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Low Serum Testosterone and High Serum Estradiol Associate With Lower Extremity Peripheral Arterial Disease in Elderly Men

The MrOS Study in Sweden

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Objectives This study sought to determine whether serum levels of testosterone and estradiol associate with lower extremity peripheral arterial disease (PAD) in a large population-based cohort of elderly men.

Background Few studies have explored the relationship between serum sex steroids and lower extremity PAD in men.

Methods The Swedish arm of the MrOS (Osteoporotic Fractures in Men) study (n = 3,014; average age 75.4 years) assessed ankle-brachial index (ABI) and defined lower extremity PAD as ABI <0.90. Radioimmunoassay measured serum levels of total testosterone, estradiol, and sex hormone-binding globulin, and we calculated free testosterone and free estradiol levels from the mass action equations.

Results A linear regression model including age, current smoking, previous smoking, diabetes, hypertension, body mass index, free testosterone, and free estradiol showed that free testosterone independently and positively associates with ABI ($p < 0.001$), whereas free estradiol independently and negatively associates with ABI ($p < 0.001$). Logistic regression analyses showed that free testosterone in the lowest quartile (vs. quartiles 2 to 4; odds ratio [OR] 1.65, 95% confidence interval [CI] 1.22 to 2.23, $p = 0.001$) and free estradiol in the highest quartile (vs. quartiles 1 to 3; OR 1.45, 95% CI 1.09 to 1.94, $p = 0.012$) independently associate with lower extremity PAD.

Conclusions This cross-sectional study shows for the first time that low serum testosterone and high serum estradiol levels associate with lower extremity PAD in elderly men. Future prospective and interventional studies are needed to establish possible causal relationships between sex steroids and the development of lower extremity PAD in men. (J Am Coll Cardiol 2007;50:1070–6) © 2007 by the American College of Cardiology Foundation

Atherosclerosis is the major cause of lower extremity peripheral arterial disease (PAD), a disease increasingly recognized as a health burden worldwide (1). Concordant with the systemic nature of atherosclerotic disease, lower extremity PAD and cardiovascular disease frequently occur together, and the risk factors for lower extremity PAD essentially parallel those of coronary artery atherosclerosis (1,2). However, some differences exist (e.g., smoking is an exceptionally powerful risk factor for lower extremity PAD, and hypertension is generally a weaker risk factor for lower extremity PAD than for coronary artery disease) (1,2).

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Several lines of evidence support a role for sex hormones in atherosclerotic disease in men. For example, several studies show an independent negative association between serum testosterone and male carotid artery atherosclerosis as well as cardiovascular disease (3–6). In addition, we recently determined that circulating estradiol independently and positively predicts progression of carotid artery intima-media thickness in middle-aged men (7). Moreover, although no current evidence suggests that testosterone treat-

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ment affects the risk of cardiovascular disease (8), high-dose estrogen treatment of men with prostate cancer associates with increased cardiovascular morbidity and mortality (9).

Few large population-based association studies have examined the relationship between endogenous sex steroids and lower extremity PAD. Indeed, only 1 previous observational study, a small nested case-control study (40 male cases and 41 control subjects) that determined no difference in serum testosterone or estradiol levels between cases and control subjects (10), investigated the influence of sex hormones on PAD in men.

We hypothesized that serum testosterone negatively and serum estradiol positively associates with lower extremity PAD in men. The current study investigated a possible link between serum testosterone and estradiol levels and lower extremity PAD in a large population-based cohort of elderly men.

Abbreviations and Acronyms

ABI = ankle-brachial index
BMI = body mass index
CI = confidence interval
CV = coefficient of variation
IRMA = immunoradiometric assay
OR = odds ratio
PAD = peripheral arterial disease
RIA = radioimmunoassay
SHBG = sex hormone-binding globulin

Methods

Study population. The MrOS (Osteoporotic Fractures in Men) study is a multicenter study including elderly men in Sweden (n = 3,014), Hong Kong (n = 1,999), and the U.S. (n = 5,995). The design of MrOS has been previously described (11). In the Swedish arm of MrOS we investigated possible links between sex steroid levels and ankle-brachial index (ABI). With national population registers, we randomly identified and enrolled men 69 to 80 years of age in 3 Swedish cities: Malmö, Uppsala, and Göteborg (n ≃ 1,000 in each city). Eligibility for study participation required the ability to walk unassisted; subjects with bilateral hip prosthesis were excluded. There were no other exclusion criteria. The MrOS study in Sweden was approved by the ethics committees at Göteborg, Lund, and Uppsala Universities. Informed consent was obtained from all study participants.

Both ABI and serum levels of all sex steroids (testosterone, estradiol, and sex hormone-binding globulin [SHBG]) were available for 2,812 subjects. After excluding 28 subjects with ABI >1.40 (see comment in following text), the current analysis encompassed 2,784 subjects.

Assessment of covariates. A standardized questionnaire (11) collected information about present and previous smoking habits. Diabetes was defined as subjects' report of diabetes diagnosis by their doctors. Of subjects with self-reported diagnosis of diabetes, 78% reported hypoglycemic medication. Hypertension was defined as self-reported hypertension diagnosis with either self-reported antihypertensive treatment or systolic blood pressure ≥140 mm Hg. Of subjects with self-reported diagnosis of hypertension, 90% reported antihypertensive medication. We used standard equipment to measure height and weight and calculated body mass index (BMI) according to the formula BMI (kg/m^2) = weight (kg)/height $(m)^2$.

Measurement of ABI. The ABI is a quick and noninvasive measurement that provides objective information on the presence and severity of lower extremity PAD. With a standard mercury sphygmomanometer and a Doppler probe, we conducted duplicate measures of supine blood pressure in the right arm and both ankles after subjects rested in a quiet room for at least 10 min. The study coordinator placed appropriately sized cuffs over the right upper arm and around each ankle, proximal to the malleolus, rapidly inflated them to 30 mm Hg above the audible systolic pressure, and then slowly deflated them over each artery. A hand-held Doppler (Huntleigh Mini Dopplex Model D900, Huntleigh Healthcare AB, Limhamn, Sweden) recorded the pressure in each artery as the first audible systolic pressure. After performing 2 measurements at each site, we used the mean value of systolic pressure taken first in the right brachial artery and then in the right and left posterior tibial artery. We calculated ABI for each leg by dividing the posterior tibial systolic pressure by the upper extremity pressure and used the lowest ABI to determine the extent of lower extremity PAD.

A low ABI indicates the presence of arterial disease in the lower extremities. In accordance with the current standard, we defined lower extremity PAD as ABI <0.90 (1,2,12), a level 95% sensitive and 99% specific for angiographically diagnosed lower extremity PAD (12). Because ABI >1.40 might represent falsely high values due to incompressible arteries (12), we excluded 28 potential subjects with ABI >1.40 from our study.

An earlier study of 69-year-old men showed a linear association between ABI and intima-media thickness as well as the occurrence of atherosclerotic plaques in the common femoral artery (13). Therefore, we considered ABI as both a continuous and dichotomous variable (ABI less than or exceeding 0.90).

Assessment of sex hormones. Ultrasensitive radioimmunoassay (RIA) (Orion Diagnostics, Esboo, Finland, limit of detection 5 pmol/l [140 pg/dl], intra-assay coefficient of variation [CV] 3%, interassay CV 6%) measured total estradiol, and an RIA (Orion Diagnostics, limit of detection 0.1 nmol/l [3 ng/dl], intra-assay CV 6%, interassay CV 6%) measured total testosterone. We used immunoradiometric assay (IRMA) (Orion Diagnostics, limit of detection 1.3 nmol/l, intra-assay CV 3%, interassay CV 7%) to measure SHBG. Two subjects had undetectable estradiol levels and 1 had undetectable testosterone levels. Taking the concentrations of total testosterone, total estradiol, and SHBG into account and assuming a fixed albumin concentration of 43 g/l, we calculated free testosterone and free estradiol accord-

ing to the method described by Vermeulen et al. (14) and Van den Beld et al. (15). We analyzed all samples in duplicate in 1 laboratory and averaged the duplicates for further analyses.

Statistical analysis. Spearman rank correlation assessed univariate associations among variables. With linear regression equations, we adjusted the hormone–ABI relationships (Fig. 1) for selected covariates (the free estradiol–ABI relationship was adjusted for free testosterone, and the free testosterone–ABI relationship was adjusted for free estradiol). Analysis of variance followed by Tukey's post hoc test examined differences in crude and adjusted ABI between quartiles of sex hormone levels. We performed trend analyses through linear regression analysis. Linear regression analysis with sex hormone levels divided into quartiles determined the independent predictors of ABI. Logistic regression examined predictive values of low testosterone (lowest quartile vs. quartiles 2 to 4) and high estradiol (highest quartile vs. quartiles 1 to 3) for PAD (defined as ABI <0.9) (1,2,12) with and without adjustment for vascular risk factors. Variables not normally distributed (i.e., BMI and sex hormone levels) were log transformed undergoing regression analyses. We performed statistical analyses with SPSS for Windows (version 13.0, SPSS, Chicago, Illinois).

Results

Characteristics of the study population. Table 1 shows the characteristics of the study population, including serum sex hormone levels. Among the 2,784 subjects, 213 (7.7%) had a self-reported history of prostate cancer. Exclusion of these subjects did not alter the results or conclusions of this study.

Serum testosterone associates positively and serum estradiol associates negatively with ABI. In univariate association analyses, total and free testosterone correlated positively with ABI, and total and free estradiol correlated negatively (Table 2). To further explore the relation between sex hormones and ABI, we plotted mean ABI against quartiles of free testosterone and free estradiol (Figs. 1A and 1B). Subjects within the lowest quartile of free testosterone had a lower mean ABI compared with subjects within quartiles 2 to 4; subjects within the highest quartile of free estradiol levels had a lower mean ABI compared with quartiles 1 to 2. There was no linear association between SHBG and ABI (Table 2), and there was no evidence of a threshold effect in the relation between SHBG and ABI with SHBG quartiles (data not shown).

As previously described (16), serum levels of free testosterone and free estradiol clearly correlated positively (Pearson's $r = 0.60$, $p < 0.001$). Therefore, we plotted mean ABI

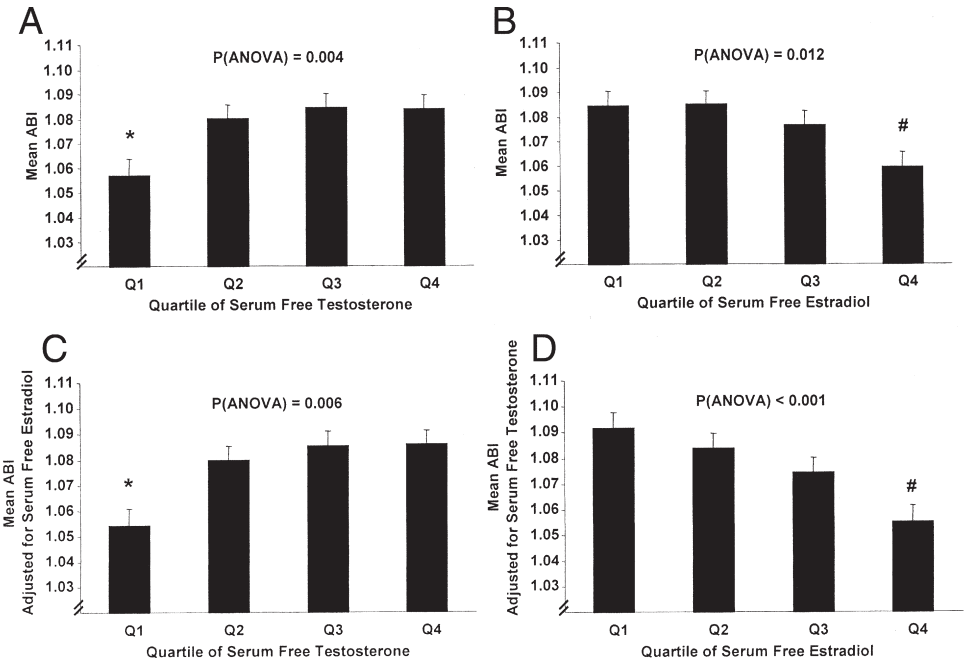

Figure 1 Mean ABI According to Quartile of Serum Free Testosterone and Free Estradiol Levels

Mean ankle-brachial index (ABI) (±SE) according to quartile (Q) of serum free testosterone **(A)** and free estradiol **(B)** levels. Limits in free testosterone for different quartiles were as follows: Q1 0.003 to 0.23, Q2 0.23 to 0.30, Q3 0.30 to 0.39, and Q4 0.39 to 1.43 nmol/l. Limits for free estradiol were Q1 0.05 to 1.20, Q2 1.20 to 1.60, Q3 1.60 to 2.12, and Q4 2.12 to 9.04 pmol/l. **(C)** Mean ABI (±SE) adjusted for free estradiol according to quartile of serum free testosterone levels. **(D)** Mean ABI (±SE) adjusted for free testosterone according to quartile of serum free estradiol levels. Statistical analyses were performed by analysis of variance followed by Tukey's post hoc test. *$p < 0.05$ versus Q2, Q3, and Q4; #$p < 0.05$ versus Q1 and Q2.

Table 1 Characteristics of the Study Population

Factor	Prevalence or Mean ± SD
Age (yrs)	75.4 ± 3.2
Smoking (%)	
Never	35.2
Previous	56.2
Current	8.6
Body mass index (kg/m^2)	26.4 ± 3.5
Hypertension (%)	35.4
Diabetes (%)	9.3
ABI	1.08 ± 0.17
PAD (ABI <0.9), %	10.9
Estradiol (pmol/l)	98.4 ± 40.8
Free estradiol (pmol/l)	1.72 ± 0.78
Testosterone (nmol/l)	16.9 ± 7.0
Free testosterone (nmol/l)	0.31 ± 0.14
SHBG (nmol/l)	43.2 ± 21.9

n = 2,784.
ABI = ankle-brachial index; PAD = lower extremity peripheral arterial disease; SHBG = sex hormone-binding globulin.

adjusted for free estradiol against quartiles of free testosterone (Fig. 1C) and mean ABI adjusted for free testosterone against quartiles of free estradiol (Fig. 1D). Interestingly, after adjusting ABI for free testosterone levels, the negative relation between quartiles of free estradiol and ABI appeared more linear. Indeed, p values for linear trend across quartiles 1 to 3 of free estradiol levels (not including quartile 4 in the analyses) were 0.37 before and 0.049 after adjusting ABI for free testosterone levels. In contrast, adjusting ABI for free estradiol did not influence substantially the association between ABI and quartile of free testosterone.

Because ABIs 1.30 to 1.40 might also reflect stiff arteries (1), the univariate relation between sex steroid levels and ABI was explored without this group (n = 102), yielding similar results as if included (data not shown). Furthermore, sex steroid levels did not differ in the 1.30 to 1.40 group as compared with a reference group within the MrOS Sweden population (n = 1,327) with normal ABIs of 1.10 to 1.29 (12) (data not shown).

Serum free testosterone independently and positively associates with ABI, whereas free estradiol independently and negatively associates with ABI. With linear regression models including Swedish MrOS site, age, current smoking, previous smoking, diabetes, hypertension, BMI, and quartile of free testosterone and free estradiol, we examined the likelihood that sex hormone levels and ABI associate independently. Free testosterone independently and positively associates with ABI, and free estradiol independently and negatively associates with ABI (Table 3). As expected, age, smoking, diabetes, and hypertension negatively associate with ABI (Table 3).

Table 2 Univariate Associations Between Serum Sex Hormone Levels and ABI

	r_s	p Value
Estradiol	−0.070	<0.001
Free estradiol	−0.061	0.001
Testosterone	0.051	0.008
Free testosterone	0.050	0.008
SHBG	0.013	0.51

ABI = ankle-brachial index; r_s = Spearman rank correlation coefficient; SHBG = sex hormone-binding globulin.

Table 3 Serum Free Testosterone Positively and Independently Associates With ABI, and Estradiol Negatively and Independently Associates With ABI

	Standardized Beta Coefficient	p Value
Age	−0.138	<0.001
Current smoking	−0.213	<0.001
Previous smoking	−0.125	<0.001
Diabetes	−0.055	0.004
Hypertension	−0.125	<0.001
Body mass index	0.008	0.68
Free testosterone	0.072	<0.001
Free estradiol	−0.080	<0.001

Multiple linear regression analysis with ankle-brachial index (ABI) as the dependent variable and age, MrOS (Swedish Osteoporotic Fractures in Men) study site, current smoking, previous smoking, diabetes, hypertension, body mass index, and quartiles of free testosterone and free estradiol as independent variables.
ABI = ankle-brachial index.

Low serum free testosterone and high free estradiol levels independently associate with lower extremity PAD. We next evaluated the independent predictive values of low free testosterone and high free estradiol for lower extremity PAD, defined as ABI <0.90 (1,2,12). Logistic regression analyses showed that serum levels of free testosterone in the lowest quartile as well as serum levels of free estradiol in the highest quartile associate with lower extremity PAD (Table 4, Models 1 and 2). Further adjustment for smoking, diabetes, hypertension, and BMI demonstrated that low serum free testosterone and high serum free estradiol independently associate with lower extremity PAD (Table 4, Model 3). The corresponding odds ratios (ORs) of low/high total hormone levels for lower extremity PAD were included for reference. Although low free and total testosterone yielded similar ORs for lower extremity PAD, the relation for high total was weaker than for high free estradiol.

Combining serum levels of free testosterone in the lowest quartile with free estradiol in the highest quartile (n = 69) strongly associates with lower extremity PAD (model with adjustment for age and Swedish MrOS site: OR 3.72, 95% confidence interval [CI] 2.15 to 6.41, p < 0.001; model with adjustment for age, Swedish MrOS site, smoking, diabetes, hypertension, and BMI: OR 2.95, 95% CI 1.66 to 5.24, p < 0.001).

Discussion

Accumulating data support a role for sex hormones in atherosclerotic disease in men. However, the relationship between serum sex steroids and lower extremity PAD

Table 4 Low Serum Free Testosterone as Well as High Free Estradiol Independently Associate With Lower Extremity PAD

	Model 1 (Adjusted for Age, MrOS Site)		Model 2 (Adjusted for Age, MrOS Site, the Other Hormone*)		Model 3 (Adjusted for Age, MrOS Site, the Other Hormone*, Body Mass Index, Current Smoking, Previous Smoking, Diabetes, Hypertension)	
	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
Free testosterone (Q1 vs. Q2–4)	1.53 (1.18–1.98)	0.001	1.78 (1.34–2.36)	<0.001	1.65 (1.22–2.23)	0.001
Total testosterone (Q1 vs. Q2–4)	1.75 (1.36–2.27)	<0.001	1.94 (1.49–2.54)	<0.001	1.70 (1.28–2.28)	<0.001
Free estradiol (Q4 vs. Q1–3)	1.48 (1.13–1.93)	0.004	1.61 (1.22–2.12)	<0.001	1.45 (1.09–1.94)	0.012
Total estradiol (Q4 vs. Q1–3)	1.38 (1.06–1.80)	0.017	1.48 (1.12–1.94)	0.005	1.33 (1.00–1.76)	0.052

Logistic regression analysis with lower extremity peripheral arterial disease (PAD) as the dependent variable. *The model of free testosterone was adjusted for free estradiol; the model of total testosterone was adjusted for total estradiol; the model of free estradiol was adjusted for free testosterone; the model of total estradiol was adjusted for total testosterone.
CI = confidence interval; OR = odds ratio; MrOS = Osteoporotic Fractures in Men study; Q = quartile.

requires further study. We show here that circulating free testosterone positively associates with ABI in a large population-based cohort of elderly men, indicating a negative association between testosterone and the degree of atherosclerotic disease in the lower extremities. In contrast, free estradiol negatively associates with ABI in the same population, indicating that higher estradiol levels are associated with more atherosclerosis. Furthermore, when lower extremity PAD was defined as an ABI <0.90, we found that low serum testosterone (in the lowest quartile) and high serum estradiol (in the highest quartile) associate with lower extremity PAD.

Few studies have examined endogenous estrogens and PAD in men (10), and the current study shows for the first time a positive association between serum estradiol levels and lower extremity PAD in men. Our data are in accordance with accumulating evidence suggesting that endogenous estrogens detrimentally affect atherosclerosis and cardiovascular disease in men. For example, we recently found that circulating estradiol independently predicts the progression of carotid intima-media thickness in middle-aged men (7). Furthermore, serum estradiol levels increased in subjects with coronary heart disease in a case-control study of men in the Framingham cohort (17). In addition, another study coupled the CC genotype of the estrogen receptor alpha c.454-397T>C polymorphism, possibly associated with enhanced receptor function (18), with increased incidence of myocardial infarction in men (19,20).

The notion that endogenous estradiol detrimentally influences atherosclerotic disease concurs with the association between high-dose estrogen treatment of men with prostate cancer and increased cardiovascular morbidity and mortality (9). These studies employed various estrogen substances including parenterally administered estradiol (21,22). Additionally, the Coronary Drug Project showed excessive deaths and recurrent infarction in men treated with conjugated equine estrogens after myocardial infarction (23). Currently, no clinical trial has investigated the effect of estrogenic hormones on PAD in men (24). The adverse cardiovascular effects of high-dose oral estrogen in men often have been ascribed to the prothrombotic effects of estrogen (9,23). Our finding of a positive association between serum estradiol levels and PAD supports the notion that estrogens, besides possibly increasing the risk for thrombosis and thereby cardiovascular events, also influence atherogenesis in men.

Likewise, there are data in women supporting adverse effects of estrogens on atherosclerotic disease. In accordance with the results of the Coronary Drug Project (23), treatment with conjugated equine estrogens increased the risk of myocardial infarction and stroke in postmenopausal women (25,26). Furthermore, serum estradiol associates positively with atherosclerosis in postmenopausal women (27).

Thus, the results of the present as well as previous studies challenge the paradigm that estradiol protects against atherosclerosis (28). The "estrogen protection hypothesis" is supported by the beneficial effects of estrogens on, for example, serum lipids, vascular function, and experimental atherosclerosis in both genders (8,28). Furthermore, a recent population-based study suggested that endogenous estradiol levels associate with lower risk for cardiovascular events in men (29). In postmenopausal women, treatment with estradiol slowed the progression of carotid intima-media thickness (30). Thus, opposing results suggesting protective as well as adverse effects of estradiol on atherosclerotic disease are found in both genders and might not only be explained by differences between endogenous and exogenous hormones. One might speculate that the effects of estradiol on atherosclerosis might differ depending on stage of the atherosclerotic process. In addition to species differences, this might partly explain why the atheroprotective effects of estradiol on atherosclerosis in male mouse models are not repeated in men. Clearly, the impact of estradiol on atherosclerotic disease in men (and women) requires further examination.

The present study reports for the first time a negative association between serum testosterone levels and lower extremity PAD in men. This result is in agreement with previous studies reporting a negative association between serum testosterone levels and carotid intima-media thickness (3 to 5) as well as cross-sectional studies showing a consistent inverse relationship between endogenous testosterone and male cardiovascular events (6). However, no studies have established a significant relationship between

circulating testosterone and incident cardiovascular events in men (6,8). In most animal studies, testosterone treatment inhibits atherosclerosis in males; and testosterone (high-dose) is a coronary vasodilator in men with established atherosclerosis (6). However, no current interventional study has sufficient power to assess a possible protective effect of testosterone on human atherosclerosis or cardiovascular disease (6,24).

Interestingly, although serum testosterone and estradiol associate positively, the relationship between testosterone and ABI contradicts the association between estradiol and ABI. In comparison, our previous data from the MrOS Sweden cohort demonstrate that both testosterone and estradiol associate positively with bone mineral density in elderly men (16). Although earlier studies suggested that testosterone confers protection against atherosclerosis through local or systemic aromatization to estradiol (6,8,31), this notion is not supported by the present study, demonstrating that free testosterone associated negatively and free estradiol associated positively with PAD. The more precise role of testosterone and estradiol as well as their precursors and derivatives in the human atherosclerotic process requires future research.

Although low free and total testosterone yielded similar ORs for lower extremity PAD, the relation was weaker for high total than for high free estradiol. This result is in accordance with previous results from this cohort on the association between total and free estradiol levels and bone mineral density (16). Thus, it might be important to calculate the bioactive estradiol concentration, which might reflect the clinical situation more accurately than the total hormone levels (15).

Limitations of the present study include its cross-sectional design, which did not allow determination of the temporal relationship between serum sex steroids and PAD. Thus, our results could reflect hormonal changes from the PAD disease process rather than vice versa. Furthermore, our results are limited to elderly Caucasian men. Because the radioimmunoassay technique might result in artifacts at the lowest estradiol levels, another possible limitation involves the use of this assay for estradiol measurements (32). Because analyses were not adjusted for lipid disorders or therapy for hyperlipidemia and confirmation of self-reported diagnoses with medical records was not performed, there is a possibility of residual confounding as a possible explanation for at least part of the observed associations. The strengths of the present study include its population-based nature and the large number of men investigated.

Conclusions

We show here that low serum testosterone and high serum estradiol associate with lower extremity PAD in elderly men. Future prospective and interventional studies are needed to establish possible causal relationships between sex steroids and the development of lower extremity PAD in men.

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