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ORIGINAL ARTICLE

Chronic Exercise Reduces CETP and Mesterolone Treatment Counteracts Exercise Benefits on Plasma Lipoproteins Profile: Studies in Transgenic Mice

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Abstract Regular exercise and anabolic androgenic steroids have opposing effects on the plasma lipoprotein profile and risk of cardio-metabolic diseases in humans. Studies in humans and animal models show conflicting results. Here, we used a mice model genetically modified to mimic human lipoprotein profile and metabolism. They under-express the endogenous LDL receptor gene (R1) and express a human transgene encoding the cholesteryl ester transfer protein (CETP), normally absent in mice. The present study was designed to evaluate the independent and interactive effects of testosterone supplementation, exercise training and CETP expression on the plasma lipoprotein profile and CETP activity. CETP/R1 and R1 mice were submitted to a 6-week swimming training and mesterolone (MEST) supplementation in the last 3 weeks. MEST treatment increased markedly LDL levels (40%) in sedentary CETP/R1 mice and reduced HDL levels in exercised R1 mice (18%). A multifactorial ANOVA revealed the independent effects of each factor, as follows. CETP expression reduced HDL (21%) and increased non-HDL (15%) fractions. MEST treatment increased the VLDL concentrations (42%) regardless of other interventions. Exercise training reduced triacylglycerol (25%) and free fatty acids (20%), increased both LDL and HDL (25–33%), and reduced CETP (19%) plasma levels. Significant factor interactions showed that the increase in HDL induced by exercise is explained by reducing CETP activity and that MEST blunted the exercise-induced elevation of HDL-cholesterol. These results reinforce the positive metabolic effects of exercise, resolved a controversy about CETP response to exercise and evidenced MEST potency to counteract specific exercise benefits.

Keywords Lipoproteins · Exercise · Testosterone · CETP · Transgenic mice

Abbreviations

AAS	Anabolic androgenic steroids
ANOVA	Analysis of variance
Apo	Apolipoprotein
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
CETP	Cholesteryl ester transfer protein
CETP/R1	Mice over-expressing CETP and expressing only one allele of the endogenous LDL receptor gene
CHOL	Cholesterol
CVD	Cardiovascular diseases
FFA	Plasma-free fatty acids
FPLC	Fast protein liquid chromatography
HDL	High-density lipoproteins
HL	Hepatic lipase
LDL	Low-density lipoprotein
LP	Lipoproteins
LPL	Lipoprotein lipase

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MEST	Mesterolone (1-alpha-methyl-5-alpha-androstan-17beta-ol-3-one)
R1	Mice expressing only one allele of the endogenous LDL receptor gene
TAG	Triacylglycerol
VLDL	Very low-density lipoprotein

Introduction

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone. Athletes often use AAS in attempt to improve physical performance given their putative capacity for enhancing muscle mass and strength [1]. The abuse of AAS by healthy athletes has been linked to many severe toxic effects, including the incidence of premature cardiovascular diseases (CVD) [2]. A change to an atherogenic lipoprotein profile may explain, at least in part, the increased CVD associated with AAS self-administration. It generally induces decreases in plasma levels of high-density lipoprotein (HDL)-cholesterol and apolipoprotein Al and increases in apoB-containing lipoproteins, particularly in low-density lipoprotein (LDL)-cholesterol [1, 3]. In addition, several studies have shown a close relationship between AAS use and hepatic disorders, including hepatocyte structural disarrangements, cholestatic jaundice and hepatocellular tumors [4, 5], which may also be involved in the disturbances of lipoprotein metabolism.

It is generally accepted that regular exercise modulates favorably the plasma lipoprotein profile, thus reducing the risk of cardio-metabolic diseases such as diabetes, atherosclerosis and obesity [6, 7]. There are evidences showing that being physically active lowers total or LDL-cholesterol and/or elevates HDL-cholesterol [7], thereby protecting against atherosclerosis. However, many studies fail to show these beneficial effects of exercise, probably because there is a graded response of the plasma lipoprotein levels to increasing amounts (duration and intensity) of exercise [8], in addition to differences in basal phenotype and genotype [9, 10].

Cholesteryl ester transfer protein (CETP) mediates the transfers of cholesteryl ester (CE) from HDL to apolipoprotein B-containing lipoproteins (apoB-LP), thus remodeling HDL composition in the plasma of several species, such as humans, primates and rabbits. Epidemiological and experimental evidences have shown that CETP may play an important role in the development of atherosclerosis [11]. Inhibitors of CETP have been tested in humans showing elevation of HDL-cholesterol and reduction of LDL-cholesterol and apoB. However, clinical trials using CETP inhibitors have not shown benefits regarding CVD mortality and morbidity. Other CETP inhibitors are currently being tested in humans for their potential anti-atherogenic actions [12].

Mice and rats do not express CETP [13]. A genetically engineered CETP transgenic mice model has been very useful to study lipoprotein metabolism and atherogenesis. Studies in these CETP transgenic mice have shown neutral, pro- or anti-atherogenic effects of this protein, depending upon the metabolic context [11]. Contrary to humans, wild-type mice show very low levels of LDL and high levels of HDL. These differences may be attributed to the lack of expression of the CETP (that raises HDL) and to a high number of LDL receptors (that decreases LDL) in the wild-type mice. Thus, by introducing the expression of the CETP gene and reducing the expression of the LDL receptor gene, the lipemic phenotype of these transgenic mice resembles those of humans [3, 14, 15].

Because of the potential dual role (pro- or anti-atherogenic) of CETP, its interference on exercise-induced changes of lipoprotein profile is an intriguing question. Intervention studies showed inconsistent results, suggesting that exercise could decrease, increase or have no effects on the CETP levels [16–19]. In addition, genetic variants of CETP in humans may explain part of the inter-individual differences in lipoprotein responses to exercise, particularly in HDL levels [9, 10, 20]. On the other hand, testosterone status affects lipoproteins in a manner dependent on the CETP levels. For instance, testosterone deficiency in mice resulted in increased plasma levels of very low-density lipoprotein (VLDL) and LDL, and decreased HDL, but transgenic mice that over-express CETP had such effects significantly attenuated [15]. Besides, the presence of CETP reduced atherosclerosis by 50% in castrated male and female CETP transgenic mice [14, 15]. Therefore, the lipoprotein profile may be influenced by CETP, androgens and exercise, either acting independently or in combination.

The present study was designed to evaluate the effects of exercise training and use of AAS on the CETP levels as well as the interplay of exercise, AAS and CETP expression on the lipid and lipoprotein profiles of a "humanized" mouse model. For this purpose, we used the progenies of CETP transgenic mice crossbred with LDL receptor knockout mice, treatment with mesterolone (MEST, a synthetic steroid not metabolized in the body) and a moderate exercise protocol that does not cause cardiac hypertrophy or other deleterious consequences of overtraining.

Materials and Methods

Animal and Treatments

Animal protocols were approved by the University's Committee for Ethics in Animal Experimentation (CEUA/UNICAMP, Protocol #040-2) and all experiments were performed in accordance with relevant guidelines and

regulations. Mice were housed in a temperature-controlled room (22 ± 2 °C) on a 12-h light/dark cycle and had free access to food (rodent chow diet; Nuvital CR1, Colombo, Brazil) and water. Hemizygous human CETP transgenic mice (line 5203, C57BL6/J) [21] expressing a human CETP minigene under the control of natural flanking sequences were crossbred with LDL receptor knockout mice (C57BL6/J) purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CETP-expressing mice were genotyped by assaying plasma CETP activity [22]. CETP-expressing(CETP/R1) and non-expressing (R1) male mice of 4–6 months of age were used in this study. R1 and CETP/R1 mice were randomly assigned to sedentary and exercised groups; $n = 24$ of each genotype in each group. While sedentary groups remained in their cages, exercised groups were submitted to a swimming training protocol during 6 weeks as previously described [23]. Mice started training with 20 min of swimming in a tank with heated water (30–31 °C) in individualized lanes. No weight loads were used. To prevent floating during the swimming session, tubes connected to an air pump system produced water bubbling from the tank bottom. The swimming time was increased 10 min/day until the animals reach 60 min of training. The sessions were carried out once a day, five times a week, during 6 weeks. This protocol does not cause cardiac hypertrophy [23]. In the last 3 weeks of the experimental protocol, the mice under exercise training and the sedentary mice were divided in two sub-groups ($n = 12$) and started daily treatment with MEST or arabic gum (vehicle) orally, 2 µg/g of body weight of MEST (1-alpha-methyl-5-alpha-androstan-17beta-ol-3-one) (Schering do Brazil, São Paulo, SP, Brazil). A diagram of the experimental groups and protocol is shown in Fig. 1. Twenty-four hours after the last session of physical training, the animals were deeply anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and total blood was obtained through the retro-orbital plexus.

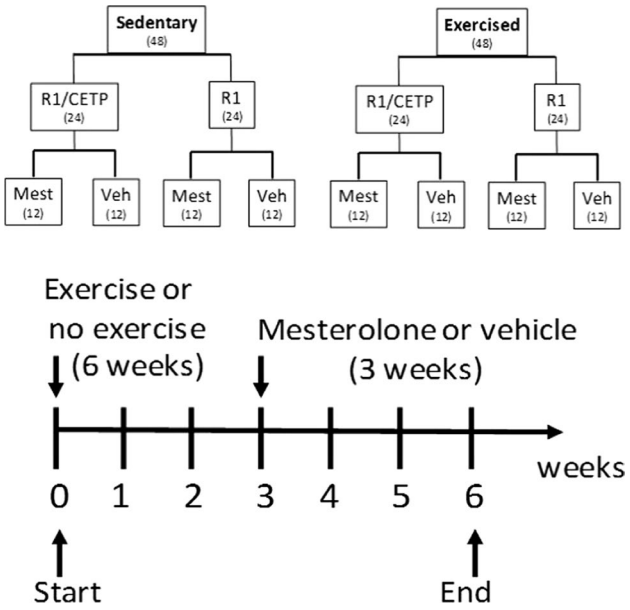

Fig. 1 Diagram of experimental groups and protocol. R1 and CETP/R1 mice were randomly assigned to sedentary and exercised groups; $n = 24$ of each genotype in each group. While sedentary groups remained in their cages, exercised groups were submitted to a swimming training protocol during 6 weeks as previously described [23]. In the last 3 weeks, mice under training and sedentary mice were divided in two sub-groups ($n = 12$) and started daily treatment with mesterolone (MEST) or arabic gum (vehicle) orally, 2 µg/g of body weight

Plasma Biochemical Analyses

Lipoproteins from pooled or individual plasmas of mice were separated by fast-protein liquid chromatography (FPLC; Amersham-Pharmacia Biotech, Uppsala, Sweden) as described previously [22]. The FPLC HR10/30 Superose 6 column separates three well-defined cholesterol peaks from mouse plasma: fractions 10–15, VLDL ($d < 1.006$, > 30–80 nm), fractions 16–26, intermediate- and LDL (IDL + LDL, $d = 1.006$–1.063, 16–30 nm), and fractions 27–37, HDL ($d = 1.063$–1.21, 8–16 nm). Total cholesterol and triacylglycerols (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany) and plasma free fatty acids (Wako Chemical, Neuss, Germany) were determined by enzymatic-colorimetric methods according to the manufacturer's instructions. Plasma glucose concentrations were determined by the glucose oxidase method using the Merck Diagnostic Biotrol®kit (Chennevires-les-Louvres, France).

Plasma CETP Activity

CETP activity assay using exogenous substrates, which reflects the plasma CETP concentration, was measured as described previously [22]. Briefly, a mixture of human VLDL and LDL (100 µg of proteins) was incubated with 10,000 dpm of human HDL_3 labeled with [^{14}C]cholesteryl ester [24] and 5 µl of mouse plasma as the source of CETP in a final volume of 100 µl. Blanks were prepared with tris-saline-EDTA buffer and negative controls with non-transgenic mouse plasma. The incubations were carried out at 37 °C for 2 h and stopped in an ice bath. The apoB-containing lipoproteins were precipitated using 1.6% dextran sulphate and 1 M $MgCl_2$ solution (1:1) and 100 µg of human LDL as carrier. The remaining radioactivity was measured in the supernatant (HDL) in a LS6000 Beckman beta counter.

Intravascular Lipase Activities

Total lipase activity was determined according to Ehnholm and Kuusi [25]. Overnight-fasted mouse plasmas obtained before (basal) and 10 min after subcutaneous injection of

heparin (100 µU/kg body weight) were incubated with a [^{3}H]triolein/gum arabic substrate ([9,10-^{3}H (N)]triolein, New England Nuclear, Boston, MA, USA) for 1 h. Hepatic lipase (HL) activity was determined in tubes in which the lipoprotein lipase (LPL) was inhibited by 2 M NaCl. The hydrolyzed labeled free fatty acids were extracted with methanol-chloroform-heptane (1.4:1.25:1) and dried under N_2 and radioactivity was determined in a LS6000 Beckman beta counter. LPL activity was calculated as the difference between the total lipase and the hepatic lipase activities.

Statistical Analyses

Data were expressed as mean ± SE. Two statistical analyses were applied. Segregated analysis by physical activity state, within sedentary or exercised groups (two mean comparisons by Mann–Whitney test, InStat GraphPad software, version 3.0) was used to determine the effects of MEST. Global analysis of all groups, using multi-factorial ANOVA (software SAS version 8) was used to determine the effects of genotypes (presence of CETP or not), hormonal treatment (MEST vs. vehicle) and physical activity (exercise vs. sedentary), as well as the interaction between these factors. Differences between groups were considered significant at $p \leq 0.05$.

Table 1 Fasting plasma levels of cholesterol, non-esterified fatty acids, triacylglycerol and glucose in sedentary LDL receptor-deficient mice expressing CETP (CETP/R1) or not (R1), after 3 weeks of mesterolone (MEST) or vehicle (arabic gum) treatment

	R1		R1/CETP	
	MEST	Vehicle	MEST	Vehicle
CHOL (mg/dl)	109 ± 4.3	101 ± 4.5b	98 ± 3.9a	81 ± 3.0ab
NEFA (nmol/l)	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
TAG (mg/dl)	80 ± 8.5	75 ± 6.5	67 ± 2.4	63 ± 3.8
Glucose (mg/dl)	60 ± 3.6	64 ± 2.5	67 ± 2.1	69 ± 2.9

Mean ± SE ($n = 12$). The same letters indicate the pair comparisons that are significantly different, $p < 0.05$

CHOL cholesterol, *NEFA* non-esterified fatty acids, *TAG* triacylglycerol

Results

Effects of Mesterolone (MEST) in Sedentary Mice Expressing or Non-expressing CETP

Cholesterolemia was significantly increased ($p = 0.002$) in the MEST group that expressed CETP (CETP/R1) when compared to the vehicle (Table 1). This was not verified in the R1 group of mice that had already a basal cholesterol concentration higher than the CETP/R1 vehicle ($p = 0.0003$); MEST treatment did not further increase it. As for the plasma levels of free fatty acid (FFA), triacylglycerol (TAG) and glucose, there were no significant variations between genotypes or hormone treatment (Table 1).

Cholesterol distribution among plasma lipoprotein fractions is presented in Table 2, where it can be observed that VLDL-cholesterol did not change among the groups. However, the treatment with MEST induced a marked elevation of LDL-cholesterol of the CETP/R1 (+ 37%) and of HDL-cholesterol in both genotype groups (+ 30% in CETP/R1 and + 20% in R1 mice). As expected, HDL-cholesterol levels were significantly lower in CETP-expressing groups (CETP/R1) than in non-expressing (R1) under both conditions, vehicle and MEST treatment. LDL-cholesterol elevation induced by MEST treatment in the presence of CETP was reflected in their non-HDL fraction (+ 33%). Taking into account the [V + LDL]/HDL ratio, CETP-expressing mice treated with MEST exhibited a potentially more atherogenic lipid profile among the four sedentary groups (+ 31% vs. R1 vehicle).

We also determined the plasma activities of the two major modulators of HDL metabolism, CETP and HL. We verified

Table 2 Plasma lipoprotein concentrations, cholesteryl ester transfer protein and hepatic lipase activities in LDL receptor-deficient mice expressing CETP (CETP/R1) or not (R1), after 3 weeks of mesterolone (MEST) or vehicle (arabic gum) treatment

	R1		R1/CETP	
	MEST	Vehicle	MEST	Vehicle
VLDL-chol	2.3 ± 0.4	1.7 ± 0.3	2.2 ± 0.3	1.8 ± 0.4
LDL-chol	22 ± 0.9a	20 ± 2.8	26 ± 2.0ab	19 ± 1.9b
HDL-chol	86 ± 3.9cd	72 ± 1.7ce	73 ± 3.9fd	56 ± 2.9fe
non-HDL	24 ± 0.9g	22 ± 2.8	28 ± 2.2gh	21 ± 1.8h
V + LDL/HDL	0.29 ± 0.01i	0.30 ± 0.04	0.38 ± 0.02i	0.36 ± 0.03
HL (nmol/ml/h)	1203 ± 41	1210 ± 46	1345 ± 49	1191 ± 42
CETP (% CE transfer)	–	–	28 ± 2.1	26 ± 1.9

Mean ± SE ($n = 6$–12). Lipoproteins (VLDL, LDL, HDL, non-HDL, V + LDL/HDL ratio). The same letters indicate the pair comparisons that are significantly different: (a) $p = 0.02$, (b) $p = 0.03$, (c) $p = 0.008$, (d) $p = 0.01$, (e) $p = 0.0007$, (f) $p = 0.03$, (g) $p = 0.04$, (h) $p = 0.03$ and (i) $p = 0.02$

chol cholesterol, *CETP* cholesteryl ester transfer protein, *HL* hepatic lipase

that the amounts of CETP and of HL were not altered by MEST treatment (Table 2). Therefore, we cannot attribute the increased plasma levels of HDL-cholesterol induced by MEST to modifications in the levels of these two proteins.

Effect of Testosterone in Exercised Mice Expressing or Non-expressing CETP

In exercised mice, the plasma concentrations of cholesterol, TAG, FFA and glucose did not vary as a function of MEST treatment, nor of CETP expression (Table 3). However, significant changes were observed in the lipoprotein profiles of the exercised mice (Table 4). MEST treatment significantly reduced the concentration of HDL (− 18%) in R1 mice, what was not observed in the CETP/R1 mice (− 9%, non-significant). The presence of CETP led to a higher concentration of non-HDL-cholesterol and, therefore, a higher atherogenic index in CETP/R1 groups, in both vehicle- and MEST-treated mice. The activities of CETP and HL (Table 4) did not change significantly between the genotypes or hormone treatment in exercised mice. Therefore, in exercised mice, the testosterone treatment reduced HDL fraction in CETP non-expressing mice (R1). The LP profile of exercised mice expressing CETP was significantly more atherogenic, regardless the MEST treatment, as shown by lower HDL, higher non-HDL and higher [V + LDL]/HDL ratio.

Table 3 Fasting plasma levels of cholesterol, non-esterified fatty acids, triacylglycerol and glucose in LDL receptor-deficient mice expressing CETP (CETP/R1) or not (R1), after 3 weeks mesterolone (MEST) and vehicle (arabic gum) treatment and 6 weeks of swimming training

	R1		R1/CETP	
	MEST	Vehicle	MEST	Vehicle
CHOL (mg/dl)	115 ± 5.1	122 ± 4.3	114 ± 4.5	120 ± 6.2
NEFA (nm/l)	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
TAG (mg/dl)	51 ± 1.7	52 ± 2.1	50 ± 2.8	52 ± 2.4
Glucose (mg/dl)	71 ± 5.1	69 ± 4.5	68 ± 5.7	68 ± 6.0

Mean ± SE ($n = 12$). No significant differences were observed

CHOL cholesterol, *NEFA* non-esterified fatty acids, *TAG* triacylglycerol

Independent Effects of CETP Genotype, Mesterolone Treatment and Exercise Training

The study design was carefully planned to allow a robust statistical analysis (multifactorial analysis of variance) to determine independent and interactive effects of the three factors (CETP genotype, MEST treatment and exercise training). The multifactorial statistical analysis provides conclusions that cannot be inferred from the data, as grouped in the previous tables [1–4]. Instead, data were treated collectively and grouped in distinct ways. The detailed statistical analysis for each dependent variable is provided as supplementary information. Next, we describe the independent effects of CETP genotype, MEST treatment and exercise training shown in Figs. 2, 3 and 4.

Concerning CETP genotype effects, multifactorial ANOVA showed that CETP expression, independently of exercise training and hormone treatment, reduces HDL (21%, $p = 0.0001$) and increases non-HDL cholesterol (15%, $p = 0.06$) fractions (Fig. 2). In this analysis of all groups, we verified that the presence of CETP increased the [VLDL + LDL]/HDL ratio in 36% ($p < 0.0001$), indicating that, in this context of partial LDL receptor deficiency, the presence of CETP favors an atherogenic LP profile.

Mesterolone treatment increased the concentrations of VLDL-cholesterol in 42% ($p = 0.02$), regardless of the presence of CETP and status of physical activity (Fig. 3).

Exercise training had the independent effects of elevating LDL (+ 33%, $p = 0.0013$), non-HDL (+ 31%, $p = 0.0005$), HDL (+ 25%, $p = 0.0001$) and decreasing plasma CETP levels (− 19%, $p = 0.01$) as shown in Fig. 4. In addition, exercise reduced the plasma concentrations of TAG in 25% ($p < 0.0001$) and FFA in 20% ($p < 0.0004$).

Table 4 Plasma lipoprotein concentrations, cholesteryl ester transfer protein and hepatic lipase activities in LDL receptor-deficient mice expressing CETP (CETP/R1) or not (R1), after 6 weeks of swimming training and 3 weeks mesterolone (MEST) or vehicle (arabic gum) treatment

	R1		R1/CETP	
	MEST	Vehicle	MEST	Vehicle
VLDL-chol	2.7 ± 0.5	1.6 ± 0.3	3.2 ± 0.5	2.7 ± 0.4
LDL-chol	23 ± 2.9	25 ± 1.8	30 ± 2.1	35 ± 3.6
HDL-chol	86 ± 3.7a	105 ± 4.4ab	83 ± 3.2	91 ± 4.9b
non-HDL	28 ± 3.3	27 ± 1.9c	33 ± 2.0	38 ± 3.7c
V + LDL/HDL	0.32 ± 0.03d	0.27 ± 0.02e	0.41 ± 0.03d	0.42 ± 0.03e
HL (nmol/ml/h)	1219 ± 103	1409 ± 107	1282 ± 32	1239 ± 66
CETP (% CE transfer)	–	–	21 ± 1.9	22 ± 1.6

Mean ± SE ($n = 6$–12). The same letters indicate the pair comparisons that are significantly different: (a) $p = 0.008$, (b) $p = 0.03$, (c) $p = 0.05$, (d) $p = 0.06$ and (e) $p = 0.05$

CETP cholesteryl ester transfer protein, *HL* hepatic lipase

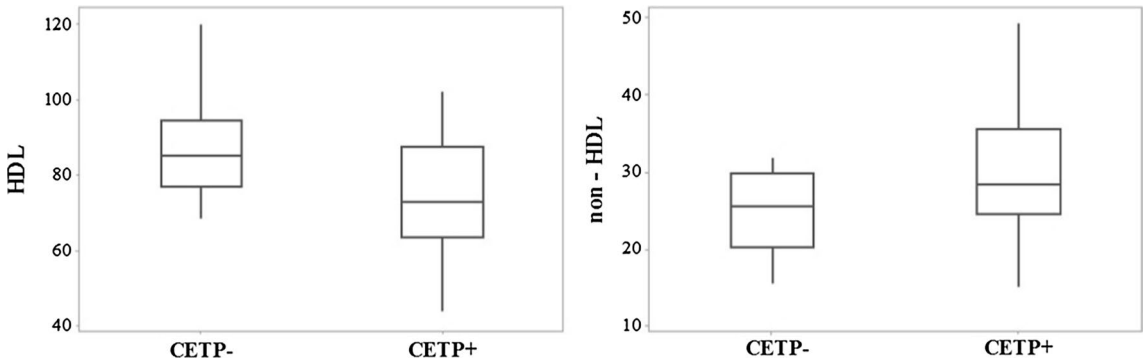

Fig. 2 Independent effects of CETP expression on HDL (decrease) and non-HDL (increase) plasma levels that were identified by multifactorial analysis of variance (SAS, version 8). Box plot with median, 1st and 3rd quartile, minimum and maximum limits

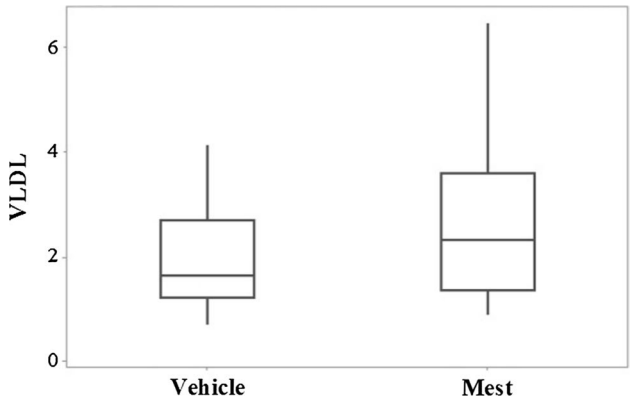

Fig. 3 Independent effect of mesterolone treatment on VLDL (increase) plasma levels that was identified by multifactorial analysis of variance (SAS, version 8). Box plot with median, 1st and 3rd quartile, minimum and maximum limits

Interactions of Exercise, CETP and Mesterolone

The total cholesterol elevation due to exercise-CETP interaction is explained by elevations in the HDL fraction that were more intense in the group expressing CETP (+ 46%, $p < 0.0001$) than in the R1 (+ 20%, $p = 0.01$). Thus, the interactions between exercise and CETP show that exercise induced an elevation of HDL-cholesterol fraction in CETP/R1 mice likely as a consequence of decreasing CETP.

Interactions of exercise-MEST show that exercise increased all lipoprotein fractions in mice not treated with MEST (vehicle groups), as follows: LDL-cholesterol (60%, $p = 0.0006$), non-HDL-cholesterol (+ 56%, $p < 0.0009$) and HDL-cholesterol (+ 59%, $p < 0.05$). On the other hand, MEST treatment increased HDL-cholesterol levels in sedentary groups by + 27%, ($p < 0.0001$), while exercised groups had already much higher HDL-cholesterol concentrations. Therefore, interactions of exercise-MEST evidences that exercise increases HDL, particularly in mice that were not treated with MEST.

Discussion

Studies in humans and animal model have provided conflicting results regarding the isolated or combined effects of exercise and anabolic androgenic steroids (AAS) on the plasma lipids and lipoproteins fractions. Most variations in the studies outcomes are related to distinct models, protocols (exercise type and volume, AAS type and time of use), and metabolic and genetic background of the studied species or individuals. Genetic variations in individuals have gained attention regarding differential responses to exercise [10]. In the present study, we employed a well-defined genetic and metabolic context, the genetically modified mice expressing CETP and half of the endogenous LDL receptors, to determine independent and interactive effects of CETP expression, MEST treatment and exercise training on the plasma lipoprotein profile and CETP activity. These genetic modifications make the lipoprotein metabolism in this model more similar to humans. The type of exercise chosen was a very moderate one, previously shown to not cause cardiac hypertrophy recommended for cardiovascular protection. We concluded that: (1) CETP expression, as expected, independently of any other intervention, reduced HDL and augmented non-HDL cholesterol fractions. The presence of CETP resulted in a potentially more atherogenic lipoprotein profile in both states, sedentary and exercised; (2) MEST treatment had the independent effect of increasing VLDL-cholesterol. In addition, it blunted the effect of exercise on increasing HDL-cholesterol (interactive effect); and finally, (3) exercise training reduced TAG and FFA, increased both LDL and HDL, and reduced CETP plasma levels. Interactive effects showed that the exercise-induced reduction of CETP activity contributed to increase HDL-cholesterol levels. The increase of LDL-cholesterol with exercise was unexpected. This may be related to the stimulation of the TAG-rich lipoprotein (VLDL) metabolism, as evidenced by the exercise-independent effect of reducing TAG levels. Since these mice have partial deficiency of LDL receptors, they accumulate

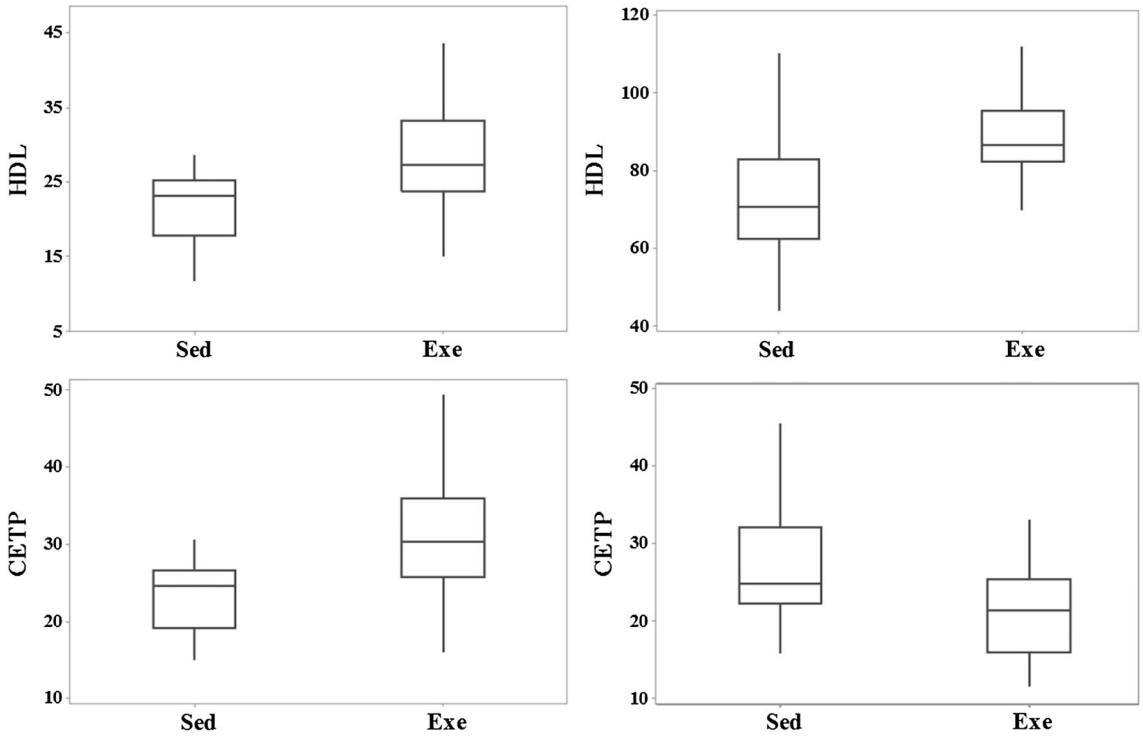

Fig. 4 Independent effects of exercise training on the lipoproteins and CETP plasma levels as identified by multifactorial analysis of variance (SAS, version 8). Exercise increased LDL-cholesterol, non-HDL-cholesterol and HDL-cholesterol levels and decreased CETP plasma activity. Box plot with median, 1st and 3rd quartile, minimum and maximum limits

these particles in the plasma. It is important to emphasize that partial deficiency of LDL receptors is a very common condition in the human population [26].

The interactions of exercise-CETP and exercise-MEST helped to conciliate some of the previous discordant results. Several authors reported that total cholesterol plasma levels in exercised men did not change, was reduced or even increased [7]. Reduction of plasma cholesterol was also observed in studies with exercised men treated with testosterone [27, 28]. Here, we showed that CETP expression worsens the elevation of plasma cholesterol associated with exercise training. However, alterations in specific lipoprotein fractions are more relevant to understand changes in cholesterol metabolism, as discussed next.

VLDL-cholesterol plasma levels are, in general, decreased as a function of physical training [29]. Here, we found no direct independent effect of exercise on VLDL-cholesterol. However, fasting plasma TAG levels reflect directly the rate of VLDL metabolism, and we found an independent effect of exercise on reducing TAG levels, suggesting an acceleration of VLDL lipolysis. Accordingly, most reports [17, 29, 30], but not all [31, 32], show that exercise reduces plasma TAG levels, basically by increasing lipoprotein lipase activity, and thus, VLDL clearance rate [33, 34]. Testosterone supplementation or replacement in several animal models, such as rats, mice, rabbits and monkeys, showed quite variable responses of their VLDL-cholesterol plasma levels [31, 35–37]. In men, testosterone does not seem to have an impact on the VLDL metabolism [38, 39]. In the present study, MEST treatment had an independent effect of increasing VLDL-cholesterol. In a previous study with CETP/R1 mice, we also observed that MEST induced increases in VLDL- and LDL-cholesterol in sedentary but not in treadmill-exercised mice [3].

In most human studies, physical training either decreased or did not alter the plasma levels of LDL-cholesterol. However, in animal models, the effects on the LDL fraction are more variable; for instance, Von Duvillard *et al*. [40] observed an increase in the LDL-cholesterol fraction in pigs fed a hyperlipidemic diet and exercised. High-intensity resistance exercise increased non-HDL cholesterol in hamsters fed a hypercholesterolemic diet [41]. On the other hand, wild-type and LDL receptor knockout mice fed with an atherogenic diet had reduced LDL by exercise training [42, 43]. Therefore, this effect seems to be related to the species background and/or to the presence of a high-fat diet. In the present work, the partial deficiency of the LDL receptor is probably responsible for the elevation of LDL induced by exercise, since no unbalanced diet was used.

One of the most acknowledged beneficial roles of exercise is its action on raising HDL. However, that is not always the case. Niebauer *et al*. [44] did not observe an increase in the HDL of apoE knockout and wild-type female mice after 4 weeks of treadmill training. On the contrary, von Duvillard *et al*. [40] observed an increase in HDL fraction in exercised pigs. In humans, exercise may or may not increase the HDL levels and the reasons for these variable responses are likely related to the different types of exercises performed, their intensity and regularity [8] and also the genetic background. The relationship of testosterone and HDL is complex and studies on this matter are more scarce. The endogenous testosterone in middle-aged men is positively linked with the levels of both HDL- and apoB-containing lipoproteins [45]. In other studies investigating the association between exercise and testosterone, the HDL levels were decreased. Body builders using AAS showed lower plasma concentrations of HDL when compared to former users [46], non-user athletes [28, 47] and sedentary users [47]. Here, exercise independently induced elevation of HDL, but MEST treatment interfered negatively in this effect.

CETP is one of the most important endogenous negative regulators of HDL. Thus, in the 90s, it was hypothesized that the effects of exercise on HDL levels could be mediated by alterations in CETP. Seip *et al*. [17] showed a decrease in the activity of the CETP in normolipidemic men and women subjected to physical training during 9–12 months when compared to their own pre-training values. Foger *et al*. [48] also observed decreased mass and activity of CETP in endurance athletes after a biking marathon when compared to pre-competition values. On the contrary, Gupta *et al*. [16] observed increased CETP activity in athletes when compared to sedentary men. Wilund *et al*. [20] studied sedentary men subjected to endurance training that showed variable responses in the concentrations of HDL according to their CETP gene polymorphism Taq1B. The individuals with the CETP-B1B2 genotype responded to exercise with an increase of HDL, while this did not occur in B1B1 subjects. In CETP transgenic mice with preserved LDL receptor expression, Rocco *et al*. [19] reported no changes in CETP activity before and after a 6-week aerobic exercise training program. Here, it was shown that in mice with partial LDL receptor deficiency, exercise independent of other interventions reduces CETP activity, which is in agreement with most human studies. The reasons that might link exercise and reduction in CETP activity are unknown. Several hormonal and metabolic factors elicited by exercise may have influenced CETP levels. Although beyond the scope of this study, we could speculate about a few factors, such as increased growth hormone [49], decreased FFA or PPARa activity [50] or other factors modulated by exercise, for instance, systemic/tissue microRNAs or anti-inflammatory factor release/expression.

In conclusion, in the present work, it was possible to verify independent effects of exercise, AAS treatment and CETP expression based on comparisons of well-defined genetic and metabolic contexts. Interactions of exercise-CETP and exercise-MEST helped to conciliate some discrepant results in the literature. The results reinforce the positive metabolic effects of exercise (reduced triacylglycerol and free fatty acids and increased HDL), resolved a controversy about CETP response to exercise (reduced CETP plasma levels) and evidenced that MEST counteracts specific exercise benefits such as elevation of HDL. Since pharmacological inhibition of CETP has been questioned, the present findings show that exercise is a safe intervention to reach this anti-atherogenic goal. Overall, the present findings show how the CETP genetic background and the hormonal state interact with exercise to define a final lipoprotein profile outcome.

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Compliance with Ethical Standards

Conflict of interest The authors have no competing interests to disclose.

References

1. Hartgens F, Kuipers H (2007) Effects of androgenic–anabolic steroids in athletes. Sports Med 34:513–554
2. Turillazzi E, Perilli G, Di Paolo M, Neri M, Riezzo I, Fineschi V (2011) Side effects of AAS abuse: an overview. Mini Rev Med Chem 11:374–389
3. Fontana K, Oliveira HC, Leonardo MB, Mandarim-de-Lacerda CA, da Cruz-Hofling MA (2008) Adverse effect of the anabolic–androgenic steroid mesterolone on cardiac remodelling and lipoprotein profile is attenuated by aerobic exercise training. Int J Exp Pathol 89:358–366
4. Soe KL, Soe M, Gluud C (1992) Liver pathology associated with the use of anabolic–androgenic steroids. Liver 12:73–79
5. Fontana K, Aldrovani M, de Paoli F, Oliveira HC, de Campos Vidal B, da Cruz-Hofling MA (2008) Hepatocyte nuclear phenotype: the cross-talk between anabolic androgenic steroids and exercise in transgenic mice. Histol Histopathol 23:1367–1377
6. Thompson P (2003) Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease. Arterioscler Thromb Vasc Biol 23:1319–1321
7. Enkhmaa B, Surampudi P, Anuurad E, Berglund L (2015) Lifestyle changes: effect of diet, exercise, functional food, and obesity treatment, on lipids and lipoproteins. In: De Groot LJ et al (eds)

Source endotext [Internet]. MDText.com, Inc., South Dartmouth, pp 2000–2015
8. Tall AR (2002) Exercise to reduce cardiovascular risk—how much is enough? N Engl J Med 347:1522–1524
9. Hagberg JM (1985) Do genetic variations alter the effects of exercise training on cardiovascular disease and can we identify the candidate variants now or in the future? J Appl Physiol 111:916–928
10. Roth SM, Rankinen T, Hagberg JM, Loos RJ, Perusse L, Sarzynski MA, Wolfarth B, Bouchard C (2012) Advances in exercise, fitness, and performance genomics in 2011. Med Sci Sports Exerc 44:809–817
11. Oliveira HC, de Faria EC (2011) Cholesteryl ester transfer protein: the controversial relation to atherosclerosis and emerging new biological roles. IUBMB Life 63:248–257
12. Barter PJ, Rye KA (2012) Cholesteryl ester transfer protein inhibition as a strategy to reduce cardiovascular risk. J Lipid Res 53:1755–1766
13. Ha YC, Barter PJ (1982) Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. Comp Biochem Physiol B 71:265–269
14. Cazita PM, Berti JA, Aoki C, Gidlund M, Harada LM, Nunes VS, Quintao EC, Oliveira HC (2003) Cholesteryl ester transfer protein expression attenuates atherosclerosis in ovariectomized mice. J Lipid Res 44:33–40
15. Casquero ACBJ, Salerno AG, Bighetti EJ, Cazita PM, Ketelhuth DF, Gidlund M, Oliveira HC (2006) Atherosclerosis is enhanced by testosterone deficiency and attenuated by CETP expression in transgenic mice. J Lipid Res 47:1526–1534
16. Gupta AK, Ross EA, Myers JN, Kashyap ML (1993) Increased reverse cholesterol transport in athletes. Metabolism 42:684–690
17. Seip RL, Moulin P, Cocke T, Tall A, Kohrt WM, Mankowitz K, Semenkovich CF, Ostlund R, Schonfeld G (1993) Exercise training decreases plasma cholesteryl ester transfer protein. Arterioscler Thromb 13:1359–1367
18. Vislocky LM, Pikosky MA, Rubin KH, Vega-Lopez S, Gaine PC, Martin WF, Zern TL, Lofgren IE, Fernandez ML, Rodriguez NR (2009) Habitual consumption of eggs does not alter the beneficial effects of endurance training on plasma lipids and lipoprotein metabolism in untrained men and women. J Nutr Biochem 20:26–34
19. Rocco DD, Okuda LS, Pinto RS, Ferreira FD, Kubo SK, Nakandakare ER, Quintao EC, Catanozi S, Passarelli M (2011) Aerobic exercise improves reverse cholesterol transport in cholesteryl ester transfer protein transgenic mice. Lipids 46:617–625
20. Wilund K, Ferrell RE, Phares DA, Goldberg AP, Hagberg JM (2002) Changes in high-density lipoprotein-cholesterol subfractions with exercise training may be dependent on cholesteryl ester transfer protein (CETP) genotype. Metabolism 51:774–778
21. Jiang XC, Agellon LB, Walsh A, Breslow JL, Tall A (1992) Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. J Clin Invest 90:1290–1295
22. Berti JA, Amaral ME, Boschero AC, Nunes VS, Harada LM, Castilho LN, Oliveira HC (2001) Thyroid hormone increases plasma cholesteryl ester transfer protein activity and plasma high-density lipoprotein removal rate in transgenic mice. Metabolism 50:530–536
23. Evangelista FS, Brum PC, Krieger JE (2003) Duration-controlled swimming exercise training induces cardiac hypertrophy in mice. Braz J Med Biol Res 36:1751–1759
24. Oliveira HC, Quintao EC (1996) 'In vitro' cholesteryl ester bidirectional flow between high-density lipoproteins and triglyceride-rich emulsions: effects of particle concentration and composition, cholesteryl ester transfer activity and oleic acid. J Biochem Biophys Methods 32:45–57
25. Ehnholm C, Kuusi T (1986) Preparation, characterization, and measurement of hepatic lipase. Methods Enzymol 129:716–738
26. Futema M, Kumari M, Boustred C, Kivimaki M, Humphries SE (2015) Would raising the total cholesterol diagnostic cut-off from 7.5 to 9.3 mmol/l improve detection rate of patients with monogenic familial hypercholesterolaemia? Atherosclerosis 239:295–298
27. Zmuda JM, Fahrenbach MC, Younkin BT, Bausserman LL, Terry RB, Catlin DH, Thompson PD (1993) The effect of testosterone aromatization on high-density lipoprotein cholesterol level and postheparin lipolytic activity. Metabolism 42:446–450
28. Sader MA, Griffiths KA, McCredie RJ, Handelsman DJ, Celermajer DS (2001) Androgenic anabolic steroids and arterial structure and function in male bodybuilders. J Am Coll Cardiol 37:224–230
29. Gordon B, Chen S, Durstine JL (2014) The effects of exercise training on the traditional lipid profile and beyond. Curr Sports Med Rep 13:253–259
30. Foger B, Chase M, Amar MJ, Vaisman BL, Shamburek RD, Paigen B, Fruchart-Najib J, Paiz JA, Koch CA, Hoyt RF, Brewer HB Jr, Santamarina-Fojo S (1999) Cholesteryl ester transfer protein corrects dysfunctional high density lipoproteins and reduces aortic atherosclerosis in lecithin cholesterol acyltransferase transgenic mice. J Biol Chem 274:36912–36920
31. Herd SL, Kiens B, Boobis LH, Hardman AE (2001) Moderate exercise, postprandial lipemia, and skeletal muscle lipoprotein lipase activity. Metabolism 50:756–762
32. Katzmarzyk PT, Leon AS, Rankinen T, Gagnon J, Skinner JS, Wilmore JH, Rao DC, Bouchard C (2001) Changes in blood lipids consequent to aerobic exercise training related to changes in body fatness and aerobic fitness. Metabolism 50:841–848
33. Magkos F (2009) Basal very low-density lipoprotein metabolism in response to exercise: mechanisms of hypotriacylglycerolemia. Prog Lipid Res 48:171–190
34. Kersten S (2014) Physiological regulation of lipoprotein lipase. Biochim Biophys Acta 1841:919–933
35. Greger NG, Insull W Jr, Probstfield JL, Keenan BS (1990) High-density lipoprotein response to 5-alpha-dihydrotestosterone and testosterone in Macaca fascicularis: a hormone-responsive primate model for the study of atherosclerosis. Metabolism 39:919–924
36. Larsen BA, Nordestgaard BG, Stender S, Kjeldsen K (1993) Effect of testosterone on atherogenesis in cholesterol-fed rabbits with similar plasma cholesterol levels. Atherosclerosis 99:79–86
37. Bruck B, Brehme U, Gugel N, Hanke S, Finking G, Lutz C, Benda N, Schmahl FW, Haasis R, Hanke H (1997) Gender-specific differences in the effects of testosterone and estrogen on the development of atherosclerosis in rabbits. Arterioscler Thromb Vasc Biol 17:2192–2199
38. Nielsen S, Karpe F (2012) Determinants of VLDL-triglycerides production. Curr Opin Lipidol 23:321–326
39. Høst C, Gormsen LC, Christensen B, Jessen N, Hougaard DM, Christiansen JS, Pedersen SB, Jensen MD, Nielsen S, Gravholt CH (2013) Independent effects of testosterone on lipid oxidation and VLDL-TG production: a randomized, double-blind, placebo-controlled, crossover study. Diabetes 62:1409–1416
40. von Duvillard SP, Foxall TL, Davis WP, Terpstra AH (2000) Effects of exercise on plasma high-density lipoprotein cholesteryl ester metabolism in male and female miniature swine. Metabolism 49:826–832
41. Frajacomo FT, Demarzo MM, Fernandes CR, Martinello F, Bachur JA, Uyemura SA, Perez SE, Garcia SB (2012) The effects of high-intensity resistance exercise on the blood lipid profile and liver function in hypercholesterolemic hamsters. Appl Physiol Nutr Metab 37:448–454
42. Meilhac O, Ramachandran S, Chiang K, Santanam N, Parthasarathy S (2001) Role of arterial wall antioxidant defense in

beneficial effects of exercise on atherosclerosis in mice. Arterioscler Thromb Vasc Biol 21:1681–1688
43. Wen S, Jadhav KS, Williamson DL, Rideout TC (2013) Treadmill exercise training modulates hepatic cholesterol metabolism and circulating PCSK9 concentration in high-fat-fed mice. J Lipids 2013:908048
44. Niebauer J, Maxwell AJ, Lin PS, Tsao PS, Kosek J, Bernstein D, Cooke JP (1999) Impaired aerobic capacity in hypercholesterolemic mice: partial reversal by exercise training. Am J Physiol 276:H1346–H1354
45. Oettel M (2003) Testosterone metabolism, dose-response relationships and receptor polymorphisms: selected pharmacological/toxicological considerations on benefits versus risks of testosterone therapy in men. Aging Male 6:230–256
46. Urhausen A, Torsten A, Wilfried K (2003) Reversibility of the effects on blood cells, lipids, liver function and hormones in former anabolic–androgenic steroid abusers. J Steroid Biochem Mol Biol 84:369–375
47. McKillop G, Ballantyne D (1987) Lipoprotein analysis in bodybuilders. Int J Cardiol 17:281–288
48. Foger B, Wohlfarter T, Ritsch A, Lechleitner M, Miller CH, Dienstl A, Patsch JR (1994) Kinetics of lipids, apolipoproteins, and cholesteryl ester transfer protein in plasma after a bicycle marathon. Metabolism 43:633–639
49. Carrilho AJ, Cunha-Neto MB, Nunes VS, Lottenberg AM, Medina WL, Nakandakare ER, Musolino NR, Bronstein MD, Quintão EC (2001) Plasma cholesteryl ester transfer protein and lipoprotein levels during treatment of growth hormone-deficient adult humans. Lipids 36(6):549–554
50. Raposo HF, Patrício PR, Simões MC, Oliveira HC (2014) Fibrates and fish oil, but not corn oil, up-regulate the expression of the cholesteryl ester transfer protein (CETP) gene. J Nutr Biochem 25(6):669–674