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Development of models to predict anabolic response to testosterone administration in healthy young men

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Woodhouse, Linda J., Suzanne Reisz-Porszasz, Marjan Javanbakht, Thomas W. Storer, Martin Lee, Hrant Zerounian, and Shalender Bhasin. Development of models to predict anabolic response to testosterone administration in healthy young men. *Am J Physiol Endocrinol Metab* 284: E1009–E1017, 2003. First published January 7, 2003; 10.1152/ajpendo.00536.2002.—Considerable heterogeneity exists in the anabolic response to androgen administration; however, the factors that contribute to variation in an individual's anabolic response to androgens remain unknown. We investigated whether testosterone dose and/or any combination of baseline variables, including concentrations of hormones, age, body composition, muscle function, and morphometry or polymorphisms in androgen receptor could explain the variability in anabolic response to testosterone. Fifty-four young men were treated with a long-acting gonadotropin-releasing hormone (GnRH) agonist and one of five doses (25, 50, 125, 300, or 600 mg/wk) of testosterone enanthate (TE) for 20 wk. Anabolic response was defined as a change in whole body fat-free mass (FFM) by dual-energy X-ray absorptiometry (DEXA), appendicular FFM (by DEXA), and thigh muscle volume (by magnetic resonance imaging) during TE treatment. We used univariate and multivariate analysis to identify the subset of baseline measures that best explained the variability in anabolic response to testosterone supplementation. The three-variable model of TE dose, age, and baseline prostate-specific antigen (PSA) level explained 67% of the variance in change in whole body FFM. Change in appendicular FFM was best explained (64% of the variance) by the linear combination of TE dose, baseline PSA, and leg press strength, whereas TE dose, log of the ratio of luteinizing hormone to testosterone concentration, and age explained 66% of the variation in change in thigh muscle volume. The models were further validated by using Ridge analysis and cross-validation in data subsets. Only the model using testosterone dose, age, and PSA was a consistent predictor of change in FFM in subset analyses. The length of CAG tract was only a weak predictor of change in thigh muscle volume and lean body mass. Hence, the anabolic response of healthy, young men to exogenous testosterone administration can largely be predicted by the testosterone dose.

testosterone dose response; testosterone effects on muscle; predicting lean body mass response to testosterone; androgen receptor genetic polymorphism; polyglutamine repeats; polyglycine repeats

TESTOSTERONE SUPPLEMENTATION increases fat-free mass (FFM) and muscle size in healthy, hypogonadal men (5, 8, 18, 29, 35, 36), human immunodeficiency virus (HIV)-infected men with low testosterone levels (3, 6, 15, 16), and older men with low testosterone levels (19, 30, 31, 33). However, there are striking qualitative and quantitative differences in this anabolic response to testosterone administration among the various studies. Of the six, placebo-controlled, clinical trials evaluating testosterone administration in HIV-infected men, two (11, 12) reported no significant difference in the change in FFM between the placebo- and testosterone-treated men. Among the studies that did demonstrate significant gains in lean body mass (LBM) after androgen administration, the magnitude of increase varied considerably (3, 6, 15, 16). In one study (3) in which HIV-infected men with low testosterone levels were treated with placebo or testosterone patches, the mean gain in LBM in the testosterone-treated men was 1.4 kg, whereas in another study (6) 100 mg testosterone enanthate (TE) weekly was associated with a larger (mean 2.9 kg) gain in LBM.

Studies of testosterone supplementation in older men have also demonstrated similar variability in results (19–21, 30, 31, 33). Although Sih et al. (21) reported no significant gains in LBM during testosterone administration, others (30, 31) found greater gains in older men treated with testosterone than in those treated with placebo. These data are similar to anecdotal reports that athletes using androgenic steroids differ significantly in their anabolic response to these agents. We do not know whether these varying responses in HIV-infected and older men in different studies are because of differences in testosterone dose, baseline characteristics of the subjects, or methods of body composition assessment.

We recently completed a clinical trial to determine the effects of graded doses of testosterone on several androgen-dependent processes. In the current investigation, we used multiple linear regression analyses to identify those baseline variables that could best explain the variability in the anabolic response to testos-

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terone supplementation. We explored statistical models to best explain the dose-response relationship between serum testosterone and change in various androgen-dependent processes over a range of serum testosterone concentrations from sub- to supraphysiological levels (7). We further examined whether, in addition to the testosterone dose administered, any single baseline variable or combination of baseline variables, including age, measures of body composition, serum hormone and growth factor concentrations, skeletal muscle function, and morphometry or polymorphisms in androgen receptor gene, could explain the variability in anabolic response. We operationally defined anabolic response as the change in LBM measured by dual-energy X-ray absorptiometry (DEXA) scan, a change in appendicular (arm + leg) LBM, measured by DEXA scan, and a change in thigh muscle volume, measured by magnetic resonance imaging (MRI). A second objective of this study was to develop equations that could be used to predict the change in FFM and muscle volume during testosterone administration.

We treated healthy, young, eugonadal men with a long-acting gonadotropin-releasing hormone (GnRH) agonist plus one of five doses (25, 50, 125, 300, or 600 mg/wk) of TE. The study design and the main findings of this study have been published elsewhere (19). Using this model of combined GnRH plus TE administration, we were able to suppress endogenous testosterone production, resulting in more homogeneous levels of circulating testosterone within each of the five dose groups and more diverse testosterone concentrations, ranging from subphysiological to supraphysiological levels, among the five groups.

METHODS

Study Design

The protocol for this double-blind, randomized, testosterone dose-response study was approved by the institutional review boards of Charles Drew University and the Research and Education Institute at Harbor-University of California Los Angeles Medical Center. The entire trial consisted of a 4-wk control period for baseline studies, followed by 20 wk of treatment with a long-acting GnRH agonist plus TE and 16 wk of recovery. Details of the study design have been published previously (7, 22, 24).

Participants

Participants included eugonadal men, 18–35 yr of age, who had not used anabolic agents and did not exercise train or participate in competitive athletics for 12 mo before the study.

Randomization

Sixty-one eligible, healthy young men were randomized to receive one of five weekly doses (25, 50, 125, 300, or 600 mg/wk) of TE in combination with monthly injections of a long-acting GnRH agonist. To ensure compliance, participants received their injections in the General Clinical Research Center.

Diet and Exercise

Diet and exercise stimulus were standardized 2 wk before and throughout the treatment period. Caloric and protein intake were maintained at 36 $kcal \cdot kg^{-1} \cdot day^{-1}$ and 1.2 $g \cdot kg^{-1} \cdot day^{-1}$, respectively. Adherence was monitored using 24-h food recall and 3-day food records every 4 wk. Participants were asked not to engage in strength training or moderate-to-intense aerobic exercise throughout the study.

Outcome Measures

The independent variables examined included testosterone dose; age; measures of body composition (weight, height, body mass index, total body fat, and lean mass); leg muscle performance (leg press strength, power and local muscular endurance); skeletal muscle morphometry (mean fiber area and fiber type from biopsies of vastus lateralis); serum hormone concentrations [total and free testosterone concentrations, sex hormone-binding globulin (SHBG), luteinizing hormone (LH), LH-to-testosterone (LH/T) concentration ratio, log (LH/T) ratio and follicle-stimulating hormone (FSH)]; concentrations of IGF-I and its major binding protein IGFBP-3; lipid profile, including high- and low-density lipoprotein cholesterol concentrations; Hct and Hb; prostate-specific androgen (PSA) levels; and lengths of the polyglutamine (CAG) and polyglycine (GGC) trinucleotide tracts in exon 1 of the androgen receptor gene. These measures have been shown previously to be androgen responsive. We used LH/T as a marker of the feedback and feedforward relationship between the gonadotrope and the Leydig cell. Variations in the lengths of the CAG and GGC trinucleotide tracts have been reported to correlate inversely with the variation in the transcriptional activity of the androgen receptor protein and the risk of prostate cancer and infertility in men (13, 32). We examined the extent to which the variation in the biological response to testosterone could be explained on the basis of polymorphisms in the length of these tracts.

Independent Variables Explored

All independent variables were measured at baseline, within 2 wk before initiating treatment.

Whole body and appendicular fat and LBM. Total body fat and lean mass were measured by DEXA scanning (model QDR 4500A; Hologic, Waltham, MA). Appendicular skeletal muscle and fat masses were determined by adding the bilateral arm and leg lean and fat masses, respectively (17).

Leg muscle performance. Unilateral leg press strength was measured as the maximum amount of weight that could be lifted one time [1 repetition maximum (1RM)] on a Keiser leg press device. Measures were repeated two times (2–5 days apart) and a third time (if the difference between the first 2 days exceeded 5%). Muscular endurance was measured as the maximum number of repetitions that could be performed using a load equal to 80% of each individual's 1RM. Leg power was measured as the maximum rate of force produced (watts) using a Nottingham leg extension rig.

Vastus lateralis muscle biopsies. Percutaneous needle biopsies of the right vastus lateralis muscle were obtained using the Bergström method. Muscle fibers were identified as either type I or type II fibers by immunohistochemical staining, as previously described (24). At least 500 muscle fibers were counted for each biopsy specimen. The relative abundance of type I and II fibers was expressed as a percentage of the total number of fibers counted. The cross-sectional area of the muscle fibers was determined by point counting (1, 23). A

minimum of 150 type II fibers and 100 type I fibers was analyzed in each biopsy specimen.

Hormonal assays. Serum total testosterone and estradiol concentrations were measured by immunoassays (3–7), free testosterone by equilibrium dialysis (25), LH, SHBG, and PSA by immunoradiometric assays (4–7), and IGF-I after acid-ethanol extraction. Serum IGF-binding protein-3 (IGFBP-3) was measured by a specific RIA that does not cross-react with other currently known IGFBPs. The sensitivities and intra- and interassay coefficients of variation were as follows: total testosterone, 0.6 ng/dl, 8.2 and 13.2%; free testosterone, 0.22 pg/ml, 4.2 and 12.3%; estradiol, 2.5 pg/ml, 6.5 and 12.4%; LH, 0.05 U/l, 10.7 and 13.0%; SHBG, 6.25 nmol/l, 4 and 6%; PSA 0.01 ng/ml, 5.0 and 6.4%; and IGF-I, 80 ng/ml, 4 and 6%, respectively (3, 6, 18, 29).

Lengths of CAG and GGC repeats. In a subset of participants ($n = 38$), genomic DNA was isolated from the muscle biopsies using TRIzol reagent (GIBCO-BRL). PCR amplifications were performed in a final volume of 25 μl reaction mix containing 50–100 ng genomic DNA, 25 pmol primers, dNTPs, and Ampli*Taq* polymerase (Perkin-Elmer). For the CAG repeat analysis, we used two pairs of oligonucleotide primers. The first set was 5′-GCGCGAAGTGATCCAGAAC-3′ and 5′-CTTGGGGAGAACCATCCTC-3′. The primer previously described by Edwards et al. (13) was modified slightly based on a computer search (website: www-genome.wi.mit.edu/primer3). Thermocycling consisted of 95°C for 5 min, 5 × 4 cycles under "touchdown" conditions, reducing the annealing temperature by 2°C from 64 to 56°C, and 20 cycles at 54°C annealing temperature followed by 10 min of extension. The 5′ primers were labeled with 1-dimethoxytrityloxy-3-[*O*-(*N*-carboxy-(di-*O*-pivaloyl-fluorescin)-3 aminopropyl)] propyl-2-*O*-succinoyl-long-chain alkylamino-CPG, and the 200-bp-long PCR products were analyzed by the short tandom repeat (STR) method. The second primer set was 5′-GCCTGTTGAACTCTTCTGAGC-3′ and 5′-GCTGTGAAGGTTGCTGTTCCTC-3′. Reaction mix consisted of 5 nmol of each dNTP, 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, and 1 unit Ampli*Taq* polymerase. The PCR conditions included denaturation at 95°C for 5 min, 35 temperature cycles each for 1 min at 95°C, 2 min at 65°C, and 1.5 min at 72°C, followed by extension at 72°C for 8.5 min. Samples were loaded on 1.2% agarose gel. The 450-bp PCR products were cloned into a TA-cloning vector (pGEM-T Easy; Promega). After transformation of DH5α-bacteria, plasmids from 5–5 white colonies were analyzed by sequencing with T7 and SP6 sequencing primers.

Primers for GGC repeat analyses were designed as described above. The optimal annealing conditions were pretested using a RoboCycler Gradient Temperature Cycler between 57 and 68°C by using three different primer sets. The best amplification was obtained by using primers 5′-CTCATCCTGGCACACTCTCT-3′and 5′-CAGGGTACCACACATCAGGT-3′and an annealing temperature of 63°C. The 25-μl reaction mix consisted of 50–100 ng genomic DNA, 10× buffer, Q solution, dNTPs, and *Taq* polymerase (QIAGEN; Taq PCR Core Kit). The 5′ primer was labeled with 6-FAM dye, and the expected 220-bp PCR product was analyzed by the STR method. To validate the procedure and verify that the correct product was being amplified, the PCR products obtained from amplification of CAG- and GGC-containing segments were subcloned and sequenced.

Dependent Variables

Whole body DEXA and MRI scans of the thigh were obtained at baseline and after 20 wk of treatment. The anabolic response was operationally defined as change in *1*) total LBM measured by DEXA scan, *2*) appendicular LBM measured by DEXA scan, and *3*) thigh muscle volume measured by MRI. Change in each of these 3 variables was calculated as the *week 20* value minus the baseline value. These dependent variables were selected because they are known to be androgen responsive and can be measured with a high degree of precision and accuracy.

Thigh muscle volume. Thigh muscle volume was measured from MRI scans of the thigh by using a Signa Horizon LX Scanner (General Electric Medical Systems, Milwaukee, WI) at baseline and after 20 wk of treatment, as previously described (19). Cross-sectional MRI scans of the thigh were obtained with the first slice taken at the inferior border of the lateral femoral condyle. A total of 17 slices was obtained (10 mm thick, 15 mm apart). Thigh volumes were determined for three sequential transverse slices of the right thigh, including the slice with the largest cross-sectional area on axial imaging plus the slice immediately above and below, for each subject, with commercially available software (AW version 3.1; General Electric Volume Analysis Software). Accuracy of the volume analysis software was determined by scanning and analyzing a phantom cylinder of known dimensions. Duplicate manual tracings were drawn around the outermost edge of the entire thigh (total thigh), the skeletal musculature (to subtract out subcutaneous fat), and the femur (to subtract out femoral bone areas). The quadriceps musculature was measured by manually tracing around the vastus lateralis, vastus medialis, rectus femoris, and sartorius muscles. All analyses were done by the same investigator, in a blinded fashion.

Statistical Analyses and Model Development

SigmaStat (Science version 2.03; SPSS, Chicago, IL) and Statistical Analysis Systems (SAS version 8.1; SAS Institute, Cary, NC) were used for all statistical analyses. Correlations were examined using the Pearson product-moment correlation coefficient. Testosterone dose and all baseline variables were screened, using univariate regression analyses, to identify those variables to be further examined using multiple regression models. Only variables that were correlated, using a conservative significance level of $P \leq 0.3$, with the dependent variables (change in total LBM, change in appendicular LBM, or change in thigh muscle volume) were included in subsequent multiple regression analyses. The following baseline variables met this criteria and were examined further in multiple regression analysis: testosterone dose, age, body mass index, total and free testosterone concentrations, log LH/T, SHBG and FSH concentrations, triglycerides, very low density lipoprotein-C, Apo-CIII, leg press strength and local muscular endurance, PSA, Hb, and lengths of CAG and GGC repeats. Best subset regression analyses were used to identify the model that best predicted the change in each of the dependent variables. The multiple regression model that maximized R^2 (the square of the multiple regression correlation coefficient) and optimized Mallow's C_p statistic for each of the dependent variables was selected. The level of significance was set at $P < 0.05$.

To ensure that possible collinearity of independent variables did not affect the analysis, the method of ridge regression was employed to reassess each model. The prediction models that emerged from the multivariate analyses were cross-validated in seventeen randomly selected subsets of data in which a small number of randomly selected subjects (<25%) was left out.

Table 1. *Baseline characteristics of the participants*

	All Groups	Monthly GnRH Agonist + Testosterone Enanthate Dose, mg/wk					
		25	50	125	300	600	*P* value
Age, yr	27 ± 4	28 ± 4	29 ± 4	28 ± 4	24 ± 5	25 ± 4	0.039
Height, cm	176 ± 7	174 ± 4	176 ± 6	178 ± 7	176 ± 6	174 ± 9	0.603
Weight, kg	75.4 ± 10.6	68.0 ± 8.6	77.4 ± 8.2	78.4 ± 9.8	79.0 ± 10.5	74.9 ± 12.4	0.098
Body mass index, kg/m^2	24 ± 3	22 ± 3	25 ± 3	25 ± 3	26 ± 3	25 ± 3	0.152
Serum testosterone, nmol/l	585 ± 189	603 ± 145	533 ± 73	562 ± 188	607 ± 207	606 ± 216	0.888
Free testosterone, pg/ml	60.4 ± 21.7	61.4 ± 19.0	57.1 ± 22.2	49.0 ± 17.8	72.4 ± 29.0	62.8 ± 17.7	0.148
SHBG, nmol/l	32.8 ± 14.8	30.1 ± 10.9	24.8 ± 8.9	33.9 ± 15.1	31.1 ± 15.0	40.0 ± 18.0	0.200
LH, U/l	3.4 ± 1.2	3.5 ± 1.5	3.8 ± 0.5	3.6 ± 1.7	2.9 ± 1.0	3.4 ± 1.0	0.596
N	54	11	8	12	10	13	

Data represent baseline mean ± SD values for the 54 participants (*N*) in the study. GnRH, gonadotropin-releasing hormone; SHBG, sex hormone-binding globulin; LH, luteinizing hormone.

RESULTS

Participant Characteristics

Details of the study design have been published previously (7). Fifty-four of the 61 men who were randomized completed the entire study. One man discontinued treatment because of acne; the other six withdrew because of an inability to meet the demands of the protocol. Eleven were randomized to receive 25 mg TE/wk (*group 1*), 8 to receive 50 mg TE/wk (*group 2*), 12 to receive 125 mg TE/wk (*group 3*), 10 to receive 300 mg TE/wk (*group 4*), and 13 to receive 600 mg TE/wk (*group 5*). There were no significant differences among the five treatment groups with respect to baseline characteristics (Table 1). Compliance with GnRH agonist injections was 100%. Only one individual in the 125-mg group missed one testosterone injection.

Hormone Levels

As previously reported (7), minimum serum total ($r = 0.82$, $P < 0.0001$) and free ($r = 0.71$, $P < 0.0001$) testosterone levels measured during *week 20*, 1 wk after the previous testosterone injection, were correlated linearly with testosterone dose. Estradiol levels paralleled those of total testosterone. Serum LH levels were suppressed across all five groups.

Development of Univariate Regression Models

We tested linear, log linear, and growth models to describe the relationship between steady-state testosterone concentrations and change in FFM and muscle volume. The linear and log linear models provided the best fit for these data. Because the linear model provided as good a fit as the log linear model, correlations between testosterone dose or concentrations and change in FFM and muscle volume are derived using the linear regression model.

We examined the change in total body and appendicular FFM as a function of serum total and free testosterone levels during treatment. There was a strong linear relationship between TE dose and change in total body FFM ($r = 0.81$, $P < 0.0001$; Fig. 1*A*). The nadir serum total ($r = 0.62$, $P < 0.0001$) and free ($r = 0.59$, $P < 0.0001$) testosterone concentrations during

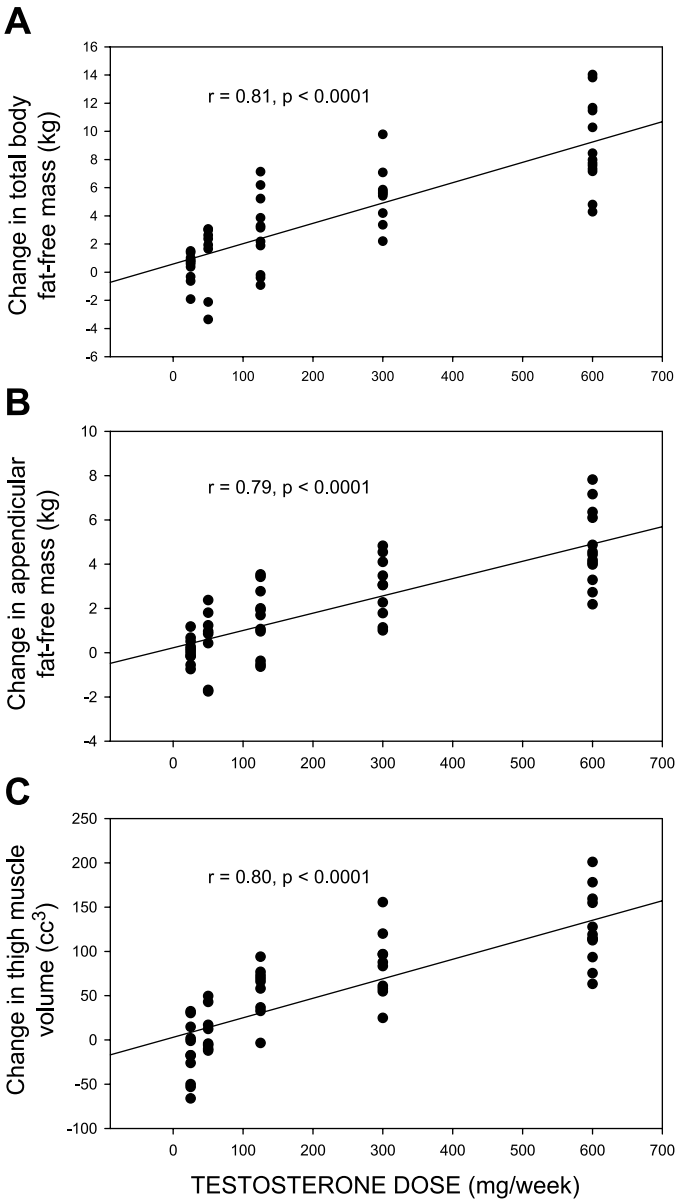

Fig. 1. Linear regression of the testosterone enanthate (TE) dose and change in total body fat-free mass (*A*), appendicular fat-free mass (*B*), and thigh muscle volume (*C*) demonstrates the heterogeneity of individual responses within each TE dose despite strong and significant correlations overall (Pearson product-moment correlation coefficients of $r = 0.79$–0.81, $P < 0.0001$ for each model).

treatment were also linearly related to change in total body FFM. Changes in appendicular LBM were highly correlated with testosterone dose ($r = 0.79, P < 0.0001$; Fig. 1*B*) and steady-state, nadir serum total ($r = 0.63$, $P < 0.0001$) and free ($r = 0.55, P < 0.0001$) testosterone concentrations during treatment. Based on univariate linear regression analyses, steady-state serum total testosterone concentrations during treatment accounted for 38% ($P < 0.001$) and 39% ($P < 0.001$) of the variation in whole body and appendicular FFM, respectively. Similarly, steady-state free testosterone levels accounted for 35% ($P < 0.001$) and 30% ($P < 0.001$) of the variation in total and appendicular FFM responses, respectively (Tables 2 and 3). The equations for each model were as follows

change in whole body FFM (kg)

= 1.434 + 0.00243 (steady-state total testosterone concentration in ng/dl)

and

2.097 + 0.0170 (steady-state free testosterone concentration in pg/ml)

and

change in appendicular FFM (kg)

= 0.633 + 0.00136 (steady-state total testosterone concentration in ng/dl)

and

1.092 + 0.00877 (steady-state free testosterone concentration in pg/ml).

We also examined the change in thigh muscle volume as a function of serum total and free testosterone levels during treatment. The changes in thigh muscle volume were significantly correlated with testosterone dose ($r = 0.80$, $P < 0.0001$; Fig. 1*C*) and serum total ($r = 0.62$, $P < 0.0001$) and free ($r = 0.57$, $P < 0.0001$) testosterone concentrations after treatment. On the basis of univariate linear regression analyses, serum total testosterone levels during treatment accounted for 39% ($P < 0.001$) of the variation, whereas free testosterone levels accounted for 32% ($P < 0.001$) of the variation in change in muscle volume (Table 4). The equation for each model was as follows

change in thigh muscle volume (cm^3)

= 14.283 + 0.0371 (steady-state total testosterone concentration in ng/dl)

and

26.367 + 0.247 (steady-state free testosterone concentration in pg/ml)

Table 2. *Steady-state serum total and free testosterone concentrations after 16 wk of TE treatment as a predictor of change in total fat-free mass*

Variable	Parameter Estimate (β)	SE	*P* Value
Intercept for total testosterone	1.434	0.642	0.030
Steady-state total testosterone, nmol/l	0.00243	0.000430	<0.001
Intercept for free testosterone	2.097	0.589	<0.001
Steady-state free testosterone, pg/ml	0.0170	0.00325	<0.001

A linear regression model was used to investigate how much of the variance in anabolic response, change in total fat-free mass measured by DEXA (kg), could be explained by total and free testosterone concentrations during treatment. Serum total and free testosterone levels were measured after 16 wk of testosterone enanthate (TE) treatment at nadir, 1 wk after the previous injection. For total testosterone: $R^2 = 0.38$, F statistic = 31.77, $P < 0.001$. For free testosterone: $R^2 = 0.35$, F statistic = 22.28, $P < 0.001$.

Table 3. *Steady-state serum total and free testosterone concentrations after 16 wk of TE treatment as a predictor of change in appendicular fat-free mass*

Variable	Parameter Estimate (β)	SE	*P* Value
Intercept for total testosterone	0.633	0.353	0.08
Steady-state total testosterone, nmol/l	0.00136	0.000236	<0.001
Intercept for free testosterone	1.092	0.337	0.002
Steady-state free testosterone, pg/ml	0.00877	0.00186	<0.001

A linear regression model was used to investigate how much of the variance in anabolic response, change in appendicular fat-free mass measured by DEXA (kg), could be explained by total and free testosterone concentrations during treatment. Serum total and free testosterone levels were measured after 16 wk of TE treatment at nadir, 1 wk after the previous injection. For total testosterone: $R^2 = 0.39$, F statistic = 33.15, $P < 0.001$. For free testosterone: $R^2 = 0.30$, F statistic = 22.21, $P < 0.001$.

Multivariate Prediction Model Based on Subject Characteristics and Testosterone Dose

Predicting change in whole body FFM (DEXA). In the multivariate analysis, the testosterone dose alone ex-

Table 4. *Steady-state serum total and free testosterone concentrations after 16 wk of TE treatment as a predictor of change in thigh muscle volume*

Variable	Parameter Estimate (β)	SE	*P* Value
Intercept for total testosterone	14.283	10.237	0.17
Steady-state total testosterone, nmol/l	0.0371	0.007	<0.001
Intercept for free testosterone	26.367	9.603	0.008
Steady-state free testosterone, pg/ml	0.247	0.052	<0.001

A linear regression model was used to investigate how much of the variance in anabolic response, change in thigh muscle volume measured by magnetic resonance imaging (ml^3), could be explained by total and free serum testosterone concentrations during treatment. Serum testosterone levels were measured after 16 wk of TE treatment at nadir, 1 wk after the previous injection. For total testosterone: $R^2 = 0.39$, F statistic = 30.44, $P < 0.001$. For free testosterone: $R^2 = 0.32$, F statistic = 22.47, $P < 0.001$.

Table 5. *Results of the best subset multiple regression to assess determinants of androgen responsiveness defined as change in fat-free mass and thigh muscle volume*

	Dependent Variable	Variable(s) in Equation	Model R^2	*P* Value
Model 1	Change in total body FFM	Testosterone dose, mg/wk	0.64	
		Age, yr	0.66	
		PSA, ng/ml	0.67	<0.001
Model 2	Change in appendicular FFM	Testosterone dose, mg/wk	0.61	
		PSA, ng/ml	0.63	
		Leg press, lbs	0.64	<0.001
Model 3	Change in thigh muscle volume	Testosterone dose, mg/wk	0.65	
		Log_{10} [LH/T]	0.68	
		Age, yr	0.70	<0.001

FFM, fat-free mass; PSA, prostate-specific androgen; LH/T, leuteinizing hormone-to-testosterone ratio. Equations for models as follows: *model 1*: change in total body FFM (kg) = −3.242 + 0.015 (T dose in mg/wk) + 0.189 (age in yr) + 1.873 (PSA in ng/ml); *model 2*: change in appendicular FFM (kg) = −1.732 + 0.0078 (T dose in mg/wk) + 0.861 (PSA in ng/ml) + 0.0014 (leg press in kg); and *model 3*: change in thigh muscle volume (ml^3) = −93.999 + 0.227 (T dose in mg/wk) − 27.683 (log [LH/T]) + 1.241 (age in yr).

plained 64% of the variance of change in whole body FFM measured by DEXA. The best three-variable model that explained >67% of the variation in anabolic response included testosterone dose, age, and baseline levels of PSA (Table 5). The model was developed such that the change in total body FFM = β_o + β_1 (testosterone dose) + β_2 (age) + β_3 (baseline PSA). The equation for the preferred model was as follows: change in total body FFM (kg) = −3.242 + 0.015 (TE dose in mg/wk) + 0.189 (age in yr) + 1.873 (PSA in ng/ml) (Table 5).

When testosterone dose was excluded from the multiple linear regression analysis, only 17% of the variability in change in whole body FFM could be explained using a three-variable model that included baseline measures of triglycerides, leg press endurance, and Hb (Table 6).

Predicting change in appendicular FFM (DEXA). The best model that explained ~64% of the variation in change in appendicular FFM was a three-variable model that included testosterone dose, PSA level at baseline, and leg press strength (Table 5). The equation for the preferred model was as follows: change in appendicular FFM (kg) = −1.732 + 0.0078 (TE dose in mg/wk) + 0.861 (PSA in ng/ml) + 0.0014 (leg press in kg) (Table 5). When testosterone dose was excluded from the multiple linear regression analysis, only 16% of the variability in change in appendicular FFM could be explained using a three-variable model that included baseline measures of VLDL-C, Hb, and leg press endurance (Table 6).

Predicting change in thigh muscle volume (MRI). In the multivariate analysis, testosterone dose alone explained 64% of the variance. None of the other baseline subject characteristics in and of themselves were good predictors of the change in muscle volume. The best three-variable model that explained 70% of the variation in the change in muscle volume included testosterone dose, the log[LH/T] concentration ratio at baseline, and age (Table 5). The equation for the preferred model was as follows: change in thigh muscle volume (cm^3) = −93.999 + 0.227 (TE dose in mg/wk) − 27.683 (log[LH/T]) + 1.241 (age in yr) (Table 5). When testosterone dose was excluded from the multiple linear regression analysis, only 18% of the variability in the change in thigh volume could be explained using a three-variable model that included baseline measures of triglycerides, Hb, and log[LH/T] (Table 6).

Relationship Between Change in FFM or Thigh Muscle Volume and Polymorphisms in Androgen Receptor Gene

Linear regression analyses were used to explore the relationship between the lengths of CAG and GGC

Table 6. *Results of the best subset multiple regression to assess determinants of androgen responsiveness defined as change in fat-free mass and thigh muscle volume when testosterone dose was excluded*

	Dependent Variable	Variable(s) in Equation	Model R^2	*P* Value
Model 1	Change in total body FFM	Hemoglobin, g/l	0.10	
		Triglycerides, mg/dl	0.15	
		Leg press endurance, reps	0.17	0.029
Model 2	Change in appendicular FFM	VLDL, mg/dl	0.11	
		Hemoglobin, g/l	0.15	
		Leg press endurance, reps	0.16	0.032
Model 3	Change in thigh muscle volume	Triglycerides, mg/dl	0.12	
		Hemoglobin, g/l	0.16	
		Log_{10} [LH/T]	0.18	0.028

VLDL, very low density lipoprotein; reps, repetitions. Equations for models as follows: *model 1*: change in total body FFM (kg) = 16.373 − 0.024 (triglycerides in mg/dl) − 0.080 (leg press endurance in reps) − 0.058 (hemoglobin in g/l); *model 2*: change in appendicular FFM (kg) = 9.195 − 0.067 (VLDL in mg/dl) − 0.035 (hemoglobin in g/l) − 0.034 (leg press endurance in reps); and *model 3*: change in thigh muscle volume (ml^3) = 149.456 − 0.393 (triglycerides in mg/dl) − 1.070 (hemoglobin in g/l) − 43.522 (log_{10} [LH/T]).

trinucleotide tracts and change in each of the dependent variables. There were no significant relationships between change in total body FFM and length of CAG ($r = 0.20$, $P = 0.23$) or GGC ($r = 0.19$, $P = 0.28$) repeats. This lack of association was also true for the change in appendicular FFM (vs. CAG: $r = 0.19$, $P = 0.26$; vs. GGC: $r = 0.17$, $P = 0.32$) and thigh muscle (vs. CAG: $r = 0.18$, $P = 0.27$; vs. GGC: $r = 0.25$, $P = 0.14$) volume.

Ridge Analysis for the Effects of Multicollinearity

Regression analyses can produce estimates with large mean square error when predictor variables are multicollinear. Ridge regression can reduce the impact of multicollinearity in ordinary least-squares regressions. Because some of the predictor variables in each of the prediction models were correlated, we performed ridge regression analyses to evaluate the effects of multicollinearity. Ridge regression results were similar to those of the best subset regression analyses with no appreciable change in mean square error for any of the three prediction models developed.

Cross-Validation of the Models

The prediction models that emerged from the multivariate analyses were cross-validated in 17 randomly selected subsets of data in which a small number of randomly selected subjects (<25%) was left out of the analysis for each run. Best subset regression analysis, to maximize R square, was run on these 17 subsets of data. The frequency with which each of the three-variable models occurred on the 17 runs was used to confirm the selection of the best predictor variables in each of the three prediction models developed. In all cases, testosterone dose was a significant predictor. For change in total body FFM, in addition to testosterone dose, age also appeared in almost all of the cross-validation runs. For change in appendicular FFM, testosterone dose and the PSA level were significant predictors for the majority of runs, whereas the baseline leg press strength appeared less frequently. Finally, for the change in thigh muscle volume, testosterone dose and log[LH/T] appeared in a substantial number of runs, as did age to a lesser extent.

DISCUSSION

The mean changes in whole body FFM, appendicular FFM, and muscle volume were strongly correlated with testosterone dose and steady-state testosterone concentrations during the period of exogenous testosterone administration. Testosterone dose was the best predictor of the anabolic response to testosterone administration and accounted for a major part (61–65%) of the variance in the anabolic response. A regression equation that takes into account testosterone dose can largely predict the anabolic response, as measured by the change in whole body FFM, appendicular FFM, or thigh muscle volume.

Serum total and free testosterone concentrations are a function of the administered testosterone dose, the absorption kinetics, and plasma clearance. Therefore, it is not surprising that steady-state total and free testosterone concentrations during treatment explained 32–39% of the variance in change in the anabolic response. In spite of the high degree of correlation between testosterone dose or testosterone concentrations and the anabolic response, considerable heterogeneity existed when within-dose group responses were considered. Regardless of the dependent variable used to evaluate androgen responsiveness (change in whole body FFM, appendicular FFM, or thigh muscle volume), testosterone dose explained 61–64% of the variance in response. The addition of age and baseline PSA to the model for change in total body FFM; baseline PSA and leg press strength to the model for change in appendicular LBM; and log[LH/T] and age to the model for change in thigh muscle volume accounted for only an additional 3–5% of the variance in anabolic response. When testosterone dose was excluded from the independent variables assessed, only 10–18% of the variance in anabolic response could be explained. Furthermore, in cross-validation subset analyses, only testosterone dose and age were consistent predictors of the anabolic response. It is possible that one or more additional variables, including combinations of genetic polymorphisms, might have small effects on the natural variation in androgen responsiveness. Studies with sample size of 1,000–2,000 men would be needed to detect the small contribution of these polymorphisms to the variation in response.

Our data were derived in healthy young men; we do not know whether similar dose-response relationships exist in older men or in men with chronic illness. It is also unknown whether women have different androgen responsiveness from men. The study population was ethnically heterogeneous; it is possible that individuals of different ethno-genetic background might respond differently to androgen administration; our study was not designed or powered to address that issue. These empirically derived prediction equations need to be tested in other populations and prospectively in another study in this subject population.

One disadvantage of using testosterone esters is that testosterone levels vary in temporal relation to the time of testosterone administration (28). Currently, it is only with the use of testosterone esters that one can achieve a wide range of circulating testosterone concentrations, particularly very high concentrations. The testosterone concentrations reported here are nadir concentrations; total and free testosterone levels at other time points must have been higher. We used nadir testosterone concentrations only as a marker of "androgen exposure." We did not measure peak or average testosterone concentrations; it is possible that peak or average testosterone concentrations achieved during the interval after each TE injection might be better predictors of the outcomes than nadir testosterone concentrations.

Baseline body composition, muscle size, and muscle morphology did not affect the subsequent response to testosterone administration. It is possible that factors

that determine skeletal muscle mass and fiber composition are different from those that determine the magnitude of the anabolic response to androgen. We only examined the influence of CAG and GGC repeat lengths in the androgen receptor gene; it is possible that polymorphisms in other genetic determinants of skeletal muscle mass, such as myostatin, angiotensin-converting enzyme, IGF-I receptor, growth hormone receptor, or other genes, might explain some of the variance in anabolic response to testosterone.

The transcriptional activity of the androgen receptor protein has been linked to the length of two polymorphic trinucleotide tracts in exon 1: the CAG repeat, which codes for a polyglutamine tract; and the GGC repeat, which codes for a polyglycine tract (2, 9, 10, 26, 27). The length of the CAG repeat is usually between 8 and 35 in healthy, young men, whereas the number of GGC repeats varies from 10 to 30. Individuals with >40 CAG repeats suffer from a neurological disorder, spinobulbar muscular dystrophy. The changes in the length of GGC are not as well characterized as those in the CAG repeat, but deletions of the GGC repeat reduce the transcriptional activity of the androgen receptor by one-third (14). Our data did not reveal a major effect of either the polyglutamine or polyglycine tract length on the change in FFM or muscle volume in response to testosterone administration. Conflicting data exist on the relationship between polymorphisms in androgen receptor gene and infertility, prostate cancer risk, cardiovascular risk, and bone density (2, 9, 10, 26, 27, 34, 37), likely resulting from the small effect size and suboptimal sample size of these studies.

On the basis of these prediction models, we considered the possibility that the differences in testosterone dose and concentrations might account for the differences in anabolic response observed in HIV-infected men and in older men. Indeed, we observed greater gains in FFM in HIV-infected men treated with TE injections, 100 mg weekly (6), than those receiving testosterone replacement by a 5-mg testosterone patch (12). Because the 100-mg weekly dose of TE delivers a larger amount of testosterone than that delivered by the 5-mg patch, our model would predict greater accretion of lean tissue with this higher dose.

Similarly, most studies of testosterone supplementation in older men used relatively small doses of testosterone that raised serum testosterone concentrations in the lower end of the normal male range; therefore, it is not surprising that the increments in FFM and muscle strength in these studies have been modest. Our prediction model suggests that increasing testosterone concentrations in the higher end of the normal male range would be associated with greater gains in muscle mass and likely muscle strength; the safety and effectiveness of such an approach should be tested in prospective clinical trials.

Our analysis shows that the biological response to exogenously administered testosterone can be predicted largely by taking into account the administered dose and age; PSA level, $\log_{10}$(LH/T), and leg press strength only marginally improve the model. Because a large part of the variance is accounted for by these variables that can be measured easily, it would be possible to predict the change in LBM and muscle size. The regression equations developed from these data in healthy young men should be tested prospectively in other populations to establish their validity and generalizability.

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REFERENCES

1. **Alway SE, Grumbt WH, Gonyea WJ, and Stray-Gundersen J.** Contrasts in muscle and myofibers of elite male and female bodybuilders. *J Appl Physiol* 67: 24–31, 1989.
2. **Beilin J, Ball EM, Favaloro JM, and Zajac JD.** Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. *J Mol Endocrinol* 25: 85–96, 2000.
3. **Bhasin S, Storer TW, Asbel-Sethi N, Kilbourne A, Hays R, Sinha-Hikim I, Shen R, Arver S, and Beall G.** Effects of testosterone replacement with a nongenital, transdermal system, Androderm, in human immunodeficiency virus-infected men with low testosterone levels. *J Clin Endocrinol Metab* 83: 3155–3162, 1998.
4. **Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, and Casaburi R.** The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med* 335: 1–7, 1996.
5. **Bhasin S, Storer TW, Berman N, Yarasheski KE, Clevenger B, Phillips J, Lee WP, Bunnell TJ, and Casaburi R.** Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *J Clin Endocrinol Metab* 82: 407–413, 1997.
6. **Bhasin S, Storer TW, Javanbakht M, Berman N, Yarasheski KE, Phillips J, Dike M, Sinha-Hikim I, Shen R, Hays RD, and Beall G.** Testosterone replacement and resistance exercise in HIV-infected men with weight loss and low testosterone levels. *JAMA* 283: 763–770, 2000.
7. **Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, and Storer TW.** Testosterone dose-response relationships in healthy young men. *Am J Physiol Endocrinol Metab* 281: E1172–E1181, 2001.
8. **Brodsky IG, Balagopal P, and Nair KS.** Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men: a clinical research center study. *J Clin Endocrinol Metab* 81: 3469–3475, 1996.
9. **Chamberlain NL, Driver ED, and Miesfeld RL.** The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res* 22: 3181–3186, 1994.
10. **Choong CS, Kemppainen JA, Zhou ZX, and Wilson EM.** Reduced androgen receptor gene expression with first exon CAG repeat expansion. *Mol Endocrinol* 10: 1527–1535, 1996.
11. **Coodley GO and Coodley MK.** A trial of testosterone therapy for HIV-associated weight loss. *AIDS* 11: 1347–1352, 1997.
12. **Dobs AS, Cofrancesco J, Nolten WE, Danoff A, Anderson R, Hamilton CD, Feinberg J, Seekins D, Yangco B, and Rhame F.** The use of a transscrotal testosterone delivery system in the treatment of patients with weight loss related to human immunodeficiency virus infection. *Am J Med* 107: 126–132, 1999.

13. **Edwards SM, Badzioch MD, Minter R, Hamoudi R, Collins N, Ardern-Jones A, Dowe A, Osborne S, Kelly J, Shearer R, Easton DF, Saunders GF, Dearnaley DP, and Eeles RA.** Androgen receptor polymorphisms: association with prostate cancer risk, relapse and overall survival. *Int J Cancer* 84: 458–465, 1999.
14. **Gao T, Marcelli M, and McPhaul MJ.** Transcriptional activation and transient expression of the human androgen receptor. *J Steroid Biochem Mol Biol* 59: 9–20, 1996.
15. **Grinspoon S, Corcoran C, Askari H, Schoenfeld D, Wolf L, Burrows B, Walsh M, Hayden D, Parlman K, Anderson E, Basgoz N, and Klibanski A.** Effects of androgen administration in men with the AIDS wasting syndrome. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 129: 18–26, 1998.
16. **Grinspoon S, Corcoran C, Parlman K, Costello M, Rosenthal D, Anderson E, Stanley T, Schoenfeld D, Burrows B, Hayden D, Basgoz N, and Klibanski A.** Effects of testosterone and progressive resistance training in eugonadal men with AIDS wasting. A randomized, controlled trial. *Ann Intern Med* 133: 348–355, 2000.
17. **Heymsfield SB, Smith R, Aulet M, Bensen B, Lichtman S, Wang J, and Pierson RN Jr.** Appendicular skeletal muscle mass: measurement by dual-photon absorptiometry. *Am J Clin Nutr* 52: 214–218, 1990.
18. **Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal DI, Anderson EJ, and Klibanski A.** Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. *J Clin Endocrinol Metab* 81: 4358–4365, 1996.
19. **Kenny AM, Prestwood KM, Gruman CA, Marcello KM, and Raisz LG.** Effects of transdermal testosterone on bone and muscle in older men with low bioavailable testosterone levels. *J Gerontol A Biol Sci Med Sci* 56: M266–M272, 2001.
20. **Morley JE, Perry HMd, Kaiser FE, Kraenzle D, Jensen J, Houston K, Mattammal M, and Perry HM Jr.** Effects of testosterone replacement therapy in old hypogonadal males: a preliminary study. *J Am Geriatr Soc* 41: 149–152, 1993.
21. **Sih R, Morley JE, Kaiser FE, Perry HM III, Patrick P, and Ross C.** Testosterone replacement in older hypogonadal men: a 12-month randomized controlled trial. *J Clin Endocrinol Metab* 82: 1661–1667, 1997.
22. **Singh AB, Hsia S, Alaupovic P, Sinha-Hikim I, Woodhouse L, Buchanan TA, Shen R, Bross R, Berman N, and Bhasin S.** The effects of varying doses of T on insulin sensitivity, plasma lipids, apolipoproteins, and C-reactive protein in healthy young men. *J Clin Endocrinol Metab* 87: 136–143, 2002.
23. **Sinha Hikim AP, Bartke A, and Russell LD.** Morphometric studies on hamster testes in gonadally active and inactive states: light microscope findings. *Biol Reprod* 39: 1225–1237, 1988.
24. **Sinha-Hikim I, Artaza J, Woodhouse L, Gonzalez-Cadavid N, Singh AB, Lee MI, Storer TW, Casaburi R, Shen R, and Bhasin S.** Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. *Am J Physiol Endocrinol Metab* 283: E154–E164, 2002.
25. **Sinha-Hikim I, Arver S, Beall G, Shen R, Guerrero M, Sattler F, Shikuma C, Nelson JC, Landgren BM, Mazer NA, and Bhasin S.** The use of a sensitive equilibrium dialysis method for the measurement of free testosterone levels in healthy, cycling women and in human immunodeficiency virus-infected women. *J Clin Endocrinol Metab* 83: 1312–1318, 1998 [published erratum in *J Clin Endocrinol Metab* 83: 2959, 1998].
26. **Sleddens HF, Oostra BA, Brinkmann AO, and Trapman J.** Trinucleotide repeat polymorphism in the androgen receptor gene (AR) (Abstract). *Nucleic Acids Res* 20: 1427, 1992.
27. **Sleddens HF, Oostra BA, Brinkmann AO, and Trapman J.** Trinucleotide (GGN) repeat polymorphism in the human androgen receptor (AR) gene (Abstract). *Hum Mol Genet* 2: 493, 1993.
28. **Snyder PJ and Lawrence DA.** Treatment of male hypogonadism with testosterone enanthate. *J Clin Endocrinol Metab* 51: 1335–1339, 1980.
29. **Snyder PJ, Peachey H, Berlin JA, Hannoush P, Haddad G, Dlewati A, Santanna J, Loh L, Lenrow DA, Holmes JH, Kapoor SC, Atkinson LE, and Strom BL.** Effects of testosterone replacement in hypogonadal men. *J Clin Endocrinol Metab* 85: 2670–2677, 2000.
30. **Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Lenrow DA, Holmes JH, Dlewati A, Santanna J, Rosen CJ, and Strom BL.** Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *J Clin Endocrinol Metab* 84: 2647–2653, 1999.
31. **Tenover JS.** Androgen replacement therapy to reverse and/or prevent age-associated sarcopenia in men. *Baillieres Clin Endocrinol Metab* 12: 419–425, 1998.
32. **Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, and Yong EL.** Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. *J Clin Endocrinol Metab* 82: 3777–3782, 1997.
33. **Urban RJ, Bodenburg YH, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, and Ferrando A.** Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. *Am J Physiol Endocrinol Metab* 269: E820–E826, 1995.
34. **Van Pottelberg I, Lumbroso S, Goemaere S, Sultan C, and Kaufman JM.** Lack of influence of the androgen receptor gene CAG-repeat polymorphism on sex steroid status and bone metabolism in elderly men. *Clin Endocrinol (Oxf)* 55: 659–666, 2001.
35. **Wang C, Eyre DR, Clark R, Kleinberg D, Newman C, Iranmanesh A, Veldhuis J, Dudley RE, Berman N, Davidson T, Barstow TJ, Sinow R, Alexander G, and Swerdloff RS.** Sublingual testosterone replacement improves muscle mass and strength, decreases bone resorption, and increases bone formation markers in hypogonadal men: a clinical research center study. *J Clin Endocrinol Metab* 81: 3654–3662, 1996.
36. **Wang C, Swedloff RS, Iranmanesh A, Dobs A, Snyder PJ, Cunningham G, Matsumoto AM, Weber T, and Berman N.** Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameters in hypogonadal men. Testosterone gel study group. *J Clin Endocrinol Metab* 85: 2839–2853, 2000.
37. **Zitzmann M, Brune M, Kornmann B, Gromoll J, Junker R, and Nieschlag E.** The CAG repeat polymorphism in the AR gene affects high density lipoprotein cholesterol and arterial vasoreactivity. *J Clin Endocrinol Metab* 86: 4867–4873, 2001.