# Atherosclerosis

# Voluntary wheel running increases bile acid as well as cholesterol excretion and decreases atherosclerosis in hypercholesterolemic mice

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## ARTICLE INFO

## ABSTRACT

*Objective:* Regular physical activity decreases the risk for atherosclerosis but underlying mechanisms are not fully understood. We questioned whether voluntary wheel running provokes specific modulations in cholesterol turnover that translate into a decreased atherosclerotic burden in hypercholesterolemic mice.

*Methods:* Male LDLR-deficient mice (8 weeks old) had either access to a voluntary running wheel for 12 weeks (RUN) or remained sedentary (CONTROL). Both groups were fed a western-type/high cholesterol diet. Running activity and food intake were recorded. At 12 weeks of intervention, feces, bile and plasma were collected to determine fecal, biliary and plasma parameters of cholesterol metabolism and plasma cytokines. Atherosclerotic lesion size was determined in the aortic root.

*Results:* RUN weighed less (∼13%) while food consumption was increased by 17% ($p = 0.004$). Plasma cholesterol levels were decreased by 12% ($p = 0.035$) and plasma levels of pro-atherogenic lipoproteins decreased in RUN compared to control. Running modulated cholesterol catabolism by enhancing cholesterol turnover: RUN displayed an increased biliary bile acid secretion (68%, $p = 0.007$) and increased fecal bile acid (93%, $p = 0.009$) and neutral sterol (33%, $p = 0.002$) outputs compared to control indicating that reverse cholesterol transport was increased in RUN. Importantly, aortic lesion size was decreased by ∼33% in RUN ($p = 0.033$).

*Conclusion:* Voluntary wheel running reduces atherosclerotic burden in hypercholesterolemic mice. An increased cholesterol turnover, specifically its conversion into bile acids, may underlie the beneficial effect of voluntary exercise in mice.

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## 1. Introduction

Atherosclerosis is a complex vascular disease, which is characterized by major abnormalities in systemic factors, such as circulating lipids and lipoproteins, and concomitant inflammation of the vascular wall.

It has long been known that exercise is a deterrent of atherosclerosis. Numerous clinical and experimental studies report on the beneficial effects of physical activity on atherosclerosis [1–7] and various effects of physical activity on different processes involved in the pathogenesis and progression of atherosclerosis have been reported. For example, it has been shown that physical activity improves the antioxidant system [4], plaque composition as well as plaque stability [3,6] and favorably modulates the inflammatory response [1]. However, despite these recent efforts it remains unclear how exactly physical activity decreases the atherosclerotic process. We hypothesize that the enterohepatic system, which plays a critical role in several aspects of cholesterol metabolism, may be of great relevance herein.

Increasing cholesterol excretion into feces as neutral sterols or bile acids represents an efficient strategy in the amelioration of atherosclerosis, as it improves the pro-atherogenic state by modulating lipid content in plasma [8,9]. The liver secretes free cholesterol into bile, which is released into the intestine upon ingestion of a meal. In the small intestine, biliary cholesterol mixes with dietary cholesterol and is partially reabsorbed. The remainder is lost in the feces within the neutral sterol fraction. Bile acids are synthesized from cholesterol exclusively in the liver and enter

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the intestinal lumen after a meal. Bile acids are important for the emulsification and absorption of dietary fats in the intestine [10]. About 95% of the bile acids are reabsorbed from the terminal ileum, transported back to the liver for re-secretion into bile (enterohepatic circulation). The fraction of bile acids that escapes reabsorption is lost in feces and constitutes an important part of cholesterol turnover, since fecal bile acid loss is compensated for by *de novo* synthesis from cholesterol to maintain the bile acid pool size [11]. Under steady state conditions, fecal bile acid loss equals hepatic *de novo* bile acid synthesis.

We have recently shown that exposing healthy chow-fed mice to a voluntary running wheel for two weeks enhanced fecal neutral sterol and bile acid excretion with specific changes in biliary, plasma and intestinal parameters contributing to an increased cholesterol turnover upon running [12]. To our knowledge, no previous studies have examined the effects of exercise on cholesterol and bile acid metabolism in a hypercholesterolemic mouse model. Thus, the purpose of this study was to investigate whether the recently observed effects of voluntary running wheel exercise on whole body cholesterol turnover in healthy chow-fed mice [12] extend to the hypercholesterolemic LDLR-deficient mouse model. We hypothesized that voluntary wheel running beneficially modulates cholesterol and bile acid metabolism in hypercholesterolemic mice and thereby mediates a reduction in atherosclerotic burden.

## 2. Methods

All experiments were approved by the Animal Care and Use Committee of the University of Groningen, The Netherlands. The University of Groningen is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals.

### 2.1. Animals and voluntary cage-wheel exercise

Sixteen 5-week-old male LDLR deficient (B6.129S7-$LDLR^{tm1Her}$/J) mice were purchased from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME, USA). Upon arrival, mice were singly housed in a cage ($47 \times 26 \times 14.5$ cm) in a temperature-controlled room with a 12:12 h light–dark cycle and had access to standard commercial pelleted laboratory chow (RMH-B, ABDiets, Woerden, The Netherlands). At 8 weeks of age, mice were switched to a western-type diet (0.25% cholesterol, 16% fat, Purified Western Diet, 4021.06, ABDiets, Woerden, The Netherlands) and were randomly selected to either voluntary cage wheel running (RUN, $n = 9$) or to remain sedentary (CONTROL, $n = 7$) for 12 weeks. Throughout the study, mice had *ad libitum* access to food and water. The voluntary running wheel set-up has been described previously [12]. Twice a week, mice were weighed and food intake was recorded. Two mice in the running group were excluded from all analyses because they showed no activity on the running wheel.

### 2.2. Experimental procedures

Fecal, plasma, biliary, hepatic and intestinal parameters were collected at the endpoint of the experiment after 12 weeks of RUN or CONTROL, i.e., at 20 weeks of age.

### 2.3. Fecal parameters

Forty-eight hours feces were collected before and at 12 weeks running wheel exposure. Feces were dried, weighed and homogenized to a powder. Aliquots of fecal powder were used for analysis of total bile acids by an enzymatic fluorimetric assay [13]. Neutral sterols and bile acid profiles were determined according to Arca et al. [13] and Setchell et al. [14], respectively [15].

### 2.4. Determination of biliary parameters of cholesterol and bile acid metabolism

After 12 weeks of CONTROL or RUN, all mice underwent gallbladder cannulation for continuous collection of bile [16]. Briefly, mice were anaesthetized by intraperitoneal injection with Hypnorm® ($1 \text{ ml kg}^{-1}$) and diazepam ($10 \text{ mg kg}^{-1}$). During the 30 min bile collection period, mice were placed in a humidified incubator to ensure maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of $1 \text{ g ml}^{-1}$ for bile. Bile was stored at $-20\,^{\circ}\text{C}$ until analysis. Total biliary bile acids were determined by an enzymatic fluorimetric assay [17]. Biliary cholesterol and phospholipids levels were measured as described by Kuipers et al. [18].

### 2.5. Determination of plasma markers of cholesterol metabolism

Immediately after bile collection, blood was drawn via the orbital sinus. Plasma was collected by centrifugation and stored at $-20\,^{\circ}\text{C}$ until analyzed. Plasma total cholesterol, free cholesterol and triglyceride levels were measured by standard enzymatic methods using commercially available assay kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma pro- and anti-inflammatory makers were analyzed using BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Diego, CA). Utilizing gaschromatography, as described by Windler et al. [19], we analyzed plasma plant sterols (campesterol and sitosterol) relative to plasma cholesterol levels as marker of intestinal cholesterol absorption in pooled plasma samples of each group. Pooled plasma samples from each group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) in a superose 6 column using an Akta Purifier (GE Healthcare, Diegem, Belgium)

### 2.6. Tissue collection

Mice were opened immediately after blood collection. The heart was slowly perfused with PBS at physiological pressure. Then, the liver was excised, weighed and snap frozen in liquid nitrogen. The small intestine was excised, flushed with ice cold PBS ($4\,^{\circ}\text{C}$) and divided into three sections of equal lengths and subsequently snap-frozen in liquid nitrogen. Lastly, the thoracic aorta was excised and epididymidal fat pads were removed and weighed. Thoracic aorta, liver and intestine were stored at $-80\,^{\circ}\text{C}$ for later analysis. Hearts were flushed with PBS to remove the excess of blood before fixation in formaldehyde 1% (Formal-Fixx, Thermo Electron Corporation, Pittsburgh, PA) for 24 h, cut in an angle eventually revealing the aortic sinus and stored at $-80\,^{\circ}\text{C}$ embedded in OCT (Tissue-Tek O.C.T., Sakura, Zoeterwoude, The Netherlands).

### 2.7. Determination of atherosclerotic lesion size and aortic cholesterol content

Frozen sections from the aortic sinus were prepared according to Paigen et al. [20]. Surface lesion area was measured after Oil Red O staining by computer-assisted image quantification with Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Leica DFC 420 video camera. At least 5 sections per mouse were examined for each staining. Due to technical difficulties, we were able to analyze atherosclerotic lesion size in 4 of 7 running mice and 4 of 7 sedentary mice. Aortic cholesterol content was utilized as an alternative method to assess atherosclerotic

**Table 1**
Biometrical data.

|  | CONTROL | RUN |
|---|---|---|
| Body weight (g) | 29.6 ± 2.3 | 25.9 ± 1.0[*] |
| Liver weight (g) | 1.35 ± 0.14 | 1.23 ± 0.05 |
| Liver weight/body weight (%) | 4.6 ± 0.7 | 4.7 ± 0.2 |
| Small intestine length (cm) | 33.3 ± 1.8 | 31.5 ± 1.8 |
| Epididymal white adipose weight (g) | 0.85 ± 0.25 | 0.23 ± 0.13[*] |
| Food Intake (g/day) | 3.4 ± 0.4 | 4.0 ± 0.1[*] |

Values represent mean ± SD at 12 weeks of running in CONTROL (*n* = 7) and RUN (*n* = 7).
[*] *p* < 0.05 vs. CONTROL.

**Table 2**
Plasma and liver lipids.

|  | CONTROL | RUN |
|---|---|---|
| Plasma lipids (mmol L$^{-1}$) |  |  |
| Total cholesterol | 27.7 ± 1.5 | 23.6 ± 2.5[*] |
| Free cholesterol | 8.80 ± 1.4 | 7.65 ± 0.7 |
| Cholesterol esters | 18.9 ± 0.9 | 15.9 ± 1.8[*] |
| Triglyce rides | 8.52 ± 1.54 | 6.59 ± 0.8[*] |
| Liver lipids (nmol mg$^{-1}$ liver) |  |  |
| Triglycerides | 78 ± 2 | 41 ± 10[*] |
| Total cholesterol | 16.3 ± 2.0 | 13.1 ± 1.3[*] |
| Free cholesterol | 5.5 ± 0.9 | 4.7 ± 0.3 |
| Cholesterol esters | 10.9 ± 1.7 | 8.4 ± 1.1 |
| Phospholipids | 26.7 ± 2.9 | 23.8 ± 4.5 |

Values represent mean ± SD at 12 weeks of running in CONTROL (*n* = 7) and RUN (*n* = 7).
[*] *p* < 0.05 vs. CONTROL.

lesion burden [21,22] in all mice (*n* = 7/group) and measured following the same procedure as for hepatic cholesterol. Percent smooth muscles cells in lesions were determined using anti-alpha smooth muscle actin antibody (1A4, Abcam) as primary antibody (diluted 1:100 in TBS–1% BSA–0.01% Tween-20) and goat anti-mouse IgG 2α-HRP (SouthernBiotech, Birmingham, USA) and Sirius red was used to stain collagen fibers in lesions.

### 2.8. Determination of hepatic lipids

Hepatic lipids were determined after extraction according to Bligh and Dyer [23] and redissolving in Triton–2% H$_2$O using the same kits as for plasma lipids.

### 2.9. RNA isolation and PCR procedures

Total RNA was isolated from liver and intestine using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers' protocol. cDNA was produced as described by Plosch et al.[16]. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to β-actin.

### 2.10. Statistics

Statistical analysis was performed using the Mann–Whitney *U* test (SPSS 12.0.1 for Windows). The Wilcoxon-signed-rank test was used to analyze differences in running wheel activity between the beginning and end of the intervention. All data are expressed as means ± SD. *P*-values of <0.05 were considered statistically significant

## 3. Experimental results

### 3.1. Running wheel activity and morphometric parameters

Mice exposed to a voluntary running wheel progressively ran less during the 12 week running wheel intervention. While a daily average running distance of ∼10 km and average running duration of ∼6.5 h was observed at the start of the experiment, it dropped to ∼5.5 km/day and ∼4.0 h at the end of the experiment (Supplemental Fig. 1). Despite a 17% increase in food intake compared to control mice, running mice displayed a ∼13% lower bodyweight and ∼73% lower epididymal white adipose tissue weight at 12 weeks of running (Table 1). Liver weight, body weight/liver weight ratio and small intestinal length were not different between running and control mice (Table 1).

### 3.2. Effect of voluntary wheel running on atherosclerotic lesion size and inflammatory markers

First, we investigated whether 12 weeks of voluntary wheel running beneficially affected lesion size area in atherosclerosis-prone LDLR-deficient mice. Indeed, quantification of atherosclerotic lesions in the aortic sinus showed a significant reduction in running mice (Fig. 1A–C). In addition, also aortic cholesterol content as an alternative approach to quantify aortic atherosclerosis was decreased by 33% in running mice compared to CONTROL (Fig. 1D). Because inflammation plays an important role in the pathogenesis of atherosclerosis, we also assessed the effect of running on plasma levels of inflammatory markers. Interestingly, voluntary wheel running had no effect on plasma levels of inflammatory markers at 12 weeks of running (Supplemental Fig. 2).

### 3.3. Voluntary wheel running beneficially affects plasma lipoprotein profile

Elevated lipid levels are key factors in the development of atherosclerosis. Running mice displayed a small but significant reduction in plasma levels of total cholesterol, esterified cholesterol and triglycerides (Table 2). Importantly, we found improved plasma lipoprotein profiles with reduced levels of VLDL- and LDL-sized lipoproteins in running compared to control mice (Supplemental Fig. 3A and B). Paralleling these running-induced improvements in lipoprotein profiles, we not only found a reduction in hepatic expression of microsomal triglyceride transfer protein, indicative of a decreased hepatic production of VLDL, but also observed an increased hepatic lipoprotein lipase expression (Table 3), indicative of an increased lipoprotein clearance. More beneficial effects of running were observed on hepatic lipid storage. First, control mice displayed substantial hepatic triglyceride stores, which were reduced by almost half in running mice (Table 2). Second, running resulted in a significantly lower hepatic storage of cholesterol, paralleled by a running-induced decrease in Hmgcr, the rate-limiting enzyme in cholesterol biosynthesis. Hepatic contents of cholesterol esters tended to be reduced in running mice with a lower expression of Acat2, an enzyme required for cholesterol esterification (Table 3). Moreover, the beneficial changes in hepatic lipid content were paralleled by decreased expression levels of key lipogenic genes Fasn and Scd1 (Table 3). Collectively, these data show that running provokes favorable changes in plasma and liver lipid metabolism.
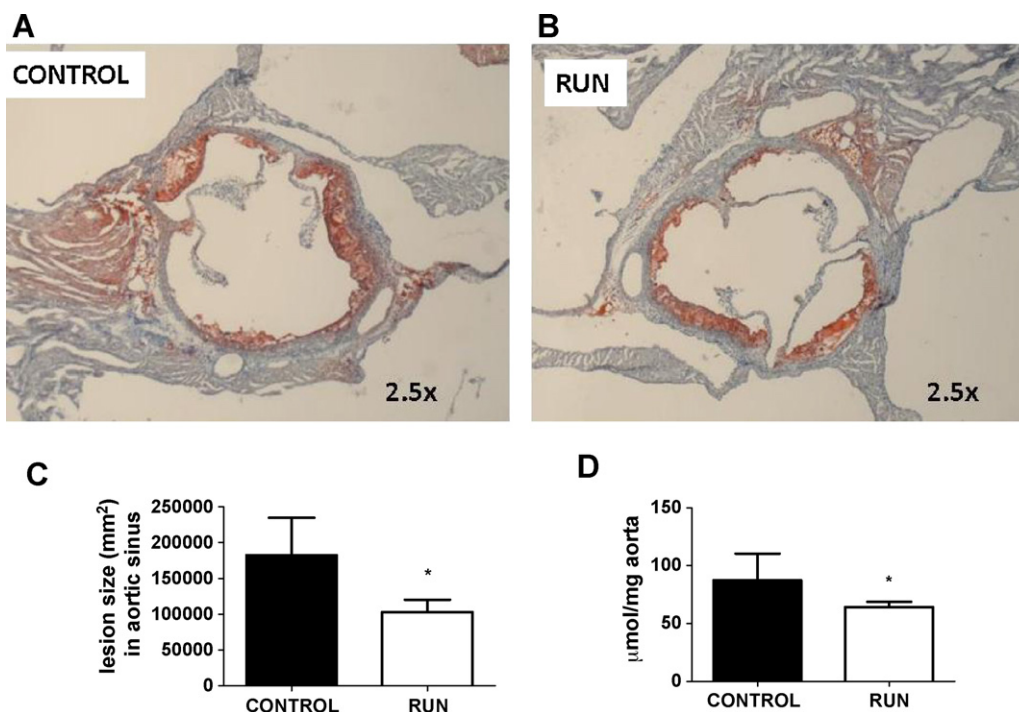
**Fig. 1.** Voluntary wheel running reduces atherosclerotic lesion formation. Representative morphological section of aortic root stained with Oil Red Oil of CONTROL (A) and RUN (B); (C) quantification of lesion size in aortic sinus of CONTROL ($n = 4$) and RUN ($n = 4$) and (D). Total aortic cholesterol content in CONTROL ($n = 7$) and RUN ($n = 7$) at 12 weeks of running. *$p < 0.05$ vs. CONTROL.

### 3.4. Voluntary wheel running increased fecal sterol output

Next, we assessed whether the running-induced beneficial changes in plasma and liver lipid metabolism were accompanied by changes in cholesterol and bile acid metabolism. First, we assessed fecal parameters of cholesterol and bile acid metabolism and feces were collected quantitatively from all mice during the last 48 h of the experiment. Hypercholesterolemic running mice had significantly increased feces production (+19%, data not shown) as well as fecal neutral sterol (+33%) and fecal bile acid output rates (+93%) compared to sedentary controls (Table 4). No major differences

in fecal bile acid composition (Supplemental Table 1) and any of these parameters were observed before the running wheel intervention (data not shown). Next, we investigated whether voluntary wheel running increased fecal neutral sterol output by modulating cholesterol absorption. Running had no effect on the plasma plant sterol/cholesterol ratio (Supplemental Fig. 4A), a marker of cholesterol absorption. No effect of voluntary wheel running was found on jejunal Npc1l1 mRNA expression (Supplemental Fig. 4B), while, intriguingly, the expression of the ATP-cassette binding transporters g5 and g8 (Supplemental Fig. 4C and D), which are known to promote efflux of cholesterol and plant sterols from the enterocyte back into the intestinal lumen for elimination into feces, was increased.

### 3.5. Voluntary wheel running increases bile flow and biliary bile acid secretion

To evaluate whether physical activity modulates biliary parameters under hypercholesterolemia, mice were subjected to gallbladder canulations for collection of hepatic bile at 12 weeks of running. Indeed, running mice had a 24% increased bile flow

**Table 3**
Hepatic genes involved in lipid metabolism.

|  | CONTROL | RUN | $p$-value |
|---|---|---|---|
| Cholesterol metabolism |  |  |  |
| Clearance |  |  |  |
| Lpl | $1.0 \pm 0.2$ | $1.6 \pm 0.1$* | 0.006 |
| Production |  |  |  |
| Mttp | $1.0 \pm 0.3$ | $0.7 \pm 0.2$* | 0.029 |
| Hmgcr | $1.0 \pm 0.3$ | $0.6 \pm 0.1$* | 0.020 |
| Acat2 | $1.0 \pm 0.2$ | $0.7 \pm 0.1$* | 0.024 |
| Efflux |  |  |  |
| Abca1 | $1.0 \pm 0.3$ | $1.1 \pm 0.3$ | 0.061 |
| Abcg5 | $1.0 \pm 0.2$ | $0.9 \pm 0.2$ | 0.426 |
| Abcg8 | $1.0 \pm 0.1$ | $1.0 \pm 0.1$ | 0.420 |
| Fat metabolism |  |  |  |
| Fasn | $1.0 \pm 0.2$ | $0.7 \pm 0.1$* | 0.048 |
| Scd1 | $1.0 \pm 0.3$ | $0.6 \pm 0.1$* | 0.029 |

Hepatic mRNA expression levels of lipoprotein lipase (Lpl), microsomal triglyceride transfer protein (Mttp), 3-hydroxy-3-methyl-glutaryl-CoA reductase (Hmgcr), acetyltransferase 2 (Acat2), ATP-binding cassette transporter 1 (Abca1), ATP-binding cassette transporter g5 (Abcg5), ATP-binding cassette transporter 8 (Abcg8), fatty acid synthase (Fasn), stearoyl-CoA desaturase-1 (Scd1), Values are relative to β-actin and represent mean ± SD in CONTROL ($n = 7$) and RUN ($n = 7$).
* $p < 0.05$ vs. CONTROL.

**Table 4**
Fecal and biliary parameters.

|  | CONTROL | RUN |
|---|---|---|
| Fecal outputs (μmol/24 h/100 g BW) |  |  |
| Neutral sterols | $47.8 \pm 5.7$ | $64.0 \pm 4.1$* |
| Bile acids | $5.3 \pm 1.6$ | $10.2 \pm 3.1$* |
| Biliary secretions (μmol/24 h/100 g BW) |  |  |
| Cholesterol | $0.9 \pm 0.3$ | $1.6 \pm 0.9$ |
| Bile acids | $249 \pm 49$ | $417 \pm 134$* |
| Phospholipids | $26 \pm 3$ | $25 \pm 4$ |
| Bile flow (ml/24 h/100 g BW) | $6.1 \pm 1.1$ | $7.6 \pm 1.1$* |

Values represent mean ± SD at 12 weeks of running for CONTROL ($n = 7$) and RUN ($n = 7$).
* $p < 0.05$ vs. CONTROL.

($p = 0.011$), a 67.5% increase in biliary bile acid secretion and a trend towards increased biliary cholesterol secretion ($p = 0.179$), while no differences in the rate of biliary phospholipid secretion was found (Table 4). Compared to sedentary mice, running mice displayed an increase biliary secretion of cholate-derived bile acids and tended to decrease in chenodeoxycholate-derived bile acids (Supplemental Table 1). Moreover, no differences in the expression levels of important genes involved in cholesterol efflux from the hepatocyte towards the plasma (Table 3) were observed. Our data suggest that voluntary wheel running increases cholesterol turnover to promote its fecal excretion as cholesterol and bile acids, indicating increased cholesterol excretion out of the body in the absence of changes in endogenous cholesterol synthesis.

## 4. Discussion

In the present study, we tested the hypothesis that voluntary wheel running ameliorates atherosclerosis possibly by modulating cholesterol metabolism. By using hypercholesterolemic LDLR-deficient mice on a western-type diet, we were able to show for the first time that voluntary wheel running provokes specific changes in cholesterol metabolism, particularly by promoting its conversion into bile acids likely contributing to reduced plasma lipid levels, and that these alterations coincide with a reduction in atherosclerosis.

First, we show running-induced increases in fecal neutral sterol and bile acid excretion. The fecal bile acid loss in running mice is massive and reflective of an increased *de novo* bile acid synthesis. Second, we found that the increased fecal bile acid loss was paralleled by specific changes in biliary parameters consistent with an increased cholesterol turnover. Specifically, running mice had a higher bile flow, an increased biliary bile acid secretion and a trend towards an increased biliary cholesterol secretion. Third, hepatic cholesterol content was reduced in running mice, indicating cholesterol turnover over storage.

To the best of our knowledge, this is the first study linking the effects of voluntary exercise on sterol metabolism with atherosclerotic lesion development. While we show here a 33% reduction in atherosclerotic lesion size upon voluntary wheel running, previous studies in LDLR-deficient mice undergoing forced exercise, like treadmill running or swimming, reported a reduction by ∼40% [4,24]. Additionally, reductions of atherosclerotic lesion size of ∼54–30% have been observed in another hypercholesterolemic mouse model, the ApoE-deficient mouse, when forced to swim for different durations [5,7,25]. Moreover, using a different atherosclerotic mouse model and a different study design to focus on atherosclerosis regression even a reduction in pro-inflammatory markers was reported [1], however, no literature is available describing the effects of exercise (forced or voluntary) on plasma pro-inflammatory markers in atherosclerosis development in mice. We show here that voluntary wheel running reduced atherosclerosis with no concomitant improvements in inflammatory markers. However, due to the study design we were not able to measure inflammatory markers at earlier time points. Further, in contrast to another study exposing ApoE-deficient mice to a long-time swim training [26] no differences in parameters of plaque stability were observed in our study at 12 weeks of voluntary running (percentage of collagen content in lesions was $21.4 \pm 8.2\%$ for CONTROL and $24.2 \pm 8.8\%$ for RUN; percentage of smooth muscle cells content in lesions was $21.8 \pm 9.2\%$ for CONTROL and $26.3 \pm 4.1\%$ for RUN (mean $\pm$ SD)). Differences in the respective study design (duration of the exercise intervention) and exercise protocols (voluntary vs. forced) likely account for these discrepancies.

The mechanisms behind the beneficial effects of exercise on atherosclerosis are not yet understood. Being a major physiological process for the body to clear excess cholesterol, the fecal excretion of cholesterol as neutral sterol or bile acid plays a critical role in the maintenance of whole-body cholesterol homeostasis. Increasing cholesterol excretion into feces as neutral sterol or bile acid is long known as an efficient strategy in the amelioration of atherosclerosis, as it improves the pro-atherogenic state by reducing lipid content in plasma [8,9]. Furthermore, it has been demonstrated that patients with coronary artery disease have a reduced fecal excretion of bile acids [27]. Strikingly, studies describing the effects of physical activity on the enterohepatic metabolism of sterols in hypercholesterolemic mice and men are thus far lacking. However, the fecal bile acid loss upon running observed in our study parallels earlier work in healthy humans. One initial study [28], not specifically designed to elucidate the effects of exercise on sterol metabolism did not find an effect of exercise on fecal sterol excretion. But subsequent studies specifically designed to investigate the impact of exercise on fecal sterol excretion in humans indicated that exercise increased feces production and fecal sterol output [29,30], consistent with the mouse data of our present study.

Our observations of running-induced increases in fecal neutral sterol and bile acid outputs also confirm our earlier results in chow-fed mice running for 2 weeks [12]. It is, however, noteworthy that the effects are more striking in hypercholesterolemic mice running for 12 weeks. For example, while we found a ∼30% increase in both fecal neutral sterol and bile acid outputs in chow-fed mice upon 2 weeks running, we report here a similar increase in fecal neutral sterol loss in 12 week running hypercholesterolemic mice (∼33%) yet because the diet already contained 0.25% the amount of extra cholesterol excreted by running is massive. In addition there was a strong increase in fecal bile acid secretion (∼93%) compared to 30% in control mice. This increase in fecal bile acid excretion in running mice is remarkable and reflects an increase in *de novo* bile acid synthesis. Intriguingly, we observed a negative correlation between atherosclerotic lesion size and fecal neutral sterol output ($r = -0.602$; $p = 0.024$) and a strong negative correlation between atherosclerotic lesion size and fecal bile acid output ($r = -0.88$; $p = 0.007$), indicating that there is a crosstalk between the reduction in atherosclerosis and the increase in cholesterol turnover.

Consistent with our previous study [12] in chow-fed mice, we did not observe changes in any of the major genes involved in bile acid synthesis in running mice (data not shown). Thus, within this and our previous study, the increase in bile acid synthesis upon voluntary wheel running as demonstrated by fecal bile acid loss did not result in an upregulation of key genes involved in bile acid synthesis, such as Cyp7a1 and Cyp8b1 indicating that regulation via the nuclear receptor FXR and its downstream target FGF15 is not operational here. Increases in *de novo* bile acid synthesis without concomitant increases in any of the major bile acid genes have also been reported by others [31,32] and are most likely induced by posttranscriptional mechanisms. It is speculative, therefore, that physical activity enhances bile acid synthesis by metabolic mechanisms. For example, physical activity increased dietary fatty acid absorption (data not shown). During physical activity, energy is depleted in energy expending tissues, such as skeletal muscle. Thus, a high demand to recover this expended energy manifests in these tissues. In this regard, physical activity might, hypothetically, increase bile acid synthesis to increase the capacity for micelle formation, thereby fatty acid absorption and thus energy delivery to energy expending tissues.

Paralleling our observations of fecal bile acid excretion, running appears to induce more drastic effects on biliary parameters in hypercholesterolemic mice than in chow-fed healthy mice. Compared to control mice, the increase in biliary bile acid secretion was ∼20% in healthy chow-fed mice running for 2 weeks [12]. Furthermore, we report here an increase in bile flow, which was not observed in running chow-fed mice, but has been previously reported in exercising rats [33].

Next, it is intriguing that the livers of running mice display ∼20% less cholesterol stores and ∼45% less triglyceride stores than control mice do. Control LDLR-deficient mice on a western-type diet display hepatic triglyceride and cholesterol contents more than 2.5 times that of chow-fed wildtype mice [12]. Yet, despite their increased food intake, running mice had significantly reduced hepatic cholesterol and triglyceride stores demonstrating an enhanced turnover rather than their storage. No data are available describing the effects of exercise, either forced or voluntary, on hepatic lipids in hypercholesterolemic mouse models. Yet, limited data show that high fat and low fat-fed wildtype mice displayed reduced hepatic triglycerides levels after treadmill exercise training [34] and that swim training reduced hepatic fatty acid synthesis in C57BL/6J mice [35]. Moreover, we previously found reduced hepatic triglyceride content and a trend towards reduced hepatic cholesterol content in chow-fed mice running for 2 weeks [12]. Collectively these observations demonstrate favorable effects of physical activity on hepatic lipid storage.

We have previously reported indications for impaired cholesterol absorption in chow-fed C57BL/6J mice exposed to a voluntary running wheel as the jejunal expression levels of a crucial protein in cholesterol absorption, Npc1l1, and the plasma plant sterol/cholesterol ratio were decreased in running mice [12]. In contrast, we did not observe an effect on jejunal Npc1l1 expression nor on the plasma plant sterol/cholesterol ratio here. In contrast, we show a running-induced increased expression of jejunal Abcg5/8 the heterodimer cholesterol efflux transporter implicated in the excretion of cholesterol from the intestine [36]. Thus, running appears to differentially affect parameters of cholesterol absorption under low dietary cholesterol, normo-cholesterolemic *versus* high dietary cholesterol, hypercholesterolemic conditions. It is also possible that the previously observed running-induced decreases in expression of Npc1l1 upon 2 weeks of running might underlie transient adaptations in the intestine, which could be modulated further during longer periods of running.

Noteworthy is that we also found improvements in plasma cholesterol levels and plasma lipoprotein profile and thereby reduced atherosclerotic lesion formation in running mice. Similar small improvements in plasma cholesterol levels have previously been reported in swimming [4] and treadmill-running [24] LDLR-deficient mice and may underlie at least part of the antiatherosclerotic effect.

Intriguing is the running-induced improvements in plasma lipoprotein profiles, showing a marked reduction in the apoB-containing lipoprotein particles VLDL and IDL/LDL. Decreased LDL and VLDL levels have been reported for physically active men [37], however, no such descriptions are available in hypercholesterolemic mice. Furthermore, the improved plasma lipoprotein profiles parallel the running-induced increase in hepatic lipoprotein lipase expression levels and the running-induced decrease in hepatic microsomal transfer protein, suggesting an increased lipoprotein clearance and decreased production, respectively. However, what exactly the role of running in lipoprotein clearance and reduced production is and how this relates to the human situation remains to be explored in future studies.

An interesting observation of this study was the progressive drop in running wheel activity during the experimental period which does not occur in wildtype mice at 20 weeks of age [38]. However, a progressive drop in running wheel activity during the last 4 weeks of an 8 week intervention has previously also been observed in ApoE-deficient mice that started running at 20 weeks of age [1] but has not been published thus far in the model used in the current study, the LDLR-deficient mouse.

Altogether, the present study shows that voluntary wheel running is a feasible means to decrease atherosclerotic burden in hypercholesterolemic mice and that an enhanced turnover of

cholesterol into bile acids might be the underlying mechanism herein.

## Disclosure

The authors have nothing to disclose.

## Acknowledgment

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.06.040.

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