, leaving 46 patients evaluable for treatment response. Of the patients enrolled, 68% were originally treated by radical prostatectomy, 10% by external beam radiotherapy, 10% by brachytherapy, 7% by surgery and radiation, and 5% by cryotherapy. The original Gleason score were read as

intermediate (5–7) in 94% of patients, whereas 6% had Gleason 4 cancers. All patients were clinical or pathologic N_0 and M_0, and 63% were clinically or pathologically staged with organ-confined disease, whereas 37% had locally advanced cancers extending into the periprostatic or seminal vesicle tissues. At study entry, median PSA for the cohort was 1.05 ng/mL (average, 2.23 ± 2.58 ng/mL).

In stage I, 24 patients were treated of whom 2 patients were objective responders, with PSA declines of 85% and 50%. In the first responder, the PSA rose to a maximal value at 6 months on study, followed by the 85% decrease in PSA level, which did not return to its peak until 21 months. The second responder continues to maintain a 57% reduction in peak PSA at 33 months on study. In addition to these 2 subjects, 6 of the first 24 patients progressed by PSA criteria. Of the 46 patients analyzed from both stages, 16 (35%) achieved a decrease in PSA during treatment (median, 18%; average, 27%; range, 5-85%). Of these 46 patients, a total of 4 met objective response criteria and achieved a PSA decline >50%, meeting efficacy criteria to justify future clinical testing. The slope of their mean log PSA decreased 35%, from 0.066 ± 0.007 (mean λ ± SE) at baseline down to 0.043 ± 0.007 (mean λ ± SE) on treatment ($P < 0.001$; Fig. 1).

Comparisons between baseline and posttreatment PSADT were calculated at multiple time points. At 15 months, there were 28 patients with sufficient data points for meaningful analysis. At 15 months, the average pretreatment baseline PSADT was estimated to be 14.3 ± 10.8 months, whereas the posttreatment PSADT was 25.5 ± 33.5 months ($P = 0.048$). At 24 months, the average PSADT for 39 patients before intervention was 15.0 ± 11.1 months (median, 11.5 months). At 24 months, 7 subjects had negative slopes (PSA was decreasing); therefore, no PSADT could be estimated in these patients who were therefore excluded from analysis at this time point. Note that by excluding patients with a declining PSA from the PSADT calculation the actual PSADT will be underestimated. In the remaining evaluable patients, the PSADT was 37.0 ± 53.0 months (median, 19.9 months). The 22-month prolongation in PSADT reached statistical significance (signed rank sum test, $P = 0.0001$). Due to the large number of patients that continued to have stable disease at 18 months of treatment, the protocol was amended to permit patients to continue treatment until meeting disease progression criteria. At 33 months, 3 additional patients were able to be included for analysis, as their PSA values had increased to the point that they developed positive PSA slopes, allowing their PSADT to be estimated. At 33 months, the estimate of the average PSADT before intervention was 15.6 ± 10.8 months (median, 11.5 months), whereas the average posttreatment PSADT was 54.7 ± 102 months (median, 28.7 months; $P < 0.001$). During the study, 83% of patients achieved an improvement in PSADT (i.e., either prestudy PSADT > 0 and poststudy PSADT < 0 or poststudy PSADT > prestudy PSADT) after intervention (signed rank sum test; $P < 0.0001$). Baseline T stage showed some correlation with the change of PSADT, but this did not reach statistical significance. There were no serious adverse events reported and the treatment was well tolerated, and no patients developed metastatic disease on study.

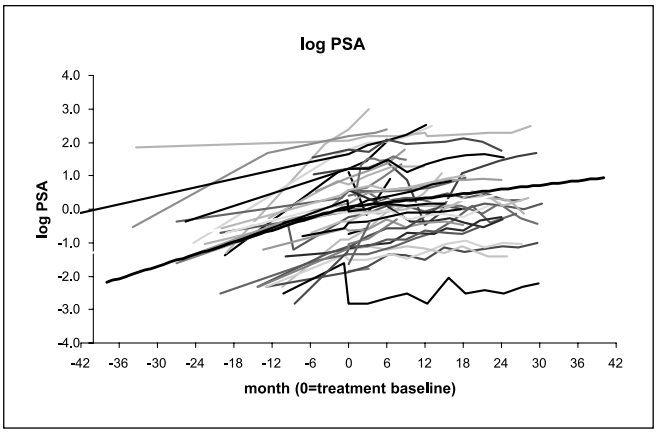

Fig. 1. Each individual thin line represents the log PSA by time for one subject, pretreatment and posttreatment (month 0 = baseline treatment), with the average slope of the entire cohort plotted in thick black line. The PSA values tend to increase, but the increase rate (slope) decreased. The slope of the mean log PSA of the entire cohort decreased 35%, from 0.066 ± 0.007 (mean λ ± SE) at baseline down to 0.043 (mean λ ± SE) on treatment ($P < 0.001$).

The effects of pomegranate juice treatment on *in vitro* cancer cell growth from baseline and posttreatment patient serum was compared using a cell culture assay system designed to measure the effects of medium supplemented with individual serum on the growth of human prostate cancer cells. This cell culture assay, developed in our laboratory (W.A. and R.J.B.), tests in an exploratory manner whether pomegranate juice consumption results in serum changes that reduce the growth or increase apoptosis in prostate cancer cell lines *in vitro*. To perform this assay, fetal bovine serum in LNCaP tissue culture medium was replaced with subject serum as described previously (26). Compared with baseline, at 9 months, there was a 12% reduction (Fig. 2) in the growth of LNCaP (signed rank sum test; $P = 0.0048$), with 84% of patients showing a month 9 value that was less than their respective baseline (mean decrease in proliferation posttreatment, −0.024 ± 0.075). Interestingly, the postintervention serum in 6 subjects, of which 3 were classified as having progressive disease, increased the proliferation of LNCaP cells. The results of these bioassays theoretically represent the total patient growth factor milieu comprising all proapoptotic/antiapoptotic factors in the subjects fasting serum, with multiple factors in the serum not related to pomegranate intake also potentially affecting their results. These changes noted in cellular proliferation were associated with a corresponding 17.5% increase in apoptosis at 9 months (Fig. 3) compared with baseline (signed rank sum test; $P = 0.0004$), with 75% of patients tested showing an increase in apoptosis (mean increase in apoptosis posttreatment, 0.010 ± 0.018). Baseline apoptosis and proliferation were not significantly correlated with baseline PSA level.

The *in vitro* serum antioxidant effect of pomegranate juice consumption was determined by evaluating the basal serum oxidative state and the sensitivity to AAPH-induced oxidation of the patients' serum at baseline and after 9 months of pomegranate juice consumption using the lipid peroxides method. Compared with baseline, patients' serum showed a significant 40% reduction in the basal oxidative state and a significant 15% reduction in the resistance of their serum samples to AAPH-induced lipid peroxidation after pomegranate juice consumption ($P < 0.02$ for both tests; Fig. 4).

Compared with baseline, there was a 23% increase in serum NO metabolites measured in patients serum at 9 months

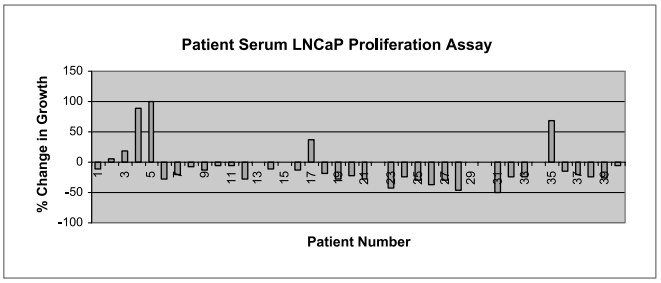

Fig. 2. Percent change in the growth of human prostate cancer cells (LNCaP) using culture medium supplemented with individual serum, comparing proliferation observed using posttreatment versus baseline patient serum. Compared with baseline, at 9 months, there was an average 12% reduction in the growth of LNCaP (signed rank sum test, $P = 0.0048$).

(mean of difference, 8.56 μmol/L; $P = 0.0085$), with two thirds of patients assayed having an increase compared with baseline (Fig. 5). There was a significant negative correlation between baseline PSA and baseline nitrite level (Spearman correlation coefficient = −0.40; $P = 0.0281$).

Pomegranate polyphenols (i.e., ellagic acid) were detected in the urine of all participants by liquid chromatography-mass spectrometry (Fig. 6).

There was no significant difference in pretreatment and posttreatment patient hormone levels (testosterone, estradiol, sex hormone-binding globulin, dehydroepiandrosterone, insulin-like growth factor, and androstenedione) measured (data not shown).

Discussion

In this study of 46 men with recurrent prostate cancer, treatment with pomegranate juice was associated with statistically significant prolongation of PSADT (albeit it, with relatively few objective PSA responses), durable prolongation of disease stabilization, and significant effects on exploratory laboratory assays, such as the patients' serum antioxidant status, and on prostate cancer *in vitro* cell growth and apoptosis. The pattern of achieving a slowing of PSA progression without significant PSA declines is consistent with a cytostatic rather than a cytotoxic treatment effect.

There has been a great deal of interest regarding the potential role of diet in altering the incidence, progression, or mortality of prostate cancer. Much of the research that has shown an inverse relationship between diet and these prostate cancer end points has been epidemiologic in nature, primarily observational cohort and case-control studies (8). The largest prospective study of this type, the European Prospective Investigation into Cancer and Nutrition, is ongoing. European Prospective Investigation into Cancer and Nutrition is the largest study of the relationship between diet, nutritional status, environmental factors, and incidence of cancer, having recruited >500,000 participants in 10 European countries beginning in 1992. Preliminary, early results from the European Prospective Investigation into Cancer and Nutrition study have not shown a link between the self-reported amount of

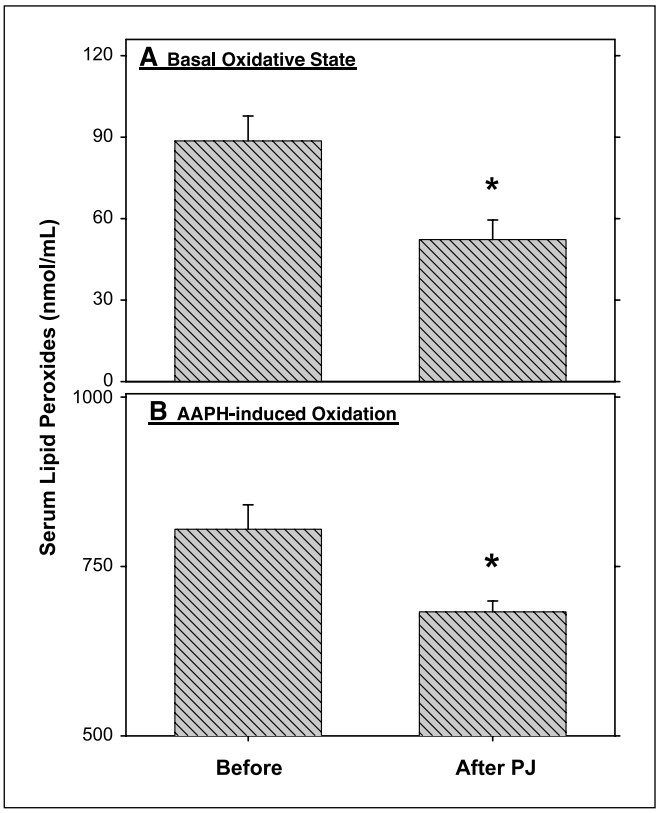

Fig. 4. Oxidative state and sensitivity to oxidation of serum from prostate cancer patients before and after pomegranate juice consumption. *A,* basal oxidative state was analyzed in the patients' serum samples by the lipid peroxide method before and after pomegranate juice (*PJ*) consumption. *B,* resistance of the serum samples to the free radical generator AAPH-induced lipid peroxide formation was analyzed by the lipid peroxides method. *Columns,* mean; *bars,* SD. *, $P < 0.02$.

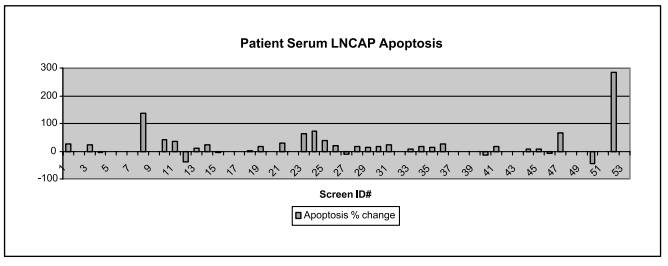

Fig. 3. Percent change in apoptosis of human prostate cancer cells (LNCaP) using culture medium supplemented with individual serum, comparing cell death observed using posttreatment versus baseline patient serum. Compared with baseline, at 9 months, there was a 17.5% increase in apoptosis at 9 months compared with baseline (signed rank sum test, $P = 0.0004$).

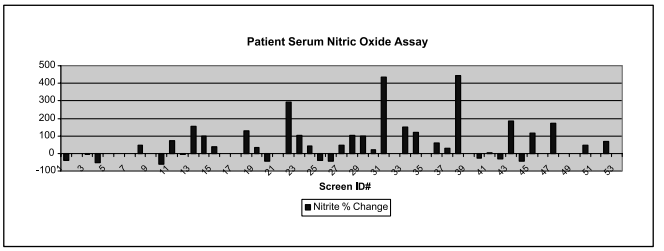

Fig. 5. Percent change in circulating serum NO metabolites. Compared with baseline, there was a 23% increase in serum NO metabolites measured in patients serum at 9 months ($P = 0.0085$).

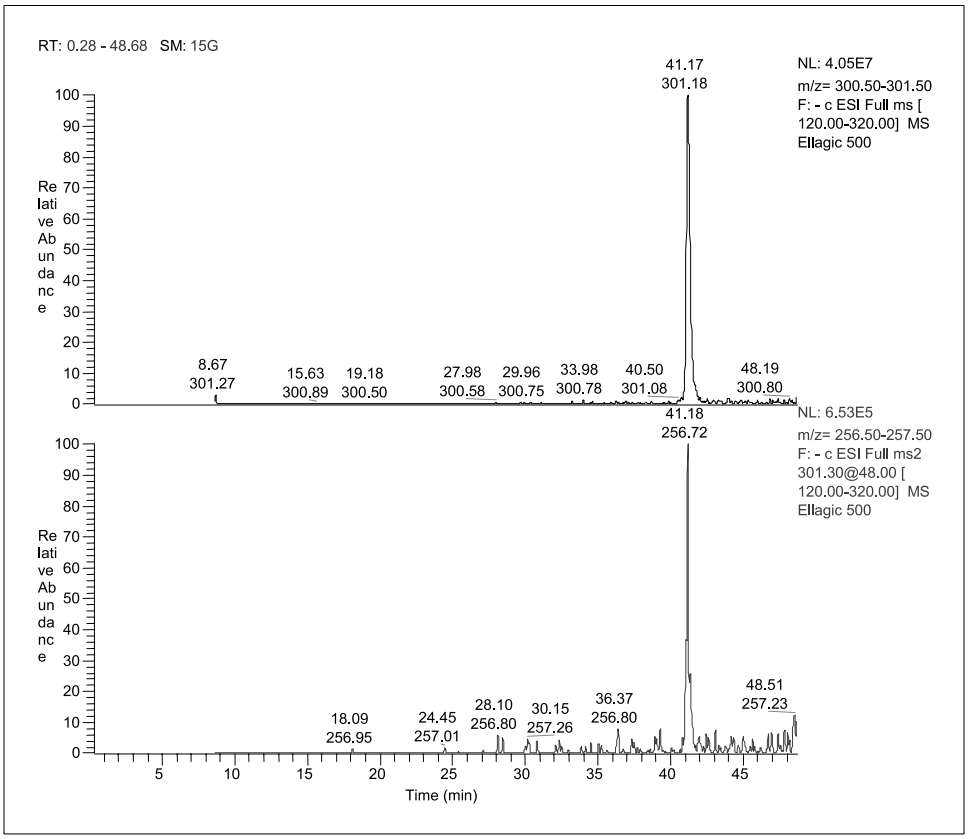

Fig. 6. Liquid chromatography-electrospray ionization/mass spectrometry spectra of posttreatment patient urine showing extracted ion chromatogram of ellagic acid, M-H *m/z* 301. Spectra were obtained by electrospray ionization in negative mode acquiring ions between 120 and 1,500 a.m.u.

fruits and vegetables that men eat and their risk of developing prostate cancer at 4.8 years (31). Several large, prospective, randomized, placebo-controlled experimental studies to study dietary prevention of prostate cancer are ongoing, including the Selenium and Vitamin E Chemoprevention Trial sponsored by the NIH, and a study of selenium in men with high-grade prostatic intraepithelial neoplasia organized by the Southwest Oncology Group. Although much focus has been on identifying primary chemopreventive agents that might delay or prevent cancer from developing *de novo*, there is no *a priori* reason to assume that such an agent would be as effective in the treatment of established cancer or vice versa. Unfortunately, as of yet, there have been no comparably sized clinical intervention studies of dietary treatments for established prostate cancer, although several small, PSA-based phase II studies have been reported. These studies include foods, such as soy protein (32, 33), mushroom extract (34), and multi-ingredient dietary antioxidant nutritional supplements (35, 36). Although with few exceptions (33, 36) PSA kinetics were unaffected by the dietary treatment studied, these studies have tended to feature small cohorts of patients, treated for short periods of time, and without placebo control comparison.

In the current study, in addition to the significant prolongation of PSADT, pomegranate juice intake was associated with antiproliferative and proapoptotic effects in our exploratory bioassays. It should be recognized that the clinical relevance of these unvalidated assays is as yet unclear. However, although the specific factors mediating these effects will be a subject of future studies in a prospective randomized trial comparing pomegranate juice with placebo, it is interesting to speculate on the potential mechanisms of action of pomegranate polyphenols on the growth dynamics observed in the *in vivo* prostate cancer assays. The lack of any observed effect on serum androgen levels suggests that the effect is not primarily hormonal in nature. Further, there is no reason to believe that polyphenols artifactually affect circulating serum PSA measurement. This is supported by a separate pharmacokinetic study (data not shown), in which there was no relationship between circulating ellagic acid and PSA when both were serially and simultaneously measured after pomegranate juice consumption. Chief, however, among the potentially explanatory hypotheses is the role of inflammation in cancer and the antioxidant and anti-inflammatory effects of pomegranate polyphenols. Chronic inflammation has been linked to the incidence of many cancers, including that of the prostate (37). Studies investigating samples of human tissues have shown that epithelial cell proliferation is increased by inflammation (38). An increased risk of cancer is associated with inflammatory mechanisms, as ~15% of all cancers can be related to chronic inflammation (38). Epidemiologic studies have found an increased risk for prostate cancer in men who have a prior history of sexually transmitted disease or prostatitis (39). Inflammation in the microenvironment of the prostate cancer cell may stimulate the multistep process of carcinogenesis by up-regulating the production of proinflammatory cytokines and their signaling pathways. In fact, proliferative inflammatory atrophy has recently been proposed as a precursor to prostatic intraepithelial neoplasia and prostate cancer (40).

Inflammation can result in persistent oxidative stress in cancer cells and the reactive oxygen species may lend cancer

cells a survival advantage (41–43). Mild levels of oxidative stress stimulate cancer cell proliferation (42) and increase mutation rates through DNA damage and/or epigenetic changes (44). De Marzo et al. (40) have shown the loss of glutathione *S*-transferase P1 as an early event in prostate tumors that sets the stage for stimulation of growth by oxygen radicals. Oxidative stress has also been shown to increase cancer cell proliferation by increasing the sensitivity of growth factor receptors and by altering transcription factor activity. Inflammatory cells, such as macrophages and mast cells, release angiogenic factors and cytokines like tumor necrosis factor-α, interleukin-1, and vascular endothelial growth factor, which signal cell growth and proliferation. Additionally, cytokines regulate signaling pathways that control proliferation, apoptosis, differentiation, and metastasis.

Oxidative stress is thought to be a factor associated with many human diseases either as a cause or as an effect. Diverse diseases, including cancer, cardiovascular disease, and diabetes mellitus, are associated with oxidative damage mediated presumably via reactive oxygen and nitrogen species (reactive oxygen and nitrogen species, respectively). The main cellular components susceptible to damage by free radicals are lipids (peroxidation of fatty acids in membranes), proteins (denaturation), and nucleic acids. The role of antioxidants derived from the diet in protection against oxidative stress and the development or progression of cancer remains a topic of significant controversy. The antioxidative properties of plant polyphenols are thought to arise from their reactivity as hydrogen or electron donors, from their ability to stabilize unpaired electrons, and from their ability to terminate Fenton reactions (45). Although many dietary antioxidants have been shown to function as antioxidants *in vitro*, the ability of these substances to decrease oxidative damage to biomolecules *in vivo* in humans has been less frequently studied, and long-term intervention studies using manipulation of dietary antioxidants have been done even less frequently mainly with disappointing results (46, 47).

Potent antioxidant and prostaglandin-inhibitory activities have been suggested previously for polyphenols extracted from pomegranate juice and seed oil, although the literature on this topic remains relatively small (48). Likewise, however, these polyphenols have been shown to promote apoptosis, to inhibit proliferation and invasion, and to inhibit 7,12-dimethylbenz(*a*)anthracene–initiated carcinogenesis *in vitro* and *in vivo* models of human and murine breast cancer (22). In skin cancer models, pomegranate seed oil significant decreased tumor incidence and multiplicity in CD_1 mice and was associated with an inhibition of 12-*O*-tetradecanoylphorbol-13-acetate–induced ornithine decarboxylase activity (49) as well as modulation of mitogen-activated protein kinase and NF-κB pathways (18). The suppression of NF-κB is notable and has been noted that pomegranate can suppress NF-κB activation in other models, such as vascular endothelial cells (50). Activation of the NF-κB transcription factor results in up-regulation of antiapoptotic genes, and NF-κB is thought to be a key factor related involved in the control of cell proliferation, inhibition of apoptosis, and oncogenesis in many cancer, including prostate cancer (51). Pomegranate phytochemicals have been shown to inhibit the *in vitro* proliferation of three prostate cancer cell lines, LNCaP, PC3, and DU145, mediated by changes in both cell cycle distribution and induction of apoptosis and showed *in vivo* inhibition of xenograft growth in athymic mice (20). Also in PC3, Malik et al. (52) showed inhibition of cell growth by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and induction of apoptosis as determined by DNA fragmentation, induction of Bax and Bak, and down-regulation of Bcl-X_L and Bcl-2. Likewise, we have presented preclinical *in vitro* studies[6] that have shown 59% to 75% growth inhibition, delayed progression into S phase, and generation of low levels of apoptosis in the PC3 prostate cancer cell line, whereas s.c. and orthotopic *in vivo* studies of LAPC-9 tumors in severe combined immunodeficient mice have shown 52% growth inhibition tumors 70% reduction in PSA.[7]

Although a plethora of assays are available to measure oxidative stress and antioxidant status (53, 54), currently no standard methods are available for estimating oxidative stress, making the choice of assay somewhat arbitrary. To date, the overwhelming majority of these assays have not been subjected to rigorous validation checks, and none can currently be classified as a true clinical efficacy measure or as a validated surrogate end point. This is true even for perhaps the most commonly used and popular assays of oxidative damage, 8-oxo-7,8-dihydroguanine, which measures the extent of DNA base oxidation. Although the European Standards Committee on Oxidative DNA Damage was set up to resolve the problems associated with the measurement of background levels of oxidative DNA damage, large sources of uncertainty still exist in estimating concentrations of 8-oxo-7,8-dihydroguanine.

In the current study, two assays were used to estimate oxidative stress, one directly through a conventional assay (lipid peroxidation) and the other indirectly through an estimate of NO, which has well-defined anti-inflammatory properties (55). It has been shown previously that intervention with antioxidants can increase endothelial NOS expression both in cultured endothelial cells and in hypercholesterolemic mice (56). More recently, it has been reported that polyphenol flavonoids contained in pomegranate juice are capable of eliciting similar effects, enhancing the biological actions of NO by virtue of their capacity to stabilize NO. By protecting against the oxidative destruction of NO by reactive oxygen species and other radicals, pomegranate juice has produced higher and more prolonged cellular NO concentrations and biological actions (57). The role of NO as a potential chemopreventive agent remains controversial, with both inhibitory and promoting effects on neoplasia being reported secondary to the NO/inducible NOS system (58). In studies *in vitro*, NO-donating aspirin, consisting of aspirin plus an -ONO(2) moiety linked via a molecular spacer, has been shown to inhibit the growth of colon, pancreatic, lung, skin, leukemia, breast, and prostate cancer cells (59), owing both to inhibition of cellular proliferation by blocking the G_1-S cell cycle transition and to induction of apoptosis at least partially through inhibition of NF-κB. In this current study, there was a significant negative correlation between baseline PSA and baseline serum nitrite level (Spearman correlation coefficient = −0.40; $P = 0.0281$).

[6] Unpublished data.

[7] A.J. Pantuck and A.S. Belldegrun. Prostate Cancer Foundation Scientific Retreat, Lake Tahoe, NV, 2004.

From a practical standpoint, the prevention of disease progression to a metastatic state, where the risk of cancer related mortality is increased, is also a desirable outcome. This objective can be achieved by eliminating the disease but also by slowing the growth of the tumor. It remains controversial whether modulation of PSA levels represents an equally valid clinical end point. Although the Food and Drug Administration has not yet accepted a PSA end point to support drug approval, evaluation of additional data and further discussions of PSA end points are planned in future workshops and Oncologic Drugs Advisory Committee meetings. PSADT, however, is increasingly being seen by some as an important surrogate biomarker for prostate cancer mortality. Recently, it was shown that a posttreatment PSADT of <3 months seems to be a surrogate end point for prostate cancer-specific mortality (60). Freedland et al. recently published the outcomes of 379 prostate cancer patients who developed an increasing PSA level after radical prostatectomy, finding that in this setting PSADT is the clinical factor most consistently correlated with death from prostate cancer (61). Because men with a greater PSADT can expect a longer survival, implementation of strategies that could prolong PSADT, thereby delaying the time to treatment with hormone therapy and to death, would of great benefit to patients. Previously, several small single-arm phase II studies of agents in prostate cancer have shown stabilization of PSA variables (62–64), but at least one such study failed to show a statistically significant effect on the PSADT when compared in a placebo-controlled study (65). Interestingly, in the randomized study of the peroxisome proliferator-activated receptor-γ inhibitor rosiglitazone in men with prostate carcinoma and a rising serum PSA level, 38% of men in the placebo arm experienced a favorable outcome, defined as a posttreatment PSADT >150% of the baseline PSADT. Although this incidence of PSADT prolongation was smaller than that seen in this study, these studies highlight the potential limitations of PSA variables in monitoring patients and the need for confirmatory prospective studies using a blinded control arm.

Conclusions

We report the first clinical trial of pomegranate juice in patients with recurrent prostate cancer. This study shows statistically significant effects on PSADT coupled with corresponding effects on prostate cancer *in vitro* cell growth and apoptosis. These proposed benefits, however, are in assays that are as yet unvalidated, and further research is needed to prove the validity of these tests and to determine whether improvements in such biomarkers (including PSADT) are likely to serve as surrogates for clinical benefit. The present results, therefore, are being further tested in a randomized, double-blind, three-arm, placebo-controlled study, in which the ability of two pomegranate juice doses to produce a predefined alteration in PSA kinetics is compared with the change observed in a control group. This future study, which began in April 2006, addresses several limitations of the current study, with the inclusion of two treatment arms in a dose-response design as well as the use of a placebo control. Furthermore, the planned study will be used to prospectively evaluate the performance of the exploratory biomarkers used in the current protocol and to permit further study of potential serum factors mediating the antiproliferative and proapoptotic effects observed in the present study. Finally, should such a study be positive, a strong rationale would exist for research on other plant polyphenols (red wine resveratrol, etc.) that might mediate similar effects.

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