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Increased High-Density Lipoprotein Cholesterol Levels in Mice With XX Versus XY Sex Chromosomes

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Objective—The molecular mechanisms underlying sex differences in dyslipidemia are poorly understood. We aimed to distinguish genetic and hormonal regulators of sex differences in plasma lipid levels.

Approach and Results—We assessed the role of gonadal hormones and sex chromosome complement on lipid levels using the four core genotypes mouse model (XX females, XX males, XY females, and XY males). In gonadally intact mice fed a chow diet, lipid levels were influenced by both male–female gonadal sex and XX–XY chromosome complement. Gonadectomy of adult mice revealed that the male–female differences are dependent on acute effects of gonadal hormones. In both intact and gonadectomized animals, XX mice had higher HDL cholesterol (HDL-C) levels than XY mice, regardless of male–female sex. Feeding a cholesterol-enriched diet produced distinct patterns of sex differences in lipid levels compared with a chow diet, revealing the interaction of gonadal and chromosomal sex with diet. Notably, under all dietary and gonadal conditions, HDL-C levels were higher in mice with 2 X chromosomes compared with mice with an X and Y chromosome. By generating mice with XX, XY, and XXY chromosome complements, we determined that the presence of 2 X chromosomes, and not the absence of the Y chromosome, influences HDL-C concentration.

Conclusions—We demonstrate that having 2 X chromosomes versus an X and Y chromosome complement drives sex differences in HDL-C. It is conceivable that increased expression of genes escaping X-inactivation in XX mice regulates downstream processes to establish sexual dimorphism in plasma lipid levels. **(*Arterioscler Thromb Vasc Biol*. 2015;35:1778-1786. DOI: 10.1161/ATVBAHA.115.305460.)**

Key Words: diet ■ female ■ HDL cholesterol ■ male ■ mice

Plasma lipid levels are used as both clinical predictors and as therapeutic targets for cardiovascular disease. As such, substantial effort has been expended to identify genetic factors that influence plasma lipid levels.[1–4] A key genetic determinant of lipid levels is male–female sex. Inherent sex differences in lipid levels have led to distinct standards for the diagnosis of hyperlipidemia in men and in women, but the underlying mechanisms that contribute to differences in lipid levels are not well understood. Men tend to have higher low-density lipoprotein (LDL) and triglyceride levels, and lower high-density lipoprotein (HDL) levels, than premenopausal women.[5,6] After menopause, women often have proatherogenic lipid levels that reach or exceed those in men.[5–7] These observations support a role for gonadal hormones as a key determinant of sexual dimorphism in lipid levels.[7] But the relationship seems to be complex, with postmenopausal hormone therapy affording greatest cardioprotection in women when provided within 10 years of menopause.[8–12] Other studies suggest that the basis for sex differences in lipid metabolism are multifactorial, with gonadal hormones acting together with direct or indirect contributions from other sex-specific factors.[6]

Besides gonadal secretions, another fundamental difference between males and females is the presence of an XX or XY sex chromosome complement. Differences because of gonadal sex versus sex chromosome complement have been difficult to discriminate because, typically, female gonads occur together with XX chromosomes, and male gonads with XY chromosomes. In this study, we used the four core genotypes (FCG) mouse model to identify independent effects on plasma lipid levels of gonadal sex (testes versus ovaries) and sex chromosome complement (XX versus XY). The FCG model consists of 4 types—or sexes—of mice: gonadal male mice with either XX or XY sex chromosomes, and gonadal female mice with XX or XY sex chromosomes.[13–15] In the FCG model, the Y chromosome is deleted for the testis-determining *Sry* gene, which is provided instead by an *Sry* transgene inserted into an autosome. As a result, gonadal sex segregates independently from the sex chromosome complement. Sex

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Nonstandard Abbreviations and Acronyms	
apoB	apolipoprotein B
FCG	four core genotypes
FFA	free fatty acid
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol

differences observed between gonadal males and females can be attributed to the action of gonadal hormones, whereas differences between XX and XY mice can be ascribed to the number of X or Y chromosomes. In addition, by comparing intact and gonadectomized mice, further distinction can be made between the effects of gonadal hormones during development and those resulting from acute effects of hormones in adulthood.

We recently used the C57BL/6 FCG mouse model to determine how sex chromosome complement contributes to sex differences in metabolic traits, such as body weight, adiposity, and hepatic lipid content. Specifically, when gonadectomized as adults to remove acute gonadal effects, XX mice have increased obesity and fatty liver compared with XY mice, regardless of whether they originally had male or female gonads.[16] We hypothesized that sex chromosome complement may also contribute to sex differences in plasma lipid profiles. Here, we report that gonadal hormones and sex chromosome complement have independent effects on plasma lipoprotein levels. These results have implications for understanding the basis for sex differences in plasma lipid levels, and may inform about key risk factors in the metabolic syndrome.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Acute Gonadal Hormones and the Sex Chromosome Complement Influence Plasma Lipid Levels

To analyze sex differences, we measured fasting lipid levels (total cholesterol, HDL cholesterol [HDL-C], triglyceride, and free fatty acids [FFA]) in the 4 genotypes of FCG mice. We defined HDL-C levels as the cholesterol present in particles that lack apolipoprotein B (apoB), and LDL/VLDL (very low-density lipoprotein) cholesterol levels as that from all non-HDL particles (Materials and Methods section of this article). Statistical analyses were performed by 2-way ANOVA, with gonadal sex (male or female) and sex chromosome complement (XX or XY) as covariates.

We first assessed plasma lipid levels in gonadally intact mice fed a chow diet. Total cholesterol levels were similar to those reported previously for C57BL/6 mice, with HDL-C accounting for the majority of plasma cholesterol, as is typical in mice.[17,18] Compared with females, male mice had higher levels of total and HDL-C, as well as triglyceride and FFA (Figure 1A). Males also had slightly higher amounts of unesterified cholesterol (UC in Figure 1). Notably, however, animals of both gonadal sexes with XX chromosomes had

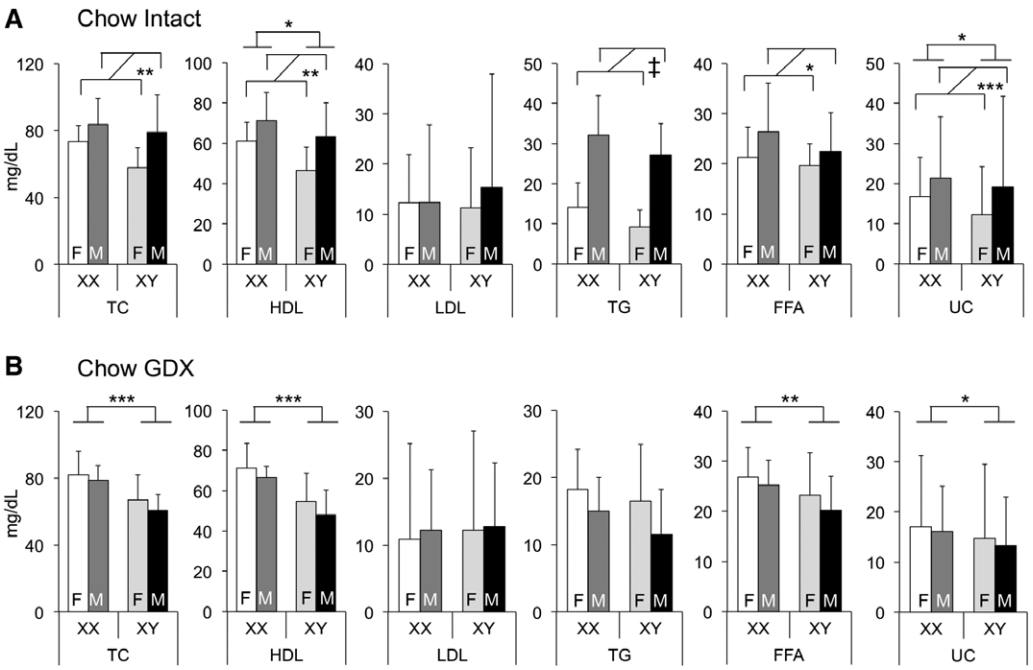


Figure 1. Plasma lipid levels are regulated by both gonadal sex and sex chromosome complement. **A** and **B**, Concentrations of total cholesterol (TC), unesterified cholesterol (UC), high-density lipoprotein (HDL) cholesterol, triglycerides (TG), and free fatty acids (FFA) were measured in 7.5-month-old gonadally intact (**A**) and gonadectomized (GDX; **B**). Four core genotypes mice fed a standard chow diet (n=8). Low-density lipoprotein (LDL) cholesterol values were calculated by subtracting HDL from TC. Values represent the mean±SD. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by Int. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ‡$P \leq 0.000001$. F indicates gonadal female; and M, gonadal male.

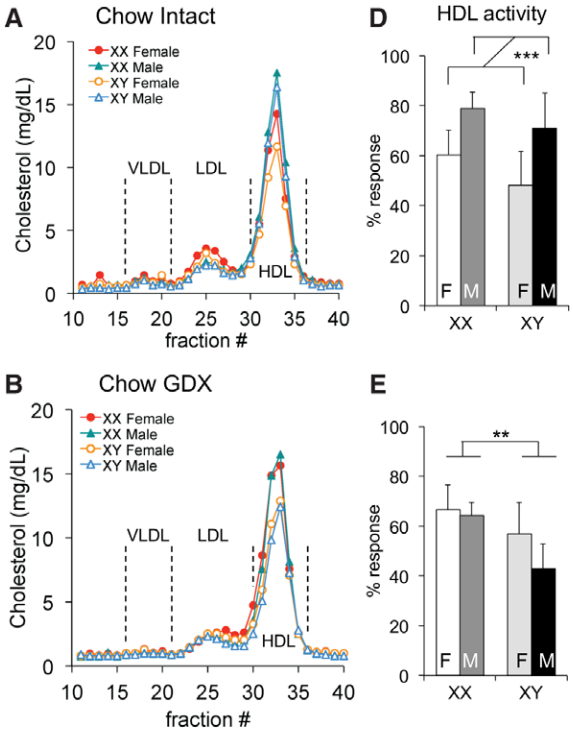
20% higher HDL-C levels than XY mice ($P<0.02$). These results indicate that male–female gonads are a determinant of sex differences in plasma lipid levels, but also reveal that the sex chromosome complement influences HDL-C levels, even in the presence of normal gonadal hormone levels.

The sex differences in lipid levels that were observed between males and females could result from either long-term or short-term effects of gonadal secretions.[19] To distinguish between these, we gonadectomized mice after they reached adulthood (75 days of age) and determined lipid levels 5 months later, at which point acute effects of gonadal hormones should be absent, but long-term effects might persist. Gonadectomized mice did not exhibit the male–female differences in lipid levels that were present in gonadally intact mice indicating that much of the male–female dimorphism in plasma lipid levels is related to acute effects of gonadal secretions. However, as observed in intact mice, HDL and UC levels were higher in XX compared with XY mice ($P<0.0003$ and $P<0.04$), regardless of original gonad type (Figure 1B). After gonadectomy, total cholesterol levels and FFA levels were also higher in XX than in XY mice ($P<0.007$ and $P<0.003$). Thus, the effects of XX chromosome complement on HDL and UC levels are robust, occurring in both the presence and absence of gonadal secretions, and gonadectomy exposes underlying effects of chromosome complement on total cholesterol and FFA levels.

Lipoprotein Composition Differs in XX and XY Mice

As described above, HDL-C values (determined after fractionation of apoB-containing lipids) are higher in mice with XX compared with XY chromosome complement. We wondered if sex chromosome complement influences HDL characteristics, such as particle size, apolipoprotein content, or HDL–apolipoprotein (apo)A-I exchange activity (a measure of HDL function). To assess whether HDL size distribution differs among the FCG genotypes, we fractionated plasma samples by fast protein liquid chromatography and quantified cholesterol content of the resulting fractions. HDL-C peaks directly mirrored the results of biochemical fractionation, with highest HDL-C levels in the XX mice within each sex (XX males>XY males and XX females>XY females), and higher levels in males than in females in gonadally intact mice (Figure 2A). In mice that had been gonadectomized as adults, the HDL-sized particles were more abundant in XX compared with XY mice, and male–female differences were not detectable (Figure 2B). Minor sex differences were observed in cholesterol levels in LDL-sized particles, with slightly higher levels in females than in males in both intact and gonadectomized mice (Figure 2A and B).

We assessed the relative plasma apolipoprotein levels in plasma from the FCG mice. Consistent with the higher HDL-C levels in intact male mice, levels of the major HDL apolipoprotein, apoA-I, were slightly higher in males than



C

	ApoAI	ApoAII	ApoAIV	ApoE
Chow Intact	M > F* XY > XX*	n.s.	XY > XX**	M > F*** XY > XX*
Chow GDX	XY > XX** Interaction**	Interaction**	n.s.	Interaction*

Figure 2. High-density lipoprotein (HDL) lipoprotein composition and apoA-I exchange activity is influenced by sex. Plasma was collected from 7.5-month-old gonadally intact (**A** and **D**) and gonadectomized (GDX; **B** and **E**) chow-fed mice. **A** and **B**, Three representative plasma samples from each genotype were pooled and assayed using fast protein liquid chromatography. **C**, Plasma levels of apolipoproteins were quantified by immunoblot densitometry. Direction of statistically significant comparisons for gonadal sex and for sex chromosomes are shown. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Interaction." **D** and **E**, HDL–apoA-I dissociation activity was measured by electron paramagnetic resonance and represented as % response. Values represent the mean±SD. *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$. F indicates gonadal female; LDL, low-density lipoprotein; M, gonadal male; n.s., not significant; and VLDL, very low-density lipoprotein.

in females ($P<0.02$; Figure 2C; Table I and Figure I in the online-only Data Supplement). A similar male–female difference was observed for apoE, a component of multiple lipoprotein classes ($P<0.0005$). Apolipoprotein levels were also influenced by sex chromosome complement. The levels of apoA-I, apoA-IV, and apoE were higher in XY compared with XX mice of both gonadal sexes ($P<0.02$, $P<0.03$, and $P<0.01$, respectively). This was unexpected, given the higher HDL-C levels in XX compared with XY mice. Gonadectomy abolished the male–female differences in apolipoprotein levels, but maintained the higher apoA-I levels in XY compared with XX mice ($P<0.006$). Removal of the gonads also uncovered interactions between sex chromosome complement and the original gonadal sex (Figure 2C; Table I in the online-only Data Supplement). ApoB levels were low in all chow-fed mice, and neither apoB nor apoA-II levels differed among the 4 genotypes (Table I and Figure I in the online-only Data Supplement). Overall, our results reveal complex effects of sex chromosome complement on plasma lipoprotein composition, with XX chromosome complement favoring higher HDL-C content, but lower total levels of apolipoproteins that are often associated with HDL, including apoA-I and apoA-IV.

The lower apolipoprotein/cholesterol ratio of HDL from XX compared with XY mice could influence HDL function. One assessment of HDL function is the degree to which apoA-I present on HDL can be dissociated from the lipoprotein particle (HDL–apoA-I exchange).[20] A reduced HDL–apoA-I exchange rate correlates with metabolic syndrome and acute coronary syndrome in humans, and with increased atherosclerotic plaque burden in rabbits.[21] We assessed the HDL–apoA-I exchange rate using site-directed spin-label electron paramagnetic resonance.[21] In both intact and gonadectomized mice, the HDL–apoA-I exchange activity in plasma mirrored HDL-C concentrations, with male>female in intact mice ($P=0.001$) and XX>XY in gonadectomized mice ($P<0.003$; Figure 2D and 2E). Thus, sex differences in HDL–apoA-I exchange rates parallel those in HDL-C levels, and are influenced by both gonadal and chromosomal sex determinants.

Sex Chromosome–Diet Interactions Influence Cholesterol Levels and HDL Activity

Lipid levels are highly responsive to diet. We investigated the factors underlying sexual dimorphism in lipid levels in response to dietary cholesterol by feeding FCG mice, a diet containing 1.25% cholesterol (in contrast to 0.02% in chow). As expected, the cholesterol-enriched diet caused substantial increases in the absolute levels of total and LDL cholesterol (LDL-C) in all genotypes compared with levels in mice fed the chow diet. Although both sex chromosomes and gonadal sex influenced lipid levels on a high cholesterol diet, specific effects differed from chow diet. As we observed on chow diet, sex chromosome complement remained an important determinant of HDL-C levels, with XX>XY (Figure 3A). Unlike chow diet, however, sex chromosome complement also influenced triglyceride and FFA levels, with XY>XX. HDL and UC levels were both influenced by gonadal sex in intact mice fed a cholesterol-enriched diet, with female>male. Thus, in some cases, the determinants of sexual dimorphism in lipid traits are responsive to diet.

Removal of the acute effects of gonadal secretions by gonadectomy of adult mice produced unique patterns of lipid

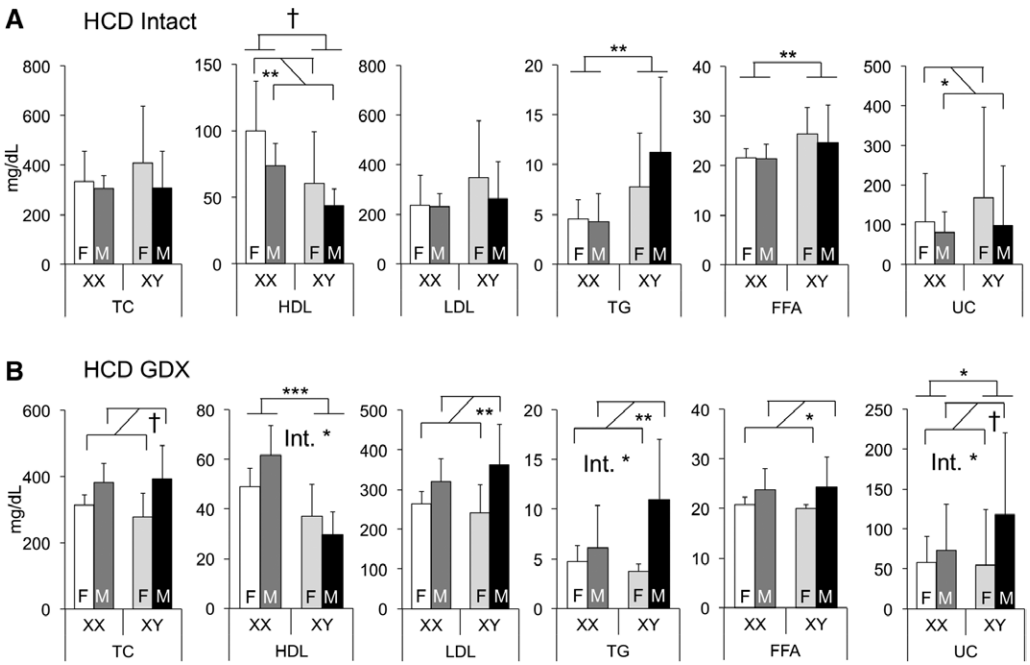
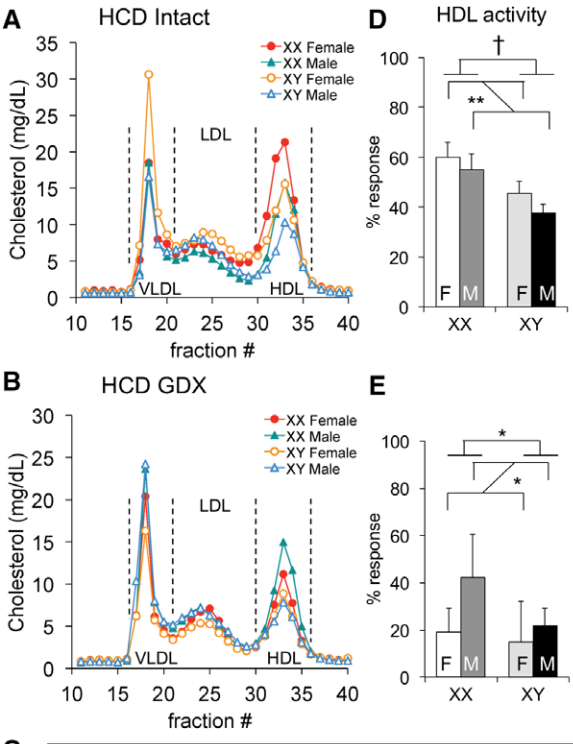


Figure 3. Diet interacts with gonadal sex and sex chromosome complement to modulate plasma lipid levels. **A** and **B**, Concentrations of total cholesterol (TC), unesterified cholesterol (UC), high-density lipoprotein (HDL) cholesterol, triglycerides (TG), and free fatty acids (FFA) were determined in 7.5-month-old gonadally intact (**A**) and GDX (**B**) FCG mice fed a high cholesterol diet (HCD, n=4–10). Low-density lipoprotein (LDL) cholesterol values were calculated by subtracting HDL from TC. Values represent the mean±SD. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by Int. *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$, $P\leq0.0001$. F indicates gonadal female; and M, gonadal male.

levels among the 4 genotypes compared with chow diet or intact mice fed cholesterol diet. HDL-C and UC levels were higher in XX compared with XY mice (Figure 3B). Thus, HDL and UC cholesterol levels were consistently influenced by sex chromosome complement across diets (chow and high cholesterol) and gonadal state (intact and gonadectomized). Unexpectedly, in gonadectomized mice, the cholesterol diet uncovered male–female differences in several lipid traits that were not apparent in gonadally intact mice. Thus, males had higher levels than females of total cholesterol, LDL-C, UC, triglyceride, and FFA (Figure 3B). Interestingly, the only condition examined in which LDL-C levels exhibited sexual dimorphism was in mice gonadectomized and fed a cholesterol-enriched diet. Detection of male–female lipid level differences in gonadectomized mice suggests a role for either long-lasting (organizational) effects of gonadal hormones present in early life, or an effect of *Sry* acting outside of the gonads. Furthermore, the emergence of male–female dimorphism in cholesterol traits exclusively in gonadectomized animals suggests that acute effects of gonadal secretions in intact mice may counteract these organizational hormone effects.

As described above, the presence of XX sex chromosomes was associated with higher HDL-C levels than XY chromosome complement. On the cholesterol-enriched diet, HDL-C levels were ≈60% higher in XX than in XY mice in both intact and gonadectomized states (Figure 3). In the intact mice on both diets, the sex chromosome effect was overlaid with male–female sex differences. The XX>XY differences in HDL-C levels of mice fed a cholesterol-enriched diet were recapitulated when HDL particles were defined by size via fast protein liquid chromatography fractionation (Figure 4A and 4B). Analysis of apolipoprotein content on the cholesterol-enriched diet showed XX>XY for several HDL apolipoproteins (apoA-I, apoA-II, and apoA-IV) in intact mice; female>male effects were also evident for apoA-IV and apoE (Figure 4C; Table I and Figure I in the online-only Data Supplement). In gonadectomized mice fed a cholesterol-enriched diet, the sex chromosome effects on apolipoprotein content were less pronounced, but still apparent for apoA-II and apoE.

Assessment of HDL–apoA-I exchange activity revealed a strong effect of diet. On a chow diet, HDL activity in gonadally intact mice was higher in males than in females (Figure 2D); after cholesterol feeding, XX mice had higher HDL–apoA-I exchange activity than XY mice, and female mice had higher activity than males (Figure 4D). Gonadectomy in combination with dietary cholesterol reduced the absolute levels of HDL–apoA-I exchange activity compared with all other dietary–gonadal hormone conditions, particularly in females (Figure 4E); in chow-fed mice, gonadectomy reduced HDL–apoA-I exchange activity only in males (Figure 2E). These results suggest that acute effects of gonadal hormones are a determinant of HDL–apoA-I exchange capacity, with distinct sexually dimorphic effects that respond to diet.



C

	ApoAI	ApoAII	ApoAIV	ApoB	ApoE
HCD Intact	XX > XY* Interaction*	XX > XY**	F > M* XX > XY*	n.s.	F > M**
HCD GDX	n.s.	XX > XY**	F > M**	Interaction**	M > F** XY > XX*

Figure 4. Diet interacts with sex to influence high-density lipoprotein (HDL) lipoprotein composition and apoA-I exchange activity. Plasma was collected from 7.5-month-old gonadally intact (**A** and **D**) and gonadectomized (GDX; **B** and **E**) mice fed the high cholesterol diet (HCD). **A** and **B**, Three representative plasma samples from each genotype were pooled and assayed using fast protein liquid chromatography. **C**, Plasma levels of apolipoproteins were quantified by immunoblot densitometry. Direction of statistically significant comparisons for gonadal sex and for sex chromosomes are shown. A significant interaction of sex chromosome complement and gonadal sex is denoted by Interaction. **D** and **E**, HDL–apoA-I dissociation activity was measured by electron paramagnetic resonance and represented as % response and as activity per unit of HDL cholesterol. Values represent the mean±SD. **P*≤0.05, ***P*≤0.01, †*P*≤0.0001. F indicates gonadal female; M, gonadal male; and n.s., not significant.

Table. Sex Differences in Hepatic Gene Expression

	Chow Intact	Chow GDX	HCD Intact	HCD GDX
Cholesterol synthesis and transport				
Abca1	M>F**	n.s.	F>M**	XY>XX* Int.**
Abcg1	F>M† XY>XX*** Int.**	XY>XX† Int.*	F>M** XY>XX**	XY>XX**
ApoB	XX>XY**	M>F*	M>F†	n.s.
Hmgcr	n.s.	n.s.	M>F** XY>XX**	n.s.
Lcat	F>M*	M>F*	M>F†	F>M* XX>XY***
Ldlr	XX>XY*	n.s.	M>F†	XX>XY*
Lipc	F>M* Int.**	XX>XY**	M>F†	n.s.
Mvk	n.s.	n.s.	Int.*	n.s.
Pltp	F>M*** XY>XX*	n.s.	F>M† XY>XX**	XY>XX**
Scarb1	n.s.	n.s.	M>F**	XX>XY*
Bile acid synthesis				
Cyp7a1	F>M*** XX>XY**	n.s.	F>M* Int.*	n.s.
Cyp8b1	M>F** XX>XY**	F>M**	n.s.	n.s.
Cyp27a1	XX>XY***	XX>XY* Int.*	M>F**	n.s.
X-inactivation escape				
Ddx3x	XX>XY***	M>F*	M>F** XX>XY** Int.***	XX>XY***
Eif2s3x	M>F** XX>XY ‡ Int.**	M>F* XX>XY**	XX>XY†	XX>XY† Int.*
Kdm5c	M>F** XX>XY ‡	M>F*	M>F** XX>XY ‡ Int.*	XX>XY† Int.**
Kdm6a	XX>XY**	F>M* XX>XY***	XX>XY‡	M>F** XX>XY**

Hepatic gene expression was measured by quantitative polymerase chain reaction. *P*-values are represented by direction of sex difference and magnitude of significance. F indicates gonadal female; GDX, gonadectomized; HCD, high cholesterol diet; M, gonadal male; and n.s., not significant.

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, †$P \leq 0.0001$, ‡$P \leq 0.000001$.

Gene Expression Levels for Components of Cholesterol Synthesis and Metabolism Do Not Explain Sex Differences in Plasma Cholesterol Levels

In all dietary and gonadal conditions examined here, HDL-C levels were higher in mice with XX compared with XY chromosome complement. To investigate potential mechanisms, we examined hepatic gene expression levels for key players in cholesterol synthesis and metabolism. These included determinants of cholesterol biosynthesis (*Hmgcr* and *Mvk*), cellular lipoprotein uptake (*Ldlr* and *Scarb1*), cholesterol conversion to bile acids (*Cyp7a1*, *Cyp8b1*, and *Cyp27a1*), and HDL lipid accumulation (*Lcat, Pltp, Abca1,* and *Abcg1*). We searched for patterns of gene expression that mirrored the elevated HDL-C levels in XX compared with XY genotypes across the 4 cohorts of mice, all of which had higher HDL-C levels in XX compared with XY mice. In some cases (eg, *Pltp, Abcg1*), male gonadal secretions appeared to inhibit gene expression, as increased expression of these genes was observed in chow-fed males after gonadectomy. And although we identified some instances of XX versus XY differences in gene expression, we did not detect patterns that are likely to explain the elevated levels of HDL in XX compared with XY mice (Table; Figures II–IV in the online-only Data Supplement).

Differences in X Chromosome Gene Dosage Associate With Plasma HDL-C Levels

The association of HDL-C levels with XX chromosome complement suggests a mechanism that is directly related to the presence of a second X chromosome or the absence of a Y chromosome. To distinguish between these 2 possibilities, we measured plasma lipid levels in a mouse model differing in the number of sex chromosomes.[14,22] The abnormal Y* chromosome undergoes recombination with the X chromosome to produce XX, XX^{Y*} (similar to XXY), and XY* (similar to XY) mice. Chow-fed, gonadectomized mice with 2 X chromosomes (XX and XXY) had higher levels of total,

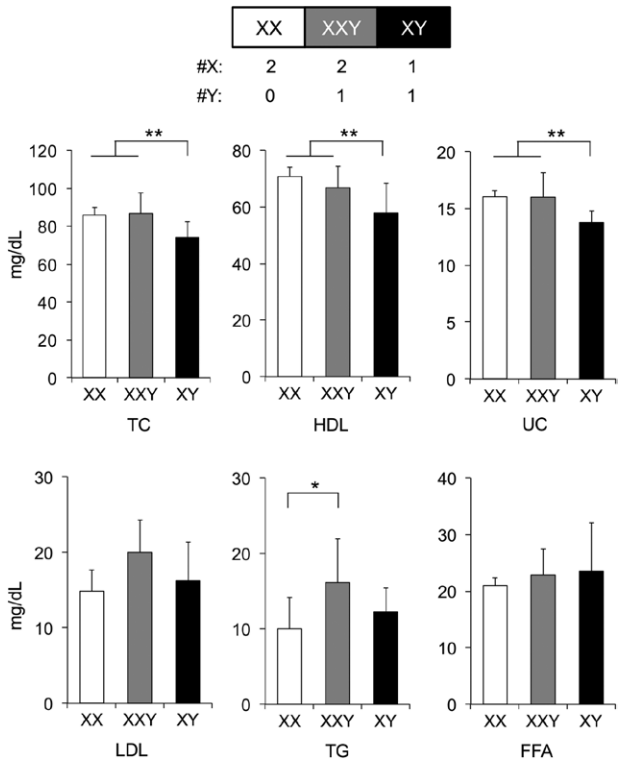


Figure 5. Elevated total and high-density lipoprotein (HDL) cholesterol levels are associated with presence of 2 X chromosomes. Concentrations of total cholesterol (TC), unesterified cholesterol (UC), HDL cholesterol, triglyceride (TG), and free fatty acids (FFA) were measured in gonadectomized XY* chow-fed mice with the sex chromosome genotypes indicated (n=7–8). Low-density lipoprotein (LDL) cholesterol values were calculated by subtracting HDL from TC. Values represent the mean±SD. Significant comparisons by 1-way ANOVA with Duncan multiple-comparison test are denoted by brackets. *$P \leq 0.05$, **$P \leq 0.01$.

unesterified, and HDL-C than mice with a single X chromosome (XY; $P<0.007$, $P<0.006$, and $P<0.04$, respectively). The presence of the Y chromosome did not affect HDL-C levels (compare XX with XXY; Figure 5). By contrast, mice with a Y chromosome (XXY and XY) had higher LDL-C and triglyceride levels than mice without a Y ($P<0.03$ and $P<0.02$, respectively), regardless of the number of X chromosomes. These data indicate that the presence of X and Y chromosomes have distinct effects on lipid species, with HDL-C influenced by the number of X chromosomes, and LDL-C influenced by the presence of the Y chromosome.

In general, dosage of X chromosome gene expression is normalized between XX and XY cells through inactivation of 1 X chromosome in XX cells. However, a small subset of genes escape X chromosome inactivation and exhibit higher expression levels in XX compared with XY cells.[23] Genes that are well established to escape inactivation in both mice and humans include *Ddx3x*, *Eif2s3x*, *Kdm5c*, and *Kdm6a*. The expression levels of these genes have the potential to influence numerous downstream cellular processes through their roles as histone methylases (*Kdm5c* and *Kdm6a*), a DNA helicase (*Ddx3x*), and a translation initiation factor (*Eif2s3x*). To assess whether the X-inactivation escapee genes have enhanced expression levels in the 4 cohorts of mice studied here in a relevant metabolic tissue, we quantitated gene expression in liver of both intact and gonadectomized FCG mice on chow and high cholesterol diets. These genes were expressed at higher levels in XX mice compared with XY mice in nearly all cohorts (Table; Figure V in the online-only Data Supplement). In some cases, male–female dimorphism was also observed. The higher hepatic expression levels of X chromosome escapee genes in XX compared with XY liver raise the possibility that altered X chromosome gene dosage may contribute to sexual dimorphism in HDL-C levels, and probably other metabolic traits.

Discussion

We used the FCG mouse model to investigate the relative contributions of gonadal secretions and sex chromosome complement to lipid levels. Using this model, we were able to detect sex chromosome complement as a determinant of sexual dimorphism in plasma lipids and in lipoproteins, particularly HDL-C.

In gonadally intact mice fed a chow diet, total and HDL-C, as well as triglyceride and FFA levels, were higher in male mice (XX and XY) compared with female mice (XX and XY). In addition, HDL-C levels were influenced by sex chromosome complement, with higher levels in XX compared with XY mice. Thus, even in the presence of endogenous gonadal hormones, the effect of sex chromosomes on HDL-C levels was apparent. To further explore the influence of sex chromosome complement on lipid levels, we reduced hormone levels by gonadectomy, which eliminated male–female differences observed in the intact mice, and amplified the XX versus XY effects on HDL-C levels. Gonadectomy also revealed that XX chromosome complement promotes higher total cholesterol and FFA levels. These results suggest that sex chromosome complement may become a particularly important determinant of lipid levels under conditions characterized by reduced gonadal hormones, such as middle age and postmenopause in humans.

We detected interactions between sex chromosome complement and diet. Gonadally intact XX mice fed a diet enriched in cholesterol had higher HDL-C levels than XY mice, as was observed in chow-fed mice. In addition, the cholesterol-enriched diet brought out a novel sex chromosome effect on triglyceride and FFA levels, with higher levels in XY compared with XX mice. After gonadectomy, male mice had significantly higher levels than females for total cholesterol, LDL-C, triglyceride, and FFA. Because mice were gonadectomized 5 months before lipid measurements, the observed male–female dimorphism must be a result of long-lasting (ie, organizational) effects of gonadal hormones, or nongonadal effects of *Sry* that are confounded with gonadal sex in this model. Together, our results indicate that sex differences in lipid levels are determined by a combination of hormonal and sex chromosome effects, and further, these differences are dependent on hormonal (high or low gonadal hormone concentrations) and diet (chow or high cholesterol) context (Figure 6A).

Regardless of diet or sex hormone milieu, HDL-C levels were elevated in mice with 2 X chromosomes. The potential benefits of elevated HDL-C levels include cholesterol efflux

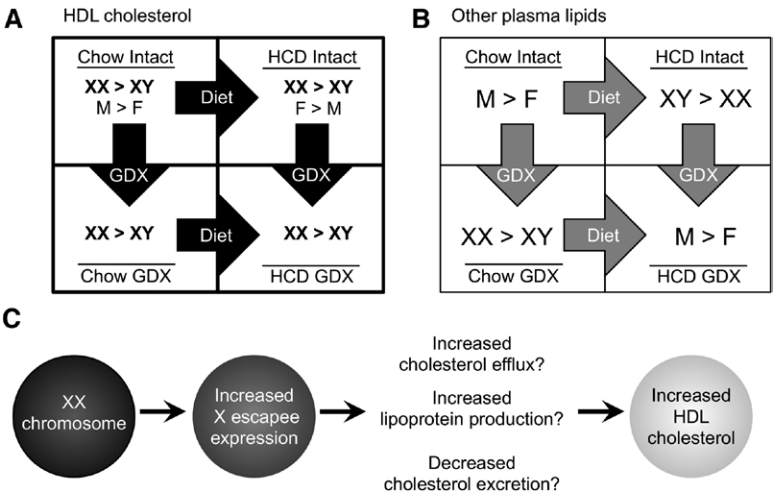


Figure 6. Factors influencing circulating high-density lipoprotein (HDL) cholesterol. **A**, HDL cholesterol levels are higher in XX mice compared with XY mice, regardless of diet or sex hormone presence status. Male/female gonad status also influences HDL cholesterol levels under some conditions. **B**, Plasma lipid other than HDL exhibit sex differences that depend on the diet and gonadal status. **C**, Mice with 2 X chromosomes have increased X escapee gene expression in metabolic tissues such as liver. These genes may influence several metabolic pathways to increase circulating HDL cholesterol. F indicates gonadal female; GDX, gonadectomy; HCD, high cholesterol diet; and M, gonadal male.

from peripheral tissues, anti-inflammatory and antioxidative activities, and protection from infection.[24,25] One metric of HDL activity that has been correlated with metabolic syndrome in humans and atherosclerosis in rabbits is the efficiency of apoA-I exchange from HDL particles.[21] We found that total HDL–apoA-I exchange activity was greater in XX mice than in XY mice, concordant with the increased HDL-C concentration in XX animals. These results suggest that XX chromosome complement may be a factor in determining both the levels and the protective activity of HDL.

By altering the number of X and Y chromosomes using the Y* mouse model, we determined that the presence of 2 X chromosomes is associated with increased HDL-C. We previously determined that the presence of 2 X chromosomes also leads to increased adiposity and fatty liver development on a high-fat diet.[16] Thus, a future goal of broad importance is to understand how increased X chromosome dosage impacts metabolism. Here, we demonstrate that genes that escape X chromosome inactivation are expressed at higher levels in XX compared with XY tissues. The proteins encoded by these genes—which have roles in transcriptional regulation, RNA processing, and protein translation[26–33]—could conceivably influence lipid homeostasis (Figure 6B). The specific targets of X chromosome escapee gene activity are not well characterized at present, but ongoing studies are focused on their identification using large-scale transcriptional and epigenomic profiling.

Our data using the Y* mouse model revealed that in addition to effects of 2 X chromosomes on HDL-C levels, the presence of the Y chromosome may influence LDL-C levels. The Y chromosome has traditionally been viewed to harbor genes restricted to male gonad development and spermatogenesis. However, a recent study using consomic mouse strains with Y chromosomes derived from distinct inbred strains suggests that genetic variation in Y chromosome genes influences plasma lipoprotein levels.[34] The Y chromosome carries a set of genes that encode Y-specific proteins that are similar to paralogous genes on the X chromosome. These include the Y chromosome counterparts of the X chromosome escapee genes that exhibit increased dosage in mice with 2 versus a single X chromosome. In our study, all X and Y chromosomes were genetically identical, derived from the C57BL/6 strain, so that dosage alone was manipulated. It is interesting to speculate that both dosage and genetic variation in these X and Y chromosome genes may influence lipid levels.

Our genetic analysis of sexual dimorphism of lipoprotein levels has limitations imposed by the necessity of using a mouse model. It is well established that mice and humans differ in several aspects of lipoprotein metabolism.[35] When fed a chow diet, mice carry approximately two thirds of plasma cholesterol in HDL particles, whereas humans carry a similar proportion in LDL particles. In this study, some mice were fed a cholesterol-enriched diet to increase the levels of LDL cholesterol, but because mice do not express cholesteryl ester transfer protein, which promotes the transfer of cholesteryl esters and triglycerides between lipoprotein particles, species differences in the metabolism of circulating lipoproteins persist. Relevant to the effects of gonadal hormones on lipoprotein metabolism, it has been demonstrated that estradiol administration produces different effects on HDL-C levels in mice and humans.[36,37] Despite the known species differences in lipoprotein metabolism, the genetic loci that control HDL-C levels in mouse and human exhibit a substantial degree of overlap,[38] raising the possibility that fundamental genetic influences on lipoprotein physiology are shared between mouse and man.

In conclusion, our studies demonstrate that sexual dimorphism in lipid levels is a result of interactions between gonadal hormones, sex chromosome complement, and diet. The results further indicate that XX chromosome complement has a major influence on HDL-C levels, irrespective of diet or gonadal status. Future studies with FCG mice will facilitate the identification of sex-dependent biomarkers of disease associated with altered lipid levels, such as atherosclerosis. Such studies are crucial for improving the assessment and treatment of cardiovascular disease risk in men and women.

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Disclosures

M.N. Oda is a founder and holds an ownership stake in Seer BioLogics, Inc. This did not influence his or his laboratory's interpretation or presentation of results.

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Significance

Lipid profiles are an important indicator of the metabolic syndrome. Reports of sexually dimorphic low-density lipoprotein and high-density lipoprotein cholesterol levels suggest regulation by sex hormones. Here, we show that the sex chromosome complement is also a key factor in modulating plasma lipid levels. High-density lipoprotein cholesterol is consistently elevated in mice with 2 X chromosomes compared with mice with XY sex chromosomes, regardless of diet or circulating sex hormone levels. These findings are important for understanding cardiovascular disease risk in both men and women.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Increased High-Density Lipoprotein Cholesterol Levels in Mice With XX Versus XY Sex Chromosomes

Jenny C. Link, Xuqi Chen, Christopher Prien, Mark S. Borja, Bradley Hammerson, Michael N. Oda, Arthur P. Arnold and Karen Reue

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Supplemental Table I. Apolipoprotein quantification

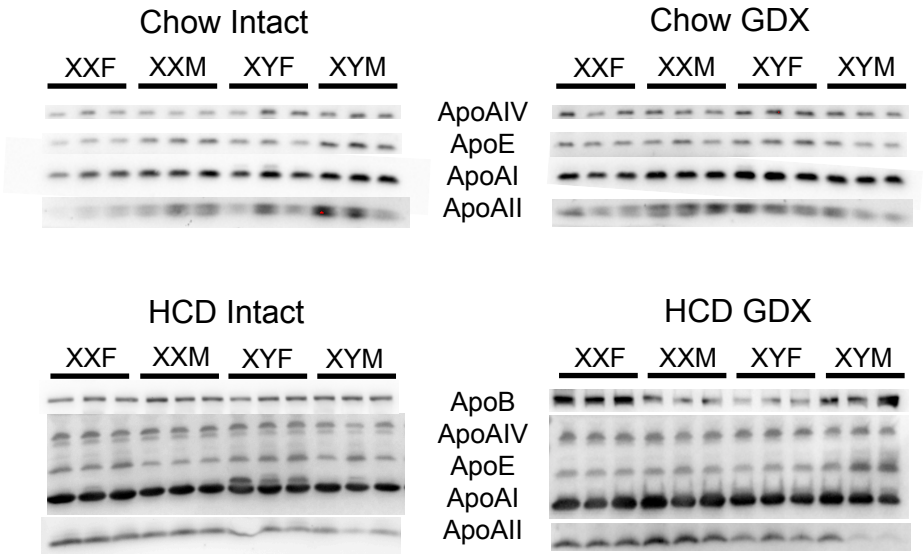
		ApoAI	ApoAII	ApoAIV	ApoB	ApoE
Chow Intact	XXF	1.10 (0.32)	0.61 (0.39)	0.63 (0.19)	not detected	0.62 (0.14)
	XXM	1.70 (0.19)	1.98 (0.58)	0.59 (0.07)		1.39 (0.19)
	XYF	1.72 (0.37)	1.85 (1.23)	1.09 (0.41)		1.01 (0.24)
	XYM	2.11 (0.12)	3.14 (1.60)	1.07 (0.14)		1.79 (0.34)
Chow GDX	XXF	1.77 (0.28)	1.80 (0.42)	1.03 (0.37)	not detected	1.07 (0.12)
	XXM	2.30 (0.07)	3.24 (0.64)	1.06 (0.06)		1.09 (0.28)
	XYF	3.10 (0.40)	3.18 (0.76)	1.53 (0.14)		1.61 (0.27)
	XYM	2.12 (0.19)	1.78 (0.80)	1.19 (0.27)		0.99 (0.25)
HCD Intact	XXF	1.27 (0.13)	7.14 (1.76)	3.20 (0.35)	4.79 (0.44)	3.41 (0.33)
	XXM	1.12 (0.08)	5.78 (0.39)	2.76 (0.16)	5.29 (0.88)	2.08 (0.16)
	XYF	0.91 (0.07)	3.39 (0.84)	2.83 (0.10)	4.66 (0.83)	3.16 (0.37)
	XYM	1.15 (0.17)	3.28 (1.29)	2.15 (0.49)	5.08 (0.06)	2.66 (0.66)
HCD GDX	XXF	3.18 (0.46)	2.62 (0.21)	0.75 (0.02)	6.52 (2.76)	0.69 (0.03)
	XXM	3.59 (0.55)	3.07 (0.66)	0.65 (0.05)	2.16 (0.52)	1.12 (0.19)
	XYF	3.64 (0.20)	1.63 (0.54)	0.78 (0.03)	3.31 (0.68)	0.76 (0.14)
	XYM	3.46 (0.63)	0.97 (1.08)	0.68 (0.07)	7.47 (0.49)	1.78 (0.63)

Supplemental Table I. Quantification of apolipoproteins. Three representative plasma samples from each genotype and from each cohort were separated by gel electrophoresis. Protein levels of apolipoproteins were quantified by densitometry and given as mean values with standard deviation in parentheses. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Table II. Mouse primer sequences for qPCR

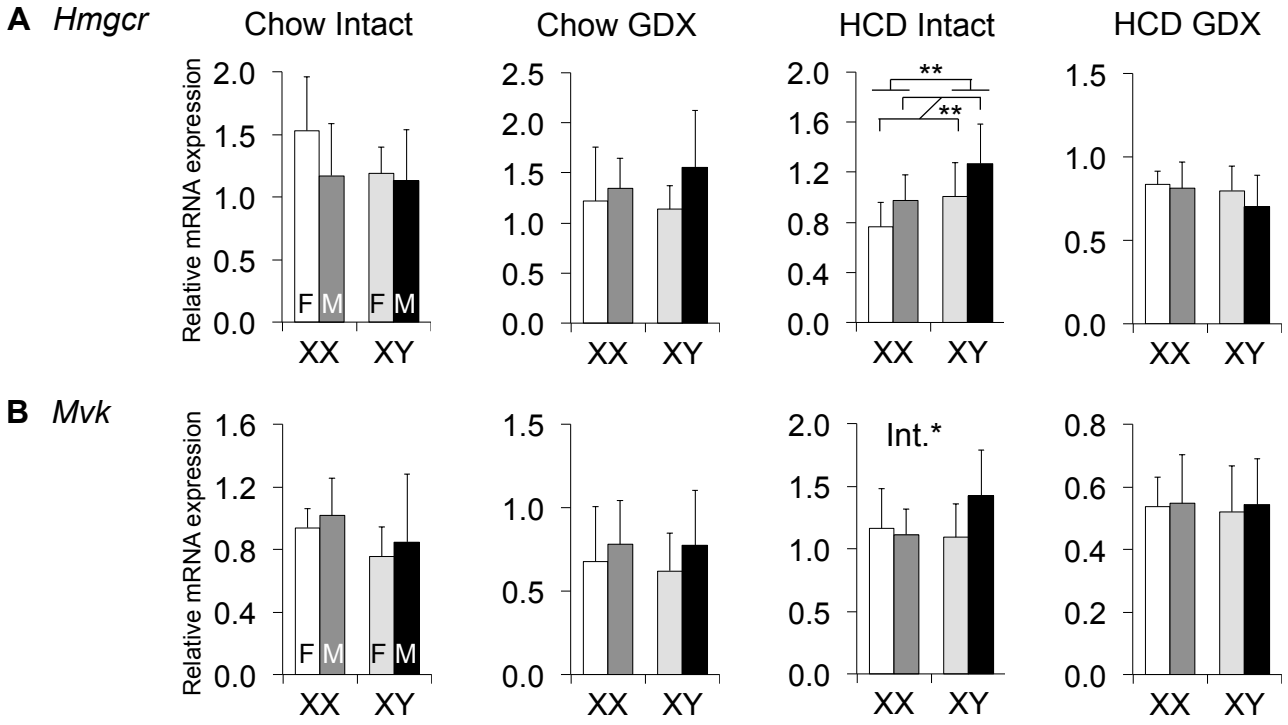
Genes	Forward primer	Reverse primer
β2m	CAGCATGGCTCGCTCGGTGAC	CGTAGCAGTTCAGTATGTTCG
Tbp	ACCCTTCACCAATGACTCCTATG	ATGATGACTGCAGCAAATCGC
Abca1	ACCAGCTTCCATCCTCCTTGT	TTGGTCCTTGGCAAAGTTCAC
Abcg1	CCTGCTCTTCTCCGGATTCTT	ATGTCGCAGTGCAGGTCTTCT
ApoB	CAGTATTCTGCCACTGCAACC	AGGACTTCACTAGATAAGGTCC
Hmgcr	ATGCCTTGTGATTGGAGTTGG	TGGACGACCCTCACGGCTTTC
Lcat	CCCACCAGCAGGATGAATACTAC	AGGCTATGCCCAATGAGGAA
Ldlr	CTTCTCCTTGGCCATCTATGAGG	CATTGGGGAGGAGGGCTGTTGT
Lipc	TGGAACACAGTGCAGACCATC	TGGAGGTCATCCAGATTTTCG
Mvk	TGACCAAGTTCCCTGAGATTG	CTTGCTCTAGACCTGGCTTC
Pltp	GGCCGTCTCAGTGCTAAGTTG	ATCACTCCGATTTGCAGCAGT
Scarb1	CGTACCTCCCAGACATGCTTC	TCTTGCTGAGTCCGTTCCATT
Cyp7a1	CAATGAAAGCAGCCTCTGAAG	AGCCTCCTTGATGATGCTATC
Cyp8b1	AAGGCTGGCTTCCTGAGCTT	AACAGCTCATCGGCCTCATC
Cyp27a1	CCACAAGGGCCTCACCTATG	GCACCTGGTCCAGCCGGGTG
Ddx3x	GGATCACGGGGTGATTCAAGAGG	CTATCTCCACGGCCACCAATGC
Eif2s3x	TTGTGCCGAGCTGACAGAATGG	CGACAGGGAGCCTATGTTGACCA
Kdm5c	ACCCACCTGGCAAAAACATTGG	ACTGTCGAAGGGGGATGCTGTG
Kdm6a	CCAATCCCCGCAGAGCTTACCT	TTGCTCGGAGCTGTTCCAAGTG

Supplemental Figure I



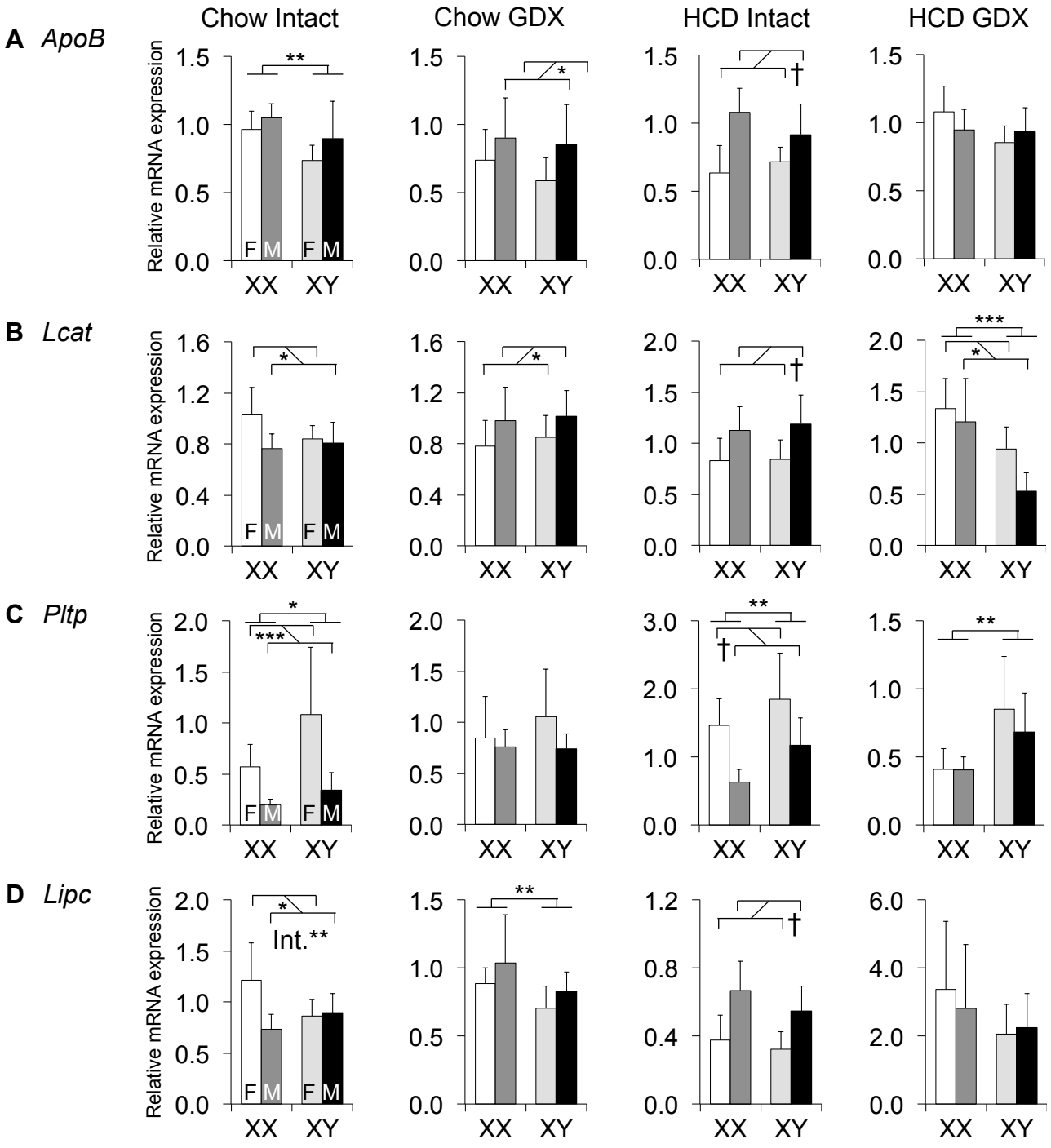
Supplemental Figure I. Plasma levels of apolipoproteins. Three representative plasma samples from each genotype and from each cohort were separated by gel electrophoresis. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure II

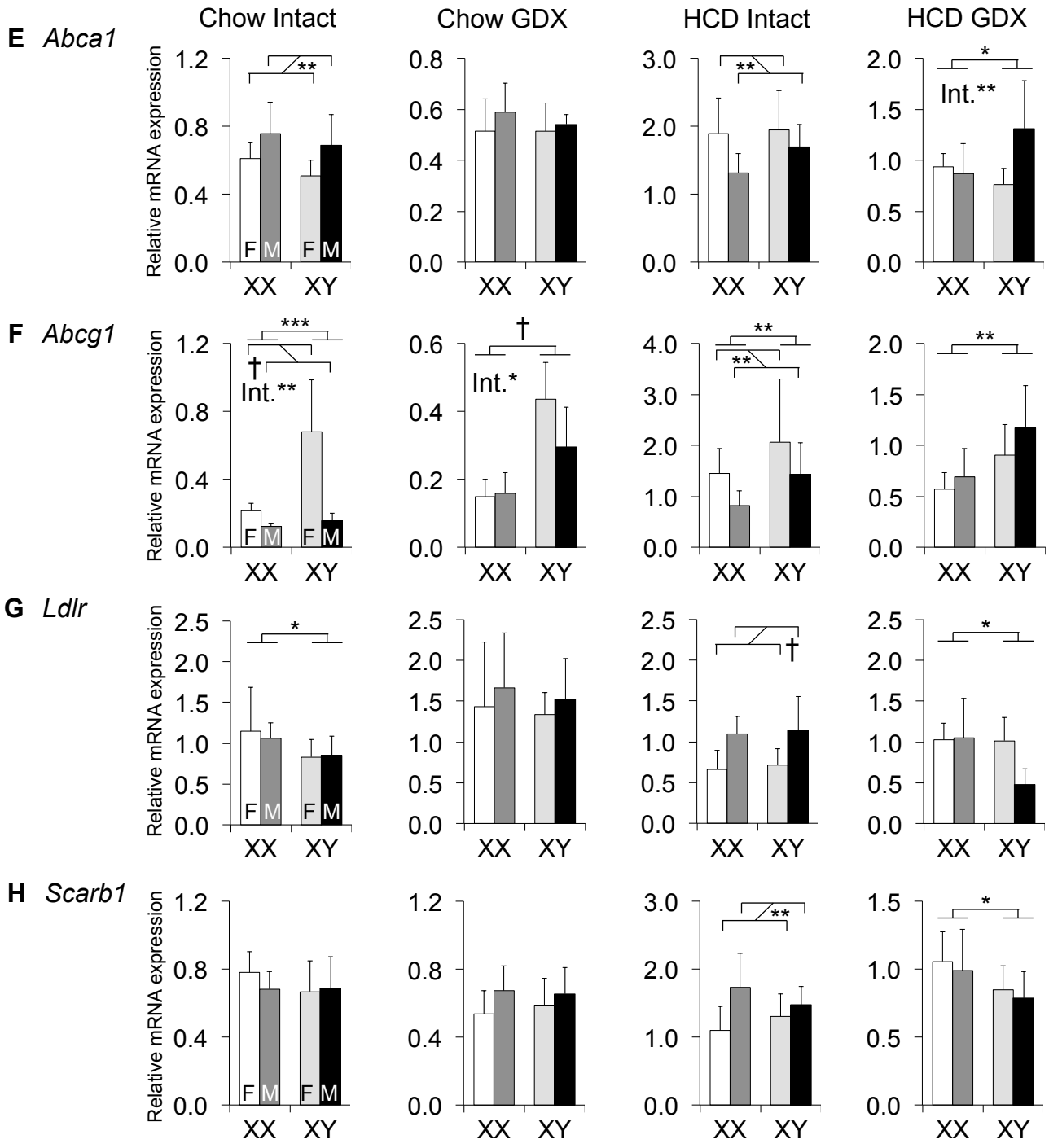


Supplemental Figure II. Key enzymes of cholesterol synthesis are not associated with sex differences in plasma cholesterol levels. Hepatic levels of *Hmgcr* (**A**) and *Mvk* (**B**) were measured by quantitative PCR. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure III

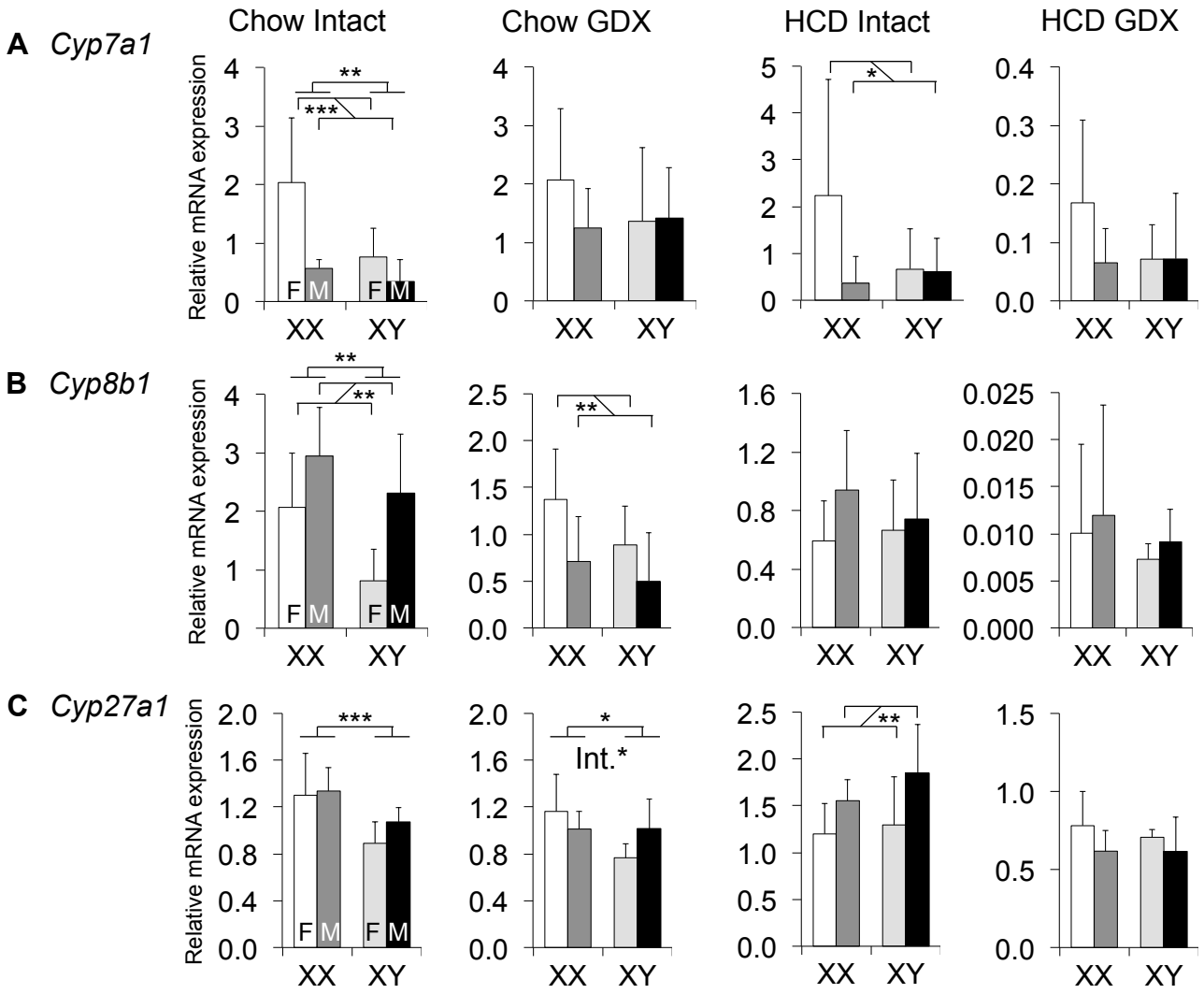


Supplemental Figure III, continued



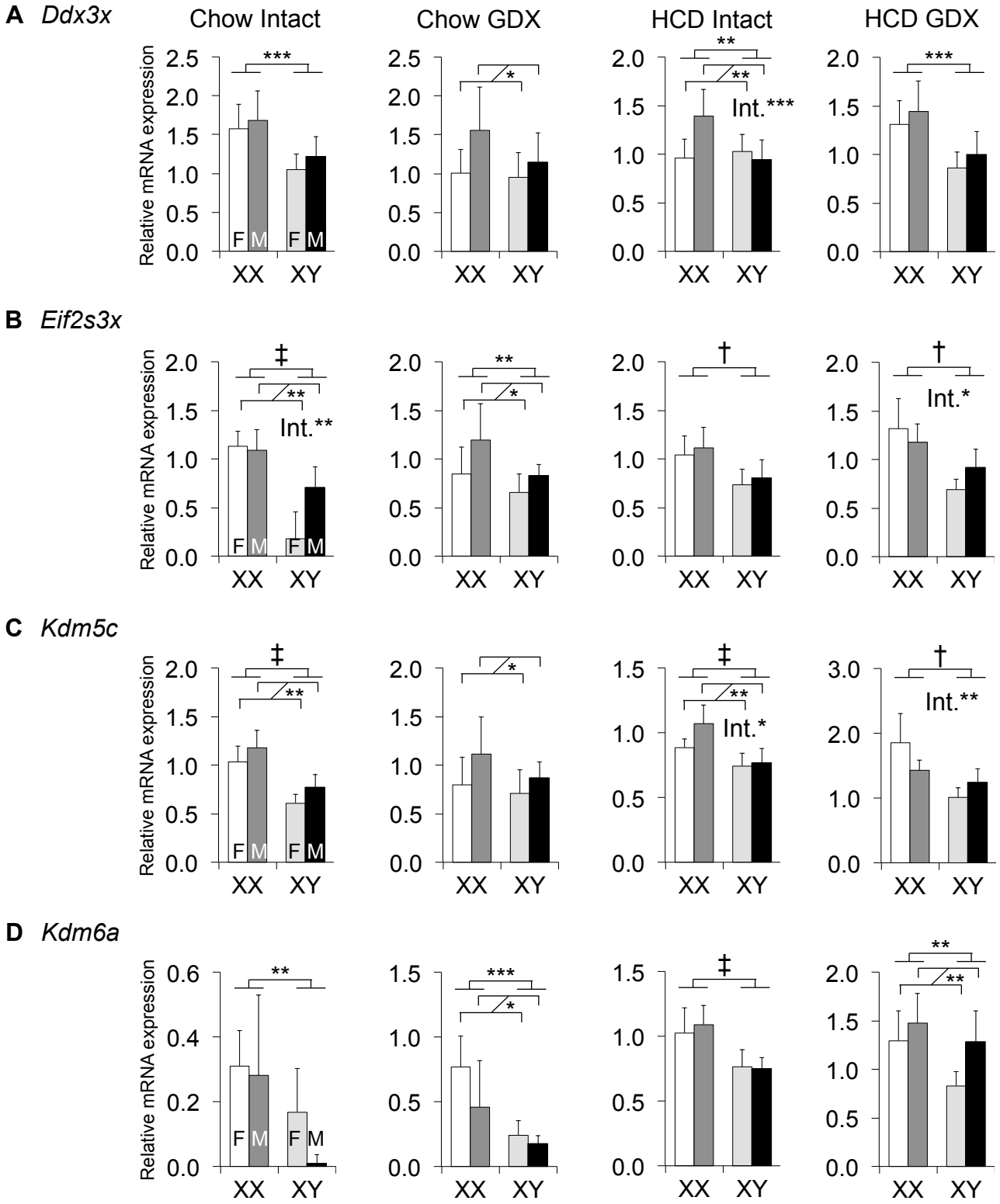
Supplemental Figure III. Components of lipoprotein synthesis, remodeling, and uptake are not associated with plasma HDL cholesterol levels. Relative mRNA expression was measured by quantitative PCR. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$; †, $P \leq 0.0001$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure IV



Supplemental Figure IV. Key enzymes of bile acid synthesis do not explain sex differences in plasma cholesterol levels. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure V



Supplemental Figure V. Genes escaping X-inactivation are consistent with XX–XY differences in HDL cholesterol levels. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$; †, $P \leq 0.0001$; ‡, $P \leq 0.000001$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

SUPPLEMENTAL MATERIAL

The presence of XX versus XY sex chromosomes is associated with increased HDL cholesterol levels in the mouse

Jenny C. Link, Xuqi Chen, Christopher Prien, Mark S. Borja, Bradley Hammerson, Michael N. Oda, Arthur P. Arnold, and Karen Reue

MATERIALS AND METHODS

Mice

Four Core Genotypes (FCG) C57BL/6 mice were bred and genotyped as described previously.[1] Briefly, XX female mice were mated with XY–(Sry+) male mice to generate XX, XX(Sry+), XY–, and XY–(Sry+) offspring, and genotyping was performed by PCR to detect presence of the Sry transgene (forward: AGCCCTACAGCCACATGATA; reverse: GTCTTGCCTGTATGTGATGG) and Y-chromosome–specific sequence (forward: CTGGAGCTCTACAGTGATGA; reverse: CAGTTACCAATCAACACATCAC).[1] Where indicated, gonadectomy was performed at 75 days of age, as previously described.[1] XY* mice, backcrossed to strain C57BL/6EiJ for >10 generations, were bred as described previously.[1,2] XY* males have the Y* chromosome that recombines aberrantly with the X chromosome. Mating XY* males with XX females generates the three genotypes included in this study: XX, XX^{Y*}, and XY*, which are similar to XX, XXY, and XY mice, respectively. Progeny of XY* mice were gonadectomized at 75 days of age, as previously described.[1]

Gonadal males and females were housed in separate cages and maintained at 23°C with a 12:12 hour light:dark cycle. All mice were initially fed Purina mouse chow diet containing 5% fat (Purina 5001; PMI Nutrition International, St. Louis, MO). Where specified, mice were fed a chow diet until age 3.5 months of age (4 weeks after gonadectomy), and then fed an atherogenic diet for 16 weeks (diet TG90221 containing 7.5% cocoa butter, 1.25% cholesterol, 0.5% sodium cholate; Teklad Research Diets, Madison, WI).

For all studies, blood samples were obtained at 7.5 months of age (FCG mice) or 14 months of age (XY* mice) after fasting 0800–1300. Plasma was collected after centrifugation of whole blood at 3,400 x g for 10 minutes at 4°C. Mouse studies were conducted in accordance with and approved by the Institutional Animal Research Committee of the University of California, Los Angeles.

Measurement of plasma lipid

Total cholesterol, HDL cholesterol, free cholesterol, triglycerides, and free fatty acid levels were determined by enzymatic colorimetric assays.[3] Combined LDL cholesterol and VLDL cholesterol concentration was determined by subtracting HDL cholesterol values from total cholesterol values. Lipoproteins were fractionated from 150 µL of plasma pooled from 3 mice of each genotype by fast protein liquid chromatography at the Mouse Metabolic Phenotyping Center (Vanderbilt University, Nashville, TN).

HDL-ApoA-I Exchange Assay

The HDL-ApoA-I exchange assay was performed on freshly thawed plasma using site-directed spin-label electron paramagnetic resonance (EPR) as described by Borja

et al.[4] Briefly, plasma samples (in triplicate) were diluted by a factor of 4 in PBS and PEG 6000 was added to a final concentration of 4%. ApoB-containing lipoproteins were removed by centrifugation (13,000 rpm, 10 min, 4°C), and clarified plasma was combined with spin-labeled apoA-I. EPR measurements were performed on each sample at 6°C and again after 15 min at 37°C using a Bruker eScan EPR spectrometer with temperature controller (Noxygen). HDL-apoA-I exchange activity was defined as the value obtained at 6°C (normalized to an internal standard) from value obtained at 37°C (normalized to same internal standard) followed by subtracting the baseline spectra for spin-labeled apoA-I in PBS. Additional calculations were performed as described.[4]

Immunoblotting

Plasma aliquots (0.5 uL) were fractionated by SDS-PAGE in a 4-20% Tris-glycine gel and transferred onto a nitrocellulose membrane. Rabbit anti-mouse antibodies against ApoA-I, ApoA-II, and ApoA-IV were described previously[5,6] and used at 1:4000 dilution. Rabbit anti-mouse antibody against ApoE (Cat. K23100R, Meridian Life Science, Memphis, TN) was used at 1:2000. A mouse monoclonal antibody against ApoB[7] was used at 1:1000. HRP-conjugated rabbit anti-mouse antibody against IgG or HRP-conjugated goat anti-rabbit antibody against IgG was used at 1:10,000 (Cat. Sc-2030, Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescence (ECL2, Cat. 80196, Thermo Fisher, Rockford, IL) was detected using ChemiDoc XRS+ and quantified by ImageLab 4.0.1 (Bio-Rad, Hercules, CA).

Quantitative RT-PCR

Mouse livers were dissected, flash frozen in liquid nitrogen, and stored at –80°C. RNA was isolated from tissues using Ribozol (Cat. N580, Amresco, Solon, OH). First strand cDNA was generated by reverse transcription with iScript (Cat. 170-8840, Bio-Rad). Quantitative RT-PCR was performed with a Bio-Rad CFX Connect Real-Time PCR Detection System using SsoAdvanced SYBR Green Supermix (Bio-Rad). β2 microglobulin and TATA box-binding protein mRNA were amplified in each sample as normalization controls. All primer sequences are shown in Supplemental Table II.

Statistical Analysis

Groups were compared using two-way ANOVA (NCSS 2001; Number Cruncher Statistical Systems, Kaysville, UT) with main factors of sex (gonadal male vs. gonadal female) and sex chromosome complement (XX vs. XY). In the XY* study, the three groups were compared using one-way ANOVA with Duncan's multiple comparison test. Statistically significant comparisons or interactions are presented ($p<0.05$). All error bars represent one standard deviation.

Methods References

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