ANIMAL MODELS

Sex-Specific Differences in an $ApoE^{-/-}$: $Ins2^{+/Akita}$ Mouse Model of Accelerated Atherosclerosis

Daniel E. Venegas-Pino,^{*,†} Pei-Wen Wang,[†] Heidi K. Stoute,^{†‡} Nicholas A. Singh-Pickersgill,[†] Brian Y. Hong,[†] Mohammad I. Khan,[†] Yuanyuan Shi,[†] and Geoff H. Werstuck^{*,†‡}

From the Departments of Biochemistry and Biomedical Sciences* and Medicine[†] and the Thrombosis and Atherosclerosis Research Institute,[‡] McMaster University, Hamilton, Ontario, Canada

Accepted for publication
September 28, 2015.

Address correspondence to
Geoff H. Werstuck, Ph.D.,
Thrombosis and Atherosclerosis
Research Institute, Hamilton
General Campus, McMaster
University, 237 Barton St E,
Hamilton, ON, Canada L8L
2X2. E-mail: geoff.werstuck@taari.ca.

Diabetic patients have a twofold to fourfold increased risk of cardiovascular disease. Despite a vast amount of research, the underlying mechanisms that predispose individuals with diabetes to the development of cardiovascular disease are unclear. To further our understanding of how diabetes promotes atherosclerosis, we have established, characterized, and manipulated a new model of hyperglycemia-induced atherosclerosis: the apolipoprotein E-deficient ($ApoE^{-/-}$): $Ins2^{+/Akita}$ mouse. All mice were fed a standard chow diet. Male $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice developed chronic hyperglycemia, which significantly accelerated atherosclerosis. Female $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice presented hyperglycemia that normalized by 15 weeks of age. Despite the transient hyperglycemia, advanced atherosclerosis was observed at 15 weeks of age compared with $ApoE^{-/-}$ females. To better understand these differences, subsets of mice were castrated or ovariectomized at 5 weeks of age. Castrated $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice showed a reduction in blood glucose levels that correlated with the amelioration of atherosclerosis. Interestingly, castrated normoglycemic $ApoE^{-/-}$ mice developed larger atherosclerotic lesions than sham-operated controls. Ovariectomized $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice presented chronic hyperglycemia, and atherosclerosis appeared to be advanced. We have characterized the distinctive sex-specific phenotypes exhibited by the $ApoE^{-/-}$: $Ins2^{+/Akita}$ mouse model and present evidence for the action of sex hormones on pancreatic β -cell function and the vasculature that affect the regulation of blood glucose levels and the development of atherosclerosis. This model will provide a test bed to further delineate these effects. (*Am J Pathol* 2016, 186: 67–77; <http://dx.doi.org/10.1016/j.ajpath.2015.09.009>)

A strong correlation exists between diabetes mellitus (DM) and the prevalence and severity of large-vessel disease. Angiographic and autopsy studies have reported that, compared with nondiabetic patients, individuals with diabetes have a greater degree of both high-grade and subclinical atherosclerosis, the underlying cause of most coronary artery disease.^{1,2} DM is associated with a cardiovascular disease mortality rate that exceeds 70%, and diabetic patients have a twofold to fourfold increased risk of dying from myocardial infarction or stroke.³

Our understanding of the molecular mechanisms that link diabetes to the accelerated development and progression of atherosclerosis is complicated by the interdependence of circulating glucose and insulin concentrations, as well as the close association of DM with other cardiovascular risk factors,

including dyslipidemia, obesity, and hypertension.⁴ To facilitate the investigation and identification of the relevant molecular mechanisms and pathways, several different mouse models of diabetes-induced atherosclerosis have been developed. Most of these models involve the induction of hyperglycemia, in the presence or absence of insulin resistance, in dyslipidemic, atherosclerosis-prone apolipoprotein E-deficient ($ApoE^{-/-}$) or low-density lipoprotein receptor-deficient mice.⁵

The most common, and direct, strategy to induce hyperglycemia in rodents and other experimental animals has been

Supported by the Canadian Institutes of Health Research (CIHR, operating grant number: MOP-142248) and the Canadian Diabetes Association (CDA, operating grant number: OG-3-12-3852-GW), and the Comisión Nacional de Investigación Científica y Tecnológica (Chile) scholarship (D.E.V.-P.).

Disclosures: None declared.

through a single, or multiple, i.p. injection of streptozotocin (STZ). STZ is a DNA-alkylating agent that is selectively toxic to pancreatic β cells, resulting in severe insulinopenia.⁶ Many studies have shown that STZ-induced hyperglycemia can promote atherosclerosis in $ApoE^{-/-}$ mice.^{7–10} However, there are several disadvantages associated with the use of STZ, including possible toxicity to other tissues and organs,^{11,12} the severity of the hyperglycemia,^{7,8} and the potential for subsequent β -cell regeneration.¹³

The $Ins2^{+/Akita}$ mouse represents a genetic alternative to chemically induced hyperglycemia. This mouse carries a point mutation (C96Y) in one allele of the insulin 2 gene, which disrupts a disulfide bond between the insulin A and B chains. The resulting proinsulin polypeptide cannot be properly processed and, therefore, accumulates, causing endoplasmic reticulum (ER) stress and β -cell dysfunction.¹⁴ It has been postulated that ER-stress-induced β -cell death is the major cause of insulinopenia in $Ins2^{+/Akita}$ mice. Subsequent studies have suggested that the formation of mutant proinsulin-derived aggregates sequester the wild-type proinsulin, leading to ER retention and degradation of mutant and wild-type proinsulin, as the underlying cause of the decrease in circulating insulin and the development of hyperglycemia.^{15–19}

We have independently generated and extensively characterized an $ApoE^{-/-}:Ins2^{+/Akita}$ mouse strain in a C57BL/6J genetic background. Herein, we show that glucose regulation and atherosclerotic progression vary significantly between male and female $ApoE^{-/-}:Ins2^{+/Akita}$ mice. We use castration and ovariectomy to expose the roles played by sex hormones in the development of hyperglycemia and atherosclerosis.

Materials and Methods

Mice

Male $ApoE^{+/+}:Ins2^{+/Akita}$ mice (Jackson Laboratory, Bar Harbor, ME) were crossed with female $ApoE^{-/-}:Ins2^{+/+}$ mice. The resulting male $ApoE^{+/+}:Ins2^{+/Akita}$ offspring were crossed with female $ApoE^{-/-}:Ins2^{+/+}$ mice. The male $ApoE^{-/-}:Ins2^{+/Akita}$ offspring produced from this cross and female $ApoE^{-/-}:Ins2^{+/+}$ were set up as breeding pairs to produce the $ApoE^{-/-}:Ins2^{+/Akita}$ and control $ApoE^{-/-}:Ins2^{+/+}$ littermates that were used in the following experiments. Genotypes were confirmed by PCR using primers specific for *Ins2* and *ApoE* genes (Supplemental Figure S1). Primers used were as follows: *Ins2*, 5'-TGC-TGATGCCCTGGCCTGCT-3' (forward) and 5'-TGGTC-CCACATATGCACATG-3' (reverse); *ApoE*, 5'-GCCGC-CCCGACTGCATCT-3' (forward); wild type, 5'-TGTG-ACTTGGGAGCTCTGCAGC-3' (reverse); and knockout, 5'-GCCTAGCCGAGGGAGAGCCG-3' (reverse). The restriction enzyme Fnu4HI was used to identify the presence of the $Ins2^{Akita}$ mutation. All of the mice used in this study were fed a standard chow diet (2018 Teklad Global 18%

Protein Rodent Diet; Harlan Teklad, Madison, WI) *ad libitum* with free access to water. Atherosclerosis was assessed at 5, 15, and 25 weeks of age in male $ApoE^{-/-}$ ($n = 25$), male $ApoE^{-/-}:Ins2^{+/Akita}$ ($n = 25$), female $ApoE^{-/-}$ ($n = 21$), and female $ApoE^{-/-}:Ins2^{+/Akita}$ ($n = 25$) mice. For castration and ovariectomy experiments, mice were stratified by glucose levels and body weight, and then randomly allocated into each group. All of the animal procedures were approved by the McMaster University Animal Research Ethics Board.

Castration

Five-week-old mice were anesthetized with isoflurane and administered a dose of buprenorphine (100 μ L of a 0.015 mg/mL solution) for pain control. A 5-mm incision was made through the skin along the midline of the scrotal sac. After crossing the skin, another 5-mm incision was made on the left side on the scrotal sac membrane. The left testis was then dissected. The incision made on the scrotal sac membrane and the skin was then sutured. The process was repeated to remove the right testis ($n = 12$ $ApoE^{-/-}$ and 12 $ApoE^{-/-}:Ins2^{+/Akita}$ mice). Identical incisions were made on the sham-operated mice, but testicles were not removed ($n = 9$ $ApoE^{-/-}$ and 8 $ApoE^{-/-}:Ins2^{+/Akita}$ mice). After surgery, mice recovered on a heating bed, to stabilize corporal temperature, and were returned to sterile cages where they were monitored daily.

Ovariectomy

Five-week-old mice were anesthetized with isoflurane and administered a dose of buprenorphine for pain control. Hair was removed from the incision site, and skin was cleaned with 70% ethanol. A 10-mm lateral incision was made through the skin of the left side of the back. After crossing the skin, another 10-mm incision was made to cross the muscle layer. The ovary and oviduct were located, and the ovary was removed. Then, the muscle and skin incisions were sutured. The process was repeated to remove the right ovary ($n = 8$ $ApoE^{-/-}$ and 9 $ApoE^{-/-}:Ins2^{+/Akita}$ mice). Identical incisions were made on the sham-operated groups, but ovaries were not removed ($n = 7$ $ApoE^{-/-}$ and 9 $ApoE^{-/-}:Ins2^{+/Akita}$ mice). After surgery, mice recovered on a heating bed, to stabilize corporal temperature, and were returned to sterile cages where they were monitored daily.

Harvesting and Storing

Mice were anesthetized with isoflurane, and blood was extracted. Mice were sacrificed by cervical dislocation, and the vasculature was rinsed with 5 mL of 0.9% saline. Liver and fat pad were removed, and the vasculature was perfusion fixed with 10% neutral-buffered formalin. The heart, pancreas, and aorta were extracted. All of the

organs were stored in 10% neutral-buffered formalin at room temperature.

Analysis of Atherosclerosis

Paraffin-embedded hearts were divided into sections with a microtome, and serial sections (4.5 µm thick) from the aortic sinus were collected onto glass slides until atherosclerotic lesions were no longer observed. Sections were stained with hematoxylin and eosin (Sigma, St. Louis, MO), and lesion areas and volumes were determined, as previously described.²⁰ The necrotic core of the atherosclerotic lesions was estimated by Masson's trichrome staining (Sigma).²¹ Calcified lesion areas were detected and quantified by von Kossa staining.²² Smooth muscle cells and macrophage/foam cells were quantified in the atherosclerotic lesions by immunofluorescence staining for α -actin (Santa Cruz Biotechnology, Santa Cruz, CA) and Mac-3 (BD Pharmingen, San Diego, CA), respectively. All images were captured with an Olympus DP71 digital camera (Olympus Imaging, Center Valley, PA) mounted on a Leitz Laborlux S bright-field microscope (Leica Microsystems, Concord, ON), and assessed using ImageJ software version 1.48v (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

En Face Aortas

The fixed aortas were cleaned of surrounding muscle and adventitial fat. They were longitudinally opened and stained for lipid content with Sudan IV (Sigma). Images of the whole aorta were captured using a L320 digital camera (Nikon, Mississauga, ON, Canada), and the percentage of the atherosclerotic area was assessed using ImageJ software.

Analysis of Pancreata

Paraffin-embedded pancreata were divided into sections (6 µm thick), and consecutive sections were collected onto glass slides. The antibody against GADD153 (growth arrest and DNA damage-inducible protein; Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect the percentage of cells under ER stress.²³ Images were taken with an Olympus DP72 digital camera (Olympus) mounted onto an Olympus BX41 microscope (Olympus).

Analysis of Plasma

Nonfasting and fasting (6 hours) blood glucose levels were measured using a glucometer (One Touch Ultra; Life Scan, Burnaby, BC, Canada). Plasma lipid levels were determined in nonfasting and fasting conditions using the colorimetric diagnostic kit for total cholesterol and triglyceride (Thermo Scientific, Middletown, VA). Fasting plasma lipoproteins were separated by fast protein liquid chromatography, and

total cholesterol was measured in each fraction. Enzyme-linked immunosorbent assay kits were used to measure insulin (Crystal Chem, Downers Grove, IL), estradiol (Calbiotech, Spring Valley, CA), progesterone, and testosterone (ALPCO Diagnostics, Salem, NH) under fasting conditions.

Surrogate indexes of β -cell function, insulin resistance, and sensitivity were calculated from fasting blood glucose and plasma insulin concentrations as follows:

$$\text{HOMA } \% \beta = [(20 \times I_0)/(G_0 - 3.5)] \quad (1)$$

where HOMA is Homeostasis Model Assessment, G_0 is fasting glucose (mmol/L), and I_0 is fasting insulin (μ IU/mL);

$$\text{HOMA IR} = [(G_0 \times I_0)/22.5] \quad (2)$$

where HOMA is Homeostasis Model Assessment, IR is Insulin Resistance, G_0 is fasting glucose (mmol/L), and I_0 is fasting insulin (μ IU/mL); and

$$\text{QUICKI} = (1/[\log(I_0) + \log(G_0)]) \quad (3)$$

where QUICKI is Quantitative Insulin Sensitivity Check Index, I_0 is fasting insulin (μ IU/mL) and G_0 is fasting glucose (mg/dL).^{24,25}

OGTT and PITT

Independent groups of mice were prepared for the oral glucose tolerance test (OGTT) and the peritoneal insulin tolerance test (PITT) in male *ApoE*^{-/-} ($n = 11$) and *ApoE*^{-/-}:*Ins2*^{+Akita} ($n = 15$) or female *ApoE*^{-/-} ($n = 12$) and *ApoE*^{-/-}:*Ins2*^{+Akita} ($n = 12$) mice. Twenty-week-old mice were fasted for 4 hours and then blood samples were collected from the tail vein. After the first collection (time 0), glucose (2 g/kg body weight of a 200 mg/mL solution; Sigma) was administered by oral gavage, and blood samples were collected at time points of 15, 30, 60, and 120 minutes. Glucose levels were determined using a colorimetric glucose assay reagent (Sigma).

For PITT, 20-week-old mice were fasted for 4 hours and then blood glucose levels were measured with a glucometer (time 0). After the first measurement, bovine insulin (Sigma) was then injected i.p. (0.75 U/kg body weight of a 200 mU/mL solution) and blood glucose was measured at time points of 15, 30, 60, and 120 minutes.

Data Analysis

Data were analyzed by *t*-test to compare two groups. To compare multiple groups, one- or two-way analysis of variance was used, followed by the Bonferroni multiple comparison test between all groups. Data are expressed as arithmetic means $\pm$ SEM. For all experiments, $P < 0.05$ was considered statistically significant.

Figure 1 Analysis of metabolic parameters in male and female apolipoprotein E-deficient (*ApoE*^{-/-}):*Ins2*^{+/Akita} mice and age-matched controls. **A:** Nonfasting blood glucose levels. **B:** Oral glucose tolerance test (OGTT). **C:** Peritoneal insulin tolerance test (PITT). **D:** Nonfasting plasma cholesterol and triglycerides (**E**). **F:** Body weight. **G:** Fat pad. ****P* < 0.001 ♀*ApoE*^{-/-} versus ♀*ApoE*^{-/-}:*Ins2*^{+/Akita}, †*P* < 0.05 ♂*ApoE*^{-/-} versus ♂*ApoE*^{-/-}:*Ins2*^{+/Akita}, ††*P* < 0.01 ♂*ApoE*^{-/-} versus ♂*ApoE*^{-/-}:*Ins2*^{+/Akita}, and †††*P* < 0.001 ♂*ApoE*^{-/-} versus ♂*ApoE*^{-/-}:*Ins2*^{+/Akita}, ‡*P* < 0.05 ♂*ApoE*^{-/-}:*Ins2*^{+/Akita} versus ♀*ApoE*^{-/-}:*Ins2*^{+/Akita}, ‡‡*P* < 0.01 ♂*ApoE*^{-/-}:*Ins2*^{+/Akita} versus ♀*ApoE*^{-/-}:*Ins2*^{+/Akita}, and ‡‡‡*P* < 0.001 ♂*ApoE*^{-/-}:*Ins2*^{+/Akita} versus ♀*ApoE*^{-/-}:*Ins2*^{+/Akita}; §§*P* < 0.01 ♂*ApoE*^{-/-} versus ♀*ApoE*^{-/-}, §§§*P* < 0.001 ♂*ApoE*^{-/-} versus ♀*ApoE*^{-/-}. *n* = 8 to 12 per group (**A** and **F**); *n* = 4 to 5 per group (**B**); *n* = 7 to 10 per group (**C**); *n* = 5 to 11 per group (**D** and **E**); *n* = 6 to 12 per group (**G**).

Results

Sex-Specific Effects on Hyperglycemia in *ApoE*^{-/-}:*Ins2*^{+/Akita} Mice

Male and female *ApoE*^{-/-}:*Ins2*^{+/Akita} mice developed hyperglycemia spontaneously by 5 weeks of age relative to age-matched *ApoE*^{-/-} controls. Nonfasting blood glucose levels for 5-week-old male mice were 20.4 ± 1.1 versus 8.7 ± 0.4 mmol/L (*P* < 0.001), respectively. For female mice, blood glucose levels were 18.0 ± 1.1 versus 8.8 ± 0.2 mmol/L (*P* < 0.001). Hyperglycemia was sustained for the entire lifespan of the male mice. The blood glucose levels of female *ApoE*^{-/-}:*Ins2*^{+/Akita} mice normalized by 15 weeks of age (Figure 1A). Normalization of blood glucose in female *ApoE*^{-/-}:*Ins2*^{+/Akita} mice was at least partially dependent on *ApoE* deficiency because blood glucose levels in

heterozygous *ApoE*^{+/-}:*Ins2*^{+/Akita} females remained significantly elevated at 15 weeks of age (Supplemental Figure S2).

OGTT and PITT were performed at 20 weeks of age. Male *ApoE*^{-/-}:*Ins2*^{+/Akita} mice were clearly glucose intolerant (Figure 1B) and also exhibited an impaired response to exogenous insulin (Figure 1C). Female *ApoE*^{-/-}:*Ins2*^{+/Akita} mice showed marginally impaired glucose tolerance and normal insulin sensitivity (Figure 1, B and C). In addition to chronic hyperglycemia, male *ApoE*^{-/-}:*Ins2*^{+/Akita} mice also developed enhanced hypercholesterolemia (Figure 1D), but showed no defined changes in triglycerides (Figure 1E) by 25 weeks of age. There were no differences in plasma lipid levels in female *ApoE*^{-/-}:*Ins2*^{+/Akita} mice relative to *ApoE*^{-/-} controls (Figure 1, D and E).

At the end of the study, the average body weight of the hyperglycemic male mice was 5 g less than male

Figure 2 Chronically hyperglycemic male apolipoprotein E-deficient ($ApoE^{-/-}$): $Ins2^{+/Akita}$ and transiently hyperglycemic female $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice develop accelerated atherosclerosis. Atherosclerotic lesion area and volume were quantified in the aortic sinus (A), and lipid accumulation was determined in *en face* aortas with Sudan-IV staining (B) in 25-week-old male mice. Atherosclerotic lesion area and volume were quantified in the aortic sinus (C), and lipid accumulation was determined in *en face* aortas with Sudan-IV staining (D) in 15-week-old female mice. * $P < 0.05$ $\delta ApoE^{-/-}$ versus $\delta ApoE^{-/-}:Ins2^{+/Akita}$, ** $P < 0.01$ $\delta ApoE^{-/-}$ versus $\delta ApoE^{-/-}:Ins2^{+/Akita}$, and *** $P < 0.001$ $\delta ApoE^{-/-}$ versus $\delta ApoE^{-/-}:Ins2^{+/Akita}$; † $P < 0.05$ $\delta ApoE^{-/-}$ versus $\delta ApoE^{-/-}:Ins2^{+/Akita}$, †† $P < 0.01$ $\delta ApoE^{-/-}$ versus $\delta ApoE^{-/-}:Ins2^{+/Akita}$. $n = 12$ per group (A); $n = 5$ per group (B); $n = 9$ to 10 per group (C and D). AUC, area under the curve.

$ApoE^{-/-}$ controls (Figure 1F). The hyperglycemic male mice had virtually no epididymal fat pad (Figure 1G). There was no significant difference in liver weight between the male hyperglycemic mice and controls (data not

shown). Transiently hyperglycemic $ApoE^{-/-}:Ins2^{+/Akita}$ and normoglycemic $ApoE^{-/-}$ female mice presented similar metabolic parameters throughout the study (Figure 1, F and G).

Figure 3 Female apolipoprotein E-deficient ($ApoE^{-/-}$): $Ins2^{+/Akita}$ mice develop chronic hyperglycemia after ovariectomy. **A:** Fasting blood glucose levels in ovariectomized (Ovx) and sham-operated on (sham) mice. **B:** GADD153 expression in pancreatic islets at 25 weeks of age. **C:** Homeostasis Model Assessment Insulin Resistance (HOMA-IR). **D–G:** Body weight progression (**D**), fat pad weight (**E**), fasting plasma cholesterol (**F**), and triglycerides (**G**) at 25 weeks of age. * $P < 0.05$ ♀ $ApoE^{-/-}$: $Ins2^{+/Akita}$ -sham versus ♀ $ApoE^{-/-}$: $Ins2^{+/Akita}$ -Ovx, ** $P < 0.01$ ♀ $ApoE^{-/-}$: $Ins2^{+/Akita}$ -sham versus ♀ $ApoE^{-/-}$: $Ins2^{+/Akita}$ -Ovx, and *** $P < 0.001$ ♀ $ApoE^{-/-}$: $Ins2^{+/Akita}$ -sham versus ♀ $ApoE^{-/-}$: $Ins2^{+/Akita}$ -Ovx; † $P < 0.05$ ♀ $ApoE^{-/-}$ -sham versus ♀ $ApoE^{-/-}$ -Ovx, ††† $P < 0.001$ ♀ $ApoE^{-/-}$ -sham versus ♀ $ApoE^{-/-}$ -Ovx. $n = 7$ to 9 per group (**A** and **D–G**); $n = 3$ per group (**B**); $n = 4$ to 6 per group (**C**).

Accelerated Atherosclerosis in $ApoE^{-/-}$: $Ins2^{+/Akita}$ Mice

Atherosclerosis was analyzed in 5-, 15-, and 25-week-old male and female $ApoE^{-/-}$ and $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice. No atherosclerotic lesions were detected in any of the 5-week-old mice (data not shown). Atherosclerotic lesions in the aortic sinus of 15-week-old male $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice were relatively small and not significantly different compared with controls (Supplemental Figure S3A). However, the examination of the whole aortas revealed an increase of lipid accumulation, predominantly in the aortic arch of the hyperglycemic male mice (Supplemental Figure S3B). At 25 weeks of age, hyperglycemic male $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice presented with advanced atherosclerosis when compared with age- and sex-matched normoglycemic $ApoE^{-/-}$ controls. Atherosclerotic lesion volume from male $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice was at least 4× larger ($P < 0.001$) in the aortic sinus (Figure 2A). Similarly, *en face* aortas from male $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice showed 3× ($P < 0.05$) more atherosclerotic lesion area (Figure 2B).

Transiently hyperglycemic female $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice developed accelerated atherosclerosis in the aortic sinus compared with female $ApoE^{-/-}$ controls, at 15 weeks of age ($P < 0.01$) (Figure 2C). A similar trend was observed when the whole aortas were examined for lipid accumulation (Figure 2D). Lesion volume in the aortic sinus (Supplemental Figure S3C) and lipid accumulation in the descending aorta (Supplemental Figure S3D) increased in female $ApoE^{-/-}$ and $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice by 25 weeks of age; however, there were no longer statistically significant differences in area or volume.

Ovx $ApoE^{-/-}$: $Ins2^{+/Akita}$ Mice Develop Chronic Hyperglycemia

To further examine the effects of sex, $ApoE^{-/-}$: $Ins2^{+/Akita}$ and $ApoE^{-/-}$ mice were ovariectomized (Ovx) at 5 weeks of age. Sham-operated on female $ApoE^{-/-}$: $Ins2^{+/Akita}$ and $ApoE^{-/-}$ mice were used as controls. In contrast to the transient hyperglycemia observed in female $ApoE^{-/-}$: $Ins2^{+/Akita}$ -sham mice, Ovx $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice remained hyperglycemic for the duration of the study (Figure 3A). Examination of the pancreatic islets of 25-week-old mice showed that ovariectomy resulted in a significant increase of GADD153-stained nuclei (40% stained nuclei per islet) (Figure 3B). Consistent with this finding, β -cell function, evaluated by HOMA % β (Supplemental Figure S4A), and the glucose-induced insulin secretion (Supplemental Figure S5) were impaired in the Ovx group. HOMA IR and QUICKI indexes were also significantly altered in the Ovx $ApoE^{-/-}$: $Ins2^{+/Akita}$ mice relative to sham-operated on controls (Figure 3C and Supplemental Figure S4B). Ovariectomy had no significant effect on fasting blood glucose levels, GADD153 expression, insulin sensitivity, or β -cell function in the $ApoE^{-/-}$ control mice (Figure 3, A–C, and Supplemental Figure S4, A and B).

Examination of other metabolic parameters showed no significant differences in body weight, fat pad, liver weight, or plasma lipid levels between 25-week-old $ApoE^{-/-}$: $Ins2^{+/Akita}$ -Ovx and $ApoE^{-/-}$: $Ins2^{+/Akita}$ -sham mice (Figure 3, D–G, and Supplemental Figure S6A). Twenty-five-week-old $ApoE^{-/-}$ -Ovx mice showed a significant increase of body weight with respect to all of the other groups (Figure 3D). This increase of body weight was accompanied

Figure 4 Ovariectomized (Ovx) apolipoprotein E-deficient (*ApoE*^{-/-}):*Ins2*^{+/-}/*Akita* mice have more advanced atherosclerotic lesions than sham-operated controls. Atherosclerotic lesion area and volume (A), necrotic core (B), calcification in the aortic sinus (C), and lipid accumulation in *en face* aortas (D) were quantified in 25-week-old female mice. **P* < 0.05 ♀*ApoE*^{-/-}-sham versus ♀*ApoE*^{-/-}:*Ins2*^{+/-}/*Akita*-Ovx. *n* = 7 to 9 per group (A–D). AUC, area under the curve.

by an increase in epididymal fat pad (Figure 3E) but no changes in liver weight (Supplemental Figure S6A). *ApoE*^{-/-}-Ovx mice also had increased fasting plasma cholesterol levels (Figure 3F) that correlated with an elevation of the very-low-density lipoprotein (VLDL)-cholesterol and LDL-cholesterol peaks (Supplemental Figure S7A). As expected, Ovx mice had significantly reduced plasma estrogen (estradiol) and progesterone levels compared with sham-operated on controls (Supplemental Figure S8, A and B).

Ovx *ApoE*^{-/-}:*Ins2*^{+/^{Akita}} Mice Develop Advanced Atherosclerosis

No differences in atherosclerosis were observed at the aortic sinus among 25-week-old Ovx and sham-operated on mice (Figure 4A, Supplemental Figure S9A, and Supplemental Figure S10A). However, the atherosclerotic lesions found in 25-week-old *ApoE*^{-/-}:*Ins2*^{+/+}*Akita*-Ovx mice showed a trend toward larger necrotic cores (Figure 4B) and increased artery calcification (Figure 4C). Similarly, examination of *en face*

aortas showed that 25-week-old *ApoE*^{-/-}:*Ins2*^{+/^{Akita}}-Ovx mice presented with an increased atherosclerotic area in the descending aorta (Figure 4D).

Castration Reduces Blood Glucose Levels in *ApoE*^{-/-}:*Ins2*^{+/*Akita*} Mice

Male *ApoE*^{-/-}:*Ins2*^{+/*Akita*} and *ApoE*^{-/-} mice were castrated (Cx) at 5 weeks of age. After the surgery, *ApoE*^{-/-}:*Ins2*^{+/*Akita*}-Cx mice presented with a moderate, but significant, decrease in fasting glucose levels when compared with male *ApoE*^{-/-}:*Ins2*^{+/*Akita*}-sham mice. At 25 weeks of age, this reduction in blood glucose was no longer observed (Figure 5A). In 25-week-old male *ApoE*^{-/-}:*Ins2*^{+/*Akita*} mice, castration did not alter the percentage of GADD153-positive nuclei in pancreatic islets (Figure 5B). β -Cell function, evaluated by HOMA % β index, was significantly impaired in both the sham-operated on and castrated *ApoE*^{-/-}:*Ins2*^{+/*Akita*} mice (Supplemental Figure S4C). However, castration did result in improved HOMA IR and

Figure 5 Blood glucose levels are reduced in male apolipoprotein E-deficient ($ApoE^{-/-}$); $Ins2^{+/Akita}$ mice after castration. **A:** Fasting blood glucose levels in castrated (Cx) and sham-operated on (sham) mice. **B:** GADD153 expression in pancreatic islets. **C–G:** Homeostasis Model Assessment Insulin Resistance (HOMA-IR; **C**), body weight progression (**D**), fat pad weight (**E**), fasting plasma cholesterol (**F**), and triglycerides (**G**) at 25 weeks of age. * $P < 0.05$ $\delta ApoE^{-/-}$; $Ins2^{+/Akita}$ -sham versus $\delta ApoE^{-/-}$; $Ins2^{+/Akita}$ -Cx, ** $P < 0.01$ $\delta ApoE^{-/-}$; $Ins2^{+/Akita}$ -sham versus $\delta ApoE^{-/-}$; $Ins2^{+/Akita}$ -Cx, and *** $P < 0.001$ $\delta ApoE^{-/-}$; $Ins2^{+/Akita}$ -sham versus $\delta ApoE^{-/-}$; $Ins2^{+/Akita}$ -Cx; † $P < 0.05$ $\delta ApoE^{-/-}$ -sham versus $\delta ApoE^{-/-}$ -Cx, †† $P < 0.01$ $\delta ApoE^{-/-}$ -sham versus $\delta ApoE^{-/-}$ -Cx, and ††† $P < 0.001$ $\delta ApoE^{-/-}$ -sham versus $\delta ApoE^{-/-}$ -Cx. $n = 8$ to 12 per group (**A**); $n = 3$ per group (**B**); $n = 4$ to 6 per group (**C**); $n = 7$ to 12 per group (**D–G**).

QUICKI indexes (Figure 5C and Supplemental Figure S4D). In $ApoE^{-/-}$ mice, castration had no effect on fasting blood glucose levels, the percentage of GADD153-positive nuclei, insulin resistance or sensitivity, and β -cell function (Figure 5, A–C, and Supplemental Figure S4, C and D).

Castration did not affect body weight of 25-week-old $ApoE^{-/-}$; $Ins2^{+/Akita}$ -Cx mice (Figure 5D), but they recovered the capability to store fat in the abdominal cavity (Figure 5E). In addition, these mice presented a decrease of liver weight (Supplemental Figure S6B), which was accompanied by lower plasma cholesterol levels (Figure 5F). Consistent with these findings, there was a reduction of the VLDL-cholesterol and LDL-cholesterol peaks in the lipid profile (Supplemental Figure S7B). Twenty-five-week-old $ApoE^{-/-}$ -Cx mice had reduced body weight, epididymal fat pad, and liver weight (Figure 5, D and E, and Supplemental Figure S6B). No changes in plasma lipids were detected (Figure 5, F and G, and Supplemental Figure S7B). Castration effectively reduced plasma testosterone levels. However, testosterone levels were already significantly reduced in the hyperglycemic $ApoE^{-/-}$; $Ins2^{+/Akita}$ -sham relative to normoglycemic $ApoE^{-/-}$ -sham mice (Supplemental Figure S8C).

Atherosclerosis Is Delayed in Castrated $ApoE^{-/-}$; $Ins2^{+/Akita}$ Mice

At 25 weeks of age, the atherosclerotic lesions in the castrated $ApoE^{-/-}$; $Ins2^{+/Akita}$ mice were significantly smaller at the aortic sinus (Figure 6A). In addition, these lesions were less advanced, having smaller necrotic cores (Figure 6B), less calcification (Figure 6C), and a reduction in the number of smooth muscle cells but not macrophages (Supplemental Figure S9B and Supplemental Figure S10B), relative to the sham-operated on $ApoE^{-/-}$; $Ins2^{+/Akita}$ mice. Descending

aortas of $ApoE^{-/-}$; $Ins2^{+/Akita}$ -Cx mice had significantly less atherosclerosis relative to sham-operated on controls (Figure 6D). Conversely, the castration in the $ApoE^{-/-}$ mice significantly accelerated the development of atherosclerosis (Figure 6, A and B, and Supplemental Figure S9B).

Discussion

Herein, we have characterized the sex-specific differences of the $ApoE^{-/-}$; $Ins2^{+/Akita}$ mouse model of hyperglycemia-accelerated atherosclerosis. Specifically, male $ApoE^{-/-}$; $Ins2^{+/Akita}$ mice develop chronic hyperglycemia and significantly accelerated atherosclerosis by 25 weeks of age. Castration slows the development of atherosclerosis in $ApoE^{-/-}$; $Ins2^{+/Akita}$ mice but enhances disease progression in the normoglycemic controls. Female $ApoE^{-/-}$; $Ins2^{+/Akita}$ mice are only transiently hyperglycemic, with glucose levels normalizing by 15 weeks of age. Still, transiently hyperglycemic female mice develop accelerated atherosclerosis. Ovx $ApoE^{-/-}$; $Ins2^{+/Akita}$ mice are chronically hyperglycemic and show indications of advanced atherosclerosis.

Differences in the incidence and the prevalence of DM between sexes do exist.^{26,27} In fact, sex differences in prevalence extend to dyslipidemia,^{28,29} obesity,^{30,31} hypertension,³² atherosclerotic development,³³ and the risk of myocardial infarction.³⁴ Premenopausal women have a lower incidence of DM, and evidence suggests that estrogen protects against pancreatic β -cell failure in rodent models.^{35,36} Other studies have focused on the multiple benefits of androgens in men.³⁷ Testosterone levels tend to decrease with age,³⁸ and this decrease could explain the increased prevalence of age-related disease. In the STZ-hyperglycemic male rats,³⁹ the lack of circulating insulin leads to the decrease of the circulating pituitary hormones, follicle-stimulating hormone and luteinizing

Figure 6 Castration (Cx) delays atherosclerotic development in apolipoprotein E-deficient ($ApoE^{-/-};Ins2^{+/Akita}$) mice. Atherosclerotic lesion area and volume (**A**), necrotic core (**B**), calcification in the aortic sinus (**C**), and lipid accumulation in *en face* aortas (**D**) were quantified in 25-week-old male mice. * $P < 0.05$ $\delta ApoE^{-/-};Ins2^{+/Akita}$ -sham versus $\delta ApoE^{-/-};Ins2^{+/Akita}$ -Cx, ** $P < 0.01$ $\delta ApoE^{-/-};Ins2^{+/Akita}$ -sham versus $\delta ApoE^{-/-};Ins2^{+/Akita}$ -Cx; $^{\dagger}P < 0.05$ $\delta ApoE^{-/-}$ -sham versus $\delta ApoE^{-/-}$ -Cx. $n = 8$ to 12 per group (**A**, **C**, and **D**); $n = 7$ to 12 per group (**B**). AUC, area under the curve.

hormone, which has a direct impact in the availability of testosterone. Similarly, in human males, an inverse correlation between testosterone levels and type 2 DM has been described,^{40,41} where an increase of the insulin resistance is associated with a deficiency of testosterone.

We observe that castration significantly accelerates the development of atherosclerosis in normoglycemic $ApoE^{-/-}$ mice, which is consistent with a protective vascular effect of androgens.^{42–44} When the $Ins2^{Akita}$ mutation is incorporated into the male $ApoE^{-/-}$ mice, we and others⁴⁵ have observed the early onset of chronic hyperglycemia, enhanced hypercholesterolemia, and accelerated progression of atherosclerosis. We also observed a significant reduction in insulin sensitivity. In these hyperglycemic mice, the testosterone levels are significantly lower than in normoglycemic controls. This is consistent with previous observations in hyperglycemic rodents³⁹ and humans.^{40,41} Decreased testosterone combined with chronic hyperglycemia and enhanced hypercholesterolemia would exacerbate the development of atherosclerosis. Interestingly, when the castration is performed in the hyperglycemic $ApoE^{-/-};Ins2^{+/Akita}$ mice, atherosclerosis is attenuated. This effect may be explained by the corresponding reduction in blood

glucose and non-high-density lipoprotein-cholesterol levels and the improved insulin sensitivity associated with castration. Together, these results support a model in which testosterone confers vascular protection in the male normoglycemic $ApoE^{-/-}$ mice, but in diabetic mice, testosterone appears to exacerbate hyperglycemia, hypercholesterolemia, and ultimately atherosclerosis. Further investigations are required to fully delineate the underlying mechanisms of these effects.

Female $Ins2^{+/Akita}$ mice have been reported to be hyperglycemic, but with lower blood glucose levels than male $Ins2^{+/Akita}$ mice.¹⁹ In this study, we show that, by 15 weeks of age, blood glucose levels in female $ApoE^{-/-};Ins2^{+/Akita}$ mice are similar to $ApoE^{-/-}$ controls. This phenomenon is partially dependent on $ApoE$ deficiency because significant hyperglycemia is sustained at 15 weeks of age in heterozygous $ApoE^{+/-};Ins2^{+/Akita}$ females. The underlying mechanisms by which female (but not male) mice can produce functional insulin in the presence of the $Ins2^{Akita}$ mutation have not been explained. We show that female $ApoE^{-/-};Ins2^{+/Akita}$ mice have significantly less β -cell stress (indicated by GADD153 staining) compared with males. We also demonstrate that the transient hyperglycemia

in female *ApoE*^{-/-}:*Ins2*^{+Akita} mice does not exacerbate the already dyslipidemic profile of *ApoE*^{-/-} mice, but does promote accelerated atherosclerosis. Ovariectomy of *ApoE*^{-/-}:*Ins2*^{+Akita} mice induces sustained hyperglycemia, effectively abolishing the observed protection from β -cell stress (chronically induced by the *Ins2*^{Akita} mutation) in the pancreatic β cells and reducing the ability to produce functional insulin. The atherosclerotic lesion volumes of the Ovx *ApoE*^{-/-}:*Ins2*^{+Akita} mice are similar to those observed in the sham-operated on *ApoE*^{-/-}:*Ins2*^{+Akita} and the *ApoE*^{-/-} mice, but specific characteristics of the lesions appear to be advanced in addition to the lipid accumulation in the whole aortas.

The effects of estrogen on pancreas^{35,36} and the vasculature⁴⁶ have been elegantly demonstrated in animal models and in *in vitro* studies. However, the protective effects of estrogen replacement therapy in menopausal women are questioned because of disparate results⁴⁷ and the potential cancerogenic effects of estrogens.⁴⁸ Finan et al⁴⁹ have recently overcome these obstacles and repositioned estrogens as a real alternative to the treatment of the metabolic syndrome.

In addition to the tissue- or cell-specific effects described for sex hormones, alterations in their circulating levels may also affect vascular health through indirect mechanisms. Specifically, evidence suggests that sex hormones modulate levels of lipoprotein particles, including high-density lipoprotein, LDL, and VLDL as well as levels of ApoA1, ApoB100, ApoB48, and ApoC-III.^{50–53} In addition, modulations in hormone levels have been linked to alterations in the concentration of vasodilators, vasoconstrictors, inflammatory cytokine profiles, and the response of immune cells to modified LDL particles.^{54,55} These multiple targets and interactions contribute to the complex array of effects observed when sex hormone levels are manipulated.

In conclusion, we have characterized the sex-specific differences of the *ApoE*^{-/-}:*Ins2*^{+Akita} mouse model of hyperglycemia-accelerated atherosclerosis. By using this model, we have shown that the hormonal component from each sex is playing an important role in diabetes and the development of atherosclerosis by affecting the function of the pancreas as well as pathogenesis in the artery wall. More important, in this model, testosterone can have atheroprotective or proatherogenic effects, depending on the glycemic status of the mouse. These findings may have important clinical implications with regard to androgen replacement therapies in the context of chronic disease.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.09.009>.

References

1. Goraya TY, Liebson CL, Palumbo PJ: Coronary atherosclerosis in DM: a population based study. *J Am Coll Cardiol* 2002, 40:946–953
2. D'Souza A, Hussain M, Howarth F, Woods NM, Bidasee K, Singh J: Pathogenesis and pathophysiology of accelerated atherosclerosis in the diabetic heart. *Mol Cell Biochem* 2009, 331:89–116
3. Haffner SM, Lehto S, Ronnema T, Tyorala K, Laakso M: Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998, 339:229–234
4. Steinberger J, Daniels SR; American Heart Association Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young); American Heart Association Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism): Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). *Circulation* 2003, 107:1448–1453
5. Wu KK, Huan Y: Diabetic atherosclerosis mouse models. *Atherosclerosis* 2007, 191:241–249
6. Lenzen S: The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia* 2008, 51:216–226
7. Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, Stern D, Schmidt AM: Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* 1998, 4:1025–1031
8. Werstuck GH, Khan MI, Femia G, Kim AJ, Tedesco V, Trigatti B, Shi YY: Glucosamine-induced endoplasmic reticulum stress is associated with accelerated atherosclerosis in a hyperglycemic mouse model. *Diabetes* 2006, 55:93–101
9. Veerman KJ, Venegas-Pino D, Khan MI, Shi Y, Gerstein HC, Werstuck GH: Hyperglycaemia is associated with impaired vasa vasorum neovascularisation and accelerated atherosclerosis in apolipoprotein-E deficient mice. *Atherosclerosis* 2013, 227:250–258
10. Bowes AJ, Khan MI, Shi Y, Robertson L, Werstuck GH: Valproate attenuates accelerated atherosclerosis in hyperglycemic ApoE-deficient mice: evidence in support of a role for endoplasmic reticulum stress and glycogen synthase kinase-3 in lesion development and hepatic steatosis. *Am J Pathol* 2009, 174:330–342
11. Lee JH, Yang SH, Oh JM, Lee MG: Pharmacokinetics of drugs in rats with diabetes mellitus induced by alloxan or streptozotocin: comparison with those in patients with type I diabetes mellitus. *J Pharm Pharmacol* 2010, 62:1–23
12. Muller YD, Golshayan D, Ehrchiou D, Wyss JC, Giovannoni L, Meier R, Serre-Beinier V, Puga Yung G, Morel P, Bühler LH, Seebach JD: Immunosuppressive effects of streptozotocin-induced diabetes result in absolute lymphopenia and a relative increase of T regulatory cells. *Diabetes* 2011, 60:2331–2340
13. Grossman EJ, Lee DD, Tao J, Wilson RA, Park SY, Bell GI, Chong AS: Glycemic control promotes pancreatic beta-cell regeneration in streptozotocin-induced diabetic mice. *PLoS One* 2010, 5:e8749
14. Wang J, Takeuchi T, Tanaka S, Kubo SK, Kayo T, Lu D, Takata K, Koizumi A, Izumi T: A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the Mody mouse. *J Clin Invest* 1999, 103:27–37
15. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M: Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* 2002, 109:525–532
16. Ron D: Proteotoxicity in the endoplasmic reticulum: lessons from the Akita diabetic mouse. *J Clin Invest* 2002, 109:443–445
17. Gupta S, McGrath B, Cavener DR: PERK (EIF2AK3) regulates proinsulin trafficking and quality control in the secretory pathway. *Diabetes* 2010, 59:1937–1947
18. Liu M, Hodish I, Rhodes CJ, Arvan P: Proinsulin maturation, misfolding, and proteotoxicity. *Proc Natl Acad Sci U S A* 2007, 104:15841–15846

19. Yoshioka M, Kayo T, Ikeda T, Koizumi A: A novel locus, *Mody4*, distal to *D7Mit189* on chromosome 7 determines early-onset NIDDM nonobese C57BL/6 (*Akita*) mutant mice. *Diabetes* 1997, 46:887–894
20. Venegas-Pino DE, Banko N, Khan MI, Shi Y, Werstuck GH: Quantitative analysis and characterization of atherosclerotic lesions in the murine aortic sinus. *J Vis Exp* 2013, 82:50933
21. Thim T, Hagensen MK, Wallace-Bradley D, Granada JF, Kaluza GL, Drouet L, Paaske WP, Bøtker HE, Falk E: Unreliable assessment of necrotic core by virtual histology intravascular ultrasound in porcine coronary artery disease. *Circ Cardiovasc Imaging* 2010, 3:384–391
22. Jeziorska M, McCollum C, Woolley DE: Calcification in atherosclerotic plaque of human carotid arteries: associations with mast cells and macrophages. *J Pathol* 1998, 185:10–17
23. Oyadomari S, Mori M: Roles of *CHOP/GADD153* in endoplasmic reticulum stress. *Cell Death Differ* 2004, 11:381–389
24. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985, 28:412–419
25. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, Quon MJ: Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000, 85:2402–2410
26. Gale EA, Gillespie KM: Diabetes and gender. *Diabetologia* 2001, 44: 3–15
27. Meisinger C, Thorand B, Schneider A, Stieber J, Döring A, Löwel H: Sex differences in risk factors for incident type 2 diabetes mellitus: the MONICA Augsburg cohort study. *Arch Intern Med* 2002, 162: 82–89
28. Freedman DS, Jacobsen SJ, Barboriak JJ, Sobocinski KA, Anderson AJ, Kissebah AH, Sasse EA, Gruchow HW: Body fat distribution and male/female differences in lipids and lipoproteins. *Circulation* 1990, 81:1498–1506
29. Goff DC Jr, Bertoni AG, Kramer H, Bonds D, Blumenthal RS, Tsai MY, Psaty BM: Dyslipidemia prevalence, treatment, and control in the Multi-Ethnic Study of Atherosclerosis (MESA): gender, ethnicity, and coronary artery calcium. *Circulation* 2006, 113: 647–656
30. Ogden CL, Carroll MD, Kit BK, Flegal KM: Prevalence of obesity in the United States, 2009–2010. *NCHS Data Brief* 2012, 82:1–8
31. Lovejoy JC, Sainsbury A; Stock Conference 2008 Working Group: Sex differences in obesity and the regulation of energy homeostasis. *Obes Rev* 2009, 10:154–167
32. Reckelhoff JF: Gender differences in the regulation of blood pressure. *Hypertension* 2001, 37:1199–1208
33. Joakimsen O, Bonna KH, Stensland-Bugge E, Jacobsen BK: Age and sex differences in the distribution and ultrasound morphology of carotid atherosclerosis: the Tromsø Study. *Arterioscler Thromb Vasc Biol* 1999, 19:3007–3013
34. Njølstad I, Arnesen E, Lund-Larsen PG: Smoking, serum lipids, blood pressure, and sex differences in myocardial infarction: a 12-year follow-up of the Finnmark Study. *Circulation* 1996, 93:450–456
35. Tiano JP, Delghingaro-Augusto V, Le May C, Liu S, Kaw MK, Khuder SS, Latour MG, Bhatt SA, Korach KS, Najjar SM, Prentki M, Mauvais-Jarvis F: Estrogen receptor activation reduces lipid synthesis in pancreatic islets and prevents β cell failure in rodent models of type 2 diabetes. *J Clin Invest* 2011, 121:3331–3342
36. Le May C, Chu K, Hu M, Ortega CS, Simpson ER, Korach KS, Tsai MJ, Mauvais-Jarvis F: Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. *Proc Natl Acad Sci U S A* 2006, 103:9232–9237
37. Jones RD, Nettleship JE, Kapoor D, Jones HT, Channer KS: Testosterone and atherosclerosis in aging men: purported association and clinical implications. *Am J Cardiovasc Drugs* 2005, 5:141–154
38. Vermeulen A: Clinical review 24: androgens in the aging male. *J Clin Endocrinol Metab* 1991, 73:221–224
39. Ballester J, Muñoz MC, Domínguez J, Rigau T, Guinovart JJ, Rodríguez-Gil JE: Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *J Androl* 2004, 25: 706–719
40. Corona G, Monami M, Rastrelli G, Aversa A, Sforza A, Lenzi A, Forti G, Mannucci E, Maggi M: Type 2 diabetes mellitus and testosterone: a meta-analysis study. *Int J Androl* 2011, 34:528–540
41. Tomar R, Dhindsa S, Chaudhuri A, Mohanty P, Garg R, Dandona P: Contrasting testosterone concentrations in type 1 and type 2 diabetes. *Diabetes Care* 2006, 29:1120–1122
42. Bosco C, Bosnyak Z, Malmberg A, Adolfsson J, Keating NL, Van Hemelrijck M: Quantifying observational evidence for risk of fatal and nonfatal cardiovascular disease following androgen deprivation therapy for prostate cancer: a meta-analysis. *Eur Urol* 2015, 68: 386–396
43. Vlachopoulos C, Ioakeimidis N, Miner M, Aggelis A, Pietri P, Terentes-Printzios D, Tsekoura D, Stefanadis C: Testosterone deficiency: a determinant of aortic stiffness in men. *Atherosclerosis* 2014, 233:278–283
44. Doğan BA, Karakılıç E, Tuna MM, Arduç A, Berker D, Güler S: Effect of androgen replacement therapy on atherosclerotic risk markers in young-to-middle-aged men with idiopathic hypogonadotropic hypogonadism. *Clin Endocrinol (Oxf)* 2015, 82:422–428
45. Jun JY, Ma Z, Segar L: Spontaneously diabetic *Ins2(+/-Akita):apoE*-deficient mice exhibit exaggerated hypercholesterolemia and atherosclerosis. *Am J Physiol Endocrinol Metab* 2011, 301:145–154
46. Bourassa PA, Milos PM, Gaynor BJ, Breslow JL, Aiello RJ: Estrogen reduces atherosclerotic lesion development in apolipoprotein E-deficient mice. *Proc Natl Acad Sci U S A* 1996, 93:10022–10027
47. Mikkola TS, Clarkson TB: Estrogen replacement therapy, atherosclerosis, and vascular function. *Cardiovasc Res* 2002, 53: 605–619
48. Persson I, Weiderpass E, Bergkvist L, Bergström R, Schairer C: Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. *Cancer Causes Control* 1999, 10:253–260
49. Finan B, Yang B, Ottaway N, Stemmer K, Müller TD, Yi CX, Habegger K, Schriever SC, García-Cáceres C, Kabra DG, Hembree J, Holland J, Raver C, Seeley RJ, Hans W, Irmeler M, Beckers J, de Angelis MH, Tiano JP, Mauvais-Jarvis F, Perez-Tilve D, Pfluger P, Zhang L, Gelfanov V, DiMarchi RD, Tschöp MH: Targeted estrogen delivery reverses the metabolic syndrome. *Nat Med* 2012, 18: 1847–1856
50. Surra JC, Guillén N, Arbonés-Mainar JM, Barranquero C, Navarro MA, Arnal C, Orman I, Segovia JC, Osada J: Sex as a profound modifier of atherosclerotic lesion development in apolipoprotein E-deficient mice with different genetic backgrounds. *J Atheroscler Thromb* 2010, 17:712–721
51. Gardner CD, Tribble DL, Young DR, Ahn D, Fortmann SP: Population frequency distributions of HDL, HDL(2), and HDL(3) cholesterol and apolipoproteins A-I and B in healthy men and women and associations with age, gender, hormonal status, and sex hormone use: the Stanford Five City Project. *Prev Med* 2000, 31:335–345
52. Wolfe BM, Barrett PH, Laurier L, Huff MW: Effects of continuous conjugated estrogen and micronized progesterone therapy upon lipoprotein metabolism in postmenopausal women. *J Lipid Res* 2000, 41:368–375
53. Khoo C, Campos H, Judge H, Sacks FM: Effects of estrogenic oral contraceptives on the lipoprotein B particle system defined by apolipoproteins E and C-III content. *J Lipid Res* 1999, 40: 202–212
54. Tam NN, Leav I, Ho SM: Sex hormones induce direct epithelial and inflammation-mediated oxidative/nitrosative stress that favors prostatic carcinogenesis in the noble rat. *Am J Pathol* 2007, 171:1334–1341
55. Caligiuri G, Nicoletti A, Zhou X, Törnberg I, Hansson GK: Effects of sex and age on atherosclerosis and autoimmunity in apoE-deficient mice. *Atherosclerosis* 1999, 145:301–308