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The effect of exercise training on cholesterol and bile acid metabolism in ovariectomized rats

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Résumé

Il existe un nombre grandissant de preuves au cours des dernières années que la diminution de la sécrétion des œstrogènes chez les animaux ovariectomisés (Ovx) et chez les femmes ménopausées conduit à une accumulation importante de triglycérides (TG) dans le foie. Cependant, les évidences de perturbations dans le métabolisme du cholestérol, en lien avec la diminution des œstrogènes, sont limitées à des observations de niveaux élevés de cholestérol total dans le plasma trouvés chez la femme ainsi que chez les animaux. En fait, l'impact de la suppression des œstrogènes sur le métabolisme du cholestérol dans le foie a reçu peu d'attention et montre quelques controverses. Par conséquent, les trois études présentées dans cette thèse ont été réalisées chez des rats Ovx, comme modèle animal de femmes post-ménopausées, afin de documenter les effets du retrait des œstrogènes sur les marqueurs moléculaires clés du métabolisme du cholestérol et des acides biliaires dans le foie et dans l'intestin et des effets potentiels de l'entraînement physique. Il a été en effet démontré que l'entraînement physique peut réduire le niveau plasmatique de cholestérol. Une amélioration du transport du cholestérol en périphérie vers le foie pour sa sécrétion subséquente dans la bile et pour son l'excrétion de l'organisme a été suggérée, bien que les mécanismes sous-jacents ne soient pas entièrement compris.

Dans la première étude, nous avons démontré que les rattes Ovx nourris avec une diète standard et une diète standard + cholestérol avait un taux de cholestérol total dans le foie plus élevé ($P < 0,05$) que les rattes avec une ovariectomie simulée (Sham) nourris avec ces deux derniers types de diète, tandis que la teneur en triglycérides du foie était plus élevée chez les rattes Ovx que chez les rattes Sham nourris avec une diète standard, une diète standard + cholestérol et aussi une diète riche en grasses + cholestérol. Étonnement, la diète standard + cholestérol a été associée à un niveau plasmatique plus faible ($P < 0,001$) de cholestérol total et de triglycérides chez les rats Ovx que les rats Sham, ce qui suggère une diminution de la sécrétion de lipoprotéines à très basses densités (VLDL). Par conséquent, la transcription de plusieurs marqueurs clés de la synthèse des VLDL, y compris la microsomal triglyceride transfer Protein (MTP) et apoB-100, ont été réduites ($P < 0,05$) chez les rattes Ovx par rapport aux rattes Sham nourris avec tous les trois types diètes et cette diminution de MTP et apoB-

100 était plus prononcée chez les rats nourris avec la diète standard + cholestérol. Pour aller un peu plus loin, dans la deuxième étude, nous avons déterminé les effets de l'entraînement physique sur les marqueurs clés hépatiques de la voie farnesoid X receptor (FXR) - small heterodimer partner (SHP) - de cholestérol 7 alpha-hydroxylase (CYP7A1) (FXR-SHP-CYP7A1) impliquée dans la conversion de cholestérol en acides biliaires et de leur excrétion chez les rat Ovx nourris avec une diète standard + cholestérol.

Notre groupe expérimental principal comprenait des rats Ovx nourris avec une diète riche en cholestérol (Ovx-Chol). Ce groupe a été comparé à un groupe de rats Ovx nourris avec une diète standard (Ovx-SD) et un groupe de rats Sham nourris avec une diète riche en cholestérol (Sham-Chol) pour observer, respectivement, l'effet de l'alimentation et l'effet du retrait de l'œstrogène. Les résultats de cette étude ont démontré que les niveaux de cholestérol total dans le plasma et dans le foie ne sont pas affectés par l'entraînement physique dans aucune des conditions expérimentales. L'alimentation en cholestérol a induit une accumulation plus importante chez les rats Sham et Ovx a mené à une accumulation du cholestérol dans le foie significativement plus élevée ($P < 0,001$) que chez les rats Ovx-SD. Un effet principal d'entraînement physique ($P < 0,05$) a été trouvée dans l'expression génique du SHP et de CYP7A1. Ce dernier gène est reconnu pour son implication majeure sur le contrôle de la biosynthèse des acides biliaires à partir du cholestérol. De plus, cette étude a montré que le récepteurs des LDL (LDL-R) et proprotein convertase subtilisin/kexin type 9 (PSCK9) au foie, qui sont impliqués dans l'absorption du cholestérol de la circulation, ne sont pas influencés par l'entraînement physique. Ces résultats suggèrent que l'entraînement physique module le métabolisme du cholestérol chez les animaux Ovx par un réglage positif de la formation des acides biliaires. Un nombre croissant de preuves récentes suggèrent que le transport inverse du cholestérol (RCT) peut également passer par une voie non-biliaire connue sous le nom « transintestinal cholesterol excretion » (TICE). En effet, le foie et l'intestin sont impliqués dans l'excrétion du cholestérol excédentaire du corps. Dans cette optique, dans la troisième étude, nous avons élargi nos recherches afin de déterminer si l'entraînement physique module l'expression des récepteurs de cholestérol de la membrane intestinale qui sont impliqués dans TICE chez les rats intacts et Ovx nourris avec une diète standard et une diète riche en cholestérol. Les résultats de cette étude ont montré que l'entraînement physique a augmenté (P

<0,01) l'expression génique intestinale de LDL-R et de PCSK9 impliquées dans la captation du cholestérol intestinal de la circulation et de leur récepteur nucléaire, « sterol regulatory element-binding protein 2 » (SREBP2) (P <0,05) chez les rats Sham et Ovx par rapport aux rats sédentaires (Sed). D'autre part, l'expression des gènes hépatiques de LDL-R et de PCSK9 ont été supprimées (P <0,01) par l'alimentation riche en cholestérol, mais pas affectée par l'entraînement physique. L'expression du gène « flavin monooxygénase 3 » (FMO3), en tant que régulateur de l'équilibre du cholestérol dans le foie, a été diminuée de façon significative (P <0,01) par le cholestérol alimentaire chez les rats Sham et Ovx par rapport aux rats nourris avec la diète standard, mais demeure inchangée suite à l'entraînement physique et le retrait des œstrogènes. Un réglage positif de l'expression de gènes du LDL-R et PCSK9 intestinale par l'entraînement physique chez les rats intacts et Ovx suggère que l'entraînement physique peut contribuer à l'accroissement de l'élimination de cholestérol par la voie TICE.

Dans l'ensemble, nos résultats indiquent qu'une combinaison d'une diète riche en cholestérol et un retrait des œstrogènes a mené à une diminution de l'expression des gènes des marqueurs essentiels de la synthèse de VLDL, ce qui implique une réduction de l'excrétion du cholestérol du foie. Il semble que la réduction de LDL-R hépatique pourrait être due à l'accumulation du cholestérol dans le foie. De plus, nos résultats ont présenté l'entraînement physique comme une intervention non pharmacologique appropriée pour stimuler l'excrétion du cholestérol excédentaire de l'organisme par le réglage positif des gènes impliqués dans la biosynthèse des acides biliaires dans le foie et les récepteurs intestinaux de cholestérol dans la voie TICE.

Mots-clés : Ovariectomie, Rat, Diète riche en cholestérol, l'assemblage VLDL, LDL-R, PCSK9, Accumulation de cholestérol dans le foie, Entraînement physique, CYP7A1, la voie TICE.

Abstract

There has been accumulating evidence in recent years that the estrogen deficient state in ovariectomized (Ovx) animals and in postmenopausal women results in substantial liver triglyceride (TG) accumulation. However, evidence of disturbances in cholesterol metabolism in link with estrogen deficiency is limited to observations of higher plasma total cholesterol levels found in human as well as in animals. In fact, the impact of estrogen withdrawal on liver cholesterol metabolism has received little attention and shows some controversies. Therefore, the three studies presented in this thesis have been conducted in Ovx rats, as an animal model of post-menopausal women, to investigate the effects of estrogen withdrawal on key molecular markers of cholesterol and bile acid metabolism in liver and in transintestinal cholesterol excretion (TICE), and also to determine the potential role of exercise training as a positive alternative intervention. It has been shown that exercise training can improve plasma cholesterol levels. An enhanced transport of peripheral cholesterol toward the liver for subsequent secretion into bile and excretion from the body has been suggested; however, the underlying mechanism for this action is not fully understood.

In the first study, we showed that estrogen withdrawal was associated with higher ($P < 0.05$) liver total cholesterol under the standard diet and the standard diet + cholesterol diet, while liver triglyceride (TG) content was higher in Ovx than in Sham rats in all three dietary conditions which are the standard diet, the standard diet + cholesterol and the high fat diet + cholesterol. Surprisingly, the standard diet + cholesterol was associated with lower ($P < 0.001$) plasma total cholesterol and TG levels in Ovx than in Sham rats, suggesting a decrease in very low-density lipoprotein (VLDL) secretion. Accordingly, several transcripts of key markers of VLDL synthesis including microsomal triglyceride transfer protein (MTP) and apoB-100 were decreased ($P < 0.05$) in Ovx compared to Sham rats under the three dietary conditions and even more so for MTP and apoB-100 when rats were fed the standard diet + cholesterol. To go one step further, in the second study we determined the effects of exercise training on hepatic key markers of farnesoid X receptor (FXR)-small heterodimer partner (SHP)-cholesterol 7 alpha-hydroxylase (CYP7A1) (FXR-SHP-CYP7A1) pathway, involved in cholesterol conversion into bile acid and excretion from the body, in Ovx cholesterol fed rats. Our main

experimental group was Ovx rats fed a high cholesterol diet (Ovx-Chol) that was compared, on one hand, to a group of Ovx rats fed a standard diet (Ovx-SD) to observe the effects of the diet and, on the other hand, compared to a group of Sham operated rats fed the cholesterol diet (Sham-Chol) to observe the effect of estrogen withdrawal. Results of this study showed that plasma and liver total cholesterol levels were not affected by exercise training in any of the experimental conditions. Cholesterol feeding in both Sham and Ovx rats resulted in significantly (P<0.001) higher hepatic cholesterol accumulation than in Ovx-SD rats. A main effect of training (P< 0.05) was, however, found for transcripts of SHP and CYP7A1. The SHP and CYP7A1 transcripts were increased by training. These results suggest that exercise training through up-regulation of genes involved in bile acid formation may modulate cholesterol metabolism in Ovx animals. Finally, a recent growing body of evidence suggests that reverse cholesterol transport (RCT) can also proceed through a non-biliary pathway known as transintestinal cholesterol excretion (TICE). Indeed, both liver and intestine are involved in excretion of the excess cholesterol from the body. Based on this concept, we expanded our research to determine whether exercise training has an effect on intestinal membrane cholesterol receptors involved in TICE pathway in intact and Ovx rats fed a normal and a high cholesterol diet. Results of the third study showed that exercise training increased (P< 0.01) transcripts of intestinal LDL-R and PCSK9, which are involved in intestinal cholesterol uptake from circulation, and their nuclear transcription factor, intestinal sterol regulatory element-binding protein 2 (SREBP2) (P< 0.05) in both Sham and Ovx rats compared to rats remaining sedentary (Sed). On the other hand, hepatic LDL-R and PCSK9 gene expression was suppressed (P< 0.01) by cholesterol feeding but not affected by exercise training. Flavin monooxygenase 3 (FMO3) gene expression, as a cholesterol balance regulator in liver, was significantly decreased (P<0.01) by cholesterol feeding in both Sham and Ovx rats compared to rats were fed the SD diet but unchanged following exercise training and estrogen withdrawal. An up-regulation of intestinal gene expression of LDL-R and PCSK9 following voluntary wheel running in intact and Ovx rats suggests that exercise training may contribute to increased cholesterol elimination through the TICE pathway.

Overall, our results indicate that a high cholesterol diet and ovariectomy combine to decrease the gene expression of key markers of VLDL synthesis suggesting a reduction in

cholesterol excretion from the liver. Alternatively, it seems that reduced hepatic LDL-R transcript found in Ovx animals might be due to hepatic cholesterol accumulation. Moreover, our findings introduced exercise training as an appropriate non-pharmacological intervention to stimulate the excretion of the excess cholesterol from the body through upregulation of genes involved in bile acid biosynthesis in liver and intestinal basolateral cholesterol transporters in TICE.

Keywords: Ovariectomy, Rat, High cholesterol diet, VLDL assembly, LDL-R, PCSK9, Hepatic cholesterol accumulation, Exercise training, CYP7A1, TICE pathway.

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Abbreviations

ABCA1: Adenosine triphosphate binding cassette transporter A1

ABCB1 a/b: ATP-binding cassette transporter B1 a and b

ABCG5/G8: Adenosine triphosphate binding cassette transporters G5/G8

ACAT-2: Acyl-CoA:cholesterol acyltransferase

apoA-1: Apolipoproteins A-1

apoB: Apolipoprotein B

apoER2: Apolipoprotein E receptor 2

ASBT: Apical sodium-dependent bile acid transporter

BSEP: Bile salt export pump

CE: Cholesteryl ester

CEPT: Cholesteryl ester transfer protein

Cideb: Cell death-inducing DNA fragmentation factor alpha (DFFA)-like effector B

Chol: Cholesteryl

CPF: CYP7A1 promoter binding factor

CYP7A1: Cholesterol 7 alpha-hydroxylase

DGAT2: Diacylglycerol acyltransferase 2

E2: 17β-estradiol

ER: Endoplasmic reticulum

ERα: Estrogen receptor α

EX: Exercise

FGF15/19: Fibroblast growth factor 15/19

FGFR4: Fibroblast growth factor receptor 4

FMO3: Flavin monooxygenase 3

FC: Free cholesterol

FXR: Farnesoid X receptor

HC: High cholesterol

HDL: High density lipoprotein

HF: High fat

HFHC: High fat high cholesterol

HMGCoA-r: 3-hydroxy 3-methylglutaryl coenzyme A reductase

LPL: Lipoprotein lipase

iBABP: Ileal bile acid binding protein

IBAT: Ileal bile acid transporter

IDL: Intermediate-density lipoprotein

KO: Knockout

LCAT: Lecithin:cholesterol acyltransferase

LDL: Low density lipoprotein

LDL-C: Low density lipoprotein cholesterol

LDL-R: Low-density lipoprotein receptor

LIMP2: Lysosomal integral membrane protein-2

LRP1: LDL receptor-related protein

LRH-1: Liver related homologue-1

LXR: Liver X receptor

MDR2: Multidrug resistance protein 2

MTP: Microsomal triglyceride transfer protein

NAFLD: Non-alcoholic fatty liver disease

NASH: Nonalcoholic steatohepatitis

NPC1: Niemann-Pick type C1

NPC2: Niemann-Pick type C1

NPC1L1: Niemann-Pick C1-Like 1

NTCP: Na+- dependent taurocholate cotransport peptide

Ostα-Ostβ: Organic solute transporter α and β

Ovx: Ovariectomized

PCSK9: Proprotein convertase subtilisin/kexin type 9

PLTP: Phospholipid transfer protein

PXR: Pregnane X receptor

RCT: Reverse cholesterol transport

Sar1a: Small GTPase a

Sed: Sedentary

SD: Standard diet

Sham: Sham operated

SHP: Small heterodimer partner

SR-BI: Scavenger receptor class B class I

SREBP-2: Sterol regulatory element binding protein-2

TC: Total cholesterol

TG: Triglyceride

TICE: Transintestinal cholesterol excretion

VLDL: Very low density lipoprotein

VLDLR: Very low-density lipoprotein receptor

WT: Wild type

To my parents & In memory of my Grandmother

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Introduction

Menopause is defined by the progressive decrease of estrogen production resulting in cessation of menses (Mastorakos et al. 2010). Menopause as well as ovariectomy in animals is associated with diverse metabolic consequences including ectopic fat deposition mainly in the liver and this condition is known as non-alcoholic fatty liver disease (NAFLD) (Brunt 2001; Volzke et al. 2007). There has been accumulating evidence in recent years showing that the estrogen deficient state in ovariectomized (Ovx) animals and in postmenopausal women results in substantial liver triglyceride (TG) accumulation, indicating the protective role of estrogens against NAFLD and also perturbation in TG metabolism in the absence of estrogens (Picard et al. 2000; Paquette et al. 2007; Volzke et al. 2007). In addition to TG accumulation, lipidomic analyses indicate that hepatic free cholesterol content was also increased in hepatic steatosis (Puri et al. 2007) suggesting that cholesterol metabolism is also affected in liver diseases. The situation of hepatic cholesterol content in Ovx animals is controversial. It was reported that hepatic total cholesterol content was not affected by estrogen withdrawal in female C57BL/6J mice. Despite the fact that they showed higher plasma cholesterol levels, hepatic cholesterol content was not changed probably due to a reduced liver cholesterol uptake by LDL-R and a reduced hepatic de novo cholesterol production by 3-hydroxy 3-methylglutaryl coenzyme A reductase **(**HMGCoA-r) (Kamada et al. 2011). On the other hand, other studies showed that hepatic total cholesterol content was increased in Ovx rats (Kato et al. 2009; Ngo Sock et al. 2014a) suggesting the vulnerability of Ovx animals to develop hepatic cholesterol accumulation. Liver is known as a master regulator of cholesterol metabolism in terms of cholesterol synthesis, uptake from circulation and excretion from the body. Estrogens through its interaction with HMGCoA-r and LDL-R, genes involved in cholesterol synthesis and uptake respectively, play a critical role in cholesterol homeostasis (Bruning et al. 2003). In fact, the information on the impact of the absence of estrogens on cholesterol metabolism in liver is scarce and is mostly limited to observations of hypercholesterolemia in human as well as in animals. Estrogen deficiency state has been repeatedly reported to result in the development of an atherogenic lipid profile characterized by an increase in plasma total cholesterol (TC) and low density lipoprotein cholesterol (LDL-

C) levels (Matthews et al. 1989; Kimura et al. 2006; Park et al. 2011b; Chaudhuri et al. 2012; Kaur et al. 2013). Elevated plasma concentration of LDL-C is the primary risk factor for coronary artery disease and atherosclerosis, which constitute the largest cause of mortality in developed countries (Mozaffarian et al. 2016). It is important to note that incidence of cardiovascular diseases increases with age in women, with a noticeable increase after menopause (Sharp et al. 1997). This situation raises the hypothesis that perhaps disturbances in different aspects of cholesterol metabolism in liver might be the root of hypercholesterolemia in an estrogen deficient state. Indeed, plasma cholesterol level is tightly determined by a complex homeostatic network which requires the accurate metabolic interplay between hepatic and intestinal processes, to regulate efficiently cholesterol homeostasis (Oram et al. 2006). Recent studies on newly identified transintestinal cholesterol excretion (TICE) pathway have revealed that cholesterol homeostasis in the body depends on a dynamic interplay between liver and intestine (Temel et al. 2012). An interesting intervention to metabolic deterioration due to estrogen deficiency is exercise training. For instance, in an intervention study where Ovx rats were submitted to the treadmill training for 12 weeks, reduced plasma LDL-C and total cholesterol levels were observed (Oh et al. 2007). An increase in fecal cholesterol excretion accompanied by lower plasma cholesterol levels was also reported in exercise trained animals (Meissner et al. 2011). However exactly how exercise exerts such beneficial actions is largely unknown.

These observations highlight the importance of a need for more physiological and molecular information to better understand how liver, as a master regulator of cholesterol metabolism, is affected by estrogens withdrawal. The three studies presented in this thesis have been conducted to provide molecular information on how liver regulates cholesterol metabolism in Ovx rat model of menopause and whether exercise training could provide some beneficial effects on cholesterol metabolism. Rodent ovariectomy is an experimental model of human post-menopausal state. Ovariectomy eliminates the interference of endogenous estrogens and mimics post-menopausal condition which makes it possible to study the metabolic consequences of loss of ovarian functions. On the other hand, ovariectomy can result in metabolic changes in very short period of time, while post-menopausal state is a natural process that happens gradually over several years. We also used the high cholesterol

diet as a nutritional tool to investigate the role of liver in regulating cholesterol metabolism in our series of experiments.

In the first study, we investigated the effects of high dietary cholesterol on hepatic key markers of VLDL and cholesterol/bile acid metabolism in Ovx rats. There is some evidence that exercise training is one of the best non-pharmacological strategies to attenuate hepatic cholesterol accumulation; however, the molecular information on how this action takes place is lacking. In line with this first approach, in the second study we determined the effect of exercise training on key markers of hepatic cholesterol and bile acid metabolism by targeting the FXR-SHP-CYP7A1 gene markers in Ovx rats submitted to the high cholesterol diet. In the third study, we expanded our research to determine the effect of exercise training on key intestinal cholesterol receptors involved in TICE pathway in intact and Ovx rats fed a normal and a high cholesterol diet. We targeted gene expression of key molecules involved in TICE via cholesterol uptake and excretion at the intestinal basolateral and apical membrane, respectively.

The present thesis consists of three chapters. Chapter 1 is devoted to the review of the literature which is divided into two sections. The objective of the first section is to provide the reader with an overview of reverse cholesterol transport (RCT) with an emphasis on the major molecular markers of liver and intestine associated with it. This first section is subdivided into two parts: hepatobiliary pathway, non-hepatobiliary TICE pathway. In the second section, we review the effects of a high cholesterol diet, estrogen withdrawal, and exercise training on RCT by subdividing it in two parts: hepatobiliary and non-hepatobiliary TICE pathways. Chapter 2 introduces the original research articles of this thesis that are presented according to the format required by the journals to which they are published or submitted. Finally, chapter 3 provides a general discussion and conclusion on the findings of the thesis.

Chapter 1: Review of Literature

Cholesterol is an essential key component of vertebrate cell-membrane structure and function. It permits cells to keep their permeability and fluidity which is fundamental for cell viability (Ikonen 2008; Maxfield et al. 2010). In addition to its structural function, cholesterol is a precursor for many substances such as bile acids, steroid hormones and vitamin D (Maxwell et al. 2003). The regulation of cholesterol metabolism relies on a complex homeostatic network, which requires accurate metabolic cross-talks between hepatic and intestinal processes, to regulate efficiently cholesterol homeostasis (Oram and Vaughan 2006). Indeed, both liver and intestine have a key role in cholesterol metabolism, particularly in cholesterol excretion. Consequently, there is an increasing interest in investigating the pathways involved in the elimination of surplus cholesterol from the body. The process of reverse cholesterol transport and cholesterol excretion from the body is an effective way to decrease LDL-C, which eventually declines the risk of atherosclerosis (Temel and Brown 2012).

In the first part of the present review of literature, the pathways and key molecules involved in cholesterol removal from the body both through liver and intestine will be reviewed. In the second part, the effects of the high cholesterol diet, estrogen withdrawal, and exercise training on different aspects of cholesterol and bile acid metabolism in Ovx animal model will be discussed.

1.1 Reverse Cholesterol Transport (RCT)

There are two main excretory pathways for cholesterol disposal from the body, which are named the hepatobiliary and non-biliary route (Temel and Brown 2012). The hepatobiliary route transfers cholesterol from peripheral cells and macrophage foam cells in the artery wall plaque to the liver for secretion (Glomset 1968; Rong et al. 2001), while in the non-biliary pathway the cholesterol is directly secreted into the intestine (Figure 1) (van der Velde et al. 2007). Therefore, both the liver and intestine are involved in cleansing the body from the excess cholesterol, and indeed, both the biliary and non-biliary pathways are part of the reverse cholesterol transport (RCT).

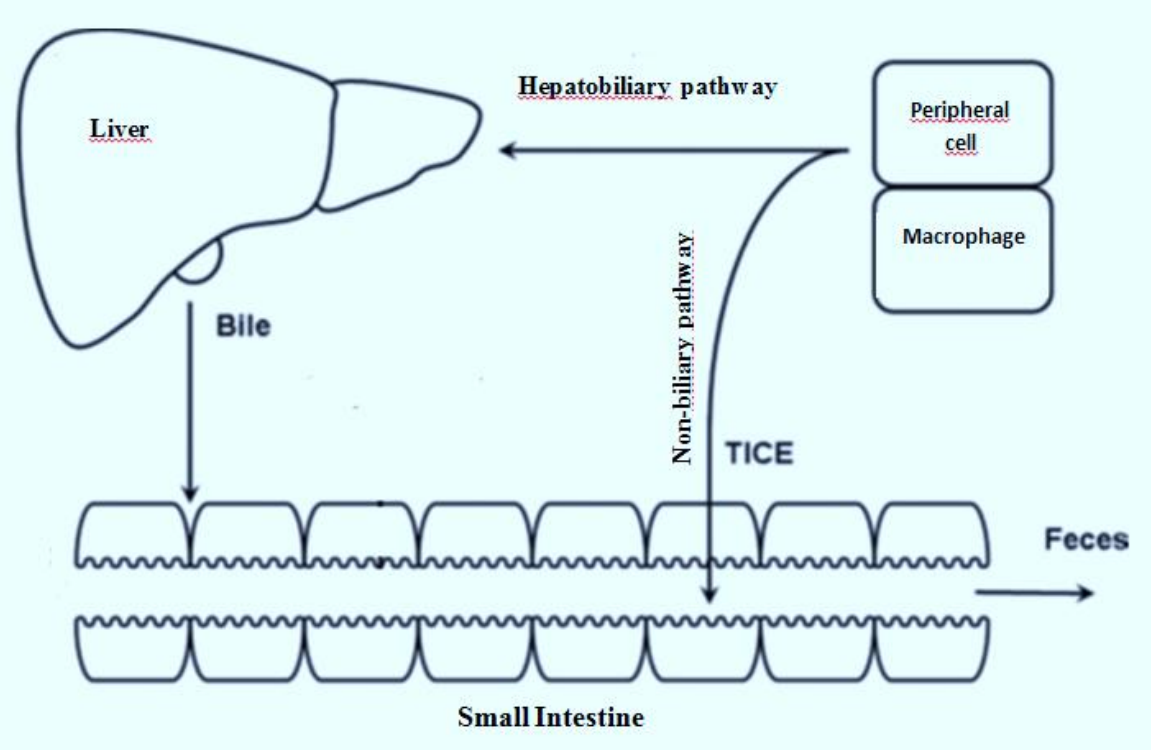

Figure 1. Schematic representation of the main pathways of cholesterol excretion. Adapted from (Brufau et al. 2011).

1.1.1 Hepatobiliary pathway

The classic model of cholesterol secretion, which is called the hepatobiliary pathway, was presented by John Glomset almost 40 years ago (Glomset 1968). In this process, cholesterol, from peripheral tissues and macrophage foam cells in the artery wall plaques, is returned to the liver via high-density lipoproteins (HDLs), for secretion into bile and excretion through the feces (Glomset 1968; Rong et al. 2001).

Different types of molecules such as transporters and/or receptors both in peripheral cells and hepatocytes (e.g., the HDL receptor), apolipoproteins (e.g., apoA1), and plasmatic enzymes (e.g., lecithin:cholesterol acyltransferase (LCAT)) are involved in this process. They are responsible for transporting the cholesterol from peripheral tissues, moving it through plasma, and finally delivering it to the liver for excretion (Jolley et al. 1998). In the following pages, this pathway will be explained in details.

1.1.1.1 Cholesterol influx into liver

HDL is the main lipoprotein involved in removing excess cholesterol from cells and transporting it through the circulation into the liver. The apolipoprotein A1 (apoA1) is the main structural protein component of the HDL. HDL formation starts when apoA1 is synthesized and secreted as a lipid-poor protein mainly by the liver and also to some extent by the intestine (Figure 2) (Oram and Vaughan 2006). ApoA1 interacts with the membrane-embedded ATP binding cassette transporter A1 (ABCA1) and incorporates small amounts of unesterified cholesterol and phospholipids into the apoA1 molecule (Zannis et al. 2006). ABCA1 changes lipid-poor apoA1 to partially lipidated “nascent” lipoproteins. Then, these nascent HDLs become effective acceptors for cholesterol secreted by peripheral and foam cells (Oram and Vaughan 2006; Vaughan et al. 2006). ABCA1 mediates the rate-limiting step in HDL particle formation and maintains plasma HDL levels (Oram and Vaughan 2006). Maturation of these nascent HDLs happen in the circulation through the activity of the enzymes lecithin: cholesterol acyltransferase (LCAT) and phospholipid transfer protein (PLTP). LCAT converts free cholesterol of nascent HDL to cholesteryl ester and PLTP transfers phospholipids from remnant particles to HDL (Jolley et al. 1998). Then, ABC transporters (ABCA1 and ABCG1), in peripheral and foam cells, by interacting with HDL,

transfer the unesterified excess cholesterol (Wang et al. 2004; Tall et al. 2008). ABCG1 is highly expressed in macrophages whereas ABCA1 is more ubiquitous (Oram and Vaughan 2006). Targeted disruption of ABCA1 and ABCG1 resulted in a total ablation of cholesterol efflux *in vitro*, decreased reverse cholesterol transport into feces *in vivo,* and accelerated atherosclerosis (Out et al. 2006; Yvan-Charvet et al. 2007). Moreover, higher expression of ABCG1 was observed in cholesterol-loaded macrophages, providing an explanation for the reverse relationship between HDL levels and risk of atherosclerosis (Wang et al. 2004; Brufau et al. 2011). Mature HDL particles are remodeled by cholesteryl ester transfer protein (CEPT) in the circulation. CEPT facilitates the transport of cholesterol esters (CEs) and TG between the lipoproteins. It transfers CE molecules from HDL to very low density lipoprotein (VLDL) and chylomicron in exchange for TG (Curtiss et al. 2006). Ultimately, cholesteryl esters are delivered to the liver (Jolley et al. 1998).

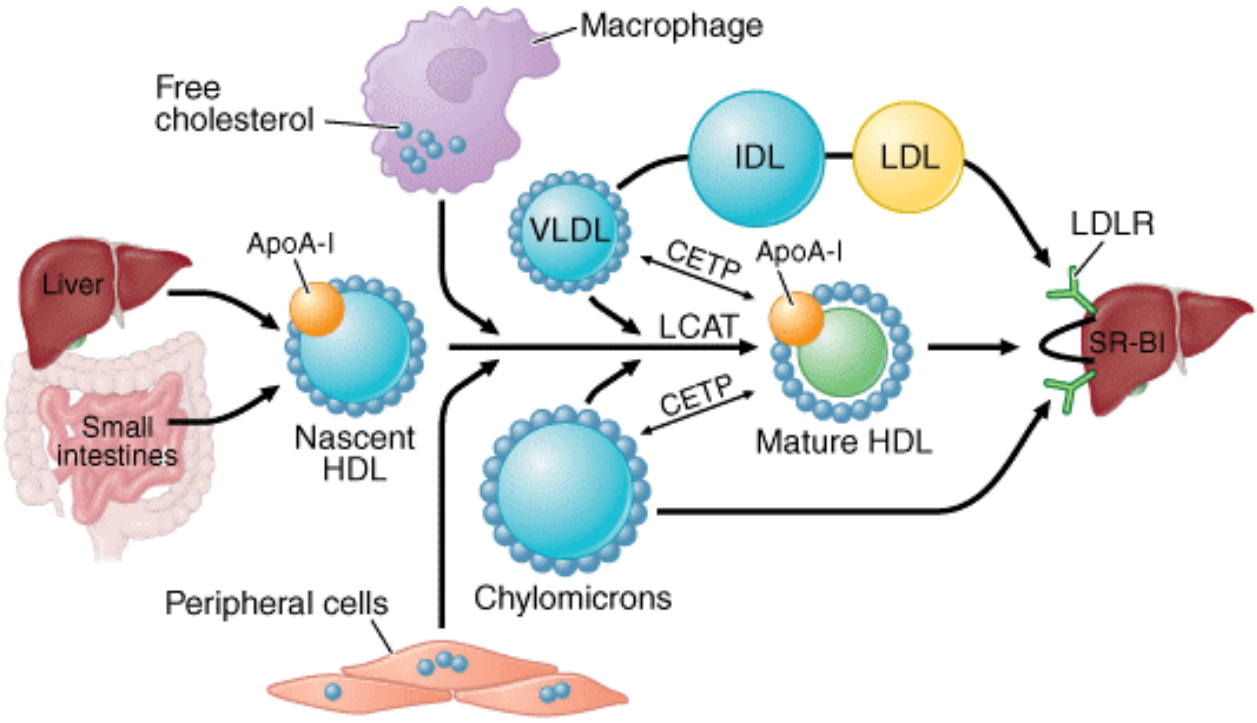

Figure 2. HDL formation and cholesterol influx into liver. Taken from (Wiener et al. 2012)

1.1.1.2 Hepatic cholesterol uptake from circulation

The liver first clears the cholesterol from HDL at the basolateral side of the hepatocytes via scavenger receptor class B type 1 (SR-B1)-dependent selective uptake which is predominant pathway of cholesterol uptake in rodents (Jolley et al. 1998). It is important to note that in CETP containing species like human, monkey and rabbit a large portion of HDL's CE cargo is transferred by CETP to apolipoprotein B (apoB)-containing lipoproteins, which are cleared by the liver through hepatic low-density lipoprotein (LDL) receptors (LDL-R) (Figure 3) (Morton et al. 2014; Temel et al. 2015). Approximately 70% of total LDL-R found in the body are present at the basolateral membrane of hepatocytes. The major function of LDL-R is mainly to bind ApoB and/or ApoE containing lipoproteins, such as LDL, VLDL, and chylomicron remnants to remove the highly atherogenic LDL particles from the circulation (Ikonen 2008). Therefore, hepatic LDLRs have a crucial role in the removal of LDL cholesterol particles from the circulation (Ouguerram et al. 2004). The LDL-R activity is downregulated post-transcriptionally by proprotein convertase subtilisin/kexin type 9 (PCSK9) (Abifadel et al. 2003).

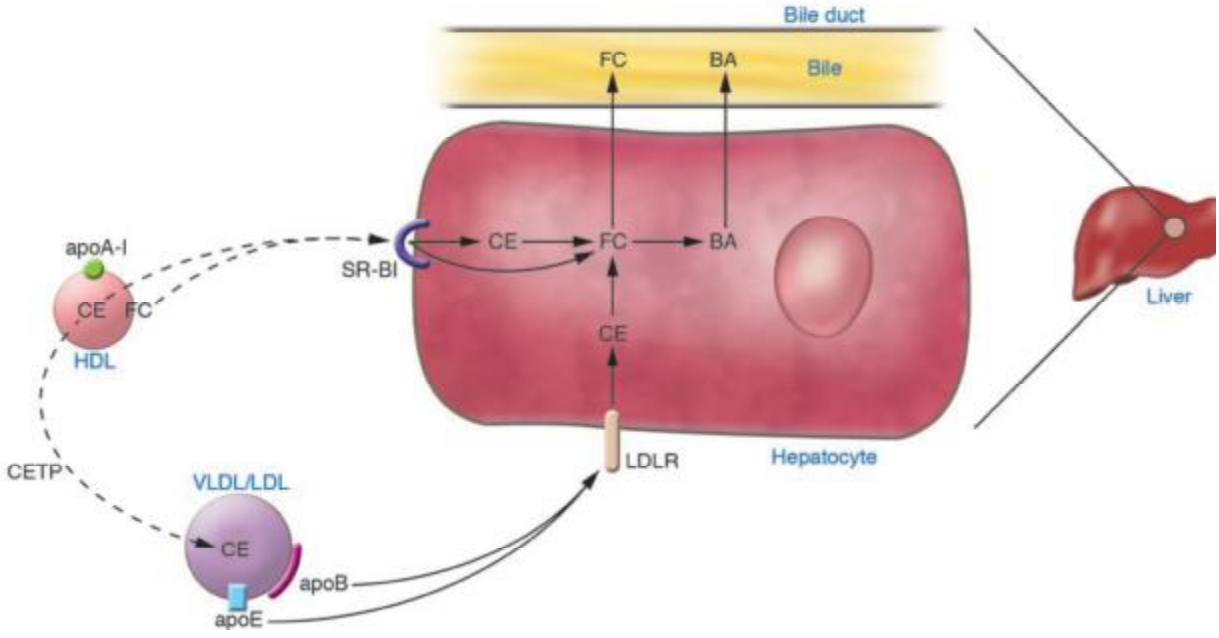

Figure 3. Cholesterol uptake by the liver. Taken from (Rader 2006)

Cholesterol may also be taken up from circulation through LDL receptor-related protein 1 (LRP1). LRP1, as a multifunctional receptor, is expressed in the liver and has close structural and biochemical similarities to LDL-R. LRP1 is responsible for the removal of VLDL remnants (IDL) and chylomicron remnants from the circulation. The lipoprotein remnants are enriched with cholesterol, therefore, their prolonged stay in the bloodstream could be atherogenic as well (Moon et al. 2012). LRP1 thus plays a key role in clearing these atherogenic particles along with LDLRs (Rohlmann et al. 1998). Hepatic LRP1 gene expression has been found to be negatively associated with intracellular cholesterol levels (Moon et al. 2011). Moreover, lower hepatic gene expression of LRP1 has been reported in HepG2 cells in a diabetic condition which was associated with the development of an atherogenic dyslipidemia (Moon et al. 2012). Sterol regulatory element-binding protein 2 (SREBP-2), as a nuclear receptor involved in cholesterol metabolism, regulates the gene expression of LDL-R, PCSK9 and LRP1 in the liver (Moon et al. 2011). When cellular cholesterol levels are low, SREBP-2 are transported to the Golgi, cleaved, and translocated to the nucleus in which gene targets are activated, while cellular cholesterol levels are high, the SREBP-2 remain uncleaved and attached to the endoplasmic reticulum and their gene expression is suppressed (Engelking et al. 2005).

Interaction between PCSK9 and LDL-R

PCSK9, a member of the subtilisin serine protease family, is synthesized by the liver as a precursor in the endoplasmic reticulum (ER), and then transformed to an active protease in the Golgi apparatus and is subsequently secreted into circulation (Seidah et al. 2003). PCSK9 is coded as a natural inducer of LDL-R degradation (Maxwell et al. 2004). PCSK9 binds to the LDL-R, internalizes it, and the receptor along with the LDL particle are subsequently destroyed (Horton et al. 2009). Indeed, the tight binding of PCSK9 to LDL-R and its degradation in lysosome compartments prevents LDL-R recycling to the cell surface (Gent et al. 2004). Reduced LDL-R levels result in a reduction of LDL cholesterol uptake, which could lead to hypercholesterolemia. Therefore, PCSK9 has an important role in cholesterol metabolism (Maxwell et al. 2003). In the absence of PCSK9, the apoB–LDL receptor complex undergoes endocytosis then the LDL-R dissociates from the ligand. The

ligand is sent to the lysosome for degradation, and the LDL-R is recycled back to the cell surface to clear more LDL-C from the circulation (Figure 4) (Gent and Braakman 2004). It is important to note that there are different gene variants of PCSK9 which vary in their affinity for LDL-R and consequently leads to diverse changes in the circulating levels of LDL-C (Cunningham et al. 2007; Kwon et al. 2008). PCSK9 loss-of function (LOF) mutations are associated with lower plasma LDL-C levels and decreased risk of cardiovascular diseases. It means that PCSK9 does not bind LDLR to induce degradation. Therefore, LDLR can return to the cell surface and clear the cholesterol from circulation. Whereas PCSK9 gain-of function (GOF) mutations lead to higher LDL-C levels due to LDLR degradation and consequently, hypercholesterolemia and an increased risk of CVD (Abifadel et al. 2003; Cohen et al. 2006). PCSK9 shares a mutual regulatory pathway with LDL-R through the SREBP-2 (Maxwell et al. 2003). Indeed, both PCSK9 and LDL-R gene expression are up regulated transcriptionally by the transcription factor, SREBP-2 (Smith et al. 1990; Dubuc et al. 2004). Regulation of cholesterol metabolism is mainly modulated by SREBP-2. For instance, high dietary cholesterol prevents maturation of SREBPs and cuts off cholesterol and LDL receptor synthesis (Goldstein et al. 2006).

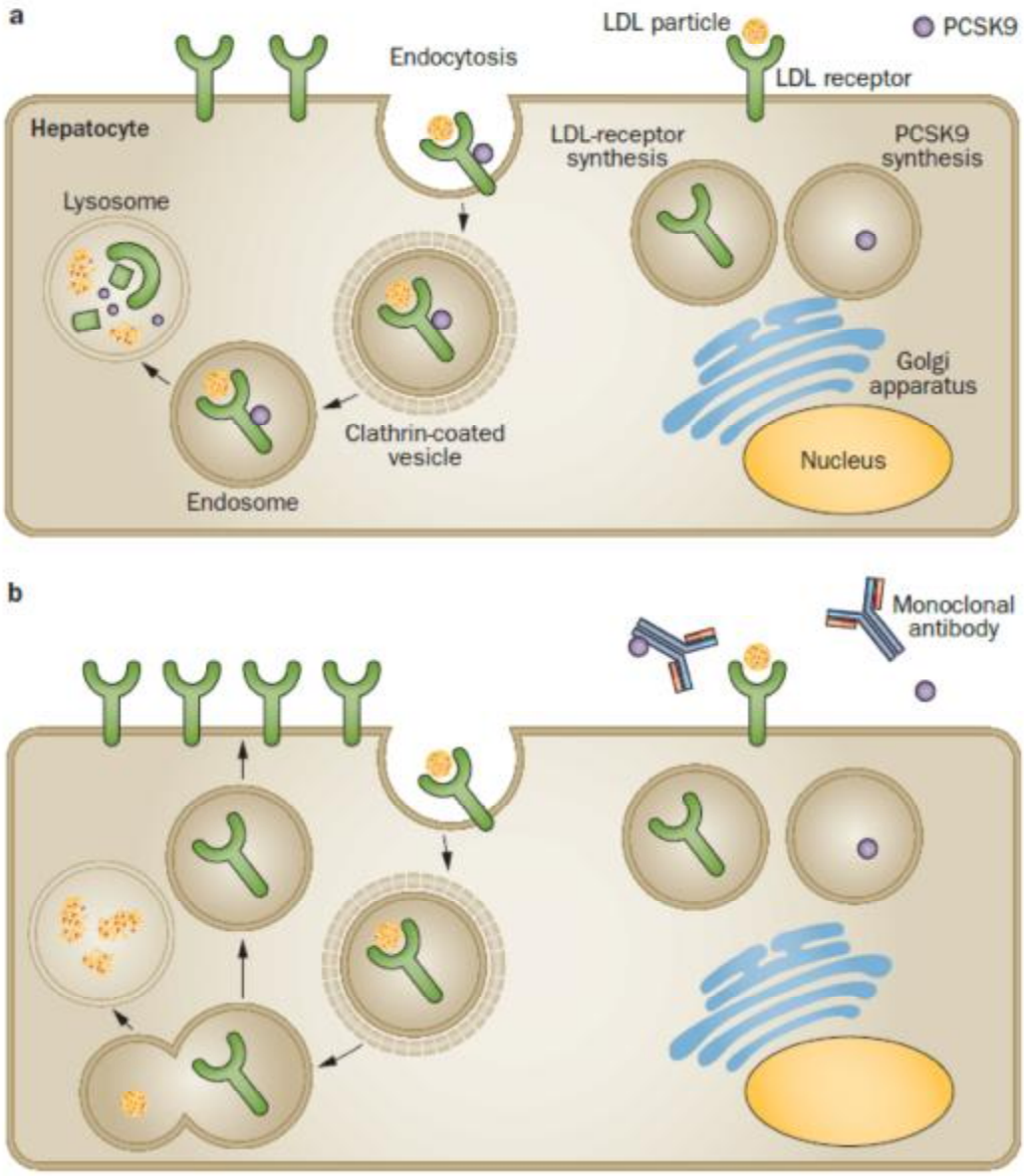

Figure 4. LDL-cholesterol metabolism in the presence (a) or absence of PCSK9 (b). Taken from (Dadu et al. 2014).

Transcriptional control of cholesterol levels in the liver

Cholesterol synthesis, uptake and clearance from the body are chiefly regulated via two nuclear receptors, sterol regulatory element binding proteins (SREBPs) and liver X receptors (LXRs) in the liver (Ikonen 2008).

The molecular mechanism of how hepatocytes maintain cholesterol homeostasis has become more precise with the discovery of the transcription factors sterol regulatory element binding proteins (SREBPs) (Weber et al. 2004). Sterol regulatory elements (SREs) are nucleotidic sequences in the gene promoters, encoding proteins involved in cholesterol homeostasis such as HMGCoA-r and LDL receptor (LDL-R). These sequences are recognized by a family of transcription factors called SREBP. The SREBP family members, SREBP-1 (a and c) and SREBP-2, are synthetized as membrane protein in the endoplasmic reticulum. SREBP-2 is considered to be largely involved in the regulation of cholesterol metabolism (Goldstein et al. 2006)

SREBP2 modulates cholesterol metabolism through the activation of transcription of genes involved in cholesterol synthesis and liver uptake from the circulation (Goldstein et al. 2006). 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMGCoA-r) catalyzes the rate limiting step in cholesterol synthesis in the liver and its gene expression is regulated by SREBP2 (Horton et al. 1998). As mentioned above, LDLR, PCSK9, and LRP1 transcripts are also regulated by nuclear factor SREBP2.

LXR acts as whole body cholesterol sensors. Oxysterols as ligands activate LXR and its activation contributes in reverse cholesterol transport through stimulation of transcripts involved in cellular cholesterol efflux and hepatic cholesterol secretion (Tontonoz et al. 2003). ABC transporters have been identified as LXR target genes, including ABCA1, ABCG1, and also ABC transporters G5/G8 (ABCG5/G8). The ABCA1 and ABCG1 play a critical role in the efflux of excess cellular cholesterol to apoA1, the first step in reverse cholesterol transport. The ABCG5/G8 proteins form a dimer that resides in the apical membrane of the hepatocyte and functions to pump cholesterol into bile (Venkateswaran et al. 2000; Tontonoz and Mangelsdorf 2003). In addition to ABC transporters, cholesterol 7 alpha-hydroxylase

(CYP7A1) is also an LXR target gene. CYP7A1 catalyzes the rate-limiting step in bile acid synthesis in the liver (Peet et al. 1998).

1.1.1.3 Cholesterol excretion from the liver

A large portion of cholesterol delivered to the liver can be directly discarded via ABCG5/G8 in the bile canaliculus (Berge et al. 2000). The presence of a biliary acceptor 'micelle' is necessary for cholesterol movement into bile. Micelles are assembled during the simultaneous transport of bile acids and phospholipids into bile by transporters such as the bile salt export pump (BSEP) and the multidrug resistance protein 2 (MDR2), respectively. MDR2 is also encoded by the ABCB4 gene (Voshol et al. 1998). Furthermore, cholesterol can be converted to bile acids by an array of enzymes including CYP7A1 and then secreted in the bile canaliculus (Figure 5) (Myant et al. 1977). The excess free cholesterol can also move to the endoplasmic reticulum (ER) in the liver where it is repackaged onto nascent apoB-containing lipoproteins that are ultimately secreted from the liver into the bloodstream (Pramfalk et al. 2005).

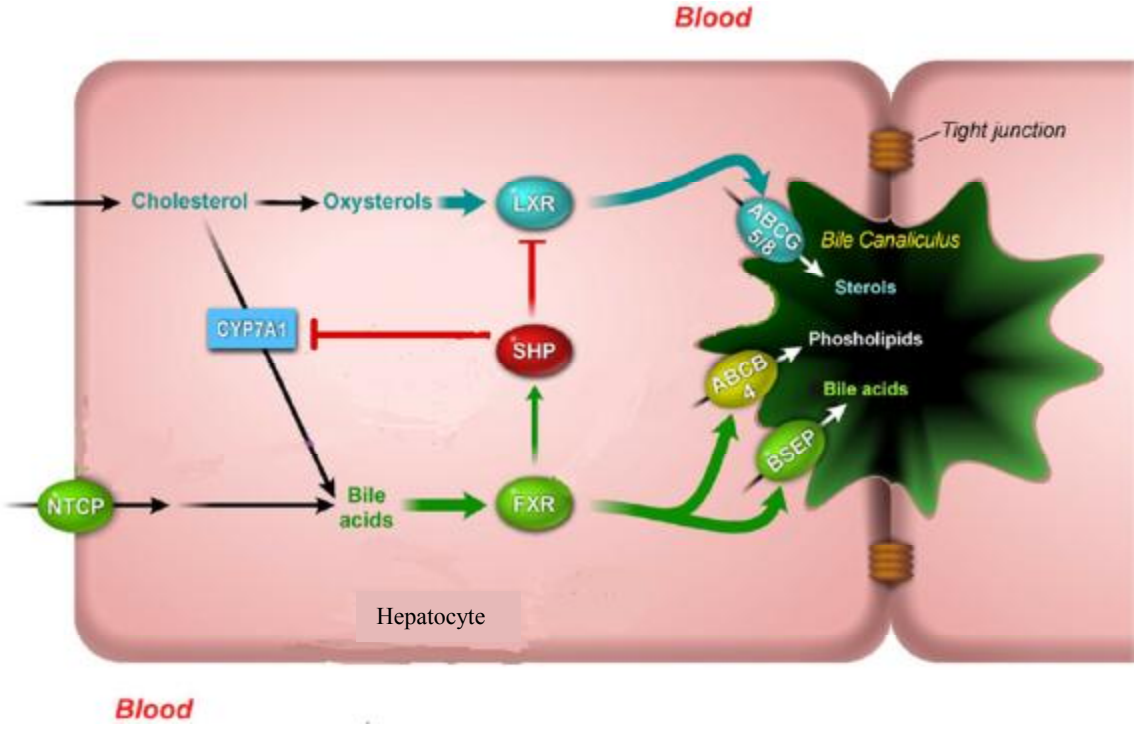

Figure 5. Cholesterol excretion from the liver. Adapted from (Jonker et al. 2009).

Once cholesterol is secreted into bile, a large portion of this pool is delivered to the lumen of the small intestine via the common bile ducts (Temel and Brown 2015). Cholesterol excretion in the form of neutral sterols or bile acids via the bile into the feces is the main mechanism of elimination of excess cholesterol from the body (Brufau et al. 2011). Therefore, the hepatobiliary pathway is an atheroprotective route that decreases the risk of atherosclerosis (Yu et al. 2002b; Meissner et al. 2011).

1.1.1.3.1 Bile acids formation and the enterohepatic circulation

Bile acids are amphipathic steroids that are formed from cholesterol in the liver. Conversion of cholesterol to bile acids is critical for maintaining cholesterol homeostasis and preventing cholesterol accumulation in liver. Primary bile acids are conjugated to either taurine or glycine to increase hydrophilicity. They are secreted into the bile and stored in the gallbladder. Upon ingestion of a meal, they are discharged into the small intestine to promote nutrient digestion and absorption in the proximal intestine. Bile acids are re-absorbed with an efficiency of 95% at the distal ileum and transported back to the liver through the portal vein and re-secreted into bile which results in the accumulation of a pool of bile acids (Russell 2003; Dawson et al. 2009). This bile acid pool cycles between the liver and the intestine and is called the enterohepatic circulation (Hofmann 2009) and the non-absorbed (5%) bile acids are eliminated from the body in the feces (Dawson et al. 2009)

Role of FXR in regulation of bile acid synthesis and transport

There is accumulated evidence indicating that farnesoid X receptor (FXR) exerts a key role in bile acid metabolism through the regulation of bile acid synthesis, bile acid secretion, intestinal bile acid absorption, and hepatic uptake of bile acids (Sinal et al. 2000; Eloranta et al. 2008; Lefebvre et al. 2009; Modica et al. 2010).

Bile acid absorption in the intestine is regulated by the nuclear factor FXR through the regulation of bile acid transporters from the intestine to the portal system (Wang et al. 1999; Matsubara et al. 2013). These acid transporters are involved in bile acid reabsorption at the apical and basolateral membranes of the ileum (Figure 6). The apical sodium-dependent bile

acid transporter (ASBT) is expressed at the apical membrane of enterocytes in the terminal ileum and mediates the reabsorption of bile acids from the ileum. The ileal bile acid binding protein (iBABP) shuttles the bile acids from the apical side to the basolateral membrane of ileum. Heteromeric organic solute transporter α and β (Ostα-Ostβ) are ileal basolateral bile acid transporters. They transport the bile acids from the basolateral side of the ileum toward the liver (Shneider 2001; Dawson et al. 2005). Bile acids are then re-circulated via the portal circulation to the hepatocytes, where a sinusoidal Na^{+-} dependent taurocholate cotransport peptide (NTCP) takes them up into hepatocytes. NTCP transporters are involved in bile acid uptake at the basolateral membrane of the hepatocytes. It is the major uptake system to transport bile salts from the portal circulation into the liver cells (Stieger et al. 1994).

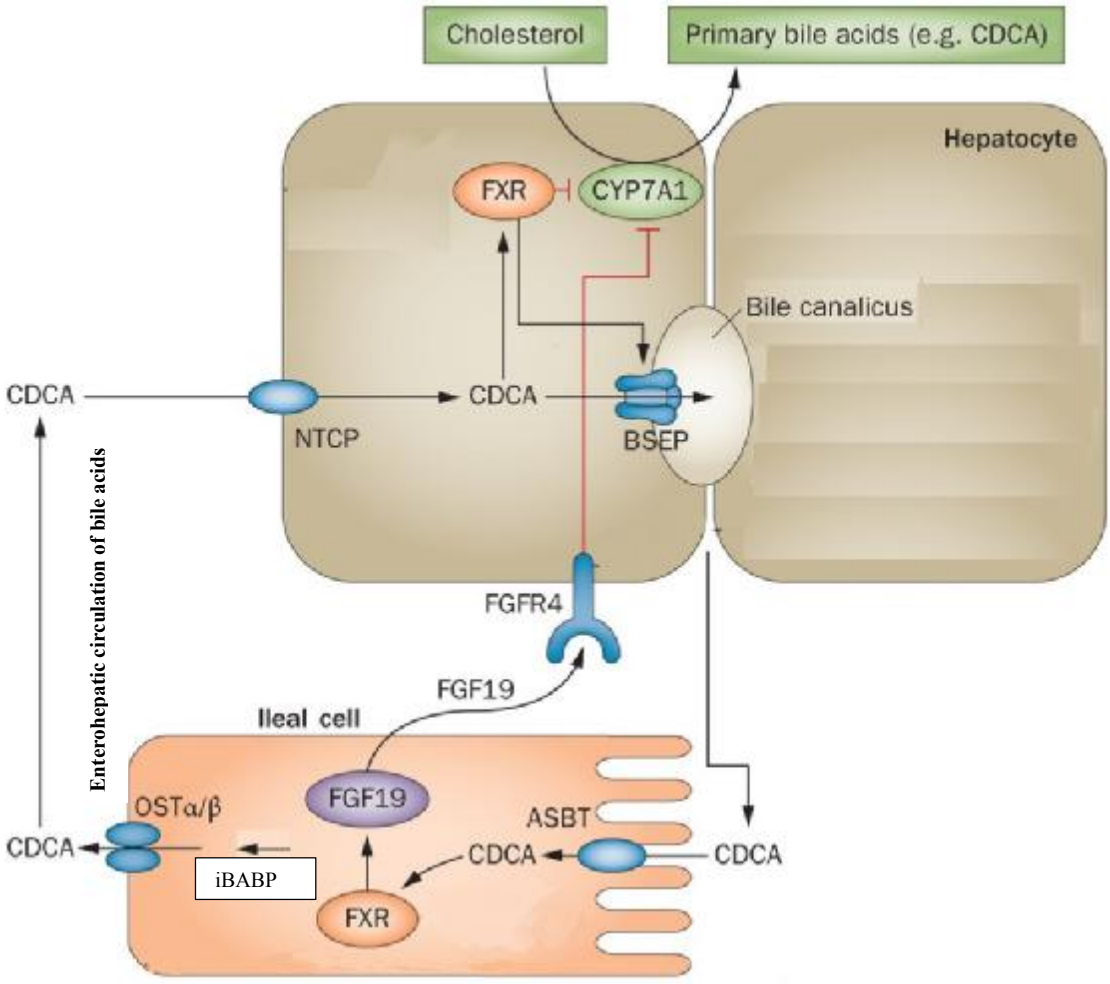

Figure 6. Transporters involved in bile acid reabsorption in the ileum. Adapted from (Schaap et al. 2014)

Bile acids function as natural ligands for the transcription factor FXR (also known as bile acid receptor or nuclear receptor subfamily 1 group H member 4) (Parks et al. 1999; Wang et al. 1999). Bile acids induce FXR activation both in the liver and the intestine which leads to a set of interactions resulting in suppression of bile acid biosynthesis (Wang et al. 1999). Activation of FXR is a major mechanism in suppressing bile acid biosynthesis by reducing the expression levels of CYP7A1. FXR-mediated induction of hepatic small heterodimer partner (SHP) and intestinal fibroblast growth factor 15/19 (FGF15/19) (FGF19 in humans) has been shown to be responsible for this suppression (Kerr et al. 2002; Holt et al. 2003).

a. Hepatic FXR/SHP/CYP7A1 pathway

FXR knockout mice showed an increase in bile acid synthesis and CYP7A1 gene expression suggesting FXR-mediated bile acid inhibition of CYP7A1 (Figure 7) (Sinal et al. 2000). CYP7A1 catalyzes the rate-limiting step of cholesterol conversion into bile acids in the liver (Jelinek et al. 1990). FXR inhibits CYP7A1 gene transcription by indirect mechanism. Bile acid-activated FXR induces SHP gene expression that inhibits the activity of liver related homologue-1 (LRH-1), and results in inhibiting CYP7A1 gene transcription (Goodwin et al. 2000). The FXR/SHP mechanism is supported by the finding that SHP and CYP7A1 mRNA expression levels have an inversed relationship, and CYP7A1 expression and bile acid synthesis are induced in SHP knockout mice. Paradoxically, bile acid feeding to SHP null mice inhibits CYP7A1 expression and bile acid synthesis suggesting that redundant pathways may exist for bile acid inhibition of CYP7A1 (Kerr et al. 2002; Wang et al. 2002).

SHP

The SHP gene is expressed in different tissues, including liver, heart, pancreas, kidney, adrenal gland, spleen, stomach, and small intestine (Lee et al. 1998). SHP is an atypical receptor without a DNA-binding domain, but has a putative ligand-binding domain, which makes SHP a member of the nuclear receptor family (Seol et al. 1996; Seol et al. 1997). Furthermore, SHP interacts with several nuclear receptor family members. Through these interactions, SHP is involved in diverse metabolic pathways, including cholesterol, bile acid,

triglyceride, and glucose homeostasis (Lee et al. 2007; Zhang et al. 2011). For instance, hepatic SHP overexpression in transgenic mice led to liver steatosis due to an indirect activation of SREBP-1c (Boulias et al. 2005). Besides, deletion of SHP decreased TG accumulation in ob/ob obese mice, which was associated with increased hepatic VLDL secretion and elevated expression of microsomal triglyceride transfer protein (MTP), the rate limiting enzyme in VLDL assembly and secretion (Huang et al. 2007).

Not only SHP can affect diverse biological responses, but also several genetic variations and mutations of SHP were identified in obese and diabetic subjects in population based studies showing that there might be a relationship between SHP genetic variations and increased risk of obesity and type 2 diabetes (Nishigori et al. 2001; Hung et al. 2003; Echwald et al. 2004; Enya et al. 2008).

b. Intestinal FXR/FGF15/19 /FGFR4 pathway

It has been suggested that intestine-derived FGF15/19 acts as an enterohepatic signal to activate hepatic fibroblast growth factor receptor 4 (FGFR4) signaling, which inhibits CYP7A1 expression in the hepatocytes (Figure 7) (Inagaki et al. 2005). Bile acids induce FXR activation in the intestine which results in stimulation of FGF15/19 and then, FGFR4 acts as a hepatic receptor for intestinal FGF15/19. FGFR4 mediates the effects of intestinal FGF15/19 on suppression of bile acid biosynthesis in liver (Holt et al. 2003). FGFR4 deficient mice showed higher mRNA levels of CYP7A1 suggesting that FGFR4 is also involved in suppression of bile acid biosynthesis (Kong et al. 2012).

Intestinal FXR-dependent mechanism is based on the observation that GW4064 (a synthetic FXR agonist) induces an intestinal hormone FGF15/19, which activates a hepatic FGFR4 signaling and through that inhibits bile acid biosynthesis in the liver (Holt et al. 2003). Intestinal FXR activation by bile acids leads to the release of FGF15/19 from the ileum and the expression of FGF15/19 mRNA is negatively correlated to the CYP7A1 mRNA expression levels in mouse liver (Inagaki et al. 2005).

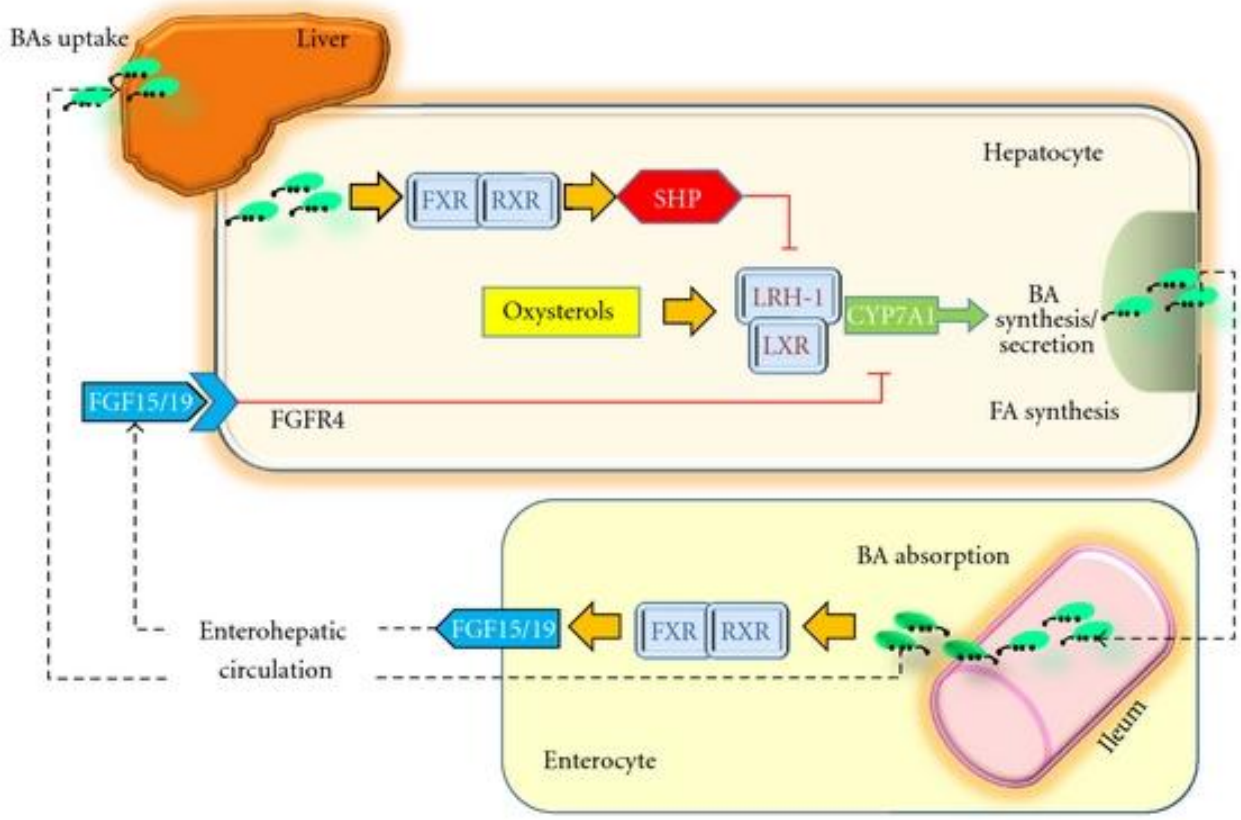

Figure 7. The molecular mechanisms of FXR pathway in bile acid synthesis in liver and intestine. Adapted from (Ory 2004; Inagaki et al. 2005)

1.1.1.3.2 VLDL assembly

Since liver constantly takes up circulating triglycerides and cholesterol from both endogenous and exogenous sources, VLDL production and secretion by hepatocytes is a crucial step in preventing hepatic steatosis (Alger et al. 2010; Flamment et al. 2010).

When lipids are available in the ER, the newly synthesized apo-B polypeptide interacts co-translationally with MTP, which is a rate-limiting molecule in VLDL assembly and secretion, and transfers triglycerides (TG) into the apo-B (Figure 8) (Rava et al. 2006). ApoB-100 is an essential structural protein that translocates into the luminal side of the endoplasmic reticulum (Cianflone et al. 1990). Diacylglycerol acyltransferase 2 (DGAT2) plays a role in VLDL assembly by converting fatty acids into TG. DGAT2 catalyzes the last step of the synthesis of TGs that are going to be incorporated into VLDL. Acyl-CoA:cholesterol acyltransferase (ACAT-2) has also a key role in the hepatic storage and packaging of cholesteryl ester into apoB-containing lipoproteins (VLDL), by converting free cholesterol into cholesterol esters (Pramfalk et al. 2005; Chang et al. 2009). Cell death-inducing DNA fragmentation factor alpha (DFFA)-like effector B (Cideb) is a lipid droplet-associated protein contributing to further lipidation of lipoprotein particles after they exit the endoplasmic reticulum compartment (Ye et al. 2009). The last step of VLDL production is mediated by small GTPase a (Sar1a), which is required for Golgi trafficking events. Sar1a, an intracellular vesicular trafficking protein, facilitates the movements of VLDL particles toward the Golgi apparatus where they are secreted in the plasma (Asp et al. 2000). Since VLDL carries both TG and cholesterol into circulation, appropriate assembly and secretion of VLDL is important for both liver cholesterol contents and plasma cholesterol levels.

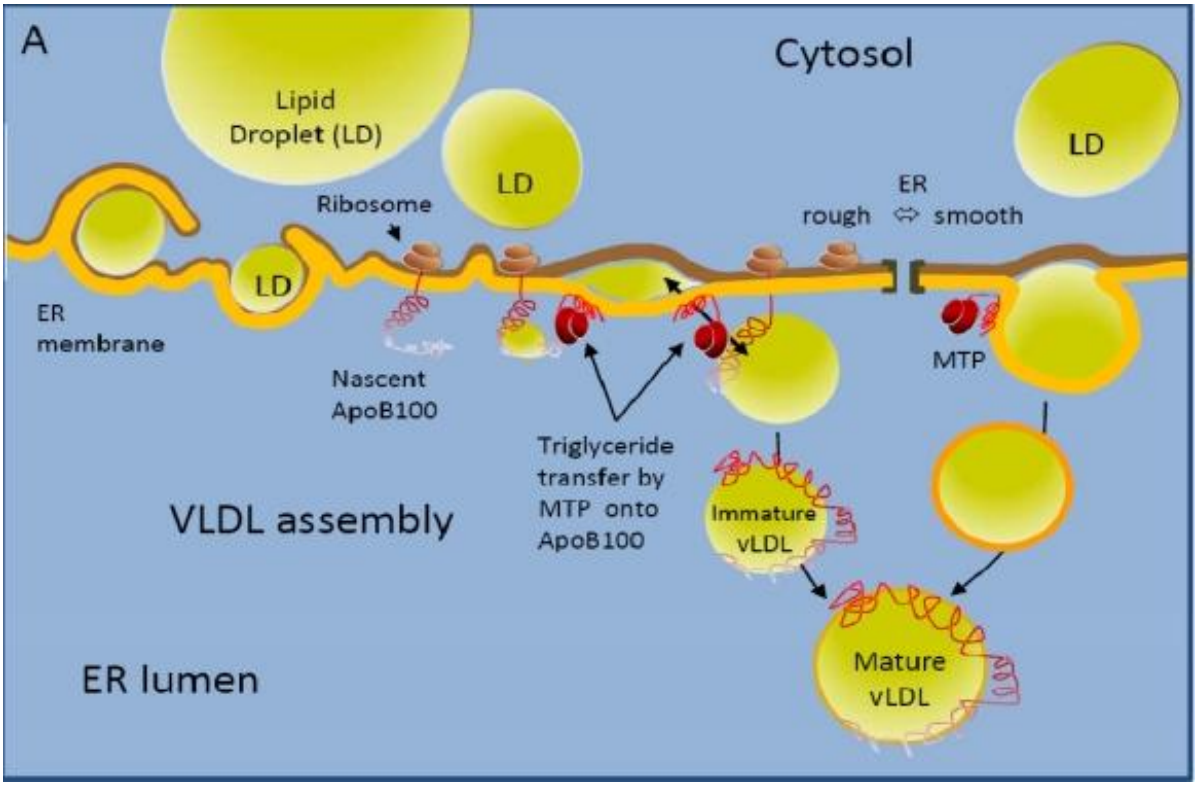

Figure 8. VLDL assembly in liver. Taken from (Bartosch et al. 2010)

1.1.2 Non-biliary TICE pathway

Transintestinal cholesterol excretion (TICE) presents a non-hepatobiliary direct route of cholesterol secretion "from blood to gut" (van der Velde et al. 2007). The highest levels of TICE have been reported to take place in the proximal small intestine (Brown et al. 2008). Under normal physiological conditions the biliary route is a predominant pathway for cholesterol excretion while TICE accounts for nearly 20–30% of fecal neutral sterols in both human and animal (Temel and Brown 2012). However, there is evidence showing that the TICE pathway can be stimulated by both pathophysiologic and pharmacologic stimuli (Temel and Brown 2012). For example, pharmacological activation of liver X receptor (LXR) (van der Veen et al. 2009) or high fat diet (van der Velde et al. 2008) resulted in increased intestinal cholesterol disposal. There is evidence showing that the set point of cholesterol excretion is sustained by the crosstalk between biliary and non-biliary pathways (Kruit et al. 2005). In a recent study hepatic flavin monooxygenase 3 (FMO3) was identified as a key cholesterol regulator of both biliary and non-biliary RCT pathways (Warrier et al. 2015). Indeed, cholesterol disposal from the body requires contribution of both biliary and non-biliary TICE pathways (Temel and Brown 2015). Since TICE like the hepatobiliary route is involved in cholesterol removal from the body, it can be considered an atheroprotective pathway.

However, to date our knowledge about the molecular mechanisms that define TICE is limited. The identification of non-biliary route largely stems from multiple observations indicating that biliary cholesterol excretion does not match with the amount of cholesterol in the feces. According to the classic biliary model, both biliary cholesterol secretion and fecal cholesterol loss should precisely be predictable by plasma HDL levels. In contrast, several studies showed that biliary and fecal sterol losses are quite normal in mice with extremely low HDL levels (Jolley et al. 1998; Groen et al. 2001; Xie et al. 2009). Similarly, biliary obstruction or diversion did not avoid the appearance of neutral sterols in the feces (Pertsemlidis et al. 1973; van der Velde et al. 2008). For instance, in hepatic ABCG5/G8 and MDR2 knockout mice, which lack the ability to normally secrete cholesterol into the bile, fecal cholesterol loss remains either unchanged or, in some cases, increased (Voshol et al. 1998; Yu et al. 2002a). These findings have led to the discovery of a non-hepatobiliary route of cholesterol excretion termed transintestinal cholesterol excretion (TICE) (van der Velde et

al. 2007). Earlier findings of the existence of an additional pathway of cholesterol excretion go back to the beginning of the last century. In 1927, Sperry reported that bile diversion in mice did not lessen fecal neutral excretion (Sperry 1927). However, this observation was only confirmed 50 years later by Pertsemlidis (Pertsemlidis et al. 1973).

TICE as its name implies, relies on the intestine for cholesterol secretion. Through this pathway, cholesterol-derived from plasma lipoprotein is directly secreted via the small intestine into the lumen (van der Velde et al. 2008; Temel and Brown 2012). The intestine is responsible for receiving the cholesterol from the blood via its cholesterol receptors at the basolateral membrane. Possibly, there are molecules involved in transferring the cholesterol through the basolateral to the apical side of enterocytes, and finally there are cholesterol transporters at the apical membrane of enterocytes to discard cholesterol into the lumen. In order to address this pathway effectively, the molecules and receptors involved in the TICE pathway will be discussed in details in four steps.

1.1.2.1 Step1: The role of lipoproteins in the TICE pathway

TICE requires plasma lipoproteins to transport the cholesterol from either peripheral tissues and/or the liver to the small intestine for secretion (Temel and Brown 2012). However, it is still not clear which type of lipoproteins play the main role in this pathway.

As mentioned before, normal biliary and fecal cholesterol loss was observed in apoA-1 or ATP-binding cassette transporter A1 (ABCA1) null mice, although these mice present extremely low plasma HDL levels (Jolley et al. 1998; Xie et al. 2009). These findings imply two important points; firstly, the existence of non-biliary cholesterol excretion in addition to hepatobiliary route and secondly the non-biliary route does not depend on HDL to be proceeded. In line with these studies, recently Vrins et al showed that the rate of TICE did not change significantly in ABCA1 deficient mice with very low levels of HDL compared to the wild type (WT) littermates (Vrins et al. 2012). Moreover, intestinal perfusions of a modified Krebs solution supplemented with bile salts and phospholipids in SR-B1-deficient mice, SR-B1 is a well-known HDL receptor, were significantly associated with a twofold increase in TICE suggesting that HDL might not have a main role in the TICE pathway (Acton et al.

1996; van der Velde et al. 2008). It thus seems that despite the predominant role of HDL in the hepatobiliary route (Tall et al. 2008), its role in non-hepatobiliary TICE pathway is unclear.

Based on these findings, it seems that liver-derived apoB-containing lipoproteins have a main role in delivering cholesterol to the proximal part of the intestine. The excess free cholesterol in the liver is shifted to the ER where it is packed onto nascent apoB-containing lipoproteins. The first apoB-containing lipoprotein secreted from the liver into the circulation is the VLDL. The VLDL then changes to other apoB-containing lipoproteins such as the IDL and/or the LDL. The liver-derived apoB-containing lipoproteins are then recognized by the proximal small intestine through lipoprotein receptors such as LDL-Rs and probably other receptors like LDL-Rs family (Temel and Brown 2015). For instance, Brown et al showed that liver-specific depletion of Acetyl-CoA acetyltransferase 2 (ACAT2) resulted in increased fecal sterol loss through non-biliary pathway without changes in HDL levels (Brown et al. 2008). ACAT2 converts free cholesterol into cholesterol esters to pack them in VLDL molecules. Liver-specific inhibition of ACAT2 via antisense oligonucleotide (ASO) prevents cholesterol esterification and secretion of CE in apoB-containing lipoproteins into the plasma. In this study, a reduction in ACAT2 protein and activity of 99.3% was observed and they found that even a small amount (less than 1% of normal) of ACAT2 may be sufficient to support packaging of hepatic CE into apoB-containing lipoproteins and these lipoproteins drives the cholesterol toward the proximal small intestine for secretion, suggesting that apoB-containing lipoproteins deliver cholesterol esters to the TICE pathway for excretion (Brown et al. 2008). In line with this study, Marshal et al recently showed that acutely reducing hepatic ACAT2 expression resulted in packaging the hepatic cholesterol onto nascent apoB-containing lipoproteins that feed cholesterol into the TICE pathway for fecal excretion (Marshall et al. 2014). All in all, these studies suggest that hepatic apoB-containing lipoproteins have a key role in delivering cholesterol into the TICE pathway for fecal excretion.

On the other hand, Le May et al. recently reported that both LDL and HDL can provide cholesterol for TICE in human and mice jejunal explants at the basolateral side of the enterocytes. They radiolabeled both LDL and HDL with ^{3}H-free cholesterol (^{3}H-LDL, ^{3}H-HDL) and observed that both these lipoproteins can deliver cholesterol to the proximal part of

the small intestine. According to Le May's finding, both HDL and LDL can be involved in TICE (Le May et al. 2013).

Based on these findings, various lipoproteins might participate in TICE and provide cholesterol for secretion through this pathway. Regarding the role of lipoproteins as the cholesterol carriers in TICE, the liver function is undeniable in this pathway. Indeed, the liver plays a central role in TICE by providing requisite lipoproteins. Taking all together, these two organs, the intestine and the liver, collaborate for cholesterol secretion through the TICE pathway.

1.1.2.2 Step 2: Cholesterol receptors at intestinal basolateral membrane

Several studies have been conducted to investigate the role of potential intestinal basolateral receptors involved in cholesterol uptake from circulation in TICE route based on which lipoproteins are involved in delivering cholesterol to the basolateral side of the small intestine.

Recently, Le May et al reported that deletion of PCSK9 increases TICE. PCSK9 deletion means no degradation effect on LDL-R and that results in a higher number of intestinal LDL-R as a cholesterol acceptor in the TICE and consequently higher levels of TICE (Le May et al. 2013). This group had previously showed that PCSK9 deficient mice have higher amounts of LDL receptors in their intestine (Le May et al. 2009). Plasma PCSK9 induces LDL receptor degradation. Overall, this study showed that LDL receptors at the intestinal basolateral membrane are involved in cholesterol uptake from LDL (Le May et al. 2013). However, intestinal cholesterol uptake from apoB-containing lipoprotein does not merely depend on LDL receptors because there is evidence showing that LDL receptor deficient mice still have normal or increased levels of TICE (Brown et al. 2008; Le May et al. 2013).

Seemingly, other members of the LDL-R family can also play a role as cholesterol acceptors at the basolateral membrane of enterocytes. Other receptors including low-density lipoprotein receptor-related protein 1 (LRP1), very low-density lipoprotein receptor (VLDLR), and apolipoprotein E receptor 2 (apoER2), which are all members of the LDL receptor family, are also expressed in the gut (Herz et al. 1988; Garcia-Miranda et al. 2010). As a result, it

would be reasonable to assume that they can function as the main or secondary receptors for cholesterol delivering lipoproteins.

Based on these data, it can be concluded that LDL receptor and/or LDL receptor family, including LRP1, VLDLR, and apoER2 could participate in the intestinal cholesterol uptake from both apo E and/or apoB-containing lipoproteins.

1.1.2.3 Step 3: Cholesterol trafficking from the basolateral to the apical membrane of enterocytes

The trafficking itinerary of TICE-derived cholesterol within the enterocyte is not fully understood. It is probable that apoB-containing lipoproteins, which are the TICE cholesterol donor particles, are eventually degraded in lysosomes. It is tempting to assume that the trafficking itinerary would involve endosomal/lysosomal compartments (Temel and Brown 2012). Thus, TICE would need Niemann-Pick type C1 (NPC1) and NPC2 proteins to move the cholesterol out of the lysosomal compartment. These two cholesterol-binding proteins act in the removal of unesterified cholesterol from lysosomes (Vance et al. 2011; Temel and Brown 2012).

Vrins et al introduced other factors possibly involved in trafficking cholesterol. They showed that endosomal Rab protein 9 (Rab9) and lysosomal integral membrane protein-2 (LIMP2) could also play a role in intracellular trafficking of cholesterol derived from TICE (Vrins et al. 2009). Rab is a family of Ras-like small G proteins that control membrane trafficking (Ikonen 2008). The intestinal expression of these two genes and TICE were significantly increased in mice upon peroxisome proliferator-activated receptor delta (PPAR δ) activation (Vrins et al. 2009). Furthermore, earlier Van der Veen et al. showed that PPAR δ activation led to higher fecal neutral sterol excretion without affecting the hepatobiliary cholesterol secretion (van der Veen et al. 2005). As a consequence, increased expression of Rab9 and LIMP2 might be a sign of cholesterol trafficking. Nevertheless, the relationship between the proteins encoded by these genes and TICE is still not clear (Vrins et al. 2009).

1.1.2.4 Step 4: Cholesterol efflux via intestinal apical transporters into the lumen

After being moved to the intestinal apical membrane, cholesterol is excreted via apical transporters into the lumen. ABCG5/G8 transporters are involved in TICE, regarding to their indispensable role in cholesterol excretion. Van der Veen et al. showed that TICE is impaired in ABCG5 deficient mice, suggesting that ABCG5/G8 contributes to TICE. However, in this study ABCG5 deficient mice still had a substantial contribution of TICE, suggesting that other apical transporters might be involved (van der Veen et al. 2009). On the other hand, Le May et al observed that TICE was reduced by 26.5% in ABCB1a/b deficient mice (Le May et al. 2013). It seems, therefore, that both apical transporters, ABCG5/G8 and ABCB1a/b, contribute to the excretion of cholesterol into the gut lumen. It is reasonable to suppose that the secreted cholesterol into the gut requires luminal acceptors. A mixture of bile salts and phospholipids has been suggested as the luminal cholesterol acceptors in TICE (van der Velde et al. 2007). This finding is in the line with the presence of high concentration of bile-derived phospholipids in the proximal small intestine, a region of the gut that has been reported to have the highest levels of TICE (Temel and Brown 2012).

In summary, mainly plasma apoB-containing lipoproteins deliver cholesterol to the intestine for excretion through the TICE route. LDL receptor and/or LDL receptor family are responsible for cholesterol uptake from circulation at the intestinal basolateral membrane. The exact molecules involved in trafficking the cholesterol from basolateral to apical membrane, are unknown at the present time. ABCG5/G8 and ABCB1a/b are the main transporters at the intestinal apical membrane responsible for the secretion of cholesterol into the lumen in the TICE pathway (Figure 9).

Taken together, TICE is a non-biliary route of the cholesterol elimination. It directly discards the cholesterol into the lumen. Thus, TICE could be considered an anti-atherogenic pathway; however, a better understanding of this newly identified pathway will require further investigations.

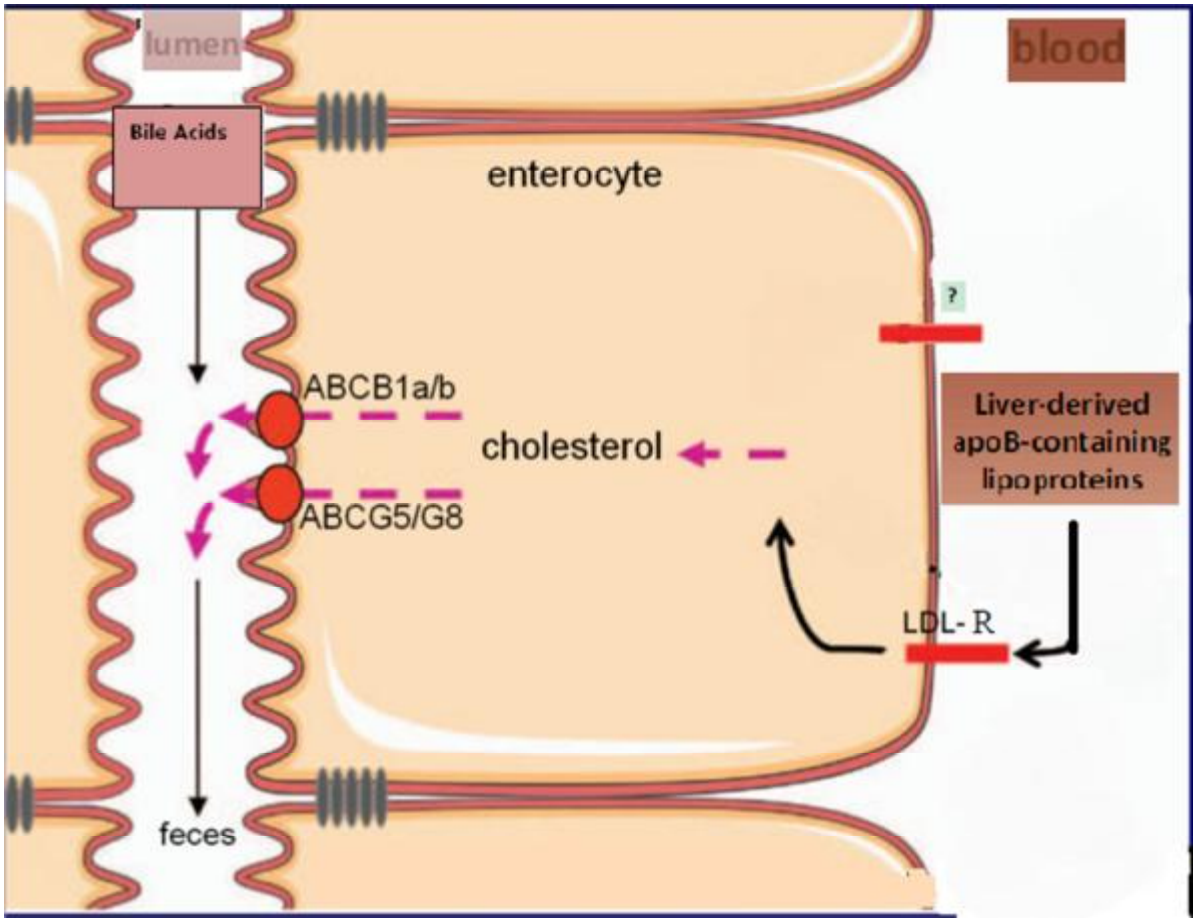

Figure 9. A model of non-biliary transintestinal cholesterol excretion (TICE). Adapted from (Le May et al. 2013).

Cholesterol absorption in intestine

In addition to *de novo* cholesterol synthesis which is tightly regulated by HMGCoA-r, dietary intake is another source of cell cholesterol supply. The Niemann–Pick C1-like-1 (NPC1L1) protein which is located at the apical membrane of enterocytes is the main transporter responsible to facilitate the dietary cholesterol uptake across the brush border membrane. It is also the target of the cholesterol absorption inhibitor ezetimibe (Altmann et al. 2004). The process of cholesterol absorption by intestinal NPC1L1 is the opposite of biliary and non-biliary RCT flux (Sehayek et al. 2008). The intestinal ABCG5/G8 transporter functions at the apical membrane of enterocytes to export the absorbed cholesterol back into the lumen (Wang 2007). In ER of enterocytes, the absorbed cholesterol is esterified by ACAT2, which forms the nascent chylomicron particles. Then Apo-B48, as the main apolipoprotein of chylomicron, is used for packaging absorbed lipids and cholesterol to form chylomicrons. The particles leave the ER in COPII-coated vesicles and then are secreted through the Golgi complex to the basolateral side of the enterocyte. Chylomicrons travel through lymphatic vessels to reach the venous circulation (Figure 10) (Shoulders et al. 2004). The majority of cholesterol absorbed by the intestine is directed to the liver (Turley et al. 2003), as a result it affects *de novo* cholesterol synthesis, cholesterol esterification and also packaging into lipoproteins (Dietschy et al. 2002; Horton et al. 2002; Turley and Dietschy 2003). This indicates that cholesterol homeostasis requires dynamic interactions between the liver and the intestine in different aspects of cholesterol metabolism.

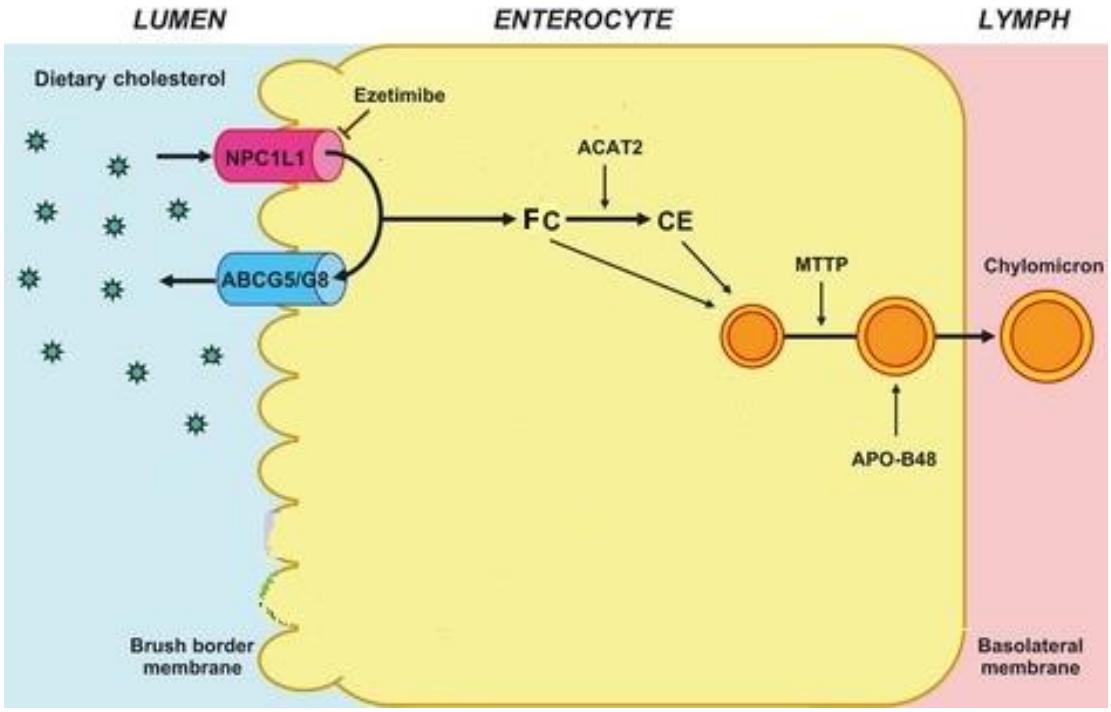

Figure 10. Dietary cholesterol absorption. Adapted from (Wang et al. 2013).

1.2 The effects of high cholesterol diet, estrogen withdrawal and exercise training on reverse cholesterol transport (RCT)

1.2.1 Hepatobiliary pathway

1.2.1.1 Hepatic cholesterol accumulation

a. The effects of high cholesterol diet

It has been reported that cholesterol feeding (0.2%) for 6 weeks increased cholesterol ester storage and triglyceride accumulation in mice hepatocytes. The authors explained that the presence of cholesteryl ester in liver interferes with TG hydrolysis and mobilization and this can lead to hepatic triglyceride accumulation. It was also mentioned that hepatic triglyceride accumulation observed following the cholesterol diet is independent of lipogenesis. Using ACAT2 KO mice, the authors suggested that ACAT2 depletion is consistently associated with a major reduction in hepatic cholesteryl ester and with a decrease in liver triglycerides (Alger et al. 2010). ACAT2 is the main cholesterol-esterifying enzyme in the liver, and genetic deletion of this enzyme in mice has been shown to decrease hepatic cholesteryl ester accumulation when mice are challenged with a high cholesterol diet (Willner et al. 2003; Lee et al. 2005). The data of Alger's study provided evidence that there is a limitation of triglyceride mobilization from the liver in the form of VLDL-TG in the presence of cholesteryl ester accumulation. It also explains underlying mechanisms for dietary cholesterol-driven NAFLD (Alger et al. 2010). Savard et al also showed that feeding a high fat high cholesterol (HFHC) diet (15% fat and 1% cholesterol) for 30 weeks led to hepatic steatosis with profound deposition of TGs and also cholesterol esters in the liver of male C57BL/6J mice (Savard et al. 2013). In this study, hepatic lipid accumulation caused by HFHC was greater than the HF or HC diet alone (Savard et al. 2013). A HFHC (0.25% and 0.5%) diet in Ovx rats led to increased hepatic TC accumulation which was accompanied by reduced plasma cholesterol levels, suggesting an impaired mobilization of cholesterol from the liver (Cote et al. 2014). Moreover, hepatic TC accumulation following the HFHC diets was higher than the HF diet alone, suggesting the critical role of the cholesterol in a diet (Cote et al. 2014). It seems that a diet rich in cholesterol alone or combined with a HF diet has a

determinant role in cholesterol accumulation in liver and hepatic cholesterol accumulation might be the main reason for limitation of TG mobilization and accumulation in liver.

b. The effects of estrogen withdrawal

It has been reported that liver TC content was not increased in Ovx animal models. The authors concluded that similar hepatic cholesterol levels in Sham and Ovx mice despite the higher serum cholesterol levels in cholesterol fed groups might be a reflection of a reduced liver cholesterol uptake by LDL-R and a reduced hepatic *de novo* cholesterol production by HMGCR (Kamada et al. 2011). However, recent studies conducted in Ovx rats showed an increase in hepatic cholesterol accumulation (Kato et al. 2009; Ngo Sock et al. 2014a) that was more pronounced when Ovx rats were fed a HFHC diet suggesting a determinant role of the cholesterol diet (Cote et al. 2014). It might be assumed that hepatic TC accumulation is the result of hepatic cholesterol biosynthesis in Ovx animals. However, there is accumulating evidence indicating that hepatic HMGCoA-r mRNA expression is lower in Ovx than in Sham rats (De Marinis et al. 2008; Kato et al. 2009; Ngo Sock et al. 2013; Cote et al. 2014). HMGCoA-r catalyzes the rate limiting step in cholesterol biosynthesis in liver (Horton et al. 1998). Furthermore, low expression of HMGCoA-r mRNA was reported in Ovx mice fed a HFHC diet (containing 15% fat, 1.25% cholesterol) for 6 weeks compared to Sham mice fed the same HFHC diet (Kamada et al. 2011). In contrast, estrogen administration resulted in an increase in hepatic HMGCoA-r protein content in normal female animal models (Di Croce et al. 1996; Di Croce et al. 1997). These studies support the concept that estrogens play an important role in regulating cholesterol synthesis and homeostasis. Indeed, HMGCoA-r promoter has an estrogen-responsive element-like sequence and HMGCoA-r activity is stimulated by estradiol (Di Croce et al. 1999). In addition to the HMGCoA-r, gene expression of SREBP2, the nuclear factor involved in the regulation of HMGCoA-r expression, was also reduced in Ovx animals (Cote et al. 2014; Ngo Sock et al. 2014b) indicating that hepatic cholesterol accumulation is not a consequence of elevated cholesterol biosynthesis in liver of Ovx animals.

Taken together, estrogens have a key role in regulation of cholesterol biosynthesis through their effects on HMGCoA-r promoter. Therefore, high hepatic TC accumulation in Ovx animals is not the result of an increased cholesterol biosynthesis in liver. On the other

hand, hepatic cholesterol accumulation might subsequently interfere in cholesterol biosynthesis in Ovx rats. Accumulation of cholesterol in the ER membrane of hepatocyte, the place of cholesterol biosynthesis by HMGCoA-r, was reported in previous studies (Di Croce et al. 1996; Di Croce et al. 1997). It has been reported that ER stress caused by cholesterol accumulation in ER of hepatocytes can lead to disturbances in VLDL secretion and through that might exacerbate hepatic cholesterol accumulation in Ovx rats (Hager et al. 2012).

c. The effects of exercise training

There is conflicting information about the effect of exercise training on cholesterol accumulation in liver. For instance, male rats trained on a voluntary wheel running for two weeks showed a decrease in hepatic TG accumulation without any change in hepatic TC content compared to sedentary rats. An increase in hepatic cholesterol synthesis via higher mRNA levels of HMGCoA-r to compensate for fecal sterol loss was explained as a reason for unchanged hepatic TC content between trained and sedentary rats (Meissner et al. 2010a). However, 12 and 8 weeks of running training resulted in a reduction of hepatic TC content in LDL-R deficient mice and HF fed mice (Meissner et al. 2011; Wen et al. 2013). Excretion and elimination of cholesterol from the liver in the form of VLDL or bile acids in response to exercise can be put forward as possible reasons to explain decreased or unchanged hepatic TC accumulation. These will be discussed in the following pages.

1.2.1.2 Hepatic cholesterol uptake from circulation

a. The effects of high cholesterol diet and estrogen withdrawal

In addition to HMGCoA-r, a reduction in hepatic LDL-R transcripts was also reported in Ovx animals (Di Croce et al. 1997; Ge et al. 2006; Kato et al. 2009; Kamada et al. 2011; Cote et al. 2014; Ngo Sock et al. 2014a). Inversely, estrogen supplementation has been reported to upregulate levels of hepatic LDL-R mRNA and protein levels in animals (Di Croce et al. 1996; Di Croce et al. 1997; Parini et al. 2000). Estrogen stimulates expression of the LDL-R gene through the estrogen receptor (ER-α), which can activate transcription of the LDL-R promoter through its interaction with Sp1 (Bruning et al. 2003).

Along with HMGCoA-r and LDL-R transcripts, a reduction in hepatic PCSK9 transcripts and plasma PCSK9 levels were also observed in Ovx rats (Ngo Sock et al. 2014a). As aforementioned PCSK9 induces LDL-R degradation (Maxwell and Breslow 2004). Therefore, a paralle reduction in PCSK9 and LDL-R transcripts was not expected. The rate of hepatic LDL-R recycling on the cell surface might be an explanation for similar reductions in PCSK9 and LDL-R transcripts in Ovx animals (Roubtsova et al. 2015). Furthermore, it seems that PCSK9 like LDL-R might have an estrogenic-like promoter site on its gene. That might explain somehow the reduction of both PCSK9 and LDL-R transcript in Ovx animals. Moreover, as mentioned before, there are different gene variants of PCSK9 with different affinity for LDL-R. It need to be clarified what type of gene mutations exist in Ovx animal model.

On the other hand, feeding the rats with dietary cholesterol (2%) for 8 days decreased PCSK9 gene expression, resulting in an increased number of hepatic LDL-Rs proteins despite a reduction in LDL-R mRNA levels suggesting posttranscriptional regulation of LDL-R by PCSK9 (Persson et al. 2009). High-dose ethinylestradiol treatment resulted in a 50% decrease in PCSK9 gene expression in male rats accompanied with an increase in the number of hepatic LDL-R. The authors concluded that the increase in hepatic LDL-R by high-dose estrogen treatment in the male rat was due to a combination of posttranscriptional regulation, i.e. a reduced PCSK9 expression together with a strong transcriptional increase of the LDL-R gene. (Persson et al. 2009). Surprisingly, different studies reported similar impacts on PCSK9 transcripts by estrogen treatment and estrogen deficiency state. The difference in response to estrogens in male and female animals might explain this similar effect.

It is important to note that in addition to LDL-R, LRP1 has also been identified as a target for PCSK9 induced degradation in HepG2 cells. It seems that these receptors compete for PCSK9 activity (Canuel et al. 2013). Moreover, a reduction in gene expression and protein content of LRP1 has been reported in Ovx rats (Ngo Sock et al. 2014a; Ngo Sock et al. 2016) along with a decrease in the SREBP-2 transcription factor transcript levels (Moon et al. 2011). Reduced transcript levels of both LDL-R and LRP1 could cause high plasma cholesterol levels in Ovx rats through the decrease in LDL cholesterol and lipoprotein remnants uptake from the circulation.

Taking together, these findings suggest that the increase in plasma LDL cholesterol concentrations found in Ovx rats is associated with a decrease in hepatic LDL-R activity.

b. The effects of exercise training on **LDL-R**

Twelve week of treadmill exercise training increased LDL-R gene expression and attenuated the gallstone development in gallstone-sensitive male mice fed a lithogenic diet (21% fat, 1.25% cholesterol, 0.5% cholic acid) (Wilund et al. 2008). The lithogenic diet is a supersaturation of bile with cholesterol which is a prerequisite of the development of gallstones (Reihner et al. 1996). Moreover, it has been reported that treadmill exercise (EX) for 8 weeks increased the mRNA expression of LDL-R, PCSK9, and protein content of SREBP2 in mice fed a high fat (HF) diet compared to sedentary HF fed mice. A decrease in plasma PCSK9 levels was also observed in HF+EX group. These results suggested that treadmill exercise reduces circulating non-HDL cholesterol through higher cholesterol uptake by LDL-R (Wen et al. 2013). Furthermore, CETP transgenic (CETP-tg) mice showed an elevated content of hepatic LDL-R protein after six weeks of treadmill exercise. This enhanced LDL receptor protein levels suggests that exercise has a positive effect on RCT through increased hepatic cholesterol uptake from circulation (Rocco et al. 2011). CETP transfers esterified cholesterol from HDL to apoB-containing lipoproteins that are ultimately removed by the hepatic LDL-R. CETP-tg mice were used as an animal model to study RCT similar to human lipoprotein pattern (Yin et al. 2012; Temel and Brown 2015). Recently, it was reported that LDL-R protein content was higher in male mice on treadmill exercise for 6 weeks (Pinto et al. 2015). Trained mice for 2 weeks also showed a dramatic increase in hepatic LDL-R gene expression, suggesting that exercise might favor reverse cholesterol transport and lipoprotein clearance from the circulation (Wei et al. 2005).

Based on these findings, it appears that exercise training has a positive key role in RCT by reducing the circulating LDL cholesterol through an increase in LDL-R and consequently an elevated hepatic cholesterol uptake from the circulation, a potential mechanism for the anti-atherosclerotic effect of exercise (Wei et al. 2005; Halverstadt et al. 2007; Meissner et al. 2010a; Meissner et al. 2011; Rocco et al. 2011).

On the other hand, treadmill exercise for 8 weeks did not change the gene expression of LDL-R, PCSK9, and LRP1 in Sham and Ovx rats, although its positive effects on plasma TC concentrations in Ovx rats. On the opposite, estradiol replacement markedly corrected changes in gene expression in almost all markers of hepatic cholesterol metabolism. The authors concluded that exercise might have no impact on hepatic expression of genes involved in cholesterol uptake from circulation in Ovx rats (Ngo Sock et al. 2014a; Ngo Sock et al. 2016).

c. The effects of exercise training on **HDL**

Higher ABCA1 mRNA and plasma HDL content were reported in rats following treadmill exercise for 6 weeks. It seems that elevated plasma HDL levels induced by exercise might be the result of higher hepatic ABCA1 gene expression, suggesting a positive effect of exercise training on RCT process (Ghanbari-Niaki et al. 2007). Moreover, increased hepatic expression of SR-B1 was observed following two weeks of exercise training in mice. SR-B1 is known as a hepatic HDL receptor (Wei et al. 2005). Furthermore, it was reported that an increase in SR-B1 mRNA was accompanied with a reduction in gallstone development in gallstone sensitive mice fed a lithogenic diet after 12 weeks of exercise training (Wilund et al. 2008). An increase in hepatic SR-B1 protein content with evidence of increased macrophage cholesterol flux into the liver has been also reported in male mice trained for 6 weeks (Pinto et al. 2015). Six weeks of treadmill exercise resulted in an increase in hepatic ABCA1 protein content in CETP transgenic mice suggesting high levels of circulating HDL and a positive effect of exercise on the hepatobiliary route. However, treadmill exercise had no influence on SR-B1 expression in CETP transgenic mice (Rocco et al. 2011). On the other hand, exercise training had no impact on ABCA1 and SR-B1 mRNA expression in Ovx rats (Ngo Sock et al. 2014a).

Taken together, it seems that exercise training has a positive influence on molecular markers of HDL metabolism and that might favor an increase in circulating HDL levels and reverse cholesterol transport (Halverstadt et al. 2007).

1.2.1.3 Cholesterol excretion from the liver

1.2.1.3.1 VLDL assembly and secretion

a. The effects of high cholesterol diet

There are some data indicating that overconsumption of cholesterol induces the overproduction of hepatic lipoproteins. For instance, cholesterol feeding (2%) for 3 months resulted in a four-fold increase in hepatic secretion of newly synthesized VLDL in perfused liver of Japanese monkey (Teramoto et al. 1987). In another study, 2 weeks of 2% cholesterol supplementation versus a standard diet increased the hepatic production of VLDL-cholesterol fourfold, VLDL-triglyceride two and one-half-fold, and also enhanced apo-B protein levels tenfold in isolated hamster liver (lChen et al. 1996). On the other hand, there is other evidence pointing toward a reduction in VLDL assembly and secretion following a cholesterol diet. Alger et al reported that a six week cholesterol diet (0.2%) led to a reduction in hepatic VLDL–TG secretion, resulting in neutral lipid retention within the hepatocytes. However ACAT2 deficient mice showed an increase in mobilization and use of stored hepatic triglyceride for VLDL secretion. This study suggests that there is an association between hepatic cholesterol ester accumulation and reduced VLDL secretion. The authors explained that hepatic cholesterol accumulation results in limitation of TG mobilization and use of TG in VLDL assembly (Alger et al. 2010). Alger's study provided data supporting the hypothesis that accumulation of hepatic cholesteryl ester in lipid droplets can limit the mobilization of hepatic triglyceride (Demel et al. 1985; Ghosh et al. 1995; Alger et al. 2010) and consequently reduce VLDL secretion (Alger et al. 2010). Moreover, Savard et al. showed that high cholesterol (1%) diet feeding for 30 weeks decreased transcripts of MTP and ACAT2, involved in VLDL assembly and secretion, in mice compared to SD fed mice. It is important to note that similar differences in these gene expressions were observed when the HFHC-fed mice were compared to the HF-fed mice. Therefore, the authors concluded that dietary cholesterol causes consistent changes in gene expression of MTP and ACAT2, whether it was added to the control diet or to the HF diet, suggesting that cholesterol is a determinant component in a diet regarding VLDL assembly and production (Savard et al. 2013).

b. The effects of estrogen withdrawal

It has been reported that estrogen deficiency state results in a reduction in VLDL secretion (Lemieux et al. 2005; Barsalani et al. 2010). Therefore common high plasma cholesterol levels in Ovx animal are hardly a consequence of elevated cholesterol secretion in the form of VLDL from the liver (Kato et al. 2009; Ngo Sock et al. 2013). Disruption to VLDL production is a mechanism that may explain the accumulation of TG and TC found in the liver of Ovx rats fed a HFHC diet compared with those fed other diets (Cote et al. 2014). In addition to a decrease in VLDL production, expression of several genes involved in hepatic VLDL synthesis and assembly including MTP and apo-B were also reduced in Ovx rats fed a standard diet (Barsalani et al. 2010; Cote et al. 2014). It seems that estrogen withdrawal in Ovx animals or the blockage of estrogen receptors results in a reduction of MTP transcript and impaired VLDL secretion (Barsalani et al. 2010; Cote et al. 2012).

On the other hand, hepatic VLDL secretion was unaffected by ERα overexpression in female *ob/ob* mice (Wang et al. 2015), suggesting that estrogens have no direct impacts on VLDL secretion. Disturbance in VLDL assembly and production in Ovx rats might be induced by ER stress as a result of cholesterol accumulation in ER of hepatocytes (Hager et al. 2012). ER stress through apoB degradation impairs VLDL assembly and secretion (Ota et al. 2008) and as a result, this exacerbates hepatic triglyceride and cholesterol accumulation in Ovx animals.

c. The effects of exercise training

Barsalani et al reported that VLDL-TG production is lower in Ovx compared to Sham rats and using treadmill exercise for 5 weeks as an intervention also resulted in lower plasma VLDL-TG levels in Ovx rats. It seems that exercise training is unable to restore VLDL-TG production in estrogen deficient state. However, in this study reduced VLDL-TG production by training in Ovx rats did not lead to hepatic TG accumulation probably due to an increase in fat oxidation as a source of energy (Barsalani et al. 2010). In addition to reduced VLDL-TG production induced by exercise training, there is evidence that hepatic MTP protein content

was also diminished by exercise training in mice and female rats fed a SD and HF diet (Chapados et al. 2009; Meissner et al. 2011).

On the other hand, a lower rate of VLDL secretion and reduced gene expression of apoB and MTP observed in sedentary tumour-bearing male Wistar rats were restored to control values when the animals were submitted to the treadmill exercise for 8 weeks (Lira et al. 2008). The authors suggest that exercise training promoted the re-establishment of hepatic VLDL assembly and secretion and this subsequently resulted in decreased hepatic TG accumulation (Lira et al. 2008).

1.2.1.3.2 Bile acid biosynthesis

a. The effects of high cholesterol diet

Another plausible explanation for hepatic cholesterol accumulation in Ovx rats, especially when they were fed the HFHC diets, is a decrease in cholesterol excretion from the liver in the form of bile acids. For instance, lower FXR gene expression, the regulator of hepatic bile acid metabolism, and its target gene CYP8b1 were observed in rats fed HFHC diet for 6 weeks (40% fat, 0.5% cholesterol) compared to rats fed a SD diet (Cote et al. 2014). It seems that dietary interventions can result in a disruption to bile acid metabolism and, in turn, exacerbate cholesterol accumulation in liver.

A 2-week cholesterol feeding (2%) decreased bile salt secretion by 28% but increased cholesterol secretion 118% in bile in the isolated perfused hamster liver, however phospholipid and bile volume did not change in this study (lChen et al. 1996). Low bile salt secretion might be consequence of a reduction in cholesterol conversion to bile acid and to some extent disturbance in bile acid production.

Increased hepatic ABCG5/8 and CYP7A1 mRNA expression, involved respectively in cholesterol secretion into bile duct and bile acid biosynthesis, and also higher fecal cholesterol excretion were observed in male mice fed cholesterol diet (1%) for 30 weeks compared to control diet fed mice. FXR transcript was significantly low in cholesterol fed mice (Savard et al. 2013). It seems that the effect of cholesterol diet on bile acid metabolism is different in short and long term course of study. Increased cholesterol secretion and bile acid production at

the gene levels might be kind of an adaptation to the diet that happened over a longtime study (30 weeks).

b. The effects of estrogen withdrawal

There is growing evidence showing that bile acid formation and consequently cholesterol excretion in the form of bile acid are reduced in Ovx animals. For instance, CYP7A1 and CYP8B1 gene expression, involved in bile acid biosynthesis in liver, were decreased in Ovx rats and mice (Kato et al. 2009; Kamada et al. 2011; Ngo Sock et al. 2013; Cote et al. 2014). This reduction was higher when Ovx rats were fed a high fat diet (42%) (Ngo Sock et al. 2013). Previously, it was reported that CYP7A1 activity and bile acid biosynthesis in short term was increased by 17 β-estradiol administration in rat hepatocyte monolayers in vitro (Chico et al. 1996). Recently it has been observed that 17 β-estradiol administration restored reduced hepatic CYP7A1 gene expression in Ovx mice fed a HFHC diet (1.25% cholesterol, 15% cocoa butter, and 0.5% cholic acid) (Kamada et al. 2011). Furthermore, mRNA levels of BSEP and MDR2, involved respectively in bile acid and phospholipid secretion from hepatocytes into bile canaliculi, were also decreased in Ovx rats. Moreover, estrogen deficiency in this study was associated with lower transcripts of nuclear receptor FXR (Cote et al. 2014), suggesting that there is no bile acid accumulation in the liver of Ovx rats. FXR has a key role in preventing bile acid accumulation and toxicity through inhibition of genes involved in bile acid biosynthesis and stimulation of genes involved in bile acid excretion. In fact, bile acids act as ligands for FXR and hepatic bile acid accumulation induces FXR activation which leads to a set of interactions resulting in the suppression of bile acid biosynthesis (Wang et al. 1999). It seems that not only bile acid biosynthesis but also its secretion via the hepatobiliary route are decreased by estrogen deficiency. Since biliary cholesterol excretion is the main way of cholesterol elimination from the body, disturbances in bile acid metabolism result in hepatic cholesterol accumulation.

Furthermore, in a series of studies conducted by Czerny et al. a decrease in total bile production was found in Ovx rats, supporting the hypothesis that biliary metabolic pathways are disrupted by estrogen withdrawal (Czerny et al. 2005; Czerny et al. 2006; Czerny et al. 2011). Since disrupted biliary metabolic pathways restrain hepatic cholesterol output, the

repression of key enzymes involved in bile acid excretion by ovariectomy is consistent with the massive accumulation of liver TC in Ovx rats.

On the other hand, hepatic ABCG5/G8 transcripts, involved in the secretion of cholesterol into the bile duct, were not altered in Ovx compared to Sham rats (Ngo Sock et al. 2013; Cote et al. 2014) and in aromatase KO mice implying that these transporters may not be modulated by estrogens (Hewitt et al. 2003).

Relationship between SHP and CYP7A1

SHP knockout (KO) mice showed an increase in bile acids synthesis and accumulation due to the removal of the suppressive effect of SHP on CYP7A1 and CYP8B1 (Kerr et al. 2002). Studies conducted on mice lacking SHP showed that under normal physiological conditions, the inputs of FXR and SHP are the major known pathway that mediates inverse feedback regulation of bile acid synthesis (Kerr et al. 2002). However, beyond normal physiological conditions such as liver damage (cholestasis, hepatosteatosis), additional mechanisms of regulation such as pregnane X receptor (PXR) and its target gene, CYP7A1 promoter binding factor (CPF), which is independent of FXR-SHP pathway, might come into play to modulate bile acid output in a way to protect against liver injury (Staudinger et al. 2001; Xie et al. 2001; Kerr et al. 2002)

It has also been reported that estrogen treatment directly through activation of SHP promoter induces SHP gene expression in mouse and rat liver and in human HepG2 cells (Evans et al. 2002; Lai et al. 2003). Wang et al recently reported that mRNA levels of hepatic SHP were reduced in Ovx mice while estrogen upregulated SHP expression through binding to its proximal promoter. SHP promoter has an estrogen receptor responsive element (ERE) site. In fact, SHP is a target gene for estrogen/estrogen receptor α (ERα) in the liver, suggesting a novel role of estrogen in improving hepatosteatosis through upregulation of SHP expression (Wang et al. 2015). Moreover, this ERα binding site overlaps with the known FXR binding site on the SHP promoter. The combination of ethynylestradiol plus FXR agonists did not produce an additive induction of SHP expression in Ovx mice, suggesting that simultaneous occupancy of this site by both estrogen receptor and FXR could not happen. To

test this hypothesis, Ovx mice were given either a control diet or a cholic acid diet (to activate FXR) along with subcutaneous ethynylestradiol treatment. Ethynylestradiol administration stimulated SHP expression in the mice consuming the control diet but not in mice fed the cholic acid diet. The lack of ethynylestradiol effect on the stimulation of SHP expression in animals in which was activated by FXR is consistent with the overlapping of ethynylestradiol and FXR response elements site on the SHP promoter. Surprisingly, it has been reported that induction of SHP by ethynylestradiol did not inhibit expression of the known SHP target genes CYP7A1 or CYP8B1 (Lai et al. 2003). It is expected that activation of SHP inhibits expression of CYP7A1 (Goodwin et al. 2000) which was not observed under estrogen treatment, suggesting that the stimulation of SHP by estrogens may not result in suppression of CYP7A1 transcript. Furthermore, it seems that SHP may act independently of FXR.

c. The effects of exercise training

A 30% increase in fecal bile acid and cholesterol excretion were reported by submitting healthy and LDL-R deficient male mice to voluntary wheel running for 2 and 12 weeks respectively. However the expression of key hepatic genes involved in bile acid synthesis including CYP7A1, CYP8B1, and CYP27A1 were not affected by exercise training in these studies. It was mentioned that changes in these genes might be at the posttranscriptional levels (Meissner et al. 2010b; Meissner et al. 2011). Nevertheless, Rocco et al. reported a decrease in hepatic CYP7A1 mRNA levels in 6 weeks trained CETP-tg mice. CETP-tg mice were used as an animal model to study RCT (Rocco et al. 2011).

On the other hand, Pinto et al. recently reported that CYP7A1 mRNA was increased by 6 weeks of training in male mice (Pinto et al. 2015). It seems that elevated gene expression of CYP7A1 as an enzyme involved in conversion of extra cholesterol to bile acids is a reflection of the beneficial effect of exercise training on RCT. Furthermore, higher CYP27A1 gene expression was observed in mice fed a lithogenic diet after 12 weeks of exercise training (Wilund et al. 2008).

Taken together, these data indicate that exercise training through upregulation of key hepatic genes involved in bile acid biosynthesis and increased fecal cholesterol and bile acid

excretion from the liver promotes cholesterol excretion from the liver. This would depict a mechanism by which exercise has a positive effect on RCT via the hepatobiliary pathway.

1.2.2 Non-biliary TICE pathway

Studies regarding the effects of cholesterol diet, estrogen withdrawal, and exercise training on TICE pathway have not been conducted so far. In fact, the findings discussed in this part are presenting the effects of above mentioned factors on intestinal genes involved in cholesterol metabolism including the intestinal genes of TICE pathway.

Bile acid content in small intestine was increased in Ovx rats as a consequence of a reduction in bile acid reabsorption through its transporter, IBAT in the terminal ileum (Kato et al. 2009). On the other hand, ABCA1 transcripts were higher in jejunum of Ovx rats which means an increase in intestinal cholesterol efflux through HDL (Ngo Sock et al. 2014a). Exercise training for 8 weeks had no impact on ABCA1 mRNA expression in the jejunum of Ovx rats (Ngo Sock et al. 2014a). On the other hand, ABCA1 gene expression was increased by 8 weeks training in the ileum of female rats (Ngo Sock et al. 2014b). An increase in ABCA1 transcripts in the proximal part of the small intestine following exercise training was also reported in Wistar rats (Khabazian et al. 2009). The intestine, similar to the liver, has a role in apoA1 lipidation through ABCA1. It seems that the effect of training on genes involved in HDL synthesis is the same for both liver and intestine, suggesting an increase in HDL synthesis and enhanced the cholesterol efflux following exercise training.

Increased small intestinal ABCG8 gene and protein expression were reported in treadmill-trained female rats after 8 weeks (Ghanbari-Niaki et al. 2012). However, Ngo Sock et al reported a reduction in NPC1L1 and ABCG5/G8 mRNA in the ileum of female rats trained for 8 weeks, suggesting less cholesterol accumulation in trained rats (Ngo Sock et al. 2014b). Furthermore, 12 weeks of exercise training resulted in a decrease in NPC1L1 and ABCG5/G8 transcript in duodenum of male mice. Lower NPC1L1 and less cholesterol absorption by enterocytes were proposed as a reason for the decrease in ABCG5/G8 transcripts as a transporter involved in cholesterol excretion from enterocytes into the lumen (Wilund et al. 2008).

Increased fecal bile acid and cholesterol loss and decreased jejunal expression of NPC1L1 were also reported following two weeks of voluntary wheel running in male mice, suggesting a decrease in intestinal cholesterol absorption (Meissner et al. 2010a). Moreover, lower ileal OSTα/β and hepatic NTCP mRNA, involved in bile acid enterohepatic circulation, were observed in 2 weeks voluntary running mice. Jejunal gene expression of ABCG5/G8 was not changed by running in this study. Reduced NPC1L1 transcript involved in cholesterol absorption was explained as a reason for unchanged jejunal ABCG5/G8 transcript. ABCG5 and G8 are responsible for sterol efflux from the enterocytes into the lumen, whereas NPC1L1 is involved in sterol entry from the lumen into the enterocyte (Kidambi et al. 2008). The authors concluded that regular exercise increases cholesterol turnover and through this action contributes to reduce the risk of cardiovascular diseases (Meissner et al. 2010a). In another study from this group, increased fecal bile acid loss and a 33% reduction in the aortic lesion size were also reported in LDL-R deficient mice after 12 weeks of running, implying some beneficial effects of exercise training in reducing the risk of atherosclerosis (Meissner et al. 2011). LDL-$R^{-/-}$ mice were submitted to the HFHC (0.15%) diet for 3 months and after that placed on 3 months treadmill exercise program with a normal diet which resulted in a 50% reduction in the aortic lesion area in LDL-$R^{-/-}$ mice compared to sedentary LDL-$R^{-/-}$ mice (Ramachandran et al. 2005). In addition to a reduction of the aortic lesion size, prevention of aortic valve sclerosis was also reported following exercise training in LDL-R deficient mice compared to sedentary group (Matsumoto et al. 2010).

A reduction in ileal FXR transcription factor transcripts along with its downstream genes ileal OSTα/β have been also reported in female rats trained for 8 weeks (Ngo Sock et al. 2014b). Since FXR has a key role in protecting the intestine against bile acid accumulation, reduced FXR transcript suggests that there is less bile acid accumulation in trained animals. It seems that the need to protect the intestine against bile acid overload is reduced in trained rats fed a normal diet (Ngo Sock et al. 2014b).

Taken together, it seems that reverse cholesterol efflux through intestinal genes of TICE similar to hepatobiliary pathway can be affected by exercise training and through excretion of the cholesterol from enterocytes into the lumen has a key role in reducing the risk of atherosclerosis.

1.3 General objective of the thesis and the presentation of manuscripts

The general objective of the present thesis is to provide physiological and molecular information to shed some light on how cholesterol and bile acid metabolism is affected by estrogens withdrawal in liver and intestine. It was also intended to introduce an appropriate non-pharmacological intervention (exercise training) to stimulate cholesterol excretion from the body and consequently decrease the risk of atherosclerosis. The three studies presented in this thesis have been conducted in an ovariectomized (Ovx) rat model as an experimental model of human post-menopausal state to study the metabolic consequences of loss of estrogens on cholesterol and bile acid metabolism. We also used a diet rich in cholesterol as a nutritional tool to investigate the role of liver and intestine in regulating cholesterol metabolism in our series of experiments.

The first study was undertaken to investigate the effects of high dietary cholesterol on key hepatic markers of VLDL assembly and some other molecular markers of cholesterol/bile acid metabolism including BSEP, MDR2, and NTCP, in Ovx rats. In this study, Ovx and sham operated (Sham) rats were given either a standard (SD), a SD supplemented with 0.25% cholesterol (SD+Chol), or a high fat supplemented with 0.25% cholesterol (HF+Chol) diets for 5 weeks. There is growing evidence showing that exercise training, as one of the best non-pharmacological strategies, improves cholesterol metabolism and diminishes the risk of atherosclerosis; however, the molecular mechanisms have not been fully explored. Therefore, in the second study, it was of interest to determine the effect of exercise training on key markers of hepatic cholesterol and bile acid metabolism by targeting the molecular markers of the FXR-SHP-CYP7A1 pathway in Ovx rats under cholesterol feeding. In the second study, the main experimental group was composed of Ovx rats fed a high cholesterol diet (Ovx-Chol) that was compared, on one hand, to a group of Ovx rats fed a standard diet (Ovx-SD) to observe the effects of the diet and, on the other hand, compared to a group of Sham operated rats fed the cholesterol diet (Sham-Chol) to observe the effect of estrogen withdrawal. These groups of Ovx and Sham rats were subdivided into either voluntary wheel running or sedentary groups for 5 weeks. Recent studies on newly identified transintestinal cholesterol

excretion (TICE) pathway showed that this pathway contributes in cholesterol excretion along with the hepatobiliary route indicating that cholesterol homeostasis in the body depends on a dynamic interplay between liver and intestine. In line with this concept, in the third study, we expanded our research to determine whether exercise training has effects on key intestinal cholesterol receptors involved in the TICE pathway in intact and Ovx rats fed a normal and a high cholesterol diet. We targeted key molecular markers involved in TICE via cholesterol uptake and excretion at the intestinal basolateral and apical membrane, respectively. To reach our goal, Sprague-Dawley rats were first divided into 4 groups: Sham operated and Ovx rats fed a standard diet (Sham-SD; Ovx-SD), or a high cholesterol diet (Sham-Chol; Ovx-Chol). These 4 groups were subsequently subdivided into either sedentary (Sed) or voluntary wheel running (Tr) groups for 6 weeks.

Chapter 2: Original research articles

2.1 Article 1 : High dietary cholesterol and ovariectomy in rats repress gene expression of key markers of VLDL and bile acid metabolism in liver.

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High dietary cholesterol and ovariectomy in rats repress gene expression of key markers of VLDL and bile acid metabolism in liver

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*Short title***:** Cholesterol, ovariectomy, and VLDL assembly

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Abstract

Background

The purpose of the study was to evaluate the effects of high dietary cholesterol in ovariectomized (Ovx) rats on several key markers of hepatic cholesterol and bile acid metabolism.

Method

Ovx and sham operated (Sham) rats were given either a standard (SD), a SD supplemented with 0.25% cholesterol (SD+Chol), or a high fat supplemented with 0.25% cholesterol (HF+Chol) diets for 5 weeks.

Results

Ovx was associated with higher ($P<0.05$) liver total cholesterol (TC) under the SD and the SD+Chol diet, while liver triglyceride (TG) content was higher in Ovx than in Sham rats in all 3 diet conditions. Surprisingly, the SD+Chol diet was associated with lower ($P<0.001$) plasma TC and TG levels in Ovx than in Sham rats, suggesting a decrease in VLDL secretion. Accordingly, several transcripts of key markers of VLDL synthesis including microsomal TG transfer protein (Mttp) and Apob-100 were decreased ($P<0.05$) in Ovx compared to Sham rats under the three dietary conditions and even more so for Mttp and Apob-100 when rats were fed the SD+Chol diet. Transcripts of bile acid transporters including bile salt export pump (Bsep) and Na+-taurocholate cotransporting polypeptide (Ntcp) were decreased by the addition of cholesterol to the SD diet in both Ovx and Sham rats.

Conclusion

These results indicate that a high cholesterol feeding and ovariectomy combine to reduce the gene expression of key markers of hepatic cholesterol/bile acid metabolism suggesting a reduction in excretion of cholesterol from the liver.

Key words: liver cholesterol, cholesterol diet, VLDL synthesis, LDL receptors

Introduction

There has been accumulating evidence in recent years that estrogens deficient state in ovariectomized (Ovx) animals and in postmenopausal women results in substantial liver triglyceride (TG) accumulation[1-4]. On the other hand, evidence of disturbances of cholesterol metabolism in link with estrogens deficiency is limited to observations of higher plasma levels of total cholesterol found in human as well as in animal models[5-8]. Liver cholesterol metabolism in Ovx animals has received little attention and shows some controversies. For instance, liver total cholesterol (TC) level was reported not to be affected by Ovx in previous studies[9-10] although we recently found an increase in rats ovariectomized for 8 wks[11]. There is, therefore, a need for more physiological and molecular information to better understand how liver, as a master regulator of cholesterol metabolism, is affected by estrogens withdrawal.

Nutritional approaches have been used frequently as a tool to investigate the role of the liver in regulating TG and cholesterol metabolism[12,13]. In Ovx animals, our group recently observed a large cholesterol accumulation in liver of rats fed a high fat (HF) diet, suggesting a vulnerability to cholesterol accumulation of Ovx animals when fed a HF diet[14]. The vulnerability of Ovx animals to dietary cholesterol has also been recently enlighten by the demonstration that gene expression of several molecular markers of VLDL assembly were reduced following high fat/high cholesterol diets[15]. However, dietary fat and dietary cholesterol have been reported to result in a positive synergistic interaction on the development of for instance hypercholesterolemia[13]. We, therefore, postulated that a better understanding of how Ovx animals regulate hepatic cholesterol metabolism would be obtained if the animals were fed a high cholesterol diet without the confounding effect of dietary fat.

In an attempt to shed some light on how liver of Ovx animals respond to high dietary cholesterol, we targeted key molecular markers of pathways involved in cholesterol/bile acids metabolism/transport that have recently been found to be affected by estrogens deficiency[15]. We first looked at molecular markers of VLDL assembly, including microsomal TG transfer protein (*Mttp*), a rate limiting molecule in VLDL assembly and secretion, *Apob-100* an essential structural protein that translocates into the luminal side of the endoplasmic reticulum, diacylglycerol acyltransferase 2 (*Dgat2*) involved in converting fatty acids into triglyceride

(TG), acyl-Coa: cholesterol acyl transferase (*Acat*2) that converts free cholesterol into cholesterol ester, cell death-inducing like-effector type B (*Cideb*) a protein involved in lipidation of particles, and small GTP-binding protein a (Sar1a) a protein that facilitates the movements of VLDL particles toward the Golgi apparatus.

Furthermore, we investigated the gene expression of molecular markers of bile acids metabolism/transport, a pathway that is tightly associated with elimination of cholesterol from the liver. These included ATP-cassette binding protein G5 and G8 (*Abcg5/Abcg8*) that export cholesterol from hepatocytes to the bile duct, bile salt export pump (*Bsep*) and multidrug resistance-associated transporter 2 (*Mdr2*) which stimulate bile acid and phospholipid transport from hepatocytes to bile canaliculi, Na+-taurocholate cotransporting polypeptide (*Ntcp*) involved in bile acid uptake in the basolateral membrane of the hepatocytes, farnasoid X receptor (*Fxr*) a nuclear receptor involved in regulation of hepatic bile acid biosynthesis, and cytochrome P450 7A1 (*Cyp7a1*) the main enzyme that catalyses the conversion of cholesterol into bile acids. Finally, we complemented our approach by investigating the response of hepatic LDL-receptor (*Ldl-r*), a major determinant of removal of LDL-cholesterol particles from the circulation, LDL receptor-related protein-1 (*Lrp-1*) involved in the removal of plasma remnant lipoproteins[16,17], and sterol regulatory element-binding protein-2 (*Srebp2*) a transcription factor involved in the regulation of cholesterol.

The aim of the present study was to determine the effects of high dietary cholesterol on hepatic key markers of VLDL and cholesterol/bile acid metabolism in Ovx rats.

Methods

Animal care

Female Sprague-Dawley rats (*n* 48; Charles River, St Constant, PQ, Canada) weighing 180-200 g upon arrival were housed individually. Food and water were supplied *ad libitum.* Their environment was controlled in terms of light (12 h light–dark cycle starting at 06:00 AM), humidity and room temperature (20–23°C). Body weight and food intake were monitored two times per week. All experimental procedures were conducted according to the protocols approved by the directives of the Canadian Council on Animal Care after institutional approval.

Diets and surgery

Rats were first acclimated to their environment for a period of one week while fed a chow diet (12.5 % lipid, 63.2 % CHO and 24.3 % protein; kJ from Agribrands Canada, Woodstock, Ontario, Canada). Thereafter, rats underwent either a bilateral ovariectomy (Ovx, *n* 24) or a bilateral sham-operation (Sham, *n* 24) according to the technique described by Robertson *et al.* under isoflurane anaesthesia[18]. After surgery, animals were injected with antibiotics (Tribrissen 24%; 0.125 cc/kg, subcutaneously) and analgesic (Carprofen; 4.4 mg/kg, subcutaneously) for 3 days. Thereafter, the Ovx and Sham rats were assigned to one of three following diets: standard (SD), SD supplemented with 0.25% cholesterol (SD+Chol), or high fat supplemented with 0.25% cholesterol (HF+Chol) for 5 weeks (Table 1). The 0.25% cholesterol dose was chosen to increase the dietary cholesterol without the atherogenic effects of higher doses used in several other studies[12,13].

Blood and tissue sampling

Rats were fasted overnight and sacrificed between 09:00 and 12:00 AM. Immediately after complete anaesthesia with isoflurane, the abdominal cavity was opened following the median line of the abdomen and approximately 4 ml of blood was collected from the abdominal vena cava (<45 s) into syringes pre-treated with ethylenediaminetetraacetic acid (15%; EDTA). Blood was centrifuged (3000 rpm; 4°C; 10 min; Beckman GPR Centrifuge) and the plasma kept for further analyses. Immediately after blood collection, the liver median lobe was removed and freeze-clamped. This sample was used for triacylglycerol (TG), cholesterol, and mRNA determinations. Several organs and tissues were removed and weighed (Mettler AE 100) in the following order: uterus, mesenteric, urogenital, and retroperitoneal fat deposits. The mesenteric fat pad consisted of adipose tissue surrounding the gastrointestinal tract from the gastroesophageal sphincter to the end of the rectum. The urogenital fat pad included adipose tissue surrounding the kidneys, bladder as well as ovaries, oviducts and uterus. The retroperitoneal fat pad was taken as that distinct deposit behind each kidney along the lumbar muscles. All tissue samples were frozen in liquid nitrogen immediately after being weighed (Mettler AE-100). All tissue samples were stored along with plasma samples at −80 ◦C until analyses were performed.

Biochemical analyses

Commercial kits from Sigma (Sigma; St-Louis, Missouri, USA) were used to determine plasma and liver TG by colorimetric method. Liver TG concentrations were estimated from glycerol released after KOH hydrolysis. Total liver cholesterol concentrations were determined with some adaptations of the procedure described by Folch *et al.*[19]. Briefly, 0.1g of liver was homogenized with chloroform–methanol mixture (2:1, v/v). The chloroform layer was collected and evaporated overnight. After adding 10% Triton X-100 in isopropanol, the sample was assayed for total cholesterol using commercial kits according to the manufacturer's instructions (Wako Diagnostics and Chemicals USA, Richmond, VA, USA). Plasma total cholesterol was determined using the same kits supplied by Wako.

Molecular analyses

Total RNA was extracted from frozen liver with the use of RNA extraction Mini kit (Invitrogen) according to the manufacturer's protocol. Then RNA was treated with DNase (Invitrogen) in order to avoid genomic contamination. Total RNA (2 μg) was reverse-transcribed into complementary DNA using high capacity complementary DNA reverse transcription kits (Applied Biosystems). RT samples were stored at -20°C. Gene expression for *β-actin* was determined using a pre-validated Taqman Gene Expression Assay (Applied Biosystems, Rn0146*26*61, Foster City, CA). Gene expression level for target genes was determined using assays designed with the Universal Probe Library from Roche. The primer sets and UPL probe numbers are presented in Table 2. To validate the efficiency of the qPCR assays, we used a mix of the samples tested in the study.

The ABI PRISM® 7900HT (Applied Biosystems) was used to detect the amplification level and was programmed with an initial step of 3 min at 95˚C, followed by 40 cycles for 5 s at 95˚C and 30 s at 60˚C. All reactions were run in triplicate and the average values of threshold cycle *(C_T)* were used for quantification. *β-actin* was used as endogenous control. The relative quantification of target genes was determined using the $\Delta\Delta C_T$ method. Briefly, the C_T values of target genes were normalized to an endogenous control gene (*β-actin*) ($\Delta C_T = C_{T\ target} - C_{T\ \beta\text{-actin}}$) and compared with a calibrator: ($\Delta\Delta\ C_T = \Delta\ C_{T\ Sample} - \Delta C_{T\ Calibrator}$). Relative expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) and the formula is $RQ = 2^{-\Delta\Delta C_T}$.

Statistical analysis

All data are presented as mean ± SE. Statistical significance *(P*<0.05*)* was determined using a 2-way ANOVA for non-repeated measures with ovariectomy and diets as main factors. Fisher LSD *post hoc* test was used in the event of a significant interaction effect. For a significant diet effect without interaction, Fisher LSD from a one-way ANOVA was used.

Results

Body weight, intra-abdominal fat pad weight and food intake measured at the end of the experiment were all significantly (*P*<0.01) higher in Ovx than in Sham animals in the three dietary groups (Table 3). The addition of Chol to the SD diet did not result in any changes in both Ovx and Sham rats in body weight, intra-abdominal fat pad weight, and food intake when compared to animals fed the SD diet. However, food intake was significantly (*P*<0.05) higher in rats fed the HF+Chol diet as compared to the two other diets in both Sham and Ovx animals. Body weight and intra-abdominal fat pad weight also showed a strong tendency to be higher in rats fed the HF+Chol diet with respect to the Sham and Ovx conditions (*P*=0.06 and *P*=0.08, respectively). Uterus weight was lower (*P*<0.001) in Ovx than in Sham rats throughout the dietary conditions confirming total ovariectomy (Table 3).

Plasma and liver lipid profile

Liver TC levels in Sham rats were progressively higher (*P*<0.01) following the SD, SD+Chol and HF+Chol diets order (Fig. 1(a)). This was not observed, however, in Ovx animals. Liver TC levels were on the whole higher (*P*<0.001) in Ovx than in Sham rats but not under the HF+Chol diet (Fig. 1(a)). Liver TG levels were higher (*P*<0.001) in Ovx than in Sham rats in all three dietary conditions (Fig. 1(b)). Liver TG levels were not affected by the SD+Chol diet while it was higher (*P*<0.01) in the HF+Chol diet compared to the SD diet in both Ovx and Sham animals. A completely different picture was observed for the TC and TG concentrations measured in plasma. Plasma TC levels were higher (*P*<0.05) in Ovx than in Sham rats under the SD and HF+Chol diets but largely lower (*P*<0.001) in Ovx than in Sham animals under the SD+Chol diet (Fig. 1(c)). On the whole, plasma TC levels in Ovx rats were lower (*P*<0.05) under the two Chol diets as compared to the SD diet. On the opposite of liver TG, plasma TG levels were lower (*P*<0.05) in Ovx than in Sham rats under the SD and SD+Chol diets (Fig.

1(d)). Plasma TG values were also lower (P<0.001) in Sham rats fed the HF+Chol diet as compared to Sham rats in the 2 other diets. On the whole, TC and TG levels under the SD+Chol feeding were higher in liver of Ovx vs Sham animals while the opposite was found in plasma.

Hepatic gene expression

Gene expressions of key molecules involved in VLDL synthesis are presented in Fig. 2. On the whole, mRNA levels of 5 out of the 6 genes involved in VLDL synthesis (including *Sar1a* and *Cideb* and with the exception of *Acat2*) were lower (P<0.05) in Ovx than in Sham rats in all dietary conditions (only under the SD+Chol diet for *Dgat2*). The lowest (P<0.01) values for *Mttp*, *Dgat2,* and *Apob-100* transcripts were found in Ovx rats under the SD+Chol (as compared to the SD diet), suggesting that Ovx and the SD+Chol diet combine to decrease VLDL synthesis. Interestingly, we found that the decreased responses in gene expression of *Mttp*, *Dgat2*, *Acat2*, and *Apob-100* under the SD+Chol diet were statistically attenuated (as compared to the SD diet) when rats were fed the HF+Chol diet (Fig. 2).

To complete the information we investigated genes involved in hepatic cholesterol and biliary acids transport (Fig. 3). With the exception of *Mdr2* transcripts, we found no difference between Ovx and Sham rats in any of the measured gene expression in all dietary conditions (Fig. 3). However, we observed that several of the genes (*Abcg8*, *Mdr2*, *Bsep*, *Fxr,* and *Ntcp*) had their transcripts decreased when animals were fed the SD+Chol compared to the SD diet. On the other hand, *Abcg5/g8* and *Cyp7a1* mRNA levels were higher (P<0.01) in HF+Chol compared to SD+Chol fed animals.

To further explore hepatic cholesterol metabolism, we measured gene transcripts of key molecules involved in cholesterol uptake from cholesterol rich lipoproteins. We first found a lower (P<0.001) *Lrp1* gene expression in Ovx than in Sham animals in all dietary groups (Fig. 4(b)). Interestingly, we observed that gene expression of *Ldlr* and *Srebp2* were highly decreased (P<0.001) when rats were fed either the SD+Chol or the HF+Chol diets as compared to rats fed the SD diet, while *Lrp1* transcripts were decreased (P<0.05) only under the SD+Chol diet (Fig. 4).

Discussion

The main finding of the present study is that high dietary cholesterol represses gene expression of key molecular markers of VLDL synthesis (*Mttp, Acat2, Apob-100*) in Sham rats and even more so in Ovx rats (*Mttp* and *Apob-100*). In addition, the sole addition of cholesterol to the SD diet reduced gene expression of several liver markers of bile acid and phospholipid transport (*Bsep, Ntcp, Mdr2*) in both Sham and Ovx animals. Finally, gene expression of key markers involved in liver LDL cholesterol uptake (*Ldl-r* and *Lrp1*) was also decreased by the sole addition of cholesterol to the diet in Sham as well as in Ovx rats. These results first indicate that the cholesterol component in a mixed diet is a determinant factor that regulates liver cholesterol metabolism in sham as well as in Ovx rats. In addition, the present molecular responses to high cholesterol diet converge to indicate a reduction in hepatic TG and cholesterol excretion from the liver, this being to a certain extent, accentuated by the absence of estrogens.

We recently reported data showing an impairment of VLDL assembly following ovariectomy and high fat/high cholesterol diets in rats[15]. The present study extends these findings by indicating that the sole high content of cholesterol in the diet impaired VLDL assembly in Sham and even more so in Ovx animals. Ovx has been previously associated with a decrease in VLDL production in rat via *Mttp* regulation[20]. Accordingly, molecular expression of several genes related to VLDL assembly, including *Mttp* and *Apob*, was reduced in Ovx rats under the present SD diet. That high cholesterol diet decreases even more so VLDL assembly/production in Ovx animals suggests an additive effect of these two stimuli. This additive effect on VLDL assembly/production is corroborated by the higher accumulation of TC and TG in liver along with lower levels of plasma TC and TG found in Ovx compared to Sham animals under the SD+Chol diet.

It is not clear at this point the mechanism by which a high cholesterol diet would repress VLDL assembly/production. Hepatic VLDL production has been reported to be reduced by competitive inhibitors of HMGCoA-r reductase, the main enzyme responsible for cholesterol biosynthesis, through the regulation of the SREBP family transcription factors[21,22]. SREBP-2 gene expression was repressed by the cholesterol diet in the present study. Although there is evidence that several steps of the VLDL assembly/secretion are under the control of SREBP-

1, there are also indications that upon the cell type and physiological conditions, SREBP1 and SREBP2 may mediate changes in lipoprotein assembly and secretion[22]. An alternative explanation for the decrease in VLDL synthesis/production following high dietary cholesterol and Ovx would be the endoplasmic reticulum (ER) stress. There are indications that cholesterol can induce hepatic ER stress through free cholesterol accumulation in the ER[23], and that ER stress limits VLDL assembly and secretion through apoB degradation[24].

In addition of reducing VLDL synthesis/production, high cholesterol feeding in Ovx and Sham animals also repressed gene expression of key markers of bile acid metabolism. The present finding of a reduction in *Bsep* and *Mdr2* gene expression suggests a reduction in bile acid and phospholipid excretion, while the reduction in *Ntcp* mRNA suggests a reduction in bile acid uptake from the entero-hepatic circulation. These observations may be taken as an indication of a reduction of the entero-hepatic circulation of bile acids. Furthermore, the reduction in *Fxr* gene expression in the SD+Chol fed rats suggests that even though cholesterol level was increased in liver, there was no accumulation of bile acids since the role of hepatic *Fxr* is to prevent bile acid hepatotoxicity. Dietary interventions such as high cholesterol/high fat diets have been reported to repress *Fxr* gene expression in liver[13,15]. The gene expression of the present key molecules thus supports the previous suggestion that high cholesterol feeding in rats disrupts bile acid metabolism [15].

In addition to a decrease in gene expression of markers of VLDL synthesis and bile acid transport, dietary cholesterol in the present study resulted in a down regulation in gene expression of *Ldl-r* and *Lrp1* in Sham and Ovx animals suggesting a decrease in cholesterol uptake from circulation. This response was even more pronounced in Ovx rats for *Lrp1* transcripts. Ovx has been previously reported to be associated with a reduction in the expression of several genes involved in the uptake of lipoprotein molecules[11,25]. The decrease in LDL receptors was most likely linked to the excess hepatic cholesterol level through the decrease in *Srebp2* gene expression[26,27]. Taken together, the present results suggest an association between reduced VLDL synthesis/production, reduced bile acids transport and reduced LDL receptors under high dietary cholesterol in Sham as well as in Ovx rat.

The interest of comparing a high cholesterol diet with and without the addition of a high fat content is enlighten by the observation that the sole addition of cholesterol to a SD diet had no effect on body weight in Sham and Ovx animals while the addition of fat in the diet caused an

increase in food intake and a strong tendency in higher body weight (P=0.06) and intra-abdominal (P=0.08) fat accumulation (Table 3). This implies, on a clinical point of view, that a high cholesterol diet might not be perceived as being deleterious since it does not affect body weight when in fact it causes several metabolic perturbations. One noticeable effect of adding fat to cholesterol in the diet was the increase in liver fat accumulation resulting from the diet and most likely from increased lipogenesis[28]. On the other hand, higher expression of *Abcg5/g8* and *Cyp7a1* were also observed under HF+Chol feeding, both of these genes being involved in cholesterol excretion from the liver. These responses may be taken as an indication that hepatic cholesterol metabolism may be less vulnerable to high fat/high cholesterol feeding than high dietary cholesterol alone.

In summary, results of the present study first indicate that gene expressions of key markers of VLDL synthesis/production are reduced under high cholesterol feeding and that this reduction is exacerbated in Ovx animals. In addition, the present data provide evidence that the activities of bile acid and *Ldl-r* pathways are also reduced by the sole addition of cholesterol to a SD diet in Sham as well as in Ovx animals. These results point to the direction as if the liver under high cholesterol feeding reduces its excretion of cholesterol, at least on a short term basis, thus contributing to exacerbate liver fat and cholesterol accumulation known to occur in Ovx animals.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authorship

This work was performed at the University of Montreal. ZF (conception, design, acquisition of data), IC and ETNS. (acquisition of data) JML (conception, design). All authors contributed to data interpretation and drafting/revising the article for important intellectual content. All authors approved the final version to be published.

Figure legends

Fig 1. Liver and plasma total cholesterol (TC) and triacylglycerol (TG) levels in sham (■) and ovariectomized (Ovx, □) rats fed a standard diet (SD), a SD+0.25% cholesterol diet (SD+Chol), and a high fat+0.25% cholesterol diet (HF+Chol). Values are mean ± SE with n = 8 rats per group. * Significantly different from respective Sham rats (P<0.05), **(P<0.01), *** (P<0.001); † Significantly different from respective rats fed the SD diet (P<0.05), †† (P<0.01), ††† (P<0.001); δ Significantly different from respective rats fed the SD+Chol diet (P<0.05), δδδ (P<0.001).

Fig 2. Hepatic mRNA expression of genes involved in VLDL synthesis/production in sham (■) and ovariectomized (Ovx, □) rats fed a standard diet (SD), a SD+0.25% cholesterol diet (SD+Chol), and a high fat+0.25% cholesterol diet (HF+Chol). Values are mean ± SE with n = 8 rats per group. *** Significantly different from respective Sham rats (P<0.001); † Significantly different from rats fed the respective SD diet (P<0.05), †† (P<0.01), ††† (P<0.001); δδ Significantly different from rats fed the respective SD+Chol diet (P<0.01). *Mttp* microsomal TG transfer protein; *Dgat-2*, diacylglycerol acyl transferase-2; *apoB-100*, apolipoprotein B-100; *Acat-2,* acyl-coA cholesterol acyl transferase-2; *Sar1a,* small GTP-binding protein; *Cideb*, cell death-inducing like-effector type B.

Fig. 3. Hepatic mRNA expression of genes related to bile acid metabolism in sham (■) and ovariectomized (Ovx, □) rats fed a standard diet (SD), a SD+0.25% cholesterol diet (SD+Chol), and a high fat+0.25% cholesterol diet (HF+Chol). Values are mean ± SE with n = 8 rats per group. *** Significantly different from respective Sham rats (P<0.001); † Significantly different from rats fed the respective SD diet (P<0.05), †† (P<0.01), ††† (P<0.001); δδ Significantly different from rats fed the respective SD+Chol diet (P<0.01), δδδ (P<0.001). *Abcg5/Abcg8*, ATP-cassette binding protein G5 and G8; *Mdr2,* multidrug resistance-associated transporter 2; *Bsep*, bile salt export pump; N*tcp*, Na+-taurocholate cotransporting polypeptide; *Cyp7a1*, cytochrome P450 7A1; *Fxr*, farnesoid X receptor.

Fig. 4. Hepatic mRNA expression of genes involved in uptake of cholesterol rich lipoproteins from the circulation in sham (■) and ovariectomized (Ovx, □) rats fed a standard diet (SD), a SD+0.25% cholesterol diet (SD+Chol), and a high fat+0.25% cholesterol diet (HF+Chol). Values are mean ± SE with n = 8 rats per group. *** Significantly different from respective

Sham rats ($P<0.001$); † Significantly different from rats fed the respective SD diet ($P<0.05$), ††† ($P\leq 0.001$). *Ldlr*, LDL-receptor; *Lrp-1,* LDL receptor-related protein-1; *Srebp2,* sterol regulatory element-binding protein-2.

Table 1. Diet description

	Standard Diet (SD) (D12450J)	SD + Chol (0.25%) (D13020701)	High Fat + Chol (0.25%) (D13020703)
(%)			
Protein	19.2	19.2	22.8
Carbohydrate	67.3	67.1	45.7
Fat	4.3	4.3	20.2
(g)			
Casein	200	200	200
L-Cystine	3	3	3
Corn Starch	506.2	506.2	202.5
Maltodextrin 10	125	125	125
Sucrose	68.8	68.8	68.8
Cellulose, BW200	50	50	50
Soybean Oil	25	25	25
Lard	20	20	155
Mineral Mix S10026	10	10	10
DiCalcium Phosphate	13	13	13
Calcium Carbonate	5.5	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10
Choline Bitartrate	2	2	2
Cholesterol	0.0	2.63	2.63
Kcal/g	3.85	3.84	4.56

Formulated by: Research Diets, Inc. (20 Jules Lane, New Brunswick, NJ 08901 USA)

Table 2. Oligonucleotide primers used for quantitative real-time polymerase chain reaction

Gene	Oligo FWD	Oligo REV
MDR2	ggcattctccatcatcctgt	cacttctgttgctttactgtgtca
BSEP	cggtggctgagagatcaaat	tgcgatagtggtggagaaca
ABCG5	cggagagttggtgttctgtg	caccgatgtcaagtccatgt
ABCG8	cagatgctggctatcataggg	ctgatttcatcttgccacca
ACAT-2	cctcacagatgcgtttcaca	ctctgctcacttgccatttt
Apob	gatggagatgggagatgaggt	gggctcctcatcaacaagag
Cideb	gctccaatggcctgctaag	ttatgatcacagacacggaagg
Cyp7a1	ggagcttatttcaaatgatcagg	cactctgtaaagctccactcactt
DGAT-2	aggatctgccctgtcacg	gtcttggagggccgagag
HMG-CoAr	caaccttctacctcagcaagc	acagtgccacacacaattcg
LDLr	tgctactggccaaggacat	ctgggtggtcggtacagtg
LRP-1	aatcgagggcaagatgacac	ccagtctgtccagtacatccac
Mttp	gcgagtctaaaacccgagtg	cactgtgatgtcgctggttatt
FXR	ccacgaccaagctatgcag	tctctgtttgctgtatgagtcca
Sar1a	gggcaaaccacaggaaag	cactgcacatgaacacttcca
SREBP-2	gtgcagacagtcgctacacc	aatctgaggctgaaccagga
NTCP	aaaatcaagcctccaaaggac	ttgtgggtacctttttccaga
ActB	cccgcgagtacaaccttct	cgtcatccatggcgaact
GAPDH	ccctcaagattgtcagcaatg	agttgtcatggatgaccttgg

Table 3. Anthropometric parameters and food intake

Variables	SD		SD + Chol (0.25%)		HF + Chol (0.25%)	
	Sham	Ovx	Sham	Ovx	Sham	Ovx
Final Body Weight (g)	353.9 ± 11.3	439.6 ± 14.5 ***	347.5 ± 8.7	431.6 ± 25.7***	391.6 ± 13.7	467.4 ± 15.6***
Intra-abdominal fat pad weights (g)	29.2 ± 4.4	39.3 ± 3.7 **	26.6 ± 2.6	40.2 ± 4.9 **	38.7 ± 3.8	43.8 ± 2.6 **
Food intake (kcal/day)	82.7 ± 4.3	99.8 ± 4.2 ***	81 ± 2.5	95.7 ± 7.0***	94.9 ± 4.7 † δ	110.1 ± 4.2 *** † δ
Uterus (g)	0.50 ± 0.03	0.10 ± 0.01***	0.51 ± 0.07	0.10 ± 0.01***	0.47 ± 0.05	0.11 ± 0.01***

SD, standard diet; HF + Chol (0.25%), high fat + cholesterol; Ovx, ovariectomised; Sham, sham operated.
Values are mean ± SE with n = 8 rats per group.
** Significantly different from the sham groups ($P < 0.01$), *** ($P < 0.001$)
† Significantly different from SD diet ($P < 0.05$)
δ Significantly different from SD+ Chol diet ($P < 0.05$)
Initial Body weight 190.63 ± 1.78 191.75 ± 1.32 191.25 ± 1.18 191.38 ± 2.44 193.28 ± 1.71 192.38 ± 2.09

Fig. 1

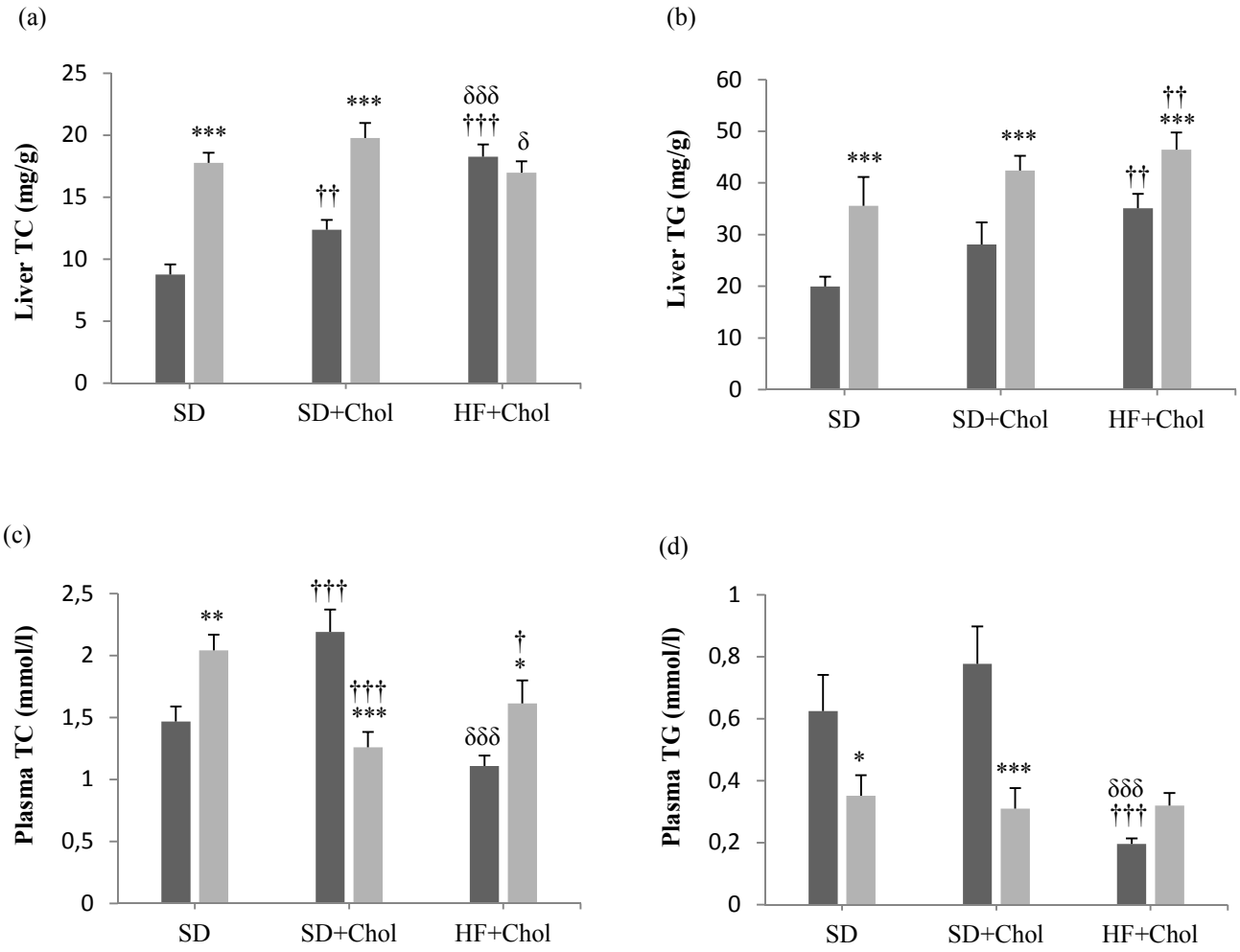
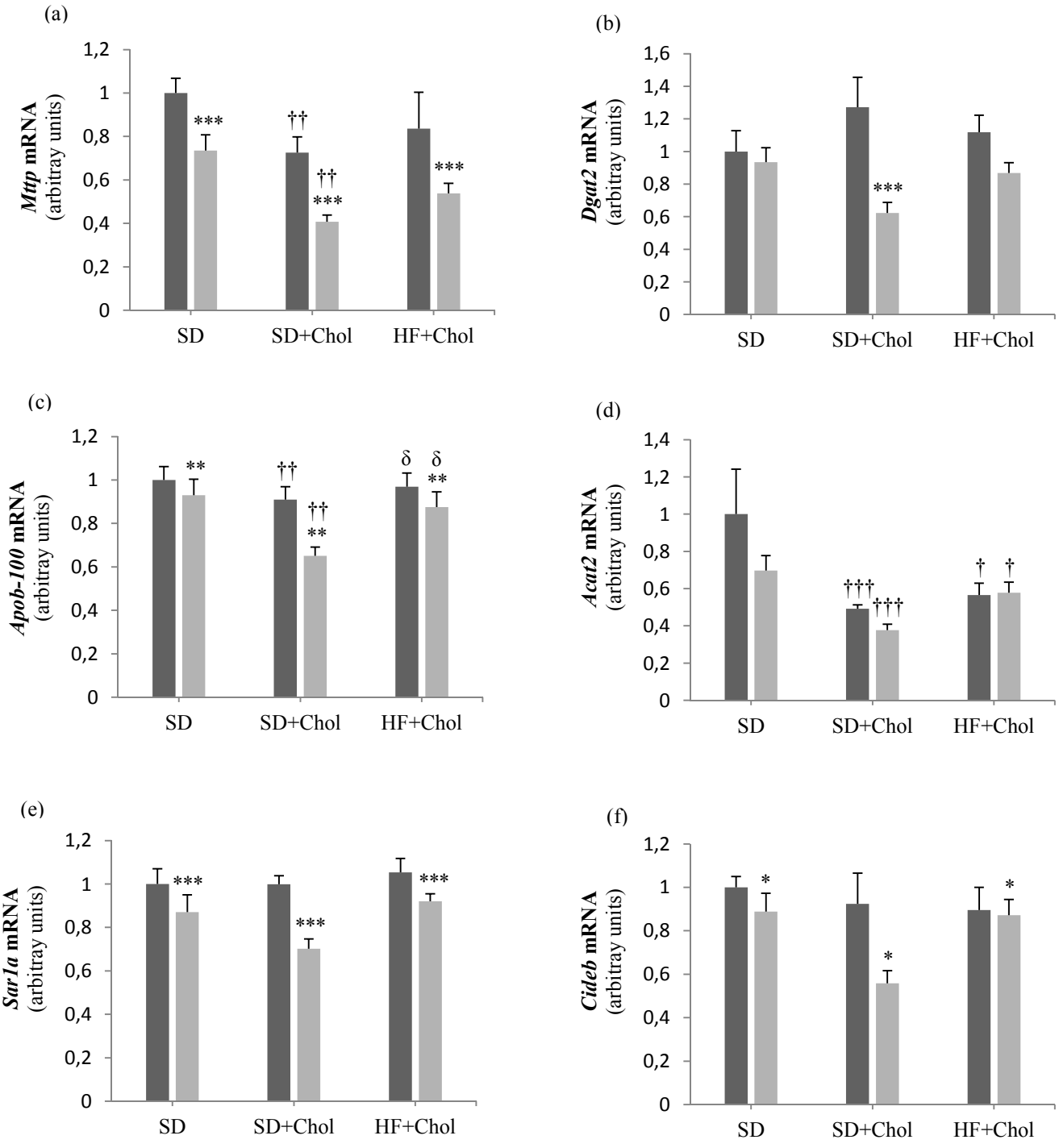

(a)
Liver TC (mg/g)
25
20
15
10
5
0
***
***
δδδ
†††
δ
††
SD
SD+Chol
HF+Chol
(b)
Liver TG (mg/g)
60
50
40
30
20
10
0
***
***
††
***
††
SD
SD+Chol
HF+Chol
(c)
Plasma TC (mmol/l)
2,5
2
1,5
1
0,5
0
**
†††
†††
***
δδδ
†
*
SD
SD+Chol
HF+Chol
(d)
Plasma TG (mmol/l)
1
0,8
0,6
0,4
0,2
0
*
***
δδδ
†††
SD
SD+Chol
HF+Chol

Fig. 2


(a)
Mtp mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
***
††
††
***
***
SD
SD+Chol
HF+Chol
(b)
Dgat2 mRNA (arbitray units)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
***
SD
SD+Chol
HF+Chol
(c)
Apob-100 mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
**
††
††
**
δ
δ
**
SD
SD+Chol
HF+Chol
(d)
Acat2 mRNA (arbitray units)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
†
†
SD
SD+Chol
HF+Chol
(e)
Sar1a mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
***
***
***
SD
SD+Chol
HF+Chol
(f)
Cideb mRNA (arbitray units)
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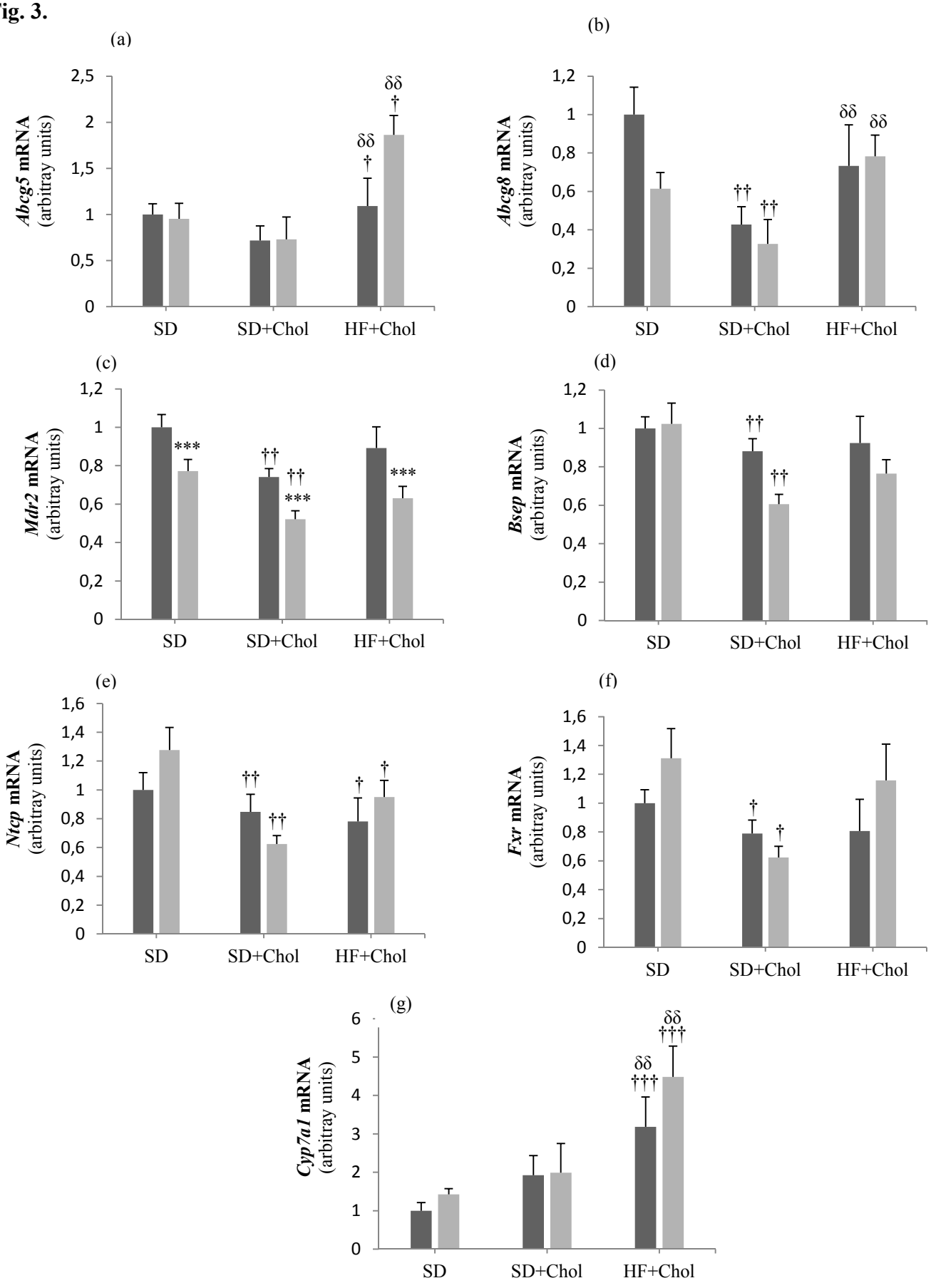
1
0,8
0,6
0,4
0,2
0
*
*
*
SD
SD+Chol
HF+Chol

Fig. 3.


(a)
Abcg5 mRNA (arbitray units)
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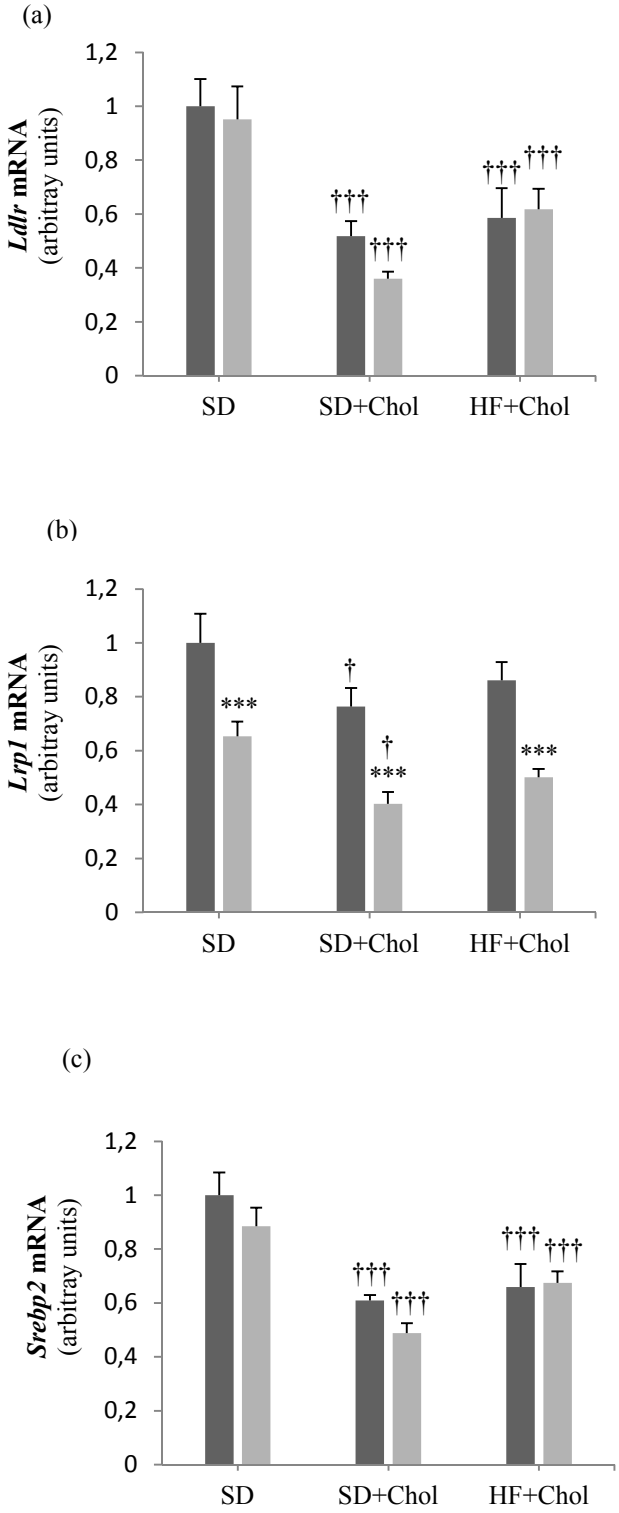
2
1,5
1
0,5
0
δδ
†
δδ
†
SD
SD+Chol
HF+Chol
(b)
Abcg8 mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
††
††
δδ
δδ
SD
SD+Chol
HF+Chol
(c)
Mdr2 mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
***
††
††
***
***
SD
SD+Chol
HF+Chol
(d)
Bsep mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
††
††
SD
SD+Chol
HF+Chol
(e)
Ntcp mRNA (arbitray units)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
††
††
†
†
SD
SD+Chol
HF+Chol
(f)
Fxr mRNA (arbitray units)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
†
†
SD
SD+Chol
HF+Chol
(g)
Cyp7a1 mRNA (arbitray units)
6
5
4
3
2
1
0
δδ
†††
δδ
†††
SD
SD+Chol
HF+Chol

Fig. 4


(a)
Ldlr mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
†††
†††
SD
SD+Chol
HF+Chol
(b)
Lrp1 mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
***
†
†
***
***
SD
SD+Chol
HF+Chol
(c)
Srebp2 mRNA (arbitray units)
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
†††
†††
SD
SD+Chol
HF+Chol

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2.2 Article 2: Exercise training counteracts cholesterol feeding in liver of Ovx rats by upgrading molecular markers of bile acid metabolism including Small Heterodimer Partner

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Journal

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Exercise training counteracts cholesterol feeding in liver of Ovx rats by upgrading molecular markers of bile acid metabolism including Small Heterodimer Partner

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Abstract

Small heterodimer partner (SHP) is an important transcriptional factor involved in the regulation of glucose, lipid, and bile acid metabolism in liver. *Shp* has been reported to be down regulated in ovariectomized (Ovx) mice and up-regulated by estrogens suggesting a link between estrogens and *Shp*. The purpose of the present study was to determine the effects of exercise training on *Shp* and key markers of cholesterol and bile acid homeostasis in Ovx rats under cholesterol feeding. Our main experimental group was composed of Ovx rats fed a high cholesterol diet (Ovx-Chol) that was compared to a group of Ovx rats fed a standard diet (Ovx-SD) and a group of Sham operated rats fed the cholesterol diet (Sham-Chol). These groups of Ovx and Sham rats were subdivided into either voluntary wheel running or sedentary groups for 5 weeks. Plasma and liver total cholesterol levels were not affected by exercise training in any of the experimental conditions. Cholesterol feeding in both Sham and Ovx rats resulted in significantly (P<0.001) higher hepatic cholesterol accumulation than in Ovx-SD. Hepatic LDL receptor (*Ldlr*) and proprotein convertase subtilisin/kexin type 9 (*Pcsk9*), which are involved in cholesterol uptake from circulation, were not influenced by training. A main effect of training (P< 0.05) was, however, found for transcripts of *Shp* and cholesterol 7 alpha-hydroxylase (*Cyp7a1*), the main gene involved in bile acid biosynthesis from cholesterol. These results suggest that voluntary wheel running may modulate cholesterol metabolism in Ovx animals through up-regulation of *Shp* and bile acid formation.

Key words: LDL receptor, *Pcsk9*, *Cyp7a1*, liver cholesterol

Introduction

Accumulated evidence from human and animal studies shows that estrogen deficient state leads to disturbances in fat and cholesterol metabolism. While most studies were limited to assessment of plasma cholesterol levels (Park et al. 2011b; Chaudhuri et al. 2012; Kaur et al. 2013) recent studies indicate that hepatic cholesterol metabolism is also affected by estrogen withdrawal (Ngo Sock et al. 2013; Ngo Sock et al. 2014a). Considering that the liver is a master regulator of cholesterol metabolism, there is a need for a better understanding of the liver response under estrogen withdrawal. Nutritional approaches have been frequently used to investigate the response of the liver to estrogen deficient conditions (Cote et al. 2013; Savard et al. 2013). For instance, a large hepatic cholesterol accumulation was observed in ovariectomized (Ovx) animals when fed a high fat and/or high fat/high cholesterol diets (Cote et al. 2014; Ngo Sock et al. 2014a). Furthermore, gene expression of different markers such as low density lipoprotein-receptor (*Ldlr*) was decreased in liver of Ovx rats fed a cholesterol diet probably due to cholesterol accumulation in liver (Farahnak et al. 2015). This study also revealed that the combined effect of cholesterol diet and ovariectomy resulted in suppression of transcripts of hepatic bile salt export pump (*Bsep*) and Na^+-taurocholate cotransporting polypeptide (*Ntcp*), two transporters of bile acids in liver. It appears that a better knowledge of the contribution of bile acid transport/metabolism, which is the main way of elimination of excess cholesterol transiting through the liver, can shed some light on how liver regulates cholesterol metabolism in Ovx animal.

In addition to nutritional approaches, there is some evidence that exercise training may also influence hepatic cholesterol metabolism. For instance, it has been reported that voluntary wheel running increases cholesterol conversion into bile acids and consequently decreases atherosclerotic burden in *Ldlr* deficient mice (Meissner et al. 2011). It seems that running modulates cholesterol metabolism through bile acid formation. Running also enhanced fecal cholesterol excretion in mice implying beneficial effects of exercise (Meissner et al. 2010b).

One of the key molecules involved in bile acid metabolism is small heterodimer partner (*Shp*). *Shp* is a main transcriptional regulator involved in the regulation of glucose, lipid and bile acid metabolism in liver. *Shp* acts through its interaction with several nuclear receptors, including farnesoid X receptor (*Fxr*), a transcription factor known to regulate *Cyp7a1* (Chiang 2004; Modica et al. 2012), the main enzyme of the biosynthesis of bile acids from cholesterol

(Jelinek et al. 1990). It was reported that *Shp* is down regulated in Ovx mice and alternatively, up-regulation of *Shp* by estrogens suggests that there is a link between estrogens and *Shp* (Wang et al. 2015).

The purpose of the present study was to determine the effect of training on key markers of hepatic cholesterol and bile acid metabolism in Ovx rats under cholesterol feeding. We targeted gene expression of molecules involved in cholesterol metabolism such as *Ldlr* and *Pcsk9*, and bile acid metabolism in liver, including *Shp* and *Cyp 7a1*.

Materials and Methods

Animal care

Female Sprague-Dawley rats (*n*=49) weighing 180–200 g were obtained from Charles River (St-Constant, PQ, Canada) and housed individually to monitor food intake in each animal. The animals had *ad libitum* access to food and tap water. Their environment was controlled in terms of light (12 h light–dark cycle starting at 06:00 AM), humidity and room temperature (20–23°C). Body weight and food intake were monitored bi-weekly from the start of experiment. All experimental procedures were conducted according to the protocols approved by the *Institutional Animal Care and Use Committee of the University of Montreal* in agreement with The Canadian Council on Animal Care's rules (CCAC-CCPA).

Diets, surgery, and exercise protocols

Rats were first acclimated to their environment for a period of one week while fed a chow diet (12.5 % lipid, 63.2 % CHO and 24.3 % protein; kJ from Agribrands Canada, Woodstock, Ontario, Canada). Thereafter, rats underwent either a bilateral ovariectomy (Ovx, *n*=34) or a bilateral sham-operation (Sham, *n*=15) according to the technique described by Robertson et al. under isoflurane anaesthesia (Robertson et al. 1984). After surgery, all animals were injected with antibiotics (Tribrissen 48%; 0.125 cc/kg, subcutaneously) and analgesics (Carprofen; 4.4 mg/kg, subcutaneously) for 3 days.

Six groups of rats were compared. Our main experimental group was composed of Ovx rats fed a high cholesterol diet (Ovx-Chol, *n*=17) that was compared to a group of Ovx rats fed a standard diet (Ovx-SD, *n*=17) and a group of Sham rats fed the cholesterol diet (Sham-Chol, *n*=15). These three groups of Ovx and Sham rats were subdivided into either voluntary wheel running (Tr) or sedentary groups (Sed). In fact, the effects of training were determined in Ovx-

Chol rats that were compared to two control groups: an Ovx-SD group and a Sham-Chol group to isolate the diet and estrogens withdrawal effects, respectively. The Chol diet consisted of a standard diet (SD) supplemented with 0.25% cholesterol (SD+Chol) (Table S1). Tr rats were placed in freely rotating wheel cages while Sed rats were placed in blocked running wheel cages. Each wheel cage was equipped with a sensor connected to a computerized data acquisition system enabling the continuous sampling of running data from individual rats. The rats were on diet and training for 5 weeks.

Blood and tissue sampling

Rats were euthanized between 09:00 and 12:00 AM. Food was removed from the cage overnight before sacrifice. Rats refrained from exercising ~24h before sacrifice. Immediately after complete anaesthesia with isoflurane, the abdominal cavity was opened following the median line of the abdomen. Approximately 4 ml of blood was collected from the abdominal vena cava (<45 s) into syringes treated with ethylenediaminetetraacetic acid (15%; EDTA). Blood was centrifuged (3000 rpm; 4°C; 10 min; Beckman GPR Centrifuge; Beckman Coulter) and the plasma kept for further analyses. Immediately after blood collection, the liver median lobe was removed and freeze-clamped. This sample was used for triacylglycerol (TG), cholesterol, and mRNA determinations. Several organs and tissues were removed and weighed (Mettler AE 100) in the following order: uterus, urogenital, retroperitoneal and mesenteric fat deposits. The urogenital fat pad included adipose tissue surrounding the kidneys, uterus and bladder as well as ovaries, oviducts and uterus. The retroperitoneal fat pad was taken as that distinct deposit behind each kidney along the lumbar muscles. The mesenteric fat pad consisted of adipose tissue surrounding the gastrointestinal tract from the gastroesophageal sphincter to the end of the rectum, with special care taken in distinguishing and removing pancreatic cells. All tissue samples were frozen in liquid nitrogen immediately after being weighed (Mettler AE-100). All tissue samples were stored along with plasma samples at −80 ◦C until analyses were performed.

Biochemical analyses

Commercial kits from Sigma (Sigma; St-Louis, Missouri, USA) were used to determine plasma and liver TG by colorimetric method. Liver TG concentrations were estimated from glycerol released after KOH hydrolysis. Liver total cholesterol concentrations were determined with some adaptations of the procedure described by Folch et al. (Folch et al.

1957). Briefly, 0.1g of liver was homogenized in a chloroform–methanol mixture (2:1, v/v). The chloroform layer was collected and evaporated overnight. After adding 10% Triton X-100 in isopropanol, the sample was assayed for total cholesterol using commercial kits according to the manufacturer's instructions (Wako Diagnostics and Chemicals USA, Richmond, VA, USA). Plasma total cholesterol was determined using the same kit supplied by Wako.

Molecular analyses

Total RNA was extracted from frozen liver using RNA extraction Mini kit (Invitrogen), according to the manufacturer's protocol. Thereafter, the RNA was treated with DNase (Invitrogen) to avoid genomic contamination. Total RNA (2 µg) was reverse-transcribed into complementary DNA using high capacity complementary DNA reverse transcription kits (Applied Biosystems). RT samples were stored at -20°C. The gene expression for *Cyclophilin B* was determined using a pre-validated Taqman Gene Expression Assay (Applied Biosystems, Rn0146*26*61, Foster City, CA). The gene expression of the target genes was determined using assays designed with the Universal Probe Library from Roche. The primer sets and UPL probe numbers are presented in Table S2. To validate the efficiency of the qPCR assays, we used a mix of the samples tested in the study. The ABI PRISM® 7900HT (Applied Biosystems) was used to detect the amplification level and was programmed with an initial step of 3 min at 95˚C, followed by 40 cycles for 5s at 95˚C and 30s at 60˚C. All reactions were run in duplicate and the average values of threshold cycle (CT) were used for quantification. *Cyclophilin B* was used as endogenous control. The relative quantification of target genes was determined using the ΔΔCT method. Briefly, the CT values of target genes were normalized to an endogenous control gene (*Cyclophilin B*) ($\Delta C_T = C_{T\ target} - C_{T\ Cyclophilin\ B}$) and compared with a calibrator: ($\Delta\Delta\ C_T = \Delta\ C_{T\ Sample} - \Delta C_{T\ Calibrator}$). Relative expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) and the formula is $RQ = 2^{-\Delta\Delta C}{}_T$.

Statistical analysis

All data are presented as mean ± SE. Statistical significance *(*$P<0.05$*)* was determined using a 2-way ANOVA for non-repeated measures with exercise and surgery-diet as main factors. Interpretation of the comparisons was made only between the Ovx-Chol and the Ovx-SD groups on one hand and between the Ovx-Chol and the Sham-Chol groups on the other hand. Fisher LSD *post hoc* test was used in the event of a significant interaction effect. For a

significant surgery-diet effect without interaction, Fisher LSD from a one-way ANOVA was used.

Results

Anthropometric parameters, food intake

Running did not significantly impact on final body weight in any of the experimental groups despite of higher ($P<0.001$) food intake (Table 1). On the other hand, final body weight ($P<0.001$) as well as food intake ($P<0.05$) were lower in Sham-Chol group compared to both Ovx groups. Intra-abdominal fat pad weight was decreased ($P<0.05$) by training in both Sham and Ovx groups fed the cholesterol diet, whereas Ovx-SD group showed slightly higher intra-abdominal fat weight under training. Similarly to body weight, intra-abdominal fat pad weight was significantly ($P<0.001$) higher in the two Ovx groups compared to Sham-Chol group. The chol diet as compared to the SD diet in Ovx animals had no effect on final body weight, food intake and intra-abdominal fat pad weight. Uterus weight was significantly ($P<0.001$) lower in Ovx groups compared to Sham rats confirming total ovariectomy (Table 1).

Liver and plasma lipid profile

Liver TC and TG levels were not affected by training in any of the nutritional conditions (Fig. 1). Nevertheless, cholesterol feeding in both Sham and Ovx rats resulted in significantly ($P<0.001$) higher hepatic cholesterol accumulation than in Ovx rats fed the SD. In addition, liver TC was significantly ($p < 0.05$) higher in Sham than in Ovx animals fed the cholesterol diet. Opposite to liver TC, hepatic TG levels were significantly ($P<0.05$) lower in Sham fed the cholesterol diet compared to both Ovx groups. As liver TC, plasma TC levels were not significantly affected by training (Fig. 1). However, plasma TC was significantly ($P<0.01$) lower in Sham cholesterol group compared to both Ovx groups. Plasma TG levels showed a tendency ($P=0.09$) to be lower in active rats fed a cholesterol diet. On the other hand, plasma TG levels were significantly ($P<0.05$) lower in both sham and Ovx groups fed the cholesterol diet compared to Ovx-SD group.

Molecular markers of hepatic cholesterol synthesis and uptake

Gene expression levels of sterol regulatory element binding protein 2 (*Srebp2*), a key regulator of hepatic cholesterol content, as well as its target genes 3-hydroxy-3-methyl-glutaryl-CoA reductase (*Hmgcoar*), LDL receptor (*Ldlr*) and proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) were not changed by training (Fig. 2). Cholesterol feeding in both Sham and Ovx rats led to lower ($P<0.01$) gene expression of *Srebp2* and all of its aforementioned target genes compared to Ovx rats fed the SD (Fig. 2). These results imply that the expression levels of the nuclear receptor and all the present genes involved in hepatic cholesterol synthesis and uptake were down regulated by the cholesterol diet.

Besides *Ldlr* and *Pcsk9*, low density lipoprotein receptor-related protein 1 (*Lrp1*) is also involved in hepatic cholesterol uptake. Running had no impact on the gene expression level of *Lrp1* similarly to *Ldlr* and *Pcsk9*. On the other hand, the expression level of *Lrp1* was lower ($P<0.01$) in Ovx-Chol than in both Ovx-SD and Sham-Chol groups (Fig. 2). These findings indicate that *Lrp1* gene expression was significantly reduced by the combination of cholesterol feeding and ovariectomy.

Molecular markers of hepatic cholesterol and bile acid excretion

Hepatic gene expression of ATP-cassette binding protein G5 and G8 (*Abcg5/g8*) and *Bsep*, involved in hepatic cholesterol and bile acid secretion, respectively, were not affected by exercise training (Fig. 3). The mRNA levels of *Abcg5/g8* were significantly ($P<0.05$) lower in both Sham and Ovx rats fed the cholesterol diet compared to animals in the Ovx-SD group, while *Bsep* gene expression was reduced ($P<0.05$) only in Ovx rats fed the cholesterol diet compared to rats in the Ovx-SD group (Fig. 3). These results suggest that cholesterol feeding can lead to a reduction in cholesterol excretion from the liver.

Molecular markers of bile acid metabolism

The most significant effects of training in the present study were found for gene expression of *Cyp7a1* and *Shp* with higher ($P<0.05$) values measured in Tr compared to Sed rats in all experimental conditions (Fig. 4). The *Cyp7a1* and *Shp* responses were not significantly affected by the surgery and the diet. Gene expression of *Ntcp*, involved in bile acids uptake at the basolateral membrane of hepatocytes, and fibroblast growth factor receptor 4 (*Fgfr4*) that mediates the effects of intestinal fibroblast growth factor 15 (*Fgf15*) on suppressing bile acid

biosynthesis in liver, along with the transcription factor *Fxr* were all decreased (P<0.001) following the cholesterol diet but their gene expressions were not affected by training (Fig. 4).

Discussion

The main finding of the present study is an increased gene expression of two key markers of bile acid metabolism (*Cyp7a1* and *Shp*) found in liver of exercise-trained rats. This finding was observed in animals fed a standard diet but more importantly when rats were fed a high cholesterol diet and that independently of estrogen levels. This observation suggests that exercise training may help overcome a cholesterol load by stimulating bile acid metabolism. On the other hand, exercise training was associated with no change in *Srebp2, Hmgcoar, Ldlr,* and *Pcsk9* transcripts indicating an absence of molecular effects on key markers of cholesterol synthesis and hepatic cholesterol uptake from the circulation.

Cyp7a1

Exercise training has for a long time being associated with changes in plasma cholesterol levels favouring a decrease in LDL- cholesterol and an increase in HDL levels, the latter being in line with an increase in the so-called reverse cholesterol transport (Durstine et al. 2002; Halverstadt et al. 2007). However, the basic pathways responsible for these exercise-induced beneficial effects are poorly understood. In recent years, Meissner et al. (Meissner et al. 2010b; Meissner et al. 2011) reported an increased fecal bile acid excretion in healthy and especially in hypercholesterolemic mice suggesting that bile acid metabolism may be involved in the action of exercise training on cholesterol metabolism. The present increase in *Cyp7a1* liver mRNA expression, the main enzyme involved in bile acid synthesis from cholesterol in liver, is in line with the concept that exercise training may regulate excess cholesterol through bile acid metabolism. In support of this, larger increase (two-fold) in *Cyp 7a1* transcripts was observed when rats, whether Ovx or Sham, were fed the high cholesterol diet. An increase in hepatic gene expression of *Cyp27a1*, a second important hepatic enzyme of bile acid formation from cholesterol, has been previously reported in exercising mice fed a lithogenic diet for 12 weeks, thus favouring the catabolism of cholesterol to bile acids and reducing gallstone formation (Wilund et al. 2008). On the other hand, Meissner et al. (Meissner et al. 2010b; Meissner et al. 2011) did not find any changes in gene expression of *Cyp7a1* in mice assigned to voluntary wheel running in spite of an increase in fecal bile acid excretion.

However, Pinto et al. (Pinto et al. 2015) recently reported an increase in hepatic *Cyp7a1* expression in liver of mice trained for 6 weeks while there were no differences in the 3H-cholesterol excretion into feces between the sedentary and exercise groups. In the present study, *Cyp7a1* transcripts were increased with exercise training in Ovx as well as in Sham rats fed the cholesterol diet indicating that this exercise effect takes place independently of estrogen levels. Taken together, the present data support the concept that higher bile acid biosynthesis following exercise training contributes to the reduction in hepatic cholesterol accumulation.

Shp

An important novel finding of the present study is the increase in mRNA expression of *Shp* with exercise training in all experimental conditions. SHP is an orphan nuclear receptor involved in the regulation of bile acids/cholesterol and also in the regulation of glucose and lipid metabolism through the modulation of several transcription factors (Chiang 2004; Boulias et al. 2005).

The importance of the present *Shp* up-regulation by exercise training is enlighten by findings of previous studies indicating that missense mutations and polymorphisms in the promoter and coding regions of *Shp* in human were associated with severe early-onset obesity and diabetes (Nishigori et al. 2001; Hung et al. 2003). SHP is, therefore, an excellent candidate that may link cholesterol/bile acid regulation to glucose and lipid adaptations known to occur with exercise training. It has been recently reported that estrogens up-regulate *Shp* liver expression and decreases hepatosteatosis in male mice fed a high-fat diet (Lai et al. 2003; Wang et al. 2015). The present finding that exercise training also up-regulated *Shp* expression in the Ovx animals extends previous findings from our lab showing that exercise training provokes estrogenic like effects on the expression of several genes involved in the regulation of lipid metabolism in liver (Pighon et al. 2011).

Relationship between Shp and Cyp7a1

Shp is known to suppress the expression of *Cyp7a1* (Nitta et al. 1999; Lee et al. 2002), the rate-limiting enzyme in conversion of cholesterol into bile acids (Jelinek et al. 1990). The lack of this relationship in our study is in concert with Lai et al's finding showing that the induction of *Shp* by ethynylestradiol did not repress the expression of *Cyp7a1* (Lai et al. 2003). In prior studies, the same researchers had shown that chronic administration of 17β-estradiol in mice

resulted in increased expression of *Shp* (Evans et al. 2002). It is, thus, likely that the induction of *Shp* by estrogens does not result in gene suppression of *Cyp7a1*. It seems that training in our study might imitate the effect of estrogens on *Shp*. On the other hand, higher expression of *Shp* and consequently *Cyp7a1* under training might be a compensatory response to overcome the hepatic cholesterol accumulation. Lack of inverse relationship between *Shp* and *Cyp7a1* in the present study suggests that based on circumstances, *Shp* acts differently. Kerr et al. suggested that under normal conditions the inputs of *Fxr* and *Shp* result in regulation of bile acid synthesis through the negative feedback on *Cyp7a1*. Beyond the normal conditions e.g. when liver is damaged, alternate pathways may regulate bile acid homeostasis (Kerr et al. 2002).

In contrast to *Cyp7a1*, gene expressions of other markers of bile acid metabolism in liver, including *Ntcp*, *Fgfr4*, and *Fxr* were all decreased by the cholesterol diet in Sham and Ovx rats but not affected by the training state. Down-regulated gene expression of *Ntcp* suggests that there is less bile acid influx to the liver from the enterohepatic circulation. Decreased transcript levels of *Fgfr4* by cholesterol feeding may indicate that the inhibitory effect of *Fgf15* on *Cyp7a1* which acts through hepatic *Fgfr4* receptor might be reduced. This lack of inhibitory effect on *Cyp7a1* would reinforce the interpretation that higher *Cyp7a1* production and consequently higher bile acid synthesis from cholesterol is a way to remove excess cholesterol from the liver.

Hmgcoar, Ldlr, and Pcsk9

Cholesterol feeding in the present study resulted in lower hepatic transcripts of *Srebp2* and its target gene, *Hmgcoar* in both Sham and Ovx animals. In addition, hepatic expression of *Ldlr* and *Pcsk9*, two other target genes of *Srebp2* which are involved in hepatic cholesterol absorption from circulation, were suppressed by the cholesterol diet. Hepatic cholesterol accumulation might be a reason for the suppression of cholesterol biosynthesis and uptake from plasma. Seemingly, it could be a protective response to prevent more cholesterol accumulation in the liver. These findings are in concert with our previous study that showed that cholesterol feeding led to a reduction in *Srebp2*, *Ldlr* and *Lrp1* expression in the liver (Ngo Sock et al. 2014a).

Meissner et al. (Meissner et al. 2010b) reported that fecal excretion of bile acids and neutral sterols in running mice was a reflection of elevated endogenous hepatic cholesterol synthesis

in running group compared to sedentary mice. This is hardly the case in the present study since cholesterol feeding resulted in lower expression of *Hmgcoar* in both Sham and Ovx rats regardless of exercise intervention. Therefore, higher *Cyp7a1* mRNA expression under training is likely a consequence of hepatic dietary cholesterol accumulation. It thus seems that exercise does not modulate a cholesterol load by reducing cholesterol synthesis, but rather by stimulating bile acid metabolism.

Moreover, a training effect was not observed in mRNA expression of *Srebp2, Ldlr, Pcsk9* and *Lrp1* in liver. Previously our group showed that exercise training increased mRNA expression of *Srebp2* in Ovx rats but had no effect on its hepatic target genes expression (Ngo Sock et al. 2014a). These data, therefore, do not provide any evidence that exercise training affect hepatic cholesterol uptake from the circulation through the *Ldlr* pathway. On the other hand, Wen's et al. reported that a high fat diet plus exercise for 8 weeks resulted in an increase in nuclear *Srebp2* protein with elevated levels of hepatic *Ldlr* and *Pcsk9* mRNA in mice due to a reduction in hepatic cholesterol accumulation (Wen et al. 2013).

Liver TC and TG

Higher *Cyp7a1* expression in response to training suggests higher bile acid synthesis and more excretion of the cholesterol from the liver in the form of bile acid. Therefore, less cholesterol accumulation might be expected inside the liver. However, liver TC and TG levels were not changed by training under all the present nutritional conditions while exercise running in other studies (Meissner et al. 2010b; Wen et al. 2013) resulted in decreased hepatic cholesterol content in male mice. It is possible that the reported effects of training in Ovx rats under the present duration of observation are only at the molecular level. Furthermore, considering that Ovx rats were fed a diet rich in cholesterol, perhaps longer time would be needed to overcome the hepatic cholesterol and TG accumulation. Nevertheless, plasma lipid profile especially TG levels were lower in trained rats fed the chol diet than rats in Ovx-SD condition. Apparently, plasma and liver TC and TG accumulation responses to exercise training follow different time courses. On the other hand, hepatic gene expression of *Abcg5/g8* and *Bsep* which are involved respectively in cholesterol and bile acid efflux from liver into the bile duct were not increased by training. This is unexpected in view of a potential increase in bile acid synthesis. It is possible the expression of hepatic gene involved in cholesterol excretion from liver need a longer period of time to respond to a training stimulus.

In summary, results of the present study indicate that exercise training modulates hepatic cholesterol metabolism through the up-regulation of *Shp* and bile acid metabolism. It seems that increased mRNA expression of *Shp* and subsequently higher expression levels of *Cyp7a1* is a positive response triggered by exercise to alleviate hepatic cholesterol accumulation and help to drive the cholesterol out from liver. Elevated cholesterol turnover induced by exercise training may contribute to improve hepatosteatosis and decrease the risk of obesity, diabetes and cardiovascular disease.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Figure captions

Fig 1. Liver and plasma total cholesterol (TC) and triacylglycerol (TG) levels in ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Ovx rats fed a standard diet + 0.25% cholesterol (Chol) (Ovx-Chol) and sham operated (Sham) rats fed the Chol diet (Sham-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed group ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective Ovx-SD group ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Ovx-Chol group ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$).

Fig 2. Hepatic mRNA expression of genes involved in hepatic cholesterol biosynthesis and cholesterol uptake from the circulation in ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Ovx rats fed a standard diet + 0.25% cholesterol (Chol) (Ovx-Chol) and sham operated (Sham) rats fed the Chol diet (Sham-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed group ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective Ovx-SD group ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Ovx-Chol group ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$). *Srebp2,* sterol regulatory element-binding protein-2; *Hmgcr,* 3-hydroxy-3-methyl-glutaryl-CoA reductase; *Ldlr*, LDL-receptor; *Pcsk9,* proprotein convertase subtilisin/kexin type 9; *Lrp-1,* LDL receptor-related protein-1.

Fig 3. Hepatic mRNA expression of ATP-cassette binding protein G5 and G8 (*Abcg5/g8*) and bile salt export pump (*Bsep*) in ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Ovx rats fed a standard diet + 0.25% cholesterol (Chol) (Ovx-Chol) and sham operated (Sham) rats fed the Chol diet (Sham-Chol) in sed (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed group ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective Ovx-SD group ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Ovx-Chol group ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$).

Fig 4. Hepatic mRNA expression of genes related to bile acid metabolism in ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Ovx rats fed a standard diet + 0.25% cholesterol (Chol) (Ovx-Chol) and sham operated (Sham) rats fed the Chol diet in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed

group ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective Ovx-SD group ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Ovx-Chol group ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$). *Cyp7a1,* cholesterol 7 alpha-hydroxylase; *Shp,* small heterodimer partner; *Ntcp,* Na+-taurocholate cotransporting polypeptide; *Fgfr4,* fibroblast growth factor receptor 4; *Fxr,* farnesoid X receptor.

Table 1. Anthropometric parameters, food intake and total distance run

Variables	Ovx-SD		Ovx-Chol		Sham-Chol	
	Sed	Tr	Sed	Tr	Sed	Tr
Final body weight (g)	438.7 ± 9.3	452.5 ± 14.1	433.2 ± 10.6	450.6 ± 11.6	372.3 ± 13.2 ††† δδδ	346.6 ± 10.6 ††† δδδ
Intra-abdominal fat pad weights (g)	37.8 ± 2.8	39.6 ± 3.9 *	39.3 ± 3.8	34.3 ± 4.3 *	30.2 ± 4.5 ††† δδδ	14.3 ± 2.8 *††† δδδ
Food intake (kcal/day)	100.1 ± 2.9	114.7 ± 3.7 ***	101.8 ± 2.2	113.0 ± 3.1 ***	92.6 ± 5.6 †δ	101.8 ± 2.5 ***†δ
Uterus (g)	0.08 ± 0.0	0.09 ± 0.0	0.09 ± 0.0	0.09 ± 0.0	0.54 ± 0.08 ††† δδδ	0.55 ± 0.07 ††† δδδ
Total distance run (km/d)	N/A	2.79 ± 0.30	N/A	2.82 ± 0.33	N/A	6.09 ± 0.39

Ovx, ovariectomised; Sham, sham operated; SD, standard diet; Chol, standard diet + 0.25% cholesterol; Sed, sedentary group; Tr, trained group. Values are mean ± SE.
* Significantly different from respective Sed group ($P < 0.05$), *** ($P < 0.001$)
† Significantly different from respective Ovx-SD group ($P < 0.05$), ††† ($P < 0.001$)
δ Significantly different from respective Ovx-Chol group ($P < 0.05$), δδδ ($P < 0.001$)

Table S1. Diet description

	Standard Diet (SD) (D12450J)	SD + Chol (0.25%) (D13020701)
(%)		
Protein	19.2	19.2
Carbohydrate	67.3	67.1
Fat	4.3	4.3
(g)		
Casein	200	200
L-Cystine	3	3
Corn Starch	506.2	506.2
Maltodextrin 10	125	125
Sucrose	68.8	68.8
Cellulose, BW200	50	50
Soybean Oil	25	25
Lard	20	20
Mineral Mix S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
Cholesterol	0.0	2.63
Kcal/g	3.85	3.84

Formulated by: Research Diets, Inc. (20 Jules Lane, New Brunswick, NJ 08901 USA)

Table S2. Oligonucleotide primers used for quantitative real-time polymerase chain reaction

Gene	Oligo FWD	Oligo REV
ABCG5	cggagagttggtgttctgtg	caccgatgtcaagtccatgt
ABCG8	cagatgctggctatcataggg	ctgatttcatcttgccacca
BSEP	cggtggctgagagatcaaat	tgcgatagtggtggagaaca
Cyp7a1	ggagcttatttcaaatgatcagg	cactctgtaaagctccactcactt
Fgfr4	ttgaggcctctgaggaaatg	tcttgctgctccgagattg
FXR	ccacgaccaagctatgcag	tctctgtttgctgtatgagtcca
HMG-CoAr	caaccttctacctcagcaagc	acagtgccacacacaattcg
LDLr	tgctactggccaaggacat	ctgggtggtcggtacagtg
LRP-1	aatcgagggcaagatgacac	ccagtctgtccagtacatccac
NTCP	aaaatcaagcctccaaaggac	ttgtgggtacctttttccaga
PCSK9	cacctagcaggtgtggtcag	gcagactgtgcagactggtg
SHP	cctcggtttgcatacagtgtt	aggttttgggagccatcaa
SREBP-2	gtgcagacagtcgctacacc	aatctgaggctgaaccagga
ActB	cccgcgagtacaaccttct	cgtcatccatggcgaact
Cyclophilin B	acgtggttttcggcaaagt	cttggtgttctccaccttcc

Fig. 1

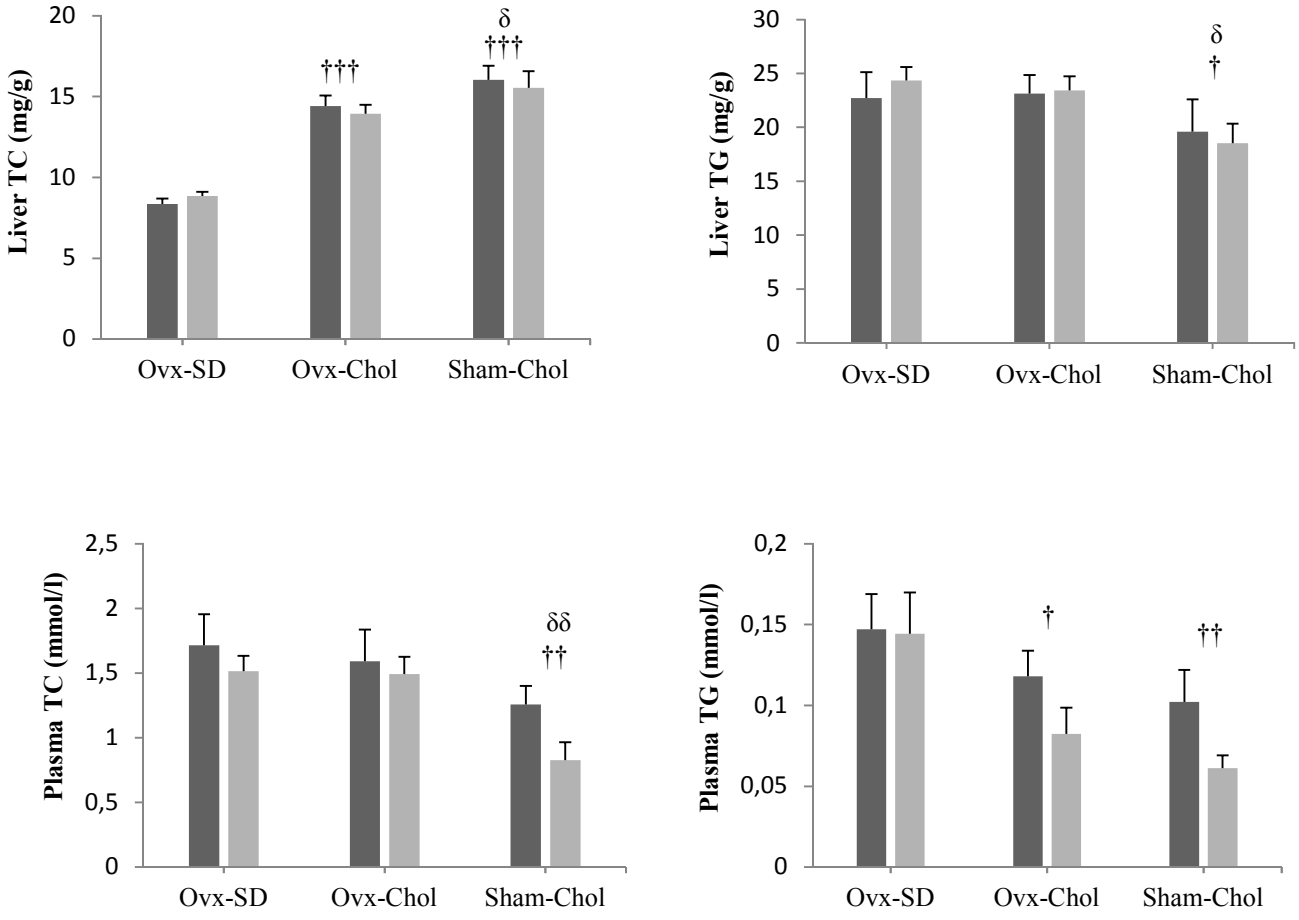

Liver TC (mg/g)
20
15
10
5
0
δ
†††
†††
Ovx-SD
Ovx-Chol
Sham-Chol
Liver TG (mg/g)
30
25
20
15
10
5
0
δ
†
Ovx-SD
Ovx-Chol
Sham-Chol
Plasma TC (mmol/l)
2,5
2
1,5
1
0,5
0
δδ
††
Ovx-SD
Ovx-Chol
Sham-Chol
Plasma TG (mmol/l)
0,2
0,15
0,1
0,05
0
†
††
Ovx-SD
Ovx-Chol
Sham-Chol

Fig. 2

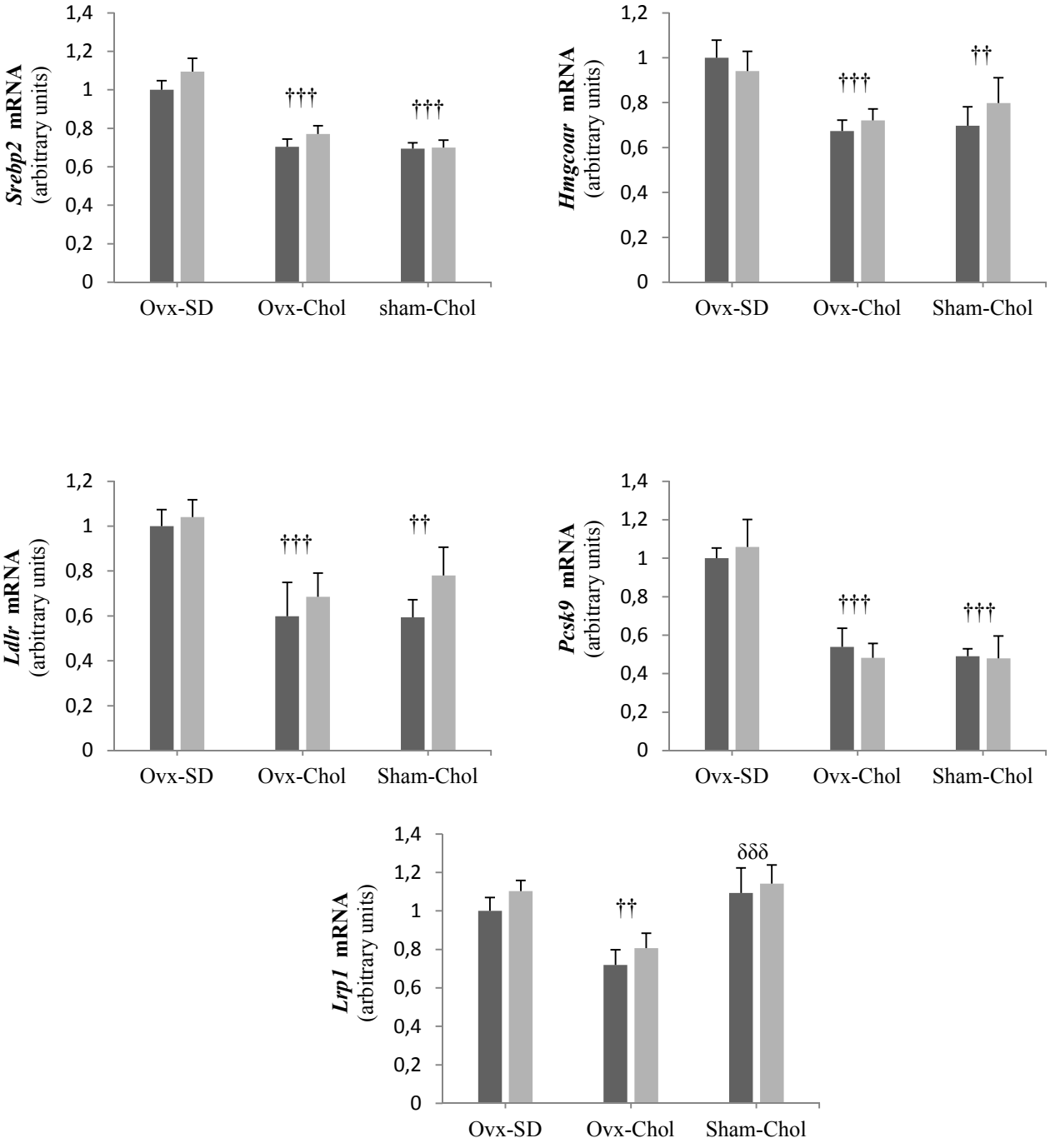

Srebp2 mRNA (arbitrary units)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
Ovx-SD
Ovx-Chol
sham-Chol
Hmgcoar mRNA (arbitrary units)
1,2
1
0,8
0,6
0,4
0,2
0
†††
††
Ovx-SD
Ovx-Chol
Sham-Chol
Ldlr mRNA (arbitrary units)
1,2
1
0,8
0,6
0,4
0,2
0
†††
††
Ovx-SD
Ovx-Chol
Sham-Chol
Pcsk9 mRNA (arbitrary units)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
Ovx-SD
Ovx-Chol
Sham-Chol
Lrp1 mRNA (arbitrary units)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
††
δδδ
Ovx-SD
Ovx-Chol
Sham-Chol

Fig. 3

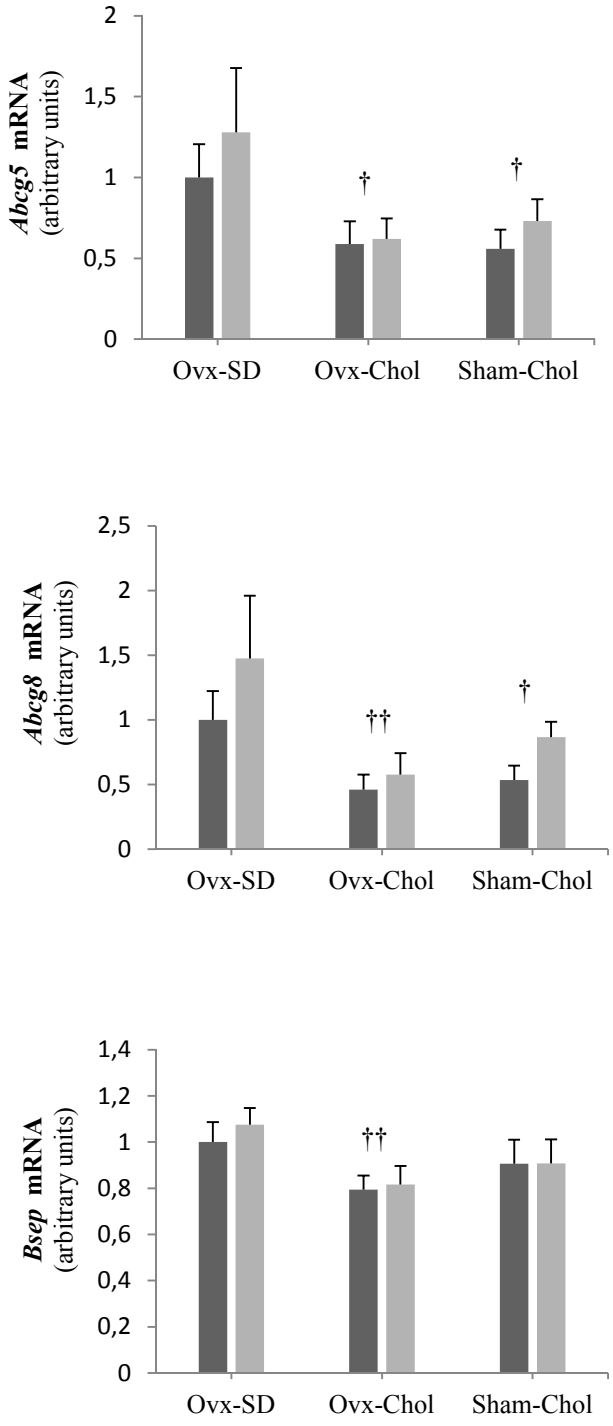
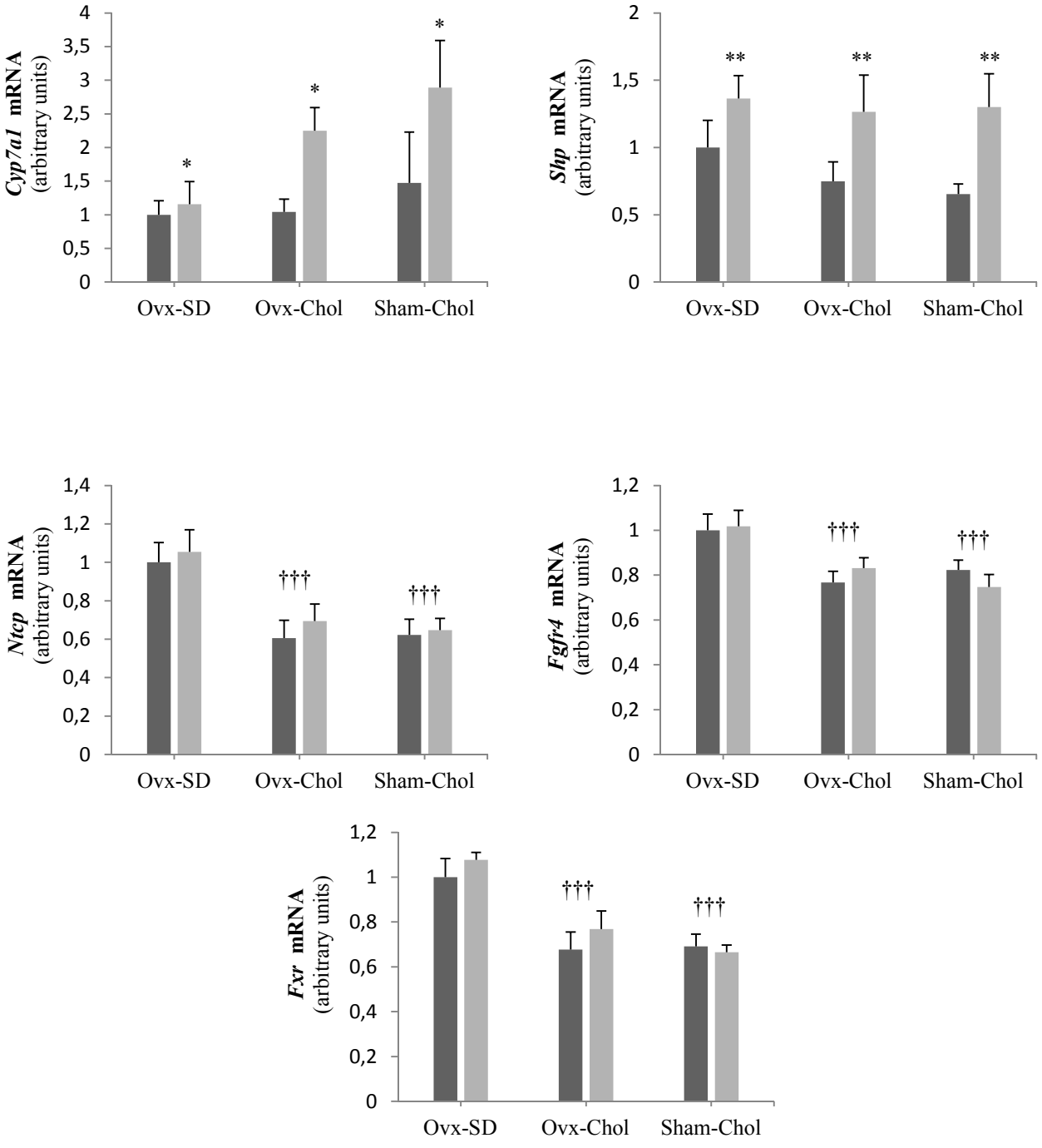

Abcg5 mRNA (arbitrary units)
2
1,5
1
0,5
0
†
†
Ovx-SD
Ovx-Chol
Sham-Chol
Abcg8 mRNA (arbitrary units)
2,5
2
1,5
1
0,5
0
††
†
Ovx-SD
Ovx-Chol
Sham-Chol
Bsep mRNA (arbitrary units)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
††
Ovx-SD
Ovx-Chol
Sham-Chol

Fig. 4


Cyp7a1 mRNA (arbitrary units)
4
3,5
3
2,5
2
1,5
1
0,5
0
*
*
*
Ovx-SD
Ovx-Chol
Sham-Chol
Shp mRNA (arbitrary units)
2
1,5
1
0,5
0
**
**
**
Ovx-SD
Ovx-Chol
Sham-Chol
Ntcp mRNA (arbitrary units)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
Ovx-SD
Ovx-Chol
Sham-Chol
Fgfr4 mRNA (arbitrary units)
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
Ovx-SD
Ovx-Chol
Sham-Chol
Fxr mRNA (arbitrary units)
1,2
1
0,8
0,6
0,4
0,2
0
†††
†††
Ovx-SD
Ovx-Chol
Sham-Chol

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2.3 Article 3 : Exercise Training Increased Gene Expression of LDL-r and PCSK9 in intestine: link to TICE

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Exercise Training Increased Gene Expression of LDL-r and PCSK9 in intestine: link to TICE

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Abstract

Aim: Transintestinal cholesterol excretion (TICE) is known as an alternate non-biliary route of cholesterol excretion. Indeed, the cholesterol excretion from the body depends on a dynamic interplay between both classic biliary and non-biliary pathways. This study was designed to determine whether exercise training has effects on intestinal membrane receptors involved in TICE in intact and ovariectomized (Ovx) rats.

Study design: Sprague-Dawley rats were first divided into 4 groups: Sham operated and Ovx rats fed a standard diet (Sham-SD; Ovx-SD), or a high cholesterol diet (Sham-Chol; Ovx-Chol). These 4 groups were subsequently subdivided into either sedentary (Sed) or voluntary wheel running (Tr) groups for 6 weeks.

Results: As expected, cholesterol diet resulted in increased hepatic cholesterol accumulation ($P< 0.001$) in both Sham and Ovx rats. Exercise training increased ($P< 0.01$) transcripts of intestinal LDL receptor (*Ldlr*) and proprotein convertase subtilisin/kexin type 9 (*Pcsk9*), which are involved in trans-intestinal cholesterol uptake from circulation, and their nuclear receptor, intestinal sterol regulatory element-binding protein 2 (*Srebp2*) ($P< 0.05$) in both Sham and Ovx rats compared to rats remaining Sed in all diet conditions except for higher *Pcsk9* mRNA expression which was found only in Chol fed rats. On the other hand, hepatic *Ldlr* and *Pcsk9* gene expression were suppressed ($P< 0.01$) by cholesterol feeding but not affected by exercise training. Flavin monooxygenase 3 (*Fmo3*) gene expression, as a cholesterol balance regulator in liver, was significantly decreased ($P<0.01$) by cholesterol feeding in both Sham and Ovx rats compared to rats were fed the SD diet but unchanged following exercise training and estrogen withdrawal.

Conclusion: The present results indicate an up-regulation of intestinal gene expression of *Ldlr* and *Pcsk9* following voluntary wheel running in intact and Ovx rats and suggest that exercise training may contribute to an increased cholesterol elimination through the TICE pathway. Exercise training appears to be an appropriate nonpharmacological intervention to stimulate TICE to excrete the excess cholesterol from the body.

Key words: Intestinal cholesterol disposal, LDL receptor, FMO3, liver cholesterol

Introduction

Although the biliary route is the main pathway for elimination of excess cholesterol from the body, several studies have recently enlighten that reverse cholesterol transport (RCT) can also proceed through a non-biliary pathway known as transintestinal cholesterol excretion (TICE) (Brown et al. 2008; van der Velde et al. 2008; Temel and Brown 2012). Indeed, in this new model of RCT, the cholesterol disposal from the body depends on an active interplay between liver and intestine (Temel and Brown 2015). In TICE pathway, cholesterol is transported through the receptors at the basolateral and apical membrane of intestine which are involved in cholesterol uptake from plasma and cholesterol secretion into the lumen, respectively. Low density lipoprotein (LDL) receptor and LDL receptor family were reported as receptors involved in cholesterol uptake from circulation at the basolateral side of intestine (Le May et al. 2013). Adenosine triphosphate binding cassette transporters G5 and G8 (ABCG5/G8) and adenosine triphosphate binding cassette transporters B1a and b (ABCB1a/b) are also introduced as receptors involved in cholesterol excretion into the lumen at the apical membrane of intestine (van der Veen et al. 2009; Le May et al. 2013). Additionally, there is evidence that the set point of cholesterol excretion is sustained by the crosstalk between biliary and non-biliary pathways (Kruit et al. 2005). In very recent study hepatic flavin monooxygenase 3 (*Fmo3*) has been identified as a key cholesterol regulator of both biliary and non-biliary RCT pathways (Warrier et al. 2015). Transport and elimination of cholesterol is particularly relevant to ovariectomized (Ovx) animals in which hepatic cholesterol metabolism has been reported to be disrupted especially when fed a high cholesterol diet (Kamada et al. 2011; Cote et al. 2013). For instance, large hepatic cholesterol content was observed in Ovx models by the cholesterol feeding suggesting that dietary cholesterol plays a critical role in development of steatohepatitis (Subramanian et al. 2011; Cote et al. 2014). In addition to hepatic cholesterol accumulation, hepatic very low density lipoprotein (VLDL) production and secretion is reduced in Ovx rats fed a cholesterol diet (Farahnak et al. 2015). Recent studies have shown that hepatic mRNA expression of adenosine triphosphate binding cassette protein G8 (*Abcg8*) and bile salt export pump (*Bsep*) transcript, involved in cholesterol and bile acid excretion from liver into bile duct respectively, were also decreased in Ovx rats fed a cholesterol diet (Cote et al. 2014; Farahnak et al. 2015). It seems that estrogen withdrawal and

high cholesterol diet act synergistically to impair different aspects of hepatic cholesterol metabolism including cholesterol excretion from the body. It has been revealed that contribution of biliary and non-biliary TICE is different under physiological or pathophysiological conditions (Temel and Brown 2015). Under normal physiological conditions, the biliary route has a predominant role and the non-biliary TICE pathway contributes to approximately 20-30% of the cholesterol disposal. However, TICE pathway can be stimulated by both pathophysiological and pharmacological stimuli (Temel and Brown 2012). For example, pharmacological activation of Liver X Receptor (LXR) (van der Veen et al. 2009) or high fat diet (van der Velde et al. 2008) resulted in increased intestinal cholesterol disposal. It seems that biliary cholesterol insufficiency can be compensated by intestine through TICE to keep normal levels of fecal cholesterol loss. Considering that hepatic cholesterol excretion is impaired in Ovx animal, thus it is important to gain knowledge about the role of TICE in cholesterol excretion in Ovx model.

There is some evidence that exercise training, as one of the best nonpharmacological strategies, attenuates hepatic cholesterol accumulation and leads to higher biliary bile acid secretion in hypercholesterolemic mice (Meissner et al. 2011). Increase in cholesterol 7 alpha-hydroxylase (*Cyp7a1*) transcript, involved in conversion of cholesterol into bile acid in liver, was also reported in trained male mice (Pinto et al. 2015). On the other hand, several studies showed diverse effects of exercise training on intestinal Abcg5/g8 transcript in healthy female rats (Ghanbari-Niaki et al. 2012; Ngo Sock et al. 2014b). Lack of evidence for training effects on TICE thus creates a great avenue to explore this pathway under training to gain a better knowledge of cholesterol disposal through intestine.

The aim of the present study was to determine the effect of exercise training on key intestinal cholesterol receptors involved in TICE in intact and Ovx rats fed a normal and a high cholesterol diet. We targeted gene expression of key molecules of TICE at intestinal basolateral membrane such as *Ldlr* and proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) and also at intestinal apical membrane like *Abcg5/g8* and *Abcb1a/b*. We also targeted gene expression of *Fmo3*, *Ldlr*, *Pcsk9* and scavenger receptor B1 (*Sr-B1*) in liver.

Materials and Methods

Animal care

Female Sprague-Dawley strain rats (*n*=65; Charles River, St Constant, PQ, Canada), weighing 187–194 g upon their arrival were housed individually and had *ad libitum* access to food and tap water. Their environment was controlled in terms of light (12 h light–dark cycle starting at 06:00 AM), humidity and room temperature (20–23°C). Body weight and food intake were monitored bi-weekly from the start of experiment. All experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Montreal in agreement with The Canadian Council on Animal Care's rules (CCAC-CCPA).

Surgery, diets, and exercise protocol

Rats were first acclimated to their environment for a period of one week while fed a chow diet (12.5 % lipid, 63.2 % CHO and 24.3 % protein; kJ from Agribrands Canada, Woodstock, Ontario, Canada). Afterwards, rats underwent either a bilateral ovariectomy (Ovx, *n*=32) or a bilateral sham-operation (Sham, *n*=33) according to the technique described by Robertson et al (Robertson 1984) under isoflurane anaesthesia. After surgery, all animals were injected with antibiotics (Tribrissen 48%; 0.125 cc/kg, subcutaneously) and analgesic (Carprofen; 4.4 mg/kg, subcutaneously) for 3 days. Ovx and Sham rats were given either a standard diet (SD) or a high cholesterol diet (Chol). The Chol diet consisted of the standard diet (SD) supplemented with 0.25% cholesterol (SD+Chol) (Table S1). The four groups composed of Sham rats fed a SD or a Chol diet (Sham-SD, *n*=17; Sham-Chol, *n*=16 and Ovx rats fed a SD or a Chol diet (Ovx-SD, *n*=16; Ovx-Chol, *n*=16) were further subdivided into either voluntary wheel running (Tr) or sedentary groups (Sed) for a total of 8 groups. Tr rats were placed in freely rotating wheel cages while Sed rats were placed in blocked running wheel cages. Each wheel cage was equipped with a sensor connected to a computerized data acquisition system enabling the continuous sampling of running data from individual rats. Rats were on diet and training for 6 weeks.

Blood and tissue sampling

Rats were fasted overnight and euthanized between 08:00 and 11:00 AM. Rats were refrained from exercising ~ 24 h before sacrifice. Immediately after complete anaesthesia with isoflurane, the abdominal cavity was opened following the median line of the abdomen.

Approximately 5 ml of blood was collected from the abdominal vena cava (<45 s) into syringes treated with ethylenediaminetetraacetic acid (15%; EDTA). Blood was centrifuged (3000 rpm; 4°C; 10 min; Beckman GPR Centrifuge; Beckman Coulter) and the plasma kept for further analyses. Immediately after blood collection, the liver median lobe was removed and freeze-clamped. This sample was used for cholesterol, and mRNA determinations. Several organs were removed and weighed (Mettler AE 100) in the following order: uterus, urogenital, retroperitoneal and mesenteric fat deposits. The urogenital fat pad included adipose tissue surrounding the kidneys, uterus and bladder as well as ovaries, oviducts and uterus. The retroperitoneal fat pad was taken as that distinct deposit behind each kidney along the lumbar muscles. The mesenteric fat pad consisted of adipose tissue surrounding the gastrointestinal tract from the gastroesophageal sphincter to the end of the rectum, with special care taken in distinguishing and removing pancreatic cells. After mesenteric fat removal, a section of approximately 5 cm of jejunum, was removed, washed in antiprotease solution (10% pepstatin A in methanol, 100% leupeptin, 1.7% phenylmethylsulfonyl fluoride in isopropanol, 0.9% NaCl), and frozen in liquid nitrogen. Liver and a fragment of jejunum along with plasma samples were stored at −78 °C until analyses were performed.

Biochemical analyses

Liver total cholesterol concentrations were determined with some adaptations of the procedure described by Folch et al. (Folch et al. 1957). Briefly, 0.1g of liver was homogenized in a chloroform–methanol mixture (2:1, v/v). The chloroform layer was collected and evaporated overnight. After adding 10% Triton X-100 in isopropanol, the sample was assayed for total cholesterol using commercial kits according to the manufacturer's instructions (Wako Diagnostics and Chemicals USA, Richmond, VA, USA). Plasma total cholesterol was determined using the same kit supplied by Wako. Plasma PCSK9 concentration was measured using Mouse/Rat PCSK9 ELISA kit from CircuLex.

RNA isolation and quantitative real-time (RT) polymerase chain reaction (PCR)

Quick-frozen tissue samples of liver and jejunum were powdered with cold mortar and pestle and ~100 mg was used for the isolation of RNA. Details of RNA extraction following by RT-PCR have been previously described (Farahnak et al. 2015). The primer sets and UPL probe numbers used to generate amplicons are presented in Table S2.

Statistical analysis

All data are presented as mean ± SE. Statistical significance *(P*<0.05*)* was determined using a 3-way ANOVA for non-repeated measures with exercise, diet and surgery as main factors. Fisher LSD *post hoc* test was used in the event of a significant interaction effect. For a significant exercise, diet and surgery effect without interaction, Fisher LSD from a one-way ANOVA was used.

Results

Anthropometric parameters, food intake

Final body weight was not affected by exercise training in any of the experimental groups whereas intra-abdominal fat pad weight was significantly decreased (*P*<0.001) by training in both Sham and Ovx rats regardless of type of the diet (Table 1). On the other hand, final body weight (*P*<0.001), intra-abdominal fat pad (*P*<0.01) and also food intake (*P*<0.001) were higher in Ovx compared to Sham rats implying the effect of estrogen withdrawal. Cholesterol diet had no impact on any of the aforementioned parameters in both Sham and Ovx rats. Uterus weight was significantly (*P*<0.001) lower in Ovx groups compared to Sham rats confirming total ovariectomy. Total running distance was approximately 2 times higher in Sham groups compared to Ovx rats (Table 1).

Molecular markers of TICE at the intestinal basolateral and apical membranes

Running resulted in significantly (*P*<0.01) higher *Ldlr* transcript in both Sham and Ovx rats compared to rats remaining Sed in all diet conditions (Fig. 1). A trend for higher *Ldlr* gene expression (*p*= 0.1) was observed under cholesterol feeding in both Sham and Ovx rats compared to rats on the SD diet. Similarly to *Ldlr*, *Pcsk9* mRNA gene expression was significantly (*P*<0.001) higher following training but only in rats fed the Chol diet. These two genes are involved in cholesterol uptake from circulation at the basolateral side of intestine. *Ldlr* and *Pcsk9* are the target genes of transcription factor *Srebp2*. Intestinal *Srebp2* transcript was also increased (*P*<0.05) by training in both Sham and Ovx rats in both dietary conditions (Fig. 1). On the other hand, estrogen withdrawal had no effect on *Ldlr* and *Pcsk9* mRNA gene expression. Based on previous studies, in addition to *Ldlr* and *Pcsk9,* scavenger receptor class B member 1 (*Sr-b1*) and very low density lipoprotein (*Vldlr*) also play a role in intestinal cholesterol uptake. Our results showed that mRNA expression of *Sr-b1* and *Vldlr* were not

affected by training, Chol diet, and or estrogen withdrawal in any of the experimental groups (Fig S1).

ATP-binding cassette protein G5/G8 (*Abcg5/g8*) and ATP-binding cassette sub-family B member 1 a/b (*Abcb1a/b*), the receptors at the apical membrane of the intestine, are involved in cholesterol excretion from the intestine into the lumen. With the exception of *Abcb1b* mRNA expression which was found to be lower ($P<0.05$) in Sham compared to Ovx rats (Fig. 2), all other receptors measured at the intestinal apical side were not affected by any of the main experimental factors (exercise, diet, surgery) (Fig. 2).

Liver and plasma total cholesterol (TC) levels

Running had no effect on liver and plasma TC levels (Fig. 3). Cholesterol diet resulted in higher ($P<0.001$) hepatic TC in both Sham and Ovx rats compared to animals on SD diet. This result indicates that cholesterol feeding led to cholesterol accumulation in liver. Plasma TC levels was not affected by the dietary intervention (Fig. 3). Estrogen withdrawal had no impact on TC levels in liver while levels of TC in plasma were higher ($P<0.01$) in Ovx compared to Sham rats fed the Chol diet.

Molecular markers of hepatic cholesterol uptake form circulation

Running had no impact on mRNA gene expression of hepatic *Ldlr*, *Pcsk9* and circulating *Pcsk9* (Fig. 4) while *Sr-b1* transcript, involved in cholesterol uptake from HDL, was higher ($P<0.05$) in Tr than in Sed animals in both Sham and Ovx groups. Cholesterol feeding significantly reduced mRNA expression of all of these three genes in both Sham and Ovx groups compared to rats fed the SD diet with the exception of *Pcsk9* gene expression which was only decreased in Sham-Chol group (Fig. 4). Ovx rats showed lower *Ldlr* transcript ($P<0.001$) compared to Sham rats in both dietary conditions. On the other hand, *Pcsk9* mRNA expression was only decreased ($P<0.05$) by estrogen withdrawal in Ovx rats on SD diet compared to Sham-SD group. *Sr-b1* mRNA expression was not affected by estrogen withdrawal in any of the experimental groups. Circulating concentration of PCSK9 showed the same trend as its hepatic gene expression (Fig. 4). Taken all together, cholesterol feeding and estrogen deficiency state had major effects on reduction of the expression of the receptors involved in cholesterol uptake from circulation in liver, especially in *Ldlr* and *Pcsk9*.

Hepatic Fmo3 and Abcg5/g8 mRNA expression

Flavin monooxygenase 3 (*Fmo3*) plays an important role in cholesterol balance in liver. It regulates the amount of cholesterol entering the biliary and non-biliary pathways. Down regulation of *Fmo3* directs the cholesterol excretion from biliary to non-biliary TICE pathway. Gene expression of *Fmo3* was not changed either by exercise training or by estrogen withdrawal (Fig. 5). On the other hand, *Fmo3* transcript was significantly decreased ($P<0.01$) by cholesterol feeding in both Sham and Ovx rats compared to respective rats fed the SD diet. Hepatic *Abcg5* and *g8* transcript, involved in hepatic cholesterol excretion into bile duct, were not affected by exercise and estrogen deficiency (Fig. 5). However like *Fmo3*, *Abcg8* mRNA expression was reduced ($P<0.05$) in rats fed Chol diet compared to animals on SD diet suggesting that liver might have less role in cholesterol secretion probably owe to deficit in hepatic cholesterol uptake as a consequence of hepatic cholesterol accumulation (Fig. 5).

Molecular marker of Vldl assembly

Microsomal triglyceride transfer protein (*Mttp*) is the rate limiting enzyme in *Vldl* assembly. The gene expression of *Mttp* was not affected by exercise training either in liver (Fig. 6a) or in intestine (Fig. 6b). Hepatic *Mttp* transcript was significantly decreased ($P<0.01$) by the Chol diet in both Sham and Ovx rats compared to their counterparts fed the SD diet implying less *Vldl* assembly in liver. Opposite to hepatic *Mttp*, intestinal *Mttp* mRNA expression was higher ($P<0.05$) in rats fed Chol diet than rats fed the SD diet. Estrogen withdrawal resulted in lower hepatic *Mttp* transcript in both SD and Chol diet groups (Fig. 6a) but had no effect on intestinal *Mttp* mRNA expression (Fig. 6b).

Discussion

The main finding of the present study was that a six week of voluntary exercise resulted in a significant increase in gene expression of intestinal *Ldlr* and *Pcsk9* at the basolateral membrane along with their regulatory transcription factor *Srebp2*. This was observed especially under the Chol-fed condition independently of the Ovx or Sham surgery. Unlike the basolateral receptors, gene expressions of the intestinal apical receptors (*Abcg5/g8* and *Abcb1a/b*) involved in cholesterol excretion from intestine into the lumen were not affected by exercise training. In addition, exercise training had no impact on hepatic *Fmo3* transcript, a cholesterol balance regulator in liver. These results indicate that intestinal cholesterol uptake from circulation might be increased at the basolateral membrane of intestine following exercise training, suggesting that TICE might be a way by which exercise training contributes to an elimination of excess cholesterol.

To the best of our knowledge, the present study is the first to report an important increase in intestinal *Ldlr* and *Pcsk9* transcripts following exercise training. The main function of LDL-receptor is to remove the circulating cholesterol from apoB-lipoproteins (Ouguerram et al. 2004). LDL-receptor activity is downregulated post-transcriptionally by Pcsk9 (Abifadel et al. 2003). Both LDL-receptor and Pcsk9 are regulated transcriptionally by the transcription factor, SREBP-2 (Smith et al. 1990; Dubuc et al. 2004). Although protein levels were not measured in the present study, there is consistency in the responses of intestinal LDL-receptor and *Pcsk9* transcripts and their nuclear transcription receptor, intestinal *Srebp2*, in response to exercise training. Higher intestinal *Pcsk9* transcript along with *Ldlr* in trained rats might raise the possibility that there is degradation of intestinal *Ldlr* through Pcsk9. However circulating concentration of Pcsk9 was significantly low in Ovx rats and Chol-fed rats compared to intact rats on SD diet suggesting less degradation effect of Pcsk9 on intestinal LDLr. This might also explain why the plasma TC levels did not increase in Chol-fed rats in our study. Indeed, despite higher intestinal Pcsk9 transcript, its protein level is low in the circulation therefore; there is less degradation effect on intestinal LDLr and as a result higher cholesterol uptake from circulation via intestinal LDLr.

Supporting the link between circulating Pcsk9 and intestinal Ldl-r is the report that circulating recombinant PCSK9 injection through degradation of LDLr content in intestine acutely decreases TICE in Pcsk9 knockout mice (Schmidt et al. 2008; Le May et al. 2013). Moreover,

it was reported that PCSK9 null mice have more LDL receptors in their gut (Le May et al. 2009)) and subsequently faster intestinal cholesterol clearance and higher TICE were observed in PCSK9 null mice compared to wild type (WT) mice (Le May et al. 2013). On the other hand, injection of recombinant PCSK9 in LDLr null mice resulted in 40% higher TICE than Pcsk9 knockout mice. The discrepancy between the effect of circulating Pcsk9 on TICE were explained by the existence of higher TICE in $LDLR^{-/-}$ mice or the difference in genetic background between strains (Le May et al. 2013). Indeed, different levels of TICE were reported in mice with different genetic backgrounds (van der Velde et al. 2007).

Le May et al recently suggested that in addition to Ldlr, other unidentified mechanisms might be involved in cholesterol uptake from plasma via TICE due to the uptake of LDL particles at the proximal part of intestine in $LDLR^{-/-}$ mice (Le May et al. 2013). In addition to intestinal Ldlr, Sr-B1 and Vldlr have been identified as cholesterol acceptors at the basolateral membrane of intestine as well. Contrary to intestinal *Ldlr*, intestinal Sr-B1 and Vldl transcripts were not altered following exercise training. Sr-B1 has a well-accepted role in RCT via hepatobiliary pathway (Tall et al. 2008), It binds with apolipoprotein A-I (apoA-I), a protein component of high-density lipoprotein (HDL), and removes esterified cholesterol from HDL (Acton et al. 1996). van der Velde AE. et al reported that upregulation of intestinal Sr-B1 expression by high-fat feeding was correlated with TICE. However in the same study they surprisingly found that intestinal perfusions resulted in twofold increase in TICE in Sr-B1 deficient compared with WT mice (van der Velde et al. 2008). It seems that despite the well-known role of Sr-B1 in hepatobiliary, its function in TICE route is unclear. In addition, Vrins CL, et al. reported that secretion of radiolabeled cholesterol from HDL via TICE did not change in WT and $Abca1^{-/-}$ and $Sr\text{-}b1^{-/-}$ mice, implying that HDL might not be the plasma cholesterol donor to intestine (Vrins et al. 2012) and consequently it is reasonable to assume that Sr-B1 might not have a significant role in cholesterol uptake from circulation at the intestinal basolateral side (Bura et al. 2013). Based on these findings and the result of the present study, it appears that LDLr might be the main cholesterol acceptor from circulation at the basolateral side of intestine influenced by exercise training.

Surprisingly, we found no effects of exercise training as well as Chol-diet and estrogen withdrawal on intestinal receptors (except *Abcb1b* transcript) at the apical side which are involved in cholesterol excretion from intestine into the lumen. Intestinal *Abcb1b* transcript

was higher in Ovx rats and particularly showed a tendency to higher expression in Ovx trained rats however it did not reach the point to be significant. Previously, van der Veen et al showed that TICE is impaired in Abcg5 null mice, implying that intestinal cholesterol transporting Abcg5/Abcg8 heterodimer contributes to TICE pathway (van der Veen et al. 2009). On the other hand, it has been shown that mice lacking Abcg5 still have an appropriate level of TICE, suggesting that other apical transporters have a role in TICE. Among the candidates, multidrug transporter ABCB1 a/b, located at the apical side of enterocyte can be involved in intestinal cholesterol excretion into the lumen (Le May et al. 2013). On the whole further studies need to be done to clarify what receptors might have the main role in cholesterol excretion at the basolateral side of intestine. In regard to exercise training, there is inconsistency in previous reports on the expression of cholesterol transporters at the intestinal apical side. Some authors reported an increase in intestinal ABCG8 gene and protein expression in treadmill-trained female rats compared to female Sed rats (Ghanbari-Niaki et al. 2012). On the other hand, reduced ABCG8 gene expression was observed in the ileum of exercise-trained female rats compared to Sed female rat (Ngo Sock et al. 2014b) probably due to a reduced need to efflux cholesterol back to the lumen as a consequence of lower cholesterol absorption (Wilund et al. 2008). Apparently, exercise training could promote TICE through increase in gene expression of receptors involved in cholesterol uptake from circulation at the basolateral side, however further works will be needed to illustrate the excretion of cholesterol from intestinal apical side into the lumen.

In contrast to intestinal *Ldlr* and *Pcsk9* transcripts, hepatic gene expression of *Ldlr* and *Pcsk9* were not altered by exercise training. Hepatic *Ldlr* transcript was, however, lowered by the ovariectomy and the Chol feeding. Unchanged hepatic transcripts of *Ldlr* and *Pcsk9* following training in the present study was in concert with the recent observation that exercise training had no effects on hepatic gene expression of *Ldl* and *Pcsk9* in Ovx rats (Ngo Sock et al. 2014a). On the other hand, it was also reported that treadmill exercise resulted in increased LDL-R, Pcsk9 and SREBP-2 mRNA expression in liver of high-fat-fed C57BL/6 mice. Reduction in hepatic cholesterol accumulation was mentioned as a main reason for higher hepatic LDL-R, Pcsk9 transcript by treadmill exercise in high-fat-fed C57BL/6 mice (Wen et al. 2013). It seems that higher hepatic cholesterol content in Chol-fed rats in our study is an underlying reason for lower hepatic LDL-R, Pcsk9 transcript and consequently suppression of

cholesterol uptake from plasma. In addition, higher expression of SR-B1, involved in cholesterol uptake from circulating HDL, by training and simultaneously its down regulation following Chol-diet led us to the interpretation that this could probably be a protective response to prevent more cholesterol accumulation in the liver of Ovx rats. Taken all together, it appears that the effects of exercise training on the management of cholesterol metabolism may happen more at the intestinal than the hepatic level in our animal model.

Considering that cholesterol uptake was reduced through the liver probably due to hepatic cholesterol accumulation, it was expected to observe higher plasma TC levels under cholesterol feeding. The absence of increased plasma TC levels in the present study might be explained by higher cholesterol uptake through intestinal Ldlr as a result of intestinal up-regulation of this receptor under training.

In addition to hepatic Ldlr and Pcsk9 transcript, hepatic ABCG8 gene expression, involved in cholesterol excretion into bile in liver, was not changed by training. Down regulation of hepatic ABCG8 transcript following the cholesterol diet was reported in the present and previous studies of Ovx model (Cote et al. 2014). The fact that there was no effect of exercise training on genes involved in cholesterol uptake and excretion in liver and simultaneously positive effects on intestinal basolateral cholesterol receptors suggests that the effects of exercise training on cholesterol uptake and excretion may take place more at the intestine than at liver tissue. Furthermore, several lines of evidence suggest that there is a mechanism of crosstalk between biliary and non-biliary TICE pathways in liver (Kruit et al. 2005; Temel et al. 2010). The new model of RCT involves the classic central role of liver but also includes extra steps where the liver decides whether to excrete the cholesterol into bile or divert it into the non- biliary TICE pathway (Temel and Brown 2015). Indeed, under normal physiological conditions, the biliary route is a main way to excrete the extra cholesterol from the body and the non-biliary TICE pathway contributes to approximately 30% of the cholesterol disposal. However it was shown that TICE pathway can be stimulated, for instance, by a high fat diet (van der Velde et al. 2008), suggesting that, based on circumstances, the TICE pathway might have the main role. To get an insight into the molecular mechanisms underlying the collaboration of liver and intestine in cholesterol excretion, we measured hepatic Fmo3 gene expression. Recently, hepatic Fmo3 was introduced as a novel regulator of cholesterol balance in biliary and non-biliary TICE pathway (Warrier et al. 2015). Similar to other hepatic genes

involved in cholesterol metabolism, hepatic Fmo3 was not affected by exercise training in the present study but lowered by the Chol feeding. Apparently, lower gene expression of hepatic Fmo3 in Chol-fed rats compared to rats on the SD diet in the present study is a mechanism through which the liver attempted to shunt a portion of cholesterol into non-biliary TICE pathway for secretion (Temel and Brown 2015). This occurrence might be a protective response from liver to avoid more cholesterol accumulation due to its overload of cholesterol. This finding is in concert with a recent study that showed that FMO3 gene expression is supressed in several mouse models of augmented TICE (Warrier et al. 2015). Moreover, in mice fed a high cholesterol diet, where the liver accumulated excess cholesterol, FMO3 knockdown caused a significant decrease in hepatic LXR target genes including ABCG5 and G8 and suppression in biliary cholesterol levels suggesting the role of FMO3 in activation of biliary route (Warrier et al. 2015). Indeed, inhibition of FMO3 reorganizes the total body cholesterol balance and redirects the cholesterol away from biliary excretion into the non-biliary TICE pathway (Warrier et al. 2015). It seems that reduced *Fmo3* transcript under the cholesterol diet might shift the responsibility for the cholesterol excretion from liver into intestine and this occurrence is probably due to impairment in cholesterol disposal from the liver as a consequence of liver overload of cholesterol. Obviously, further studies are needed to gain insights into mechanisms in which FMO3 regulates the cholesterol balance between biliary and non-biliary TICE routes.

In summary, results of the present study indicate that exercise training through up-regulation of intestinal gene expression of *Ldlr* and *Pcsk9* in intact and Ovx rats may contribute to elimination of excess cholesterol via TICE pathway. It also introduced exercise training as an appropriate nonpharmacological intervention to stimulate TICE to excrete the extra cholesterol from the body and decrease the risk of atherosclerosis.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC; 7594).

Figure legends

Fig 1. mRNA expression of genes involved in transintestinal cholesterol excretion (TICE) at the basolateral membrane of the intestine in sham operated (Sham) rats fed a standard diet (SD) (Sham-SD), ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Sham rats fed a standard diet + 0.25% cholesterol (Chol) (Sham-Chol) and Ovx rats fed the Chol diet (Ovx-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed rats ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective rats fed the SD diet ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Sham rats ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$). *Ldlr*, LDL-receptor; *Pcsk9,* proprotein convertase subtilisin/kexin type 9; *Srebp2*, sterol regulatory element-binding protein 2.

Fig 2. mRNA expression of genes involved in transintestinal cholesterol excretion (TICE) at the apical membrane of the intestine in sham operated (Sham) rats fed a standard diet (SD) (Sham-SD), ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Sham rats fed a standard diet + 0.25% cholesterol (Chol) (Sham-Chol) and Ovx rats fed the Chol diet (Ovx-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed rats ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective rats fed the SD diet ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Sham rats ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$). *Abcg5,* ATP-binding cassette protein G5; *Abcg8,* ATP-binding cassette protein G8; *Abcb1a,* ATP-binding cassette sub-family B member 1a; *Abcb1b,* ATP-binding cassette sub-family B member 1b.

Fig 3. Liver and plasma total cholesterol (TC) levels in sham operated (Sham) rats fed a standard diet (SD) (Sham-SD), ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Sham rats fed a standard diet + 0.25% cholesterol (Chol) (Sham-Chol) and Ovx rats fed the Chol diet (Ovx-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed rats ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective rats fed the SD diet ($P < 0.05$), †† ($P < 0.01$), ††† (P

< 0.001); δ Significantly different from respective Sham rats (*P < 0.05*), δδ (*P < 0.01*), δδδ (*P < 0.001*).

Fig 4. Hepatic mRNA expression of genes involved in cholesterol uptake from circulation and also plasma Pcsk9 levels in sham operated (Sham) rats fed a standard diet (SD) (Sham-SD), ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Sham rats fed a standard diet + 0.25% cholesterol (Chol) (Sham-Chol) and Ovx rats fed the Chol diet (Ovx-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed rats (*P < 0.05*), ** (*P < 0.01*), *** (*P < 0.001*). † Significantly different from respective rats fed the SD diet (*P < 0.05*), †† (*P < 0.01*), ††† (*P < 0.001*); δ Significantly different from respective Sham rats (*P < 0.05*), δδ (*P < 0.01*), δδδ (*P < 0.001*). *Ldlr*, LDL-receptor; *Pcsk9,* proprotein convertase subtilisin/kexin type 9; *Sr-b1*, Scavenger receptor class B member 1.

Fig 5. Hepatic mRNA expression of cholesterol regulator and genes involved in hepatic cholesterol secretion into bile duct in sham operated (Sham) rats fed a standard diet (SD) (Sham-SD), ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Sham rats fed a standard diet + 0.25% cholesterol (Chol) (Sham-Chol) and Ovx rats fed the Chol diet (Ovx-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed rats (*P < 0.05*), ** (*P < 0.01*), *** (*P < 0.001*). † Significantly different from respective rats fed the SD diet (*P < 0.05*), †† (*P < 0.01*), ††† (*P < 0.001*); δ Significantly different from respective Sham rats (*P < 0.05*), δδ (*P < 0.01*), δδδ (*P < 0.001*). *Fmo3*, Flavin monooxygenase 3; *Abcg5,* ATP-binding cassette protein G5; *Abcg8,* ATP-binding cassette protein G8.

Fig 6. Hepatic (a) and intestinal (b) mRNA expression of rate limiting gene involved in VLDL assembly in sham operated (Sham) rats fed a standard diet (SD) (Sham-SD), ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Sham rats fed a standard diet + 0.25% cholesterol (Chol) (Sham-Chol) and Ovx rats fed the Chol diet (Ovx-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed rats (*P < 0.05*), ** (*P < 0.01*), *** (*P < 0.001*). † Significantly different from respective rats

fed the SD diet ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Sham rats ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$). *Mttp*, Microsomal triglyceride transfer protein.

Fig S1. mRNA expression of genes involved in transintestinal cholesterol excretion (TICE) at the basolateral membrane of the intestine in sham operated (Sham) rats fed a standard diet (SD) (Sham-SD), ovariectomized (Ovx) rats fed a standard diet (SD) (Ovx-SD), Sham rats fed a standard diet + 0.25% cholesterol (Chol) (Sham-Chol) and Ovx rats fed the Chol diet (Ovx-Chol) in sedentary (Sed, ■) or trained (Tr, ■) state. Values are mean ± SE. * Significantly different from respective Sed rats ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). † Significantly different from respective rats fed the SD diet ($P < 0.05$), †† ($P < 0.01$), ††† ($P < 0.001$); δ Significantly different from respective Sham rats ($P < 0.05$), δδ ($P < 0.01$), δδδ ($P < 0.001$). *Sr-b1*, Scavenger receptor class B member 1; *Vldlr*, very-low-density-lipoprotein receptor.

Table 1. Anthropometric parameters, food intake and total distance run

Variables	Sham-SD		Ovx-SD		Sham-Chol		Ovx-Chol	
	Sed	Tr	Sed	Tr	Sed	Tr	Sed	Tr
Final body weight (g)	344.6±14.1	317.4±11.4	408.4±18.2 δδδ	378.5±24.9 δδδ	332.5±15.9	314.4±17.4	429.8±14.1 δδδ	424.9±18.5 δδδ
Intra-abdominal fat pad weights (g)	28.9±4.7	15.1±4 ***	34.7±5 δδ	26.2±5.8 *** δδ	29.8±4.5	14.1±3.7 ***	39.2±3.5 δδ	30.1±4.9 ***δδ
Food intake (kcal/day)	83.2±3.4	86.1±3.3	92.9±5.7 δδδ	95.6±5.6 δδδ	81.6±4.1	89.4±3.1	99.2±3.9 δδδ	104.9±5.7 δδδ
Uterus (g)	0.45±0.07	0.41±0.1	0.11±0.02 δδδ	0.12±0.03 δδδ	0.41±0.04	0.48±0.05	0.12±0.02 δδδ	0.11±0.04 δδδ
Total distance run (km/d)		8.9±0.8		5.3±0.7		9.1±0.8		4.4±0.5

Ovx, ovariectomised; Sham, sham operated; SD, standard diet; Chol, standard diet + 0.25% cholesterol; Sed, sedentary group; Tr, trained group.
Values are mean ± SE.
*** Significantly different from respective Sed group ($P < 0.001$)
δδδ Significantly different from respective Sham rats ($P < 0.001$)

Table S1. Diet description

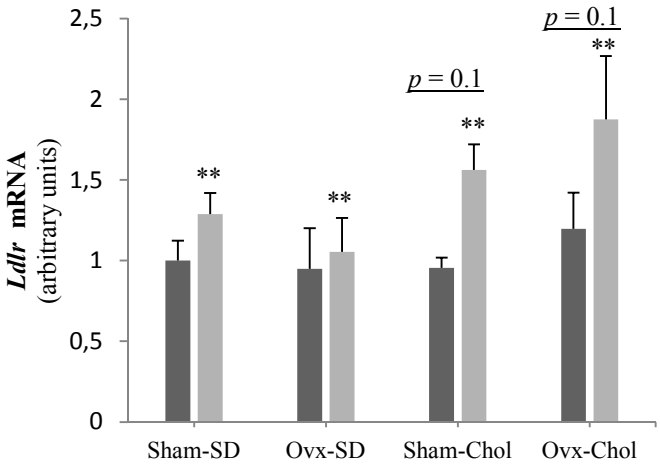
	Standard Diet (SD) (D12450J)	SD + Chol (0.25%) (D13020701)
(%)		
Protein	19.2	19.2
Carbohydrate	67.3	67.1
Fat	4.3	4.3
(g)		
Casein	200	200
L-Cystine	3	3
Corn Starch	506.2	506.2
Maltodextrin 10	125	125
Sucrose	68.8	68.8
Cellulose, BW200	50	50
Soybean Oil	25	25
Lard	20	20
Mineral Mix S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
Cholesterol	0.0	2.63
Kcal/g	3.85	3.84

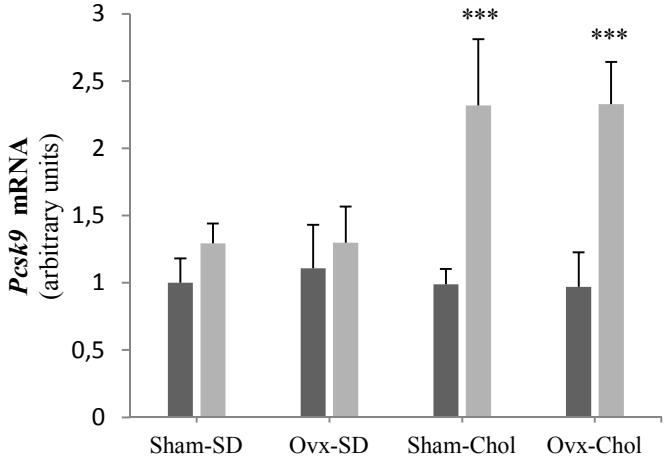
Formulated by: Research Diets, Inc. (20 Jules Lane, New Brunswick, NJ 08901 USA)

Table S2. Oligonucleotide primers used for quantitative real-time polymerase chain reaction

Gene	Oligo FWD	Oligo REV
ABCB1a	ccaccagttcatcgactcac	gatgtgaggctgtctgacga
ABCB1b	cacagaccgtcagcgaca	caatgcccgtgtaatagtaggc
ABCG5	cggagagttggtgttctgtg	caccgatgtcaagtccatgt
ABCG8	cagatgctggctatcataggg	ctgatttcatcttgccacca
FMO3	agcatgaaaactacgggttga	gctggaagctcatcattgaac
LDL-R	tgctactggccaaggacat	ctgggtggtcggtacagtg
MTP	gcgagtctaaaacccgagtg	cactgtgatgtcgctggttatt
PCSK9	cacctagcaggtgtggtcag	gcagactgtgcagactggtg
SR-B1	ggtgcccatcatttaccaac	gcgagccctttttactacca
SREBP-2	gtgcagacagtcgctacacc	aatctgaggctgaaccagga
Actb	cccgcgagtacaaccttct	cgtcatccatggcgaact
GAPDH	ccctcaagattgtcagcaatg	agttgtcatggatgaccttgg

Fig. 1


Ldlr mRNA
(arbitrary units)
2,5
2
1,5
1
0,5
0
p = 0.1
p = 0.1
**
**
**
**
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol


Pcsk9 mRNA
(arbitrary units)
3
2,5
2
1,5
1
0,5
0
***
***
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

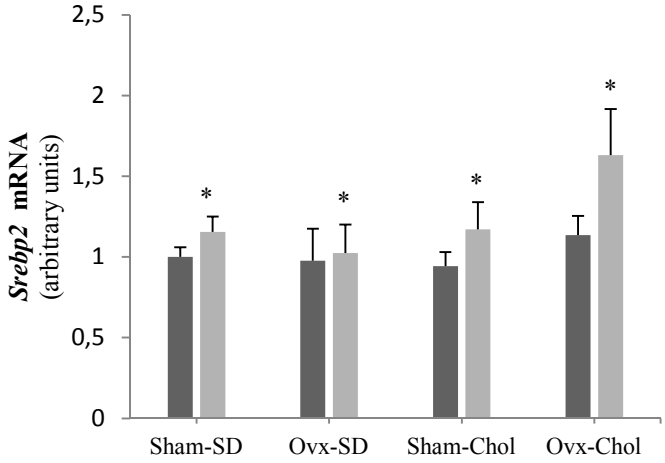
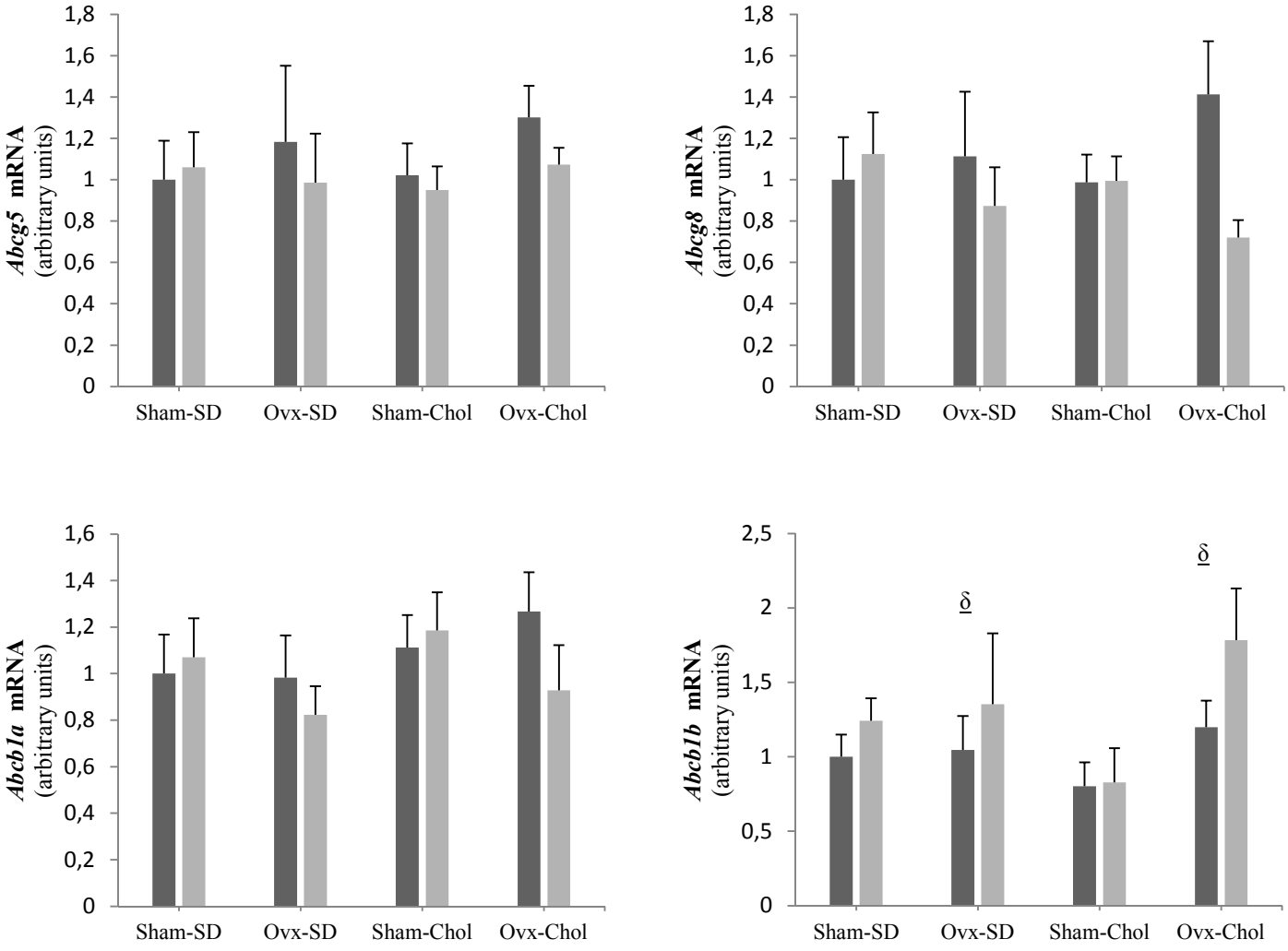

Srebp2 mRNA
(arbitrary units)
2,5
2
1,5
1
0,5
0
*
*
*
*
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

Fig. 2


Abcg5 mRNA (arbitrary units)
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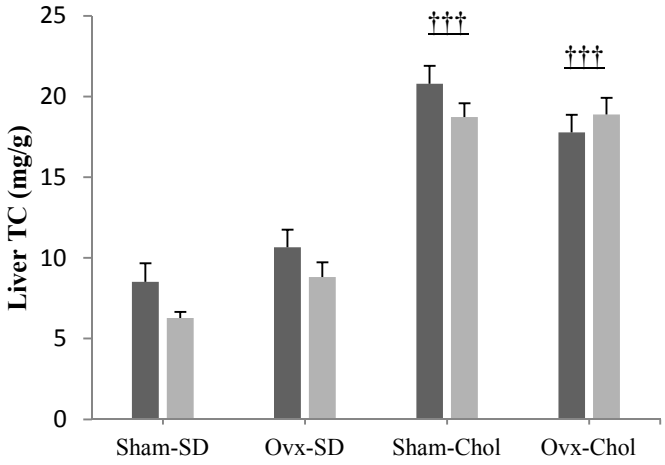
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol
Abcg8 mRNA (arbitrary units)
Abcb1a mRNA (arbitrary units)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
Abcb1b mRNA (arbitrary units)
2,5
2
1,5
1
0,5
δ
δ

Fig. 3


Liver TC (mg/g)
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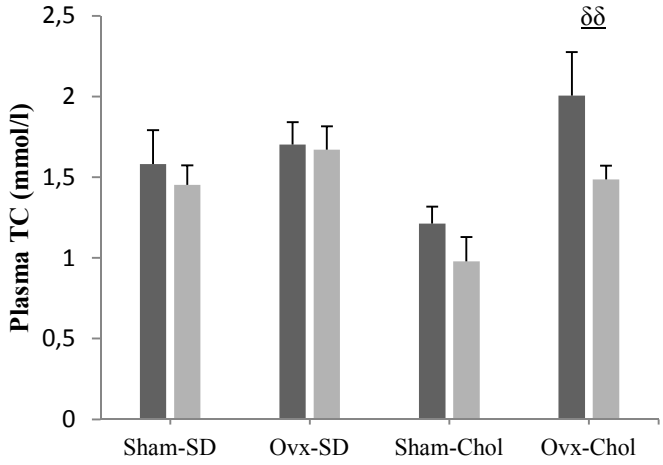
20
15
10
5
0
†††
†††
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol


Plasma TC (mmol/l)
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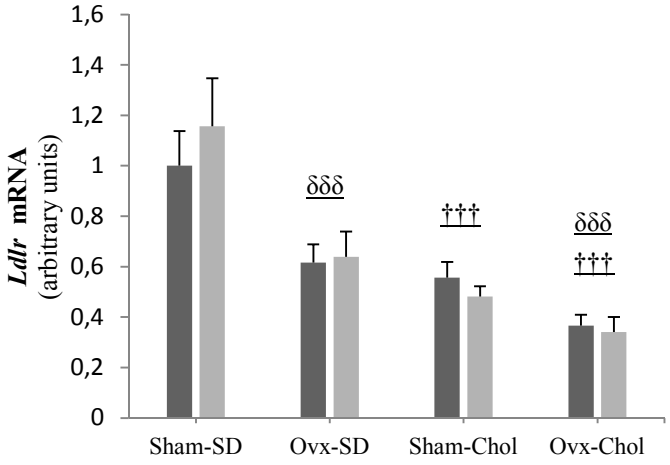
2
1,5
1
0,5
0
δδ
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

Fig. 4


Ldlr mRNA (arbitrary units)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
δδδ
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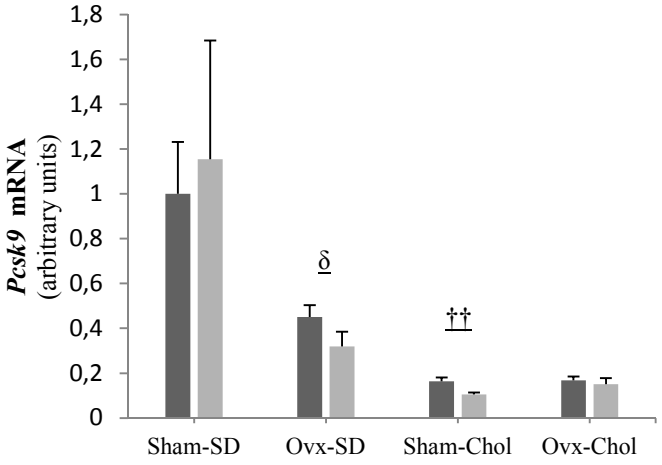
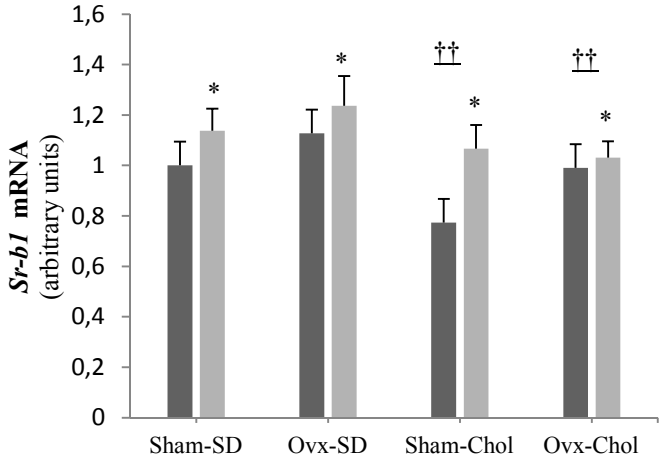
δδδ
†††
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol


Pcsk9 mRNA (arbitrary units)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
δ
††
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol


Sr-b1 mRNA (arbitrary units)
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1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
*
††
††
*
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

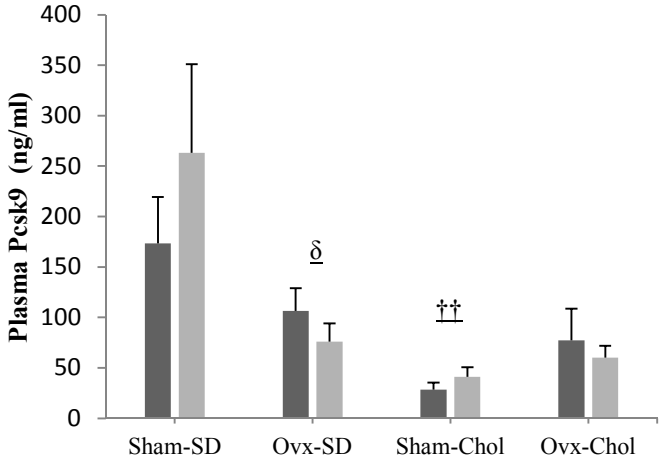
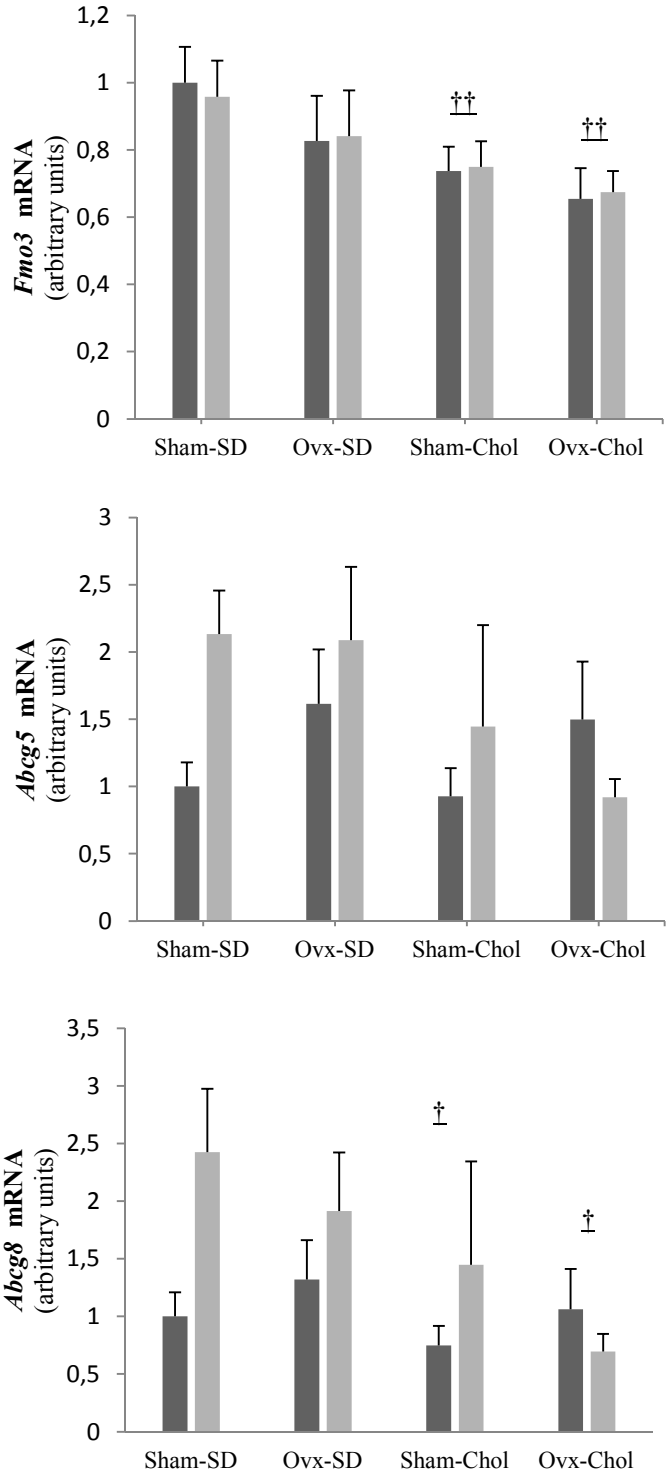

Plasma Pcsk9 (ng/ml)
400
350
300
250
200
150
100
50
0
δ
††
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

Fig. 5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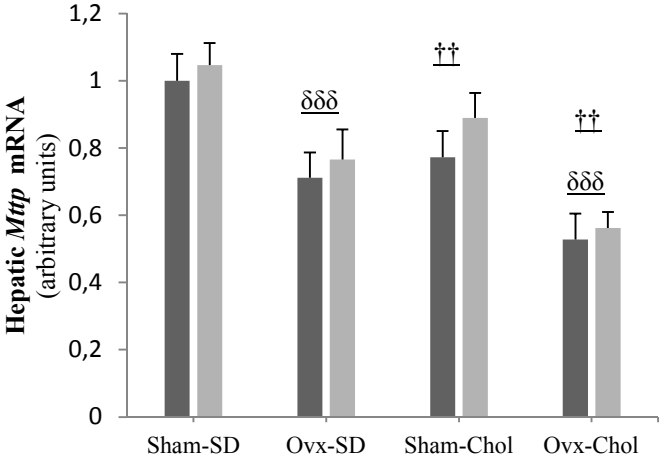
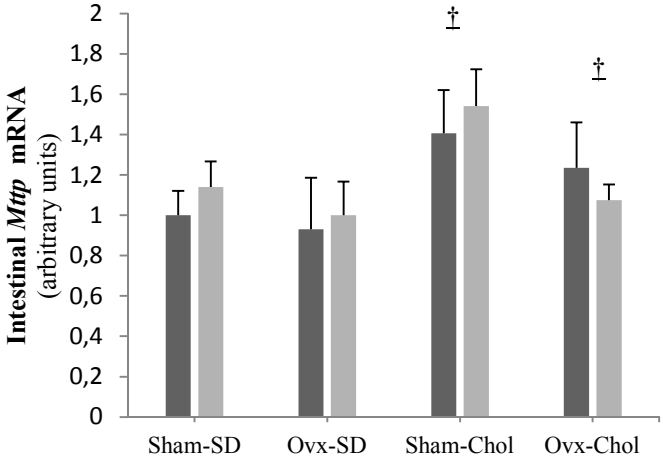
0,8
0,6
0,4
0,2
0
Fmo3 mRNA
(arbitrary units)
††
††
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol
3
2,5
2
1,5
1
0,5
0
Abcg5 mRNA
(arbitrary units)
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol
3,5
3
2,5
2
1,5
1
0,5
0
Abcg8 mRNA
(arbitrary units)
†
†
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

Fig. 6

a


Hepatic *Mtp* mRNA
(arbitrary units)
1,2
1
0,8
0,6
0,4
0,2
0
δδδ
††
††
δδδ
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

b


Intestinal *Mtp* mRNA
(arbitrary units)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
†
†
Sham-SD
Ovx-SD
Sham-Chol
Ovx-Chol

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Chapter 3: General discussion and conclusion

3.1 General discussion

The general objective of the studies presented in this thesis was to provide molecular information on how the liver and the intestine regulate cholesterol and bile acid metabolism in an ovariectomized (Ovx) rat model of menopause. It was also intended to introduce an appropriate non-pharmacological intervention (exercise training) to stimulate cholesterol excretion from the body and consequently decrease the risk of atherosclerosis.

We used an ovariectomized rat model as an appropriate research tool to mimic the postmenopausal hormonal state to investigate whether hepatic and intestinal molecular markers involved in cholesterol and bile acid metabolism are affected by estrogen withdrawal. To reach our main goal, we used a diet rich in cholesterol as a nutritional tool in our series of experiments. Nutritional approaches have been used frequently as a tool to investigate the role of the liver in regulating TG and cholesterol metabolism (Cote et al. 2013; Savard et al. 2013). The positive potential of exercise training on improvement of plasma lipoprotein profile and consequently reduction in the risk of atherosclerosis has been repeatedly reported (Durstine et al. 2002; Halverstadt et al. 2007; Butcher et al. 2008). However, the underlying mechanisms are not fully understood; therefore, it was of interest to investigate the effects of exercise training as an alternative intervention on cholesterol and bile acids pathways, especially in the context of estrogens deficiency.

In the first study, we attempted to shed some light on how liver of Ovx animals respond to high dietary cholesterol feeding by targeting the main molecular markers involved in VLDL assembly and secretion. Results of this first study indicate that a high cholesterol diet and ovariectomy combine to reduce the gene expression of key markers of VLDL assembly including MTP, apo-B, and ACAT2 suggesting disturbances in VLDL assembly from the liver. Moreover, the sole addition of cholesterol to the SD diet decreased the gene expression of ABCG8, BSEP, and MDR2 in both Sham and Ovx animals implying impairment in bile acid formation and cholesterol excretion from the liver into bile ducts.

Data of our first study support the concept that cholesterol component in a mixed diet is a determinant factor in regulation of hepatic cholesterol metabolism in both sham and Ovx rats. It has been previously reported that increasing dietary cholesterol exacerbated hepatic cholesterol accumulation and caused hepatocyte injury in different human and animal studies (Ioannou et al. 2009; Park et al. 2011a; Subramanian et al. 2011; Van Rooyen et al. 2011). Based on the findings of the first study, it seems that decreased expression of genes involved in hepatic bile acid and cholesterol excretion into bile ducts and also VLDL assembly might be the possible underlying mechanisms for impairment in cholesterol metabolism in Ovx rats leading to hepatic cholesterol loading.

There is some evidence that exercise training may influence hepatic cholesterol metabolism. For instance, it has been reported that voluntary wheel running increases cholesterol conversion into bile acids based on the observation of increased fecal bile acid excretion in LDL-R deficient mice (Meissner et al. 2011). However the underlying molecular mechanism for these observations has not been fully explored. Since hepatic cholesterol and bile acid excretion were disrupted by a high cholesterol diet, especially in Ovx rats, we conducted our second study to determine the effects of exercise training on molecular markers of hepatic FXR/SHP/CYP7A1 pathway which are involved in bile acids production, as key markers of the conversion of extra cholesterol into bile acids.

Results of second study suggest that voluntary wheel running modulates cholesterol metabolism in Ovx animals through hepatic up-regulation of SHP and CYP7A1, genes involved in bile acid biosynthesis. Surprisingly, the inverse relationship between SHP and CYP7A1 was not observed following training. Moreover, unchanged FXR transcript under training suggests that SHP can also act independently of FXR. This novel finding is in concert with findings of Lai et al showing that induction of SHP by estrogen administration did not inhibit expression of CYP7A1 or CYP8B1, the well-known SHP target genes, (Lai et al. 2003). These findings suggest that stimulation of SHP by estrogens may not result in suppression of CYP7A1 transcripts. It seems that exercise training in our study imitated the effect of estrogen therapy on SHP transcripts. In fact, the present finding that exercise training also up-regulated SHP expression in Ovx animals extends previous findings from our lab

showing that exercise training provokes estrogenic like effects on the expression of several genes involved in the regulation of lipid metabolism in liver (Pighon et al. 2011).

Results in our second study do suggest that exercise training may alleviate hepatic cholesterol load by stimulating bile acid biosynthesis. On the other hand, exercise training was associated with no changes in hepatic SREBP2, HMGCoA-r, LDL-R, and PCSK9 transcripts indicating an absence of molecular effects on hepatic key markers of cholesterol biosynthesis and hepatic cholesterol uptake from circulation. It was postulated that this might be due to hepatic overload of cholesterol.

Although the hepatobiliary route is the main pathway for elimination of excess cholesterol from the body, several studies have recently enlighten the fact that RCT can also proceed through a non-biliary pathway known as transintestinal cholesterol excretion (TICE) (Brown et al. 2008; van der Velde et al. 2008). In TICE pathway, cholesterol is transported through the receptors at the basolateral and apical membrane of intestine which are involved in cholesterol uptake from plasma and cholesterol secretion into the lumen, respectively. Indeed, in this new model of RCT, the cholesterol disposal from the body depends on an active interplay between liver and intestine (Temel and Brown 2015). Therefore, the third study was designed to determine whether exercise training may influence intestinal membrane receptors involved in TICE in intact and ovariectomized cholesterol fed rats. It was of interest to study the effect of exercise training on intestinal receptors involved in cholesterol disposal through TICE.

The results of third study indicate an up-regulation of intestinal gene expression of LDL-R and PCSK9 at the basolateral membrane along with their regulatory transcription factor SREBP2 following a six week voluntary wheel running in intact and Ovx rats. These data suggest that exercise training may contribute to increased cholesterol elimination through the TICE pathway.

In contrast to intestinal LDL-R and PCSK9 transcripts, hepatic gene expression of LDL-R and PCSK9 was not altered by exercise training. It seems that higher hepatic cholesterol content in Chol-fed rats in our study is an underlying reason for lower hepatic

LDL-R, PCSK9 transcript and consequently suppression of hepatic cholesterol uptake from plasma.

Moreover, there is evidence that the set point of cholesterol excretion is sustained by the crosstalk between hepatobiliary and non-biliary TICE pathways (Kruit et al. 2005). In very recent study hepatic flavin monooxygenase 3 (FMO3) has been identified as a key cholesterol regulator of both biliary and non-biliary TICE pathways (Warrier et al. 2015). In our study, exercise training had no impact on hepatic FMO3 transcripts. On the other hand, lower gene expression of hepatic FMO3 found in Chol-fed rats compared to rats fed a SD diet in the present study is a mechanism through which the liver attempted to shunt a portion of excess cholesterol into non-biliary TICE pathway for secretion (Temel and Brown 2015). We concluded that this occurrence might be a protective response of the liver to avoid more cholesterol accumulation due to cholesterol overload in liver.

On the whole, data of the third study indicate that intestinal cholesterol uptake from circulation might be increased at the basolateral membrane of intestine following exercise training, suggesting that TICE might be a way by which exercise training contributes to an elimination of excess cholesterol.

To the best of our knowledge, this study is the first to report an important increase in intestinal LDL-R and PCSK9 transcripts following exercise training. Our data in the third study open a new avenue for the investigation of potential mechanisms that might explain the benefits of exercise training in decreasing the hypercholesterolemia and the risk of atherosclerosis in postmenopausal women.

Taken together, it seems that estrogen withdrawal and a high cholesterol diet act synergistically to impair different aspects of hepatic cholesterol metabolism including cholesterol excretion from the body. Exercise training appears to be an appropriate non-pharmacological intervention to stimulate cholesterol excretion from the body through both hepatobiliary and non-biliary TICE pathway. Up-regulation of genes involved in bile acid formation was a predominant effect of exercise training in liver suggesting that exercise training may alleviate hepatic cholesterol load by stimulating bile acid biosynthesis. Up-regulation of intestinal basolateral receptors involved in cholesterol uptake from the

circulation following exercise training might be a mechanism through which exercise training may contribute to elimination of excess cholesterol via TICE pathway.

3.2 Conclusion

Results of the studies presented in this thesis indicate that different aspects of cholesterol metabolism including cholesterol uptake from circulation, VLDL assembly, and cholesterol excretion were disrupted in the liver of Ovx rats especially when they were fed the cholesterol diet.

Metabolic perturbations in hepatic cholesterol metabolism which were caused by the combined effect of estrogen withdrawal and a high cholesterol diet might contribute to hepatic cholesterol accumulation in addition to TG accumulation. It is also reasonable to conclude that disturbances in hepatic cholesterol metabolism might be the root of common hypercholesterolemia observed in post-menopausal women as well as in Ovx rat.

From a clinical point of view, a high cholesterol diet might not be perceived as being deleterious since it does not affect body weight. However, the fact is that at the molecular level it causes several disturbances in hepatic cholesterol metabolism. Of note, the present results generated from an animal model are intended to open research issues that need to be tested in humans.

We also introduced exercise training as an appropriate non-pharmacological intervention to alleviate perturbations in hepatic cholesterol metabolism. Exercise training modulated cholesterol metabolism through its positive effects on molecular markers involved in hepatic bile acid formation and intestinal cholesterol uptake from the circulation. This occurrence may contribute to improve NAFLD and decrease the risk of atherosclerosis and also explain the underlying mechanisms for the beneficial role given to exercise. Moreover, our results clearly indicate that cholesterol homeostasis in the body depends on a dynamic interplay between liver and intestine.

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