ORIGINAL RESEARCH

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Differences in Hypercholesterolemia and Atherogenesis Induced by Common Androgen Deprivation Therapies in Male Mice

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***Background*—**Treatment of prostate cancer often involves androgen deprivation therapy (ADT) by gonadotropin-releasing hormone (GnRH) receptor agonists, GnRH receptor antagonists, or orchiectomy. ADT may increase the rate of cardiovascular disease events, but recent clinical studies suggested that not all means of ADT carry the same risk, raising the possibility of non–testosterone-mediated effects of different forms of ADT on atherosclerosis. Here we compared effects of ADT on atherosclerosis in intact and orchiectomized *Apoe*-deficient mice.

***Methods and Results*—**Chow-fed *Apoe*-deficient mice were allocated to orchiectomy and/or monthly injections with the GnRH receptor agonist leuprolide or the GnRH receptor antagonist degarelix. Atherosclerosis was quantified at 26 weeks of age in the aortic arch by en face examination and in the aortic root by histology. In intact *Apoe*-deficient mice, all types of ADT reduced testosterone production to castration levels. Although hypercholesterolemia was accentuated in leuprolide-treated mice, the amount and composition of atherosclerosis was not different between the different types of ADT. In orchiectomized *Apoe*-deficient mice, leuprolide, but not degarelix, augmented hypercholesterolemia, changed body, thymus, and spleen weights, and increased atherosclerosis in the aortic root. No direct effects of the drugs were detectable on cytokine secretion from murine bone marrow–derived macrophages or on splenocyte proliferation.

***Conclusions*—**No differences in the development of atherosclerosis were detected among groups of intact *Apoe*-deficient mice treated with different types of ADT. A pro-atherogenic, possibly cholesterol-mediated, effect of leuprolide was seen in orchiectomized mice that might be relevant for understanding the potential cardiovascular risk associated with GnRH agonist-based ADT. **(*J Am Heart Assoc*. 2016;5:e002800 doi: 10.1161/JAHA.115.002800)**

Key Words: androgen deprivation therapy • atherosclerosis • inflammation • testosterone

Prostate cancer (PCa) is one of the most common malignancies in aging men across the world.[1] An important part of the treatment for many PCa patients is androgen deprivation therapy (ADT), which lowers testosterone either by blockade or agonist-induced desensitization of pituitary gonadotropin-releasing hormone (GnRH) receptors or the surgical removal of the testes.[2] ADT is the first-line treatment for metastatic PCa and an adjuvant therapy to radiotherapy for locally advanced PCa. Unfortunately, apart from improving survival and quality of life, ADT treatment has been associated with an excess risk of cardiovascular disease (CVD), including myocardial infarction.[2–7]

Traditionally the increased risk of CVD in patients treated with ADT has been attributed to alterations in metabolic parameters that are known or assumed to influence the development of atherosclerosis, such as an increase in subcutaneous fat, total cholesterol, triglycerides, and decreased insulin sensitivity.[3] These changes appear to result from testosterone deficiency, since testosterone supplementation in hypogonadal men has opposite effects.[8] Recent studies, however, have suggested that the number of CVD events in PCa patients differs between different types of ADT, indicating that other mechanisms than the mere lack of testosterone is involved. In observational studies and a post hoc analysis of randomized controlled trials, a higher rate of CVD events was found in patients treated with GnRH receptor agonists compared with bilateral orchiectomy or GnRH antagonists.[4,5,9] These studies are hypothesis-generating and the findings await confirmation in randomized trials with

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CVD as a prespecified end point, but the idea of a divergent effect of orchiectomy, GnRH agonists and antagonists on atherosclerosis-related disease is supported by experimental studies. Atherosclerosis is a lipoprotein-driven immunoinflammatory disease,[10] and the presence and functional role of GnRH receptors have been documented in both a murine macrophage cell line and in rat and human lymphocytes in vitro.[11–13] Furthermore, stimulation of the GnRH receptor with agonists have been shown to exacerbate lupus in castrated mice,[14] whereas treatment with an antagonist delayed the onset of autoimmune diabetes,[15] both indicating potential modulations of the immune response. Two studies on initial foam cell accumulation in hypercholesterolemic mice have recently been reported, indicating an anti-atherogenic effect of GnRH receptor antagonists compared to placebo or GnRH agonists, respectively,[16,17] but effects on more advanced atherosclerotic lesions have not been addressed.

In the present study, we addressed the hypothesis that different types of ADT may exert variable effects on the development of fibroatheromatous atherosclerosis in *Apoe*-deficient mice.

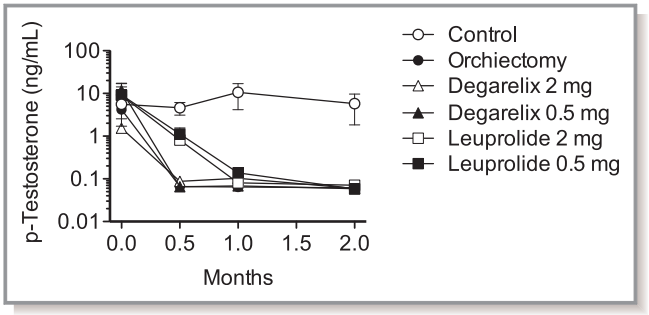

Figure 1. Effect of different doses of leuprolide and degarelix in male mice. The decline was slower with leuprolide, but both drugs led to measured testosterone levels indistinguishable from orchiectomized mice at both dosing levels tested. Number of mice in each group: Control (n=4), orchiectomy (n=4), degarelix 2 mg (n=3), degarelix 0.5 mg (n=4), leuprolide 2 mg (n=4), leuprolide 0.5 mg (n=4).

Methods

Mice

Homozygous *Apoe*-deficient mice (B6.129P2-*Apoe*^{tm1Unc}) (n=124), backcrossed more than 10 generations into the C57BL/6 background, and wild-type C57BL/6NTac mice were obtained from Taconic (Ry, Denmark). The mice had free access to tap water and were fed chow diet. All procedures were approved by the Danish Animal Experiments Inspectorate and were in accordance with institutional guidelines and the Directive 2010/63/EU of the European Parliament.

Bilateral Orchiectomy

Bilateral orchiectomy was performed in some *Apoe*-deficient mice by trained personal at Taconic (Ry, Denmark) when they were 5 weeks old. The mice were anesthetized by an intraperitoneal injection of 0.1 mL of a mixture containing xylazine (Rompun) (20 mg/mL, 0.39 mL), ketamine (Ketaminol) (100 mg/mL, 0.39 mL), acepromazine (Calmivet) (5 mg/mL, 0.24 mL), and isotonic NaCl (1.98 mL). Analgesia was secured using carprofen (50 mg/mL, 0.1 mL) injected subcutaneously.

Administration of ADT

Groups of intact and orchiectomized *Apoe*-deficient mice were injected subcutaneously with saline, 0.5 mg leuprolide (Eligard), or 0.5 mg degarelix (Firmagon) every 4 weeks commencing at 8 weeks of age (Figure 1). Both Eligard and Firmagon were reconstituted as indicated in the package insert for human use and the volume of suspension appropriate to deliver a dose of 0.5 mg was injected subcutaneously. Three mice died during the course of the study: one saline-injected orchiectomized mouse, one leuprolide-injected mouse, and one degarelix-injected mouse.

Atherosclerosis Quantification

At 26 weeks of age, the *Apoe*-deficient mice were anesthetized with an intraperitoneal injection of pentobarbital (250 mg/kg) and lidocaine (20 mg/kg) and euthanized by withdrawal of blood from the right ventricle. The mice were flushed with Cardioplex solution, perfusion-fixed with 4% formaldehyde via the left ventricle, immersed in 4% formaldehyde for 6 hours, and stored in phosphate-buffered saline at 5°C. For quantification of atherosclerosis in the aortic root, the top half of the heart was embedded in paraffin. Sections taken at 80-µm intervals from the commissures of the aortic leaflets and upward (3 levels in total) were stained with orcein and sirius red. Atherosclerotic plaque size was quantified in orcein-stained sections by computer-assisted morphometry (ImageJ, NIH). Necrotic core area was quantified in sirius red–stained sections. Necrosis was defined as the absence of collagen and viable cells, the latter judged from the orcein-stained sections.

Aortic arch lesion coverage was measured en face using a standard method. Briefly, the aorta down to the first intercostal artery was cut open, stained with Oil Red O for 10 minutes at 37°C, and mounted on a microscope slide with AquaTex. Slides were scanned and analyzed using ImageJ.

Blood Analysis

Nonfasting total plasma cholesterol was measured using a commercially available kit (Cholesterol CHOD-PAP, Roche/

Hitachi). Distribution of cholesterol across size-fractionated lipoproteins was measured in pooled samples from each group by fast protein liquid chromatography in the Gaubius Laboratory, (TNO Biosciences, the Netherlands). Testosterone was measured in plasma samples using a commercially available mouse competitive ELISA kit with a reported analytical sensitivity limit of ≈0.1 ng/mL (DRG Diagnostics).

Cell Culture Assays

Male C57BL/6NTac mice (9–12 weeks old) were euthanized by cervical dislocation. Bone marrow cells were isolated from femurs and tibias and cultured in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 10% L-cell conditioned medium for 7 days to generate bone marrow–derived macrophages (BMM) as previously described.[18] BMMs were seeded at 1.5×10^5 cells/well in 48-well plates and after 24 hours polarized towards an M1 phenotype with lipopolysaccharide (LPS, γ-irradiated, L4391, Sigma-Aldrich) at different concentrations: 1 ng/mL, 10 ng/mL, and 100 ng/mL. Leuprolide acetate (H4060, Bachem) and degarelix (Ferring Pharmaceuticals) were co-incubated with the BMMs for 24 hours at a concentration of 10^{-6} mol/L. Cytokine secretion into the cell culture medium was subsequently analyzed using IL-6, tumor necrosis factor-α, and interferon-γ ELISAs (Ready-SET-Go Mouse kits, eBioscience).

Splenocytes were isolated and seeded at 2×10^5 cells/well in 96-well plates as previously described.[19] Cells were activated with soluble anti-mouse CD3ε antibody (BioLegend, clone: 145-2C11) at different concentrations (0.3, 1.0, and 3.0 μg/mL) for 48 hours. Assessment of cell proliferation was made using the Quick Cell Proliferation Assay Kit (Abcam).

GnRH Receptor Expression in Tissue and Cultured Cells

RNA was purified using TRIzol Reagent (Ambient #15596018) from thymus, spleen, pituitary gland, cultured BMMs, and cultured splenocytes obtained from male C57BL/6NTac mice (9–12 weeks of age). BMMs and splenocytes were either naïve or activated for 24 hours with 100 ng/mL LPS or for 48 hours with 3.0 μg soluble anti-mouse CD3ε, respectively.

Conversion to cDNA was performed with iScript cDNA synthesis kit (BioRad #170-8891) on 1 μg and 700 ng total RNA from tissue samples and cultured cells, respectively. For *Gnrhr* reverse transcription polymerase chain reaction (RT-PCR), 1 μL of pituitary cDNA, and 7.5 μL of spleen or thymus cDNA were used as templates using the following PCR program: 94°C, 3 minutes for ×1 cycle, 94°C for 20 s, 56°C for 30 s, and 72°C for 1 minute ×35 cycles, and 72°C for 7 minutes ×1 cycle. β2-microglobulin was used as the reference gene. For the β2-microglobulin RT-PCR, 0.5 μL cDNA was used as template for all tissue and cell samples using the following PCR program: 94°C, 3 minutes for ×1 cycle, 94°C for 20 s, 60°C for 30 s, and 72°C for 25 s ×35 cycles, and 72°C for 7 minutes ×1 cycle. The primers 5′-TTCCACAGTGGTGGCATCAG-3′ and 5′-GTCCAGCAGACGACAAAGGA-3′ were used for amplification of the GnRH receptor, whereas β2-microglobulin was detected using the primers 5′-CTGCTACGTAACACAGTTCCACCC-3′ and 5′-CATGATGCTTGATCACATGTCTCG-3′.

Statistics

All statistical analyses were performed using the Prism statistical software (GraphPad, San Diego, CA). Data were tested for normality using D'Agostino & Pearson omnibus normality test, and normally distributed data were analyzed by 1-way ANOVA followed by Newman–Keuls post-test. Longitudinal data such as weight and plasma cholesterol were analyzed by 2-way ANOVA with repeated measures followed by Bonferroni post-test. Necrotic core size data were log-transformed before ANOVA was performed. Non-normally distributed data were analyzed by the Kruskal–Wallis test with Dunn's multiple comparison test. Data are shown as mean ± SEM. In all cases $P<0.05$ was considered to be significant and the following symbols were used to indicate the size of *P*-values: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Results

Effective Treatment Dose

First we conducted a study to determine the lowest effective dose of the GnRH agonist leuprolide and the antagonist degarelix that reduced plasma testosterone to castration levels in C57BL/6NTac mice (Figure 1). Leuprolide or degarelix was injected subcutaneously at a dose of 2 or 0.5 mg per mouse each month in line with dosing regimens reported by others.[20] Both high and low doses reduced plasma testosterone to the level of orchiectomized mice and we chose the lower dose (0.5 mg) for the study. Attempts were made to inject leuprolide at an even lower dose (0.2 mg), but at this dosing level we found little effect on plasma testosterone, which may partly be caused by difficulties in administering the small volume of the viscous depot formulation (data not shown).

ADT in *Apoe*-Deficient Mice

To analyze the effects of different forms of ADT, we allocated *Apoe*-deficient mice into 6 groups as outlined in Figure 2. This

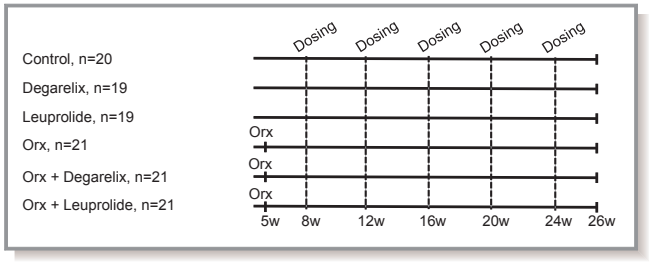

Figure 2. Outline of the study. The ages of the mice at the designated time points (weeks) are indicated at the bottom. Degarelix and leuprolide were injected subcutaneously at a dose of 0.5 mg every 4 weeks. Control mice were injected with saline. Orx, orchiectomy; n, number of mice in each group.

design allowed for 2 prespecified statistical comparisons. First, we planned to compare groups of mice subjected to either orchiectomy or monthly injections of leuprolide, degarelix, or saline to address our main hypothesis. Second, we wanted to assess for potential non–testosterone-mediated effects of both drugs by comparing orchiectomized-only mice with groups of orchiectomized mice that also received degarelix or leuprolide. Orchiectomy was performed when the mice were 5 weeks old. Administration of drugs was started at 8 weeks of age.

General Effects of Different Forms of ADT

Testosterone was measured 1, 2, and 4 months after initiation of ADT. As expected, plasma testosterone concentration decreased more slowly in the leuprolide compared to the degarelix group (Figure 3A), but the differences between ADT groups were not statistically significant. The decrease in testosterone levels within each group was accompanied by retarded weight gain during the 18 weeks of treatment (Figure 3B). Following castration, total cholesterol increased significantly in all groups (Figure 3C). The mice treated with orchiectomy or degarelix reached a plateau after 2 months and plasma concentrations stayed constant during the remainder of the study. In leuprolide-treated mice, total cholesterol increased further through the latter half of the study and ended up significantly higher than that of the other castrated groups. Size-exclusion chromatography of pooled plasma lipoproteins from each group showed that the main change in total cholesterol was due to changes in the very low-density lipoprotein–sized fraction (Figure 3D).

To evaluate the possibility for a direct effect of GnRH agonists acting through immune cell GnRH receptors, we tested for the presence of GnRH receptor mRNA by RT-PCR in thymus and spleen, but only found it faintly detectable in thymus (Figure 3E). Significant increases in thymus weight were seen with orchiectomy or degarelix, but not with leuprolide (Figure 3F). Conversely, spleen weights were unaffected in orchiectomized or degarelix-treated mice, but reduced in leuprolide-treated mice (Figure 3G).

To assess for potential non–testosterone-mediated effects of ADT, we performed similar analyses in orchiectomized mice treated with leuprolide and degarelix (Figure 4). Degarelix induced no significant changes in the measured parameters in orchiectomized mice, but similar to the results in intact mice, leuprolide was followed by increased weight gain, higher cholesterol levels, and lower thymus and spleen weights.

Impact of Different Forms of ADT on Atherosclerosis

To assess the effect of various forms of ADT on the development of atherosclerosis, we quantified the amount of disease in the aortic arch and aortic root by en face measurements and histology, respectively. Orchiectomized mice and mice receiving leuprolide or degarelix had significantly increased atherosclerosis by both measures compared to control mice (Figure 5A and 5B). In contrast to previous studies reporting effects of castration on initial necrotic core formation in hyperlipidemic mice,[17,21] we did not find significant increases in absolute or relative cross-sectional area of the necrotic core in ADT compared to saline-treated control mice (Figure 5C and 5D).

In orchiectomized mice, the addition of leuprolide, but not degarelix, led to a further increase in atherosclerosis development, which was, however, restricted to the aortic root (Figure 6A and 6B). The increase may, at least in part, be a reflection of the higher total cholesterol burden in this group. Notably, however, we were not able to detect any significant correlations between total cholesterol levels and aortic root or arch atherosclerosis within any of the groups. Necrotic core formation was not significantly affected by leuprolide or degarelix in orchiectomized mice (Figure 6C and 6D).

Cell Studies

Previous experiments with LPS-treated RAW264.7 macrophages[12] and anti-CD3 treated rat, mainly female, T lymphocytes[13,22] have reported effects of GnRH receptor stimulation on cellular activation and proliferation. These findings could be relevant for atherosclerosis. LPS activates macrophages by binding to Toll-like receptor 4, which also binds modified LDL and has been implicated in murine atherosclerosis.[23] Furthermore, T lymphocytes recognizing atherosclerosis-related auto-antigens modulate the progression of murine atherosclerosis.[24] We therefore re-investigated the impact of GnRH receptor agonists and antagonists on macrophage and lymphocyte activation using primary cells obtained from male mice. First, we cultured BMM from male C57BL/6NTac mice and assessed their activation by increasing concentrations of

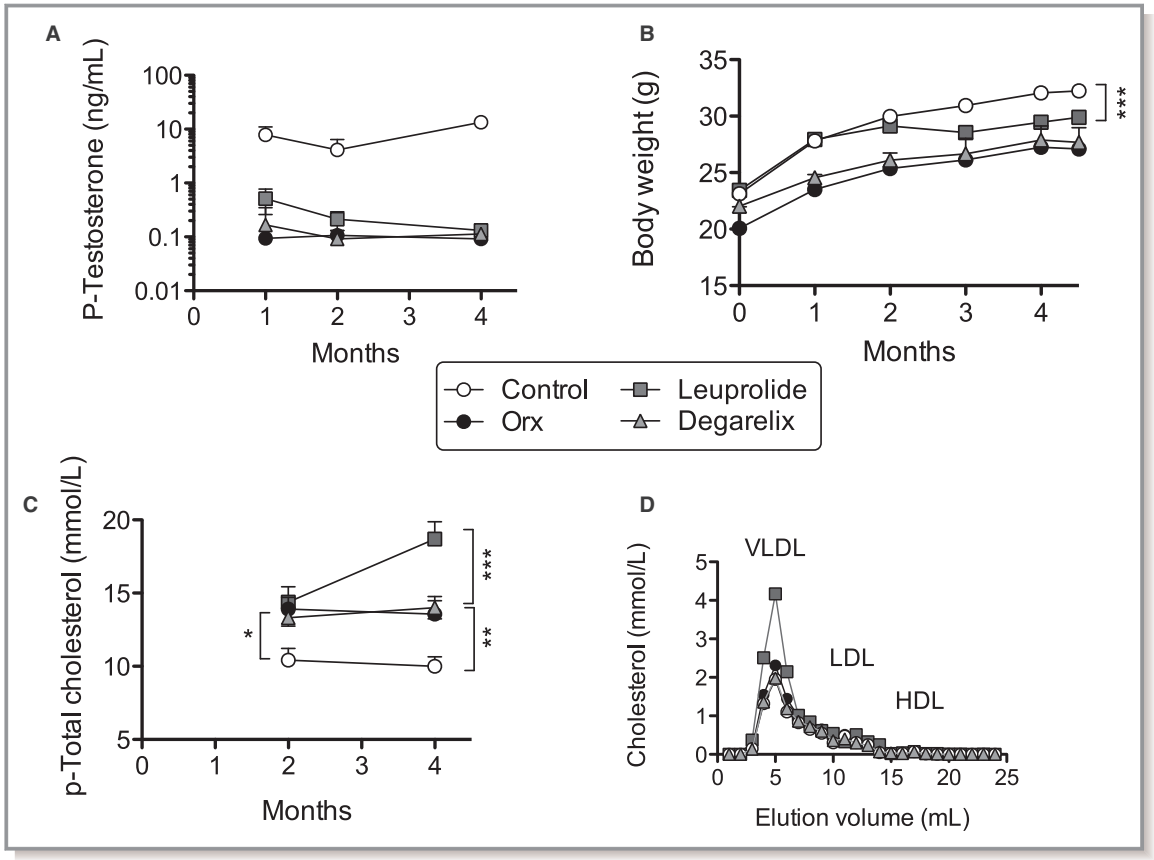

Figure 3. Effects of different forms of ADT in *Apoe*-deficient mice. A, Plasma concentration of testosterone in mice after 1, 2, and 4 months of treatment. A random subset of mice from each group was analyzed with (n=10, 8, 12), (n=9, 9, 12), (n=9, 8, 11), and (n=7, 8, 12) measurements performed in the control, leuprolide, degarelix, and orchiectomized (Orx) group at the 1-, 2-, and 4-month time points, respectively. B, Body weights in the different groups. A significant difference in weight was found between control and ADT groups, and leuprolide-treated mice had higher final body weight than mice of the other ADT groups. Number of animals in each group as indicated in Figure 2. C, Plasma total cholesterol concentration at 2 and 4 months of treatment. After 2 months of treatment, total cholesterol levels were significantly higher in ADT groups than in controls. Levels in leuprolide-treated mice increased further and were significantly higher than other ADT groups at 4 months of treatment. For controls (n=20, 20), leuprolide (n=19, 19), degarelix (n=18, 18), and orx (n=21, 21) measurements performed at 2 and 4 months, respectively. D, Distribution of cholesterol across size-fractionated lipoprotein classes measured in pooled plasma from each group obtained 4 months after treatment initiation. E, Detection of GnRH receptor mRNA in tissue samples from pituitary gland (Pit) and thymus. No GnRH receptor mRNA was detected in spleen tissue from male mice. Pit-RT, Control reaction with omission of reverse transcriptase. Expected band sizes were 237 bp (GnRH receptor) and 240 bp (β_2-microglobulin). F, Thymus weights. G, Spleen weights. *P*-values were calculated with Bonferroni post-test after 2-way ANOVA with repeated measures (B and C), with Dunn's multiple comparison test after significant Kruskal–Wallis test (F) and Neuman–Keuls post-test after significant ANOVA (G). $*P<0.05$, $**P<0.01$, $***P<0.001$. ADT, androgen deprivation therapy; GnRH, gonadotropin-releasing hormone; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.

LPS under concurrent stimulation or inhibition of the GnRH receptor with leuprolide and degarelix, respectively. Although the GnRH receptor was detectable in murine LPS-stimulated BMM (Figure 7A), no differences were found in the secretion of pro-inflammatory cytokines IL-6, monocyte chemoattractant protein-1, and tumor necrosis factor-α (Figure 7B through 7D). Furthermore, we found no differences of leuprolide or degarelix on the proliferation of anti-CD3 stimulated splenocytes (Figure 7E), and also were not able to detect GnRH receptor mRNA in such cells (Figure 7A).

Discussion

In the present study, we tested the hypothesis that different types of ADT exert differential effects on the development of atherosclerosis in *Apoe*-deficient mice. This idea was suggested by epidemiological studies and a recent meta-analysis of clinical trials conducted in ADT-treated men with preexisting CVD. We found that all forms of ADT reduced testosterone production to levels of orchiectomized mice, increased plasma total cholesterol, and accelerated atherosclerosis.

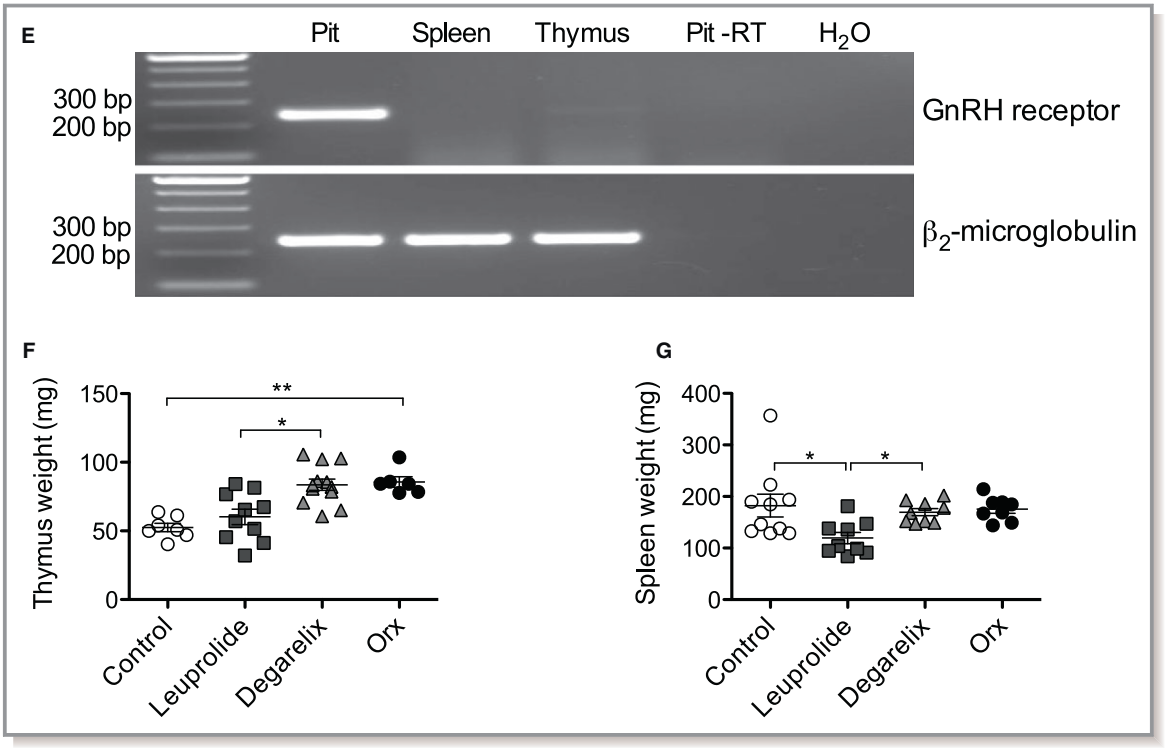

Figure 3. continued.

When applied in intact *Apoe*-deficient mice, the GnRH agonist leuprolide aggravated hypercholesterolemia, but no significant differential effects of the type of ADT on the amount or composition of atherosclerosis were detected. In orchiectomized *Apoe*-deficient mice, however, addition of leuprolide increased both hypercholesterolemia and atherosclerosis in the aortic root.

Effects of ADT on CVD

Among men with early stage PCa, atherosclerotic CVD rather than the cancer itself is the leading cause of death.[25] Epidemiological studies have implicated that ADT may be partly responsible for the increased number of CVD events, which is observed shortly after treatment initiation and most notably in patients with preexisting atherosclerotic CVD.[4,9] These observations are strongest for GnRH agonists, the most common form of ADT, whereas data on orchiectomy are less consistent and little information still exists pertaining to the newer GnRH antagonists.[9,26] In 2010, the combined observations led the U.S. Food and Drug Administration to put a warning label on GnRH agonists regarding the suspected increased risk of CVD (http://www.fda.gov/drugs/drugsafety/ucm229986.htm).

The mechanisms linking ADT to atherosclerotic CVD, however, remain poorly understood. Traditionally the effects have been ascribed to testosterone deficiency and its consequences for metabolism and lipid profile, which include increased low-density lipoprotein (LDL) and high-density lipoprotein cholesterol, as well as changes in triglycerides, fat distribution, and insulin sensitivity resembling those in patients with the metabolic syndrome.[3] However, even though LDL particles fuel the atherosclerotic disease process and it is well known that changes in LDL levels upon statin treatment can exert relatively rapid effects on CVD end points,[27] the ADT-associated LDL increase is modest (<10%),[3] and it is doubtful whether the combined metabolic changes associated with testosterone deficiency can provide a comprehensive explanation for the acutely increased CVD risk associated with ADT initiation. Interestingly, recent observational studies and a meta-analysis of randomized controlled trials suggested that non–testosterone-mediated effects of GnRH agonists may be important for the increased risk of atherosclerotic CVD.[4,5] Bosco et al, using registry data, found that GnRH agonist-based ADT was associated with a higher risk of CVD than surgical orchiectomy.[5] Furthermore, Albertsen et al, in a meta-analysis of trials comparing the GnRH agonists leuprolide and goserelin to the GnRH antagonist degarelix, found that agonist treatment was associated with a higher cardiovascular event rate in the subgroup of men with preexisting CVD.[4]

Though these data might suggest a causal relationship, they do not prove one. Observational studies are limited by selection bias because the choice of treatment is not random,

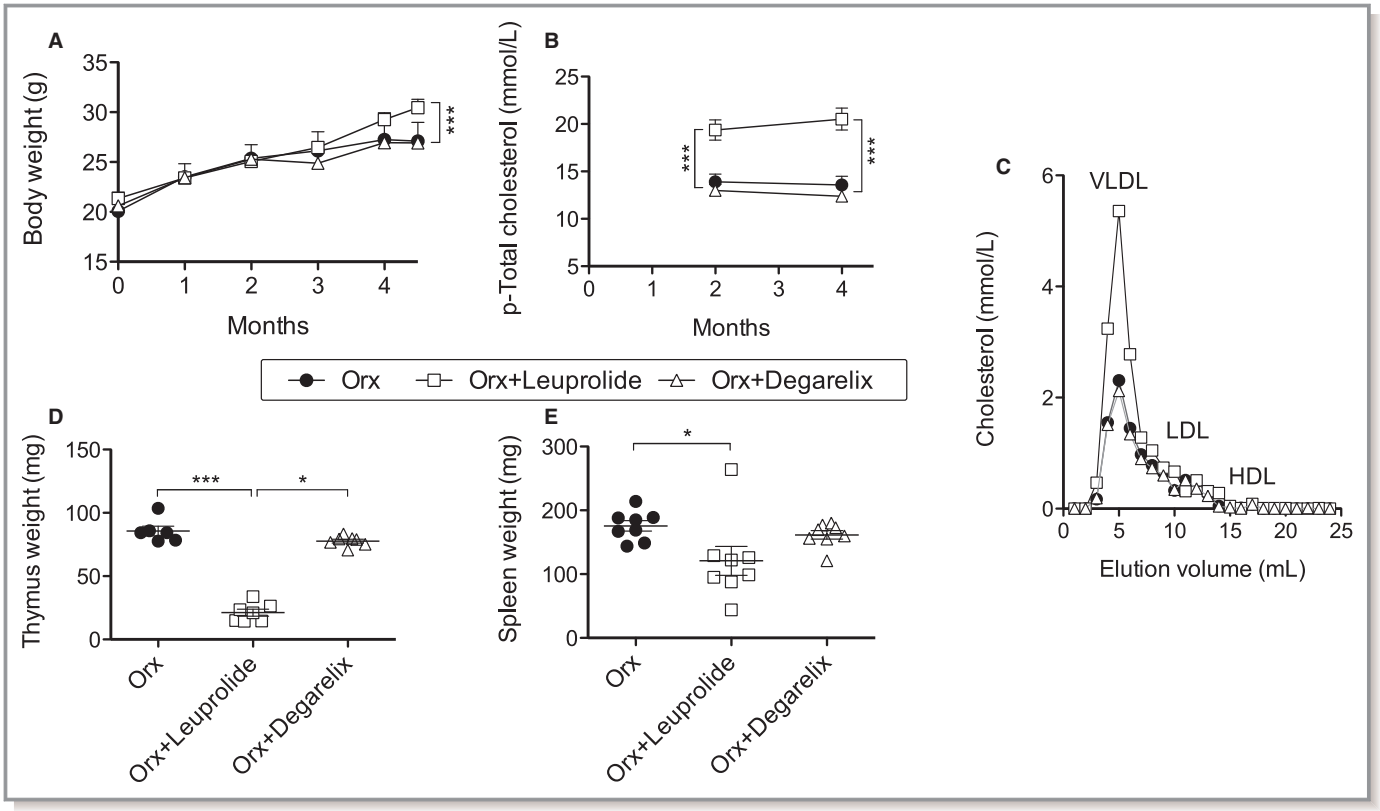

Figure 4. Effects of leuprolide and degarelix in orchiectomized *Apoe*-deficient mice. A, Body weights in orchiectomized (Orx) groups. Final mean weight of leuprolide-treated mice was significantly higher than that of the other groups. Number of animals in each group as indicated in Figure 2. B, Plasma total cholesterol measured after 2 and 4 months of treatment. At both time points, mean total cholesterol levels in the leuprolide-treated group were higher than in the other groups. Number of measurements were (n=21, 21), (n=20, 21), and (n=20, 21) in the orx, orx+leuprolide, and orx+degarelix groups at the 2- and 4-month time points, respectively. C, Distribution of cholesterol across size-fractionated lipoprotein classes measured in pooled plasma from each group obtained 4 months after treatment initiation. D, Thymus weights. E, Spleen weights. *P*-values were calculated with Bonferroni post-test after a 2-way ANOVA with repeated measures (A and B) or Dunn's multiple comparison test after significant Kruskal–Wallis test (D and E). **P*<0.05, ****P*<0.001.

and some of the characteristics of PCa patients used to guide those decisions (eg, comorbidity, age, etc) could well influence the rate of CVD events. Furthermore, being a post-hoc and subgroup analysis based on pooled data, the finding by Albertsen et al[4] needs confirmation in randomized trials with prespecified CVD end points.

Effects on ADT on Experimental Atherosclerosis

The *Apoe*-deficient mouse model, like other murine models, is not a model of vulnerable plaque,[28] but key mechanisms that increase plaque vulnerability and thus clinical CVD in humans, such as lipoprotein-driven inflammation and necrotic core formation, can be studied.[29] This supports the relevance of this experimental model to probe for the potential non–testosterone-mediated effects of GnRH agonists suggested by clinical data.

We found that different forms of ADT all accelerated atherogenesis in intact *Apoe*-deficient mice, but found no detectable differences among ADT-treated groups. This indicates that direct drug effects and other differences between the modes of castration are not important for the development of atherosclerosis in mice. In particular, it is in contrast with a previous conclusion that circulating gonadotropins (ie, follicle-stimulating hormone and luteinizing hormone levels), which are high with orchiectomy but low with GnRH antagonists, influence atherogenesis in mice.[16] On the other hand, a non–testosterone-mediated, pro-atherogenic effect of the GnRH agonist leuprolide in orchiectomized mice was detected, and it was accompanied by several other effects on total cholesterol, body weight, thymus, and spleen weights that were exclusive to this type of ADT.

Although these non–testosterone-mediated effects of leuprolide are notable, the data clearly raise several questions. Why did leuprolide only appear to be pro-atherogenic in orchiectomized mice? Leuprolide led to a slower decline in testosterone compared to orchiectomy or antagonist treatment and unlike common practice in humans, we did not compensate for this with supplemental anti-androgen treatment. Considering the possible protective effects of

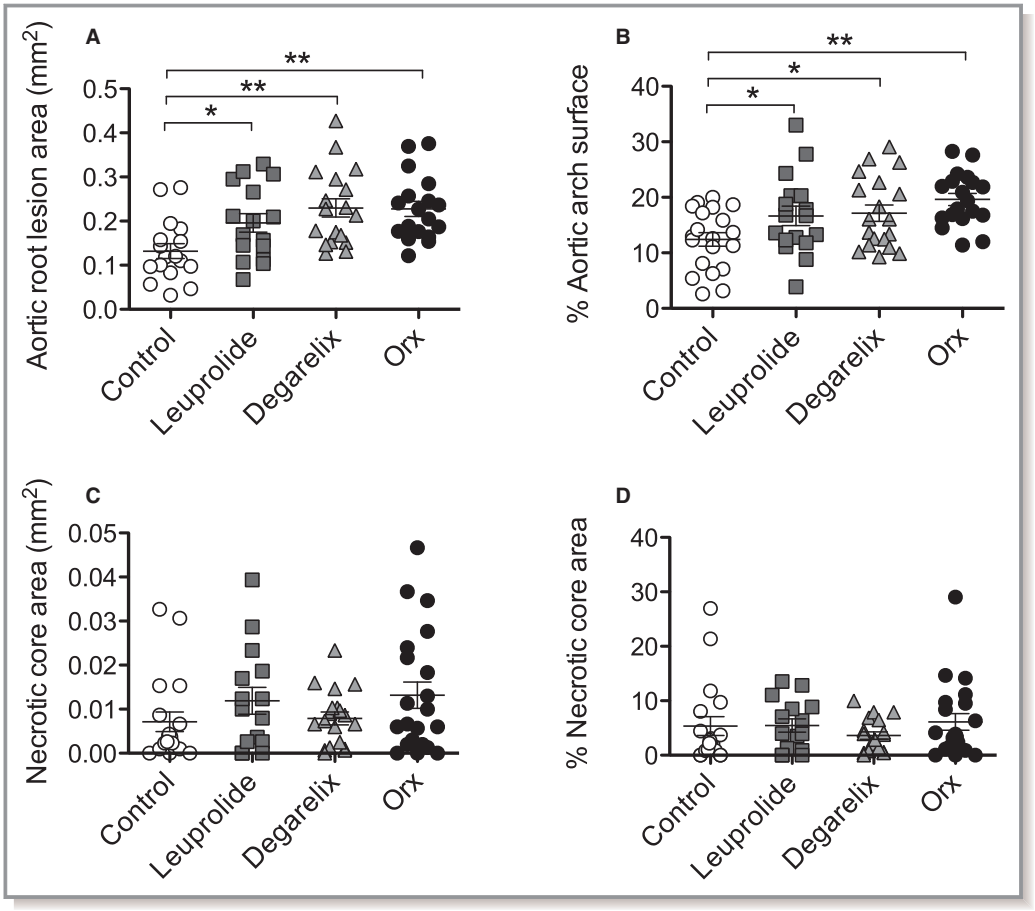

Figure 5. Atherosclerosis in *Apoe*-deficient mice subjected to different forms of ADT. A and B, All castration methods accelerated the development of atherosclerosis in the aortic root (A) and aortic arch (B) to a similar degree. C and D, Necrotic core size quantified in lesions from the aortic root. No significant differences in absolute or relative amount of necrosis were found between the groups. *P*-values were calculated using Newman–Keuls post-test after significant ANOVA (A through D). **P*<0.05, ***P*<0.01.

testosterone,[30] the higher plasma testosterone level encountered during the study period may have masked a pro-atherogenic effect of leuprolide in intact mice, but could not interfere when the comparison was done in orchiectomized mice. Importantly, unlike humans, mice do not produce androgen in the adrenal glands, which might otherwise confound the results.[31] Why was the effect only noticeable in the aortic root? It could be a Type 2 error in quantifying atherosclerosis in the aortic arch, or be caused by the later stage of lesion development in the aortic root, which would be consistent with observational studies only showing an effect in patients with known CVD.[4,9] However, it also cannot be excluded that the leuprolide-effect on atherosclerosis in the aortic root was a chance finding altogether.

Increases in total cholesterol were found in both intact and orchiectomized leuprolide-treated mice, and size-exclusion chromatography indicated that this was due to a further increase in the very low-density lipoprotein–sized remnant lipoproteins that accumulate in *Apoe*-deficient mice. Whether this finding is relevant to the clinical setting is unclear. One study conducted a pooled analysis of lipid measurements from 3 prospective studies comparing the antagonist abarelix to either leuprolide monotherapy or combined therapy with leuprolide and an androgen blocker, but found no consistent differences in LDL concentrations between groups.[32] Newer trials comparing ADTs have been conducted, but it is still not clear how different types of ADT treatment affect levels of different apoB-containing lipoproteins because surprisingly little information regarding atherosclerosis risk factors has been reported.[33] Clearly, this potentially important question warrants further investigation.

Comparison to Previous Studies

Two previous studies have investigated the impact of GnRH agonists and/or antagonist on very early atherosclerotic lesion formation.[16,17] In 12-week-old chow-fed *Apoe*-deficient mice treated with the GnRH antagonist cetrorelix for 8 weeks,

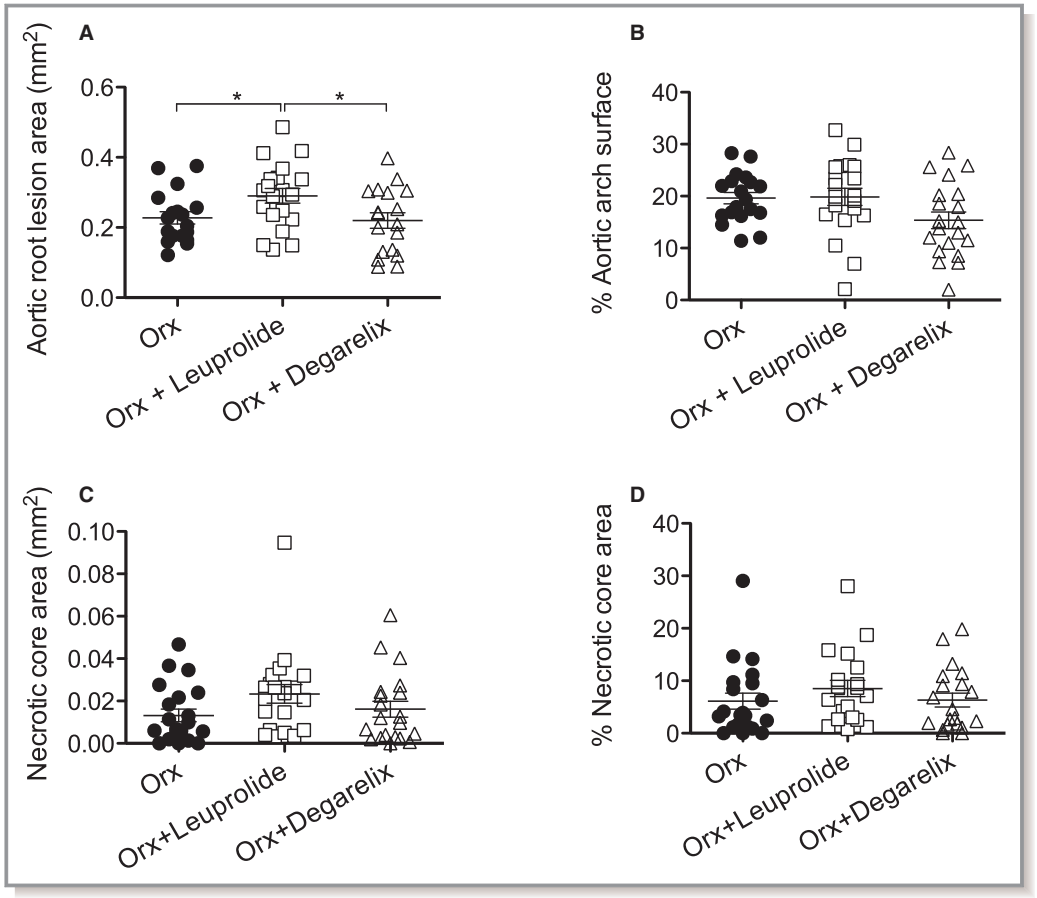

Figure 6. Atherosclerosis in orchiectomized *Apoe*-deficient mice receiving leuprolide or degarelix. A and B, Aortic root atherosclerosis was significantly increased in orchiectomized mice treated with leuprolide compared to other groups. En face analysis of the aortic arch did not demonstrate any significant effects of leuprolide or degarelix on atherogenesis. C and D, No significant differences were found in absolute or relative necrotic core areas between groups. *P*-values were calculated using Newman–Keuls post-test after significant ANOVA (A through D). **P*<0.05.

von Dehn et al found initial foam cell accumulation to be reduced compared to controls.[16] Hopmans et al[17] reported significantly increased atherosclerosis in chow-fed *Ldlr*-deficient mice following orchiectomy or treatment with leuprolide, whereas degarelix-treated animals were in-between and not significantly different from either leuprolide-treated or control mice. Notably, in both these studies, lesion size was many-fold smaller than in our study. Contrasting with our findings, Hopmans et al also found a significant difference in plaque necrosis between degarelix- and leuprolide-treated mice. Potential explanations for this discrepancy may be the much later stage of necrotic core formation in the present study or potential differences in the definition of necrosis.

ADT and the Immune System

For more than a century it has been recognized that androgen deprivation affects the immune system,[34] and the GnRH receptor has been found on immune cells from humans[35,36] and rodents.[13,37] Interestingly, stimulation of the GnRH receptor exacerbated lupus in castrated female mice[14,38] and treatment with GnRH receptor antagonist delayed the onset of autoimmune diabetes[15] and increased the survival rate among lupus-prone mice.[14] Furthermore, treatment of pregnant rats with a GnRH receptor agonist caused an increase in the cytokine interferon-γ and a decrease in IL-4 production from anti-CD3 stimulated lymphocytes,[13] indicating stimulation of pro-inflammatory Th1-type responses, which are known to be important in atherosclerosis.[39] In the present study, we tested for such effects in murine macrophage and lymphocyte cell culture assays, but failed to detect any changes following GnRH receptor blockade or stimulation. The assays used were designed to search for effects previously reported in the rat[13] or in immortalized cells,[12] and the results do not exclude that other facets of murine immune cell function could be modifiable by GnRH receptor signaling, such as the secretion of other pro-inflammatory or anti-inflammatory cytokines.

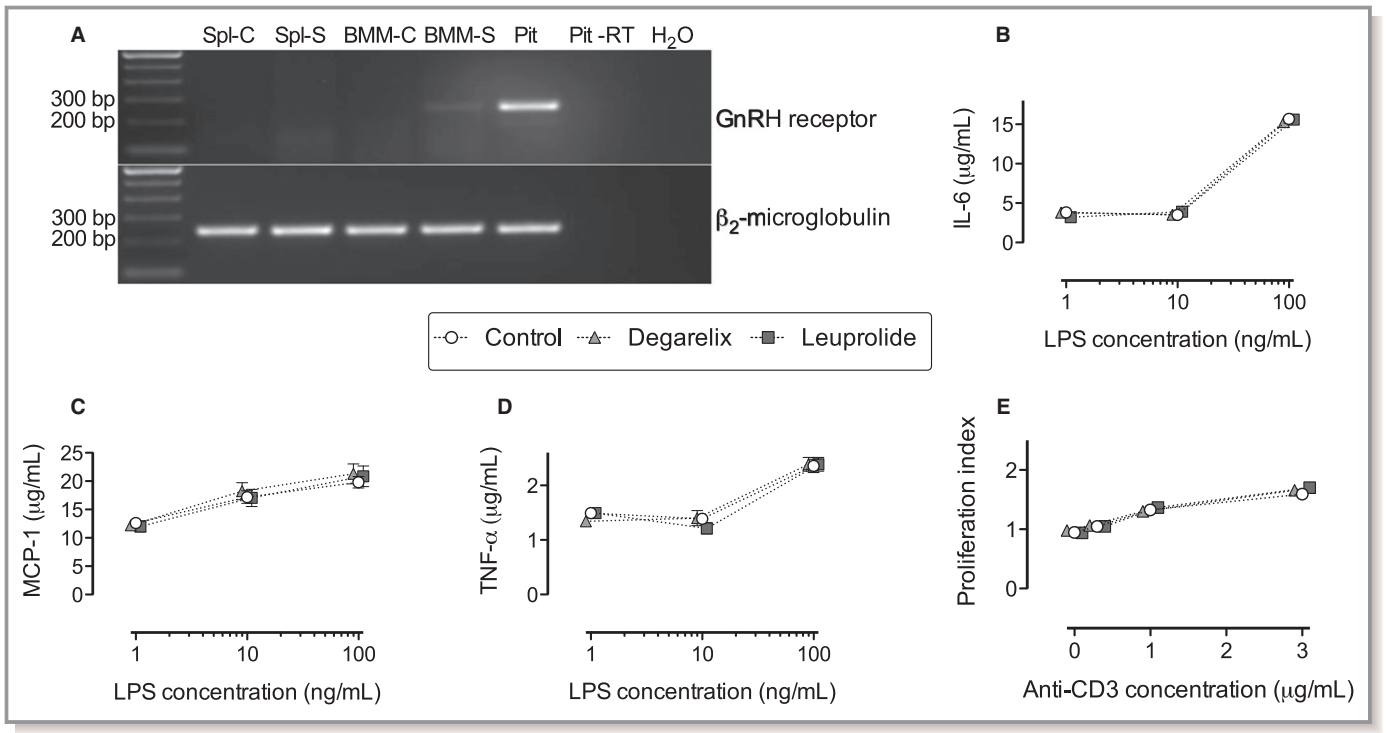

Figure 7. ADT and immune cell activation. A, Detection of GnRH receptor mRNA in cultured BMMs and splenocytes. Spl-S and Spl-C indicate splenocytes cultured with or without 3 μg/mL anti-CD3 for 48 h, respectively. BMM-S and BMM-C indicate bone marrow–derived macrophages cultured with or without 100 ng/mL LPS for 24 h, respectively. Pit, pituitary gland. Pit-RT, Control reaction with omission of reverse transcriptase. B through D, IL-6, MCP-1 and TNF-α secretion from LPS-stimulated macrophages in the presence of leuprolide or degarelix at a concentration of 10^{-6} mol/L. No significant differences were observed between the groups for any concentration of LPS. Number of biological replicates for measurements of IL-6 at LPS concentrations of 1, 10, and 100 ng/mL: Control (n=16, 26, 27); leuprolide (n=18, 25, 28); degarelix (n=16, 25, 26). For MCP-1 measurements: Control (n=14, 20, 24); leuprolide (n=14, 23, 24); degarelix (n=14, 21, 24). For TNF-α: Control (n=16, 26, 27); leuprolide, (n=18, 26, 28); degarelix (n=16, 25, 27). E, Relative number of anti-CD3 stimulated splenocytes after 48 h in the presence of degarelix or leuprolide at a concentration of 10^{-6} mol/L (n=16 in each group). ADT, androgen deprivation therapy; GnRH, gonadotropin-releasing hormone; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; TNF, tumor necrosis factor.

Deprivation of sex hormones by surgical castration or treatment with leuprolide has been found to increase thymus size and cellularity.[20,40] It involves increased proliferation of immature thymocytes,[34] but the exact mechanisms and the potential role of local GnRH receptors have not been clarified. Our study was consistent with previous literature in this field, except for leuprolide, which resulted in lower thymus and spleen weights compared to mice subjected to bilateral orchiectomy or degarelix. The reasons for this warrant further investigation, and it is apparently in conflict with a previous study using a lower dose of leuprolide in nonorchiectomized male mice.[41]

Limitations

Our experimental approach is far from exhaustive in addressing the many ways in which ADT could affect the occurrence of clinical atherosclerotic CVD. Myocardial infarction and stroke is the result of a decade-long process of plaque development suddenly complicated by thrombosis, most often precipitated by rupture of the fibrous cap and resultant exposure of the necrotic core.[42] The observed increase in risk following ADT therapy can therefore potentially be attributed to effects on any of several processes, including mechanisms in the atherosclerotic plaque that increase propensity to rupture, processes that augment plaque thrombogenicity, or factors outside the atherosclerotic lesion, such as systemic thrombotic tendency and myocardial susceptibility to ischemia that may increase the likelihood that rupture leads to a clinical event.[43]

Conclusions

Leuprolide augmented hypercholesterolemia, but no significant differences in the development of atherosclerosis were detected among groups of intact *Apoe*-deficient mice treated with different types of ADT. A pro-atherogenic, possibly cholesterol-mediated, effect of leuprolide was seen in orchiectomized mice that might be relevant for understanding

the potential cardiovascular risk associated with GnRH agonist-based ADT.

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Differences in Hypercholesterolemia and Atherogenesis Induced by Common Androgen Deprivation Therapies in Male Mice

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