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ORIGINAL RESEARCH - PRECLINICAL

Follicle-Stimulating Hormone Exacerbates Cardiovascular Disease in the Presence of Low or Castrate Testosterone Levels

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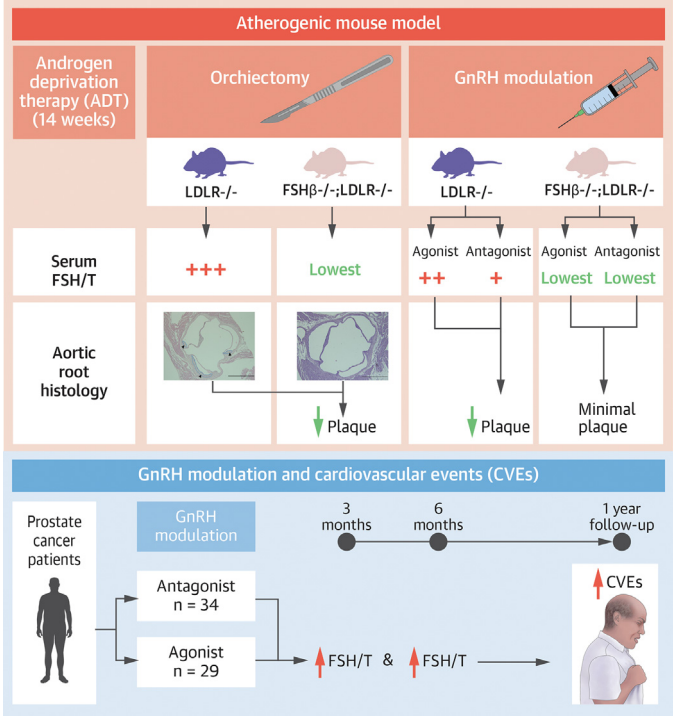
VISUAL ABSTRACT



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HIGHLIGHTS

- **Hypogonadism and PC are common in aging men and associate with cardiovascular disease. ADT to suppress T in PC patients also affects FSH.**
- **We defined a hormonal state characterized by an elevated FSH/T ratio that correlated with the amount of aortic atherosclerotic and necrotic plaque in mice after ADT and clinically associated with the risk of developing a CVE following a stressful cardiovascular trigger.**
- **ADT modalities which optimally suppress FSH (GnRH-antagonists) appear to have an inherent superior cardiovascular risk profile.**
- **Larger studies that prospectively collect CVEs and serum are needed to validate our results in ADT recipients.**

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SUMMARY

Low testosterone (T), common in aging men, associates with cardiovascular disease. We investigated whether follicle-stimulating hormone (FSH), which is affected by T, modulates the cardiovascular effects associated with low T or castration. FSH$\beta^{-/-}$:low-density lipoprotein receptor (LDLR)$^{-/-}$ mice, untreated or castrated (orchiectomy, gonadotropin-releasing hormone agonist or antagonist), demonstrated significantly less atherogenesis compared with similarly treated LDLR$^{-/-}$ mice, but not following FSH delivery. Smaller plaque burden in LDLR$^{-/-}$ mice receiving gonadotropin-releasing hormone antagonists vs agonists were nullified in FSH$\beta^{-/-}$:LDLR$^{-/-}$ mice. Atherosclerotic and necrotic plaque size and macrophage infiltration correlated with serum FSH/T. In patients with prostate cancer, FSH/T following androgen-deprivation therapy initiation predicted cardiovascular events. FSH facilitates cardiovascular disease when T is low or eliminated.

ABBREVIATIONS AND ACRONYMS

ADT = androgen-deprivation therapy

AUC = area under the receiver-operating characteristic curve

CCL21 = C-C motif chemokine ligand 21

CVD = cardiovascular disease

CVE = cardiovascular event

GnRH = gonadotropin-releasing hormone

FSH = follicle-stimulating hormone

LDLR = low-density lipoprotein receptor

MI = myocardial infarction

MINS = myocardial injury after noncardiac surgery

PC = prostate cancer

T = testosterone

Although cancer-specific survival is generally high, the overall survival of men with prostate cancer (PC) is often affected by cardiovascular disease (CVD). CVD is the leading cause of death in non-metastatic patients with PC and the risk of cardiovascular death is higher in men with metastatic PC compared with the age-matched population.[1] The main cause of CVD, atherosclerosis, often remains asymptomatic and represents subclinical disease; however, atherosclerotic plaques can rupture or erode and lead to atherothrombosis and subsequent cardiovascular events (CVEs).

Circulating testosterone (T) levels in men decline progressively from the third decade onward, resulting in high prevalence of biochemical and clinical hypogonadism in aging men.[2] Cardiovascular health in the general male population is suggested to be strongly associated with T,[3-5] and preclinical models highlight similar results.[6] We recently demonstrated this also in a population of newly diagnosed patients with PC, in whom the prevalence of low T is 42%. Hypotestosteronemic patients with PC have a higher prevalence of cardiovascular risk factors that in turn may associate with a higher projected 10-year risk of CVD than men with normal T at diagnosis.[7]

Approximately 40% of patients with PC receive androgen-deprivation therapy (ADT) within 6 months of diagnosis.[8] ADT can be achieved surgically or pharmacologically, most frequently by inhibition of the hypophyseal-testicular axis using gonadotropin-releasing hormone (GnRH)-agonists (eg, leuprolide, goserelin)[9] or antagonists (degarelix or relugolix),[10] resulting in equivalent castration, but a distinctive effect on follicle-stimulating hormone (FSH)[11] even though T and FSH levels are interrelated physiologically. FSH is one of the gonadotropins, hormones released from the anterior pituitary upon stimulation by GnRH. FSH, a glycoprotein heterodimer consisting of an alpha and unique beta subunit, supports spermatogenesis in the testes. Elevated FSH is also linked to progression of subclinical atherosclerosis in menopause.[12,13]

Clinical observations suggest that ADT in the form of GnRH-antagonist rather than the more commonly used GnRH-agonist is associated with significantly reduced cardiovascular morbidity and mortality. A recent meta-analysis found the pooled risk ratio for GnRH-antagonists compared with GnRH-agonists for CVEs to be 0.57 (95% CI: 0.39-0.81).[14] Our preclinical data corroborate these findings; GnRH-antagonists lead to less aortic necrotic plaque area in low-density lipoprotein receptor knockout (LDLR$^{-/-}$) mice than GnRH-agonist,[15] which was confirmed by Knutsson et al[16] in apolipoprotein E-deficient mice.

We hypothesized that FSH mediates the atherogenic and cardiovascular effects associated with T deficiency. Our investigations included mouse models of atherogenesis using gain and loss of FSH and ADT comparing leuprolide and degarelix, and 2 clinical studies of ADT recipients and male non-PC patients, with primary cardiovascular endpoints.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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METHODS

ANIMAL STUDIES. All protocols for animal studies were approved by the Animal Research Ethics Board of McMaster University (AUP #18-01-05). For the breeding of double-knockout ($FSH\beta^{-/-}$:$LDLR^{-/-}$) mice, without functional FSH,[17] see the Supplemental Methodology. Mice were randomly assigned to 6 groups, each with 10 to 15 male $LDLR^{-/-}$ mice[18] or $FSH\beta^{-/-}$;$LDLR^{-/-}$ mice (5 to 6 weeks old). The groups were: 1) untreated control (vehicle treated, sham operated); 2) bilateral transscrotal orchiectomy (vehicle treated); 3) GnRH-agonist (leuprolide acetate), sham operated; 4) GnRH-antagonist (degarelix), sham operated; 5) orchiectomy with testosterone supplementation; and 6) $FSH\beta^{-/-}$:$LDLR^{-/-}$ mice (vehicle treated, sham operated) supplemented with murine FSH. For detailed treatment methodology see the Supplemental Methodology and Hopmans et al.[15] Mice were weighed weekly. Serum testosterone was monitored every 2 to 4 weeks. Mice were euthanized at 20 weeks of age, 14 weeks after surgery and start of ADT. Blood, heart, ascending aorta, and testicles were collected. Serum was prepared and frozen at −20 °C. Serum assays are described in the Supplemental Methodology. ADT mice (groups 2-4) not achieving castrate T levels were excluded. The mean with 95% CI of T levels for orchiectomized mice of each strain was determined as threshold for mice not achieving castration (T >1.31 and >0.458 nmol/L (nM) for $LDLR^{-/-}$ and $FSH\beta^{-/-}$:$LDLR^{-/-}$ mice, respectively).

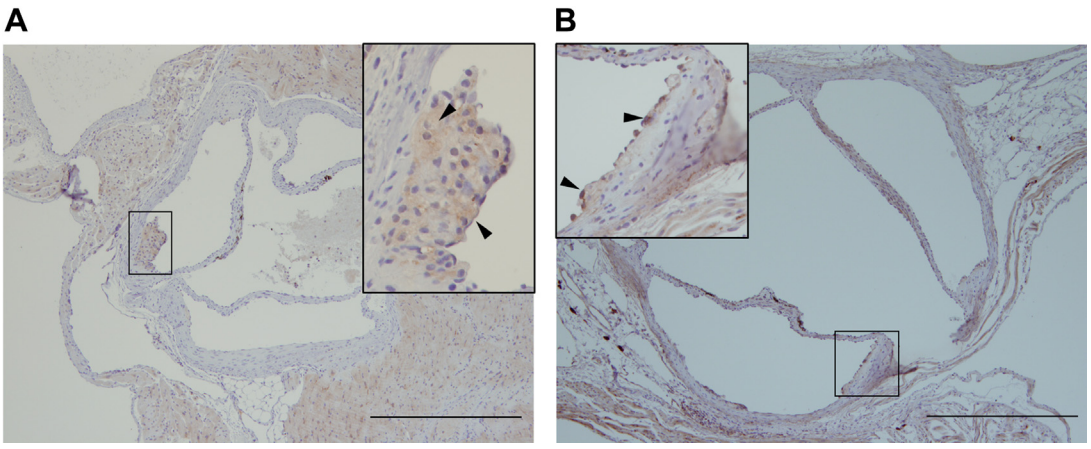
HISTOLOGY AND IMAGE ANALYSIS. Aortic tissue was processed and image analysis was performed to determine atherosclerotic and necrotic plaque size as described.[15] The area of the necrotic core was quantified by cumulative acellular areas within the atherosclerotic plaque area as described in Venegas-Pino et al.[19] Immunohistochemistry included heat-mediated antigen retrieval (citrate buffer pH = 6) and primary antibody incubation (see Kleinmann et al[20] and Supplemental Methodology), followed by horseradish peroxidase-labeled polymer (Dako Canada Inc). Omission of primary antibody served as negative control, for positive controls see Supplemental Table 1. Aortic sections were stained for CD68 and CD3 as measures for macrophage and lymphocyte infiltration, respectively, and quantified. Immunohistochemistry for CD68 and CD3 was performed on consecutive aortic sections. Macrophage infiltration was defined as the percentage of CD68-positive cellular area per atherosclerotic plaque area in the aortic root section (see Supplemental Figure 1A). Lymphocyte infiltration was defined as the percentage of CD3-positive cellular area per total atherosclerotic plaque area in the aortic root section (see Supplemental Figure 1B).

CLINICAL STUDY I: PROSPECTIVE COHORT OF PC PATIENTS. This was a randomized open-label study of the use of GnRH-antagonist compared with GnRH-agonist among men with advanced PC and preexisting CVD (Endothelial Function in Prostate Cancer Patients on Degarelix vs Luteinizing Hormone-Releasing Hormone Agonists; NCT02475057).[21] The study included men with high-risk or metastatic PC scheduled to initiate ADT for at least 1 year. All patients had to have documented history of CVD and were randomized to either GnRH-antagonist (degarelix) or GnRH-agonist. FSH and T were measured in the central lab at Rabin Medical Center, Tel Aviv, Israel, at baseline, and 3, 6, and 12 months. Patients who did not achieve castration (T >0.7 nmol/L, n = 12) were excluded. Secondary outcome of the study included the occurrence of CVEs (defined as death, myocardial infarction [MI], cerebrovascular event, transient ischemic attack, heart catheterization with or without stent insertion, and cardiac-related emergency room visits) among the 2 arms during the 12-month study period. CVEs were adjudicated by a cardiologist.[21]

CLINICAL STUDY II: PROSPECTIVE NESTED CASE-CONTROL STUDY WITHIN VISION. The study was carried out as a nested case-control study from male patients in the VISION (Vascular Events in Noncardiac Surgery Patients Cohort Evaluation Study; NCT00512109) prospective cohort study. VISION investigated the development of myocardial injury after noncardiac surgery (MINS) in a very large international cohort.[22,23] The MANAGE (Dabigatran in Patients With Myocardial Injury After Non-cardiac Surgery) trial demonstrated that MINS result in classic CVEs and death shortly after if untreated with antithrombotic medications.[24] Male patients were eligible for matching if a preoperative serum sample was available. All patients with incident cases of the composite outcome were matched 1:1 with a corresponding control. Cases were patients who developed a CVE (composite of MINS, MI, stroke, nonfatal cardiac arrest, or vascular death) within 30 days after surgery and were matched to controls who did not experience a CVE by: 1) age; 2) body mass index; and 3) history of coronary artery disease.

Levels of FSH and total T in banked serum obtained preoperatively from the identified patients were measured at the Clinical Research Laboratory and Biobank (Hamilton General Hospital, Hamilton Ontario, Canada). FSH values (IU/L) were converted

FIGURE 1 Mouse Aortic Root Immunohistochemistry for FSH Receptor



Expression of follicle-stimulating hormone (FSH) receptor in plaque macrophages in aortic root cross-sections of control (A) low-density lipoprotein receptor (LDLR)$^{-/-}$ and (B) FSHβ$^{-/-}$:LDLR$^{-/-}$ mice. Arrowheads in the magnified inserts indicate examples of FSH receptor-staining. Magnification bar = 500 μm.

to nmol/L.[25] The ratio of FSH/T was calculated as FSH (nmol/L)/total testosterone (nmol/L). Lab personnel had no knowledge of the study or groups of patients.

STATISTICAL ANALYSES. Preclinical data were not normally distributed and values are presented as median (range) or using boxplots to show the mean, median, and IQR. Mann-Whitney *U* tests were performed to determine significance. Adjustments for multiple comparisons were not performed; we report precise *P* values when <0.05. When interpreting the results, multiple comparisons need to be considered. Spearman's correlation analyses between amount of atherogenesis (total or necrotic) or macrophage/lymphocyte infiltration and serum hormone or biomarker levels at endpoint were performed. A *P* value <0.05 was considered significant. Statistical analyses were performed using SPSS version 27 (IBM Corporation).

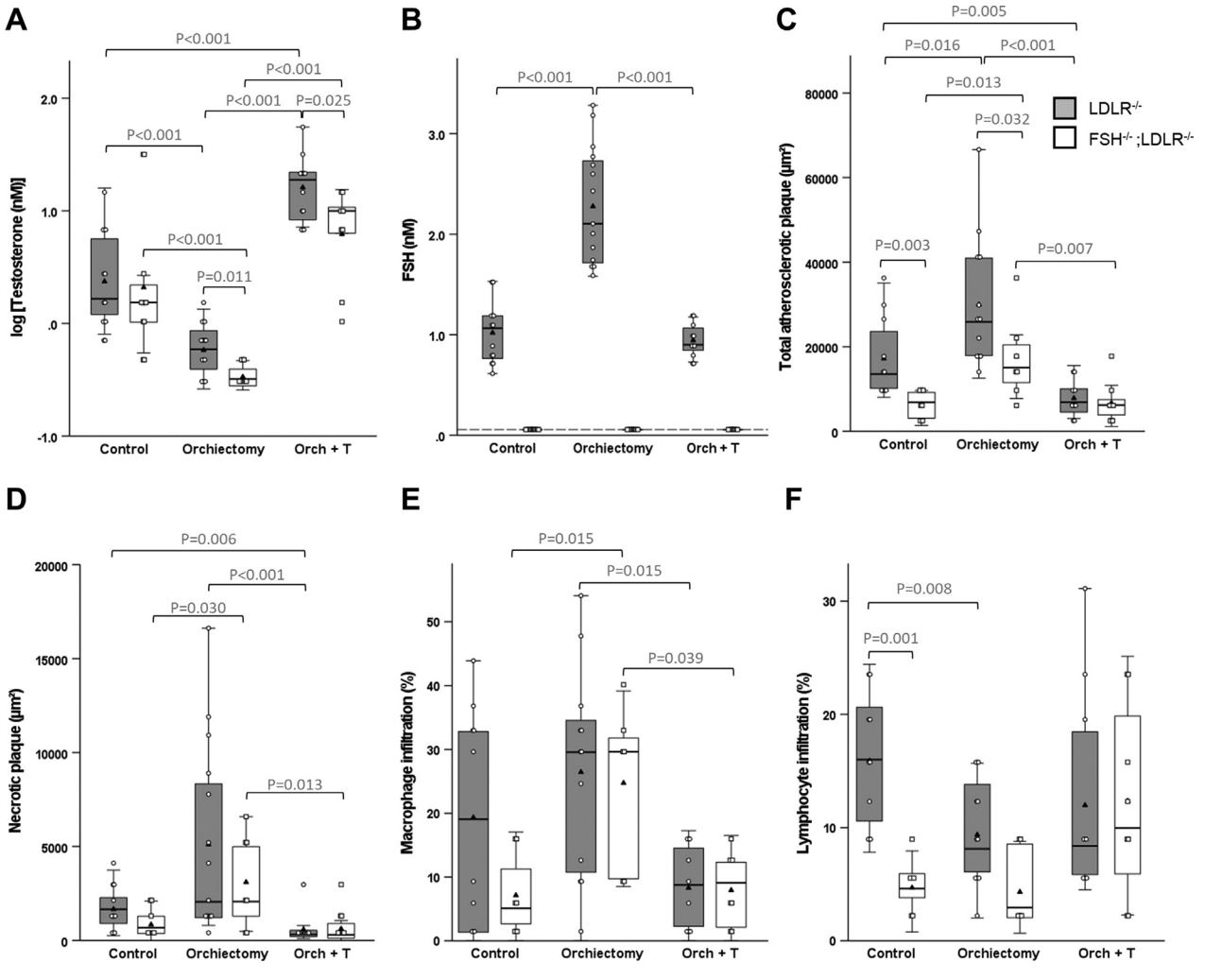
Patient characteristics were compared between patients with and without CVEs (cohort of PC patients) and between cases and controls (nested case-control within VISION) using descriptive statistics including median, minimum, and maximum for continuous variables. Variables were compared between patients with and without CVEs and between cases and controls using chi-square or Fisher's exact test for categorical variables, and Student's *t*-test or Mann-Whitney *U* test for continuous variables. Area under the receiver-operating characteristic curve (AUC) was used to determine the ability of FSH/T to predict CVE in the prospective PC patient cohort.

Conditional logistic regression was performed to compare the ratio of FSH/T in 424 cases with a CVE with 424 controls without CVEs. The dependent variable was a CVE and the prognostic factor of interest was the FSH/T ratio expressed as a continuous variable or, in a separate model, as binary (high or low using median FSH/T value as cutpoint). The association between the ratio of FSH/T and incident cases of CVEs is expressed as the OR with 95% CI. We explored potential confounders of the association between FSH/T ratio, as a binary variable, and CVEs using separate conditional logistic regression models (see Supplemental Methodology). In separate models, the association between serum FSH or T and incident cases of CVEs was evaluated, again as a continuous variable or binary (cutpoint median FSH or T value). Collinearity between variables was assessed using generalized variance inflation factors (>5 defined as significant collinearity). Conditional logistic regression was performed using R, version 4.1.0, and package survival 3.3-1.

RESULTS

ANIMAL STUDIES. To investigate the role of FSH on CVD, we generated FSHβ$^{-/-}$:LDLR$^{-/-}$ mice that develop atherogenesis on normal diet. We chose the FSH$^{-/-}$ over the FSHR$^{-/-}$ model to be able to investigate FSH treatment via exogenous delivery of FSH. Both LDLR$^{-/-}$ and FSHβ$^{-/-}$:LDLR$^{-/-}$ mice express FSH receptor in macrophages (**Figures 1A and 1B**, respectively). In LDLR$^{-/-}$ mice, castration (**Figure 2A**) via orchiectomy, which resulted in very high FSH levels

FIGURE 2 Effect of Orchiectomy With or Without T Supplementation in LDLR$^{-/-}$ and FSHβ$^{-/-}$:LDLR$^{-/-}$ Mice



(A) Serum testosterone (T) and (B) FSH levels at 20 weeks of age, 14 weeks after start of treatment. The dashed line indicates the limit of detection for serum FSH. (C) Atherosclerosis, (D) necrosis, (E) macrophage, and (F) lymphocyte infiltration after 14 weeks (n = 7-15/group). Control mice were vehicle treated and sham operated. Boxes represent the IQR, triangles the mean, whiskers the range. Significant *P* values (<0.05) obtained using Mann-Whitney *U* tests are indicated. Abbreviations as in Figure 1.

(Figure 2B), induced significant atherosclerosis, but not after concomitant T supplementation (Figure 2C). Comparatively, FSHβ$^{-/-}$:LDLR$^{-/-}$ mice demonstrated smaller atherosclerotic plaque sizes than LDLR$^{-/-}$ mice, whether untreated or orchiectomized (Figures 2 and 3). Conversely, significant atherosclerotic changes were observed in FSHβ$^{-/-}$:LDLR$^{-/-}$ mice following exogenous delivery of murine FSH (Figure 4). Mouse testicle sizes and weights, main physiological determinants of FSH, are shown in Supplemental Figure 2 and confirm the efficacy of FSH delivery and its functionality (Figure 4B).

ADT via bilateral orchiectomy, GnRH-agonists or -antagonists resulted in equivalent castration, but a distinctive effect on FSH (Figures 2A, 2B, 5A, and 5B). Orchiectomy leads to increased necrosis in LDLR$^{-/-}$ mice compared with treatment with GnRH-antagonist ($P = 0.018$). In FSHβ$^{-/-}$:LDLR$^{-/-}$ mice, however, there was no difference (Figures 2D and 5D). GnRH-antagonists vs -agonists led to significantly increased suppression of FSH (Figure 5B, Supplemental Figure 2). Treatment of LDLR$^{-/-}$ mice with GnRH-antagonists led to a 79% decrease in the amount of necrotic core and 41% decrease in total atherosclerotic plaque compared with GnRH-agonists (Figures 5C and 5D). Loss of FSH in the FSHβ$^{-/-}$:LDLR$^{-/-}$ model nullified these differences (Figures 5C and 5D), suggesting a role for FSH in plaque development in

FIGURE 3 **Mouse Aortic Root Histology and Immunohistochemistry**

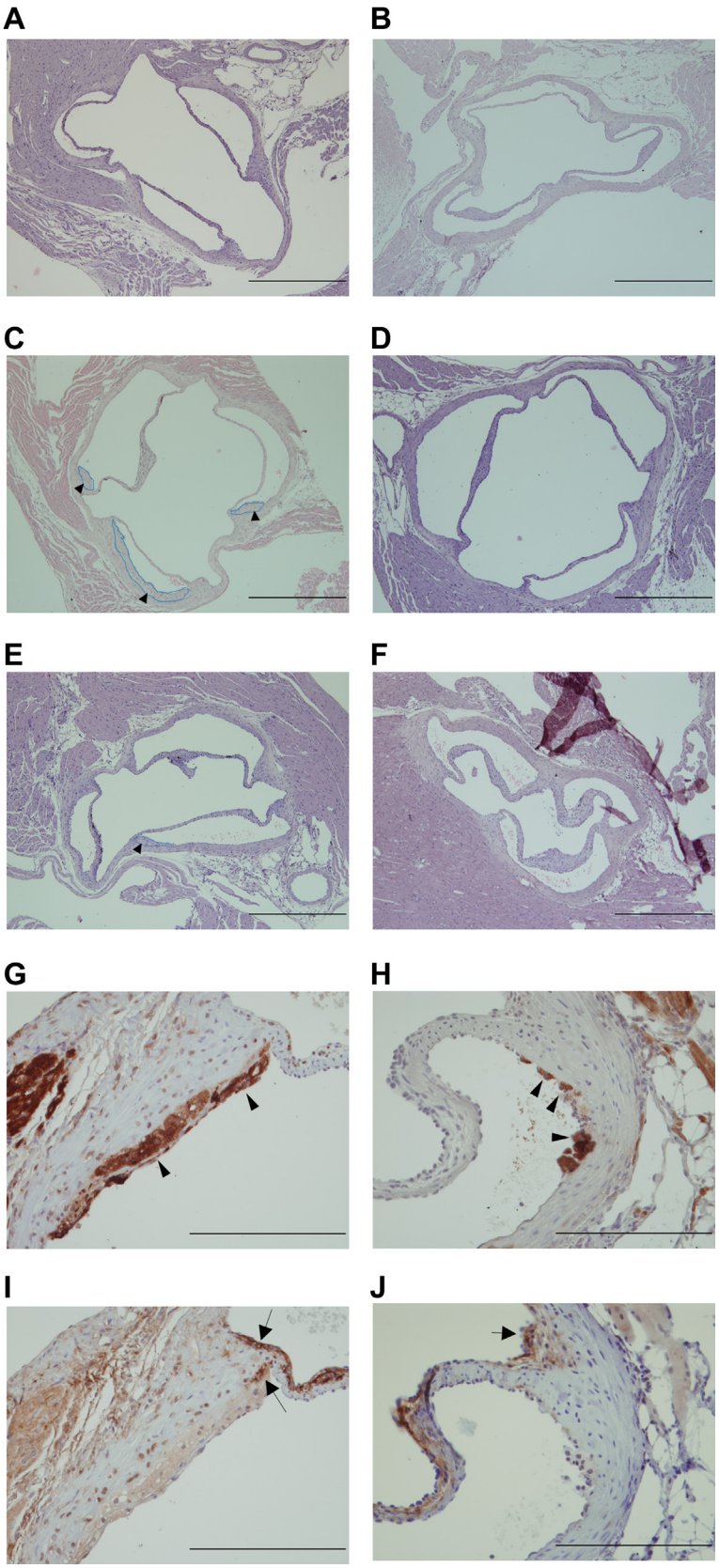


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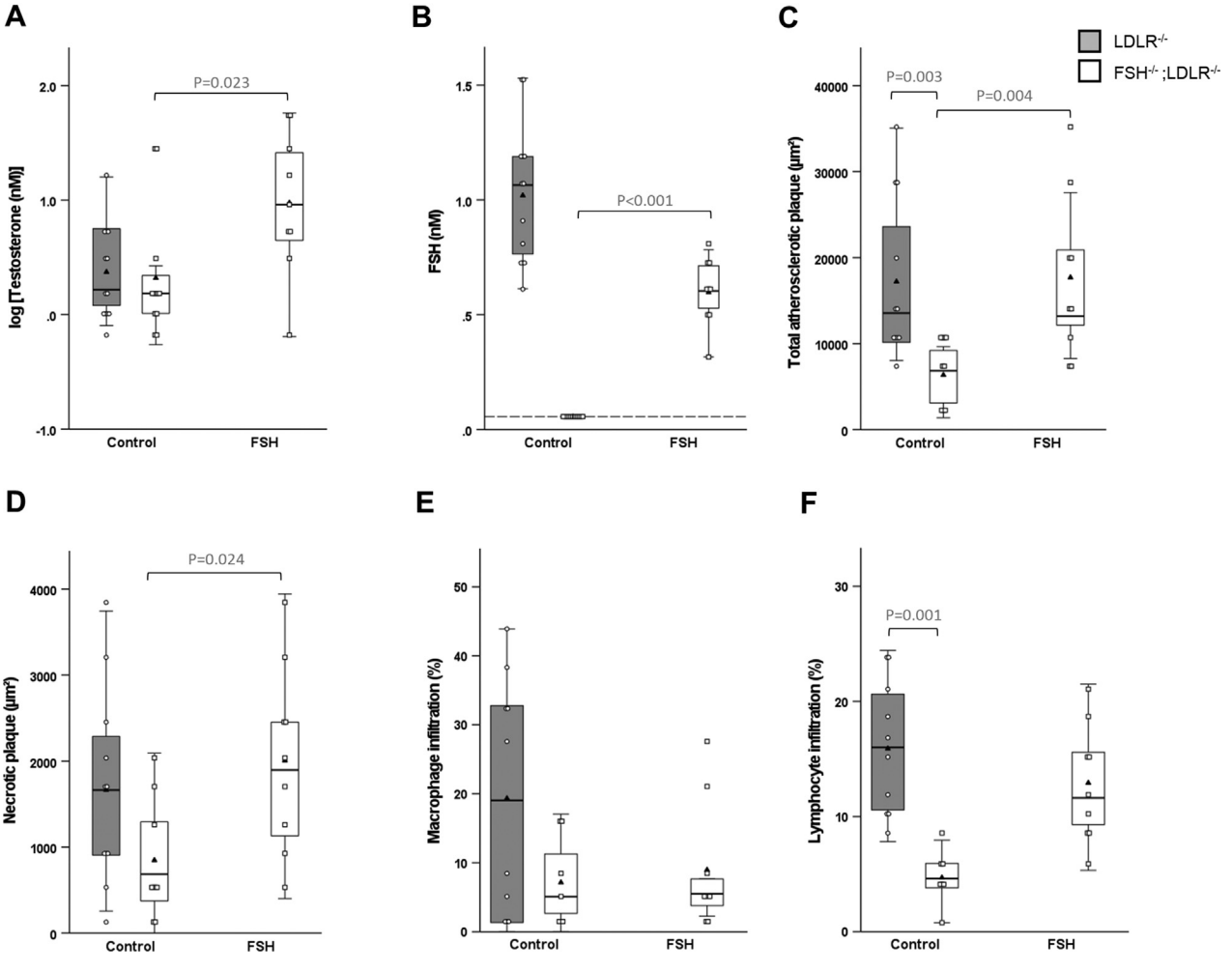
FIGURE 4 Effect of FSH Supplementation in FSHβ$^{-/-}$:LDLR$^{-/-}$ Mice Comparatively to Control LDLR$^{-/-}$ Mice



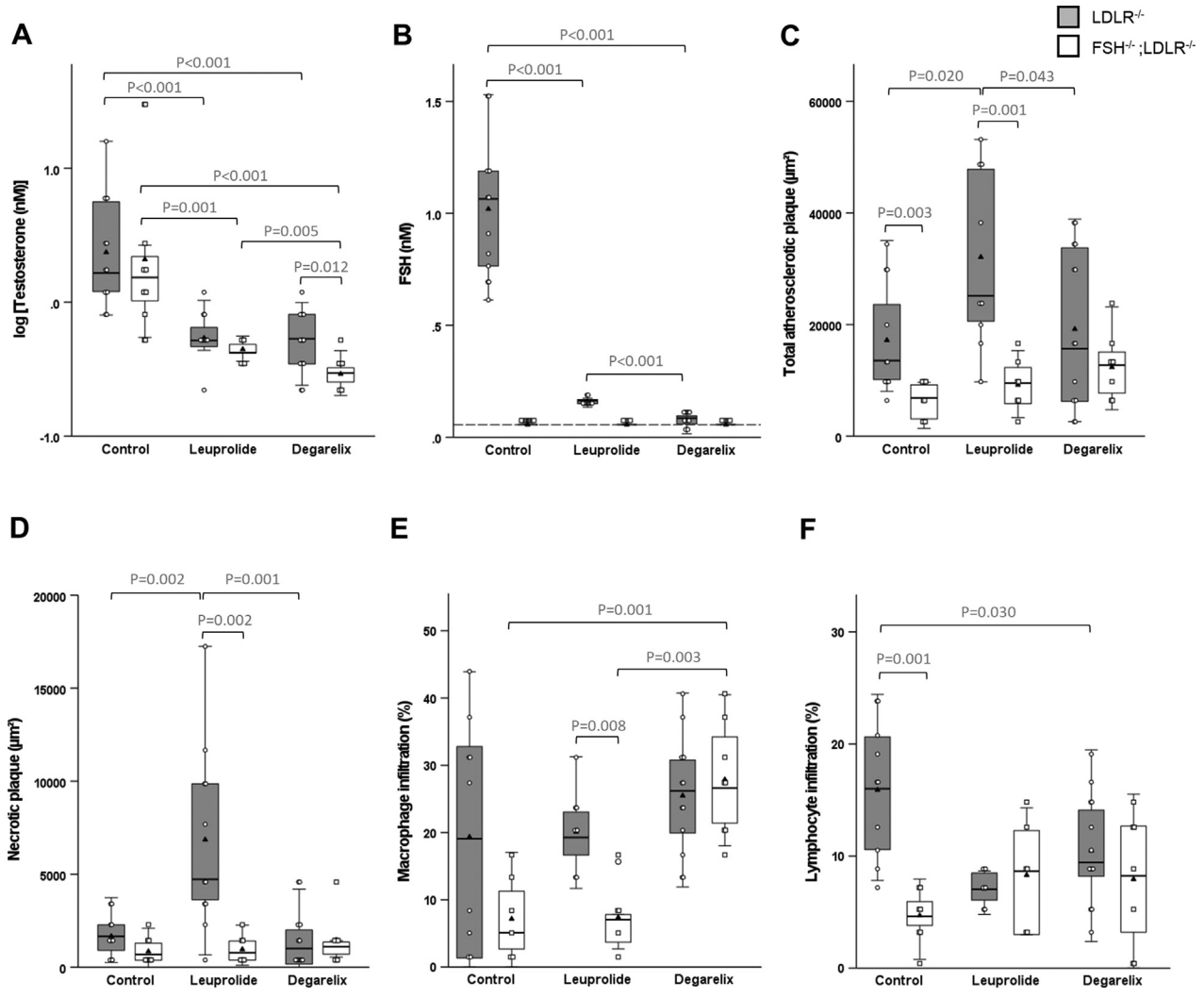
(A) Serum T and (B) FSH levels in LDLR$^{-/-}$ and FSHβ$^{-/-}$:LDLR$^{-/-}$ mice at 20 weeks of age, 14 weeks after start of treatment. The dashed line indicates the limit of detection for serum FSH. (C) Atherosclerosis, (D) necrosis, (E) macrophage, and (F) lymphocyte infiltration after 14 weeks (n = 7-11/group). Boxes represent the IQR, triangles the mean, whiskers the range. Significant *P* values (<0.05) obtained using Mann-Whitney *U* tests are indicated. Abbreviations as in Figures 1 and 2.

castrated mice. To evaluate the effects of both FSH and T, we calculated the serum FSH/T ratio. By Spearman's correlation analysis, serum FSH/T ratio correlated significantly with atherosclerotic plaque size, necrotic core, and macrophage infiltration (Figure 6A). FSH/T did not correlate with serum 17β-estradiol or luteinizing hormone and neither did these hormones correlate with atherogenesis (Figure 6A, Supplemental Table 2). We also found a significant correlation between various inflammatory

FIGURE 3 Continued

Representative hematoxylin-eosin-stained aortic root sections from untreated control (A) LDLR$^{-/-}$ and (B) FSHβ$^{-/-}$:LDLR$^{-/-}$ mice; orchiectomized (C) LDLR$^{-/-}$ and (D) FSHβ$^{-/-}$:LDLR$^{-/-}$ mice; orchiectomized and supplemented with T (E) LDLR$^{-/-}$ and (F) FSHβ$^{-/-}$:LDLR$^{-/-}$ mice after 14 weeks. The outlines of single atherosclerotic plaque areas larger than 5,000 μm^2 are marked blue, with arrowheads to indicate examples of necrotic areas within these large plaques. Magnification bar = 500 μm. Immunohistochemistry for (G and H) CD68 and (I and J) CD3 on representative serial aortic root sections of (G and I) LDLR$^{-/-}$ and (H and J) FSHβ$^{-/-}$;LDLR$^{-/-}$ mice, 14 weeks after orchiectomy. Arrowheads indicate examples of CD68-staining, arrows examples of CD3-staining. Magnification bar = 200 μm.

FIGURE 5 Effect of Treatment With GnRH-Agonist or -Antagonist in FSHβ$^{-/-}$:LDLR$^{-/-}$ and LDLR$^{-/-}$ Mice



(A) Serum T and (B) FSH levels in LDLR$^{-/-}$ and FSHβ$^{-/-}$:LDLR$^{-/-}$ mice at 20 weeks of age, 14 weeks after start of treatment. The dashed line indicates the limit of detection for serum FSH. (C) Atherosclerosis, (D) necrosis, (E) macrophage, and (F) lymphocyte infiltration after 14 weeks (n = 7-14/group). Control mice were vehicle treated and sham operated. Boxes represent the IQR, triangles the mean, whiskers the range. Significant *P* values (<0.05) obtained using Mann-Whitney *U* tests are indicated. GnRH = gonadotropin-releasing hormone; other abbreviations as in **Figures 1 and 2**.

biomarkers and plaque size, necrotic and inflammatory content (Supplemental Figure 3). However, only C-C motif chemokine ligand 21 (CCL21), a homeostatic chemokine involved in inflammatory response, associated significantly with these parameters and with the FSH/T ratio (**Figure 6A**). Notably, the receptor for CCL21, C-C motif chemokine receptor 7, is strongly expressed in mouse macrophages (Supplemental Figures 4G and 4H).

PROSPECTIVE COHORT OF PC PATIENTS WITH PREEXISTING CVD COMMENCING ON ADT. Because CVEs appear to develop early, within 1 year following ADT initiation in PC patients with known CVD,[21,26,27] it is reasonable to suspect that ADT may be a trigger for atherosclerotic plaque vulnerability.[28] Several, but not all,[29] studies suggest that GnRH-agonists are associated with higher incidence of CVEs in PC patients with preestablished CVD compared with GnRH-antagonists.[14,21,26,29,30] Given the significant differences in serum FSH/T between GnRH-agonists and -antagonists (**Figure 6B**), we hypothesize that FSH may mediate the effect of low T on atherosclerotic plaque vulnerability, increasing the risk of clinical CVEs. We explored the ratio of serum FSH/T in a

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FIGURE 6 **FSH/T Associates With Atherogenesis in Mice and CVEs in PC and Male Surgery Patients**

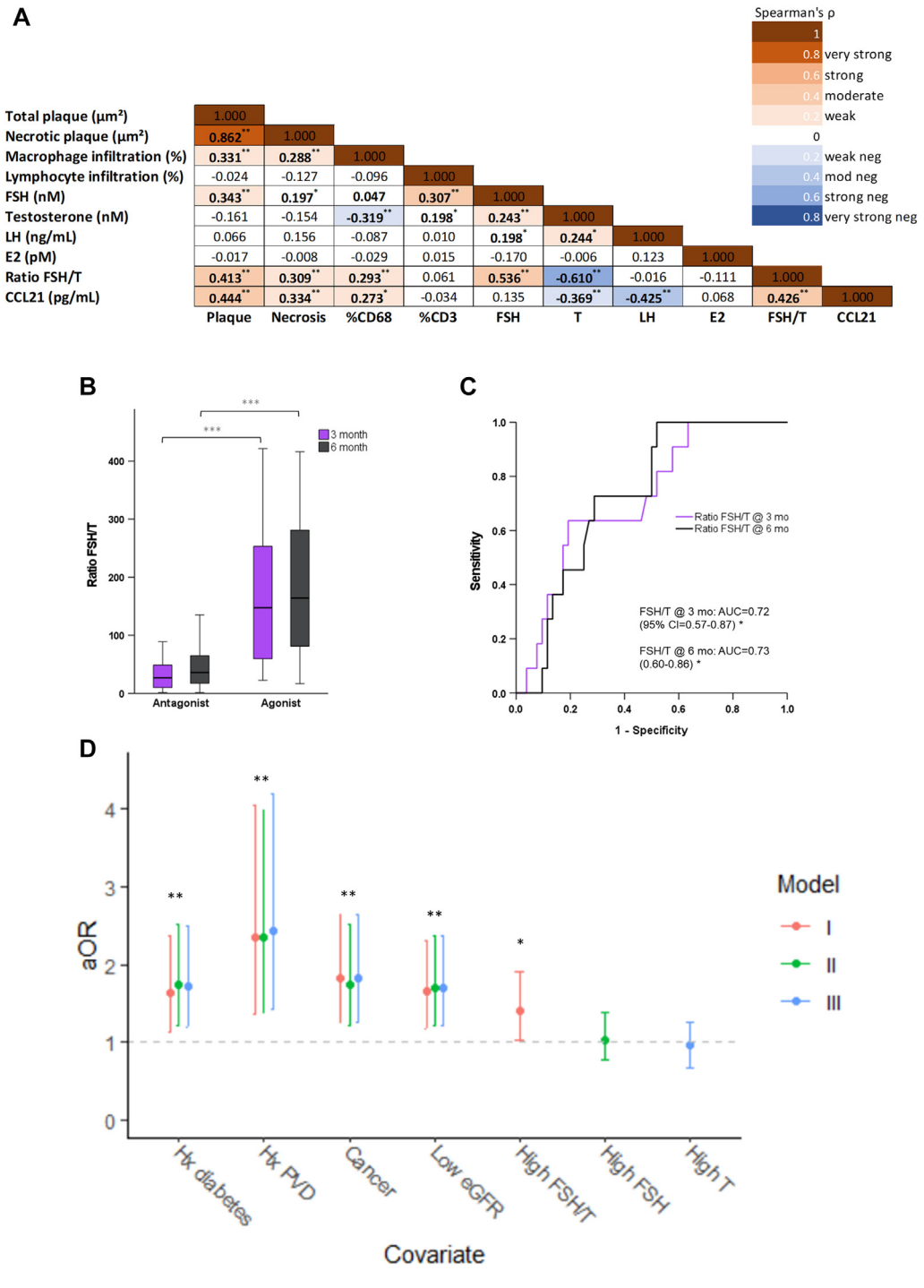

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TABLE 1 Patient Population Characteristics of the Prospective Cohort of PC Patients With Preexisting CVD Commencing on ADT

	Patients Receiving GnRH-Agonist (n = 29)	Patients Receiving GnRH-Antagonist (n = 34)	Total (N = 63)	*P* Value
Age, y	71.4 (61.7-82.7)	72.1 (58.8-87.2)	71.8 (58.8-87.2)	0.42
BMI, kg/m^2	27.4 (20.1-33.8)	28.4 (21.2-36.2)	27.5 (20.1-36.2)	0.21
Ratio FSH/T				
Baseline	3.48 (0.92-17.3)	3.20 (0.55-46.7)	3.26 (0.55-46.7)	0.34
3 months	164 (22.7-422)	27.6 (1.39-111)	48.7 (1.39-422)	**<0.001**
6 months	166 (16.8-600)	37.0 (1.47-144)	67.6 (1.47-600)	**<0.001**
Serum FSH, nM				
Baseline	30.6 (9.88-128)	43.3 (13.2-260)	37.2 (9.88-260)	0.12
3 months	23.1 (5.17-54.1)	3.29 (0.47-18.8)	7.53 (0.47-54.1)	**<0.001**
6 months	24.5 (5.17-52.2)	5.22 (0.47-23.1)	8.00 (0.47-52.2)	**<0.001**
Serum T, nM				
Baseline	12.1 (1.92-21.9)	12.6 (3.10-27.8)	12.3 (1.92-27.8)	0.16
3 months	0.130 (0.087-0.41)	0.087 (0.087-0.67)	0.090 (0.087-0.67)	0.83
6 months	0.090 (0.087-0.61)	0.087 (0.087-0.55)	0.087 (0.087-0.61)	0.55
Gleason score				0.36
7	9 (31)	16 (47)	25 (40)	
8	8 (28)	9 (26)	17 (27)	
9-10	12 (41)	9 (26)	21 (33)	
PSA baseline, ng/mL	13.3 (5.1-963)	13.2 (0.18-387)	13.3 (0.18-963)	0.45
History of MACE	27 (93)	32 (94)	59 (94)	0.87
History of hypertension	22 (76)	24 (71)	46 (73)	0.64
History of diabetes	9 (31)	8 (24)	17 (27)	0.50
History of renal failure	1 (3.4)	2 (5.9)	3 (4.8)	0.65
CVE[a] within 12 months	9 (31)	2 (6)	11 (17)	**0.009**

Values are median (range) or n (%). Significant *P* values (<0.05) are indicated in **bold**. [a]CVE is defined as death, myocardial infarction, cerebrovascular accident, transient ischemic attack, heart catheterization and cardiac-related emergency room visits. Patients who did not reach T <0.7 nM at 3 or 6 months after start of ADT (n = 12) were excluded.

ADT = androgen-deprivation therapy; BMI = body mass index; CVD = cardiovascular disease; FSH/T = follicle-stimulating hormone/testosterone; GnRH = gonadotropin-releasing hormone; MACE = major adverse cardiovascular event; PC = prostate cancer; PSA = prostate-specific antigen.

prospective cohort of PC patients with preexisting CVD commencing on ADT.[21] Patients receiving GnRH-agonist had significantly higher CVE rates (31%) compared with those receiving GnRH-antagonist (6%) (Table 1). The serum FSH/T ratio, but not FSH or T alone, at 3 and 6 months after start of ADT, was predictive of a CVE (Figure 6C): the AUC of both receiver-operating characteristics for FSH/T at 3 and 6 months amounted to 0.72 (95% CI: 0.57-0.87; $P = 0.022$) and 0.73 (0.60-0.86; $P = 0.016$), respectively.

NESTED CASE-CONTROL WITHIN VISION. Similar to ADT,[28] surgical intervention can be a trigger for CVEs. The VISION study prospectively investigated the development of MINS in a large international cohort.[22,23] MINS is associated with subsequent major CVEs and death[31] and antithrombotic and anticoagulation therapy are beneficial,[24] suggesting that MINS relates to plaque vulnerability and associated CVEs. To extend our understanding of the association of FSH/T with CVEs in the general male population,

FIGURE 6 Continued

(A) FSH/T in LDLR$^{-/-}$ and FSHβ$^{-/-}$:LDLR$^{-/-}$ mice (n = 62-149) correlated significantly with aortic atherosclerotic plaque, necrotic core, and inflammatory content as well as serum CCL21 levels. Spearman's ρ correlation coefficients in bold are significant: *$P < 0.05$; ** $P < 0.01$. (B) FSH/T ratios at 3 and 6 months after start of ADT in a prospective cohort of PC patients (n = 63) with preexisting CVD commencing on ADT. Boxes represent the IQR, whiskers the range. (C) Receiver operator curve shows that serum FSH/T was able to predict a CVE. (D) Multivariable conditional logistic regression evaluating the association between hormone levels or ratios and a CVE in men undergoing surgery in a prospective nested case-control study. Adjusted ORs for the ratio of FSH/T (model I) dichotomized into low or high ratios and a CVE and the covariates (history of diabetes and peripheral vascular disease, reduced kidney function, and active/metastatic cancer). Adjusted ORs for FSH (in model II) and T (in model III) dichotomized into low or high hormone levels. Significant *P* values are indicated: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. ADT = androgen-deprivation therapy; aOR = adjusted OR; AUC = area under the receiver-operating characteristic curve; CCL21 = C-C motif chemokine ligand 21; CVD = cardiovascular disease; CVE = cardiovascular event; eGFR = estimated glomerular filtration rate; Hx = history; LH = luteinizing hormone; PC = prostate cancer; PVD = peripheral vascular disease; other abbreviations as in Figures 1 and 2.

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we measured the preoperative levels of FSH and T in patients who experienced an event (defined as the composite of MINS, MI, stroke, nonfatal cardiac arrest, or vascular death) within 30 days after surgery and compared them with levels in patients who did not experience an event in a nested case-control study within VISION matched for age, body mass index, and history of coronary artery disease. Baseline and operative characteristics of 848 male cases and controls are presented in **Table 2**. Factors that differed significantly between cases and controls were ethnicity, presence of active or metastatic cancer, a history of congestive heart failure, peripheral vascular disease or diabetes, kidney function, and the use of aspirin. In patients who experienced an event, the ratio of serum FSH/T was higher (median 3.41, range 0.096-545) than in matched controls (median 2.66, range 0.044-346), although not statistically significant ($P = 0.056$) (**Table 2**).

To explore the relationship between the ratio FSH/T and a CVE, the baseline characteristics of the patients were stratified as high or low using the median value (3.04) of FSH/T (**Table 3**). Patients with a high ratio of FSH/T were significantly more likely to be older, have active or metastatic cancer, a history of congestive heart failure, peripheral vascular disease or diabetes, impaired kidney function, and had higher CVE rates. Furthermore, a high FSH/T ratio was significantly associated with a CVE. Conditional logistic regression showed that patients with a high FSH/T ratio had 40% increased odds of a CVE (OR: 1.40; 95% CI: 1.03-1.90; $P = 0.031$) compared with patients with a low FSH/T, after adjustment for contributing covariates (**Figure 6D**). No collinearity between variables was observed. FSH/T as a continuous variable was, however, not significantly associated with a CVE (Supplemental Table 3). Importantly, neither FSH nor T alone was associated with the development of CVEs.

DISCUSSION

Our experimental and clinical results suggest that the adverse cardiovascular effects previously attributed to low or castrate T levels may be facilitated by FSH. Our experimental results clearly suggest a role for FSH in plaque development in castrated mice. ADT, whether delivered surgically or pharmacologically, resulted in equivalent castration, but different effects on FSH and on atherogenesis. Orchiectomy yields the highest FSH/T ratio and resulted in significantly more atherosclerotic changes in $LDLR^{-/-}$ mice than in untreated mice and mice treated with GnRH-agonists or -antagonists. In the absence of FSH in the $FSH^{-/-}$;$LDLR^{-/-}$ mice, these differences were completely abolished. Moreover, GnRH-antagonists which led to significantly increased suppression of FSH compared with GnRH-agonist, led to a 79% decrease in the amount of necrotic core and 41% decrease in total atherosclerotic plaque in $LDLR^{-/-}$ mice compared with GnRH-agonists. Again, the loss of FSH in the $FSH\beta^{-/-}$;$LDLR^{-/-}$ model nullified these differences. Finally, delivery of exogenous FSH in $FSH\beta^{-/-}$;$LDLR^{-/-}$ mice restored the significant atherosclerotic changes seen in untreated $LDLR^{-/-}$ mice. These results also confirm 2 recent studies carried out on small groups of apolipoprotein $E^{-/-}$ mice which also showed that FSH leads to increased atherosclerosis on normal[32] and high-fat high-cholesterol diets.[33] However, in the latter study, serum FSH levels reached are 14.7 ng/mL (0.41 nM), at least 4-fold lower than the mean FSH levels in the orchiectomized $LDLR^{-/-}$ mice in our study. Moreover, the T levels after orchiectomy [32] are decreased by a mere 13% to 55 ng/mL (194 nM) hence not truly reflecting castration. Han et al[32] attribute the FSH-induced increase in atherosclerosis to increased secretion of interleukin (IL)-1β by macrophages, whereas Piao et al[33] implicate increased VCAM-1 expression and the PI3K/Akt/NF-κB pathway. We achieved much larger differential serum FSH levels using murine FSH, not xenogenic human FSH, and included more adequate numbers of mice per group (n = 7 vs n = 3 to 4). We did not observe an increase in serum IL-1β (median 1.77 pg/mL [range 1.28-3.74 pg/mL] in control, 1.28 pg/mL [range 0.83-4.64 pg/mL] in FHS-treated mice). Moreover, we demonstrated a significant association between FSH/T and CCL21, a chemokine involved in inflammatory response. CCL21 increases atherosclerotic macrophage recruitment and macrophage lipid accumulation,[34] and its serum levels have recently been demonstrated as a predictive marker for the development of major adverse CVEs in acute coronary syndrome patients.[35] The CCL21/C-C motif chemokine receptor 7 (CCR7) axis may therefore be a potential mechanism by which FSH facilitates cardiovascular morbidity in a low/castrate T environment.

Of all ADT modalities, orchiectomy results in the highest FSH/T ratio. We demonstrated that orchiectomy results in significantly increased amounts of necrosis in $LDLR^{-/-}$ mice than GnRH-antagonists. Although surgical castration is no longer contemporary practice, reviews and analyses of health registries show that patients with PC who undergo orchiectomy experience significantly more CVEs than patients treated with GnRH-therapy (both GnRH-antagonist and -agonist).[36] Interestingly, when only

TABLE 2 Baseline and Operative Patient Population Characteristics of the Nested Case-Control Study Within VISION

	Controls (Patients Without Event[a]) (n = 424)	Cases (Patients With Event[a] Within 30 Days) (n = 424)	Total (N = 848)	*P* Value
Age, y	74.2 (50.4-91.5)	72.0 (45.1-93.9)	73.3 (45.1-93.9)	matched
BMI, kg/m^2	27.3 (14.4-102)	27.2 (12.7-47.0)	27.2 (12.7-102)	matched
Ratio FSH/T	2.66 (0.044-346)	3.41 (0.096-545)	3.04 (0.044-545)	0.056
Serum FSH, nM	36.5 (5.55-352)	39.0 (1.36-496)	38.1 (1.36-496)	0.48
Serum T, nM	13.2 (0.38-37.9)	12.7 (0.32-48.4)	13.0 (0.32-48.4)	0.11
Ethnicity				**0.02**
Asian	50 (12)	62 (15)	112 (13)	
European	371 (88)	345 (81)	716 (84)	
Other	3 (1)	17 (4)	20 (2)	
Patient in nursing home	1 (0)	6 (1)	7 (1)	0.13
Assistance with ADLs	9 (2)	15 (4)	24 (3)	0.30
Smoker				0.30
Current	57 (13)	62 (15)	119 (14)	
Never	134 (32)	113 (27)	247 (29)	
Past	233 (55)	247 (59)	480 (57)	
Active or metastatic cancer	77 (18)	111 (26)	188 (22)	**0.006**
History of coronary artery disease	138 (32)	138 (32)	276 (32)	matched
History of congestive heart failure	13 (3)	26 (6)	39 (5)	**0.049**
History of cerebrovascular event	34 (8)	52 (12)	86 (10)	0.053
History of peripheral vascular disease	27 (6)	59 (14)	86 (10)	**<0.001**
History of diabetes	82 (19)	134 (32)	216 (25)	**<0.001**
History of hypertension	260 (61)	282 (667)	542 (64)	0.13
Preop systolic BP, mm Hg	145 (50-218)	145 (90-240)	145 (50-240)	0.98
Preop diastolic BP, mm Hg	80 (40-117)	78 (40-112)	80 (40-117)	0.24
Preop coronary revascularization	66 (16)	77 (18)	143 (17)	0.36
Preop eGFR <60 mL/min/1.73 m^2	104 (25)	159 (38)	263 (31)	**<0.001**
Medication				
Aspirin	117 (28)	151 (36)	268 (32)	**0.017**
Nitrate	22 (5)	21 (5)	43 (5)	1
Anticoagulant	31 (7)	25 (6)	56 (7)	0.49
ACE inhibitor/ARB	203 (48)	194 (46)	397 (47)	0.51
Beta-blocker	121 (29)	125 (29)	246 (29)	0.76
Statin	209 (49)	208 (49)	417 (49)	0.95
Plavix/ticlopidine	7 (2)	16 (4)	23 (3)	0.057
Alpha2-agonist	1 (0)	2 (0)	3 (0)	0.56
Rate control CCB	13 (3)	24 (6)	37 (4)	0.064
Dihydropyridine CCB	96 (23)	83 (20)	179 (21)	0.27
Therapeutic antithrombotic	8 (2)	6 (1)	14 (2)	0.59
Type of surgery				
Major vascular	35 (8)	42 (10)	77 (9.1)	0.40
Major general	70 (17)	100 (24)	170 (20)	0.10
Major thoracic	15 (4)	24 (6)	39 (4.6)	0.14
Major urologic/gynecologic	73 (17)	64 (15)	137 (16.2)	0.40
Major orthopedic	147 (35)	113 (27)	260 (31)	**0.011**
Major neurologic	13 (3)	19 (4)	32 (3.8)	0.28
Low-risk	79 (19)	76 (18)	155 (18)	0.79
Urgent/emergent	3 (1)	10 (2)	13 (1.5)	0.050

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GnRH-agonists are compared with orchiectomy, there is no difference in the CVE rate,[37] which is in support of our hypothesis that a higher FSH/T ratio after GnRH-agonist compared with -antagonist correlates with higher CVE rates. All related studies, however, are retrospective analyses of administrative data, with their inherent bias, and did not include serum FSH/T levels.

The clinical results support findings from other prospective studies, which compared the 1-year CVE

TABLE 2 Continued

	Controls (Patients Without Event[a]) (n = 424)	Cases (Patients With Event[a] Within 30 Days) (n = 424)	Total (N = 848)	*P* Value
Type of anesthesia				
General only	159 (38)	208 (49)	367 (43)	**<0.001**
Neuroaxial (spinal or epidural)	175 (41)	97 (23)	272 (32)	**<0.001**
General and nitrous oxide only	6 (1.4)	7 (2)	13 (1.5)	0.78
General and thoracic epidural only	35 (8.3)	67 (16)	102 (12)	**<0.001**
General and nerve block only	8 (1.9)	16 (4)	24 (2.8)	0.098
Other	41 (9.7)	29 (7)	70 (8.3)	0.13

Values are median (range) or n (%). Significant *P* values (<0.05) are indicated in **bold**. [a]Event is defined as myocardial injury after noncardiac surgery, stroke, myocardial infarction, nonfatal cardiac arrest, or vascular death.

ACE = angiotensin-converting enzyme; ADL = activities of daily living; ARB = angiotensin receptor blocker; BP = blood pressure; CCB = calcium channel blocker; eGFR = estimated glomerular filtration rate; preop = preoperative; other abbreviations as in Table 1.

rate between patients with PC with CVD who were randomized to receive GnRH-agonists or -antagonists and found a statistically significant lower incidence in patients receiving GnRH-antagonists.[21,26,30] In contrast to these studies that showed 1-year CVE rates of 6.2% to 20.5% and 2.4% to 8.0% for GnRH-agonists and -antagonists, respectively, Lopes et al[29] reported respective event rates of 4.1% and 5.5%. This suggests that, when cardiovascular prevention strategies including adequate delivery and monitoring in patients with a history of CVD are provided, as in Lopes et al,[29] the CVE risk is similar in patients receiving GnRH-agonist and -antagonist. Whether such rigid cardiovascular monitoring and risk factor management[29] can be routinely delivered in practice is doubtful. Nonetheless, there is a need for prospective clinical studies with CVEs as a primary endpoint. Although current clinical data in the field are conflicting, our results indicate that ADT modalities that optimally suppress FSH appear to have an inherent superior cardiovascular risk profile.

We examined the generalizability of our observation that serum FSH/T may associate with the development of CVEs in men without PC following other stressful cardiovascular triggers. Surgery has been demonstrated to be a trigger for myocardial injury[22,23] leading to CVEs[24] in the VISION cohort which represents the general population. Of the 848 men in our nested case-control study within VISION, 310 patients (36.6%) had low levels of T <11 nmol/L (definition by the European Male Aging Study[38]). Although the ratio of FSH/T as a continuous variable did not significantly associate with a CVE, we found a significant association when patients were stratified by FSH/T. Physiologically, the interplay between the effects of 2 hormones (FSH and T) is not necessarily linear, as previously suggested for T activity.[39] Using the FSH/T ratio can serve as a measure for the combined effect of both hormones rather than relating to the level of each hormone separately and reduce potential effects of saturation of FSH and androgen receptors. This may support our finding of increased odds of a CVE in patients with a high FSH/T ratio.

STUDY LIMITATIONS. Our study has several limitations. The preclinical results were not adjusted for multiple statistical comparisons and also should be considered hypothesis-generating only. They relate to atherogenesis and characteristics of atherosclerotic plaque vulnerability, not to CVEs. To our knowledge, mouse models of spontaneous CVEs do not exist. The thickness of the fibrous cap could not be included as a measure of plaque vulnerability of more advanced atherosclerotic lesions, as atherogenesis measured in our mice on a normal, not high-fat, diet, does not feature fibrous caps. Clinically, our results in ADT recipients need to be validated in larger studies that prospectively collect both serum samples and CVEs.

CONCLUSIONS

We demonstrate that the adverse cardiovascular effects previously attributed to low or castrate T levels in men may be potentiated by FSH. We defined a hormonal state characterized by an elevated FSH/T ratio in men with established CVD that associated with the risk of developing a CVE following a stressful cardiovascular trigger such as ADT or surgery. ADT using GnRH-antagonists was associated with lower necrotic plaque burden, and clinically, with significantly lower serum FSH/T levels compared with GnRH-agonists and fewer CVEs.

TABLE 3 Patient Characteristics of the Nested Case-Control Study Within VISION Stratified by Serum FSH/T

	Ratio of FSH/T			
	Low (n = 421)	High (n = 420)	Total (N = 841)	*P* Value
Age, y	70.2 (45.1-90.0)	75.5 (45.1-93.9)	73.3 (45.1-93.9)	**<0.001**
BMI, kg/m^2	27.1 (12.7-102)	27.4 (16.5-48.4)	27.2 (12.7-102)	0.82
Ratio FSH/T	1.68 (0.044-3.04)	6.21 (3.05-545)	3.04 (0.044-545)	**<0.001**
Serum FSH, nM	24.2 (2.45-90.1)	68.0 (1.36-496)	38.1 (1.36-496)	**<0.001**
Serum T, nM	15.8 (3.57-48.4)	10.3 (0.32-30.6)	13.0 (0.32-48.4)	**<0.001**
Ethnicity				**0.002**
Asian	38 (9)	73 (17)	111 (13)	
European	372 (88)	338 (80)	710 (84)	
Other	11 (3)	9 (2)	20 (2)	
Patient in nursing home	1 (0)	6 (1)	7 (1)	0.057
Assistance with ADL	8 (2)	16 (4)	24 (3)	0.096
Smoker				0.076
Current	70 (17)	47 (11)	117 (14)	
Never	120 (29)	123 (29)	243 (29)	
Past	231 (55)	248 (59)	479 (57)	
Active or metastatic cancer	79 (19)	104 (25)	183 (22)	**0.035**
History of coronary artery disease	127 (30)	148 (35)	275 (33)	0.12
History of congestive heart failure	13 (3)	26 (6)	39 (5)	**0.032**
History of cerebrovascular event	35 (8)	50 (12)	85 (10)	0.084
History of peripheral vascular disease	28 (7)	57 (14)	85 (10)	**0.001**
History of diabetes	79 (19)	135 (32)	214 (25)	**<0.001**
History of hypertension	249 (59)	289 (69)	538 (64)	**0.004**
Preop systolic BP, mm Hg	144 (50-240)	145 (90-220)	145 (50-240)	0.54
Preop diastolic BP, mm Hg	80 (43-111)	79 (40-117)	80 (40-117)	0.30
Preop coronary revascularization	40 (19)	26 (12)	142 (17)	0.23
Preop eGFR <60 mL/min/1.73 m^2	102 (24)	159 (38)	261 (31)	**<0.001**
Medication				
Aspirin	126 (30)	141 (34)	267 (32)	0.24
Nitrate	24 (6)	19 (5)	43 (5)	0.44
Anticoagulant	17 (4)	38 (9)	55 (7)	**0.003**
ACE inhibitor/ARB	193 (46)	201 (48)	394 (47)	0.54
Beta-blocker	115 (27)	130 (31)	245 (29)	0.25
Statin	205 (49)	210 (50)	415 (49)	0.71
Plavix/ticlopidine	9 (2)	14 (3)	23 (3)	0.45
Alpha2-agonist	2 (0.5)	1 (0.2)	3 (0.4)	0.57
Rate control CCB	19 (5)	18 (4)	37 (4)	0.87
Dihydropyridine CCB	75 (18)	103 (25)	178 (21)	**0.017**
Therapeutic antithrombotic	4 (1)	10 (2)	14 (2)	0.11
Composite of stroke, myocardial infarction, nonfatal cardiac arrest, vascular death within 30 days	57 (14)	76 (18)	133 (16)	0.070
Event[a] within 30 days	185 (44)	236 (56)	421 (50)	**<0.001**

Values are median (range) or n (%). Significant *P* values (<0.05) are indicated in **bold**. [a]Event is defined as myocardial injury after noncardiac surgery, stroke, myocardial infarction, nonfatal cardiac arrest, or vascular death.

Abbreviations as in Tables 1 and 2.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: GnRH-antagonists, which optimally suppress FSH, appear to have an inherent superior cardiovascular risk profile and may thus be preferred in patients with PC with or at risk of CVD. Measuring the serum FSH/T ratio in patients with PC may assist in cardiovascular risk stratification during ADT, a potential cardiovascular challenging event.

TRANSLATIONAL OUTLOOK: Preclinically, a significant association between FSH/T and CCL21 existed. Therefore, the CCL21/CCR7 axis may be a potential mechanism by which FSH facilitates cardiovascular morbidity in a low/castrate T environment. Further exploration and correlation with clinical data are needed.

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KEY WORDS androgen-deprivation therapy, cardiovascular disease, follicle-stimulating hormone, GnRH-antagonist, prostate cancer

APPENDIX For a Methodology section, and supplemental Figures and Tables, please see the online version of this article.