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XX sex chromosome complement promotes atherosclerosis in mice

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Men and women differ in circulating lipids and coronary artery disease (CAD). While sex hormones such as estrogens decrease CAD risk, hormone replacement therapy increases risk. Biological sex is determined by sex hormones and chromosomes, but effects of sex chromosomes on circulating lipids and atherosclerosis are unknown. Here, we use mouse models to separate effects of sex chromosomes and hormones on atherosclerosis, circulating lipids and intestinal fat metabolism. We assess atherosclerosis in multiple models and experimental paradigms that distinguish effects of sex chromosomes, and male or female gonads. Pro-atherogenic lipids and atherosclerosis are greater in XX than XY mice, indicating a primary effect of sex chromosomes. Small intestine expression of enzymes involved in lipid absorption and chylomicron assembly are greater in XX male and female mice with higher intestinal lipids. Together, our results show that an XX sex chromosome complement promotes the bioavailability of dietary fat to accelerate atherosclerosis.
Sex chromosomes and sex hormones are the primary determinants of biological sex. A plethora of research has focused on the role of sex hormones as mediators of sex differences in a variety of diseases, most especially cardiovascular diseases\(^{1,2}\). Generally, results from these studies suggest that estrogens have beneficial effects on circulating lipid profiles (e.g., increase HDL)\(^3\) and protect against coronary artery disease (CAD)\(^{7-10}\), and that these benefits are typically lost in post-menopausal females. Notably, some studies report that post-menopausal females exhibit a pro-atherogenic lipid profile and an increase in CAD to a level that not only catches up to, but exceeds that of age-matched males\(^{11-13}\). This suggests that female gonadal hormones, such as estrogens, are unlikely to be the only determinant of sex differences in CAD risk.

In comparison to sex hormones, genes residing on sex chromosomes have been relatively under-studied as causes of sex differences in disease development. While the Y chromosome has evolved to contain few genes, the X chromosome contains as much as 5% of the human genome, and could thus potentially mediate sex differences in a variety of factors and/or diseases\(^{14,15}\). Unfortunately, many large-scale genome-wide association studies (GWAS)\(^{16}\), including GWAS studies in subjects with CAD\(^{7,18}\), have neglected analysis of genes residing on sex chromosomes. Thus, the contribution of sex chromosome genes to CAD, and other common diseases, is not well characterized.

We use the Four Core Genotypes (FCG) mouse model\(^{19-21}\), which generates XX and XY female mice with ovaries, and XX and XY male mice with testes, to define the role of sex chromosome genotype on circulating lipids and atherosclerosis. Our results demonstrate that an XX sex chromosome genotype, relative to XY, promotes the development of atherosclerotic lesions in multiple mouse models and this is associated with profound dyslipidemia, enhanced adiposity, and augmented dietary fat bioavailability. While gonadal hormones also regulated some of these factors, the pronounced effects of XX sex chromosome genotype persist in gonadectomized (GDX) mice. Moreover, higher serum lipids and atherosclerosis are evident in XX female and male mice under different experimental paradigms (e.g., diet, genetic background, gonadectomy), and multiple linear regression analysis reveals sex chromosome genotype as an explanatory variable for the development of atherosclerosis. Our data suggest that the greater atherosclerosis susceptibility in XX compared to XY mice is associated with enhanced absorption and bioavailability of dietary fat, which likely influences serum lipid levels and adiposity. Our findings may have important ramifications for human health, particularly following menopause, when protective factors of female sex hormones are lost, and the effects of an XX sex chromosome genotype may contribute to pro-atherogenic lipid profiles and CAD.

### Results

**XX males and females have high food intake and body weight.** We fed male and female XX and XY Ldlr\(^{−/−}\) mice a Western diet for 4 months to examine effects of sex chromosome genotype on serum lipids and atherosclerosis. To separate the contribution of sex chromosomes and sex hormones, male and female mice of each sex chromosome genotype were either gonadally intact (Intact) or surgically gonadectomized (GDX) two weeks prior to initiation of the Western diet. Males (Intact) had greater body weights (Fig. 2a; \(P < 0.001\), 3-way ANOVA with Holm–Sidak test) and fat mass (Fig. 2b; \(P < 0.001\), 3-way ANOVA with Holm–Sidak test) compared to females, regardless of sex chromosome genotype. Moreover, XX female and male mice (Intact) had greater body weights (Fig. 2a, b; \(P < 0.001\), 3-way ANOVA with Holm–Sidak test), with 1.4–2.7-fold increases of the weights of white adipose tissue (Table 1) compared to XY mice of either sex. Gonadectomy decreased body weights of male, but not female Ldlr\(^{−/−}\) mice, regardless of sex chromosome genotype (Fig. 2a; \(P < 0.001\), 3-way ANOVA with Holm–Sidak test). Moreover, greater body weights (Fig. 2a, b) and fat mass (Table 1) of XX mice, relative to XY, persisted after gonadectomy.

Lipid content of serum from XX Ldlr\(^{−/−}\) mice fed a Western diet was visibly greater than XY Ldlr\(^{−/−}\) mice (Fig. 2c). Serum total cholesterol concentrations were significantly higher in male compared to female mice, regardless of sex chromosome genotype or surgery (GDX) (Fig. 2d; \(P < 0.001\), 3-way ANOVA with Holm–Sidak test). Moreover, serum total cholesterol concentrations were markedly higher (>3-fold) in XX female and male mice compared to XY mice of either sex (Fig. 2d; \(P < 0.001\), 3-way ANOVA with Holm–Sidak test).
0.001, 3-way ANOVA with Holm–Sidak test), and these effects persisted in GDX mice. However, there was no significant effect of gonadectomy of male or female Ldlr<sup>−/−</sup> mice on serum cholesterol concentrations, regardless of sex chromosome genotype. Serum concentrations of VLDL- (Fig. 2e; P < 0.001, 3-way ANOVA with Holm–Sidak test) and LDL-cholesterol (Fig. 2f; P < 0.001, 3-way ANOVA with Holm–Sidak test) were also higher in male compared to female mice, regardless of sex chromosome genotype or surgery. Notably, serum VLDL- and LDL-cholesterol concentrations were also markedly higher in XX than XY mice, regardless of sex or surgery. There was no difference in serum HDL-cholesterol concentrations between males and females,
Fig. 1 XX male and female mice have higher body weight, fat mass and food intake. a Body weight of mice of each sex chromosome complement and gonadal sex at baseline (when fed standard murine diet) or after 1 week of consumption of a Western diet (WD). Lean (b) and fat (c) mass (gm). d Food intake, normalized to lean mass, of mice fed standard murine diet. e Physical activity, normalized to lean mass, of mice fed standard murine diet. f Energy expenditure, normalized to lean mass, of mice fed standard murine diet. g Food intake, normalized to lean mass, of mice fed a Western diet (1 week). h Physical activity, normalized to lean mass, of mice fed a Western diet. i Energy expenditure, normalized to lean mass, of mice fed a Western diet. Symbols represent individual mice per group (n = 5 mice/group) per measurement, with horizontal lines representing mean ± SEM. *P < 0.05 compared to XX within gonadal sex. #P < 0.05 compared to female within sex chromosome complement. $P < 0.05 main effect of sex chromosome complement. Data were analyzed by 3-way ANOVA (A-C) with Holm-Sidak test, or by 2-way ANOVA (d-i) with Holm-Sidak test. Source data are available as a Source Data file.

Fig. 2 An XX sex chromosome complement promotes obesity and dyslipidemias. a Body weights (gm) of male and female intact and gonadectomized (GDX) mice of each genotype (Intact: FXX, n = 11; FXY, n = 14; MXM, n = 9; MXY, n = 11; GDX: FXX, n = 9; FXY, n = 8; MXM, n = 7; MXY, n = 7). b Representative pictures of serum from XX and XY male mice. d Total serum cholesterol concentrations (Intact: FXX, n = 10; FXY, n = 12; MXM, n = 9; MXY, n = 11; GDX: FXX, n = 6; FXY, n = 4; MXM, n = 6; MXY, n = 5). Concentrations of very low density lipoprotein (VLDL)-cholesterol (e) (Intact: FXX, n = 6; FXY, n = 6; MXM, n = 5; MXY, n = 6; GDX: FXX, n = 6; FXY, n = 4; MXM, n = 6; MXY, n = 5; MXM, n = 6; MXY, n = 5) and low density lipoprotein (LDL)-cholesterol (f) (Intact: FXX, n = 6; FXY, n = 6; MXM, n = 5; MXY, n = 6; GDX: FXX, n = 6; FXY, n = 4; MXM, n = 6; MXY, n = 5). Concentrations of high density lipoprotein (HDL)-cholesterol (g) (Intact: FXX, n = 6; FXY, n = 4; MXM, n = 6; MXY, n = 5; MXM, n = 6; MXY, n = 5) and TG (h) (Intact: FXX, n = 4; FXY, n = 6; MXM, n = 4; MXY, n = 5; GDX: FXX, n = 6; FXY, n = 4; MXM, n = 6; MXY, n = 5) in Ldr−/− mice fed a Western diet for 4 months. Concentrations of total serum cholesterol (i) (FXX, n = 11; FXY, n = 10; MXM, n = 12; MXY, n = 8), LDL-cholesterol (j) (FXX, n = 11; FXY, n = 10; MXM, n = 12; MXY, n = 8), HDL-cholesterol (k) (FXX, n = 11; FXY, n = 10; MXM, n = 12; MXY, n = 8) and TG (l) (FXX, n = 11; FXY, n = 10; MXM, n = 12; MXY, n = 8) in Apoe−/− mice fed a standard murine diet. Symbols represent individual mice per group per measurement, with horizontal lines representing mean ± SEM. *P < 0.05 compared to XX within gonadal sex. #P < 0.05 compared to female within sex chromosome complement. $P < 0.05 compared to XX females. Data were analyzed by 3-way ANOVA (a, d-h) with Holm-Sidak test, or by 2-way ANOVA (i-l) with Holm-Sidak test. Source data are available as a Source Data file. RPF retroperitoneal, EF epididymal, SubQ subcutaneous.
regardless of surgery (Fig. 2g). Serum HDL-cholesterol concentrations were higher in XX males, but not XX females compared to XY mice (Fig. 2g; \( P = 0.018 \), 3-way ANOVA with Holm–Sidak test). This difference in serum HDL-cholesterol concentrations between male XX and XY mice was not present in GDX mice. Serum triglyceride (TG) concentrations were also higher in male than female mice, regardless of sex chromosome genotype (Fig. 2h; \( P < 0.001 \), 3-way ANOVA with Holm–Sidak test). Moreover, XX mice had markedly higher serum TG concentrations than XY mice, and this effect persisted in GDX mice (Fig. 2h; \( P < 0.001 \), 3-way ANOVA with Holm–Sidak test). There was no effect of GDX on serum TG concentrations in male or female mice of either sex chromosome genotype.

To determine if XX effects on serum lipids and atherosclerosis were specific to an \( Ldlr^{-/-} \) background and/or required a Western diet, we examined serum cholesterol, TG, LDL- and HDL-cholesterol concentrations in FCG mice fed standard murine diet for 4 months but made hypercholesterolemia by apolipoprotein E deficiency (\( Apoe^{-/-} \)). To remove influences of sex hormones, these studies were performed in GDX mice. Similar to findings from \( Ldlr^{-/-} \) mice, serum total cholesterol and LDL-cholesterol concentrations were higher in male compared to female \( Apoe^{-/-} \) mice, regardless of sex chromosome genotype (Fig. 2i, j; \( P = 0.004 \), 3-way ANOVA with Holm–Sidak test). Moreover, XX male and female \( Apoe^{-/-} \) mice had higher serum total cholesterol (Fig. 2i; \( P < 0.001 \), 2-way ANOVA with Holm–Sidak test) and LDL-cholesterol (Fig. 2j; \( P < 0.001 \), 2-way ANOVA with Holm–Sidak test) concentrations than XY mice of either sex. However, neither serum HDL-cholesterol (Fig. 2k; \( P = 0.32 \), 2-way ANOVA with Holm–Sidak test) nor TG concentrations (Fig. 2l; \( P = 0.66 \), 2-way ANOVA with Holm–Sidak test) were different among the four genotypes.

We assessed the influence of sex chromosome complement on atherosclerosis in three independent FCG mouse genetic backgrounds: \( Ldlr^{-/-} \) mice (Intact and GDX) fed a Western diet, GDX \( Apoe^{-/-} \) mice fed standard murine diet, and C57BL/6 J mice fed a cholesterol-enriched atherogenic diet. In aortic arches of \( Ldlr^{-/-} \) mice, the percent of intimal surface area covered by atherosclerotic lesions was significantly greater in male than female mice (Intact), regardless of sex chromosome genotype (Fig. 3a; \( P = 0.029 \), 3-way ANOVA with Holm–Sidak test). Female XX, but not male XX mice (Intact) had more atherosclerosis than XY mice of the respective sex (Fig. 3a, b; \( P = 0.001 \), 3-way ANOVA with Holm–Sidak test). Gonadectomy increased atherosclerosis in female XX, but not female XY mice (Fig. 3a, b; \( P = 0.002 \), 3-way ANOVA with Holm–Sidak test), suggesting a protective role for female gonadal hormones that requires an XX sex chromosome genotype. In contrast, male XY, but not male XX mice exhibited significantly less atherosclerosis in the GDX groups relative to intact XY males (Fig. 3a, b; \( P = 0.046 \), 3-way ANOVA with Holm–Sidak test), suggesting interactions between testicular hormones and an XY sex chromosome genotype on lesion development. Following gonadectomy, atherosclerosis of XX mice was markedly greater than XY mice, regardless of sex (Fig. 3a, b; \( P < 0.001 \), 3-way ANOVA with Holm–Sidak test), demonstrating a robust effect of sex chromosome genotype.

We also quantified atherosclerosis in aortic sinus tissue sections of FCG \( Ldlr^{-/-} \) mice, where lesion areas were not significantly different between male and female mice, regardless of sex chromosome genotype or surgery (Fig. 3c). However, XX male and female mice had significantly greater atherosclerotic lesion areas compared to XY mice of either sex (Fig. 3c, d; \( P < 0.001 \), 3-way ANOVA with Holm–Sidak test), which persisted in GDX mice. In aortic sinus tissue sections from GDX \( Apoe^{-/-} \) (Fig. 3e, f) fed with standard murine diet or GDX C57BL/6 J FCG mice fed with a HF diet for 4 months, there were no differences in atherosclerotic lesion areas between male and female mice, regardless of sex chromosome genotype (Fig. 3e). However, similar to \( Ldlr^{-/-} \) FCG mice, XX female and male GDX \( Apoe^{-/-} \) FCG mice (Fig. 3e, f; \( P < 0.0001 \), 2-way ANOVA with Holm–Sidak test), as well as XX female and male GDX C57BL/6 J FCG mice fed with an atherogenic diet for 4 months (Fig. 3g; \( P = 0.001 \), 2-way ANOVA with Holm–Sidak test) had significantly greater aortic sinus lesion areas compared to XY mice of either sex. These results demonstrate the robust effect of the XX genotype on atherosclerosis under three complementary experimental paradigms.

A variety of parameters quantified in these studies could contribute to higher levels of atherosclerosis in XX compared to XY mice, including higher energy intake, body weight, fat mass or differences based on gonadal sex, sex chromosome genotype, or genetic background (e.g., C57BL/6 J vs. \( Ldlr^{-/-} \) mice). We used a multiple linear regression model with log-transformed atherosclerotic lesion area in aortic sinus as the response variable, and examined the relationship of each of the above described explanatory variables within the model to determine their relationship to atherosclerosis. For this model, reference groups for the analysis were \( Apoe^{-/-} \) mice (genetic background), GDX (surgery), females (sex), and XX sex chromosome genotype (sex

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**Table 1 Characteristics of mice of each group**

| Parameter                     | FXX                | FXY                | MXX                | MXY                |
|-------------------------------|--------------------|--------------------|--------------------|--------------------|
| Intact \( Ldlr^{-/-} \) on Western Diet |                   |                    |                    |                    |
| Retropertoneal Fat/body weight (%) | 2.04 ± 0.3         | 0.92 ± 0.2         | 2.54 ± 0.17        | 1.57 ± 0.17        |
| SubQ Fat/body weight (%)       | 2.19 ± 0.17        | 1.85 ± 0.17*       | 3.5 ± 0.27         | 1.89 ± 0.24*       |
| Gonadal Fat/body weight (%)   | 6.06 ± 0.65*       | 3.06 ± 0.4*        | 4.94 ± 0.224*      | 4.59 ± 0.38*       |
| Liver/body weight (%)          | 5.8 ± 0.23         | 6.3 ± 0.11         | 7.9 ± 0.24         | 5.8 ± 0.17         |
| Gonadectomized \( Ldlr^{-/-} \) on Western Diet |                   |                    |                    |                    |
| Retropertoneal Fat/body weight (%) | 3 ± 0.34           | 1.2 ± 0.2          | 2.1 ± 0.12         | 0.59 ± 0.09        |
| SubQ Fat/body weight (%)       | 3.0 ± 0.21         | 1.7 ± 0.2          | 3.66 ± 0.43        | 1.27 ± 0.15*       |
| Gonadal Fat/body weight (%)   | 5.1 ± 0.23         | 2.5 ± 0.3*         | 5.18 ± 0.62        | 2.01 ± 0.45*       |
| Liver/body weight (%)          | 6.3 ± 0.27         | 5.9 ± 0.1          | 5.88 ± 0.25        | 5.3 ± 0.17         |
| SubQ Fat/body weight (%)       | 0.87 ± 0.13        | 0.60 ± 0.10        | 0.85 ± 0.10        | 0.73 ± 0.05        |
| Gonadal Fat/body weight (%)   | 0.86 ± 0.23        | 0.40 ± 0.13        | 0.73 ± 0.10        | 0.55 ± 0.08        |
| Liver/body weight (%)          | 4.44 ± 0.17        | 4.70 ± 0.22        | 4.30 ± 0.10        | 4.42 ± 0.18        |
| \( Apoe^{-/-} \) on Chow Diet   |                    |                    |                    |                    |
| SubQ Fat/body weight (%)       | 0.95 ± 0.12        | 0.70 ± 0.06*       | 0.64 ± 0.05        | 0.47 ± 0.03        |
| Gonadal Fat/body weight (%)   | 2.16 ± 0.31        | 1.11 ± 0.16*       | 1.33 ± 0.23        | 0.30 ± 0.16        |
| Liver/body weight (%)          | 7.50 ± 0.40        | 9.51 ± 0.35*       | 8.45 ± 0.51        | 11.6 ± 1.0*        |

Data are mean ± SEM

*P < 0.05 compared to XX within gonadal sex. #P < 0.05 compared to female within genotype.

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An XX sex chromosome complement augments atherosclerosis in male and female mice. Atherosclerotic lesion surface area, expressed as a percentage of the aortic arch, in aortic arches from male and female XX and XY intact or gonadectomized (GDX) Ldlr−/− mice. (Intact: FXX, n = 11; FXY, n = 12; MX, n = 9; MX, n = 11; GDX: FXX, n = 5; FXY, n = 4; MX, n = 7; MX, n = 7). B Representative aortic arch, stained with Oil Red O, from Ldlr−/− mice of each group. C Atherosclerotic lesion area, quantified as Oil Red O staining, in tissue sections from the aortic sinus of male and female XX and XY intact or GDX Ldlr−/− mice. (Intact: FXX, n = 4; FXY, n = 5; MX, n = 4; MX, n = 4; GDX: FXX, n = 4; FXY, n = 4; MX, n = 5; MX, n = 5). D Representative aortic sinus tissue sections, stained with Oil Red O, from Ldlr−/− mice of each group. E Atherosclerotic lesion area in tissue sections from XX and XY male and female Ape−/− GDX mice. (FXX, n = 5; FXY, n = 4; MX, n = 5; MX, n = 5). F Representative aortic sinus tissue sections from Ape−/− GDX mice of each group. G Atherosclerotic lesion area in tissue sections from aortic sinus of XX and XY male and female C57BL/6 GDX mice fed an atherogenic diet. Symbols represent individual mice per group per measurement, with horizontal lines representing mean ± SEM. (FXX, n = 8; FXY, n = 6; MX, n = 10; MX, n = 5). *P < 0.05 compared to XX within gonadal sex. #P < 0.05 compared to female within sex chromosome complement. $P < 0.05 compared to XX females. Data were analyzed by 3-way ANOVA (A,C) with Holm-Sidak test, or by 2-way ANOVA (e, g) with Holm-Sidak test. Scale bar = 200 μm. Source data are available as a Source Data file.

| Table 2 Multiple linear regression of explanatory variables to atherosclerotic lesion area within the aortic sinus of FCG mice |
|---------------------------------------------------------------|
| Estimate | Standard Error | t-value | P-value |
| (Intercept) | 13.23 | 0.49 | 26.8 | <0.0000000000000002 |
| Body weight (g) | 0.01 | 0.02 | 0.56 | 0.58 |
| Sera cholesterol (mg/dl) | −0.00 | 0.00 | −1.43 | 0.16 |
| Gonadal fat (%) | 0.08 | 0.09 | 0.88 | 0.38 |
| Genetic background: C57BL/6 mice | −0.07 | 0.36 | −0.19 | 0.85 < 0.0000000000000002 |
| Sex | −4.62 | 0.23 | −19.95 | 0.85 < 0.0000000000000002 |
| Surgery (Intact) | −0.09 | 0.17 | −0.56 | 0.58 |
| Sex chromosome genotype | −0.99 | 0.21 | −4.77 | 0.000001 |

Reference groups were Ape−/− mouse (genetic background), female (sex), GDX (surgery), and XX genotype (sex chromosome genotype).

chromosome genotype) (Table 2). We included all mice in the analysis for which we had measurements on all variables. After adjusting for all other variables, there were two explanatory variables that were significant for the development of atherosclerosis, namely sex chromosome genotype and genetic background (Table 2). Livers of XX males and females have diverse gene expression. Elevations in serum lipids of XX mice could result from alterations in cholesterol and/or lipid homeostasis in liver, a major organ for lipoprotein synthesis, secretion and clearance. To focus on sex chromosome influences on transcriptional profiles in the absence of sex hormones, livers from GDX mice were used. We
performed transcriptome analysis on livers from GDX XX and XY male and female Ldlr−/− mice after 4 months of Western diet using Affymetrix Mouse Transcriptome Assay 1.0 assays. There was no major effect of sex on liver gene expression (Fig. 4a, Male vs. Female). However, a total of 1,399 genes exhibited highly significant differences (2-way ANOVA, False Discovery Rate < 0.01; Fig. 4a, b, Supplementary Data 1) by sex chromosome genotype (XX or XY). Volcano plots of the sex chromosome effect with highly stringent cutoffs ( > 4-fold change, \( P < 1 \times 10^{-6} \)) demonstrated that the expression of genes on sex chromosomes was strongly influenced (Fig. 4b). As expected, genes within the male-specific region of the Y chromosome (e.g., Uty, Kdm5d, Eif2s3y) were significantly greater in XY livers (Fig. 4b), while Xist (known to be expressed only in XX cells) was significantly greater in XX livers. Biological pathway analyses revealed that a large number of genes involved in the immune response (197) differed in livers from XY compared to XX male and female mice (Table 3). We did not observe an effect of sex chromosome genotype on pathways related to hepatic cholesterol synthesis.

![Fig. 4](https://doi.org/10.1038/s41467-019-10462-z)

**Table 3** XY vs XX liver transcriptional pathway over-representation

| G.O.     | Description                           | #   | \( P \) value |
|----------|---------------------------------------|-----|--------------|
| 0006955  | Immune response                        | 197 | 7.62E-52     |
| 0030029  | Actin filament-based process           | 95  | 3.51E-21     |
| 0006897  | Endocytosis                            | 82  | 2.33E-20     |
| 0007264  | Small GTPase mediated signal transduction | 72 | 7.10E-16     |
| 0005925  | Focal adhesion                         | 60  | 7.68E-13     |
| 0018212  | Peptide-tyrosine modification          | 46  | 1.18E-12     |
| 0001944  | Vasculature development                | 68  | 4.43E-08     |
| 0072593  | Reactive oxygen species metabolic process | 33 | 1.58E-07     |

Column titles: G.O. (Gene Ontology accession identifier); # - number of genes found significant with category; \( P \) value - modified Fisher's Exact Test using \# - 1 (EASE score)
(Table 3), although some individual cholesterol-related genes were different between genotypes (Supplementary Data 1).

Since serum TG and cholesterol concentrations were greater in XX female or male mice compared to XY mice, we quantified hepatic TG and cholesterol concentrations, and examined gross morphology of liver tissue. Moreover, since higher serum lipids were present in GDX XX compared to XY males and females, indicating a primary effect of sex chromosome genotype, we focused on livers from GDX mice. Hepatic TG and cholesterol contents were greater in XX than XY females, but not in livers from XX vs. XY males (Supplementary Figure 1A, B; \( P = 0.002 \), 2-way ANOVA with Holm–Sidak test). Tissue sections from livers of XX and XY male and female mice fed the Western diet for 4 months had similar gross morphology (Supplementary Figure 1C). Elevations in hepatic cholesterol concentrations could arise from increased synthesis or decreased cholesterol secretion. As transcriptome analysis did not identify cholesterol homeostasis pathways as different between livers from XX and XY mice, we quantified hepatic TG secretion as a major determinant of serum cholesterol concentration in male and female XX and XY; \( \text{LDLr}^{−/−} \) mice fed standard murine diet. Plasma TG concentrations over time in fasted XX and XY male and female mice (after injection with poloxamer to inhibit lipoprotein lipase-mediated lipolysis, Supplementary Figure 2A) and TG secretion rates (Supplementary Figure 2B) were higher in males compared to females, but there was no significant effect of sex chromosome genotype. Newly synthesized apolipoprotein B48 levels were higher in male XX and XX mice compared to female mice of each sex chromosome genotype (\( P < 0.05 \)), but there was no difference between genotypes (Supplementary Figure 2C, D). In contrast, newly synthesized apolipoprotein B100 levels were higher in XX than XY mice, regardless of sex (Supplementary Figure 2C, E; \( P = 0.029 \), 2-way ANOVA with Holm–Sidak test), consistent with higher LDL/VLDL cholesterol concentrations of XX mice.

### XX male and female intestines have augmented lipid handling.

The intestinal tract absorbs dietary fat and is an important determinant of circulating lipids. Thus, we quantified mRNA abundance of a variety of genes (cluster of differentiation 36 (Cd36), fatty acid binding protein 1 (Fabp1), fatty acid binding protein 2 (Fabp2), secretion associated Ras related GTPase 1B (Sar1b), apolipoprotein B (ApoB), diacylglycerol acyltransferases (Dgat1 and Dgat2), monoyacylglycerol O-acyltransferase 2 (Mogat2), and microsomal triglyceride transfer protein (Mttp)) involved in the absorption and synthesis of dietary fat in small intestines from XX and XY GDX male and female FCG \( \text{LDLr}^{−/−} \) mice fed the Western diet for 4 months. mRNA abundance of \( \text{Cd36}, \text{Fabp1}, \text{Fabp2}, \text{Mogat2}, \text{Sar1b}, \text{Dgat1} \) and \( \text{ApoB} \) was not different between males and females, and was not influenced by sex chromosome genotype (Supplementary Figure 3; \( P > 0.05 \), 2-way ANOVA with Holm–Sidak test). Similarly, mRNA abundance of \( \text{Dgat2} \), an enzyme that produces TG from absorbed fatty acids\(^{23,24}\) (Fig. 5a; \( P = 0.008 \), 2-way ANOVA with Holm–Sidak test), and \( \text{Mttp} \) (Fig. 5b; \( P = 0.001 \), 2-way ANOVA with Holm–Sidak test), which assembles TG into chylomicron\(^{25–27}\) was not different in small intestines of GDX male compared to female mice, regardless of sex chromosome genotype. However, mRNA abundance of both \( \text{Dgat2} \) and \( \text{Mttp} \) was higher in intestines from XX compared to XY mice of either sex. In support of sex chromosome genotype, we quantified fatty acid absorption and handling effects that may be useful for child-bearing women, could adversely influence health when protective effects of female sex hormones are lost upon menopause (Fig. 5f).

### Discussion

Our findings shed light on causes of sex differences in common cardiovascular diseases such as CAD. We demonstrate that relative to XY mice with an XX sex chromosome genotype exhibit the following: (1) markedly elevated serum cholesterol and TG concentrations, effects that were found in different experimental paradigms (e.g., diet, genetic background, gonadectomy), (2) profound elevations in atherosclerosis, (3) altered expression of hepatic genes associated with immune pathways, (4) similar hepatic TG secretion but higher levels of newly synthesized apolipoprotein B100, (5) greater expression levels of key genes in small intestine involved in lipid absorption and chylomicron assembly, higher intestinal lipid content, and modest elevations in fat absorption (Fig. 5f). These results suggest a prominent role of sex chromosomes in the control of dietary fat absorption, the regulation of serum lipids and the development of atherosclerosis. If results from these experimental studies are translatable to humans, then thrifty effects of an XX sex chromosome to promote fat absorption and handling, effects that may be useful for child-bearing women, could adversely influence health when protective effects of female sex hormones are lost upon menopause (Fig. 5f).

Sex hormones exert many effects that have been suggested to contribute to sex differences in fat storage, circulating lipids and cardiovascular diseases\(^{29}\). By comparison, the role of the other primary biological determinant of sex, namely sex chromosomes, in disease development is relatively unknown despite an association of sex chromosome abnormalities with lipid and cardiovascular disorders\(^{30–33}\). The FCG murine model allows for determination of the relative effects of sex hormones vs. sex chromosomes to a phenotype. Recent studies using this model indicate that the dose of the X chromosome influences several metabolic traits, including obesity, fatty liver, food intake and glucose homeostasis\(^{22,23,24,36}\). Results from the current study extend these findings to murine models of dyslipidemia and...
atherosclerosis, demonstrating that an XX sex chromosome genotype is associated with profound elevations in proatherogenic circulating lipids and atherosclerosis. Moreover, these effects were observed in several different mouse models of atherosclerosis on a C57BL/6 J background (\textit{Ldlr}^{-/-}, \textit{Apoe}^{-/-}, wild type), with or without consumption of experimental diets, and were largely independent of sex hormones (e.g., persisted in GDX mice). These results indicate a strong differential effect of XX vs. XY chromosomes on the development of atherosclerosis.

In preclinical models and in humans, estrogen favorably influences circulating lipid profiles and atherosclerotic lesion development\textsuperscript{7–10}. In agreement, our results demonstrate that removal of ovarian hormones increased atherosclerosis in XX mice, and extend previous results by demonstrating that this protective effect of ovarian hormones was dependent on an XX sex chromosome genotype, as XY females were not influenced by gonadectomy. Beneficial effects of estrogens have been suggested to protect females from CAD(\textsuperscript{37}). However, with advancing age (>85 years of age), the prevalence of atherosclerosis in females has been reported to not only catch up to, but in some studies exceed that of males\textsuperscript{11–13}. Our results demonstrate that in addition to regulation of atherosclerosis by sex hormones, an XX sex chromosome genotype, relative to XY, promotes atherosclerosis in male or female mice. Future studies should determine if an XX chromosome genotype contributes to a higher prevalence of atherosclerosis in aged, postmenopausal females.

Recent studies demonstrated higher HDL-cholesterol levels in XX compared to XY mice of the FCG fed standard or HF diets\textsuperscript{36}. We observed higher levels of HDL-cholesterol in XX compared to XY males, which were no longer evident in GDX males, suggesting an effect of testosterone or its metabolites to promote circulating HDL-cholesterol. In this study mice with an XX sex chromosome genotype are depicted in green, while those with XY are depicted in pink. The data show that HDL-cholesterol levels are higher in XX than XY mice. The figure includes a summary of the key findings from this study, including intestinal gene expression, triglyceride content, and fat absorption. The data were analyzed by 2-way ANOVA with Holm-Sidak test. Dietary fat absorption data were analyzed by Kruskal-Wallis. Source data are available as a Source Data file.
chromosome genotype had greater concentrations of total and LDL-cholesterol, as well as markedly increased serum TG concentrations compared to XY mice, presumably related to the hyperlipidemic background of genetically manipulated (Ldlr<sup>−/−</sup>, Apoe<sup>−/−</sup>) mice. Levels of circulating pro-atherogenic lipids in atherosclerosis-susceptible Ldlr<sup>−/−</sup> mice from the present study were far in excess of those observed in previous studies using atherosclerosis-resistant C57BL/6J mice that typically carry lipids predominately on HDL particles<sup>36</sup>. Moreover, multiple linear regression analysis demonstrated that genetic background, such as C57BL/6J mice with predominately HDL- rather than LDL-cholesterol, when compared to Apoe<sup>−/−</sup> mice as the reference background, was an experimental variable that correlated significantly to the extent of atherosclerosis.

Previous results from the Framingham Heart Study demonstrated that increases in circulating levels of TG correlated more strongly to cardiovascular disease risk in women compared to men, but mechanisms for this sex difference have not been identified<sup>33–38</sup>. In this study, serum TG concentrations were higher in XX males than XX females, but atherosclerosis in the aortic sinus was similar between XX males and females, suggesting that female XX mice have more atherosclerotic burden than XX males for a given level of circulating TG. The profound effects of an XX sex chromosome genotype to promote higher levels of circulating pro-atherogenic lipids, regardless of background genetic strain, sex hormones, or diet, could result from alterations in hepatic lipoprotein absorption. However, surprisingly, multiple linear regression did not identify serum cholesterol concentrations as an explanatory variable for the development of atherosclerosis. Thus, while serum concentrations of pro-atherogenic lipids are clearly important in the development of atherosclerosis, in these studies they were not the primary contributing variable to the extent of atherosclerosis. In contrast, sex chromosome genotype, namely an XX sex chromosome genotype, did correlate significantly to the extent of atherosclerosis.

Notably, expression levels of a large number of genes in liver were influenced by sex chromosome genotype, with greater expression of genes involved in immune function in livers from XY compared to XX mice. Surprisingly, gene pathways implicated in cholesterol synthesis and handling by liver were not altered in XX mice despite markedly higher serum cholesterol and TG concentrations. These results are in agreement with previous findings indicating that differences in plasma lipids between XX and XY mice (male or female) were not associated with alterations in liver gene expression levels for components of cholesterol synthesis and metabolism<sup>36</sup>. Moreover, these results extend previous findings by demonstrating the large impact of sex chromosome genotype on hepatic gene expression patterns. Consistent with a lack of effect of sex chromosome genotype on cholesterol handling genes, hepatic TG secretion was not altered in XX male or female mice fed standard murine diet. However, future studies should more fully characterize the contribution of hepatic production of apolipoprotein B100 to the observed effects of sex chromosome genotype on the development of atherosclerosis.

Since hepatic secretion of TG did not appear to be a primary target for regulation by sex chromosome complement, we turned to intestinal handling of lipids as a cause of higher circulating lipids and cholesterol in XX mice. Small intestine gene expression of Dgat2 and Mttp, enzymes involved in the synthesis of TG from absorbed fatty acids and assembly into chylomicrons, respectively, paralleled changes in circulating lipids, with higher levels in intestines from XX than XY males or females, and these effects were independent of gonadal hormones as they persisted in tissues from GDX mice. As these genes are not X-linked and do not reside on the X chromosome, their regulation may be indirect or downstream of X chromosomes. Moreover, elevations in gene expression of Dgat2 and Mttp in small intestines from XX compared to XY mice (male or female) were accompanied by greater intestinal TG and fatty acid content, in a manner that reflected lipids within the Western diet. Recent studies identified a role for the gut microbiome in sexual dimorphism of gene expression in mice<sup>39</sup>, sex differences in gut microbiota composition<sup>40</sup>, and differences in the composition of gut microbiota have been demonstrated between genders and between women of different hormonal status<sup>41</sup>. In agreement with previous findings<sup>39</sup>, we found that alpha diversity of gut microbiota was influenced by sex, but not necessarily by sex chromosome genotype. These results, while interesting, do not suggest a primary role for the gut microbiome in augmented fat absorption, higher serum lipids and atherosclerosis of XX compared to XY mice. Rather, absorption of dietary fat was modestly, but not significantly higher in XX compared to XY mice, indicating that altered expression levels of these pivotal lipid-regulating genes were accompanied by functional changes in fat bioavailability. The modest increases in daily fat absorption of XX mice observed in this study, which considered cumulatively over 4 months of the Western diet and in conjunction with increased energy intake, most likely contributed to the observed hyperlipidemia of XX compared to XY mice.

In conclusion, results from this study identify a profound effect of an XX sex chromosome effect to promote experimental dyslipidemias and atherosclerosis, a finding of potential relevance to increased CAD of postmenopausal females. These effects of an XX sex chromosome genotype were associated with augmented absorption and bioavailability of dietary lipid, metabolic traits that if present in humans may be important for child-bearing purposes (Fig. 5f, left). However, our findings suggest that these effects of an XX sex chromosome genotype, upon loss of protective sex hormones, may contribute to elevations of pro-atherogenic lipids and increased atherosclerotic burdens of postmenopausal females.

**Methods**

**Animals.** Male mice with deletion of the gene Sry from the Y-chromosome, but expressing Sry transgene on an autosomal (termed FCG mice) aged 8–12 weeks were bred to females of 3 different backgrounds (Ldlr<sup>−/−</sup>, Apoe<sup>−/−</sup>, and C57BL/6) to generate male and female mice with an XY or an XX sex chromosome complement. Mice genotypes were identified by amplifying DNA extracted from tail or ear clips using a Promega Maxam system and polymerase chain reaction (PCR) using a commercial PCR mix (Promega 2X Master Mix, cat#71233) and specific primers for the Sry transgene, presence of the Y-chromosome, and internal positive control. Ldlr<sup>−/−</sup> FCG mice (12–16 weeks old) were fed Western diet, 42 kcal from fat (TD88137, Harlan Teklad, Indianapolis, IN), Apoe<sup>−/−</sup> FCG mice (14–16 weeks old) were fed standard murine diet (Purina 5001; approximately 5% fat, PMI Nutrition International, St. Louis, MO), and C57BL/6 FCG mice (14–16 weeks old) were fed an atherogenic diet (7.5% cocoa butter, 1.25% cholesterol, 0.5% sodium cholate, TD90221, Harlan Teklad, Indianapolis, IN) for 16 weeks. All experiments were approved by the animal care and use committee at the University of Kentucky and the University of California, Los Angeles and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH.

**Ovariectomy (OVX).** Female FCG mice on the Ldlr<sup>−/−</sup>, Apoe<sup>−/−</sup>, and C57BL/6 background at 8–12 weeks of age were ovariectomized under anesthesia with isoflurane (3–4% for induction and 2% for maintenance). Ophthalmic ointment (Puralube vet ointment, Dechra) was applied to the eyes of mice to prevent dryness during surgery. Before surgery, mice were subcutaneously injected, and after 12–16 h of the surgery, with 10 mg/kg fluimixin for analgesia. Mice were shaved in the abdominal region at both flanks and a depilatory cream (Nair, Inc.) applied to the skin to remove hair, followed by sterilizing with povidone-iodine and ethyl alcohol. At each flank, a 1 cm incision was made to allow for locating fallopian tubes, the vascular supply to ovaries was occluded using a hemostat, and the ovaries were removed. Residual ends of fallopian tubes were ligated by cautery incision. The incision was closed by suturing (5–0 black monofilament nylon suture, Ethilon 1668G) the peritoneum and clipping the skin with wound clips (Autoclip stapler).
followed by sterilization of the site with povidone-iodine. Mice recovered on a heating pad.

**Orchiectomy.** Male mice on the Ldlrfl/fl, ApoE−/−, and C57BL/6J background at 8–12 weeks of age were orchiectomized as described previously. Briefly, mice were anesthetized (isoflurane, 2–3%) and given pre and postoperative analgesic (flunixin, 2.5 mg/kg). Mice were shaved in the scrotum region and a depilatory cream was applied to remove hair, followed by sterilizing with povidone-iodine/ethanol three times. After a small incision to this region, vas deferens are collapsed using a hemostat and the testes removed. The vasculature to the testes is cut and using a high temperature fine-tip look cautizer and the hemostat released. The surgical site was closed by wound clips and treated with povidone-iodine. For sham-surgeries, the testes are manipulated but left intact in anesthetized mice. Mice were allowed to recover for 2 weeks after surgery and to allow sufficient time to clear endogenous testicular hormones.

**16 S RNA sequencing and measures of microbiota diversity.** Male and female XX and XY FCG Ldlrfl/fl mice (n = 4–6 mice/group) were fed the Western diet for 4 weeks. Following a 6 h fast, mice were anesthetized for harvest of the feces contents. DNA was extracted from feces contents using the PowerSoil 96-well DNA Isolation Kit (Mobio, Carlsbad, CA, USA), and 16 S RNA sequencing was conducted by the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory and analyzed by Quantitative Insights Into Microbial Ecology (QIIME) as described previously. To estimate alpha diversity, Operational taxonomic units (OTUs, the count of unique OTUs found in a given sample) were chosen using open reference OTU picking against the Greengenes database (version 2013; www.greengenes.org). Phylogenetic Diversity (PD whole tree), and Shannon (information entropy of the observed OTU abundances, to account for both richness and evenness of species) were calculated.

**Measurements of plasma and serum components.** Concentrations of total serum cholesterol, triglyceride and testosterone were quantified in sera (blood collected from caudal vein puncture) using enzymatic assay kits (Total Cholesterol: FUJIFILM Wako Diagnostics USA, cat#999-02601 and Triglyceride: i-type TG cat#994-02891 color A, cat# 990–02991 color B, and Alpco, cat#55-TEMS-E01; respectively). Plasma lipoprotein cholesterol was determined on-line, high performance gel filtration chromatography using Infinity Cholesterol reagent (Thermo).

**Quantification of atherosclerosis.** Atherosclerotic lesions in the aortic arch and aortic sinus were quantified as described previously. Briefly, cleaned aortas were cut open longitudinally and mounted on a black wax background using pins (Fine Science Tools, cat# 26002-20). Lesions, appearing as white tufts on a translucent aortic wall background, were traced and the quantification of lesion area is represented as a percent of the total intimal surface.

**Quantification of whole body metabolism.** Indirect calorimetry was performed using a LabMaster system (TSE Systems Inc., St. Louis, MO). Mice were acclimated to chambers for one week, then placed on recording platforms for five days. Data from three 24-h periods were averaged and analyzed by ANCOVA plot vs. final lean mass.

**Measurement of liver VLDL secretion.** Male and female FCG XX and XY Ldlrfl/fl mice fed standard murine diet were fasted for 4 h, anesthetized with iso- flurane (4% induction & 2–3% maintenance, inhalation), and injected retro-orbitally with (1) [35S]Met/Cys (7 μCi/g body weight; Cat #: NEG772007MC, Perkin Elmer, Waltham, MA) to radionuclide labeled apoB and (2) poloxamer 407 (1,000 mg/kg, i.p.; USP grade, BASF Corporation, Florham Park, NJ) to block lipolysis. Artificial tears were applied to lubricate and protect eyes following retro-orbital injection. Blood (2 drops, about 50 μl) was drawn from the submandibular vein at 0 h (immediately prior to injection), 30 min, 1, 2, and 3 h post poloxamer injection. Blood was immediately centrifuged at 7,600 × g for 10 min, and the submandibular vein at 0 h (immediately prior to injection), 30 min, 1, 2, and 3 h post poloxamer injection. Blood was immediately centrifuged at 7,600 × g for 10 min, and then the sample (1 μl) was analyzed by gas chromatography.

**Lipidomic analysis in intestine and liver.** Lipidomic analysis was performed using an Ultimate 3000 ultrahigh performance liquid chromatography system coupled to a Thermo Q-Exactive Orbitrap mass spectrometer equipped with a heated electrospray ion source (Thermo Scientific, CA, USA). Lipid extracts were separated on a Waters ACQUITY BEH C8 column (2.1 × 100 mm, 1.7 μm) with a mobile phase consisted of 60:40 water/acetonitrile (A), and 90:10 isopropanol/acetonitrile (B), both containing 10 mM ammonium formate and 0.1% formic acid. The samples were eluted with a linear gradient from 32 to 97% B over 25 min, main- tained at 97% B for 4 min and re-equilibrated with 32% B for 6 min. The sample injection volume was 5 μL. The mass spectrometer was operated in positive ioni- zation mode, and the full scan and fragment spectra were collected at a resolution of 70,000 and 17,500, respectively. Data analysis and lipid identification were performed using the software Lipidsearch 4.1.30 (Thermo Fisher, CA, USA). Mass labeled d13-PC (18:0) was used as an internal standard.

**Quantification of dietary fat absorption.** Male and female 12 week old FCG Ldlrfl/fl mice were housed individually and fed a butterfat 5% sucrose polybehenate diet for 4 days (ad libitum) and bedding were replaced daily. Fecal pellets (5–8 g) were collected during the third and fourth day of diet consumption and the percentage of fat absorption was quantified by the University of Cincinnati Metabolic Phenotyping Center by measuring the ratio of fat to behehe in the fecal pellets described previously. Briefly, mice were housed in Suppository Table 1. mRNA abundance was calculated using a high temperature cream was applied to remove hair, followed by sterilizing with povidone-iodine/ethanol three times. After a small incision to this region, vas deferens are collapsed using a hemostat and the testes removed. The vasculature to the testes is cut and using a high temperature fine-tip look cautizer and the hemostat released. The surgical site was closed by wound clips and treated with povidone-iodine. For sham-surgeries, the testes are manipulated but left intact in anesthetized mice. Mice were allowed to recover for 2 weeks after surgery and to allow sufficient time to clear endogenous testicular hormones.

were used, covering more than 90% of the transcriptome Array data set.

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sex and sex chromosome complement. For some studies, we performed a three-way ANOVA with between group factors of gonadal sex, sex chromosomes and surgery or diet. If data were not normally distributed, they were transformed prior to ANOVA and post hoc analysis. Kruskal–Wallis ANOVA on rank was performed when data did not pass normality after transformations. Statistical analyses were performed using SigmaPlot software (Version 13) and GraphPad Prism 5. A multiple linear regression model was fit to log-transformed aortic sinus athero-sclerotic lesion area with the following main effects in the model: Body weight, Cholesterol, Gonadal Fat, Sex, Chromosome, and Sex Organs. Significance was defined as $P < 0.05$.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data are available from the corresponding author upon reasonable request. Raw microarray data (GSE64284) are available through the Gene Expression Omnibus under the accession code GSE119497. The source data underlying Figs. 1a, 2a-d, 6a, b and 7c and Supplementary Figs. 1a and 5d are provided as a Source Data file.

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Author contributions
All authors contributed to and approved the results and provided comments on the manuscript. Writing: L.A.C. Study design and supervision: L.A.C., S.E.T., KR, R.T., E.B., A.M., A.P.A., A.J.L. Performing studies: Y.A., X.C., X.W., L.C., W.K., H.M.A., M.P., P.D., K.T., P.T.

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