Reference Ranges for Testosterone in Men Generated Using Liquid Chromatography Tandem Mass Spectrometry in a Community-Based Sample of Healthy Nonobese Young Men in the Framingham Heart Study and Applied to Three Geographically Distinct Cohorts

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Context: Reference ranges are essential for partitioning testosterone levels into low or normal and making the diagnosis of androgen deficiency. We established reference ranges for total testosterone (TT) and free testosterone (FT) in a community-based sample of men.

Methods: TT was measured using liquid chromatography tandem mass spectrometry in nonobese healthy men, 19–40 yr old, in the Framingham Heart Study Generation 3; FT was calculated. Values below the 2.5th percentile of reference sample were deemed low. We determined the association of low TT and FT with physical dysfunction, sexual symptoms [European Male Aging Study (EMAS) only], and diabetes mellitus in three cohorts: Framingham Heart Study generations 2 and 3, EMAS, and the Osteoporotic Fractures in Men Study.

Results: In a reference sample of 456 men, mean (SD), median (quartile), and 2.5th percentile values were 723.8 (221.1), 698.7 (296.5), and 348.3 ng/dl for TT and 141. 8 (45.0), 134.0 (60.0), and 70.0 pg/ml for FT, respectively. In all three samples, men with low TT and FT were more likely to have slow walking speed, difficulty climbing stairs, or frailty and diabetes than those with normal levels. In EMAS, men with low TT and FT were more likely to report sexual symptoms than men with normal levels. Men with low TT and FT were more likely to have at least one of the following: sexual symptoms (EMAS only), physical dysfunction, or diabetes.

Conclusion: Reference ranges generated in a community-based sample of men provide a rational basis for categorizing testosterone levels as low or normal. Men with low TT or FT by these criteria had higher prevalence of physical dysfunction, sexual dysfunction, and diabetes. These reference limits should be validated prospectively in relation to incident outcomes and in randomized trials. **(*J Clin Endocrinol Metab* 96: 2430–2439, 2011)**

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Abbreviations: CVD, Cardiovascular disease; DM, diabetes mellitus; EMAS, European Male Aging Study; FHS, Framingham Heart Study; Gen 3, third generation; LC-MS/MS, liquid chromatography tandem mass spectrometry; MrOS, Osteoporotic Fractures in Men Study.

Androgen deficiency in men is a syndrome characterized by a constellation of symptoms and signs and low circulating testosterone levels (1). Thus, the diagnosis of androgen deficiency is predicated upon the determination of whether the circulating testosterone level is low or normal (1–3). Rigorously established reference ranges constitute the essential basis for identifying whether the circulating levels of an analyte, such as testosterone, are normal or low. The reference ranges for testosterone have been derived previously mostly from small convenience samples (2–9) or from hospital or clinic-based patients; these approaches are limited by their inherent selection bias, because patients seeking medical care are more likely to have a disease than individuals in the general population. Some recent efforts to generate reference ranges in community-dwelling men are notable; these studies included middle-aged and older men and used direct RIA (10), whose accuracy, particularly in the low range, has been questioned (3, 11, 12). In the absence of rigorously determined reference limits generated using reliable assays in community-based samples, the partitioning of total and free testosterone levels into normal or low values has been fraught with substantial risk of misclassification (2, 3, 13), relegating many healthy men to unnecessary risks of testosterone therapy and preventing others from receiving appropriate testosterone therapy because of a missed diagnosis.

We generated reference limits for total and free testosterone concentrations in a community-based sample of healthy young men in the Framingham Heart Study (FHS) third generation (Gen 3) cohort (14). Total testosterone was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS), a method with high specificity, sensitivity, and accuracy (2, 3, 11–13). We applied these reference limits to three geographically distinct cohorts of community-dwelling men: FHS Gen 2 and 3 (14), the European Male Aging Study (EMAS) (15, 16), and the Osteoporotic Fractures in Men Study (MrOS) (17). We determined whether men in these three cohorts, deemed to have low total and free testosterone levels by the proposed reference limits, had a higher prevalence of physical dysfunction, sexual symptoms, and diabetes mellitus (DM), the three categories of conditions that have been associated most consistently with low testosterone levels (18–27). We used thresholds based on a healthy young reference sample (T-score approach) because in exploratory analyses, the T-score approach and age-adjusted thresholds (Z-score approach) yielded concordant results for most outcomes. Also, the spline plots of testosterone levels against outcomes in the FHS sample did not reveal clear inflection points at which the relationship between testosterone levels and outcomes changed abruptly. The T-score approach based on limits derived in a healthy young population has been favored historically for analytes that exhibit clinically meaningful age-related trends, such as estradiol and bone mineral density.

Materials and Methods

Study sample

In 1948, to identify risk factors for cardiovascular disease (CVD), the FHS recruited 5209 men and women between the ages of 30 and 62 from Framingham, MA, that constituted the original cohort. In 1971, the study enrolled a second-generation cohort (Gen 2), 5124 of the original participants' adult children and their spouses. A third generation (4095 children of Gen 2, referred to as Gen 3) was recruited in 2002–2005 (14) to further understand how genetic factors relate to cardiovascular disease risk. The FHS design and methods have been described (http://nhlbi.nih.gov/about/framingham). The recruitment methods and the selection criteria for Gen 3 participants have been published (14) and are described briefly in the Supplemental Methods (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Of the 1912 men who attended the first Gen 3 examination (2002–2005), 1893 had total testosterone measurements, 962 were 40 yr of age or younger among whom 456 men were free of cancer (self-report of physician diagnosis supported by medical records when available), CVD (occurrence of any of the following: myocardial infarction, sudden death, stroke, congestive heart failure, coronary angioplasty or coronary artery bypass surgery, claudication, or peripheral angioplasty), DM, hypertension, hypercholesterolemia, obesity, and smoking, and constituted the reference sample (Fig. 1). Cardiometabolic disorders have been associated with low testosterone levels; therefore, men with cardiometabolic disorders were excluded from the reference sample. The men who were receiving androgen deprivation therapy or had undergone orchiectomy for prostate cancer or were taking testosterone for hypogonadism were excluded.

Application to EMAS, MrOS, and FHS broad samples

We assessed whether low total and free testosterone levels, defined as values below the 2.5th percentile of the reference sample, were associated with three categories of conditions that have been associated with low testosterone levels (18–27): physical dysfunction, sexual symptoms, and DM in the FHS broad sample (see below), the EMAS, and MrOS. The FHS broad sample was created by combining Gen 2 and Gen 3 samples (Supplemental Fig. 1A). The Gen 2 examination 7 (1998–2002) was attended by 1625 men. Exclusion of men with prostate cancer undergoing androgen deprivation therapy (n = 8) or testosterone therapy and those with missing testosterone data (n = 158) resulted in a sample of 1459 for Gen 2. This combined sample of 3352 men (1459 men in Gen 2 plus 1893 men in Gen 3) constituted the FHS broad sample. The walking speed data were available in 797 Gen 2 men who attended exam 7 and had nonmissing testosterone data.

The EMAS recruited 3369 men, aged 40–79 yr, at eight European centers (15, 16): Manchester (UK), Leuven (Belgium), Malmö (Sweden), Tartu (Estonia), Lodz (Poland), Szeged (Hun-

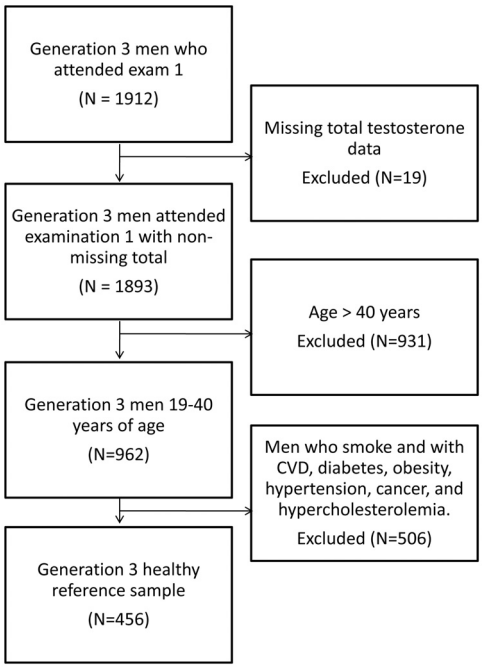

FIG. 1. The STROBE diagram: selection of the FHS reference sample. Of the 1912 men who attended the first Gen 3 examination (2002–2005), 1893 had total testosterone measurements, 962 were 40 yr of age or younger, and 456 men were free of cancer, CVD, DM, hypertension, hypercholesterolemia, obesity, and smoking.

gary), Florence (Italy), and Santiago de Compostela (Spain). The men, randomly selected from the general population, were invited for study-related assessments, including an interviewer-assisted questionnaire, several performance measures, and a fasting blood test before 1000 h. One hundred fifty men were excluded because of known pituitary, testicular, or adrenal disease or use of medications that affect sex-steroid production or action yielding an analytic sample of 3219 men (Supplemental Fig. 1B).

MrOS, an observational study of the determinants of fracture in older men, recruited 5995 community-dwelling men at least 65 yr old at six U.S. centers (17). Of 5995 men who were recruited, total testosterone was measured on fasting, morning serum specimens in 1488 randomly selected men. Among these, 19 men were excluded for missing total testosterone data, and 95 were excluded because of the use of androgens or antiandrogens or reported orchiectomy as a treatment for prostate cancer, resulting in a final analytical sample of 1488 participants (Supplemental Fig. 1C).

Physical function measures (walking speed and self-reported mobility limitation in subsets of FHS, walking speed and frailty in MrOS, and walking speed and self-reported difficulty walking or climbing stairs in EMAS) and diabetes were available in all three cohorts; data on sexual symptoms were available only in EMAS.

Ascertainment of outcomes in the FHS

The self-reported mobility limitation in FHS was determined using a modified Rosow-Breslau questionnaire (28), which has been shown to have high test-retest reliability in other large population-based studies (19, 29, 30). Participants were asked whether they were able to 1) do heavy work around the house, like shovel snow or wash windows, walls, or floors without help; 2) walk half a mile without help (about four to six blocks); and 3) walk up and down one flight of stairs. At this exam, the last item was asked as part of the Katz Activities of Daily Living scale with the following directive: during the course of a normal day, can you walk up and down one flight of stairs independently or do you need human assistance or the use of a device? Response choices included 1) no help needed, independent; 2) uses device, independent; 3) human assistance needed, minimally dependent; 4) dependent; and 5) do not do during a normal day. If the participant reported independence, he was considered able to perform the mobility task (19). A participant was considered to have a mobility limitation if he reported an inability to do one or more of the three items on the scale (19, 28).

Usual walking speed was assessed by asking the participants to walk at their usual pace over a 4-m course at an ancillary study to examination 7 in Gen 2 (19). Participants were allowed to use walking aids if necessary, but not the assistance of another person. For individuals who did not attempt or complete the walk, the value was set to the maximum value obtained by any individual.

DM was defined as a fasting blood glucose of at least 126 mg/dl and/or the use of diabetes medication. Hypertension was defined as systolic blood pressure of at least 140 or diastolic blood pressure at least 90 mm Hg and/or the use of hypertension treatment. Hypercholesterolemia was defined by total cholesterol of at least 240 mg/dl or use of cholesterol-lowering medication. Obesity was defined as body mass index of at least 30 kg/m^2.

Ascertainment of outcomes in the MrOS

For the measurement of walking speed, the participants were instructed to walk at a comfortable pace over a path of 6 m and completed two consecutively trials without a rest (31). Walking speed was calculated in meters per second using the time to complete two trials. The walking attempts were completed consecutively without a rest between attempts. Slow walking speed was defined if a participant was unable to complete the walk or scored in the slowest 20th percentile based on height-specific thresholds (0.99 m/sec for height $\leq$174.35 cm, 1.06 m/sec for height $>$174.35 cm).

Frailty was defined using modified criteria from the Cardiovascular Health Study and previous analyses in MrOS (32, 33). The Cardiovascular Health Study definition uses five components to define the presence of frailty: shrinking/sarcopenia, weakness, slowness, low activity level, and exhaustion (33). Participants with at least three components were defined as frail.

Diabetes was defined by fasting glucose above 126 mg/dl, use of oral hypoglycemic medications or insulin, or self-report of a physician's diagnosis.

Ascertainment of outcomes in EMAS

The operational definitions of conditions and symptoms in the EMAS are shown in Supplemental Table 1.

Hormone measurements

FHS samples were obtained in the morning, after an overnight fast of approximately 10 h, typically between 0730 and 0830 h. The samples were aliquoted, frozen immediately, and stored at −80 C until the time of assay. The stability of FHS samples in storage has been evaluated previously by measuring the concentrations of cholesterol, high-density lipoprotein cholesterol and

triglycerides in samples in the low, mid, and high range before freezing and storage at examination cycle 5 in 1991–1995 with repeated measurement in 2007, after storage at −80 C (34). The concentrations of these analytes were unchanged over a 15-yr period of storage at −80 C in the FHS repository using processes that are similar to those used for the collection and storage of samples included in the analyses reported here with correlation coefficients of measurements in 1991–1995 with repeated measurement in 2007 of 0.985, 0.997, and 0.948, respectively.

We measured total testosterone in the FHS Gen 2 and 3 samples using the same LC-MS/MS assay (19, 35). The functional limit of detection, defined as the lowest concentration, detected with less than 20% coefficient of variation (CV), was 2 ng/dl; no sample was outside the linear range of 2–2000 ng/dl. The recovery was calculated by adding known amounts of testosterone to charcoal-stripped serum and analyzing them by LC-MS/MS. The correlation between the amount added and the amount measured by LC-MS/MS was 0.998. The average recovery was 102 ± 3%. The cross-reactivity of dehydroepiandrosterone, dehydroepiandrosterone sulfate, and dihydrotestosterone, androstenedione, and estradiol in the testosterone assay was negligible at 10 times the circulating concentrations of these hormones. The interassay CV was 15.8% at 12.0 ng/dl, 10.6% at 23.5 ng/dl, 7.9% at 48.6 ng/dl, 7.7% at 241 ng/dl, 4.4% at 532 ng/dl, and 3.3% at 1016 ng/dl. As part of the Centers for Disease Control's (CDC) Testosterone Assay Harmonization Initiative, quality control samples provided by the CDC were run every 3 months; the CV in quality control samples with testosterone concentrations in the 100- to 1000-ng/dl range was consistently less than 6%. In addition, 28 serum samples from men and women with testosterone concentrations across the male and female range were measured in a blinded manner in the Boston University and Mayo laboratories. The Pearson correlation between values obtained in the two laboratories was higher than 0.99, and Bland-Altman plots revealed no significant differences between values obtained in the two laboratories.

Total testosterone levels in the EMAS (36) and MrOS (37) samples were measured using gas chromatography-MS/MS with sensitivities of 5 and 2.5 ng/dl, respectively. The assays used for measurement of testosterone in MrOS and EMAS have not been cross-calibrated by exchange of samples. In all cohorts, free testosterone was calculated using a published law-of-mass-action equation that uses an association constant estimated from a systematic review of published binding studies and an iterative numerical method (38). The intra- and interassay CV in the low, medium, and high pools were 4.3, 5.5, and 4.9% and 2.4, 8.1, and 2.5%, respectively.

SHBG levels were measured using a two-site immunofluorometric assay (DELFIA-Wallac, Inc., Turku, Finland) (19, 39). The interassay CV were 8.3, 7.9, and 10.9%, and intraassay CV were 7.3, 7.1, and 8.7%, respectively, in the low, medium, and high pools. The analytical sensitivity of the assays was 0.5 nmol/liter.

Statistical methods

By convention, the 2.5th percentile of the FHS reference sample defines the lower limit of the reference range (40–42); total or free testosterone concentrations below the 2.5th percentile value (total testosterone <348.3 ng/dl; free testosterone <70.0 pg/ml) were deemed low.

We determined the relationship of total and free testosterone with outcomes in three community-based samples. For these cross-sectional analyses, we related total and free testosterone levels (separate models for each) to prevalence of physical dysfunction, sexual symptoms, and diabetes (fasting glucose ≥126 mg/dl or on treatment) using multivariable logistic regression models adjusting for age and smoking. Furthermore, In the EMAS and MrOS, which were multicenter studies, the analyses were also adjusted for the study site. We did not adjust for comorbid conditions because some of the comorbid conditions (*e.g.* diabetes) were the dependent variables in these analyses. Testosterone was modeled as a binary variable (low *vs.* normal).

In exploratory analyses, we evaluated the Z-score approach, in which hormone levels were regressed on age and standardized residuals were used for continuous analysis. Low testosterone levels were established by ranking the residuals and taking the lowest 10% (or 5%) as the threshold. The T-score and Z-score approaches yielded directionally concordant results (Supplemental Table 2). Also, analyses of spline plots of testosterone levels against outcomes did not yield clear thresholds at which the relationship of testosterone and outcomes changed abruptly.

All analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC), and statistical significance was based on type I error probability of 0.05. The statistical analyses in the EMAS data were conducted using Intercooled STATA version 9.2 (StataCorp, College Station, TX).

Results

Subject characteristics

The STROBE (strengthening the reporting of observational studies in epidemiology) diagram (Fig. 1) illustrates the selection of reference sample of healthy men, 40 yr of age or younger, who were free of cancer, CVD, DM, obesity, hypertension, hypercholesterolemia, and smoking. The baseline characteristics of the samples are summarized in Tables 1 and 2. The men in the FHS broad sample were on average younger and had lower prevalence of CVD, diabetes, and cancer than those in the EMAS and MrOS samples (Table 2).

Distribution of testosterone levels in the reference sample

Table 3 describes the distribution of total and free testosterone levels in the reference sample. The mean and median total testosterone concentrations were 723.8 and 698.7 ng/dl, respectively. For free testosterone, the corresponding mean and median values were 141.8 and 134.0 pg/ml, respectively. Consistent with the approach used for defining reference limits for many other analytes (41, 42), total and free testosterone values below the 2.5th percentile (less than approximately 2 SD below the mean) were deemed low. The 2.5th percentile value for total testosterone was 348.3 ng/dl (12.1 nmol/liter), and for free tes-

TABLE 1. Characteristics of the FHS Gen 3 reference sample

	All subjects (n = 1893)	All subjects ≤40 yr (n = 962)	Reference sample ≤40 yr[a] (n = 456)
Age (yr)	40.3 (8.8)	33.3 (5.5)	32.7 (5.7)
<30	224 (11.8%)	224 (23%)	125 (27.4%)
30–39	650 (34.3%)	650 (67.6%)	297 (65.1%)
40–49	726 (38.4%)	88[b] (9.2%)	34[b] (7.5%)
50–59	274 (14.5%)	NA	NA
≥60	19 (1%)	NA	NA
Caucasian or White	1880 (99.3%)	956 (99.4%)	454 (99.6%)
Family income			
<$12,000	33 (1.8%)	17 (1.9%)	11 (2.6%)
$12,000–$24,000	60 (3.3%)	39 (4.3%)	18 (4.3%)
$25,000–$49,999	323 (18%)	192 (21.3%)	73 (17.3%)
$50,000–$74,999	448 (24.9%)	220 (24.4%)	105 (24.8%)
$75,000–$100,000	369 (20.5%)	184 (20.4%)	94 (22.2%)
>$100,000	566 (31.5%)	249 (27.6%)	122 (28.8%)
Systolic BP (mm Hg)	120.8 (12.6)	118.5 (11)	115.5 (8.9)
Diastolic BP (mm Hg)	78.3 (9.3)	76.9 (9.4)	74.1 (7.6)
Hypertension treatment	194 (10.3%)	40 (4.2%)	NA
Total cholesterol (mg/dl)	193 (37.2)	188.5 (39.5)	178.5 (30.2)
LDL cholesterol (mg/dl)	119.8 (31.6)	116.9 (31.9)	110.6 (27.4)
HDL cholesterol (mg/dl)	46.8 (12.4)	46.4 (12.0)	47.6 (11.8)
Triglycerides (mg/dl)	136.1 (109.7)	128 (102.4)	103.8 (72.3)
Cholesterol treatment	208 (11%)	45 (4.7%)	NA
Glucose (mg/dl)	98.6 (17.8)	95.6 (14.1)	93.0 (6.8)
Diabetes treatment	42 (2.2%)	8 (1%)	NA
Body mass index (kg/m^2)	28.0 (4.7)	27.4 (4.6)	25.5 (2.7)
Cancer	27 (1.4%)	14 (1.5%)	NA
Prevalent CVD	45 (2.4%)	7 (1%)	NA
Diabetes	48 (2.5%)	8 (1%)	NA
Obesity	493 (26%)	216 (22.5%)	NA
Hypertension	416 (22%)	127 (13.2%)	NA
Hypercholesterolemia	370 (19.6%)	119 (12.4%)	NA
Smoker	347 (18.3%)	183 (19%)	NA

Values are means (SD) or n (%); BP, Blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not applicable.

[a] Healthy samples from subjects free of cancers, CVD, diabetes, obesity, hypertension, and hypercholesterolemia and who were nonsmokers.

[b] These men are exactly 40 yr of age.

tosterone 70.0 pg/ml (243 pmol/liter) in the reference sample.

Distribution and categorization of testosterone levels in FHS broad sample and the EMAS and MrOS samples

The distribution of total and free testosterone levels by decades of age was similar in the three cohorts and revealed the expected age-related decline (Fig. 2 and Supplemental Tables 4 and 5). Because of the higher average age of the MrOS participants than that of the other two cohorts, the prevalence of low total and free testosterone was higher in MrOS than in the other two cohorts; 10.4% of men in the FHS broad sample, 23.5% of men in the EMAS, and 40.3% of men in the MrOS had low total testosterone. The prevalence of low free testosterone was 18.1, 24.0, and 61.4%, respectively, in the FHS, EMAS, and MrOS cohorts. In the FHS broad sample, serum total and free testosterone were associated inversely with age, body mass index, and comorbidity and positively with smoking (Supplemental Table 3); similar associations have been reported previously in the EMAS and MrOS.

Relationship of low testosterone levels with outcomes in the three cohorts

Sexual symptoms, available in the EMAS, were analyzed using multivariable logistic regression models adjusted for age, smoking, and site. Compared with men with normal testosterone levels, men with low total testosterone were more likely to report decreased morning erections (Fig. 3), and the men with low free testosterone were more likely to report decreased morning erections, erectile dysfunction, and decreased frequency of sexual thoughts.

In general, men with low total or free testosterone were more likely to have low walking speed, frailty, or physical symptoms than those with normal levels (Fig. 3). Thus, EMAS participants with low total or free testosterone were more likely to report difficulty climbing stairs or have low walking speed (in the lowest 20th percentile). In

TABLE 2. Characteristics of the participants in the three cohorts

	Broad FHS sample (Gen 2 plus Gen 3) (n = 3352)	EMAS (n = 3219)	MrOS (n = 1488)
Age (yr)	49.4 (13.8)	59.7 (11.0)	73.7 (5.8)
<30	224 (6.7%)	0 (0%)	0 (0%)
30–39	660 (19.7%)	0 (0%)	0 (0%)
40–49	872 (26.0%)	782 (24.3%)	0 (0%)
50–59	788 (23.5%)	873 (27.1%)	0 (%)
60–69	493 (14.7%)	799 (24.9%)	447 (30.0%)
70–79	289 (8.6%)	761 (23.7%)	782 (52.6%)
≥80	26 (0.78%)		259 (17.4%)
Systolic BP (mm Hg)	124.0 (15.4)	146.1 (20.9)	138.9 (18.8)
Diastolic BP (mm Hg)	77.3 (9.6)	87.3 (12.4)	NA
Hypertension treatment	736 (22.0%)		579 (38.9%)
Total cholesterol (mg/dl)	192.7 (36.4)	214.5 (48.5)	192.7 (33.2)
LDL cholesterol (mg/dl)	119.6 (31.5)	133.7 (44.1)	113.7 (29.9)
HDL cholesterol (mg/dl)	46.2 (12.7)	54.4 (14.4)	49.2 (14.6)
Triglycerides (mg/dl)	139.3 (106.1)	139.2 (103.7)	148.8 (91.9)
Cholesterol treatment	562 (16.8%)		NA
Glucose (mg/dl)	102.9 (23.2)	101.7 (25.1)	105.9 (26.9)
Diabetes treatment	166 (5.0%)		143 (9.6%)
Body mass index (kg/m^2)	28.3 (4.7)	27.7 (4.1)	27.4 (3.7)
Cancer	169 (5.0%)	170 (5.3)	424 (28.5%)
Prevalent CVD	303 (9.0%)	1137 (35.4)	434 (29.2%)
Diabetes	274 (8.2%)	236 (7.5)	149 (10.0%)
Obesity	964 (28.8%)	773 (24.5)	304 (20.4%)
Hypertension	1130 (33.7%)	895 (28.3%)	NA
Hypercholesterolemia	850 (25.4%)	786 (24.60%)	109 (7.3%)
Smoker	535 (16.0%)	681 (21.4)	57 (3.8%)

BP, Blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not applicable.

MrOS, men with low total or free testosterone were more likely to have slow walking speed than those with normal testosterone; men with low free testosterone were also more likely to have frailty. As reported previously (20), the FHS participants with low free testosterone were at higher risk of self-reported mobility limitation.

In all three cohorts, the men with low total and free testosterone levels were nearly twice as likely to have DM as those with normal levels (Fig. 3). Similarly, in all three cohorts, men with low total and free testosterone were more likely to have at least one of the following: sexual symptoms (EMAS only), a marker of physical dysfunction, or diabetes (Fig. 3). Sensitivity analyses (not shown) considering the 1st and 5th percentiles, as opposed to the 2.5th, as the threshold value for low testosterone, yielded qualitatively concordant results.

TABLE 3. Distribution of total and free testosterone in the FHS reference sample (n = 456)

	Total testosterone (ng/dl)	Free testosterone (pg/ml)
Mean	723.8	141.8
SD	221.1	45.0
Median	698.7	134.0
Quartile range (Q3–Q1)	296.5	60.0
Percentile		
99th	1322.0	266.0
97.5th	1196.6	230.0
95th	1124.0	222.0
5th	405.9	77.0
2.5th	348.3	70.0
1st	282.0	55.0

To convert total testosterone from nanograms per deciliter to nanomoles per liter, multiply concentrations in nanograms per deciliter by 0.0347. To convert free testosterone from picograms per milliliter to picomoles per liter, multiply concentrations in picograms per milliliter by 3.47.

Discussion

We generated reference limits for total and free testosterone levels in a community-based sample of healthy young men using LC-MS/MS, an accurate method with high precision and accuracy. We demonstrated that values below the proposed lower reference limits were associated with increased risk of conditions that have been associated previously with androgen deficiency (19–27) in three geographically distinct populations. Thus, men deemed to have low total or free testosterone levels had increased prevalence of sexual symptoms (15), physical dysfunction (18–21), and DM (22–27) in one or more cohorts.

Epidemiological studies such as these do not permit inferences about the causal role of testosterone in the three

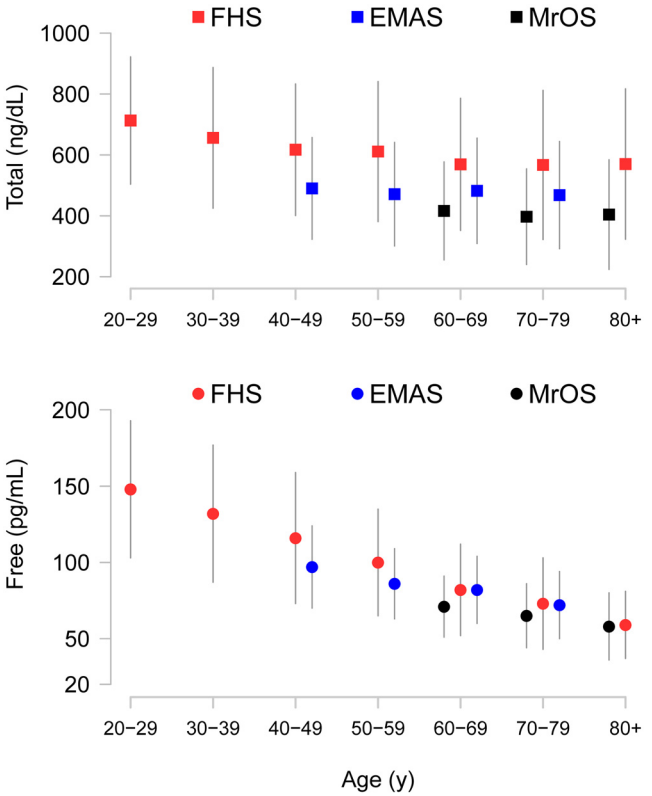

FIG. 2. Distribution of total and free testosterone levels by decades of age in the FHS broad sample as well as the EMAS and MrOS validation samples. Means and SD *bars* are shown. To convert total testosterone from nanograms per deciliter to nanomoles per liter, multiply concentrations in nanograms per deciliter by 0.0347. To convert free testosterone from picograms per milliliter to picomoles per liter, multiply concentrations in picograms per milliliter by 3.47.

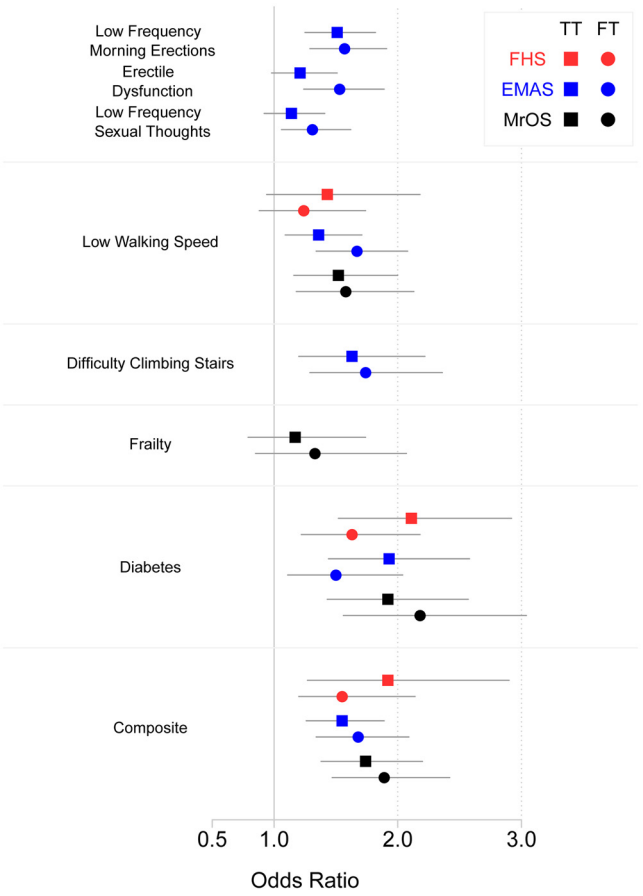

FIG. 3. Association of low total or free testosterone with sexual symptoms, physical dysfunction, DM, or any one of these conditions in the FHS, EMAS, and MrOS cohorts. The odds ratios along with the 95% confidence intervals for the association of total and free testosterone with various outcomes in the three validation cohorts are shown. The composite outcome indicates the following: in FHS, one or more of slow walking speed (walking speed in the lowest 20th percentile), self-reported mobility limitation, or diabetes; in EMAS, one or more of low frequency of morning erections, erectile dysfunction, low frequency of sexual thoughts, difficulty in climbing several stairs, limited in walking more than 1 km, slow walking speed (walking speed in the lowest 20th percentile), or diabetes; in MrOS, one or more of frailty, slow walking speed (walking speed in the lowest 20th percentile), or diabetes.

categories of conditions studied in this investigation; reverse causality is possible and cannot be excluded. These conditions should not necessarily be viewed as representative symptoms or conditions resulting from an androgen-deficient state.

The Endocrine Society defined androgen deficiency in men as a syndrome characterized by symptoms and signs and low testosterone levels (1). The occurrence of low testosterone level alone does not constitute androgen deficiency. The prevalence of low total or free testosterone in the three cohorts should not be viewed as indicative of a high prevalence of androgen deficiency in these cohorts or in the general population. Previous analyses of the EMAS (15) and Massachusetts Male Aging Study data (44) have shown that the prevalence of symptomatic androgen deficiency is substantially lower (2–5%) than the prevalence of low testosterone levels. In comparison with FHS and EMAS cohorts, MrOS participants were older and had a higher prevalence of comorbid conditions such as cancer and diabetes and also of low total and free testosterone levels.

This study has several strengths. The FHS reference cohort has many characteristics of an optimum sample described by the International Federation of Clinical Chemistry (40–42). This was a community-based sample of healthy men in sufficiently large numbers (40–42). Unlike some other epidemiological studies, which included only middle-aged and older individuals (10), the FHS included both young and older individuals. The FHS samples were drawn in the morning after overnight fast, as recommended by the Endocrine Society guidelines (1), and stored at −80 C and never thawed. The data have internal consistency, as indicated by the expected inverse association of testosterone with age, body mass index, and comorbid conditions and a positive association with smoking. We used LC-MS/MS, the method with high specificity and accuracy. The consistency of the associations of low testosterone with the prevalence of

sexual, physical and metabolic conditions across three geographically distinct samples is noteworthy.

This study also has some limitations. These reference ranges were derived from single morning samples, which discount the pulsatile, diurnal, and circannual rhythms. Symptomatic androgen deficiency designation may not be persistent over time (45). Our analyses show that early morning testosterone levels, obtained in a manner similar to that used by physicians in practice, are associated cross-sectionally with symptoms and clinical outcomes. The mass spectrometry methods used for measuring testosterone concentrations differed across the three cohorts, and the assays from EMAS and MrOS have not been cross-calibrated. The assays were performed in samples stored at −80 C; the stability of SHBG in stored samples cannot be assumed. We determined reference ranges in men 40 yr of age or younger. This age cutoff is admittedly arbitrary because there is no evidence of an inflection point in the trend line at this age. Our approach of generating the reference range in healthy young men is similar to the use of T-scores for bone mineral density. Although for some analytes, it may be appropriate to generate age-adjusted reference ranges (40–42), for others that exhibit substantial age-related change, it may be more appropriate to derive the reference ranges in a healthy, young population. However, it is difficult to determine with certainty at this time whether age-adjusted reference ranges may be needed. Given the white ethnicity of the reference sample, investigations of multiethnic cohorts to evaluate the generalizability of the proposed reference limits is important. Some studies have reported significant geographic and racial differences in sex-steroid levels (46), whereas others have not (47, 48). We calculated free testosterone concentrations using a previously published equation (38); calculated free testosterone concentrations may differ from those measured by the equilibrium dialysis method (49–51). Furthermore, different equations may yield different results depending upon the dissociation constants and the assumptions embedded in the equation. Finally, we cannot exclude the possibility that some men with putative androgen deficiency may have been included in the reference sample.

The lower limit of total testosterone levels in the FHS reference sample is slightly higher than the threshold reported historically (~300 ng/dl, 10.4 nmol/liter) but closer to the thresholds associated with sexual and physical symptoms in a recent investigation of older men (15). The thresholds for various sexual and metabolic outcomes in men supplemented with graded doses of testosterone after pharmacological suppression of endogenous testosterone production or in men with androgen deficiency receiving replacement doses of testosterone generally have been in the 250- to 400-ng/dl range (39, 52, 53). In contrast to our study, which generated the reference range in healthy men, 19–40 yr of age, using LC-MS/MS, previous epidemiological studies included middle-aged and older men and used immunoassays. We excluded men with comorbid conditions from the reference sample.

Despite these attempts to remove influences of comorbid conditions and other factors, however, there remain many sources of variation that cannot be controlled. Differences in study populations, subject selection, time of sample collection, and testosterone assays may contribute to the differences in normative ranges observed here and in other studies. These reference ranges, generated in a reference sample of healthy, lean young men of the FHS, cannot be applied to other assays in other laboratories without appropriate cross-calibration of assays. Historical experience with cholesterol and hemoglobin A1C assays indicates that the application of reference ranges across laboratories is a challenging process that requires mechanisms for standardizing assays (43, 54). The CDC testosterone standardization effort addresses this challenge and will facilitate the application of these reference ranges across laboratories.

It is likely that the results exhibited here may apply to other thresholds proposed for the lower limit to normative ranges. The proposed reference ranges represent the essential first step in defining androgen deficiency syndrome in men. The data here define only a potential reference interval from a general population; how well these discriminating thresholds can be applied to clinical diagnosis of androgen deficiency syndrome needs further validation using receiver operating characteristic curves in clinical populations. The association of low testosterone defined using these criteria with incident outcomes should be evaluated longitudinally to exclude reverse causality. Ultimately, placebo-controlled, randomized trials would be necessary to determine whether testosterone therapy improves outcomes in men deemed androgen deficient by the presence of testosterone levels below the thresholds reported here and symptoms and signs.

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