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Original Research Article

Female Gonadal Hormones are a Risk Factor for Developing Atherosclerotic Changes in C57BL/6J Mice on Atherogenic Diet

Key words

atherosclerosis;
Paigen diet;
sex;
gonadal hormones;
mouse models;
lipids and cholesterol

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Abstract: In humans, estrogens are considered protective factor against atherosclerosis because the risk increases in postmenopausal women. However, it is not clear whether estrogens are the only factor, whether sex chromosomes also have an influence, and whether estrogens play the same role in all mammals. The mouse line C57BL/6J is prone to develop atherosclerotic changes in the largest arteries after prolonged feeding of a highfat diet containing cholesterol and cholate (Paigen diet). We aimed to examine effect of sex hormones and sex chromosome complement on the development of atherosclerotic plaques using agonadal SF-1 knockout mouse on C57BL/6J background. Gonadally intact and prepubertally gonadectomized WT and agonadal SF-1 knockout C57BL/6J mice of both sexes were exposed to a Paigen diet and a control diet for 20 weeks. We monitored their body weight, food intake, and serum lipid profile. The aortas were examined by the en face method, and the cross sections of the aortic bulbs were stained for lipid content. In all groups of mice, atherosclerotic changes were small and confined to the aortic bulb. The formation of atherosclerotic plaques was sex- and hormone-dependent, as female animals with functioning ovaries developed the most prominent atherosclerotic plaques. Gonadally intact females were also the only group that gained weight comparably on control or atherogenic diet. Diet affected blood biochemistry, but there were almost no significant differences between groups in serum lipid levels. Results indicated main mechanism causing sex-dependent differences in atherosclerosis depends on sex hormones rather than sex chromosomes. Our results also suggest that a mouse model of dietary induced atherosclerosis is of limited use to study the mechanisms of atherosclerosis in humans because the presence of estrogens impairs lipid metabolism and contributes to the formation of atherosclerotic plaques.

Abbrevations: SF-1 KO: steroidogenic factor 1 (*Nr5a1* gene) knockout mice; WT: wild-type mice; CAS: castrated males; OVX: ovarectomised females; HDL: high-density lipoprotein; LDL: low-density liporotein; FFA: free fatty acids; PBS: phosphate buffered saline; FGF: fibroblast growth factor

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Introduction

Atherosclerosis is a progressive disease characterised by the accumulation of lipids and fibrous elements in large arteries and presents a serious health problem for humans in western societies with limited treatment options. Although it is wildly studied, there is no ideal animal model since other mammals are relatively resistant to the atherosclerosis and mechanisms of development of atherosclerotic lesions differ between species (1, 2). Apolipoprotein E-deficient (ApoE

KO) mouse model is probably the most commonly used rodent model, followed by LDL receptor knock-out (3–5). These mice exhibit significant hypercholesterolemia that cannot be induced by diet alone. In general, however, mice are relatively resistant to atherosclerotic changes, although there are differences between strains and each genetically modified model has some limitations for extrapolation to human disease (5–7). Mild atherogenic changes can be induced with Paigen diet containing 1.25 % cholesterol, 0.5 % cholate and polyunsaturated to saturated ratio of 0.7 in wild type C57BL/6 mice (8, 9) and combination with high-fat diet is usually preferred or even needed in testing potential therapies in other rodent models (2). The complexity of atherosclerosis as a disease arises from many genetic loci that contribute to lipoprotein levels, body fat, and other risk factors, as well as the interactions among these factors. In humans, male sex and menopause in women are considered important risk factors, although the mechanisms of this sex difference remain poorly understood and hormone-based therapies haven't produced the desired results (10–13). In animal models, the correlation is even less clear. Standard paper on methodology of atherosclerotic assessment from Paigen et al (9) already reported atherosclerotic changes more numerous or larger in female mice in comparison to male mice. Because most of the experiments in this study were performed in female mice, the sex effect was not emphasized and was usually overlooked in subsequent studies. In most other studies, only one sex was used-usually the male-to avoid variation due to the estrous cycle in females, and until recently, sex was not emphasized in basic research, including cardiovascular topics (14–16). Studies using *ApoE* knockout mice yielded equivocal results, as in some studies atherosclerotic changes were more pronounced in males (16–20) and in others in females (21–24). Possible explanations for these discrepancies are the different genetic background of the genetically modified mice and the duration of the study. In older mice, the males are more susceptible than at younger ages (reviewed in (25)).

In order to elucidate sex differences and the effects of sex hormones and sex chromosomes, we studied wild-type (WT), gonadectomized WT (OVX and CAS), and agonadal SF-1 knockout mice exposed to an atherogenic diet for 20 weeks. Steroidogenic factor 1 (SF-1) is a gene with an important function in gonadal and adrenal development. Mice lacking the *Sf-1* (*Nr5a1*) gene (abbreviated as SF-1 KO mice) are born without gonads and adrenal glands (26). Newborns die within 24 hours but can be rescued by adrenal hormone replacement and adrenal gland transplantation and are used as an agonadal adult mouse model to study possible effects of sex chromosomes on various sex-specific traits (27, 28).

Materials and methods

Animals

Heterozygous mice with a disrupted SF-1 (*Nr5a1*) allele (SF1+/-) (originally produced by dr. Keith L. Parker at DUKE University, North Carolina, USA) were backcrossed to a C57BL/6J mouse line for more than 10 generations to generate a congenic line. All mice were housed in dedicated facilities at the Faculty of Veterinary Medicine, University of Ljubljana, providing a controlled environment with relative humidity of 45-60%, temperature between 21 and 24 °C, and a 12:12 light-dark cycle. Every 2 years, the in-house breeding colony is refreshed with males of strain C57BL/6JOlaHsd (Envigo Italy). Entry and all safety measures at the animal facility are in accordance with SFP standards. Incoming animals are examined for the absence of the most common pathogens in accordance with FELASA recommendations, and internal monitoring of sentinel animals and waste bedding is conducted once a year. Animals were housed in pairs in conventional cages (Eurostandard type II or II L) on irradiated bedding (Lignocel, Rosenberg, Germany) with phytoestrogen-free feed (#2916, Harlan Teklad, Milano, Italy) and acidified water (HCl, Sigma Aldrich, Steinheim, Germany, to pH 3) ad libitum. All animal procedures were performed in accordance with the EU Directive (2010/63/EU) and approved by the Slovenian Veterinary Commission (Decision U34401-22/2015/13). SF-1 +/- mice were mated to produce homozygous SF-1 knockout (SF-1 KO) and control wild-type progeny (WT). To ensure survival of SF-1 KO mice, all newborn pups were subcutaneously injected daily with 50 µl of a corticosteroid cocktail in corn oil (400 ng/ml hydrocortisone, 40 ng/ml dexamethasone, and 25 ng/ml fludrocortisone acetate; all from Sigma Aldrich, Steinheim, Germany). Mice were genotyped between days 4 and 6 postnatal by PCR analysis of skin DNA. The primers used were *Nr5a1* F 5'-ACAAGCATTACACGTGCACC-3' and *Nr5a1* R 5'-TGACTAGCAACCACCTTG CC-3' for SF-1 WT, *Nr5a1-neo* R 5'- AGGTGA GATGACAGGAGATC-3' for disrupted SF-1 allele, and F 5'-AGGCGCCCCATGAATGCA TT-3' plus R 5'-TCCATGAGGCTGATA TTTA TAG-3' for *Sry* gene. Female WT littermates or female pups from other C57BL/6J litters born within 3 days as KO pups were used as the source of adrenal transplants. The technique was previously published (27), we omitted keeping adrenals from donors in PBS and FGF, but rather transplanted them immediately after collection. After adrenal gland transplantation on postnatal day 7, SF-1 KO mice received corticosteroid injections and then three more - on days 9, 12 and 16, weaning took place on postnatal day 21. WT mice used in the study were subjected to the same corticosteroid treatment protocol as SF-1 KO mice. After weaning, mice of the same experimental group with the same age and sex were housed in pairs until sacrifice. Half of the WT mice were gonadectomized before puberty (P23-28) to represent groups without sex hormones in adulthood: ovarectomized females – designated WT F OVX and castrated males - WT M CAS. For gonadectomies, WT mice were anaesthetized

with a mixture of ketamine (Vetoquinol Biowet 100 mg/ml, Gorzowie, Poland; 100 µg/g bw), xylazine (Xylased 20 mg/ml, Bioveta; Czech Republic; 10 µg/g bw), and acepromazine (Calmivet 5 mg/ml, Vetoquinol, France; 2 µg/g bw). The ovaries were removed trough flank incisions and the testes through bilateral inguinal incisions. Any bleeding was stopped with a battery-powered cautery, and the wound was closed with absorbable polyfilament PGA in two layers. After gonadectomy, mice received a subcutaneous injection of the analgesic butorphanol (Fort Dodge Animal Health; 1.7 µg/g body weight), followed by another injection in 4-5 hours and 100-200 µl saline subcutaneously if some blood loss occurred. SF-1 KO and intact wild-type mice (WT F and WT M) were sham-operated at P23-28 with the same anaesthetic protocol, incision and suture, according to their genetic sex. The decision of which animals were gonadectomized and which were left intact (sham operations) was made randomly by the animal caretaker, with the operator receiving the daily sequence.

Inclusion criteria were at least 7 g body mass at weaning for SF -1 KO mice, and the total number of available KO females and males was the limiting factor for the randomization strategy. The minimisation principle was applied: all WT mice were littermates or closely related young born at the same time (+/- 3 days) as KO, housed in pairs, and assigned to the diet regime on the appropriate days. Exclusion criteria included complications during surgery or prolonged recovery time after surgery, and general health problems such as persistent loss of body mass. 4 animals met the criteria for a humane end point during the diet regime, and body mass measurement and consumption data from these animals were excluded from the final analysis. Because some losses, especially in SF -1 KO mice, were expected, additional pairs were initially included in the study, in the end each experimental group consisted of 8 animals. Because of the different coloration of the diets, the researcher could not be blinded during animal and food weighing, but the order of the animals at the time of killing was randomised, and complete blinding was possible in the analysis of blood serum and tissue.

Table 1: Composition of both diets used in the study as per manufacturer's declarations (Sniff Spezi-aldiäten, Soest, Germany).

	High fat – Paigen (ssniff S1102-E124)	**Low fat – Control** (ssniff S1102-E122)
Energy ME MJ/kg	18	16
diet specific	15% cocoa butter 1.25% cholesterol 0.5% Na cholate	n.a.
crude protein %	17.6	17.6
crude fat %	16.1	7.1
crude fibre %	5.0	5.0
crude ash %	3.6	3.6
starch %	19.2	32.7
sugar %	21.0	11.0
Vitamin A (IU)	15, 000	15, 000
Vitamin D3 (IU)	1, 500	1, 500
Vitamin E (mg)	150	150
Vitamin K3 (mg)	20	20
Vitamin C (mg)	30	30
Copper (mg)	11	11
other	without phytoestrogens, sterilized 25 kGy, 10 mm round pellet	

Diets, food intake, and body mass measurement

Mice were housed in pairs and fed Paigen diet (S1102-E124) or control diet (S1102-E122, both produced on order by Sniff Spezialdiäten, Soest, Germany) for 20 weeks after reaching the second month of life - between postnatal day 65 and 75. Animals were remained on the diet until time of sacrifice, up to 22nd week. The diets were free of phytoestrogens to exclude external hormonal influences. The atherogenic diet was prepared according to the Paigen recipe with 15% cocoa oil, 1.25% cholesterol, and 0.5% sodium cholate. The control feed contained the same amount of protein, fibre, and vitamin supplements but differed in crude oils and fats. The food declaration is presented in Table 1. The stock feed was stored in the freezer and added to the animal cages in small amounts to avoid rancidity: the needed weekly amount was thawed, cages racks filled up to one tird and the remaining pelletes vacuum sealed and stored in refrigerator to be added during if needed. Every week during cages changing food was renewed from the stock.

Feed consumption was measured every month during the treatment for 1 week: Feed was weighed every other day, divided by two to determine average consumption per animal, and further divided by two to determine daily consumption. Measurements were taken at the first, fourth, 8th, 12th, 16th, and 20th weeks. Body mass was measured individually each week.

Tissue collection

The animals were euthanized at 220 to 230 days of age. The killing took place between 10 am and 2 pm, when the animals were in the second half of the light cycle and are semi-fasted by their natural behaviour (mostly sleeping). The thoracic cavity was exposed under surgical anaesthesia, a blood sample was taken from the left ventricle, followed by complete exsanguination. The incision was made in the right atrium, and the cannula was inserted in left ventricle from apex in cranial direction. The blood vessels were washed with saline (B. Braun Medical) and gentle manual pressure. Micro-scissors were used to excise the heart and the entire aorta. The *en face* aorta was prepared as previously described (29) and the entire protocol can also

be found complemented with video (30). In brief, the aorta was separated from the heart under a stereomicroscope, placed in 4% paraformaldehyde (Sigma Aldrich) in 0.05 M PBS, pH = 7.4, for 24-48 hours, washed in PBS, cleared of adventitia, pinned to a black wax support, and visualised under a stereomicroscope with 20× magnification. To quantify atherosclerotic plaques in the aortic bulb (31), the heart was sectioned transversely on a plane connecting the tips of the two atria with the line perpendicular to the aortic outlet. The lower two-thirds of the heart were discarded, and the upper portion, including the aortic bulb, was placed in a Corning microcentrifuge, covered with tissue freezing medium (Leica Biosystems, Nussloch, Germany), frozen in liquid nitrogen, and stored at -80 °C. Serial 10-µm cryosections of the heart perpendicular to the aortic bulb were cut at -20 °C with the Leica CM1850 cryotome, collected on charged glass slides (Thermo Scientific Superfrost Plus, Menzel-Glaser) and stored at -20 °C until staining. Before staining, they were air dried and fixed with 4% paraformaldehyde for staining with Oil Red O (0.3%, Sigma-Aldrich, Steinheim; Germany) and a light hematoxylin counterstain. For labelling mast cells with toluidine blue stain, they were air dried only. We excluded samples of aortic bulb if sections were too distorted due to freezing storage or not cut at appropriate angle.

Measurement of blood plasma parameters

A blood sample (200µl) was taken from the left cardiac ventricle of each animal with a 21-gauge needle washed with heparin (5000 i.e./ml Braun, Melsungen, Germany). The blood was transferred to a microcentrifuge (Eppendorf® 5415R) and spun at 3000 rpm for 10 minutes at 4 °C. Plasma was transferred to another microcentrifuge and stored at -20 °C until analysis. All analyses were performed using the Olympus AU400 analyzer (Mishima Olympus co., LTD, Japan). Cholesterol was measured using an enzymatic colour assay with chromophore detection at a wavelength of 540/600 nm (Beckman Coulter, Inc, USA). For HDL cholesterol, an enzymatic colour assay with product detection at 600/700 nm (Beckman Coulter. Inc., USA) was used. LDL cholesterol was determined using an enzymatic colour assay with cholesterol oxidase/ PAP system with detection at 600/700 nm (Beckman Coulter, Inc., USA). Triglyceride concentration was measured using an enzymatic colour assay with product detection at 660/800 nm (Beckman Coulter, Inc., USA). Non-esterified fatty acids were determined by enzymatic colorimetric method with product detection at 550 nm (Randox Laboratories Ltd, United Kingdom). Total bile acid concentration was measured indirectly by measuring the rate of thio-NADH formation in the presence of the enzyme 3-a-hydroxysteroid dehydrogenase (Diazyme Laboratories, USA).

Morphometric and Statistical Analysis

En-face aorta preparations were photographed under a stereomicroscope (Olympus SZ40, Japan), Canon 500D digital camera, and Quick-PHOTO CAMERA 3.1 software (Microscope Imaging Software 2014, Promicra, Prague, Czech Republic) and visually examined for the presence of opaque plaques. Cryosections stained with Oil red-O and hematoxylin were photographed under 100× magnification using a Nikon microscope (Nikon Microphot FXA Eclipse 80i, Japan) in conjunction with a 3CCD camera (Nikon DS -Fi1, Japan) and NIS -Elements software (F 2.20, Laboratory Imaging s.r.o. for Nikon Corporation, Praha, Czech Republic). Only sections in which all three cusps of the aortic valve were visible (2-3 per slide) were included. The area of positive staining was measured as a percentage of the total visible area using the Image J polygonal tool (v. 1.51n, NIH, Bethesda, Maryland, USA). To minimise polygonal errors, three consecutive measurements were taken at each cross-section and the mean values per animal were calculated.

Data are reported as mean ± SEM. Statistical analysis was performed using IBM SPSS Statistics v 24.00. Normality tests (Kolmogorov-Smirnov and Shapiro Wilk) rejected the null hypothesis based on median. Body weight beasurements and food consumption were analysed with repeated measurements ANOVA and Bonferroni post hoc test. For total body mass gain, cumulative calorical intake and plaque area multifactorial analysis of variance (MANOVA) with the independent variables of diet, sex, and hormones (genotype) followed by the Tukey post hoc test was used for multiple comparisons of variables between groups. For plasma parameters, a one-way ANOVA and LSD post hoc test was performed. $P < 0.05$ was considered significant.

Results

Food consumption and weight gain

The increase in body mass and estimated cumulative consumption during the experiment are shown in Fig. 1, separately for female and male animals. Intact animals (WT M and WT F) consumed more of both diets. Animals without gonads (SF-1 KO and OVX/CAS) became obese on the control diet, but on the atherogenic diet, despite its high fat content, body mass increased slowly and even stopped in some animals - the curves begin to differ between weeks 9 and 10. Gonadally intact mice continued to gain weight until the time of sacrifice. Repeated measurements ANOVA showed that the type of diet affected both body mass gain and food consumption ($p < 0.001$). The presence of sex hormones affected body mass and the interaction between hormones and diet was significant $F(1, 84)=9.8$, $p=0.002$. For food consumption also sex had an effect and the interaction between hormones and sex was significant $F(1.1, 86.6)=6.7$, $p=0.009$.

Animals that developed without hormones (SF-1 KO) significantly increased their body mass on a normal diet (as expected from previous studies(27, 32)) but on atherogenic diet the obesity was less evident. In general, most animals

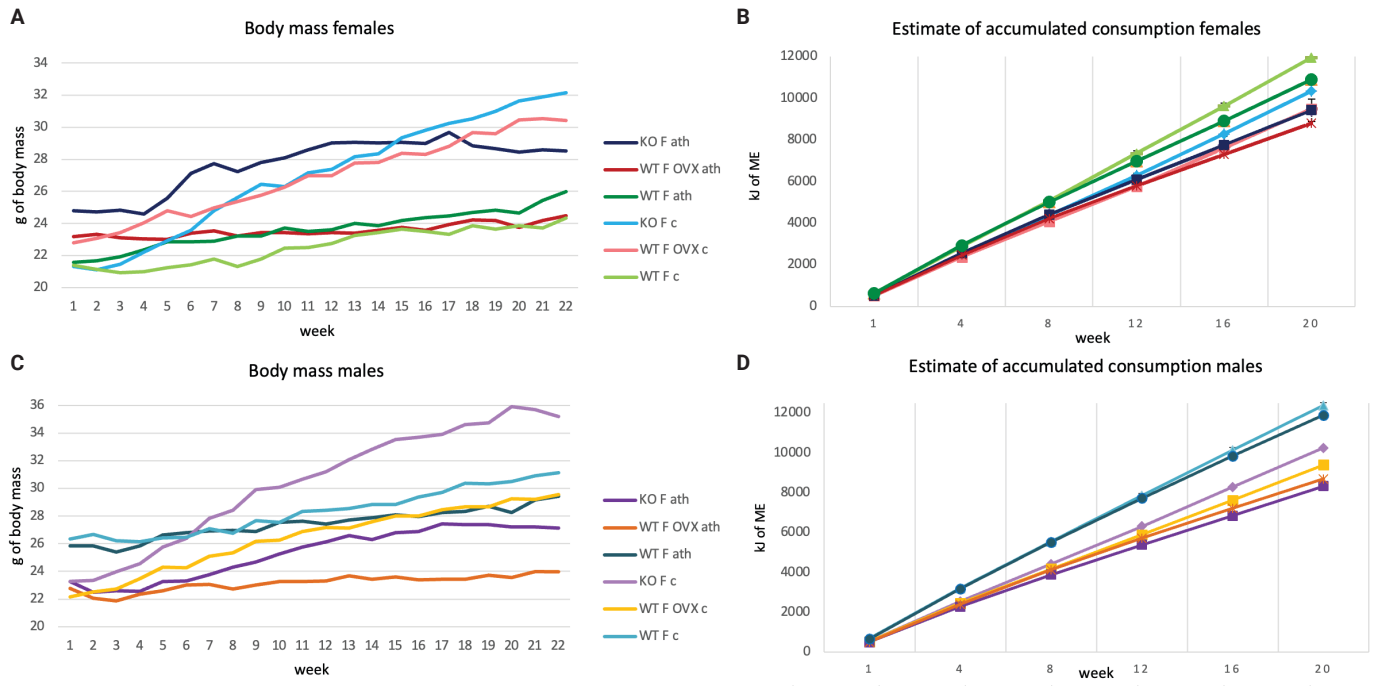

Figure 1: Graphical representation of weekly increase in body mass, separated for clarity into female (A) and male groups (C), and estimated cumulative consumption on two different diets (B, D). The colors for the groups are matched between left and right panels

gained significantly less weight in association with the reduced consumption of the atherogenic diet, except for the intact females, whose body mass increased on the atherogenic diet despite unchanged consumption.

Correlation between body mass gain and cumulative consumption was analyzed on the end-point measurements, which are also presented as bars in Figure 2. Correlation was only weak to moderate in most groups, as represented with Pearson's r in Table 2. Intact females and castrated males stand out as correlation was high on control food but Paigen diet disrupted the expected weight gain.

All groups gained significantly less body weight on the atherogenic diet, except for gonadally intact males and females (WT), in which the difference was not significant; in fact, females (WT F) appeared to utilise more energy from the high fat diet. Food consumption was significantly higher in the gonadally intact male and female groups than in the groups without sex hormones. Although consumption of atherogenic food was lower when weighted in grammes of pallets, the difference in energy value was not significant except in SF -1 KO males, whose body mass gain was also much lower on atherogenic food.

Table 2: Correlation of weight gain from the beginning to the end of the experiment with estimated cumulative consumption within groups

	Pearson Correlation body mass gain - total consumed food	
r	Paigen diet	Control diet
KO F	0, 28	0, 32
OVX	0, 45	0, 16
WT F	0, 32	0, 82
KO M	0, 49	0, 22
CAS	0, 10	0, 86
WT M	0, 17	0, 51

Atherosclerotic plaques

None of the animals had conspicuous changes in the aortic wall or visible fatty streaks on the thoracic or abdominal aorta visible as en face preparations under 20× magnification (Fig. 3). Therefore, further staining and analysis were not performed.

Oil-red-O staining revealed minimal fatty deposits on the cross-sections of the aortic bulbs of mice receiving a control diet: in couple of the SF-1 KO female group, in one ovariectomized female, and none in males without gonads (KO M, CAS), while most intact females and males on control diets exhibited minimal deposits.

On the other hand, in all groups fed an atherogenic diet histologically visible aortic root lesions in the tunica intima of the aortic wall were found (Figure 4A). Significant effect of factors sex, diet and hormones was found ($p < 0.001$) and also interaction between all three $F(1, 61)=11.13$; $p= 0.002$). The lesions were most pronounced in the intact females

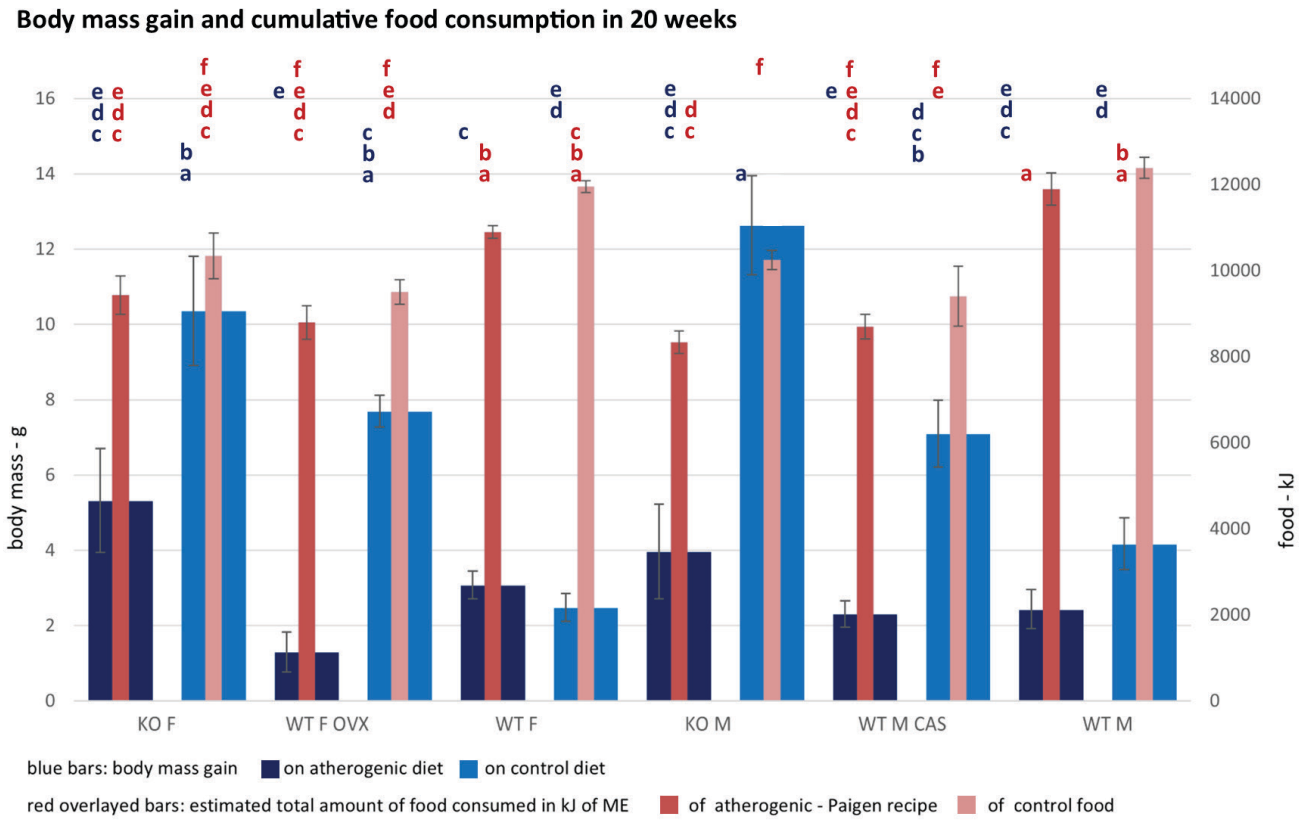

Figure 2: Direct comparison of total body mass gain (blue bars) and estimated total consumption (red overlayed bars, secondary Y axis) on atherogenic (dark red or blue) and control food (light red or blue). Different lowercase letters indicate significant differences at $p < 0.05$ probability level determined with MANOVA and Tukey's HSD test for multiple comparisons, color matched with corresponding bars. N=8 in most groups, WT F on control diet n=7, WT F OVX on atherogenic diet n=6

and measured plaque areas were significantly different from all other groups ($p < 0.001$), whereas the other groups were not significantly different from each other (Fig. 4B).

Lesions were confined to the tunica intima and rarely extended into the aortic lumen or onto the valve cusps. Most of the oil-red-O positive droplets were debris, foam cells were few (Fig. 5A) and small acellular regions were present. Necrotic cores or calcifications were not observed. Scattered mast cells, as determined by metachromatic staining with toluidine blue, were present in all samples (Fig. 5B). Their location and number were not clearly related to plaque extent, but in 50% of sections belonging to females on atherogenic diet, metachromasia was observed on the aortic valves themselves as a sign of mast cell degranulation and inflammation of the tunica intima (Fig. 5C).

Serum lipids

Measurements of total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), tryglicerides, free fatty acids, and serum bile acids in serum are shown in Fig. 6. Some differences in baseline values (animals on control diet) were observed: Total triglyceride level was higher in intact males (Fig. 6B) and HDL level was significantly lower in intact females than in intact males, SF-1 knock-out males

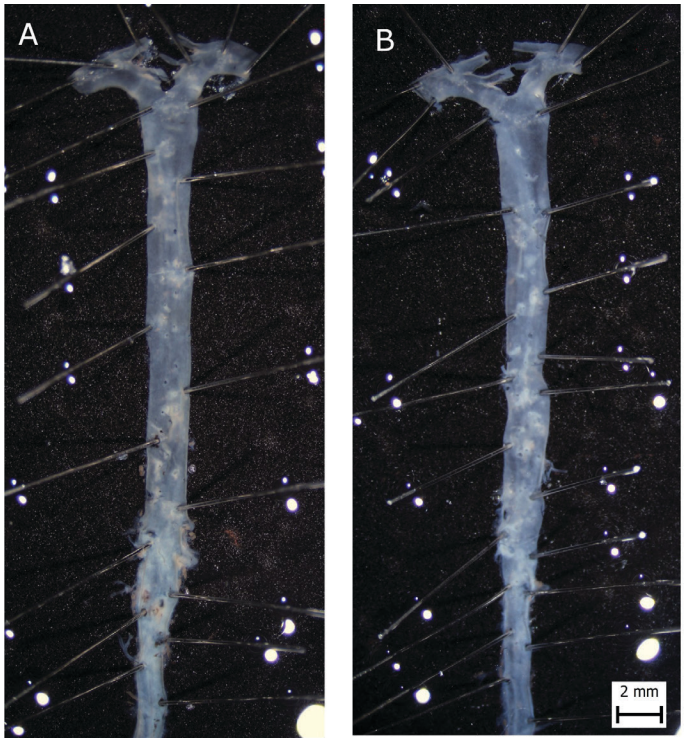

Figure 3: Photomicrograph of thoracic aorta with clear transparent wall of an intact female fed atherogenic diet (A) or control diet (B). No fatty streaks are seen, and this was similar in all groups (not shown)

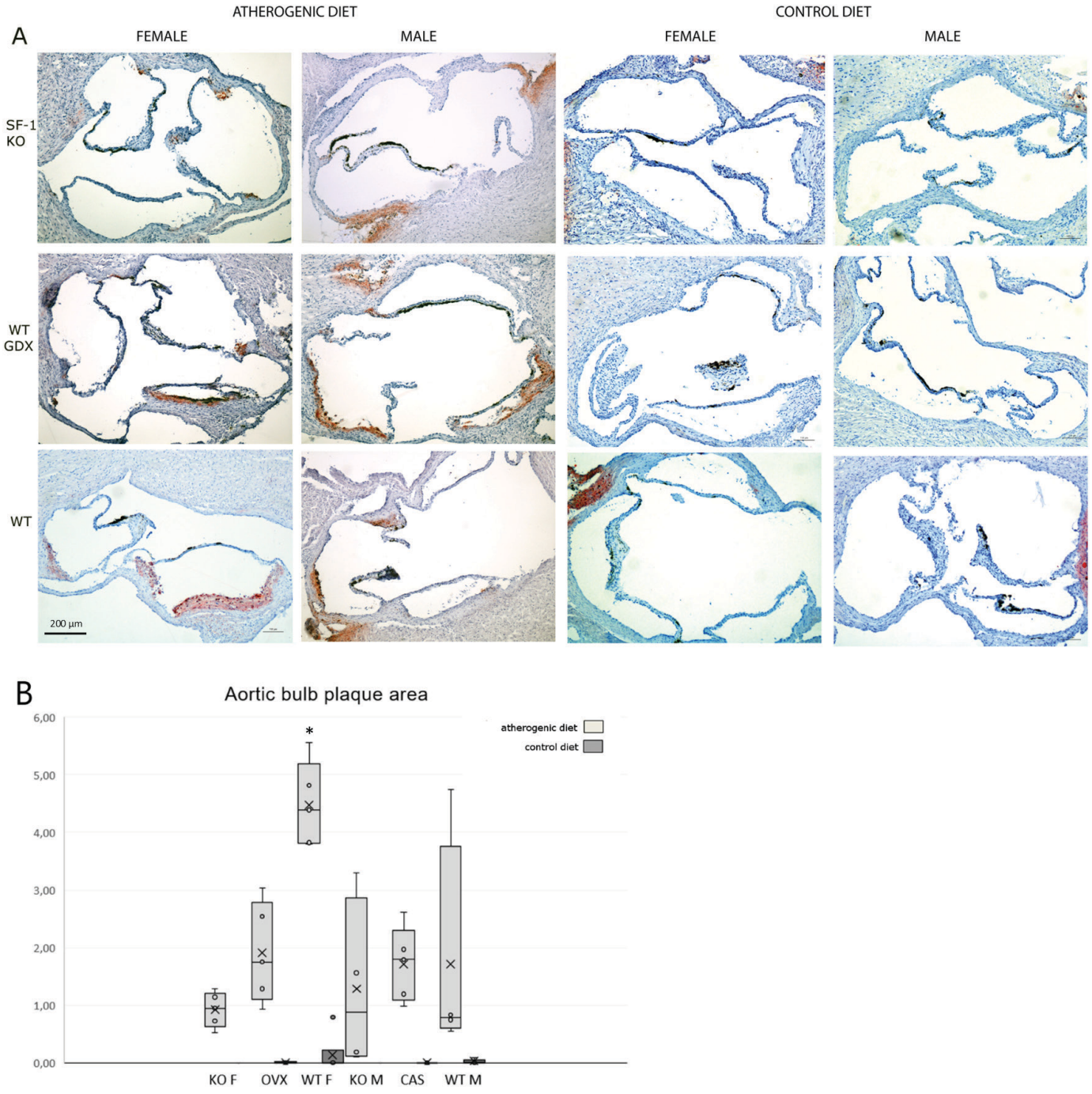

Figure 4: Atherosclerotic plaques form in the aortic root of wild-type C57BL/6J and SF-1 KO mice fed Paigen diet for 20 weeks. A) Representative photomicrographs of atherosclerotic plaques in the aortic root stained with Oil-red-O and hematoxilyn in all 6 groups fed atherogenic (two left columns) or con-trol diet (right columns). B) Quantification of plaques area as percentage of visual field with region-of-interest (aortic bulb) shows that they were most prevalent in intact female wild-type mice fed an ather-ogenic diet (* $p < 0.001$). Some minimal plaques were found in individuals from WT F and WT M (intact males and females) on control diet. (n=6 on control diet and n=5 on atherogenic diet, except for KO M and WT M on atherogenic diet n=4)

and ovariectomized females (6D, $p < 0.05$). Atherogenic diet significantly increased (nonfasting) total plasma cholesterol, LDL cholesterol, and total bile acids (Fig.6A, C, E; $p < 0.05$). No significant change in total triglycerides was observed. Free fatty acids were significantly decreased (6F) but less so in intact and castrated males, which were significantly different from all females and SF-1 KO males ($p < 0.001$). No other significant changes were observed between the atherogenic diet groups.

Discussion

The present study shows that gonadally intact female C57BL/6J mice develop more marked atherosclerotic plaques on atherogenic diet than gonadally intact males

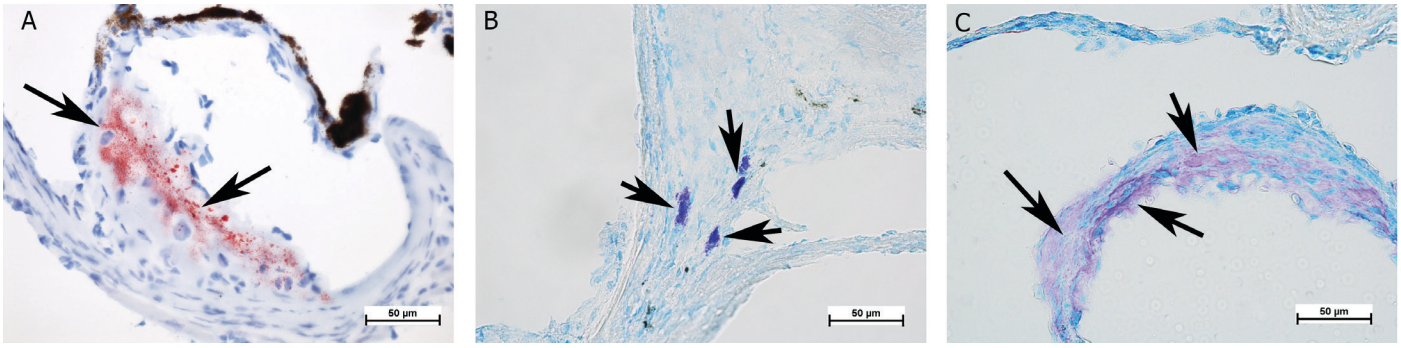

Figure 5: A) Oil- red-O stain shows lipid droplets in few foam cells (arrows) - early plaque formation in the space behind the aortic valve, dark brown are pigment cells in the valves; B) Migration of mast cells (arrows) to the aortic bulb wall, metachromatic granules (purple) in the citoplasm with toluidine blue staining, not related to extensive plaques C) mast cell degranulation (touluidine blue metachromatic reaction, arrows) in the intima of the aortic valve, found in WT females on atherogenic diet only

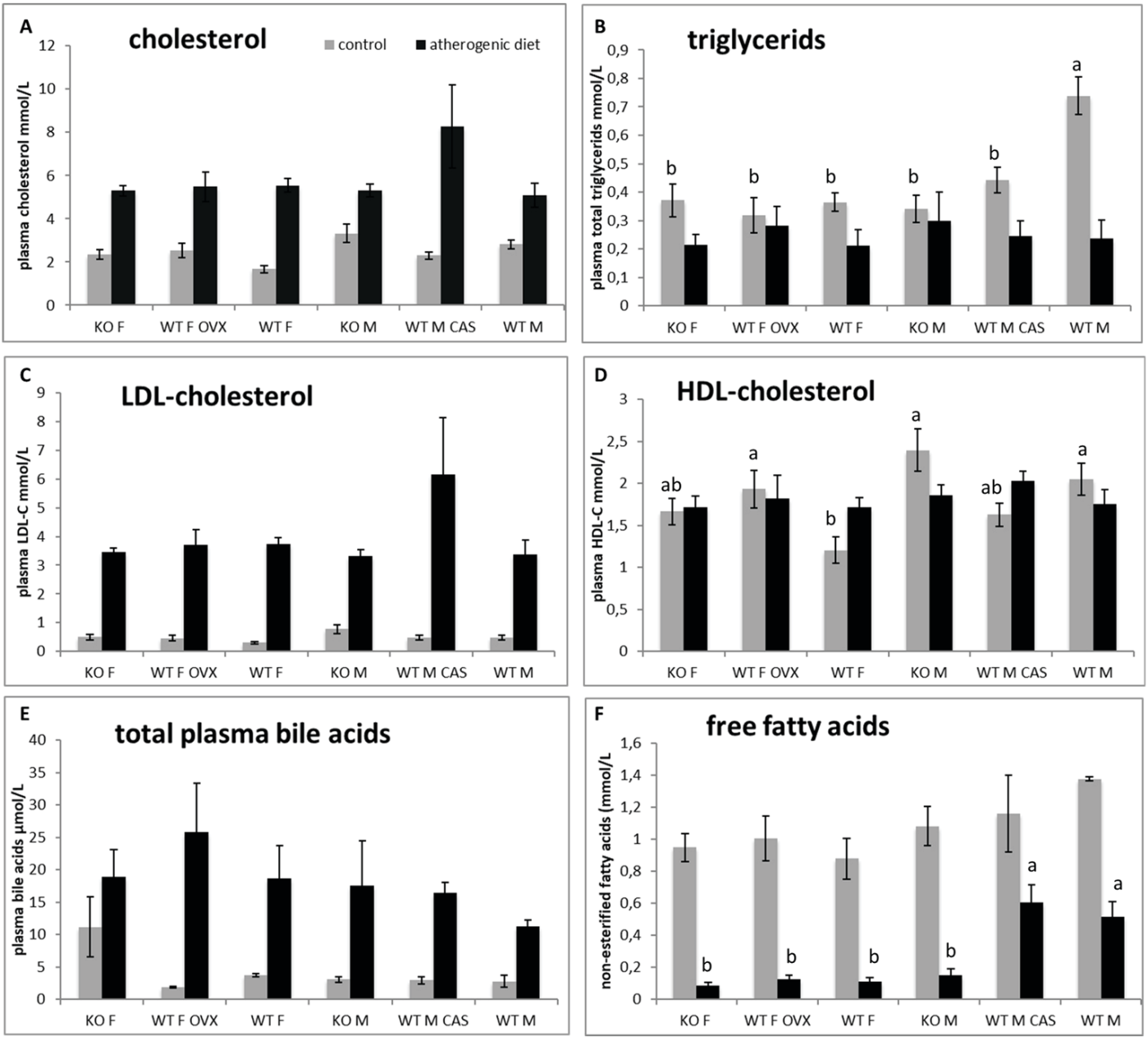

Figure 6: Effects of atherogenic diet on plasma lipid levels measured by enzymatic color assays, n=8 per group. Data are expressed as mean± SEM. Atherogenic diet significantly increased total and LDL cholesterol and total bile acids and decreased free fatty acids (significant differences between black and grey bars; $p < 0.05$). Differences between groups either on atherogenic or control diet were few and are indicated by lowercase letters at probability level $p < 0.05$

or male and female mice with suppressed sex hormones. Serum lipid profiles could not explain the increased atherosclerosis in females because there was almost no difference between the groups on atherogenic diet. Interestingly, intact females appeared to metabolise the high fat content of the diet better, as only this group consistently gained weight while fed the Paigen diet, although they didn't reach the obesity level of the agonadal mice during duration of experiment. In agonadal SF-1 knockout mice, there were no sex differences in any of the parameters studied, suggesting that the main mechanism causing sex-dependent differences in atherosclerosis depends on sex hormones rather than sex chromosomes.

In the present study, all animals fed the atherogenic diet consumed slightly less food than animals fed the control diet. When converted to estimated total consumption in caloric value of metabolic energy, the differences were not statistically significant, except for SF -1 KO males. These males also gained weight poorly on the atherogenic diet with the greatest difference from the animals on the normal diet - likely due in part to reduced consumption. Daily consumption did not change significantly over the course of the experiment and correlation between final weight gain and total amount of food consumed was only moderate. It should be noted that the Paigen diet is hepatotoxic. High cholesterol diets might not be sufficient to induce atherosclerosis, but it does lead to steatohepatitis characterised by oxidative stress and expression of inflammatory genes. The addition of cholate to induce atheroclerotic plaques also makes the diet lithogenic and specifically affects the expression of extracellular matrix deposition genes thus exacerbating liver injury (33, 34). All animals on the atherogenic diet in our study had hepatosteatosis as macroscopicaly observed at necropsy, in some individuals also large gallbladder stones and/or jaundice was observed. 4 animals reached humane endpoint between week 17 and 18 as their health detoriated with evident body mass loss. Also noted control SF-1 KO animals did not reach the usual extreme body weights observed in previous studies (27), probably due to the lower fat content of the control diet in this study, but were still heavier than gonadectomized mice. Some studies assume that only prolonged feeding of the atherogenic diet (up to a year) causes the obvious atherosclerotic changes (31), but we found that it seriously affected the general health our mouse strain already in 20 weeks, at least in SF-1 KO and gonadectomized mice. Since the animals without gonads gained significantly more weight on the control diet, we suspect that the gonadal hormones prevented the (early) deleterious effects of the Paigen diet. The average increase in body mass was slightly higher in intact females on the atherogenic diet than on the low-fat diet, although the ratio of consumation was not changed. It is known that X chromosome effects pose a risk for obesity and estrogens are generally protective against obesity (reviewed in (35)). Still the effect can be strain-dependent like it was demonstrated by Surra et al. (36) where female ApoE KO mice gained significantly more weight when on C57BL/6 background than other strains. In our study, the combination of dietary fats and normal oestrogen levels appears to have contributed to the observed sustained weight gain, but the experiment ended too early to see the long-term effect, neither total body fat content was analyzed.

In the original work by Paigen et al (9) it was reported that atherosclerotic changes were more numerous or/and extensive in female mice compared with male mice. This study was primarily a methodological study, and most experiments were performed with female mice only, so sex differences in atherosclerosis were not further emphasised. Most subsequent studies examined only one sex, and studies using ApoE-deficient mice yielded conflicting results, with some reporting atherosclerotic changes were more pronounced in male (17, 18, 20) and others in female mice (21, 22, 37). The explanation for the discrepancies may be due to the different genetic background of the modified mice (36, 38) and the duration of the study or the age of the mice. Some evidence suggests female sex hormones sensitize inflammation in atherogenesis (7) but as males get older, they are more prone to plaque formation (25). Mapping mouse atherosclerosis modifier genes in some congenic mouse strains yielded replicating results in females and males but the extent of atherosclerosis plaques appears to be slightly higher in females where the data were normally distributed but was skewed in males (39), somewhat comparable to our results. In our study, we found significant differences in atherosclerotic lesions between groups, with gonadally intact WT females having two to three times bigger atherosclerotic lesions than all other groups: gonadally intact males, prepubertally gonadectomized males and females, and agonadal SF-1 KO mice. It should be noted that the overall extent of plaques was small in the aortic bulb, not (yet) apparent in the aortic arch, and only a few foam cells (macrophage uptake of LDL) and degranulation of mast cells were observed (Fig. 5). This suggests an early inflammatory process - plaque initiation of the aortic wall (40, 41), again more pronounced in intact females. We can speculate that longer exposure to the atherogenic diet or starting the diet in much older mice would result in larger plaques and associated signs of atherosclerosis. The observation of more pronounced atherosclerotic changes in gonadally intact females is consistent with the previously reported findings of Caligiuri et al. (37) and Marek et al. (22) both of which showed more severe lesions in females compared with males and both of which used the C57BL/6 background for the ApoE mouse model. It is also consistent with some data on LDLr-deficient mice (7, 24), although these studies used only gonadally intact mice and therefore it is not possible to speculate whether testosterone plays a protective role in WT males or whether estrogens increase risk in female mice. Therefore, in our study, we used prepubertal gonadectomized WT males and females and agonadal SF-1 KO males and females for comparison and no significant difference were found among those groups. This strongly suggests that estrogens are a

risk factor for the development of diet- induced atherosclerotic plaques in mice, unlike in humans.

In general, early atherosclerotic plaque formation in all animals receiving an atherogenic diet but not in those receiving a control diet is in accordance with an overall increase in total cholesterol and LDL cholesterol in all groups. The sexual dimorphism in plasma lipid levels was associated with XX chromosome complement in four core mouse model (42) and to some extent we observed a similar effect of male sex (increased levels of tryglicerides and FFA on normal diet). However, there was no evidence that the extent of plaques can be predicted from serum cholesterol levels, because no significant differences were found between groups; in particular, gonadally intact females didn't have different LDL or HDL cholesterol levels compared with other groups on atherogenic diets. To some extent also intact males (individuals) were gaining weight and had more pronounced aortic plaques on both, atherogenic and control diet compared to males without testosterone and would possiby gain statistic significance if kept on the diet for prolonged period. Obesity has long been recognised as an important atherogenic risk factor associated with unhealthy diet, but the mouse and diet model in our study could not directly demonstrate this effect.

In humans, estrogens appear to play a protective role in the development of cardiovascular disease and the formation of atherosclerotic plaques, as both are more common in men than in women, although the risk increases in menopausal women (35). Thus, the possibility of hormone treatment (estrogens) for atherosclerosis has attracted considerable interest in medicine but has had mixed success (13). Interestingly, most studies using estrogens to treat the progression of atherosclerosis report a reduction in lesion size, although many studies show that atherosclerosis in mice is more severe in female animals (in contrast to humans). Direct comparison is complicated by the choice of mouse models, ApoE- or LDL receptor-deficient mice have been used mostly for treatments with 17beta-estradiol (5, 43)). The effects of endogenous and exogenous hormones may also differ, and whereas in some studies ovariectomy increased atherosclerosis in mice (44) other studies reported that ovariectomy did not cause vascular senescence in female C57BL/6 mice and did not exacerbate it in female ApoE KO (20). Lack of alteration in plasma cholesterol and trygliceride levels in mice is rather common observation in atherosclerosis induction or treatment (20, 44–46).

Not only ovariectomy but also complete ovarian agenesis had no effect in our SF-1 knock-out mouse model. In humans, there is some evidence that declining testosterone levels also contribute to the progression of atherosclerosis, but testosterone replacement therapies remain controversial (35). This suggests that the hormonal contribution to the development of atherosclerosis is complex, and interestingly, a study using the *ApoE-/-:Ins2+/Akita* model of accelerated atherosclerosis in mice reported that testosterone had both atheroprotective and proatherogenic effects depending on the glycemic status of the mouse. Castration accelerated atherosclerosis in normoglycemic mice but ameliorated it in diabetic mice (19). In our study, neither castration nor gonadal agenesis had significant effect on the extent of atherosclerotic plaques that developed after mice were fed a Paigen diet.

An important risk factor for atherosclerosis in humans and in mouse models is age. In C57BL/6 mice fed normal chow, vascular lipid deposits can develop spontaneously and become more prominent with age, probably because of increased oxidative stress, but only in very old animals (47). Another group also found lipid deposition on aortic valves and aortic regurgitation in old C57BL/6 mice fed normal chow, with more pronounced effects in male mice (38). In our study, individual control animals had minimal lipid deposition in the aortic root, especially intact females and males. The mice were less than 8 months old at euthanasia and thus not truly geriatric. Furthermore, no serum marker used in our study is likely to predict spontaneous/geriatric atherosclerotic changes in mice. Atherogenic diet generally affected serum lipids, but there was no difference between sexes or correlation with sex hormones. This is consistent with other studies (17, 18, 48) with the exception of study with Apo-E KO mice by Smith et al., (21) which found a similar higher incidence of atherosclerosis in females but reported a reversed lipid profile of serum lipids, as total cholesterol, tryglicerides, HDL, LDL, and VLDL were elevated in males.

The primary goal of animal studies is to determine whether drugs can evoke the regression of atherosclerotic plaques. However, many of the drugs tested have shown limited effects on plaque regression in animal studies. Quite often they have been studied in animals of only one sex, making extrapolation difficult when sexual difference in humans is long known. But at least one promising diagnostic and therapeutic agent - interleukin 19 (IL - 19) - had the same effect in male and female LDLR-deficient mice (46)

In conclusion, our study shows that atherosclerotic plaques in C57BL/6J mice on Paigen diet are exacerbated by female gonadal hormones, that female gonadal hormones also cause higher weight gain on atherogenic diet, and that serum lipid levels correspond poorly with atherosclerotic changes in mice. This suggests that estrogens are a risk factor for the development of atherosclerotic lesions and thus calls into question the validity of mouse models for the study of cardiovascular disease in humans, because in humans the situation is generally reversed and estrogens play a protective role in the development of cardiovascular disease.

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Ženski spolni hormoni predstavljajo dejavnik tveganja za nastanek ateroskleroznih sprememb pri miših linije C57BL/6J na aterogeni dieti

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Izvleček: Pri ženskah se v postmenopavznem obdobju poveča tveganje za razvoj ateroskleroze, zato je splošno sprejeto, da estrogeni hormoni varujejo ožilje pred razvojem tega žilnega obolenja. Ni pa še popolnoma raziskano, ali so estrogeni poglavitni dejavnik, ali imajo vpliv tudi spolni kromosomi in ali je vpliv spolnih hormonov enak med sesalci. Živalski modeli za proučevanje ateroskleroznega obolenja so redki, eden izmed njih so miši linije C57BL/6J, ki lahko spontano razvijejo aterosklerotične spremembe v večjih telesnih arterijah, če se jih dlje časa hrani s hrano z visoko vsebnostjo maščob, z dodatkom holesterola in holata - s t.i. aterogeno dieto po Paigenu. V raziskavi smo želeli proučiti vpliv spolnih hormonov in spolnih kromosomov na razvoj aterosklerotičnih plakov v žilah s pomočjo modela miši z izbitim genom SF-1, ki se razvijejo brez spolnih organov. 20 tednov smo mišim dajali hrano po receptu Paigen oziroma kontrolno hrano z nižjo vsebnostjo maščob. Miši obeh spolov so bile bodisi brez spolnih organov zaradi izbitega gena SF-1 (na ozadju C57BL/6J), bodisi smo jim gonade operativno odstranili pred puberteto. Tretjino samcev in samic smo pustili intaktne z gonadami. Spremljali smo telesno težo živali, povprečno porabo hrane in opravili analizo serumskih lipidov. Pregledali smo preparirane aorte po metodi *en-face* ter ocenili obseg plakov in maščob na prečnih rezih aortnega korena na nivoju aortnih zaklopk s histološkim barvanjem in analizo mikroskopske slike. Pri vseh skupinah miši, ki so bile hranjene z aterogeno dieto, so bile aterosklerotične spremembe relativno majhne in omejene na aortni koren. Obseg plakov je bil odvisen od kromosomskega spola in prisotnosti hormonov, plaki so bili najbolj očitni pri samicah z jajčniki. Istočasno so bile intaktne samice edina skupina živali, ki so podobno pridobivale na teži tako na aterogeni kot kontrolni hrani, pri ostalih skupinah so živali na aterogeni dieti priraščale bistveno manj. Vrsta hrane je imela vpliv na serumski lipidni profil, vendar praktično ni bilo statistično značilnih razlik med različnimi skupinami živali in analize krvnega seruma nismo mogli povezati z drugimi ugotovljenimi odstopanji pri samicah. Rezultati raziskave kažejo, da so glavni povod za spolne razlike pri razvoju aterosklerotičnih sprememb spolni hormoni in ne spolni kromosom. Hkrati pa rezultati postavljajo pod vprašaj uporabnost mišjih modelov za proučevanje ateroskleroze, ki jo induciramo s prehrano, saj prisotnost estrogenov - obratno kot pri ljudeh - pri miših negativno vpliva na presnovo lipidov in doprinese k izoblikovanju aterosklerotičnih plakov.

Ključne besede: ateroskleroza; dieta po Paigenu; spol; spolni hormoni; miš, lipidi in holesterol