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Clinical Research Article

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Low Progesterone and Low Estradiol Levels Associate With Abdominal Aortic Aneurysms in Men

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Abbreviations: AAA, abdominal aortic aneurysm; BMI, body mass index; cFE2, calculated free estradiol; cFT, calculated free testosterone; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; DHEA, dehydroepiandrosterone; HDL, high-density lipoprotein; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLOD, lower limit of detection; LLOQ, lower limit of quantitation; SHBG, sex hormone binding globulin; SLE, supported liquid extraction.

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Abstract

Context: Male sex is a major risk factor for abdominal aortic aneurysms (AAA) but few studies have addressed associations between sex hormone levels and AAA.

Objective: We aimed to describe the associations between serum sex steroids and early, screening-detected AAA in men.

Methods: We validated a high-sensitivity liquid chromatography–tandem mass spectrometry assay for comprehensive serum sex hormone profiling. This assay was then employed in a case-control study including 147 men with AAA (infrarenal aorta ≥ 30 mm)

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and 251 AAA-free controls recruited at the general population-based ultrasound screening for AAA in 65-year-old Swedish men. Outcomes included associations between dehydroepiandrosterone, progesterone, 17α-hydroxyprogesterone, androstenedione, estrone, testosterone, dihydrotestosterone, and estradiol and AAA presence.

Results: Dehydroepiandrosterone, progesterone, 17α-hydroxyprogesterone, testosterone, and estradiol, but not the other hormones, were lower in men with AAA. In models with adjustments for known AAA risk factors and comorbidity, only progesterone (odds ratio per SD decrease 1.62 [95% CI, 1.18-2.22]) and estradiol (1.40 [95% CI, 1.04-1.87]) remained inversely associated with the presence of AAA. Progesterone and estradiol contributed with independent additive information for prediction of AAA presence; compared with men with high (above median) levels, men with low (below median) levels of both hormones had a 4-fold increased odds ratio for AAA (4.06 [95% CI, 2.25-7.31]).

Conclusion: Measured by a high-performance sex steroid assay, progesterone and estradiol are inversely associated with AAA in men, independent of known risk factors. Future studies should explore whether progesterone and estradiol, which are important reproductive hormones in women, are protective in human AAA.

Key Words: abdominal aortic aneurysm, men, screening, progesterone, estradiol

An abdominal aortic aneurysm (AAA) is a pathological dilatation of the infrarenal aorta that may result in rupture of the aortic wall and catastrophic bleeding leading to high mortality (1). General ultrasound screening of men for AAA has been initiated in a few countries and screening for AAA in men at 65 years of age is now nationwide in Sweden (2). Surgery remains the sole treatment option and increased understanding of the pathogenesis of AAA will be important for the development of nonsurgical interventions in earlier stages of the disease (3). Known risk factors for AAA include male sex, advancing age, smoking and family history of AAA, and AAA patients also have increased frequency of hypertension, hypercholesterolemia, obesity, and atherosclerotic cardiovascular disease (1). Male sex is one of the strongest risk factors for AAA; relative to women, men have a more than 5-fold increased risk of AAA (1).

Sex hormone–mediated actions are one of the main mechanisms underlying sex differences. Testosterone, which is the main bioactive sex steroid in men, is produced predominantly by the testes and can be further metabolized in peripheral target tissues to estradiol and dihydrotestosterone (Supplementary Figure 1) (4), which is a stronger agonist to the androgen receptor than its parent steroid (5, 6). The adrenal cortex secrets sex steroids such as dehydroepiandrosterone (DHEA), androstenedione, and progesterone, which can be metabolized in target tissues to testosterone, estrone, and estradiol (5) (Supplementary Figure 1) (4). In women, progesterone and estradiol are produced by the ovaries and are important reproductive hormones, while their role in men is less clear. Sex hormone–binding globulin (SHBG) binds sex steroids in the circulation with highest affinity for androgens, but understanding of the biological role of the small free (unbound) fractions of testosterone and estradiol remains incomplete (7).

While lower sex steroid levels generally associate with cardiovascular disease as well as increasing age, body mass index (BMI), and other cardiovascular risk factors in men, their role in cardiovascular disease pathogenesis remain incompletely understood (8, 9). In AAA, a couple of genetic studies support a causal role for sex hormones. A single nucleotide polymorphism in the aromatase gene (*CYP19A1*), converting testosterone to estradiol, has been associated with presence of small AAAs in men (10) and the estrogen receptor β has been associated with AAA susceptibility in men (11), supporting a role of estradiol although the functional consequences of the studied polymorphisms are unclear. Preclinical studies consistently replicate the increased male susceptibility of AAA (12-14) and support effects of both estrogens (mainly protective) and androgens (mainly adverse) in both sexes in various experimental AAA models (13-21). Only 3 studies have evaluated the associations between serum sex hormone levels and AAA in men; a population-based cohort study and a small case-control study have reported lower free testosterone levels in men with increasing aortic diameter and/or AAA, but no independent association with total testosterone levels (22, 23). Further, a recent study, in which sex hormones were measured by immunoassays, reported higher levels of estradiol and progesterone in men with AAA (24).

Technological advancement has contributed to increasing use of mass spectrometry–based analytical methods for sex steroid measurements, which allow comprehensive profiling of sex hormone levels (5). Further, this methodology provides high-quality measurements, in contrast to immunoassays that often have low sensitivity and may be confounded by cross-reactivity, for example, with inflammatory components (25-27). To date, there is no study reporting mass spectrometry–based serum sex steroid profiles in men with AAA disease.

This case-control study, based on ultrasound screening of Swedish 65-year-old men, explored whether serum sex steroids were associated with AAA at screening. First, for accurate and comprehensive serum sex steroid measurements, we developed and validated a high-sensitivity liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay for serum sex hormone profiling. Second, this assay was used to evaluate the independent associations between serum sex steroids and AAA presence.

Methods

Study Population

In the nationwide abdominal aortic aneurysm screening program in Sweden, all men aged 65 years consecutively identified through the National Population Register (which is updated every third month) are invited to an ultrasound examination of the infrarenal aorta, regardless of any known AAA (2). The screening visit examination consists of a single ultrasound scan where the maximum infrarenal anteroposterior diameter is measured according to the leading edge-to-leading-edge method, with the ultrasound transducer placed longitudinally to the aorta. An AAA is defined as an aortic diameter ≥ 30 mm (28).

In western Sweden (1.7 million inhabitants, approximately 1/6 of the Swedish population), subjects with an AAA are invited to participate in a case-control study called Gothia 3A. The inclusion criterion of the study is an AAA diagnosed during the population-based screening examination, and the exclusion criterion is the inability to understand written Swedish study information or other reasons for not comprehending the study information. The study complies with the Declaration of Helsinki and the study has been approved by the Regional Ethical Review Board in Gothenburg, Sweden. All subjects give written informed consent. Individuals with AAA receive a study invitation at their first outpatient clinic visit within 4 weeks of the initial screening examination. Paralleling recruitment of cases, AAA-free screened controls are recruited using a randomized consecutive enrollment. Control men receive a study invitation directly at the screening site after the absence of AAA is confirmed by ultrasound examination. A separate study visit that includes blood sampling is arranged for individuals with AAA and controls within 3 months of the first screening examination.

The study population included in the present analysis consists of Gothia 3A subjects recruited from the Gothenburg screening area, comprising about half of the total screening population in western Sweden, between January 2013 and November 2018. Participants were excluded from the present analysis for the following reasons: treatment with testosterone, 5α-reductase inhibitors, gonadotropin-releasing hormone agonists or antiandrogens, or a history of surgical castration. In the Gothenburg screening area, screening invitations were sent to 26 136 men during the study period (Supplementary Figure 2) (4). Of these, 21 361 men (82%) attended the screening visit, 20 096 after the first invitation and 1265 after the second invitation. Among the screened men, 240 had an AAA. The resulting AAA prevalence among screened men was 1.12% during the study period. Individuals with an AAA had an acceptance rate of 80% for participation in the Gothia 3A study. Among AAA-free controls, 53% of those invited accepted participation (Supplementary Figure 2) (4). Ten men with an AAA who accepted the study invitation were later excluded, as follow-up examination by ultrasound, with verification by computed tomography, showed that the diameter of the abdominal aorta was less than 30 mm. Men who were taking androgens or drugs suppressing testosterone production, metabolism, or action (8 AAA, 11 controls) or men with missing serum samples (26 AAA, 39 controls; mainly due to freezer breakdown) were further excluded from the sex hormone study. The final study cohort consisted of 147 men with an AAA and 251 controls (Supplementary Figure 2) (4).

Serum Sampling and Assessment of Covariates

Serum samples for lipids were collected in the morning after overnight fasting and serum samples for storage at –80 °C and subsequent sex steroid assays were collected at 10:00 to 12:00 hours. Serum total and high-density lipoprotein (HDL) cholesterol were determined by standardized enzymatic assays (Cobas 8000, Roche Diagnostica Scandinavia AB) at the accredited clinical chemistry laboratory at the Sahlgrenska University Hospital in Gothenburg. BMI was calculated as kg/m^2 (weight [kg]/height [m]^2). A supine systolic blood pressure was measured following a 10-minute period of rest. A questionnaire was used to gather information about smoking habits, general health, and medications. Pack-years was calculated as [(number of cigarettes per day/20) * years of smoking]. Diabetes was defined as a self-reported medical diagnosis of diabetes. Prevalent cardiovascular disease was defined as a

self-reported medical diagnosis of myocardial infarction, angina pectoris, or heart failure. Prevalent asthma/chronic obstructive pulmonary disease (COPD) was defined as self-reported asthma, chronic bronchitis, or COPD. Other systemic disease was defined as self-reported prevalent advanced malignancy, rheumatic, or other inflammatory disease.

Serum Sex Steroids by LC-MS/MS

Calibrator samples and internal standard solution

Calibrator stock solutions were prepared separately for each steroid by dissolving reference steroids in ethyl acetate. The stock solutions were then pooled to a calibrator standard solution and diluted in 50% methanol/water. Eight calibrator working solutions were made within the calibration range presented in Supplementary Table 1 (4). Estradiol, estrone, dihydrotestosterone, progesterone, androstenedione, DHEA, and 17α-hydroxyprogesterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Testosterone was purchased from Fluka (Buchs, Germany). Calibration was performed by determining the peak area ratio between the target analyte and the isotope-labeled internal standard. Before each run, calibrator samples were freshly prepared by spiking 25 µL calibrator working solution to 225 µL of 0.5% BSA in PBS buffer. Internal standard stock solutions were made separately using $^{13}C_3$-labeled versions of each steroid, except for DHEA that was labeled with d6. An internal standard working solution with labeled steroid concentrations in the middle of the calibration range was prepared in 50% methanol/water and aliquoted. All calibrator and internal standard stock and working solutions were stored at –80 °C until use.

Sample preparation

Steroid hormones were extracted from samples (study, control, or calibrator samples) using supported liquid extraction (SLE) 96-well plates (Biotage, #820-0400-P01). In 1-mL 96-well plates (Waters, #186002481), 75 µL water and 25 µL of internal standard solution were added to 250 µL sample. After mixing and centrifugation, the samples were transferred to an SLE plate and samples were loaded using 5 psi pressure for 2 seconds. After 5 minutes rest, 800 µL methyl tert-butyl ether in heptane (20/80) was added to each well and the eluent was collected in a 96-well plate (Thermo Scientific Nunc, #260252). After 15 minutes of elution using gravity, 7 psi pressure for 30 seconds was applied to extract the remaining of the organic solvent. The organic solvent was then evaporated using a Martin Christ RVC 2-33 IR rotational vacuum concentrator at 35 °C, 5 mBar for 30 minutes. Finally, samples were reconstituted using 70 µL of 40% methanol in water and the plate was shaken for 15 minutes prior to analysis.

Steroid hormone assay

Steroid hormones were analyzed using a 2-dimensional liquid chromatography system consisting of Acquity UPLC system and a TQ-XS triple quadrupole mass spectrometer from Waters. Samples (50 µL) were injected from the 96-well plates using an I-Class FTN sample manager. In the first dimension, samples were fractionized using an UPLC H-Class Quaternary Solvent Manager, an XBridge C8 3.5 µm, 2.1 × 50 mm reversed phase column (Waters, #186003047), kept at 50 °C and a gradient consisting of 42% to 95% methanol in water. At 1.5 minutes, corresponding to 55% of methanol, a 1.5-minute segment from the first dimension containing the steroids was trapped on a XBridge Direct Connect HP 10 µm, 2.1 × 30 mm column at room temperature, by combining the flow from the first dimension with an isocratic flow of 5% methanol in water from an Isocratic Solvent Manager. After focusing on the trap column, the steroids were switched over to the second dimension and separated using an I-Class Binary Solvent Manager, a Kinetex Biphenyl 2.6 µ, 2.1 × 100 mm column (Phenomenex, #00D-4622-AN), kept at 40 °C and a gradient consisting of 50% to 90% methanol/0-75 µmol/L ammonium fluoride in water. For multiple reaction monitoring transitions and settings, see Supplementary Table 1 (4).

Evaluation of assay performance

Linearity of the calibration curves was determined by calculating the R^2 (Supplementary Table 1) (4). To determine the intra-assay and interassay coefficients of variation (CVs), 5 aliquots from 2 pools of human serum (with low and high levels of the analytes) were analyzed in 4 assays. The lower limit of detection (LLOD) was defined as the lowest peak having a signal at > 3 times the noise level. The lower limit of quantification (LLOQ) was defined as the lowest peak that was reproducible with a CV of less than 20% and an accuracy of 80% to 120%. To circumvent problems with endogenous steroid levels, the determinations of LLOD and LLOQ were performed in human serum pools with isotope-labeled steroids spiked at 4 different levels. The LLOD and LLOQ were then determined using the isotope-labeled steroids as quantifying signals and the unlabeled steroids as internal standards. Accuracy was evaluated on 2 different concentration levels prepared from human serum with low concentration of steroids spiked with 2 levels of calibrator standard solution containing the target analytes. Five replicates on each level and 5 on baseline were analyzed, and the accuracy was calculated as ([(observed value – baseline value)/amount spiked]) × 100% (Supplementary Table 2) (4).

The extraction recovery was evaluated on pooled human serum with low levels of steroids spiked with 2 levels of calibrator standard solution containing the target analytes and calculated as [(value in pool spiked before SLE column extraction – value in corresponding unspiked pool)/(value in pool spiked after SLE column extraction – value in corresponding unspiked pool)] × 100%. Four replicates of each level and treatment and 4 of unspiked serum (baseline) were included in the test (Supplementary Table 3) (4). The matrix effect in serum (Supplementary Table 4) (4) was determined using serum samples from 6 men (healthy blood donors). Three aliquots of each sample were analyzed before and after spiking of the extracted samples. The matrix effect (%) was calculated as ([peak area in extract after spiking – peak area in nonspiked extract – peak area in spiked blank]/peak area in spiked blank) × 100%.

Comparison of the assay vs external standards (Supplementary Table 5) (4) was performed using Conformité Européene (CE)/In Vitro Diagnostic Regulation (IVDR)-marked MassCheck Steroid Panel 2, Serum Control Level I (low) and III (high) from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). These control samples are designed to monitor the accuracy for quantitative determination of steroids in serum and plasma and are traceable to certified reference materials and primary standards. The concentrations were determined in duplicates and were compared to vendor-determined target levels and acceptance ranges. The accuracies were calculated as measured concentration/vendor target level × 100%.

Serum SHBG and Calculation of Free Hormone Fractions

Serum SHBG was measured by a chemiluminescent-based SHBG assay validated for clinical use (LIAISON SHBG, cat. code 319020, DiaSorin; RRID:AB_2895155) and the DiaSorin LIAISON immunoanalyzer was used. The sandwich assay uses 2 monoclonal antibodies for capture and detection of SHBG. The LLOQ of the assay is 0.80 nmol/L. Intra-assay CV is typically < 3% and interassay CV < 6%. We calculated free testosterone and free estradiol according to the method described by Mazer (29), taking concentrations of total testosterone and estradiol levels and SHBG into account and assuming a fixed albumin concentration (43 g/L).

Statistical Methods

Differences in frequencies of smoking, diabetes, use of antihypertensive medications, use of lipid-lowering medication, and prevalent diseases between cases and controls were tested by the chi-square test. For the continuous variables age, BMI ($\log_{10}$-transformed), systolic blood pressure ($\log_{10}$-transformed), total cholesterol, and HDL cholesterol, the corresponding analyses were performed by *t* test. Pack-years showed a skewed distribution and were analyzed with the Mann-Whitney U test. Based on distribution, sex hormones were $\log_{10}$-transformed before analysis (DHEA, progesterone, 17α-hydroxyprogesterone, androstenedione, estrone, testosterone, dihydrotestosterone, SHBG, calculated free testosterone [cFT], calculated free estradiol [cFE2]) or analyzed without log-transformation (estradiol). Correlations among sex steroids were analyzed by Pearson correlation. Levels of sex hormones in AAA cases and controls were presented in box-and-whisker plots and as mean or geometric mean (95% CI) and were compared using *t* test (all $\log_{10}$-transformed, except estradiol). Standardized hormone levels were further entered as continuous variables in logistic regression models. Power calculations show that an odds ratio of 1.34 per SD change in serum sex hormone level can be detected with 147 cases and 251 controls (significance level 0.05, power 0.80). In addition to a crude model, estimates were adjusted for current smoking, pack-years (entered as quartiles because of a nonnormal distribution of data) and BMI ($\log_{10}$-transformed), systolic blood pressure ($\log_{10}$-transformed), total cholesterol, HDL cholesterol, diabetes mellitus, use of antihypertensive medications, use of lipid-lowering medications, prevalent cardiovascular disease, asthma/COPD, and other systemic diseases.

In multivariate forward conditional logistic regression models with combinations of sex hormones, estimates were adjusted for current smoking, pack-years (quartiles), BMI ($\log_{10}$-transformed), systolic blood pressure ($\log_{10}$-transformed), total cholesterol, HDL cholesterol, diabetes mellitus, use of antihypertensive medications, use of lipid-lowering medications, prevalent cardiovascular disease, asthma/COPD, and other systemic diseases.

Progesterone and estradiol were further entered as quadratic terms in a logistic regression analysis to test for possible nonlinearity in the association between hormones and AAA. Similarly, interaction was tested by an interaction term (progesterone × estradiol). We divided subjects into 4 groups according to progesterone and estradiol status (above/below median) and studied the association with AAA in a crude logistic regression model.

All statistical analyses were performed using SPSS for Windows (version 26, SPSS, Chicago, IL).

Results

The characteristics of AAA and control men are shown in Table 1. Among men with AAA, the median diameter of AAAs was 33 mm (range, 30-98 mm; 25^{th}-75^{th} percentile,

31-40 mm); 110/147 of the AAAs (75%) were small AAA (diameter of 30-39 mm).

For accurate and comprehensive serum sex steroid measurements, we developed and validated a high-sensitivity LC-MS/MS assay for serum sex hormone profiling (for detailed description of the developed assay, see "Methods"). Sensitivity measures of the different sex steroids were excellent (LLOQ for dehydroepiandrosterone, progesterone, 17α-hydroxyprogesterone, androstenedione, estrone, testosterone, dihydrotestosterone, and estradiol were 250, 5, 20, 5, 0.5, 5, 13, and 0.5 pg/mL, respectively) and evaluation of the precision revealed that both the intra- and interassay CVs were less than 5% for all analytes when determined using a low-level and a high-level human control sample (Table 2). The accuracy for each steroid quantified over 2 spiking levels evaluated in human serum (Supplementary Table 2) (4) and the comparison of the assay vs external standards traceable to certified reference materials (Supplementary Table 5) (4) were also excellent.

In the AAA case-control study, all men had sex steroid levels above LLOQ of the LC-MS/MS assay, except 1 AAA man with DHEA level below LLOQ; this value was set to LLOQ of the assay in data analyses. All men had SHBG levels above LLOQ of the SHBG assay. A correlation matrix of sex hormone levels showed that many of the sex hormones were highly intercorrelated (Table 3). However, the correlations between the mainly adrenal-derived sex steroids (DHEA, progesterone, androstenedione, and 17α-hydroxyprogesterone) and the mainly testicular-derived sex steroids (testosterone, dihydrotestosterone, estradiol, and estrone) were in general modest. BMI associated negatively with progesterone, 17α-hydroxyprogesterone, testosterone, dihydrotestosterone, SHBG, and calculated free testosterone (cFT). Calculated free estradiol (cFE2), but not total estradiol, associated positively with BMI.

We next studied levels of sex hormones in men with AAA and AAA-free control men (Fig. 1). The serum levels of DHEA, progesterone, 17α-hydroxyprogesterone, testosterone, estradiol, cFT, and cFE2 were lower in AAA men compared with control men (Fig. 1). By contrast, levels of androstenedione, estrone, dihydrotestosterone, and SHBG did not differ significantly between men with/without screening-detected AAA.

The hormones that differed between AAA and control men were further analyzed as continuous variables in logistic regression models (Table 4). After adjustment for BMI and smoking (Model 2), progesterone, 17α-hydroxyprogesterone, estradiol, cFT, and cFE2, but not DHEA or testosterone, were significantly inversely associated with AAA. In a multivariable model (Model 3) including several known AAA risk factors and comorbidity, the associations with progesterone, estradiol, cFT, and cFE2 with AAA remained statistically significant (Table 4). The multivariate odds ratios for total testosterone adjusted for SHBG (1.53 [95% CI, 1.03-2.27]; $P = 0.034$) and total estradiol adjusted for SHBG (1.60 [95% CI, 1.17-2.19]; $P = 0.003$) were similar to the estimates for the cFT and cFE2, respectively.

Next, multivariate (as Model 3 above) odds ratios for AAA in models with combinations of sex hormones were calculated. In a model with both total testosterone and total estradiol, the odds ratio for estradiol (1.56 [95% CI,

Table 1. Characteristics of the study population

	AAA cases	Controls	
	n = 147	n = 251	*P* value
Age, years	65.31 ± 0.22	65.32 ± 0.20	0.61
BMI, kg/m^2	27.9 ± 4.2	27.0 ± 3.6	0.029
Current smoking, n (%)	47 (32.0)	16 (6.4)	<0.001
Pack-years, n	26 ± 21	9 ± 13	<0.001
Systolic blood pressure, mmHg	146.1 ± 19.7	138.6 ± 16.4	<0.001
Serum total cholesterol, mmol/L	5.01 ± 1.17	5.33 ± 1.11	0.007
Serum HDL cholesterol, mmol/L	1.25 ± 0.38	1.50 ± 0.39	<0.001
Diabetes mellitus, n (%)	18 (12.2)	21 (8.4)	0.21
Use of antihypertensive medications, n (%)	78 (53.1)	111 (44.2)	0.088
Use of lipid-lowering medications, n (%)	52 (35.4)	62 (24.7)	0.023
Prevalent cardiovascular disease, n (%)	37 (25.2)	23 (9.2)	<0.001
Prevalent asthma/COPD, n (%)	11 (7.5)	17 (6.8)	0.79
Other systemic disease, n (%)	12 (8.2)	21 (8.4)	0.94

Values are mean ± SD unless otherwise indicated. Age denotes age at blood sampling. Frequency data were analyzed by chi square test and continuous variables by *t* test, except pack-years, which was analyzed by Mann-Whitney U test.
Abbreviations: AAA, abdominal aortic aneurysm; BMI, body mass index; COPD, chronic obstructive pulmonary disease.

Table 2. LC-MS/MS assay performance

	Estradiol	Estrone	Testosterone	Dihydrotes-tosterone	Progesterone	Androstene-dione	DHEA	17α-hydroxy-progesterone
Sensitivity								
LLOD, pg/mL	0.25	0.125	2	10	2	2.5	65	15
LLOQ, pg/mL	0.5	0.5	5	13	5	5	250	20
Precision								
Intra-assay CV								
QC-low	2.3% (6.2)	1.2% (22.6)	1.1% (228)	3.0% (45)	1.8% (78)	1.1% (456)	3.8% (2236)	3.5% (177)
QC-high	1.1% (251)	1.5% (251)	1.9% (4993)	3.6% (526)	1.6% (3155)	1.7% (1458)	2.3% (4712)	1.8% (860)
Interassay CV								
QC-low	1.9% (6.2)	1.6% (22.6)	2.2% (228)	4.7% (45)	1.9% (78)	1.2% (456)	3.9% (2236)	3.8% (177)
QC-high	1.1% (251)	1.7% (251)	2.4% (4993)	4.1% (526)	1.8% (3155)	1.2% (1458)	4.3% (4712)	1.5% (860)

Values within brackets indicate the concentrations of the QC in pg/mL.
Abbreviations: DHEA, dehydroepiandrosterone; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLOD, lower limit of detection; LLOQ, lower limit of quantification; QC-low, quality control sample with low concentration; QC-high, quality control sample with high concentration.

1.08-1.86]; $P = 0.017$) but not testosterone (0.82 [95% CI, 0.55-1.21]; $P = 0.32$) was significant. In a similar analysis of cFT and cFE2, cFE2 (1.52 [95% CI, 1.06-2.17]; $P = 0.021$), but not cFT (1.07 [95% CI, 0.75-1.53]; $P = 0.70$), was independently associated with AAA.

We next evaluated whether both progesterone and estradiol were independently associated with AAA in the multivariate model including both progesterone and estradiol as well as several known AAA risk factors and comorbidity (Table 5). Indeed, both progesterone and estradiol were inversely and independently associated with AAA in this multivariable model. In a similar manner, both progesterone and cFE2 were associated with AAA in a multivariate model (Table 5).

The inclusion of a quadratic term of the evaluated hormone did not support a nonlinear association between progesterone or estradiol and AAA. Further, an interaction term of progesterone and estradiol was nonsignificant, suggesting that there was no interaction between progesterone and estradiol for the association with AAA.

As the correlation between estradiol and progesterone was marginal (variance explained, $r^2 = 1.4\%$; Table 3), these 2 hormones may contribute with independent additive information for the prediction of AAA presence. To evaluate the combined impact of low progesterone and low estradiol levels, subjects were divided into 4 groups according to both progesterone and estradiol status (above/below median) and the association with AAA presence was studied (Fig. 2). This analysis illustrated that the impact of low estradiol and low progesterone on AAA presence was additive. Men with low (below median) levels of both progesterone and estradiol had a 4-fold increased odds ratio for AAA (4.06 [95% CI, 2.25-7.31]; $P < 0.001$) compared with men with high (above median) levels of both hormones.

Discussion

Male sex is a major risk factor for AAA (1) but relatively few studies have addressed the association between sex hormone levels and AAA in men (22-24). Here we developed and validated a high-sensitivity LC-MS/MS assay for accurate and comprehensive serum sex hormone profiling and used this assay for measurements in a unique case-control study based on general population-based ultrasound screening for AAA in 65-year-old men. Our main finding was that both progesterone and estradiol were inversely associated with the presence of AAA after adjustments for known AAA risk factors and comorbidity. Men with low (below median) levels of both progesterone and estradiol had a 4-fold increased odds ratio for AAA compared with men with high (above median) levels of both hormones.

Immunoassays of sex steroids have been questioned for their limited accuracy and specificity, especially at lower hormone concentrations, such as those of progesterone and estradiol in men (25-27). Indeed, the use of estradiol immunoassays have been shown to result in false positive associations with inflammation-related cardiovascular outcomes in men (27). With the development of mass spectrometry–based assays the demand on more accurate assessment of sex hormones has increased (30), although this advanced technology may be challenging to use in clinical practice. The performance of the LC-MS/MS assay that was developed and validated in this study is excellent. The use of isotope-labeled versions of the analytes compensates for the matrix effects, resulting in an assay with low intra- and interassay CVs and high accuracy. Further, technical advances such as 2-dimensional chromatography, improved MS equipment, and ammonium fluoride as a mobile phase additive all contribute to the high performance of our assay (31). The assay is highly sensitive for the 8

Table 3. Correlation matrix of sex steroids and BMI

	DHEA	Progesterone	17α-hydroxy-progesterone	Androstene-dione	Estrone	Testosterone	Estradiol	Dihydrotestos-terone	SHBG	cFT	cFE2	BMI
DHEA	-	0.20***	0.13*	0.61***	0.30***	0.01	0.03	0.07	-0.01	0.03	0.06	-0.06
Progesterone		-	0.86***	0.23***	0.07	0.29***	0.12*	0.12*	0.26***	0.14**	-0.01	-0.33***
17α-hydroxy progesterone			-	0.18***	0.00	0.44***	0.13*	0.22***	0.36***	0.26***	-0.03	-0.37***
Androstenedione				-	0.51***	0.25***	0.27***	0.19***	0.10*	0.26***	0.22**	-0.08
Estrone					-	0.18***	0.68***	0.17**	0.12*	0.12*	0.62***	0.09
Testosterone						-	0.55***	0.77***	0.72***	0.71***	0.21***	-0.44***
Estradiol							-	0.43***	0.32***	0.46***	0.80***	-0.03
Dihydrotestosterone								-	0.65***	0.46***	0.09	-0.44***
SHBG									-	0.05	-0.24***	-0.39***
cFT										-	0.54***	-0.25***
cFE2											-	0.18***
BMI												-

Analysis by Pearson correlation, correlation coefficients are shown. All variables, except estradiol, were $\log_{10}$-transformed. Pooled analysis of 147 men with abdominal aortic aneurysm (AAA) and 251 AAA-free controls.
Abbreviations: DHEA, dehydroepiandrosterone; SHBG, sex hormone–binding globulin; cFT, calculated free testosterone; cFE2, calculated free estradiol; BMI, body mass index.
* $P < 0.05$;
** $P < 0.01$;
*** $P < 0.001$.

included sex steroids/sex steroid precursors without requiring labor-intensive derivatization of the samples. The LLOQs for estradiol and estrone in human serum are in the range of the best estrogen-optimized assays and the LLOQ for progesterone 5- to 20-times lower than many published LC-MS/MS assays (31, 32). This assay will be useful for high-throughput analyses of comprehensive sex steroid profiles in clinical research settings requiring high-sensitivity measurements of serum sex steroids (32).

To our knowledge, this is the first study describing a sex steroid profile by a mass spectrometry–based method in men with AAA. In a previous cross-sectional population-based study, Yeap et al addressed the relation between serum testosterone (by immunoassay) and SHBG and AAA in 3620 older (age 70-88 years) men, with a high (7.2%) prevalence of AAA determined from ultrasound examination of the aorta. In line with our findings, they found lower total testosterone (in univariate models only) and lower free testosterone, but similar SHBG levels, in men with AAA (22). When plasma DHEA, androstenedione, testosterone, and SHBG (all by immunoassay) were addressed in 37 male AAA cases and 91 healthy controls, SHBG but not sex steroids were found to be lower in cases (23). Contrasting with our findings, Villard et al recently reported a higher frequency of detectable levels of estradiol and progesterone in men with AAA, and lower testosterone in univariate models (24). While the setting of this study was a similarly sized case-control study of Swedish 65-year-old men recruited at AAA screening, Villard et al used immunoassays with low sensitivity, where 45% and 49% of the control men had estradiol and progesterone levels below the working range of the assay. In our study, all men had levels of these sex steroids above LLOQ of the LC-MS/MS assay. As discussed above, immunoassays also have lower specificity and may show yet unexplained associations with inflammation markers (27), which potentially may explain the higher frequency of detectable levels in AAA subjects in the study by Villard et al (24). These discrepancies further illustrate the importance of using a high-performance sex steroid assay in clinical research (30).

We found here that lower progesterone and estradiol levels were associated with AAA independently of other risk factors/markers. The correlation between estradiol and progesterone was marginal in the present study, suggesting that these 2 hormones may contribute with independent information for disease prediction. Indeed, a major finding in the present study was that the impact of low estradiol and low progesterone on AAA prediction was additive. Serum estradiol has previously been reported to associate positively with atherosclerosis and cardiovascular outcomes in men, but similar to Villard et al (24), most of these studies have used immune-based techniques with questionable

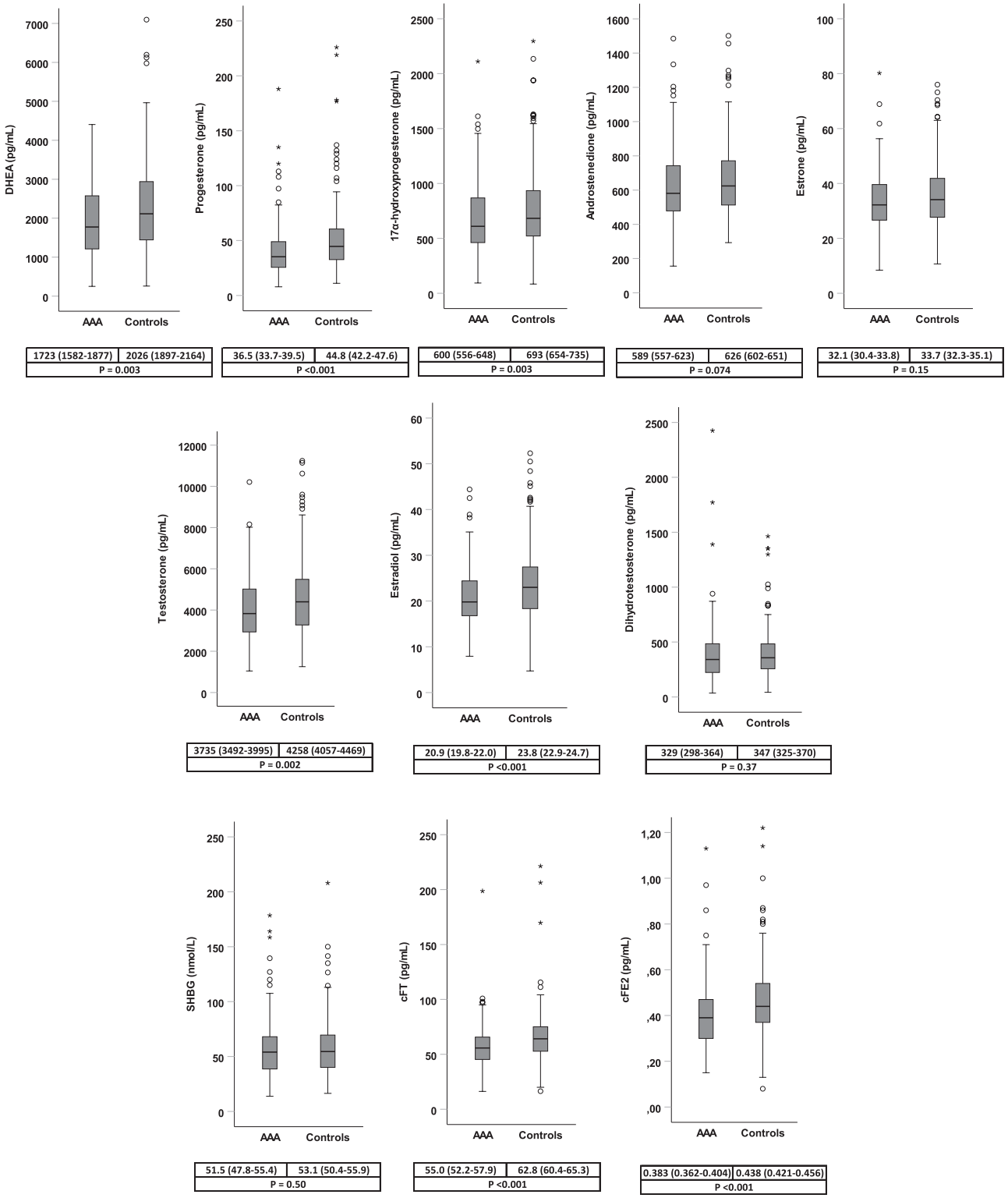

Figure 1. Sex steroid concentrations in men with AAA and controls. Box-and-whisker plots of sex steroid concentrations in men with AAA and controls. Box boundaries represent 25th–75th percentile, horizontal bars represent median values, whiskers represent minimum and maximum values, and circles and asterisks represent statistical outliers and extreme values, respectively. Below graphs, values are geometric mean (95% CI) for hormones that were log-transformed before statistical analysis (DHEA, progesterone, 17α-hydroxyprogesterone, androstenedione, estrone, testosterone, dihydrotestosterone, SHBG, free testosterone, free estradiol). For other hormones (estradiol) values are mean (95% CI). P values are from *t* test. For comparison, reference ranges provided by the Mayo Clinic (https://www.mayocliniclabs.com) are DHEA (> or = 61 years) < 5.0 ng/mL; progesterone (males > or = 18 years) < 0.20 ng/mL; 17α-hydroxyprogesterone (adult males) < 220 ng/dL; androstenedione (adult males) 40-150 ng/dL; estrone (adult males) 10-60 pg/mL; testosterone (males > or = 19 years) 240-950 ng/dL; estradiol (adult males) 10-40 pg/mL; dihydrotestosterone (males > 19 years) 112-955 pg/mL. Abbreviations: AAA, abdominal aortic aneurysm; cFE2, calculated free estradiol; cFT, calculated free testosterone; DHEA, dehydroepiandrosterone; SHBG, sex hormone–binding globulin.

Table 4. Crude and multivariate odds ratios (95% CI) for AAA in relation to sex hormone levels

	Model 1		Model 2		Model 3	
	OR (95% CI)[a]	*P* Value	OR (95% CI)[a]	*P* Value	OR (95% CI)[a]	*P* Value
DHEA	1.36 (1.10-1.67)	0.004	1.28 (1.00-1.64)	0.051	1.18 (0.90-1.55)	0.24
Progesterone	1.57 (1.25-1.96)	<0.001	1.74 (1.32-2.30)	<0.001	1.62 (1.18-2.22)	0.003
17α-hydroxyprogesterone	1.36 (1.11-1.68)	0.004	1.45 (1.12-1.87)	0.005	1.31 (0.98-1.75)	0.067
Testosterone	1.39 (1.13-1.72)	0.002	1.26 (0.96-1.65)	0.096	1.09 (0.80-1.48)	0.57
Estradiol	1.54 (1.22-1.94)	<0.001	1.54 (1.11-1.87)	0.007	1.40 (1.04-1.87)	0.024
cFT	1.56 (1.25-1.95)	<0.001	1.40 (1.08-1.81)	0.012	1.38 (1.05-1.83)	0.022
cFE2	1.53 (1.23-1.91)	<0.001	1.48 (1.15-1.92)	0.003	1.59 (1.19-2.11)	0.001

Analysis by logistic regression.
Model 1: Crude model.
Model 2: Adjusted for BMI, current smoking, and smoking burden (pack-years).
Model 3: Adjusted for BMI, current smoking, smoking burden (pack-years), systolic blood pressure, total cholesterol, HDL cholesterol, diabetes mellitus, use of antihypertensive medications, use of lipid-lowering medications, prevalent cardiovascular disease, prevalent asthma/COPD, and other systemic disease.
Abbreviations: AAA, abdominal aortic aneurysm; BMI, body mass index; cFE2, calculated free estradiol; cFT, calculated free testosterone; DHEA, dehydroepiandrosterone; OR, odds ratio.
[a]OR per SD decrease in hormone level.

Table 5. Multivariate odds ratios (95% CI) for AAA in relation to progesterone and estradiol levels

	Multivariate model	
	OR (95% CI)	*P* value
Model with progesterone and estradiol		
Progesterone, per SD decrease	1.62 (1.19-2.21)	0.002
Estradiol, per SD decrease	1.35 (1.01-1.81)	0.043
Model with progesterone and cFE2		
Progesterone, per SD decrease	1.53 (1.14-2.05)	0.005
cFE2, per SD decrease	1.62 (1.23-2.13)	<0.001

Model 3: Multivariate model with adjustment for BMI, current smoking, smoking burden (pack-years), systolic blood pressure, total cholesterol, HDL cholesterol, diabetes mellitus, use of antihypertensive medications, use of lipid-lowering medications, prevalent cardiovascular disease, prevalent asthma/COPD, and other systemic disease.
Abbreviations: AAA, abdominal aortic aneurysm; BMI, body mass index; cFE2, calculated free estradiol; COPD, chronic obstructive pulmonary disease; OR, odds ratio.

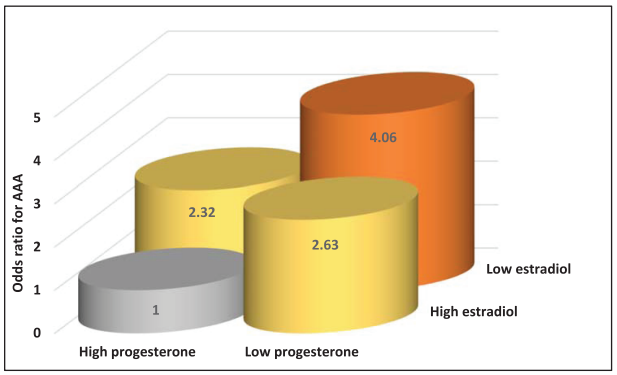

Figure 2. Odds ratios for screening-detected AAA in 65-year-old men in relation to serum progesterone and estradiol status. Crude odds ratios for AAA among 398 men (147 men with AAA and 251 control men without AAA), who were divided into 4 groups by progesterone and estradiol status. - Referent group (n = 113; 23 AAA/90 controls) with both progesterone and estradiol level above median- Group (n = 86; 32 AAA/54 controls) with low (below median) estradiol and high (above median) progesterone levels (OR for AAA 2.32 [95% CI, 1.23-4.37]; $P = 0.009$ vs referent group) - Group (n = 87; 35 AAA/52 controls) with low progesterone and high estradiol levels (OR for AAA 2.63 [95% CI, 1.41-4.93]; $P < 0.001$ vs referent group) - Group (n = 112; 57 AAA/55 controls) with low levels of both progesterone and estradiol (OR for AAA 4.06 [95% CI, 2.25-7.31]; $P < 0.001$ vs referent group).

specificity in the lower range (27). By contrast, low levels of estradiol assessed by mass spectrometry predict mortality in elderly men (33). Similar to our findings, a recent study showed that progesterone, but not estradiol, levels by mass spectrometry associate negatively with BMI in men (8). To our knowledge, serum progesterone has not previously been studied in relation to cardiovascular disease in men, likely due to the lack of cohorts with serum progesterone levels analyzed by high-performance methodology.

Importantly, AAA men are more affected by atherosclerosis-based cardiovascular disease (34) and there is ample evidence that low levels of other sex hormones, such as testosterone and DHEA, associate with cardiovascular diseases in men (35, 36). Further, any systemic disease or treatments such as corticosteroids may affect the risk of AAA as well as the production of sex hormones and adrenal precursors (37), which may be a particularly important feature of diseases with an inflammatory component such as AAA (38). Although we adjusted for prevalent diseases, there may be residual confounding in our analyses, and the underlying biology of the associations found here will require further investigation.

Although cross-sectional association studies such as this should be interpreted with caution, one possibility is that

estradiol and progesterone are protective against AAA in men. In support of this, endogenous/exogenous estradiol has been shown to exert a protective effect in both sexes in different experimental AAA models (14, 19-21), while progesterone has been little studied. The arterial vasculature expresses both progesterone and estrogen receptors, and both progesterone and estradiol directly regulate vascular responses (39-41). Further support for a modifying role of the sex steroid system in human AAA derives from genetic studies, suggesting an association between the aromatase gene, converting testosterone to estradiol, as well as the estrogen receptor β and AAA (10, 11). However, the impact of these genetic variants (rs1961177 in the aromatase gene and AluIa in the estrogen receptor β) on enzyme and receptor function remains unestablished. Furthermore, studies of vascular gene expression report lower expression of the estrogen receptor β in human aneurysm wall biopsies compared with control aorta (42). Evidently, the role of progesterone and estradiol in human AAA will require further study.

Increased understanding of the sexual dimorphism in human AAA may provide important clues to pathogenesis and potential treatment targets of AAA. In premenopausal women, levels of progesterone and estradiol show great variation across the menstrual cycle but are generally much higher than in men (5). The results of this study do not only raise the question whether high progesterone and estradiol are protective in men, but also whether women are protected against AAA by their relatively higher levels of these sex hormones during the reproductive period. Indeed, while most available studies in women are limited by small study sizes, recent data from a large cohort of postmenopausal women suggest that premature menopause may be an important risk factor for AAA in women with significant smoking history (43).

Increased knowledge of the role of sex hormones in AAA disease may also be of high clinical relevance. Prostate and breast cancer, the most common cancers in men and women respectively, are both sex hormone–dependent and drugs that suppress sex hormone production or action are foundations of their treatment. Hormone deficiencies, for example, in menopausal women and androgen-deficient men, are supplemented in large groups of patients. Increased cardiovascular risk has been reported for androgen deprivation therapy of men with prostate cancer (44) as well as for testosterone treatment of elderly men (45) and a high incidence of AAA has been detected in men with prostate cancer (46). If and how treatments that target the sex hormone system affect AAA risk should be explored in future studies.

Our study has limitations, including all those that are generally associated with a cross-sectional study design. Other limitations include self-reporting of comorbidities, that the differential study acceptance rate and missing samples among men with AAA and controls may introduce bias, and that the results may not be generalizable to men of other ages. While men with an AAA in this study showed expected risk factors vs controls, a lower prevalence of diabetes among men with AAA was not found here, contrasting the results of other studies (1). Further, calculations of free testosterone and estradiol using an arbitrary albumin concentration is a limitation of the study. The study also has notable strengths, including controls recruited parallel to cases at the same screening site, detection of early (screening-detected) disease, non-confounding by age and sex and that state-of-the-art assays were performed in a single laboratory.

In conclusion, measured by a high-performance sex steroid assay, both progesterone and estradiol are inversely associated with AAA in men, independently of known risk factors. Future studies should explore whether progesterone and estradiol, which are important reproductive hormones in women, are protective in human AAA and whether they play a role for the female protection against AAA.

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Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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