

PAPER

Estrogen receptor α -mediated adiposity-lowering and hypocholesterolemic actions of the selective estrogen receptor modulator acolbifene

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OBJECTIVE: The selective estrogen receptor (ER) modulator (SERM) acolbifene (ACOL), a potent and pure antiestrogen in the mammary gland and uterus, exerts beneficial pro-estrogenic actions on energy balance, insulin sensitivity and lipid metabolism. ACOL binds ERs α and β , both of which have been involved in the metabolic actions of estrogen. This study aimed at determining the identity of the ER involved in the beneficial metabolic actions of ACOL.

DESIGN AND MEASUREMENTS: ACOL was administered for 4 weeks to male and female wild-type and ER α knockout (KO) mice, and indices of energy balance as well as plasma and liver lipid concentrations were determined.

RESULTS: ER α KO mice were heavier, gained more fat mass and had larger adipose depots than their wild-type counterparts. In both genders, ACOL decreased fat gain (50%) and white adipose tissue mass in male and female wild-type, but not in ER α KO mice. ACOL reduced plasma cholesterol in female wild-type mice (–27%), whereas the compound remained ineffective in their ER α KO counterparts. Plasma triglycerides were unaffected by ACOL. Finally, ACOL decreased liver cholesterol and triglyceride concentrations only in wild-type female animals.

CONCLUSION: The beneficial metabolic actions of the SERM ACOL on adiposity and on plasma and liver lipids are entirely due to its interaction with the ER α .

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Introduction

Loss of estrogen is largely recognized as the principal cause of the increased risk for atherosclerosis and osteoporosis associated with menopause.^{1,2} Increased weight gain seen at menopause undoubtedly contributes to the increase in cardiovascular risk, as is the case in men throughout life.^{3,4} The plasma lipoprotein profile constitutes another important cardiovascular risk factor,⁵ and menopause is associated, among other changes, with a shift in cholesterol lipoprotein distribution toward low-density lipoproteins (LDL), away from high-density lipoproteins. Estrogen is well known to modulate adipose tissue and lipid metabolism in women, but

it remains unclear as to which of the α (ER α) or β (ER β) estrogen receptor (ER) is involved, or whether both receptors contribute to such modulation.

The study of mice invalidated for the ER α (ER α knockout (KO)), ER β (BERKO) or both (DERKO) genes has increased our understanding of the modulation of body weight and fat deposition by estrogen. Invalidation of the ER α gene increases body weight through increased adipose tissue deposition and increased food efficiency without apparent changes in food intake.⁶ Mice with inactivation of the aromatase gene (ArKO), the enzyme that favors the synthesis of estrogen, also confirm the involvement of estrogen in fat deposition.⁷ However, the implication of ER β in energy balance and lipid metabolism remains unclear. Some studies suggest that it has effects opposite to those of ER α ,⁸ whereas, conversely, others support its involvement in the anorectic action of estrogen.⁹

The changes in energy balance and lipid metabolism in women are neutralized to a certain extent by hormone

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replacement therapy (HRT).¹⁰ However, the recent findings of the Women's Health Initiative study showing that HRT does not protect women against cardiovascular disease,¹¹ along with the increased risk of breast cancer associated with HRT,^{12,13} emphasize the need for the development of alternative therapies to prevent the undesirable consequences of menopause. Selective estrogen receptor modulators (SERMs), which reduce the risk of estrogen-related cancers but at the same time display estrogen-like actions in other metabolic pathways, constitute a promising avenue for such alternative therapies.¹⁴

We have previously demonstrated beneficial changes in body weight and the lipid profile of female rats following chronic treatment with the fourth-generation SERM Acolbifene (ACOL).^{15,16} The drug was shown in rats to decrease greatly body weight gain, mostly fat mass, mainly through a reduction in food intake. ACOL also increased insulin sensitivity and brought about a major decrease in plasma cholesterol.^{15,16} The SERM binds to both ER α and ER β with much higher affinity than estrogen.¹⁴ Given this and the above-mentioned uncertainties that remain regarding the respective involvement of ER α and ER β in the modulation of energy balance and lipid metabolism, the present study was designed to determine whether the metabolic actions of ACOL were solely due to its interaction with the ERs, and if so, to establish the identity of the receptor. To this end, mice invalidated for the ER α receptor and their wild-type counterparts were treated with ACOL for 4 weeks, and variables of energy and lipid metabolism were assessed. A loss of action of ACOL in ER α KO mice would identify the ER α as the major intermediate of its metabolic actions, whereas their total or partial maintenance would indicate the involvement of either ER β or ER-independent, pleiotropic pathways.

Materials and methods

Animals and treatments

A total of 72 mice from two strains were used. The first strain, with a C57/B6 genetic background,¹⁷ was a generous gift from Dr Dennis Lubahn (University of Missouri, Columbia, MO, USA) to V Giguère. The second strain, with a 129SV/B6 background,¹⁸ was kindly provided by Dr Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP/Collège de France, Illkirch, France) to F Labrie. It is well known that the genetic background of mice can significantly influence metabolism. However, in terms of the end points of interest of the present study, both cohorts of mice displayed identical phenotypic characteristics and responses to treatment, and were therefore pooled for data analysis. In all, 19 wild-type and 20 ER α KO male mice, and 17 wild-type and 17 female ER α KO mice approximately 5 months old were housed individually in a room kept at 23 ± 1°C with a 12:12 h light–dark cycle (lights on at 0700). The animals were cared for and handled in

conformity with the Canadian Guide for Care and Use of Laboratory Animals and the protocol was approved by the institutional animal care committee. The animals were acclimated to their environment for 1 week and had *ad libitum* access to tap water and a nonpurified rodent diet (Charles River Rodent Diet #5075, Ralston Products, Woodstock, Canada). They were then fed *ad libitum* a purified high carbohydrate diet that contained, in g/100 g diet: starch, 31.2; dextrose, 31.2; corn oil, 6.4; casein, 20.0; DL-methionine, 0.3; vitamin mix (Teklad no 40060, Teklad Test Diets, Madison, WI, USA), 1.0; mineral mix (AIN-76, ICN Biochemicals, Aurora, OH, USA), 4.9; and fiber (Alphacel, ICN Biochemicals), 5.0. The diet provided 64.9% of energy as carbohydrate, 14.5% as fat, 20.6% as protein, with an energy density of 16.76 kJ/g. Mice were randomly divided into eight groups according to gender (male and female), genotype (wild type and ER α KO), and drug treatment (placebo and ACOL), with eight to 10 animals per group. As described previously,¹⁶ ACOL (EM-652.HCL) was given once daily at 0800 by oral gavage in a methylcellulose suspension at a dose of 2.5 mg/kg/day in a total volume of 0.2 ml. The control group received the same volume of vehicle alone. Mice were treated for a total of 28 days.

Body weight, body weight gain, body composition, food intake and food efficiency

Food intake and body weight were monitored every other day. At the end of the experiment, the animals were allowed access to food until 0800, at which time the last dose of ACOL or vehicle was given. Food was then removed, and mice were killed 6 h later. At the beginning and end of the experiment, body composition (fat and lean mass) and bone mass density (BMD) were determined by dual X-ray absorptiometry (DEXA, PIXImus, GE Lunar Corporation, Madison, WI, USA) under halothane anesthesia. The difference between initial and final composition was computed, and data are presented as mass gain over the 28-day experimental period. Food efficiency was calculated as g body weight gain per 100 g food ingested and is expressed as %.

Blood and tissue collection

At the time of killing, mice were anesthetized with ketamine–xylazine, blood was collected by cardiac puncture into an EDTA-containing syringe, and was then centrifuged at 1500 × g, 4°C for 15 min. Plasma was stored at –20°C for later biochemical measurements. Retroperitoneal and inguinal white adipose tissues, taken as being representative of visceral and subcutaneous fat, respectively, interscapular brown adipose tissue (BAT), the soleus and red vastus lateralis muscle (VLM) were excised and weighed. A sample of liver was also excised and immediately frozen in liquid nitrogen.

Plasma/tissue measurements

Plasma total cholesterol was quantified using a reagent kit from Boehringer Mannheim (Montreal, Quebec, Canada). Plasma triglycerides were assayed by an enzymatic method using a reagent kit from Boehringer Mannheim, which allowed correction for free glycerol. After thawing of the frozen liver samples, total lipids were extracted according to the method of Folch *et al*¹⁹ and solubilized in isopropanol. Cholesterol and triglycerides were quantified in the liver lipid extracts using the above-mentioned reagent kits.

Statistical analysis

Data are expressed as means \pm s.e.m. Some variables were log transformed before analysis to ensure homogeneity of variance. Main and interactive treatment effects were

analyzed separately for each gender using 2×2 factorial analysis of variance (ANOVA). The factors were Genotype with two levels (Wild type, ER α KO) and Drug treatment with two levels (Placebo, ACOL). Pairwise mean comparisons were performed to locate individual between-group differences using *post hoc* Fisher's protected least significant difference (PLSD) test. Differences were considered statistically significant at $P < 0.05$.

Results

The effect of the invalidation of the ER α gene and of ACOL treatment on body weight and cumulative weight gain are depicted in Figure 1. Body weight did not increase much during the course of treatment, as expected in the 5-month-old mice. In female mice, KO of the ER α gene resulted in an

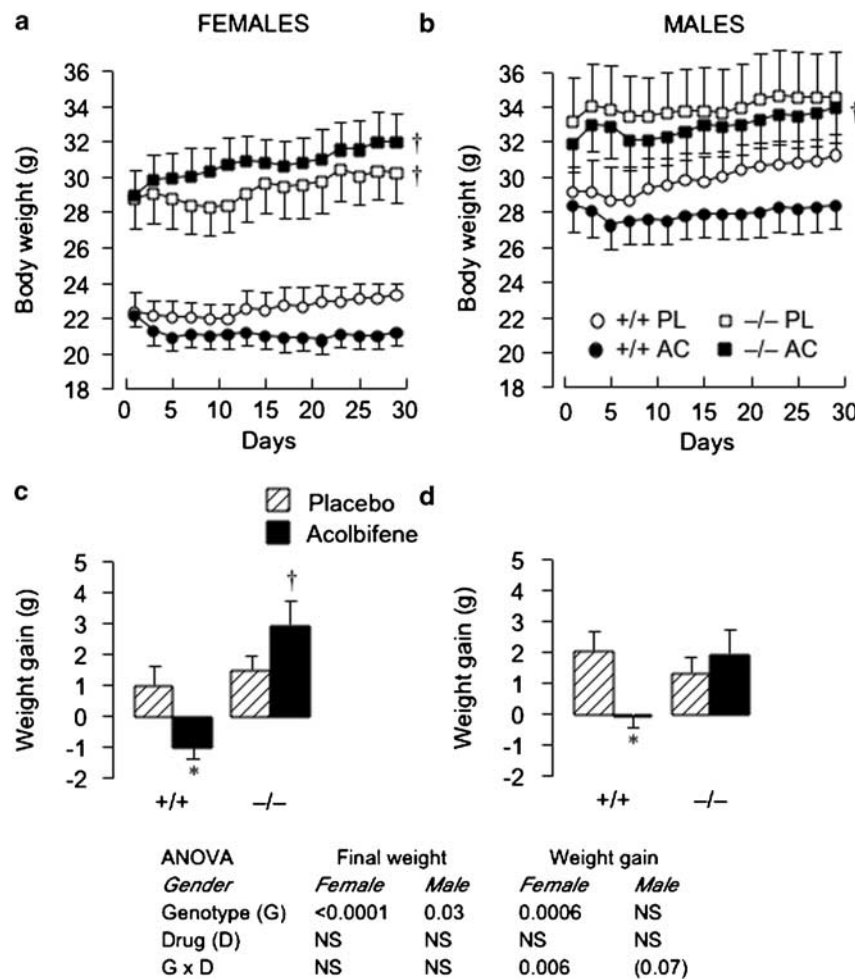


Figure 1 Body weight (a, b) and cumulative body weight gain (c, d) in female (a, c) and male (b, d) wild-type (+/+) and ER α KO mice (-/-) treated or not with ACOL for 4 weeks. Points and bars represent the mean \pm s.e.m. of eight to 10 animals. The ANOVA table depicts the level of significance of the main effects of genotype (g, with two levels: +/+ and -/-) and drug (d, with two levels: Placebo, ACOL), and their interaction (g \times d) determined by 2×2 factorial ANOVA for each gender. *Post hoc* Fisher's PLSD test was applied to localize individual pairwise between-group differences: *different from Placebo group within same genotype; †different from wild-type (+/+) group subjected to same drug treatment ($P < 0.05$). In panels a and b, symbol applies to day 29.

increased body weight (Figure 1a), the mice weighing nearly 7 g (29%) more than their wild-type counterparts of the same age at the inception of drug treatment. Final body weight remained significantly higher in KO than in wild-type mice regardless of drug treatment. ACOL tended to reduce body weight in wild-type mice, but not in the ER α KO animals, and therefore slightly amplified the genotype-related difference in body weight. In male mice, ER α KO animals weighed over 3 g (11%) more than wild-type mice, but the difference reached significance only in ACOL-treated mice (Figure 1b). In the female cohort, genotype and drug treatment interacted significantly on body weight gain over the 28 days of ACOL administration (Figure 1c). The interaction came from the fact that, whereas ACOL reduced body weight gain in $+/+$ mice, the drug tended to exert an opposite action in $-/-$ animals, such that weight gain was significantly greater in ACOL-treated $-/-$ than wild-type mice. As depicted in Figure 1a, ACOL treatment began to reduce weight gain in wild-type mice early on, the effect reaching significance at day 25 until the end of treatment. In the male cohort (Figure 1d), ACOL prevented body weight gain in wild-type, but not in ER α KO mice.

The effects of genotype and ACOL treatment on cumulative food intake, food efficiency and BMD are depicted in Figure 2. In female mice, genotype and ACOL interacted on cumulative food intake (Figure 2a). The interaction came from the fact that, whereas genotype *per se* did not influence food intake, ACOL tended to slightly reduce food consumption in $+/+$ mice but increased the latter in ER α KO mice. Detailed analysis of food intake patterns (data not shown) revealed that ACOL decreased food intake in wild-type female mice only in the first few days of treatment (corresponding to the phase during which body weights became slightly divergent), and that it increased food intake in ER α KO mice mostly during the first half of the treatment period (also corresponding to the greatest difference in body weight). In male mice, no difference in food intake related to the experimental conditions was observed (Figure 2b). In female mice, genotype and drug treatment interacted significantly on food efficiency (Figure 2c). Indeed, whereas ACOL reduced food efficiency in $+/+$ mice, the drug tended to exert an opposite action in ER α KO animals, such that food efficiency was significantly greater in ACOL-treated ER α KO than wild-type mice. In male mice (Figure 2d), the pattern of effects of genotype and ACOL on food efficiency was identical to that seen in females. In female mice, ER α gene invalidation prevented the increase in BMD observed over the course of the 28-day experimental period (Figure 2e), whereas ACOL treatment did not affect the latter. In male mice, no significant effect of genotype and drug treatment on BMD was seen (Figure 2f).

The treatment effects on body weight gain were closely reflected in those on fat mass gain and adipose depot weights (Figure 3). In fact, body weight gain was largely explained by fat gain, as evidenced by their strong correlation (females: $r=0.78$, $P<0.0001$, $n=34$; males: $r=0.87$, $P<0.0001$,

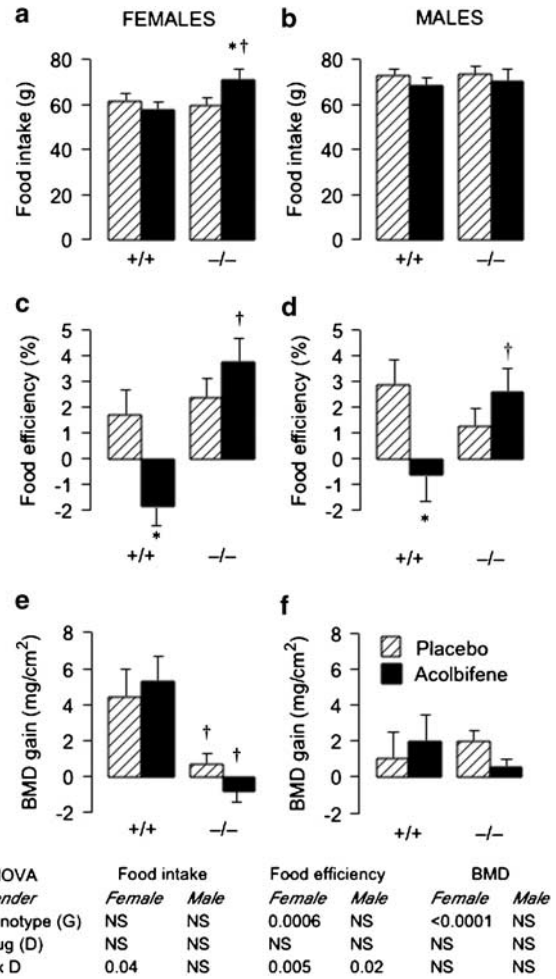


Figure 2 Cumulative food intake (a, b), food efficiency (c, d) and 28-day change in BMD (e, f) in female (a, c, e) and male (b, d, f) wild-type ($+/+$) and ER α KO mice ($-/-$) treated or not with ACOL for 4 weeks. See legend to Figure 1 for significance of ANOVA table. Bars represent the mean \pm s.e.m. of eight to 10 animals. *Different from Placebo group within same genotype; †different from wild-type ($+/+$) group subjected to same drug treatment ($P<0.05$).

$n=37$). In female mice, ER α KO animals displayed higher fat mass gain than their wild-type counterparts over the 28-day treatment period (Figure 3a), as well as larger inguinal (Figure 3c) and retroperitoneal (Figure 3d) adipose depot weights. In wild-type females, ACOL reduced all three indices of adiposity to roughly half of the values seen in untreated mice, whereas the drug remained completely ineffective in altering fat gain and depot weights in ER α KO animals. Male mice displayed more modest changes in response to ER α invalidation and ACOL treatment compared with female mice. Indeed, in untreated male mice, the absence of ER α did not affect fat mass gain over the treatment period (Figure 3b), but had an overall increasing effect on inguinal (Figure 3d) and epididymal (Figure 3f) fat

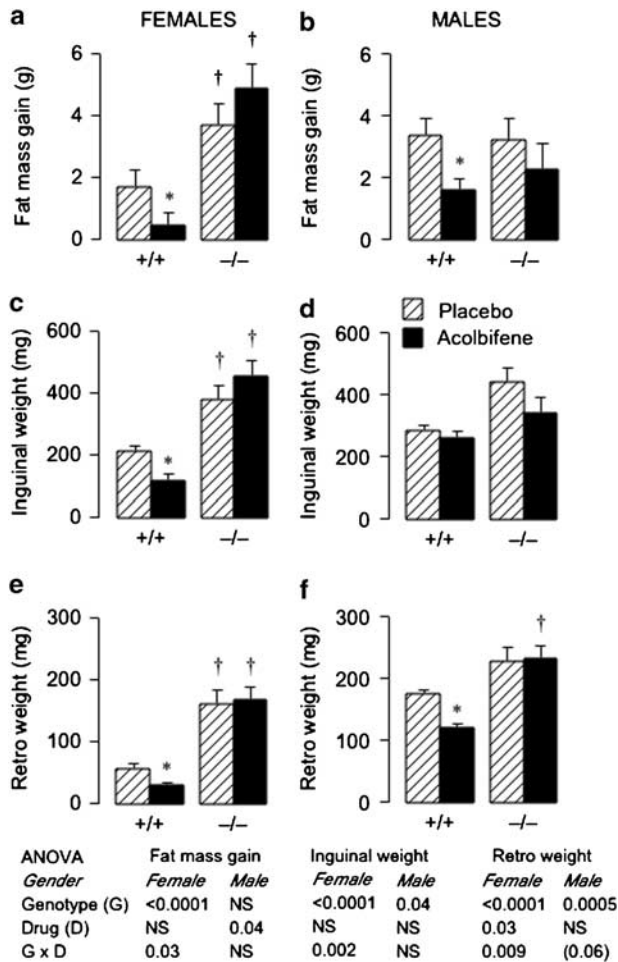


Figure 3 Total fat mass gain (28 days) (a, b), and final inguinal (c, d) and retroperitoneal (Retro) fat pad weight (e, f) in female (a, c, e) and male (b, d, f) wild-type (+/+) and ER α KO mice (-/-) treated or not with ACOL for 4 weeks. See legend to Figure 1 for significance of ANOVA table. Bars represent the mean \pm s.e.m. of eight to 10 animals. *Different from Placebo group within same genotype; †different from wild-type (+/+) group subjected to same drug treatment ($P < 0.05$).

Table 1 Weight of BAT, VLM and gastrocnemius muscle in female and male wild-type (+/+) and ER α KO mice (-/-) treated for 4 weeks with vehicle (Placebo) or ACOL

	+/+		-/-		ANOVA		
	Placebo	ACOL	Placebo	ACOL	G	D	G x D
Females							
BAT (mg)	66 \pm 5	65 \pm 8	130 \pm 18*	134 \pm 15*	<0.0001	NS	NS
VLM (mg)	70 \pm 6	68 \pm 3	66 \pm 6	81 \pm 5	NS	NS	NS
Gastrocnemius muscle (mg)	64 \pm 9	73 \pm 9	68 \pm 9	61 \pm 8	NS	NS	NS
Males							
BAT (mg)	125 \pm 8	111 \pm 12	187 \pm 26	205 \pm 41*	0.003	NS	NS
VLM (mg)	86 \pm 6	71 \pm 7	74 \pm 9	72 \pm 8	NS	NS	NS
Gastrocnemius muscle (mg)	79 \pm 10	69 \pm 8	72 \pm 4	67 \pm 7	NS	NS	NS

NS = not significant. Values are means \pm s.e.m. of eight to 10 animals. See legend to Figure 1 for significance of the ANOVA table. *Different from wild-type (+/+) group subjected to same drug treatment ($P < 0.05$) as determined by *post hoc* Fisher's PLSD test.

pad weights, as determined by ANOVA analysis, relative to their wild-type counterparts. ACOL reduced fat mass gain in wild-type, but not in ER α KO male mice (Figure 3d), an effect that was reflected in lower mass of the wild-type retroperitoneal (Figure 3f), but not the inguinal depot (Figure 3e). Differences between absolute body weight gain and fat mass gain were explained by the slight decrease in total nonfat (lean + water) mass (not shown) that occurred in all groups over the treatment period.

In female mice, BAT became larger in ER α KO animals compared with wild-type animals (Table 1), such effect, as determined visually, being apparently due to increased fat deposition. ACOL did not affect BAT mass. In male mice, similar trends were observed, except that the difference in BAT weight between untreated wild-type and ER α KO mice did not reach significance ($P = 0.08$). The weights of representative skeletal muscles were also determined, and in both female and male mice, they were found not to be notably influenced by either genotype or drug treatment (Table 1).

As shown in Figure 4a, in female mice, ER α invalidation resulted in a significant hypercholesterolemia. ACOL treatment decreased plasma cholesterol by 28% in wild-type mice, but remained without effect in ER α KO animals (genotype-drug interaction). In male mice (Figure 4b), ER α invalidation tended ($P = 0.06$) to increase cholesterolemia, whereas ACOL displayed a weak tendency to reduce the latter. In female mice, triglyceridemia was also elevated in ER α KO mice compared with wild-type animals (Figure 4c), and ACOL remained without effect. In male mice (Figure 4d), neither the genotype nor the drug impacted triglyceridemia.

Liver lipids impact lipoprotein assembly and secretion and were therefore quantified. As shown in Figure 5a, in female mice, ER α invalidation did not affect liver cholesterol concentration. The latter was significantly decreased by ACOL in +/+, but not in -/- mice (genotype-drug interaction). In male mice, ER α invalidation did increase

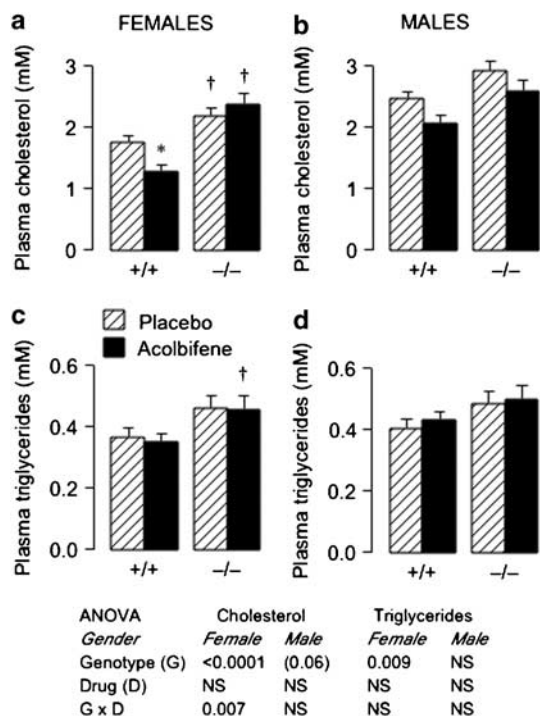


Figure 4 Plasma total cholesterol (a, b) and triglycerides (c, d) in female (a, c) and male (b, d) wild-type (+/+) and ER α KO mice (-/-) treated or not with ACOL for 4 weeks. See legend to Figure 1 for significance of ANOVA table. Bars represent the mean \pm s.e.m. of eight to 10 animals. *Different from Placebo group within same genotype; †different from wild-type (+/+) group subjected to same drug treatment ($P < 0.05$).

liver cholesterol concentration, and ACOL remained without effect in either genotype (Figure 5b). The determination of the activity of major enzymes of hepatic cholesterol synthesis, hydroxymethylglutaryl coenzyme A (HMGCoA) reductase, and of intracellular storage, acylCoA:cholesterol acyltransferase (ACAT), established that genotype and ACOL did not impact liver and plasma cholesterol concentrations through modulation of these enzymes (data not shown). In female mice, liver triglyceride concentration was higher in -/- than in +/+ animals, but such genotype-related difference was mainly due to the fact that ACOL reduced liver triglycerides in +/+, but not in -/- mice (genotype-drug interaction, Figure 5c). In male mice, the increase in liver triglycerides associated with ER α invalidation was particularly marked (~3-fold). The tendency for ACOL to decrease liver triglycerides did not reach significance.

Discussion

The objective of this study was to determine the identity of the ER involved in the beneficial metabolic actions of the SERM ACOL. To this end, the drug was administered for 4 weeks to wild-type and ER α KO mice, and indices of energy

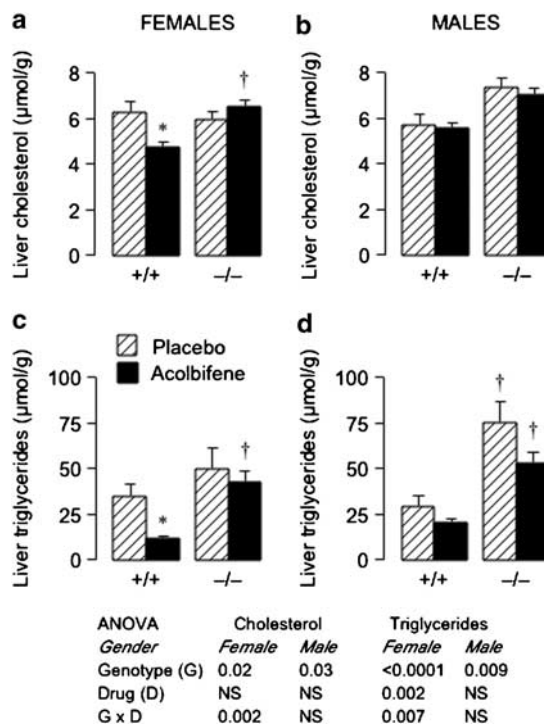


Figure 5 Liver cholesterol (a, b) and triglyceride concentration (c, d) in female (a, c) and male (b, d) wild-type (+/+) and ER α KO mice (-/-) treated or not with ACOL for 4 weeks. See legend to Figure 1 for significance of ANOVA table. Bars represent the mean \pm s.e.m. of eight to 10 animals. *Different from Placebo group within same genotype; †different from wild-type (+/+) group subjected to same drug treatment ($P < 0.05$).

balance as well as plasma and liver lipid concentrations were determined. The present data demonstrate that the marked ACOL-induced reduction in body fat accretion and cholesterolemia are entirely explained by its interaction with the ER α .

Both female and male ER α KO mice displayed an increased body weight compared with their wild-type counterparts. The increase in body weight was associated with an increased fat mass, in accordance with the ER α KO phenotype described in previous studies.^{6,20} The mice used here were 5 months old at the beginning of the study and they did not gain much weight (1–2 g) during the 28-day study period. At the onset of the study, the ER α KO mice already weighed more than the wild-type animals and, in accordance with other studies,^{6,7,20} were therefore well within the weight differentiation phase during which KO mice display higher body weight than the wild-type animals. Such phase is initiated at 3 months of age and is maximal between puberty and adulthood (4–12 months).

Treatment of +/+ mice with ACOL prevented weight gain and its associated fat accumulation. This estrogen-like effect of the SERM confirms previous findings in intact and ovariectomized rats, in which ACOL treatment was shown to reduce markedly weight (mainly fat) accretion.^{14–16} In both

female and male ER α KO mice, ACOL was unable to prevent weight and fat gain, and even tended to increase weight gain in female mice. This finding establishes that the lowering action of ACOL on weight gain is mediated via an interaction with the ER α .

Although a direct role of the ER β in the regulation of energy balance remains unclear, it is not excluded that the receptor is somehow involved in the modulation of body weight. In studies of ER α , ER β (BERKO) and double (DERKO) KO models by Ohlsson *et al*,²⁰ fat mass was found to be decreased in BERKO compared with wild-type mice, which is in clear contrast with the effect of ER α invalidation. Furthermore, in studies of ovariectomized ER α KO, BERKO and DERKO mice, gonadal fat mass was found to be reduced by estrogen administration in wild-type and BERKO, but not in ER α KO and DERKO mice.²¹ Using ovariectomized ER α KO mice, Naaz *et al*⁸ concluded that the ER β may exert a lipogenic action in adipose tissue. The findings of the present study in female animals lend some support to such a proposition; in fact, in female ER α KO mice, interaction of ACOL with the ER β tended to slightly increase body weight and fat mass gain, although not significantly so. As in the case of natural estrogen, such a lipogenic effect of ACOL through the ER β would clearly be overwhelmed in wild-type animals by the interaction of the SERM with ER α .

In rat models, the ACOL-induced reduction in fat accretion is associated with a frank reduction in food intake, with perhaps some contribution from decreased food efficiency.^{15,16} This anorectic action of ACOL is akin to that of natural estrogen, which has been shown in numerous studies to decrease food intake in rats.^{22,23} In the present study however, despite a transient initial reduction in food consumption, cumulative food intake of female and male wild-type mice was not greatly affected by ACOL treatment. The anorectic action of estrogen is less clearly established in the mouse than in the rat, and the available reports^{6,7,9,24,25} do not allow to conclude definitively regarding the regulation of food intake by estrogen and its receptors in the mouse. In the present study, the effect of ER α invalidation and ACOL treatment on food intake was rather mild, whereas their impact on body weight and fat accretion paralleled changes in food efficiency. The determinants of food efficiency that are involved remain unknown. However, ER α KO mice have been reported to display lower energy expenditure than wild-type mice,⁶ and the ArKO mouse model (without endogenous estrogen) displays decreased physical activity with no change in resting energy expenditure.⁷ Clearly, further studies specifically aimed at assessing determinants of energy balance are needed to unravel the precise mechanisms of action of ERs and of ACOL on energy balance in the mouse.

KO of the ER α increased plasma cholesterol in both male and female mice. This is in accordance with previous studies in ER α KO²⁰ and ArKO models,^{7,26} which showed that a deficit in estrogen or its α receptor increases cholesterolemia. Treatment with ACOL decreased cholesterolemia in female

wild-type mice while tending to do so in male mice. We have previously observed in the rat a very strong (~50%) cholesterol-lowering action of ACOL.^{14–16} The milder hypocholesterolemic action of ACOL in the present study is not surprising given that mice are generally more resistant to interventions aimed at reducing cholesterolemia.^{27,28} ACOL therefore shares the ability to decrease plasma cholesterol, an estrogen-like effect, with other SERMs such as tamoxifene,²⁹ raloxifene,³⁰ tibolone³¹ and miproxifene phosphate³² that have been studied in various animal models. Importantly, because ACOL remained totally ineffective in either female or male ER α KO mice, the present findings clearly establish that the hypocholesterolemic effect of ACOL is exerted through its interaction with the ER α .

The mechanisms by which SERMs in general, and ACOL in particular, modulate cholesterol metabolism remain to be defined. Estrogen is known to modulate several steps in cholesterol metabolism, including the activity of HMGCoA reductase, the rate-limiting enzyme in *de novo* cholesterol synthesis,^{33,34} the expression of the LDL receptor,^{35,36} as well as that of enzymes involved in liver cholesterol/bile acid excretion.²⁶ In the present study, HMGCoA reductase and ACAT activities were found not to be altered by invalidation of the ER α or by ACOL, pointing to pathways other than hepatic cholesterol synthesis and storage, such as increased lipoprotein or cholesterol uptake from the circulation and subsequent excretion. The involvement of these pathways in the ACOL-induced reduction in circulating cholesterol levels remains to be established experimentally. Regarding triglyceridemia, its increase by ER α invalidation may be related to the overall increase in adiposity and the stimulation of triglyceride synthesis and decrease in clearance that typically accompany obesity. The lack of effect of ACOL on hypertriglyceridemia associated with ER α invalidation is congruent with previous studies in rats.^{15,16}

Invalidation of the ER α gene appeared to have gender-specific effects on liver lipid concentrations. In female mice, the absence of ER α had no consequence on liver lipids, whereas, in male mice, it tended to increase liver cholesterol while markedly increasing liver triglycerides. Remarkably, ACOL reduced both cholesterol and triglyceride concentrations in the liver of wild-type female mice, whereas it remained without effect in male mice despite identical absolute lipid concentrations in both genders. Importantly, as in the case of fat accretion and cholesterolemia, the lipid-lowering action of ACOL in the liver of female mice was found to rely entirely upon its interaction with the ER α , as the drug remained ineffective in KO mice. It is intriguing to note that in intact mice, ACOL, through ER α -mediated mechanisms, impacted liver lipids in the one gender in which the latter were not altered by the absence of the receptor. This may be related to gender-specific differences in the degree of sensitivity of liver lipid metabolism to modulation by steroids.

Our earlier studies in rats^{15,16} have repeatedly shown that ACOL favors a mild cholesterol and triglyceride

accumulation in the liver of this species, in sharp contrast with the present study in mice. This species difference remains unexplained, particularly regarding triglycerides, but some possible explanations regarding cholesterol can be invoked. Firstly, the absence of a gallbladder in the rat, which exists in mice, may affect the enterohepatic circulation of cholesterol and bile acids and thereby impact liver cholesterol accumulation and excretion as well as their modulation by estrogen and SERMs. Secondly, although the estrogen-mediated modulation of hepatic CYP7A1 (or 7- α -hydroxylase), the rate-limiting enzyme in the conversion of cholesterol into bile acids, is similar in both rats and mice,^{26,37–39} species differences appear to exist in the modulation of the ATP-binding cassette (ABC)G5 and ABCG8 cholesterol transporters. These are members of the large ABC family of transmembrane proteins,⁴⁰ and were recently shown to be essential for cholesterol efflux from the liver to the bile canaliculus and from the intestine.⁴¹ The ABC transporters have also been implicated in mediating estrogen actions on cholesterol transport.^{42,43} In mice, although no direct assessment of the effects of estrogen on ABC transporters has yet been reported, one study²⁶ had suggested that ABCG5/G8 are not modulated by estrogen in mice. In contrast, in the rat, the well-known estrogen-induced increase in liver cholesterol content has been shown to be associated with decreased biliary cholesterol and liver ABCG5/G8 expression.⁴⁴ Therefore, it can reasonably be suggested that species-related differences in the impact of ACOL on liver cholesterol may be related to the species specificity of the modulation of ABCG5/G8 by steroids.

Several SERMs are well known to protect against bone loss related to the menopause-related deficit in estrogen, and a wide variety of studies in multiple species and conditions have demonstrated the efficacy of SERMs to reduce bone turnover, and to prevent bone or BMD loss.^{31,32,45,46} The effect of estrogen on BMD is ER α -dependent,²¹ and the ER α and ER β appear to exert opposing actions:⁴⁷ activation of the ER α promotes longitudinal bone growth, whereas that of the ER β represses it. In the present study, substantial changes in BMD over a 28-day period in 5-month-old mice were obviously not expected, but the data nevertheless point to interesting trends. Firstly, although invalidation of the ER α had no long-term effect on BMD in male mice, there was a much lower increase in BMD in KO female mice than in their wild-type counterparts during the experimental period. Secondly, ACOL tended to decrease slightly BMD gain in male and female ER α KO mice, in accordance with its binding to the ER β favoring repression of longitudinal bone growth. Although no positive effect of ACOL on BMD has been observed in wild-type mice in the present short-term study, the SERM is clearly established as one of the most potent SERMs *vis-à-vis* the prevention of bone loss.¹⁴

In conclusion, the present study demonstrates the beneficial metabolic effects of ACOL on adiposity and on plasma and liver cholesterol concentrations in a mouse model. These effects were more marked in female than in male wild-

type mice, possibly because of higher ER density in the former. In contrast, ACOL remained without beneficial effects with regard to energy balance and lipid metabolism in mice invalidated for the ER α gene, thus demonstrating that the SERM impacts the latter entirely through its interaction with ER α . The eventual demonstration that the beneficial metabolic actions of ACOL also occur in humans may broaden the therapeutic choices available for the prevention and treatment of some of the complications associated with menopause.

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References

- 1 Baker L, Meldrum KK, Wang M, Sankula R, Vanam R, Raiesdana A, Tsai B, Hile K, Brown JW, Meldrum DR. The role of estrogen in cardiovascular disease. *J Surg Res* 2003; 115: 325–344.
- 2 McClung MR. The menopause and HRT. Prevention and management of osteoporosis. *Best Pract Res Clin Endocrinol Metab* 2003; 17: 53–71.
- 3 Rosengren A, Wedel H, Wilhelmsen L. Body weight and weight gain during adult life in men in relation to coronary heart disease and mortality. A prospective population study. *Eur Heart J* 1999; 20: 269–277.
- 4 Willett WC, Manson JE, Stampfer MJ, Colditz GA, Rosner B, Speizer FE, Hennekens CH. Weight, weight change, and coronary heart disease in women. Risk within the 'normal' weight range. *JAMA* 1995; 273: 461–465.
- 5 Gohlke-Barwolf C. Coronary artery disease: is menopause a risk factor? *Basic Res Cardiol* 2000; 95 (Suppl 1): 177–183.
- 6 Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci USA* 2000; 97: 12729–12734.
- 7 Jones ME, Thorburn AW, Britt KL, Hewitt KN, Wreford NG, Proietto J, Oz OK, Leury BJ, Robertson KM, Yao S, Simpson ER. Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. *Proc Natl Acad Sci USA* 2000; 97: 12735–12740.
- 8 Naaz A, Zakroczymski M, Heine P, Taylor J, Saunders P, Lubahn D, Cooke PS. Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). *Horm Metab Res* 2002; 34: 758–763.
- 9 Liang YQ, Akishita M, Kim S, Ako J, Hashimoto M, Iijima K, Ohike Y, Watanabe T, Sudoh N, Toba K, Yoshizumi M, Ouchi Y. Estrogen receptor beta is involved in the anorectic action of estrogen. *Int J Obes Relat Metab Disord* 2002; 26: 1103–1109.
- 10 Godsland IF. Effects of postmenopausal hormone replacement therapy on lipid, lipoprotein, and apolipoprotein (a) concentrations: analysis of studies published from 1974–2000. *Fertil Steril* 2001; 75: 898–915.
- 11 McPherson R. Is hormone replacement therapy cardioprotective? Decision-making after the heart and estrogen/progestin replacement study. *Can J Cardiol* 2000; 16 (Suppl A): 14A–19A.
- 12 Jacobs HS. Postmenopausal hormone replacement therapy and breast cancer. *Medscape Womens Health* 2000; 5: E2.
- 13 Diamanti-Kandarakis E. Hormone replacement therapy and risk of malignancy. *Curr Opin Obstet Gynecol* 2004; 16: 73–78.

- 14 Labrie F, Labrie C, Bélanger A, Simard J, Gauthier S, Luu-The V, Mérand Y, Giguère V, Candas B, Luo S, Martel C, Singh SM, Fournier M, Coquet A, Richard V, Charbonneau R, Charpenet G, Tremblay A, Tremblay G, Cusan L, Veilleux R. EM-652 (SCH 57068), a third generation SERM acting as pure antiestrogen in the mammary gland and endometrium. *J Steroid Biochem Mol Biol* 1999; **69**: 51–84.
- 15 Picard F, Deshaies Y, Lalonde J, Samson P, Labrie C, Bélanger A, Labrie F, Richard D. Effects of the estrogen antagonist EM-652. HCl on energy balance and lipid metabolism in ovariectomized rats. *Int J Obes Relat Metab Disord* 2000; **24**: 830–840.
- 16 Lemieux C, Picard F, Labrie F, Richard D, Deshaies Y. The estrogen antagonist EM-652 and dehydroepiandrosterone prevent diet- and ovariectomy-induced obesity. *Obes Res* 2003; **11**: 477–490.
- 17 Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci USA* 1993; **90**: 11162–11166.
- 18 Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors α (ER α) and β (ER β) on mouse reproductive phenotypes. *Development* 2000; **127**: 4277–4291.
- 19 Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957; **226**: 497–509.
- 20 Ohlsson C, Hellberg N, Parini P, Vidal O, Bohlooly M, Rudling M, Lindberg MK, Warner M, Angelin B, Gustafsson JA. Obesity and disturbed lipoprotein profile in estrogen receptor- α -deficient male mice. *Biochem Biophys Res Commun* 2000; **278**: 640–645.
- 21 Lindberg MK, Weihua Z, Andersson N, Moverare S, Gao H, Vidal O, Erlandsson M, Windahl S, Andersson G, Lubahn DB, Carlsten H, Dahlman-Wright K, Gustafsson JA, Ohlsson C. Estrogen receptor specificity for the effects of estrogen in ovariectomized mice. *J Endocrinol* 2002; **174**: 167–178.
- 22 Geary N. Estradiol, CCK and satiation. *Peptides* 2001; **22**: 1251–1263.
- 23 Roy EJ, Wade GN. Role of food intake in estradiol-induced body weight changes in female rats. *Horm Behav* 1977; **8**: 265–274.
- 24 Geary N, Asarian L, Korach KS, Pfaff DW, Ogawa S. Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER- α null mice. *Endocrinology* 2001; **142**: 4751–4757.
- 25 Brown NM, Setchell KD. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. *Lab Invest* 2001; **81**: 735–747.
- 26 Hewitt KN, Boon WC, Murata Y, Jones ME, Simpson ER. The aromatase knockout mouse presents with a sexually dimorphic disruption to cholesterol homeostasis. *Endocrinology* 2003; **144**: 3895–3903.
- 27 Beynen AC, Meijer GW, Van der Meer R. Comparison of rats with mice concerning the response of lipid metabolism to dietary fats. *Z Ernahrungswiss* 1988; **27**: 143–149.
- 28 McGill Jr HC, Kushwaha RS. Individuality of lipemic responses to diet. *Can J Cardiol* 1995; **11** (Suppl G): 15G–27G.
- 29 Reckless J, Metcalfe JC, Grainger DJ. Tamoxifen decreases cholesterol sevenfold and abolishes lipid lesion development in apolipoprotein E knockout mice. *Circulation* 1997; **95**: 1542–1548.
- 30 Sbarouni E, Flevari P, Kroupis C, Kyriakides ZS, Koniavitou K, Kremastinos DT. The effects of raloxifene and simvastatin on plasma lipids and endothelium. *Cardiovasc Drugs Ther* 2003; **17**: 319–323.
- 31 Clarkson TB, Anthony MS, Wagner JD. A comparison of tibolone and conjugated equine estrogens effects on coronary artery atherosclerosis and bone density of postmenopausal monkeys. *J Clin Endocrinol Metab* 2001; **86**: 5396–5404.
- 32 Shibata J, Toko T, Saito H, Lykkesfeldt AE, Fujioka A, Sato K, Hashimoto A, Wierzba K, Yamada Y. Estrogen agonistic/antagonistic effects of miproxifene phosphate (TAT-59). *Cancer Chemother Pharmacol* 2000; **45**: 133–141.
- 33 Di Croce L, Bruscalupi G, Trentalance A. Independent behavior of rat liver LDL receptor and HMGCoA reductase under estrogen treatment. *Biochem Biophys Res Commun* 1996; **224**: 345–350.
- 34 Parini P, Angelin B, Stavreus-Evers A, Freyschuss B, Eriksson H, Rudling M. Biphasic effects of the natural estrogen 17 β -estradiol on hepatic cholesterol metabolism in intact female rats. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1817–1823.
- 35 Kovanen PT, Brown MS, Goldstein JL. Increased binding of low density lipoprotein to liver membranes from rats treated with 17 α -ethinyl estradiol. *J Biol Chem* 1979; **254**: 11367–11373.
- 36 Windler EE, Kovanen PT, Chao YS, Brown MS, Havel RJ, Goldstein JL. The estradiol-stimulated lipoprotein receptor of rat liver. A binding site that membrane mediates the uptake of rat lipoproteins containing apoproteins B and E. *J Biol Chem* 1980; **255**: 10464–10471.
- 37 Lai K, Harnish DC, Evans MJ. Estrogen receptor α regulates expression of the orphan receptor small heterodimer partner. *J Biol Chem* 2003; **278**: 36418–36429.
- 38 Xu G, Pan LX, Li H, Shang Q, Honda A, Shefer S, Bollineni J, Matsuzaki Y, Tint GS, Salen G. Dietary cholesterol stimulates CYP7A1 in rats because farnesoid X receptor is not activated. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G730–G735.
- 39 Bravo E, Cantafora A, Cicchini C, Avella M, Botham KM. The influence of estrogen on hepatic cholesterol metabolism and biliary lipid secretion in rats fed fish oil. *Biochim Biophys Acta* 1999; **1437**: 367–377.
- 40 Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 2001; **11**: 1156–1166.
- 41 Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC, Hobbs HH. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest* 2002; **110**: 671–680.
- 42 Srivastava RA. Estrogen-induced regulation of the ATP-binding cassette transporter A1 (ABCA1) in mice: a possible mechanism of atheroprotection by estrogen. *Mol Cell Biochem* 2002; **240**: 67–73.
- 43 Tran DQ, Jin L, Chen J, McLachlan JA, Arnold SF. Evaluation of clinical and environmental anti-estrogens with human estrogen receptor expressed in *Saccharomyces cerevisiae*: a novel role for ABC-cassette transporters in mediating anti-estrogenic activity. *Biochem Biophys Res Commun* 1997; **235**: 669–674.
- 44 Kamisako T, Ogawa H. Regulation of biliary cholesterol secretion is associated with abcg5 and abcg8 expressions in the rats: effects of diosgenin and ethinyl estradiol. *Hepatol Res* 2003; **26**: 348–352.
- 45 MacGregor JI, Jordan VC. Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev* 1998; **50**: 151–196.
- 46 Johnston Jr CC, Bjarnason NH, Cohen FJ, Shah A, Lindsay R, Mitlak BH, Huster W, Draper MW, Harper KD, Heath 3rd H, Gennari C, Christiansen C, Arnaud CD, Delmas PD. Long-term effects of raloxifene on bone mineral density, bone turnover, and serum lipid levels in early postmenopausal women: three-year data from 2 double-blind, randomized, placebo-controlled trials. *Arch Intern Med* 2000; **160**: 3444–3450.
- 47 Lindberg MK, Alatalo SL, Halleen JM, Mohan S, Gustafsson JA, Ohlsson C. Estrogen receptor specificity in the regulation of the skeleton in female mice. *J Endocrinol* 2001; **171**: 229–236.