PRELIMINARY COMMUNICATION

Effect of Testosterone Replacement Therapy on Prostate Tissue in Men With Late-Onset Hypogonadism

A Randomized Controlled Trial

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Context Prostate safety is a primary concern when aging men receive testosterone replacement therapy (TRT), but little information is available regarding the effects of TRT on prostate tissue in men.

Objective To determine the effects of TRT on prostate tissue of aging men with low serum testosterone levels.

Design, Setting, and Participants Randomized, double-blind, placebo-controlled trial of 44 men, aged 44 to 78 years, with screening serum testosterone levels lower than 300 ng/dL (<10.4 nmol/L) and related symptoms, conducted at a US community-based research center between February 2003 and November 2004.

Intervention Participants were randomly assigned to receive 150 mg of testosterone enanthate or matching placebo intramuscularly every 2 weeks for 6 months.

Main Outcome Measures The primary outcome measure was the 6-month change in prostate tissue androgen levels (testosterone and dihydrotestosterone). Secondary outcome measures included 6-month changes in prostate-related clinical features, histology, biomarkers, and epithelial cell gene expression.

Results Of the 44 men randomized, 40 had prostate biopsies performed both at baseline and at 6 months and qualified for per-protocol analysis (TRT, n=21; placebo, n=19). Testosterone replacement therapy increased serum testosterone levels to the mid-normal range (median at baseline, 282 ng/dL [9.8 nmol/L]; median at 6 months, 640 ng/dL [22.2 nmol/L]) with no significant change in serum testosterone levels in matched, placebo-treated men. However, median prostate tissue levels of testosterone (0.91 ng/g) and dihydrotestosterone (6.79 ng/g) did not change significantly in the TRT group. No treatment-related change was observed in prostate histology, tissue biomarkers (androgen receptor, Ki-67, CD34), gene expression (including *AR*, *PSA*, *PAP2A*, *VEGF*, *NXK3*, *CLU* [*Clusterin*]), or cancer incidence or severity. Treatment-related changes in prostate volume, serum prostate-specific antigen, voiding symptoms, and urinary flow were minor.

Conclusions These preliminary data suggest that in aging men with late-onset hypogonadism, 6 months of TRT normalizes serum androgen levels but appears to have little effect on prostate tissue androgen levels and cellular functions. Establishment of prostate safety for large populations of older men undergoing longer duration of TRT requires further study.

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TESTOSTERONE REPLACEMENT therapy (TRT) in aging men is a widespread, growing practice. According to pharmaceutical industry estimates, more than 1.8 million prescriptions for testosterone products were written in the United States in 2002, a 30% increase over the previous year and a 170% increase over the previous 5 years[1]; in 2005, a total of 2.3 million prescriptions were written.[2]

Serum levels of testosterone decline with age,[3] and in many aging men with low levels of the hormone, alterations such as depression, sexual dysfunction, diminished lean body mass, diminution in muscle volume and strength, and reduced bone mineral density may develop. Such changes, in association with low testosterone levels, have been called "male menopause," which is also known as male climacteric,[4] andropause,[5] androgen deficiency of the aging male syndrome,[6] or late-onset hypogonadism.[7] Aspects of the syndrome may be ameliorated with TRT,[8-10] and most testosterone prescriptions are cur-

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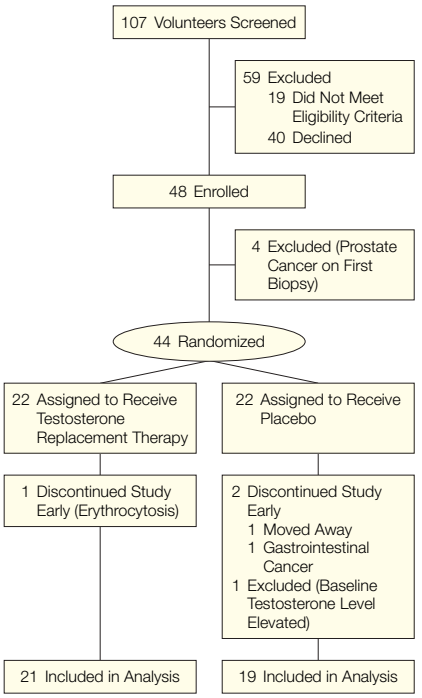
rently written for men older than 45 years,[1] a demographic in which prostate disease is most common. Between 2 and 4 million men, nearly all in this "prostatic age group," may be candidates for treatment.[11]

Figure 1. Flow of Participants Through the Study



Prostate growth, both normal and abnormal, is dependent on the presence of androgens. Without androgenic stimulation, the prostate fails to develop.[12-14] After development of the gland in adults, reduction in testosterone levels causes regression of both benign and malignant prostatic overgrowth.[15,16] Conversely, in men with advanced prostate cancer, testosterone administration often exacerbates the disease, causing increased bone pain, urinary obstruction, and even death.[17,18]

Thus, when aging men receive supplemental testosterone, a primary concern is prostate safety. Even in men with no sign of prostate cancer, the possibility of stimulating growth in subclinical disease exists. Instances of prostate cancer in men receiving testosterone supplementation have been reported.[19-21] When TRT is prescribed, careful monitoring for prostate disease is considered mandatory.[11] However, screening and follow-up of serum levels of prostate-specific antigen (PSA) may be problematic because PSA is directly regulated by androgens and might increase in the absence of disease.

Clinical trials to date have shown no evidence of any overt carcinogenicity or nonphysiological effect on prostatic growth when TRT is used in otherwise healthy aging men.[22,23] Attempts to relate endogenous serum testosterone levels to the development of prostate cancer or benign prostatic hyperplasia, with occasional exception,[24,25] have been inconclusive.[26] Experimentally, testosterone administration in laboratory animals when used alone serves only as a weak carcinogen at most.[27] One estimate suggests that to detect a 30% increase in treatment-related prostate cancers from TRT, a randomized controlled trial involving more than 6000 older men and having follow-up for at least 5 years would be required[28]; however, an expert panel of the Institute of Medicine recommended that short-term efficacy trials should be completed to justify a larger safety trial.[1]

We conducted a randomized controlled trial to assess the effects of TRT on the prostate with a focus on prostate tissue. The hypothesis tested in this serial biopsy study is that exogenous testosterone accumulates in the prostate, converts to dihydrotestosterone, and affects biological change in the gland.

METHODS

Study Participants

The study was conducted between February 2003 and November 2004 at the Urological Sciences Research Foundation, a community-based, nonprofit research center located in Los Angeles, Calif. Men aged 44 to 78 years with symptoms attributable to late-onset hypogonadism and a screening testosterone level lower than 300 ng/dL (<10.4 nmol/L) were recruited from a general urology practice of one of the authors (L.S.M.).

We screened 107 volunteers (FIGURE 1). The screening evaluation consisted of a medical history, which included the androgen deficiency of the aging male questionnaire,[7,29] physical examination, multiphasic serum panel, and measurement of total serum testosterone and PSA levels. Exclusion cri-

Table 1. Baseline Characteristics*

	Testosterone Replacement Therapy (n = 21)	Placebo (n = 19)	*P* Value†
Age, y	68 (44-78)	70 (45-78)	.47
Body mass index‡	28.34 (22.70-37.90)	29.57 (23.60-37.80)	.58
Testosterone level, ng/dL	221 (163-320)	252 (144-328)	.22
Prostate-specific antigen, ng/mL	1.55 (0.27-5.78)	0.97 (0-2.47)	.02
Prostate volume, mL§	43.75 (15.50-112.00)	36.75 (17.20-105.00)	.36
Transition zone volume, mL§	20.99 (4.76-76.50)	18.38 (6.44-54.00)	.57
International Prostate Symptom Score (voiding symptoms)	13 (0-26)	12 (0-27)	.47
Uroflowmetry rate, mL/s	14.0 (4.0-31.0)	10.6 (7.3-22.7)	.46
Race/ethnicity, No. (%)			
White	9 (43)	12 (63)	.45
Black	8 (38)	4 (21)	
Hispanic	3 (14)	3 (16)	
Asian	1 (5)	0	

SI conversion factor: To convert testosterone to nmol, multiply by 0.0347.
*Values are expressed as median (range) unless otherwise indicated.
†Calculated using the signed rank test.
‡Calculated as weight in kilograms divided by height in meters squared.
§Measured using magnetic resonance imaging.

teria included use in the past 6 months of any drug potentially affecting the pituitary-gonadal axis, serum PSA level greater than 10.0 ng/mL, or refusal or inability to undergo prostate biopsies, or presence of prostate cancer on initial biopsy results. Of the 107 men screened, 19 did not meet eligibility criteria, 40 declined to participate, and 4 were excluded after the enrollment biopsy revealed cancer (a total of 63 were excluded). The remaining 44 men were randomly assigned to receive testosterone or placebo beginning 1 week after the initial biopsy was performed (TABLE 1). Race/ethnicity was self-reported at enrollment.

Study Design

A randomized, double-blind, placebo-controlled trial was conducted to compare prostate tissue obtained before and after 6 months of biweekly treatment with either testosterone enanthate (150 mg, Delatestryl, BTG Pharmaceuticals, Iselin, NJ) or a saline placebo by intramuscular injection. Compliance with dosing exceeded 99%—only 2 doses (1 in each group) of testosterone (of 533) were given "out of window" (ie, >3 weeks after the prior dose). In these 2 participants, a new 2-week cycle was established based on the timing of the last dose but overall study duration did not exceed 28 weeks in either participant.

After baseline, blood samples were collected and the end-of-study biopsy was performed 1 week following the previous injection. All injections and clinical data collection were performed by a research coordinator (M.L.M.), who was kept blinded to group assignment. Laboratory tests and data analysis were performed by personnel who were blinded to group assignment. The randomization table was generated and maintained by a biostatistician, who had no direct patient involvement. Adverse effects were monitored by interview, physical examination, laboratory testing (including PSA levels), and by an external data and safety monitoring board review of the biopsy performed at the end of the study. At enrollment, participants provided written informed consent to participate in the study, which was approved by the Western Institutional Review Board (Olympia, Wash).

Clinical Prostate Testing

At baseline and study visits at 3 months and 6 months, patients received a digital examination of the prostate and were assessed by the research coordinator (M.L.M.) using the International Prostate Symptom Score and uroflowmetry (maximum flow rate maintained for 2 seconds with a minimal voided volume of 150 mL). Prostate volumetrics (whole and transition zone separately) were determined a few days before each biopsy session by a radiologist, who was blinded to the purpose of the study and to group assignment, using transabdominal magnetic resonance imaging (T2-weighted images) and a geometric formula (length × width × height/2). Detailed methods of clinical prostate testing have been previously published.[30,31]

Biopsy Protocol

Prostate biopsy was performed using transrectal ultrasound guidance, local anesthesia, a spring-loaded biopsy gun, and 18-gauge hollow needles.[32] At each biopsy session, cores from the mid-peripheral zone were taken from the right and left sides and quick-frozen for hormone determinations.[33] Six cores were then taken systematically from the peripheral zone of each side. These 12 cores were fixed in formalin, paraffin preserved, and then cut to exhaustion of the blocks, which usually yielded at least twenty 5-micron sections per block. A quinolone antibiotic was administered for 24 hours after each biopsy was performed.

Serum Hormones

Morning blood samples were collected at baseline and during study visits at 6 weeks, 3 months, and 6 months and were scheduled 1 week after the prior injection. All hormone studies were performed at Esoterix Endocrinology (Calabassas Hills, Calif). Testosterone level was determined by mass spectroscopy, dihydrotestosterone and estradiol levels by radioimmunoassay, sex hormone-binding globulin level by displacement of tritiated testosterone, free testosterone level by equilibrium dialysis, and luteinizing hormone level by immunochemoluminescence (methods on file, Esoterix Endocrinology). Screening testosterone levels were somewhat lower than baseline levels because the baseline levels were uniformly obtained between 8 AM and noon, while the screening levels were obtained at random times throughout the day.

Prostate Tissue Androgens

Prostate androgen determinations (testosterone and dihydrotestosterone) were performed on quick-frozen biopsy cores (5-10 mg) at the Endocrine Services Laboratory of the Oregon National Primate Research Center in Beaverton, using a technique originally developed to measure serum androgens in small aliquots (<40 uL) of primate fetal blood.[34] The technique was subsequently validated for human prostate needle biopsies.[33] Briefly, frozen tissue cores were individually thawed, weighed, homogenized, and the homogenate extracted with diethyl ether, maintaining the aqueous fraction at 4°C during the entire process. Chromatography was performed on the ether extracts using the 2.0-g Sephadex LH-20 microcolumns.

Dihydrotestosterone and testosterone fractions were collected and assayed by radioimmunoassay. Method blanks were monitored by assaying the same chromatographic fractions collected from buffer alone or with equivalent amounts of prostatic tissue obtained from a 78-year-old man pretreated with a gonadotropin-releasing hormone analog and dexamethasone. The blanks for the homogenization buffer were 6.1 pg/tube for dihydrotestosterone and 11.4 pg/tube for testosterone; for suppressed prostatic tissue, the blanks were 5.2 pg/tube for dihydrotestosterone and 7.6 pg/tube for testosterone. Extraction and chromatographic recoveries for dihydrotestosterone and testosterone were 76.1% and 75.7%, respectively. Each sample was

corrected for tissue method blank and recovery before calculating the mass per gram of the tissue. All samples were assayed in a single assay with the intraassay coefficient of variation for dihydrotestosterone and testosterone of 14.5% and 9.4%, respectively, based on a control pool (mean [SD] dihydrotestosterone: 1.05 [0.15] ng/mL assayed at 50 and 150 uL; mean [SD] testosterone: 3.33 [0.32] ng/mL assayed at 20 and 80 uL) of male macaque serum processed in parallel with the tissue samples.

Prostate Histological Studies

Routine histological examination was used to assign a tissue diagnosis and an atrophy score to each case by one of the authors (J.I.E.). Paraffin-preserved sections were used to analyze biomarkers for cell proliferation (Ki-67, MIB-1), androgen receptor, and angiogenesis (CD34) at Bostwick Laboratories (Richmond, Va), and stroma-epithelial ratio at the Brady Urological Institute of Johns Hopkins University School of Medicine (Baltimore, Md), as in a previous report.[35]

Gene Expression Profiling

Gene expression profiling was performed at the Fred Hutchinson Cancer Research Center (Seattle, Wash) using microdissected prostate epithelial cells, followed by RNA isolation, amplification, and hybridization of labeled complementary DNA to a prostate-specific custom complementary DNA microarray.[36,37] Validation of microarray findings by quantitative reverse transcriptase–polymerase chain reaction (PCR) was performed for 4 known androgen-regulated genes (*PSA*, *AR*, *NKX3.1*, *PAP2A*) and for genes related to cell survival and angiogenesis (*CLU* [*Clusterin*] and *VEGF*).

Laser Capture Microdissection and RNA Amplification. Samples in the TRT group demonstrating the highest percentage change from baseline in tissue androgen levels (for whom tissue samples were available) were selected for analysis. A similar number of samples from the placebo group were randomly selected. However, 3 samples in the placebo group demonstrated an increase in tissue testosterone of more than 100% and were excluded.

Prostate epithelial cells were microdissected from formalin-fixed, paraffin-embedded pretreatment and posttreatment prostate tissue samples using the Arcturus Veritas Laser Capture Microdissection System (Mountain View, Calif). Samples were prepared for staining, microdissection, and RNA isolation and amplification using the Arcturus Paradise Reagent System according to the manufacturer's instructions. Approximately 2000 to 3000 cells were captured per sample, yielding 20 to 40 µg of RNA after 2 rounds of amplification. Amplified RNA was quantified in a Gene-Spec III spectrophotometer (Hitachi, Tokyo, Japan) and RNA integrity was evaluated using gel electrophoresis.

Microarray Hybridization and Data Analysis. Complementary DNA probe pairs were prepared by amino-allyl reverse transcription using 3 µg of amplified RNA from microdissected samples and 30 µg of total RNA from a reference RNA pool composed of total RNA isolated from the LNCap, DU145, and PC3 prostate cell lines. Probes were labeled with either Cy5 or Cy3 fluors (Amersham Bioscience, Piscataway, NJ) and competitively hybridized to complementary DNA microarrays spotted in duplicate with approximately 6700 unique complementary DNA clones from the Prostate Expression Database as previously described.[36,37]

Arrays were normalized using a print tip-specific Lowess curve fit to the log-intensity plot compared with the log-ratio plot, using 20.0% of the data to calculate the fit at each point. The log 2 of the experimental-to-reference sample ratios were used in subsequent analyses. Duplicate complementary DNA spots on each microarray chip were averaged for purposes of spatial normalization. Data were filtered to include clones returning data for at least 75% of the samples in both the TRT group and the placebo group.

Quantitative PCR Analysis. To confirm the results of the gene expression microarrays, amplified RNA was evaluated by quantitative PCR. Complementary DNA was generated from each sample using 0.5 µg of amplified RNA in a random hexamer-primed reverse transcription reaction. Quantitative PCR reactions were performed in triplicate using an Applied Biosystems 7700 sequence detector (Foster City, Calif) with approximately 5 ng of complementary DNA, 1 µM of each primer pair, and SYBR Green PCR master mix (Applied Biosystems). Expression levels of *AR*, *PSA*, *NKX3.1*, *PAP2A*, *VEGF*, and *CLU* were evaluated. The sequences of primers used were AR forward 5′-ATCCTCATATGGCCCAGTGTCAAG-3′, reverse 5′-GCTCTCTAAACTTC-CCGTGGCATA-3′; PSA forward 5′-GCATGGGATGGGGATGAAGTAAG-3′, reverse 5′-CATCAAATCTGAG-GGTTGTCTGGA-3′; NKX3.1 forward 5′-AACCATTTCACCCAGA-CAGCCT-3′, reverse 5′-TGTGACA-GATTGGAGCAGGGTT-3′; PAP2A forward 5′-ATGCCTCTTGGATGCA-CACTTT-3′, reverse 5′-ATACAG-GTGGGGCACTGTTTTG-3′; VEGF forward 5′-TTTCCAATCTCTCTCTC-CCTGAT-3′, reverse 5′-GAGGGCA-GAGCTGAGTGTTAGC-3′; CLU forward 5′-GAGCTCTGCACGTCA-CCAAGTA-3′, reverse 5′-TTCTTC-CCATGAGCAGCAGAGT-3′.

Statistical Analysis

The study was powered to detect an approximate 25% change in mean prostate dihydrotestosterone levels based on a prior study showing mean (SD) prostate levels of dihydrotestosterone to be generally 5 to 6 (2.5) ng/g.[33] Using nQuery Advisor software version 6.0 (Statistical Solutions, Saugus, Mass), a sample size of 22 men was calculated to yield more than 80% power based on a 1-sided paired *t* test at the .05 level of significance and an effect size of 0.63. For a 2-sided test, the associated effect size was 0.77 at the same power.

Prostate dihydrotestosterone levels from the present study were similar to the levels obtained in the prior studies, indicating the power analysis to be appropriate (TABLE 2 and FIGURE 2). This study was not sufficiently powered to

detect clinical efficacy of testosterone administration (eg, effects on mood, muscle strength, urination, or sexual function). Statistical analysis was performed by one of the authors (F.J.D.).

Hormone, tissue, and clinical changes from baseline were evaluated using the nonparametric signed rank test. Changes from baseline in the 2 groups were compared using the nonparametric rank sum test. For a change to be meaningful, we established a priori a requirement for statistical significance ($P<.05$) by both tests. All participants were analyzed in the groups to which they were randomized, excluding only the 3 who failed to complete the trial (and who therefore did not have poststudy biopsies, see below) and 1 individual whose baseline testosterone level was found on end-of-study batch analysis to be a statistical outlier (701 ng/dL [24.3 nmol/L]) despite a qualifying testosterone level at the screening visit (per-protocol analysis).

Changes in gene expression were evaluated using the Statistical Analysis of Microarray program (Stanford University, Stanford, Calif) to perform unpaired 2-sample *t* tests comparing the placebo group with the TRT group and paired 2-sample *t* tests comparing prebiopsy and postbiopsy samples within the 2 groups.[38] A false discovery rate of less than 10% was considered significant.

Similarities between samples were assessed by unsupervised, hierarchical, average linkage clustering using Cluster 3.0 software (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm) and plotted using TreeView version 1.6 (http://rana.lbl.gov/EisenSoftware.htm). This pro-

Table 2. Clinical, Hormonal, and Histological Results for Baseline vs 6 Months*

	Testosterone Replacement Therapy (n = 21)			Placebo (n = 19)		
	Baseline	6 mo	*P* Value†	Baseline	6 mo	*P* Value†
Clinical						
International Prostate Symptom Score (voiding symptoms)	13.0 (0-26.0)	12.5 (0-30.0)	.43	11.0 (0-27.0)	9.5 (2.0-28.0)	.50
Uroflowmetry rate, mL/s	14.0 (4.0-31.0)	10.6 (4.8-18.9)	.09	10.6 (7.3-22.7)	8.5 (3.0-20.1)	.13
Prostate volume, mL						
Whole	43.8 (15.5-112.0)	42.0 (19.8-117.9)	.16	36.8 (17.2-105.0)	29.4 (17.8-93.0)	.20
Transition zone	21.8 (4.8-76.5)	15.4 (4.1-74.8)	.58	18.4 (6.44-54.0)	16.0 (6.9-55.2)	.47
Prostate-specific antigen, ng/mL						
Total	1.55 (0.30-5.80)	2.29 (0.40-7.10)	<.001	0.97 (0.10-2.50)	1.10 (0.02-6.90)	.006
Free	0.49 (0.20-1.60)	0.68 (0.20-2.13)	<.001	0.21 (0.04-0.66)	0.30 (0.01-5.47)	.13
Hemoglobin, g/dL	14.5 (11.0-18.0)	15.9 (12.1-20.4)	<.001	14.9 (12.6-16.1)	14.8 (12.8-16.0)	.30
Hematocrit, %	43.2 (35.2-50.5)	47.6 (38.8-57.4)	<.001	43.6 (37.4-48.2)	43.4 (37.8-47.6)	.20
Hormonal						
Testosterone						
Total, ng/dL	282 (182-444)	640 (272-1190)	<.001	282 (135-391)	273 (89-715)	.11
Free, pg/mL‡	48 (17-102)	162 (35-309)	<.001	51 (16-66)	42 (8-114)	.16
Dihydrotestosterone, ng/dL	28 (18-56)	47 (20-121)	.002	28 (11-52)	26 (7-40)	.20
Estradiol, pg/mL	22 (6-41)	37 (18-95)	.006	15 (12-36)	17 (10-19)	.67
Luteinizing hormone, IU/L	4.50 (1.10-16.00)	0.10 (0.03-13.00)	<.001	4.80 (1.80-32.00)	4.10 (1.20-40.00)	.79
Sex hormone-binding globulin, µg/dL	0.6 (0.2-2.0)	0.6 (0.1-1.2)	.005	0.7 (0.1-1.3)	0.8 (0.2-1.7)	.82
Testosterone tissue, ng/g	0.91 (0.15-16.46)	1.55 (0.10-23.11)	.29	2.00 (0.11-6.92)	0.88 (0.02-20.12)	.05
Dihydrotestosterone tissue, ng/g	6.79 (3.26-19.59)	6.82 (1.57-39.82)	.51	8.15 (1.21-18.70)	5.10 (0.70-22.37)	.01
Histological						
Carcinoma, No.	0	2		0	4	
High-grade intraepithelial neoplasia, No.	5	2		3	3	
Atrophy score, % of glands	8 (1-50)	1 (1-25)	.01	8 (1-75)	6 (1-75)	.23
Stroma-epithelial ratio	2.06 (0.86-3.80)	2.47 (0.54-5.54)	.69	2.18 (0.50-4.98)	2.65 (0.11-7.95)	.21
Biomarkers						
MIB1 (Ki-67), % of positive cells	0.53 (0.27-1.34)	0.63 (0.33-1.38)	.09	0.45 (0.23-0.86)	0.49 (0.29-1.52)	.70
Androgen receptor, % of positive cells						
Epithelium	80 (50-90)	80 (55-90)	.75	80 (60-90)	85 (65-90)	.18
Stroma	16 (5-60)	33 (8-75)	.02	24 (8-70)	48 (13-60)	.09
CD34, microvessel/200 × field	63.0 (25.0-97.5)	66.0 (48.5-89.0)	.37	65.5 (42.0-90.0)	71.5 (36.0-90.5)	.89

SI conversion factors: To convert estradiol to pmol/L, multiply by 3.671; total testosterone to nmol, multiply by 0.0347.
*Values are expressed as median (range) unless otherwise indicated. Of the 44 men randomized, 3 did not complete the trial and a fourth was excluded because baseline serum testosterone was higher than 700 ng/dL, indicating an error in screening.
†Calculated using the signed rank test.
‡Normal value for adult males is 52 to 280 pg/mL. Free testosterone at baseline was less than 70 pg/mL in 90% of participants.

gram organizes genes and samples into a tree structure based on their similarity, in which items are joined by short branches if they are similar to each other and by increasingly longer branches as their similarity decreases. In average linkage clustering, the distance between 2 items *x* and *y* is the mean of all pairwise distances between items contained in *x* and *y*, and therefore provides a visual estimate of the similarity among different items in a sample. Clustering was performed using the most variably expressed genes, defined as those with the highest interquartile range, which represents the spread between the 75th and 25th percentile of expression data obtained for each gene.

RESULTS

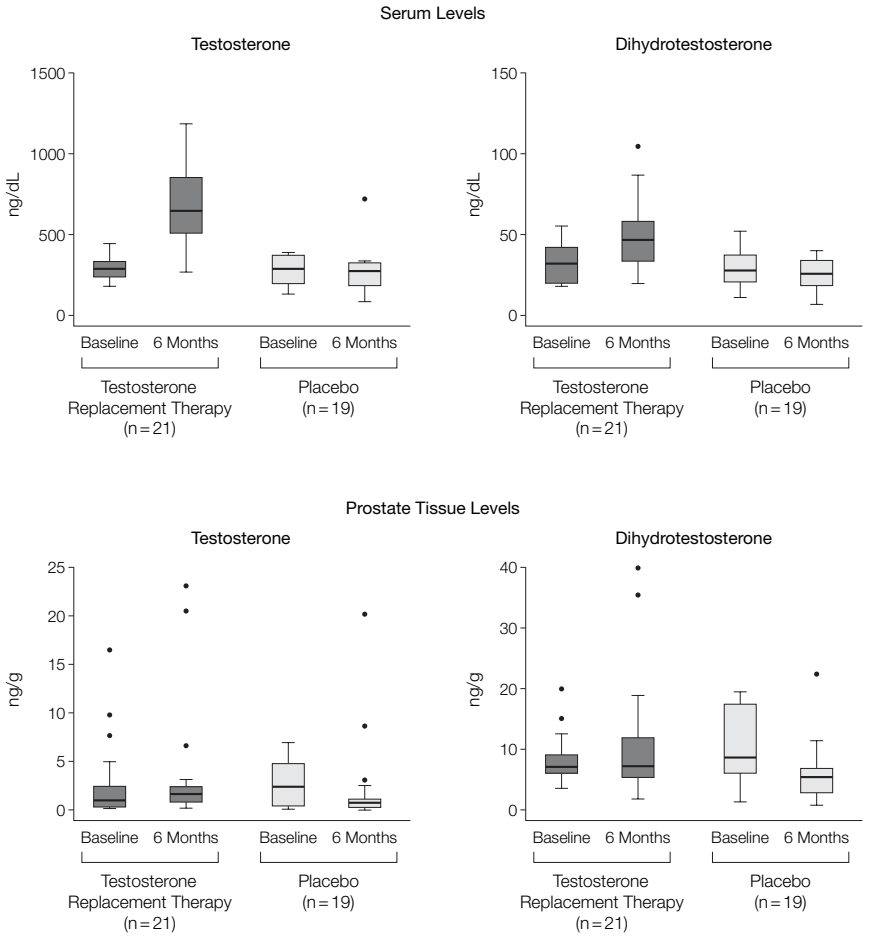
Of the 44 men randomized, 41 completed the entire protocol, including prostate biopsies performed both at baseline and at the end of the study (Figure 1). One participant assigned to receive TRT was terminated from the study early because of treatment-related erythrocytosis (hematocrit of 60% at 3-month visit), which resolved spontaneously within a few weeks of discontinuation of TRT. Two men assigned to receive placebo were also terminated from the study early: one because a gastrointestinal tract malignancy was found and the other because he relocated. The randomization resulted in 2 groups of men who were relatively well-matched by age, race, body mass index (calculated as weight in kilograms divided by height in meters squared), testosterone levels, major prostatic measures of volume, PSA (higher in the TRT group than in the placebo group), voiding symptoms, and urinary flow rate (Table 1).

Androgen Levels

The effect of TRT on prostate tissue androgen levels is shown in relation to serum levels of these hormones in Figure 2. Median serum testosterone levels at baseline indicated a mildly hypogonadal state in both groups (TRT: 282 ng/dL [9.8 nmol/L] [range, 182-444 ng/dL {6.3-15.4 nmol/L}]; placebo: 282 ng/dL [9.8 nmol/L] [range, 135-391 ng/dL {4.7-13.6 nmol/L}]) (Table 2). As a result of TRT, median serum levels of testosterone increased into the mid-normal range (640 ng/dL [22.2 nmol/L] [range, 270-1190 ng/dL {9.4-41.3 nmol/L}]) at 6 months, with no significant change in the placebo group (Table 2). At 6 months, average treatment-related increases in serum were 138% for testosterone, 214% for free testosterone, and 65% for dihydrotestosterone.

In prostate tissue, TRT increased median androgen concentrations only slightly compared with baseline levels or between the 2 groups (testosterone level of 0.91 ng/g at baseline and 1.55 ng/g at posttreatment; dihydrotestosterone level of 6.79 ng/g at baseline and 6.82 ng/g at posttreatment; $P=.29$; Table 2). At baseline (N=40), the correlation coefficient (r) between serum and tissue testosterone and dihydrotestosterone was 0.04 and 0.07, respectively, and after 6 months of TRT (n=21) it was 0.35 and 0.01, respectively. P values for these correlations ranged from .13 to .99 (ie, the correlations between serum and tissue androgens were not statistically significant).

Figure 2. Effects of Treatment on Serum and Prostatic Androgen Levels



Both testosterone and dihydrotestosterone levels increased in serum after 6 months of treatment with testosterone replacement therapy ($P<.001$ by signed rank test). However, despite an increase in serum levels for testosterone to the mid-normal range, prostate tissue levels of the androgens did not change significantly. Boxes contain 50% of data with the inside horizontal line representing the median value; whiskers contain 100% of data, except for statistical outliers shown as individual data points.

Clinical and Tissue Measures

Prostate volume (whole and transition zone) was not significantly changed by TRT. Voiding symptoms and urinary flow rates were not measurably different between the placebo group and the TRT group or between the baseline and posttreatment results. In blood, TRT resulted in significant increases from baseline in median testosterone, dihydrotestosterone, and estradiol levels and also in increases in levels of hemoglobin and hematocrit ($P<.01$ for all; Table 2). Median luteinizing hormone levels decreased significantly. Levels of PSA, which were slightly higher at baseline in the TRT group than in the placebo group ($P=.02$), increased in both groups ($P<.001$), although remaining relatively low. Among tissue measurements, the differences (Table 2) for atrophy and androgen receptor (stroma) were not significant when group changes were compared (TABLE 3). In prostate tissue, no treatment-related change was seen in stroma-epithelial ratio, percentage of atrophic glands, or in measurements of biomarkers for cell proliferation (Ki-67), androgen receptor, or angiogenesis (CD34).

Prostate Cancer

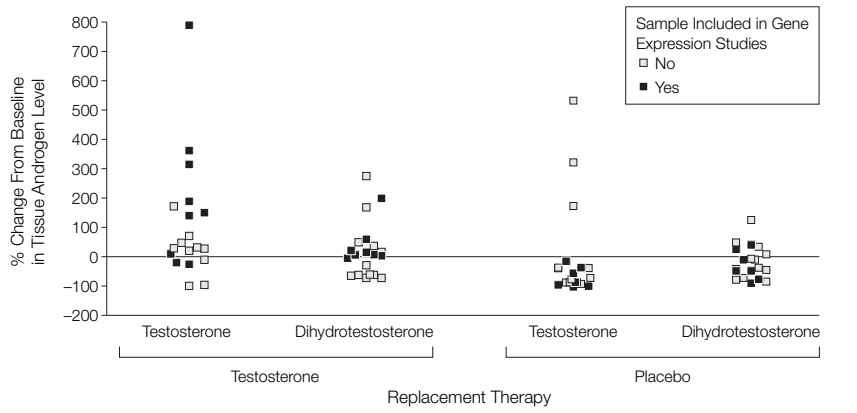
Ten low-volume prostate cancers, each involving only part of 1 biopsy core, were found. Four of the 48 men were found to have prostate cancer on the biopsy performed at enrollment and were excluded from participation at that point. Six of 41 men who completed the trial were found to have prostate cancer on the biopsy performed at the end of the study: 4 of 19 in the placebo group and 2 of 21 in the TRT group. One man in each group had a cancer of Gleason grade 7 on the biopsy performed at the end of the study, both showing a mod-

Table 3. Clinical, Hormonal, and Histological Changes From Baseline

	Testosterone Replacement Therapy (n = 21)		Placebo (n = 19)		Difference of Medians (95% CI)	*P* Value*
	Mean (SD)	Median (SIQR)	Mean (SD)	Median (SIQR)		
Clinical						
International Prostate Symptom Score (voiding symptoms)	1.43 (8.14)	0 (3.00)	−1.21 (7.74)	0 (5.50)	0 (−4.26 to 6.20)	.30
Uroflowmetry rate, mL/s	−3.66 (7.48)	−1.35 (3.85)	−3.44 (7.27)	−4.60 (4.25)	3.25 (−4.77 to 8.17)	.94
Prostate volume, mL						
Whole	3.58 (9.94)	4.19 (6.26)	−2.47 (6.51)	−1.10 (5.40)	5.30 (−1.11 to 12.09)	.07
Transition zone	1.95 (14.29)	3.15 (5.95)	−1.32 (6.40)	−2.78 (4.62)	5.93 (−6.27 to 9.09)	.45
Prostate-specific antigen, ng/mL						
Total	0.90 (0.89)	0.74 (0.55)	0.60 (1.55)	0.14 (0.16)	0.60 (0.28 to 0.83)	.008
Free	0.21 (0.24)	0.15 (0.10)	0.36 (1.25)	0.03 (0.05)	0.12 (0.06 to 0.22)	.01
Hemoglobin, g/dL	1.39 (1.00)	1.70 (0.60)	−0.22 (0.67)	−0.10 (0.50)	1.80 (1.01 to 2.27)	<.001
Hematocrit, %	4.11 (3.58)	4.90 (2.90)	−0.67 (2.30)	−1.10 (1.60)	6.00 (2.83 to 7.31)	<.001
Hormonal						
Testosterone						
Total, ng/dL	397.95 (277.71)	395.00 (221.50)	−8.56 (118.78)	−28.00 (44.50)	423.00 (296.53 to 500.62)	<.001
Free, pg/mL	111.47 (75.82)	107.00 (54.00)	−0.81 (20.84)	−6.00 (8.50)	113.00 (86.66 to 134.70)	<.001
Dihydrotestosterone, ng/dL	19.26 (16.77)	15.00 (12.50)	−3.58 (8.28)	−4.45 (3.50)	19.45 (12.07 to 26.75)	<.001
Estradiol, pg/mL	17.8 (21.3)	19.0 (9.0)	−2.7 (8.8)	−2.0 (4.8)	21.0 (9.4 to 29.3)	.01
Luteinizing hormone, IU/L	−3.93 (2.98)	−4.29 (1.54)	0.31 (2.81)	−0.50 (1.70)	−3.79 (−5.96 to −2.46)	<.001
Sex hormone-binding globulin, µg/dL	−0.16 (0.24)	0.10 (0.15)	0.03 (0.34)	0.10 (0.30)	0 (−0.35 to 0.04)	.17
Testosterone tissue, ng/g	1.01 (7.61)	0.48 (0.75)	−1.31 (5.88)	−0.74 (1.18)	1.22 (0.50 to 2.31)	.06
Dihydrotestosterone tissue, ng/g	2.83 (10.66)	0.69 (3.43)	−7.55 (19.57)	−1.45 (6.10)	2.14 (−1.13 to 8.04)	.03
Histological						
Carcinoma, No.	2		4			
High-grade intraepithelial neoplasia, No.	3		0			
Atrophy score, % of glands	−7.88 (14.42)	−4.50 (7.00)	−1.92 (7.32)	0 (1.75)	−4.50 (−7.12 to 2.20)	.06
Stroma-epithelial ratio	0.29 (1.62)	−0.25 (1.16)	0.56 (1.92)	0.46 (1.22)	−0.70 (−1.84 to 0.71)	.39
Biomarkers						
MIB1 (Ki-67), % of positive cells	0.14 (0.36)	0 (0.18)	0.01 (0.30)	0 (0.28)	0 (−0.01 to 0.01)	.29
Androgen receptor, % of positive cells						
Epithelium	1.72 (12.34)	0 (8.75)	4.62 (13.30)	10.00 (7.50)	−10.00 (−14.29 to 5.25)	.27
Stroma	11.72 (19.93)	7.50 (9.37)	18.12 (30.57)	28.75 (15.00)	−21.30 (−33.55 to −1.35)	.26
CD34, microvessel/200 × field	3.32 (21.49)	9.75 (13.25)	−1.41 (21.70)	−5.00 (25.00)	14.75 (−9.35 to 21.74)	.35

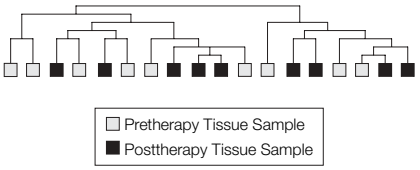
Abbreviations: CI, confidence interval; SIQR, semi-interquartile range (half the distance from the 25th to 75th percentile) constituting a nonparametric estimate of variability corresponding to the SD in parametric analyses.
SI conversion factors: To convert estradiol to pmol/L, multiply by 3.671; total testosterone to nmol, multiply by 0.0347.
*Calculated using the rank sum test.

Figure 3. Effect of Treatment on Percentage Change in Prostatic Androgen Levels



In the testosterone replacement therapy group (n=9), individuals whose genes were selected for study were those with the greatest percentage increase in tissue androgens when tissue was available; in the placebo group (n=7), 3 men with large increases in tissue androgens were intentionally avoided.

Figure 4. Gene Expression Cluster Analysis of Prostate Tissue Before and After Testosterone Replacement



The relationships of prostate tissue samples obtained at baseline and 6 months from men undergoing active treatment are grouped based on unsupervised hierarchical clustering of the 500 most-variably expressed genes (see "Methods" section for explanation of horizontal and vertical distances). Individuals included were those who at 6 months exhibited the greatest increase in tissue androgens. Testosterone replacement therapy did not cause sufficient alterations in gene expression to cluster samples based on pretreatment vs posttreatment.

est increase in serum PSA levels during the trial. The other 8 were Gleason grade 6 lesions. Serum PSA levels in the 10 cancer cases ranged from 0.62 to 6.34 ng/mL. Only 1 man had a serum PSA level greater than 4.0 ng/mL.

Gene Expression

Overall, no statistically significant gene expression changes were associated with testosterone supplementation. In participants receiving either placebo or TRT, microarray-based quantification of gene expression measured equivalent levels of transcripts encoding known prostate-specific androgen-regulated proteins such as PSA, PAP2A, and NXK3.1 in the prostatic epithelium. Paired analysis between the pretreatment and posttreatment biopsy samples from men with the largest change in tissue androgen levels (ranging from a 150%-800% increase in tissue testosterone [n=6] or a 65%-200% increase in tissue dihydrotestosterone [n=3]; FIGURE 3), identified no differentially regulated genes.

As testosterone administration might be expected to most substantially affect the expression of known androgen-regulated genes, particularly in the men with the largest changes in tissue androgens, the analysis was repeated including only those genes previously determined to be regulated by androgenic hormones. This restricted the analysis to 234 genes, but none was found to significantly change when comparing individuals treated with either placebo or TRT, or when comparing tissues from the same individuals before and after treatment with TRT. Also, we determined whether expression changes might have occurred based on whether the predominant androgen to increase had been testosterone or dihydrotestosterone. The initial analysis was performed separately on the samples that had the largest change in tissue testosterone or tissue dihydrotestosterone, again yielding no significantly changed genes.

Correlation coefficients and hierarchical clustering methods were used to further compare the effect of androgen supplementation on gene expression in samples from the TRT group. Individual gene expression measurements in the pretreatment samples were found to be highly correlated with the corresponding levels following testosterone supplementation (r=0.94), using either absolute or rank-order correlations. Thus, the gene expression profiles for the 2 groups are nearly identical. Unsupervised hierarchical clustering of all samples using the 500 most variable genes—selected to reflect those genes most substantially influenced by treatment—revealed no effect of TRT on the clustering of samples either when comparing the samples from the placebo group with the samples from the TRT group acquired at the end of study (data not shown) or when comparing the pretreatment samples with the posttreatment samples from the TRT cohort (FIGURE 4).

Confirmation of microarray findings by quantitative reverse transcriptase PCR demonstrated no significant change in the expression of known androgen-regulated genes (*PSA*, *AR*, *NKX3.1*, *PAP2A*), or in the expression of genes related to cell stress (*CLU*) or angiogenesis (*VEGF*) in the placebo group compared with the TRT group (FIGURE 5).

COMMENT

Exogenous testosterone—when administered for 6 months to men with symptomatic hypogonadism in dosages sufficient to increase serum testosterone levels to the mid-normal range—appears to have little effect on the prostate gland. In particular, prostatic androgen levels were increased only slightly by TRT. Additionally, prostate tissue composition and biomarkers of cell proliferation and angiogenesis were not altered, gene expression was not changed, and the occurrence of occult cancers was not increased. The hypothesis that testosterone supplementation would increase intraprostatic dihydrotestosterone levels and alter biological function in the gland could not be confirmed. Therefore, under the conditions herein,

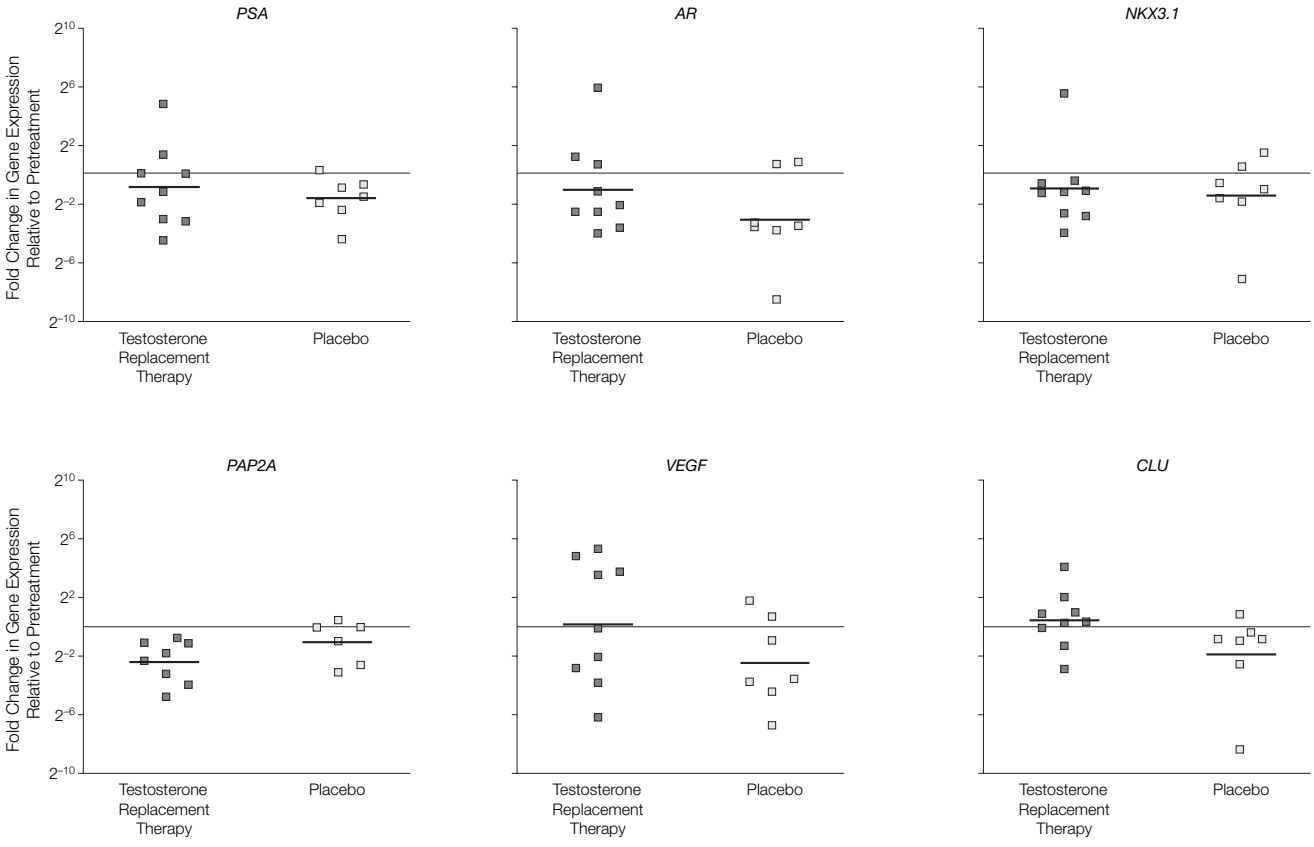
including the biopsy to detect cancer performed pretreatment, a degree of prostate safety is defined for men undergoing TRT.

The lack of prostatic uptake of exogenous testosterone may underlie the relative paucity of prostatic adverse events seen in TRT clinical trials.[22] In a low-androgen environment, constituents of the prostate may be able to import or sequester sufficient androgens for the maintenance of gene expression activity.[39] Levels above this minimal activation threshold do not appear to contribute to additional androgen receptor–mediated effects.

In the present study, the few individuals with large measurable increases in tissue androgens did not exhibit significant differences in gene expression or physiological parameters. Thus, the androgen-regulated biological functions in the prostate appear to be buffered against wide fluctuations in circulating androgens. The present study does not explain how this buffering mechanism is effected or how it dissipates in extremes (eg, following castration or with supranormal levels of circulating androgens). Importantly, other androgen-responsive tissues such as muscle may have different activity thresholds with wider concentration-response ranges that associate with compromised or enhanced function.[40] In this regard, the testosterone dose used in the present study produced the expected effects on erythropoiesis, gonadotropin suppression, and sex hormone-binding globulin levels.

In trials of testosterone replacement, prostate cancer is considered an exclusion criterion because of the known effects of testosterone administration in men with advanced cancer.[17,18] However, the effect of testosterone administration on localized subclinical prostate cancer is apparently different from its deleterious effects in men with metastatic disease. Rhoden and Morgentaler[41] showed that even in men with a predisposition to prostate cancer (high-grade intraepithelial neoplasia), 1 year of TRT did not increase cancer incidence. Testosterone replacement therapy, sufficient to raise serum levels into the mid-normal range, does not appear to entail the prostate risk that might be in-

Figure 5. Quantitative Reverse Transcriptase–Polymerase Chain Reaction Expression Analysis of Selected Genes



For each gene, the fold change in posttherapy vs pretherapy samples is depicted for the placebo group (n=7) and for the testosterone replacement therapy group (n=9) for each gene analyzed (after normalization to the housekeeping gene *RPL13A*); sample size was based on the number of samples that had adequate quality RNA to go forward. Horizontal bars represent the mean change in transcript level following therapy. No significant differences between the placebo group and testosterone replacement therapy group were observed ($P=.50$ for *PSA*; $P=.21$ for *AR*; $P=.71$ for *NKX3.1*; $P=.12$ for *PAP2A*; $P=.17$ for *VEGF*; $P=.08$ for *CLU*; based on unpaired 2 sample *t* tests).

ferred from the dramatic atrophic effects on the prostate seen with castration.

Prostate cancer was diagnosed in 10 men during our study, 4 based on the biopsy performed at enrollment (thereby excluding them from randomization) and 6 at the biopsy performed at the end of the study. Of the latter 6 men, 4 were in the placebo group and 2 were in the TRT group. Only 1 of the 10 men would have been a candidate for biopsy based on a PSA level of 4 ng/mL, which would have been considered an indication. Prostate cancer is highly prevalent in aging men, and even with a negative set of biopsy specimens, nearly a quarter of cases may escape detection.[42] The 6 men with positive results for biopsies performed at the end of the study probably had cancers present throughout the trial. From these data, TRT cannot be implicated as a cancer stimulus; nevertheless, a longer, appropriately powered study is needed to determine if rate of PSA increase (PSA velocity) can help identify men who are at special risk while receiving TRT.[43]

In previous studies of prostatic androgens, large volumes of surgically excised prostate tissue were analyzed, aiming to reduce sampling variability.[44-46] However, use of prostate biopsy cores to study tissue androgens, as used herein, has been recently validated and has considerable utility.[33] The absolute values obtained and changes in response to various treatments are essentially the same with either method as long as viable, fresh-frozen tissue is used.[31,33,44-46] For longitudinal studies that may require serial determinations of prostatic androgens, biopsy specimens offer an obvious advantage over tissues obtained by surgical excision.

To induce benign prostatic hyperplasia in canine models, both androgens and estradiol must be given together.[47] In the present study, serum levels of testosterone, dihydrotestosterone, and estradiol (aromatization) all increased after testosterone administration; however, clinical evidence of bladder outlet obstruction did not develop (ie, no significant change in voiding symptoms or urine flow resulted from treatment), and only a modest increase in prostate volume occurred.

In subhuman primates, testosterone administration has been used to induce histological and gravimetric changes in the prostate resembling human hyperplasia.[48-50] However, relevance of the primate data to the present study is not clear because in the animal studies either duration of treatment was long (33 months),[49] testosterone dosage was extreme,[48] or a castrated model was used.[50] Regarding prostate cancer, testosterone administration has not led to neoplasia in primate or canine studies. In rodent models, testosterone alone is not a reliable inducer of prostate cancer, acting mainly as a promoter in specially bred animals or as a coinducer along with nonspecific carcinogenic agents.[27] Thus, the present study is consistent with the currently available animal data.

Prostate safety issues of TRT in an aging male population in which subclinical prostate cancer and benign prostate enlargement are common and years or even decades of treatment are anticipated are different from the individual effects we documented in this small 6-month trial. To define the prostate safety of TRT at the population level, a trial with 6000 men would be required.[28]

However, in certain clinical situations, the short-term use of testosterone therapy may be of clinical benefit. For example, testosterone administration has been shown to ameliorate the profound muscle catabolism associated with severe burn injuries.[51] Perioperative testosterone supplementation appears to accelerate the postsurgical recovery of elderly men undergoing elective knee replacement surgery.[52] Furthermore, the benefits of short-term androgen administration in patients with the metabolic syndrome, a condition associated with low serum testosterone levels,[53] have been shown to extend beyond cessation of treatment.[39,54] In this context, the present study provides a degree of reassurance that short-term clinical trials designed to evaluate the beneficial effects of TRT should not entail substantial risks of stimulating occult prostate disease.

Limitations of the present study include the small sample size, the relatively short duration of treatment, and the lack of power estimates for secondary end points; thus, the present study does not establish complete safety for large populations of men receiving TRT.

Despite these limitations, the preliminary data from the present trial show that exogenous testosterone given for 6 months to men with late-onset hypogonadism in doses sufficient to increase serum concentrations to the mid-normal range does not accumulate in the prostate, does not produce abnormal levels of dihydrotestosterone, and does not appear to induce any major biological change in the gland. The prostate risks to men undergoing TRT may not be as great as once believed, especially if the results of the pretreatment biopsy are negative. However, establishment of prostate safety for large populations of older men undergoing longer duration of TRT requires further study.

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Author Contributions: Dr Marks had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Acquisition of data: Marks, Hess, Epstein, Veltri, Makarov, Partin, Bostwick, Bostwick, Mostaghel, Nelson.
Analysis and interpretation of data: Marks, Mazer, Hess, Dorey, Veltri, Makarov, Partin, Bostwick, Mostaghel, Nelson.
Drafting of the manuscript: Marks, Dorey, Veltri, Partin, Mostaghel, Nelson.
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