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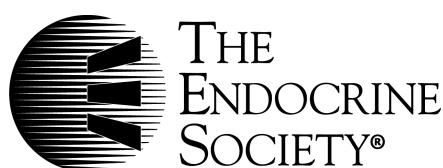
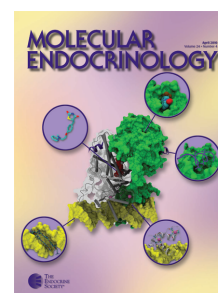
THE JOURNAL
OF CLINICAL
ENDOCRINOLOGY
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J. Clin. Endocrinol. Metab. 2004 89: 3313-3318, doi: 10.1210/jc.2003-031069

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The Effect of Testosterone Replacement on Endogenous Inflammatory Cytokines and Lipid Profiles in Hypogonadal Men

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Testosterone has immune-modulating properties, and current *in vitro* evidence suggests that testosterone may suppress the expression of the proinflammatory cytokines TNF α , IL-1 β , and IL-6 and potentiate the expression of the antiinflammatory cytokine IL-10. We report a randomized, single-blind, placebo-controlled, crossover study of testosterone replacement (Sustanon 100) vs. placebo in 27 men (age, 62 $\pm$ 9 yr) with symptomatic androgen deficiency (total testosterone, 4.4 $\pm$ 1.2 nmol/liter; bioavailable testosterone, 2.4 $\pm$ 1.1 nmol/liter). Compared with placebo, testosterone induced reductions in TNF α (–3.1 $\pm$ 8.3 vs. 1.3 $\pm$ 5.2 pg/ml; P = 0.01) and IL-1 β (–0.14 $\pm$ 0.32 vs. 0.18 $\pm$ 0.55 pg/ml; P = 0.08) and an increase in IL-10 (0.33 $\pm$ 1.8 vs. –1.1 $\pm$ 3.0 pg/ml; P = 0.01); the reductions of TNF α

and IL-1 β were positively correlated (r_s = 0.588; P = 0.003). In addition, a significant reduction in total cholesterol was recorded with testosterone therapy (–0.25 $\pm$ 0.4 vs. –0.004 $\pm$ 0.4 mmol/liter; P = 0.04). In conclusion, testosterone replacement shifts the cytokine balance to a state of reduced inflammation and lowers total cholesterol. Twenty of these men had established coronary disease, and because total cholesterol is a cardiovascular risk factor, and proinflammatory cytokines mediate the development and complications associated with atheromatous plaque, these properties may have particular relevance in men with overt vascular disease. (*J Clin Endocrinol Metab* 89: 3313–3318, 2004)

TESTOSTERONE IS THE predominant androgenic hormone and has a range of biological actions. The classical effects of testosterone are the induction and maintenance of secondary sexual characteristics and preservation of libido, sense of well-being, lean mass, and bone density. The hormone is involved in a negative biofeedback mechanism on the hypothalamic-pituitary axis to inhibit gonadotropin secretion. It has long been recognized that testosterone has an immune-modulating action. The greater incidence of immune-mediated disease in women and androgen-deficient men has been attributed to the immunosuppressive effects of androgens compared with estrogens (1). Laboratory studies using animals with experimental induced inflammatory disease have reported a beneficial response with androgens (2–4). Moreover, there are several case reports and small clinical trials of testosterone therapy in humans with rheumatoid arthritis and systemic lupus erythematosus in which major improvements of clinical status and inflammatory markers have been recorded (5–7). The mechanism by which testosterone effects these responses is uncertain, but laboratory evidence has suggested that testosterone suppresses proinflammatory cytokines and may up-regulate antiinflammatory cytokines. Despite the varied approach of

these studies, the response to testosterone appears to be similar, so that testosterone incubated in cell culture attenuates the production of inflammatory cytokines such as TNF α , IL-1 β , and IL-6 in human macrophages (8), human monocytes (9), human gingival fibroblasts (10), murine and human osteoblasts (11, 12), and human endothelial cells (13). Furthermore, antiinflammatory cytokines such as IL-10 are stimulated in the presence of testosterone (14, 15).

There have been few *in vivo* studies with highly varied experimental approaches. Injection of bacterial endotoxin into castrated male mice increased the endogenous production of TNF α , which was abrogated by testosterone replacement (16). Macrophages taken from castrated mice subjected to trauma hemorrhage had increased production of IL-1 β and IL-6 compared with sham-castrated animals subject to the same procedure (17). In the few human studies, elderly men with hypogonadism induced with gonadotropin hormone-releasing agonists developed significant increases in serum TNF α and IL-6 (18). Although testosterone replacement reduced the levels of proinflammatory serum cytokines the reduction did not achieve statistical significance. A cohort of 29 hypogonadal young men with delayed puberty was found to have increased levels of immune activation compared with age-matched healthy controls. The immune activation was normalized with androgen-stimulating therapy (19).

There have been two prospective studies of androgen therapy in men with partial androgen deficiency, but neither showed any convincing effects on inflammatory cytokines.

Abbreviations: HDL, High-density lipoprotein; LDL, low-density lipoprotein; PSA, prostate-specific antigen.

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Ng *et al.* (20) used dihydrotestosterone, recombinant human chorionic gonadotropin, or a control in healthy men with total testosterone levels below 15 nmol/liter and found no changes in C-reactive protein or the soluble cellular adhesion molecules intracellular adhesion molecule-1 and vascular cell adhesion molecule-1. Lambert *et al.* (21) used megestrol acetate with testosterone injections, resistance training, or both in elderly eugonadal men, but no treatment effects on serum TNF α or leptin were found. To our knowledge there are no published data on the inflammatory/cytokine balance in postpubertal men with symptomatic androgen deficiency, nor is there any information on the effects of testosterone replacement on the cytokine balance in these hypogonadal men.

The aim/hypothesis of this study was to assess the effects of testosterone replacement on circulating inflammatory cytokines, antiinflammatory cytokines, and plasma lipids.

Subjects and Methods

This was a randomized, single-blind, placebo-controlled, crossover trial of testosterone replacement in hypogonadal men. The primary outcome was the serum level of TNF α . Eligible androgen-deficient men were recruited from a male andrology clinic before commencing testosterone replacement. Patients provided written informed consent and underwent a screening protocol before entry and randomization. Treatment began with either testosterone or placebo for 1 month, each comprising three injections given at 0, 14, and 28 d. Assessments were performed at baseline and on d 30 of each phase; thus, the follow-up assessments were performed 2 d after the final injection in each phase. There was 1 month of no treatment (washout) between the two treatment phases.

Subjects were referred back to the andrology clinic after completion of the trial protocol for continuing management and testosterone replacement. There were no changes to concomitant medications during the study period.

Setting

This study was performed at the andrology outpatient department, Royal Hallamshire Hospital; Sheffield Cardiology Research Department, Royal Hallamshire Hospital Sheffield; and Center for Diabetes and Endocrinology, Barnsley District General Hospital, Barnsley.

Subjects

Patients were males referred to the andrology clinic who were androgen deficient and for whom androgen replacement was deemed clinically indicated by a consultant endocrinologist (T.H.J.; Table 1). Twelve of the patients had primary gonadal failure with elevation of gonadotropins above the normal range; one of these patients had Noonan's syndrome, and two had Klinefelter's syndrome. Four patients had hypogonadotropic hypogonadism with low gonadotropin levels; one of these had hemochromatosis, and the other three had no underlying diagnosis, although pituitary magnetic resonance imaging was normal in each case. The remaining patients (n = 11) had a mixed picture of hypogonadism, with low serum testosterone levels and gonadotropin levels within the normal range. The local regional ethics committee approved the protocol, and patients provided written informed consent.

Inclusion criteria for trial patients were age greater than 18 yr with a clinical indication for testosterone replacement. Exclusion criteria were elevation of prostate-specific antigen (PSA) above the age-adjusted normal range, malignancy, and any acute or chronic inflammatory condition.

Randomization, drug treatment, and blinding

We used a standard regimen of testosterone therapy in hypogonadal men, which comprised fortnightly injections of depot testosterone. Tes-

TABLE 1. Baseline characteristics

Clinical characteristic/normal range	Patients (n = 27)	Range
Age (yr)	61.6 $\pm$ 9.3	36–78
BMI (kg/m ²) (20–25)	30.9 $\pm$ 6.9	21.5–57
Total testosterone (nmol/liter) (>12 nmol/liter)	4.39 $\pm$ 1.24	0.9–17
Bioavailable testosterone (nmol/liter) (>2.5 nmol/liter)	2.42 $\pm$ 1.07	0.2–6.4
FSH (IU/liter)	12.4 $\pm$ 10.5	1.9–46.8
LH (IU/liter)	8.6 $\pm$ 12.2	1.2–59.5
SHBG (nmol/liter)	16.9 $\pm$ 7.1	8.7–32.7
Total cholesterol (mmol/liter)	4.86 $\pm$ 1.1	3.3–7.6
TNF α (pg/ml)	5.78 $\pm$ 8.4	1.05–37
IL-1 β (pg/ml)	0.41 $\pm$ 0.49	0.03–1.679
IL-6 (pg/ml)	4.7 $\pm$ 4.9	0.7–23.7
IL-10 (pg/ml)	3.3 $\pm$ 4.3	0.03–1.7
Androgen Deficiency Questionnaire Score (out of 10)	6.7 $\pm$ 2.3	4–10
Concomitant disease (n)		
Chronic heart failure	2	
Coronary heart disease	20	
Previous myocardial infarction	9	
Hypertension	10	
Diabetes mellitus	9	
Current smoker	4	
Drugs (n)		
Aspirin/clopidogrel	19/1	
HMG-CoA inhibitor	20	
Oral nitrate	13	
β -Blocker	12	
Calcium antagonist	16	
ACE/ARB	10/1	
Diuretic	4	

BMI, Body mass index; ACE, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A.

tosterone (100 mg testosterone esters/ml; Sustanon 100, Organon, West Orange, NJ) or placebo (1 ml 0.9% normal saline) in an identical syringe was given by deep im injection every fortnight by a member of the research staff (C.J.M. or P.J.P.) in the cardiology research department, with three injections given in each 1-month period. Subjects were blinded to the identity of the injection, and the drug was drawn up away from the patient. The order of drug administration was allocated using blocks of computer-generated numbers.

Subject assessment

All assessments were performed in the cardiology research department in the morning before 1000 h. At the screening visit all patients and controls were required to complete a detailed questionnaire recording their medical history and current medications. Subjects were asked to complete an androgen deficiency screening questionnaire (ADAM). The androgen screening questionnaire is a 10-statement true/false answer sheet. A positive screen is defined as a score of 3 or more out of 10, or a single positive response to any one of two highly specific questions: do you have reduced libido (sex drive)? or do you have reduced ability to maintain erections?

Blood was drawn at each visit for total testosterone, SHBG, and full lipid profiles. Blood samples for gonadotropin and PSA determinations were taken only at the screening visit. Blood for serum cytokine measurements was taken in chilled serum gel tubes and allowed to clot standing in ice. Serum was separated using a centrifuge, and plasma was stored at -80°C until analysis. Total testosterone and SHBG were measured by enzyme immunoassay (DRG Instruments, Marburg, Germany). Bioavailable testosterone was assayed using a modification of the method described by Tremblay and Dube (22). In this method testosterone is removed from the test serum by the addition of activated charcoal and is replaced by [^3H]testosterone, which binds to SHBG and other plasma proteins in equimolar concentrations as native testoster-

one. SHBG-bound testosterone is then precipitated out of the sample by the addition of cold ammonium sulfate. The remaining [^3H]testosterone, comprising the unbound fraction and the fraction weakly bound to albumin, is then measured using a β -counter. The intra- and interassay coefficients of variation of the assays were: total testosterone, less than 5% and less than 6%; SHBG, less than 9% and less than 12%; and bioavailable testosterone, less than 5% and less than 8%. PSA, gonadotropins, and lipid profiles were measured with a standard hospital assay; low-density lipoprotein (LDL) cholesterol was calculated with the Friedwald equation $\text{LDL} = \text{total cholesterol} - [\text{high-density lipoprotein (HDL)} + (\text{triglycerides}/2.2)]$. Serum TNF α , IL-1 β , and IL-6 were assayed with high sensitivity quantitative sandwich enzyme immunoassay (Quantikine, R&D Systems, Abingdon, UK). The minimum detectable level for TNF α is 0.12 pg/ml, and those for IL-1 β and IL-6 are less than 0.1 pg/ml. The intra- and interassay coefficients of variation of these assays were less than 11.5% and less than 19.5%, respectively. Serum IL-10 was measured with high sensitivity quantitative sandwich enzyme immunoassay (Amersham Pharmacia Biotech, Piscataway, NJ); the minimum detectable level is 0.1 pg/ml, and the intra- and interassay coefficients of variation were both less than 10%. Serum levels of proinflammatory cytokines should be undetectably low in 25% of young normal subjects.

Statistical analysis and sample size determination

There are no pilot data on which to base a sample size calculation. However, in a study of lipid-lowering therapy in hyperlipidemic patients, active treatment resulted in a reduction of circulating TNF α from 19.2 ± 5.1 to 9.2 pg/ml. To detect half this reduction in a placebo-controlled, crossover study with 95% power and 5% significance, it was estimated that 22 patients would be required. It was anticipated that drop-out rates would be low, and therefore, we planned to recruit 30 patients. All data are presented as the mean $\pm$ SD unless stated otherwise; analysis was performed with a statistical package (SPSS version 11.5, SPSS, Inc., Chicago, IL). Data were tested against a normal distribution using Kolmogorov-Smirnov tests. The serum levels of cytokines and most serum levels of cholesterol were nonparametric, so the Wilcoxon matched pair test was used throughout for paired data, and Mann-Whitney U tests were used for unpaired data. The correlation between data were examined using Spearman's rank correlation (r_s), and in all cases $P < 0.05$ was considered significant. Two subgroup analyses were performed, so a Bonferroni correction was made to calculate the final test statistic. Comparisons of baseline values and of treatment period interaction were performed with a t test.

Results

Twenty-nine patients agreed to take part in the study. Samples for one patient for one visit were lost, and one other patient was called for coronary artery bypass surgery sooner than expected and was withdrawn from the study. This provided data for 27 patients, but delta analysis could only be performed on 25 patients because a single visit set of sera for two patients was unsuitable for analysis. These results are

summarized in Table 2. There were no time or period treatment effects of significance, and the baseline values for each cytokine and lipid fraction in the testosterone and placebo phases were not statistically different. Testosterone caused a reduction of the serum cytokines TNF α and IL-1 β . By analysis of the delta values between testosterone and placebo, testosterone effected a reduction of TNF α (-3.1 ± 8.3 vs. 1.32 ± 5.2 pg/ml; $P = 0.012$) and an increase in IL-10 (0.33 ± 1.8 vs. -1.1 ± 3.0 pg/ml; $P = 0.014$). The reduction in IL-1 β only approached significance (-0.14 ± 0.32 vs. 0.18 ± 0.55 pg/ml; $P = 0.08$; Fig. 1). The reduction of TNF α and IL-1 β was positively correlated ($r_s = 0.588$; $P = 0.003$; Fig. 2).

Two of the hypogonadal patients had very high basal TNF α levels. A subgroup analysis was performed on the study group with these two patients removed, and the reduction in TNF α with testosterone by analysis of the delta remained significant (-0.72 ± 1.7 vs. 0.34 ± 1.6 pg/ml; $P = 0.04$, corrected).

A subgroup analysis of the response of TNF α in the 20 patients with coronary disease was also performed. The effect remained highly significant; the difference by delta analysis for testosterone was -4.1 ± 9.1 vs. 1.5 ± 5.9 pg/ml for placebo ($P = 0.016$, corrected).

The testosterone phase of treatment was also associated with a reduction of total cholesterol and serum triglycerides (Table 2), although by analysis of the delta values, only the reduction in total cholesterol remained significant (Fig. 3). The reduction of LDL cholesterol with testosterone was not significant (-0.11 ± 0.5 vs. 0.14 ± 0.98 mmol/liter; $P = 0.3$), but the reductions in total cholesterol and LDL cholesterol were positively correlated ($r_s = 0.531$; $P = 0.005$), suggesting a relationship (Fig. 4).

There were no significant effects on HDL cholesterol (-0.02 ± 0.12 vs. -0.05 ± 0.21 ; $P = 0.84$).

Discussion

This study found that 1 month of testosterone replacement given to men with symptomatic androgen deficiency resulted in statistically significant reductions of TNF α and IL-1 β compared with placebo, and there was a statistically significant increase in IL-10 by delta analysis. The baseline TNF α was higher in the testosterone phase, but not significantly so. There were no treatment/period effect of significance. Two of the hypogonadal patients had very high basal TNF α levels; both of these men had mild chronic congestive

TABLE 2. Difference by treatment in 27 hypogonadal men (Wilcoxon matched pairs test)

Variable	Placebo (n = 27)			Testosterone (n = 27)			Baseline comparison (P)
	Baseline	4 wk	P	Baseline	4 wk	P	
TNF α	2.68 ± 1.76	4.18 ± 5.0	0.4	5.77 ± 8.4	2.9 ± 2.3	0.02	0.17
IL-1 β	0.33 ± 0.37	0.5 ± 0.6	0.3	0.41 ± 0.49	0.26 ± 0.3	0.05	0.31
IL-6	4.9 ± 5.89	4.8 ± 3.5	0.23	4.66 ± 4.9	4.64 ± 4.4	0.62	0.78
IL-10	3.23 ± 3.8	2.1 ± 2.7	0.2	3.3 ± 4.3	3.75 ± 4.5	0.6	0.91
Total cholesterol	4.72 ± 1.07	4.72 ± 1.07	0.79	4.87 ± 1.1	4.6 ± 1.1	0.007	0.2
LDL	2.67 ± 1.0	2.81 ± 1.1	0.78	2.82 ± 1.1	2.71 ± 1.1	0.4	0.3
HDL	0.97 ± 0.22	0.95 ± 0.2	0.5	0.97 ± 0.19	0.94 ± 0.2	0.23	0.99
Cholesterol:HDL ratio	4.98 ± 1.24	5.09 ± 1.23	0.65	5.16 ± 1.4	5.02 ± 1.5	0.3	0.48
Triglycerides	2.4 ± 1.4	2.24 ± 1.2	0.95	2.35 ± 1.2	2.09 ± 0.8	0.04	0.96
Total testosterone	6.7 ± 4.5	8.7 ± 9.5	0.18	6.7 ± 4.8	42.6 ± 36.6	0.0001	0.98
Bioavailable testosterone	3.0 ± 1.9	3.6 ± 3.1	0.6	2.4 ± 1.3	16.5 ± 13.9	0.0001	0.11

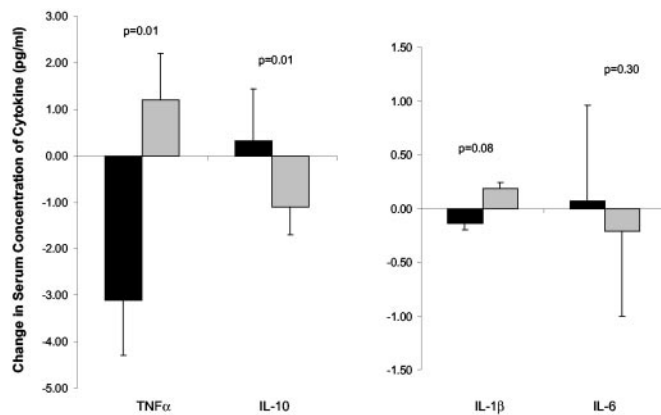


FIG. 1. Change in serum cytokines caused by treatment.

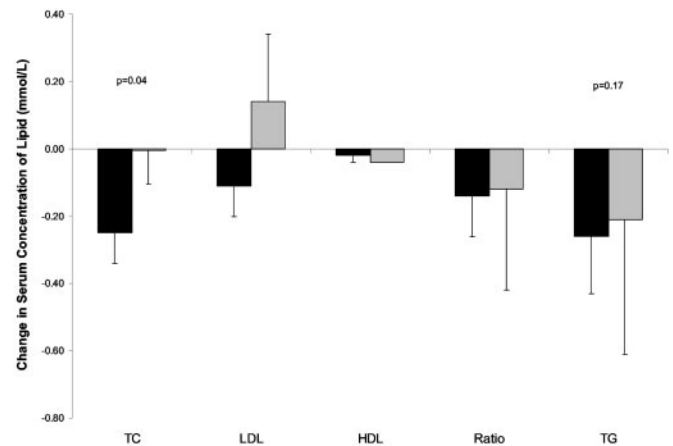


FIG. 3. Change in lipid profiles caused by treatment.

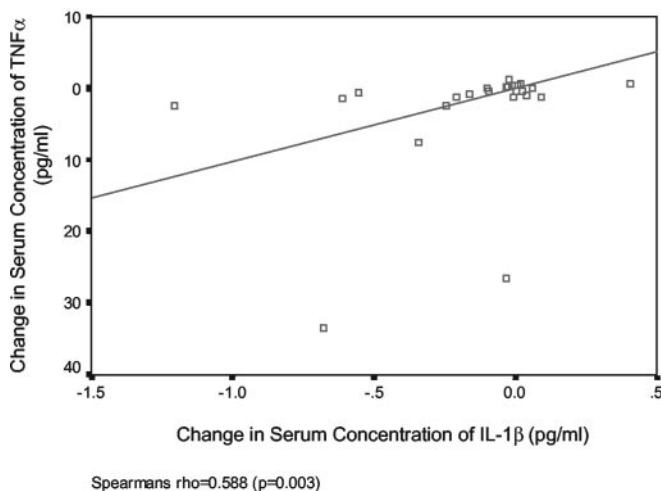


FIG. 2. Relationship of TNF and IL-1B.

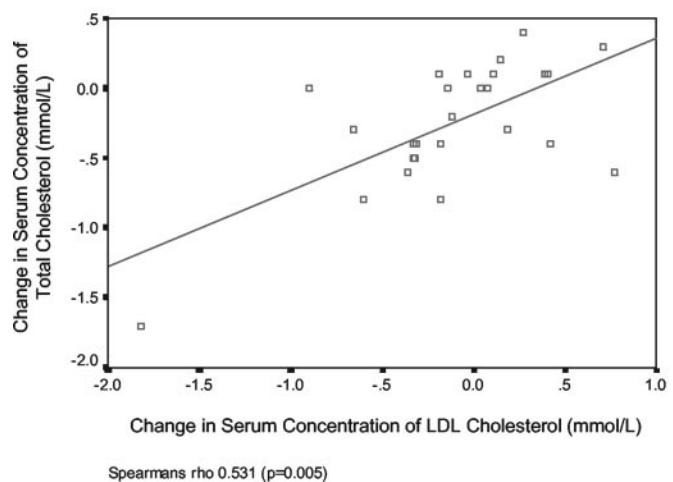


FIG. 4. Relationship of total cholesterol and LDL.

heart failure, a condition characterized by high basal $\text{TNF}\alpha$ values. A subgroup analysis was performed on the study group with these two patients removed, but the reduction in $\text{TNF}\alpha$ with testosterone by analysis of the delta remained significant. The $\text{TNF}\alpha$ response to testosterone of these patients was very sensitive, and both demonstrated large reductions compared with the effect of placebo. This is an intriguing finding, because the progression and prognosis of heart failure are linked to catabolic compounds such as $\text{TNF}\alpha$, and there have been a number of attempts to reduce $\text{TNF}\alpha$ pharmacologically as a treatment for heart failure (23), although with limited clinical benefit (24). Preliminary data suggesting reductions of $\text{TNF}\alpha$ in heart failure patients with testosterone therapy should provoke interest in further studies in this group.

The largest comorbid group was patients with coronary disease ($n = 20$); this included the two patients with congestive heart failure. Atherosclerosis is acknowledged to be a disease of chronic inflammation, and inflammatory cytokines such as $\text{TNF}\alpha$ and $\text{IL-1}\beta$ are mediators of atheromatous plaque development and complications (25). Antiinflammatory cytokines such as IL-10 can regulate inflammation and may inhibit atherosclerosis (26, 27), and elevation of IL-10 after an acute coronary syndrome confers a favorable prognosis (28). Subgroup analyses in patients with coronary dis-

ease ($n = 20$) substantially weakened the results, with only $\text{TNF}\alpha$ retaining statistical significance. This analysis suggests that testosterone had an antiinflammatory effect in all patients, not just in a subgroup with established vascular disease.

The use of 3-hydroxy-3-methyl-glutaryl-coenzyme A enzyme inhibitors was high ($n = 20$). The reason for statin therapy was secondary prevention for vascular disease in most cases. Despite the presence of vascular disease, the dyslipidemia in these patients may reflect the underlying androgen status, because there are reports of dyslipidemia associated with hypogonadism (29, 30). However, the raised body mass index and high prevalence of type 2 diabetes ($n = 9$) are also likely to contribute. Testosterone replacement caused a significant reduction in total cholesterol and triglycerides, although by delta analysis only the reduction of total cholesterol remained significant. In general, our results agree with most studies of the effect of androgen replacement in cholesterol profiles, with modest reductions of total cholesterol and LDL cholesterol reported by a recent meta-analysis (31). We did not find a reduction in HDL cholesterol in our study that was documented in the meta-analysis performed by Whitsel *et al.* (31). This effect of testosterone on

HDL cholesterol is reported to be reduced or absent in hypogonadal or obese men (32, 33), and in our study may also be masked by the positive effects of testosterone on insulin resistance that have been reported in obese men (34) and in asymptomatic men with low serum testosterone levels (35).

We have shown that testosterone replacement induces an antiinflammatory shift in cytokine balance. This study is one of the very few *in vivo* studies and the only in postpubertal men with idiopathic disease to demonstrate a convincing antiinflammatory effect of testosterone. Moreover, the observed effect has a potentially beneficial action in men with coronary disease due to atherosclerosis. An anticytokine effect in this inflammatory condition may positively affect plaque biology and stability.

The observed reduction in total cholesterol may be related to the reductions in inflammatory cytokines, as cholesterol reduction with statins is itself associated with an anticytokine effect (36, 37). However the reduction of TNF α and IL-1 β did not correlate with the reduction in total cholesterol or LDL cholesterol, suggesting the lack of a relationship. Furthermore, we cannot exclude an interaction between statin therapy and testosterone, because postmenopausal women treated with hormone replacement and statins have greater reductions in total cholesterol than those given statins alone (38, 39).

Conclusions

Testosterone replacement in men with symptomatic androgen deficiency is indicated to improve quality of life, maintain lean body mass, and preserve bone density. Although this trial enrolled a mixed population of hypogonadal men, an antiinflammatory effect of testosterone replacement may be of particular benefit to men with established vascular disease, because inflammation determines vascular risk in these individuals. Importantly, the prevalence of overt hypogonadism in men with coronary disease is high and may approach 25% (40), a figure far in excess of the background community prevalence (41). In addition, testosterone therapy has been found to improve symptoms and time to ischemic threshold in coronary disease (42–44) and congestive heart failure (45). The present study suggests that testosterone replacement in this potentially large group of men may beneficially alter cytokine balance and reduce total cholesterol. These effects may translate to prognostic benefit in addition to symptomatic improvements. Further prospective trials to determine safety and particularly cardiovascular outcomes are justified.

Acknowledgments

We acknowledge the work of Miss Joanne Hall for her expert assistance with hormone ELISAs, and Miss Gill Corlett and Miss Katherine Kerry for their assistance with the cytokine ELISAs. Acknowledgment is also made to Mrs. Bernadette Hardware for help with the recruitment and phlebotomy of patients at Barnsley District General Hospital.

Received July 10, 2003. Accepted March 18, 2004.

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This work was supported by the Central Sheffield University Hospitals Pilot Research Fund.

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