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Régulation de l'expression hépatique du récepteur LSR (*lipolysis stimulated lipoprotein receptor*): rôles de l'acide docosahexaénoïque et du récepteur PPARα (*peroxisome proliferator-activated receptor alpha*)

Regulation of the expression of hepatic lipolysis stimulated lipoprotein receptor: roles of docosahexaenoic acid and peroxisome proliferator-activated receptor α

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To my beloved family, especially my parents

To my love, Zeeshan

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LIST OF ABBREVIATIONS

ABCA1 ..... ATP-binding cassette protein A1
ACC ..... Acyl-CoA carboxylase
ACOX1 ..... Acyl-CoA oxidase 1
ACS..... Acyl-CoA synthetase
AD..... Alzheimer's disease
AF ..... Activation factor
ALA ..... α-Linolenic acid (C18:3, *n*-3)
ApoB..... Apolipoprotein B
ApoE ..... Apolipoprotein E
ARA ..... Arachidonic acid (C20:4, *n*-6)
CE ..... Cholesterol esters
CETP..... Cholesteryl ester transfer protein
CHD ..... Coronary heart disease
ChREBP..... Carbohydrate regulatory element-binding protein
CM ..... Complete medium
CPT-1..... Carnitine palmitoyl transferase-1
CVD ..... Cardiovascular disease
DBD ..... DNA-binding domain
DCE ..... Downstream core promoter element
DHA..... Docosahexaenoic acid (C22:6, *n*-3)
DHA-EE..... DHA-ethyl ester
DIO ..... Diet-induced obesity
DMEM..... Dulbecco's modified Eagle's medium
DMSO..... Dimethyl sulfoxide
dNTP..... Deoxynucleotide triphosphate
ER ..... Endoplasmic reticulum
EPA..... Eicosapentaenoic acid (C20:5, *n*-3)
FA ..... Fatty acid
FAS ..... Fatty acid synthase
FATP..... Fatty acid transport protein
FFA ..... Free fatty acid
FH ..... Familial hypercholesterolemia
FO ..... Fish oil
FXR..... Farnesoid X receptor
HDL ..... High-density lipoprotein
HL ..... Hepatic lipase
HF ..... High-fat
HNF-4α..... Hepatocyte nuclear factor-4 alpha
Hprt ..... Hypoxanthine-guanine phosphoribosyltransferase
Inr..... Initiator element

IR .....Insulin resistance
LA .....Linoleic acid
LBD .....Ligand-binding domain
LBD .....Ligand-binding domain
LC .....Long chain
LDL.....Low-density lipoprotein
LDL-R.....Low density lipoprotein-receptor
LPDS.....Lipoprotein-deficient serum
LPL .....Lipoprotein lipase
LRP .....LDL receptor-related protein
LSR .....Lipolysis-stimulated receptor
LXR .....Liver X receptors
MLX.....MAX-like factor X
MTE .....Motif ten element
MetS.....Metabolic syndrome
MetS.....Metabolic syndrome
NAFLD .....Non-alcoholic fatty liver disease
OA.....Oleic acid
PA .....Palmitic acid
PCR.....Polymerase chain reaction
PPAR .....Peroxisome proliferator-activated receptor
PPRE.....PPAR response element
PUFA .....Polyunsaturated fatty acid
RXR .....Retinoic X receptor
SR-BI .....Scavenger receptor class BI
sdLDL .....Small dense LDL
SDM.....Serum-deficient medium
SDS .....Sodium dodecyl sulfate
SREBP .....Sterol regulatory element-binding protein
SRE .....Sterol regulatory element
STAT .....Signal transducer and activator of transcription
STD.....Standard
SCD.....Stearoyl-CoA desaturase
TBP .....TATA-binding protein
TC .....Total cholesterol
T2DM.....Type 2 diabetes mellitus
TF.....Transcription factor
TFBS.....Transcription factor binding site
TG .....Triglyceride
TSS.....Transcriptional start site
VLDL.....Very low-density lipoprotein
XCPE .....X core promoter element

General Introduction

This thesis project was conducted with the "Biodisponibilité et Fonctionnalités des Lipides Alimentaires" (BFLA) team of the "Unité de Recherche Animal & Fonctionnalités des Produits Animaux" (UR AFPA) laboratory. BFLA focuses on characterizing the mechanisms that affect the bioavailability and the functions of dietary lipids which, in turn, may contribute to the development of metabolic and cellular impairments associated with aging. Indeed, the disturbances in lipid homeostasis manifest in different forms of dyslipidemias including hypertriglyceridemia and hypercholesterolemia and are considered as risk factors for various inter-related disorders including obesity, type 2 diabetes mellitus and neurodegenerative diseases such as Alzheimer's disease, all of which represent important concerns for public health worldwide. It is therefore important to understand the mechanisms underlying the regulation of lipid status.

The maintenance of normal lipid status is achieved by an intricate interplay of various lipoprotein receptors, enzymes and transport proteins that work in cooperation with one another. The lipolysis stimulated lipoprotein receptor (LSR) that was characterized in our laboratory has been shown to regulate the distribution of lipids amongst the different tissues. Indeed, it actively participates in the clearance of triglyceride (TG)-rich lipoproteins from the circulation during the postprandial phase (Bihain & Yen, 1992; Yen *et al.,* 2008; Stenger *et al.,* 2012). Reduced expression of LSR is associated with increased weight gain (Stenger *et al.,* 2010) and has been observed in obese mouse models with increased fat mass (Narvekar et al., 2009). Furthermore, LSR requires the presence of free fatty acids for its activation, and is regulated by leptin, an adipokine that is a satiety factor produced by adipose tissue (Stenger *et al.,* 2010). In view of the importance of LSR in the regulation of lipid homeostasis, my principal goal was to delineate the factors that could regulate LSR expression levels and activity, focusing specifically on the *n*-3 fatty acid, docosahexaenoic acid (DHA), and the transcriptional factor peroxisome proliferator-activated receptor alpha (PPARα) using both cell culture and animal models, as well as a bioinformatics approach.

This thesis is presented in the following manner:

Introduction: This chapter represents a review of the literature and is divided into four sections:

- ✓ The first section describes lipoprotein metabolism, including the structure and classification of lipoproteins as well as their mode of transport in the circulation. Also,

it presents an overview of the lipoprotein receptors, LDL-R, LRP1 and LSR, along with their regulation at the transcriptional level.

- ✓ The subject of the second section is the metabolism of long-chain polyunsaturated fatty acids, specifically focusing on the importance of *n*-3 fatty acids including DHA in reducing dyslipidemia and particularly hypertriglyceridemia.
- ✓ The third section describes the tissue distribution, ligand specificities, mode of transcriptional activation and functions of a class of ligand-activated transcription factors, the peroxisome proliferator-activated receptors (PPARs). The role of PPARα subtype has been described in detail, because of its function as the principal regulator of hepatic lipid metabolism.
- ✓ Finally, the fourth section discusses the disorders related to lipid metabolism that are associated with public health concerns, such as dyslipidemia, obesity, insulin resistance and metabolic syndrome, and also highlights various therapeutic strategies.

Materials and methods: This chapter describes in detail the various experimental strategies and analytical techniques that were used for these studies.

Results and discussion: This chapter is presented as four separate sections, each with its specific experimental procedures and its corresponding discussion.

- ✓ It is well-documented that DHA improves the lipid status by controlling the expression, the localization and/or the activity of various enzymes and receptors. Since LSR requires fatty acids for its activation and is mainly involved in lipoprotein metabolism, the first section is focused on determining the effects of DHA supplementation on LSR expression and activity using both cell culture and animal models.
- ✓ Since DHA exerts its hypotriglyceridemic effects *via* various transcription factors involved in lipid metabolism, we next sought to determine which transcription factors could be involved in the control of *lsr* gene expression by identifying the response elements present in the upstream regulatory region. As the promoter sequence of *lsr* gene is not yet known, we performed *in silico* analysis of the 5' regulatory regions of mouse, rat and human *lsr* gene. This second section hence describes the identification

of various core promoter elements present in the potential upstream region of *lsr* gene, along with the putative transcription factor elements including PPAR response elements.

- ✓ Following the *in silico* analysis, the third section focuses on a study designed to determine whether PPARα is involved in the transcriptional regulation of LSR in *in vitro* models.
- ✓ The fourth section details the results following qPCR array of liver mRNA obtained from $LSR^{+/+}$ and $LSR^{+/-}$ mice placed on standard or high-fat diets. A comparative analysis is presented highlighting the changes in mRNA expression of various transcription factors, enzymes and transport proteins related to hepatic lipid metabolism.

Conclusions and perspectives: The last chapter summarizes the principal findings of the Results and discussion chapter and presents the conclusions as well as the perspectives supported by this experimental work.

Review of Literature

I. Lipoprotein biology and metabolism

1. Structure of lipoproteins

Being hydrophobic in nature, lipids are transported in the blood as soluble complexes called lipoproteins, consisting of cholesterol, triglycerides (TGs), phospholipids and proteins. Plasma lipoproteins are spherical particles consisting of a hydrophobic core containing TG and cholesterol esters as well as small quantities of fat-soluble vitamins (e.g., vitamins A, D, E, and K). The outer layer of lipoproteins is made up of the amphipathic molecules phospholipids, unesterified cholesterol and apolipoproteins. While the hydrophobic regions of these molecules are oriented towards the inner side, the hydrophilic regions are towards the outer side, rendering these particles soluble in their surrounding aqueous environment (Figure 1).

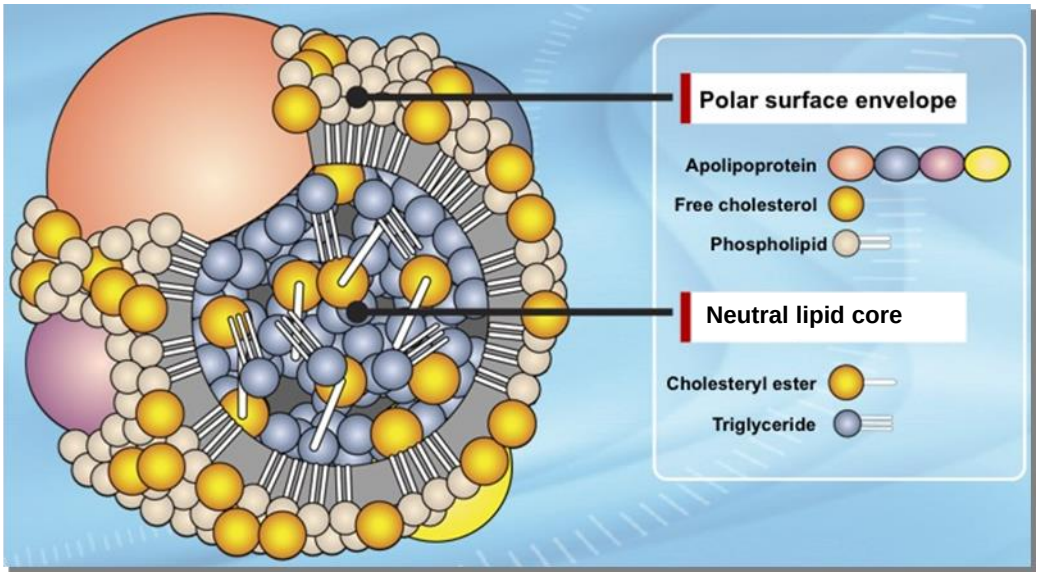


Figure 1: Schematic representation of the structure of a lipoprotein
Source: International Chair on Cardiometabolic Risk (www.cardiometabolic-risk.org)

1.1. Apolipoproteins

The apolipoprotein or apoprotein content of a lipoprotein determines its structural and functional properties by playing a significant role in lipid solubilization, maintaining the structural integrity of lipoproteins as well as serving as ligands for many lipoprotein receptors and as cofactors for many enzymes (Davis & Wagganer, 2005). Being amphipathic in nature, the apolipoproteins interact both with the hydrophobic portions of lipoproteins and the aqueous environment. Plasma apolipoproteins are grouped into the exchangeable (ApoA-I, ApoA-II, ApoA-IV, ApoC-I, ApoC-II, ApoC-III, and ApoE) and the non-exchangeable (ApoB-100 and

ApoB-48) apolipoproteins (Segrest *et al.*, 1992 & 2001). Exchangeable apolipoproteins attach reversibly with lipids and are, therefore, able to dissociate from a lipoprotein particle and reassociate to another lipoprotein in the circulation. In contrast, the non-exchangeable apolipoproteins remain attached to the TG-rich lipoproteins from the beginning of lipoprotein assembly and secretion to the end of remnant particle clearance. This occurs mainly because of their hydrophobic β-sheet elements that remain embedded within the lipid aggregate of lipoprotein particles, thus ensuring a tight association of these apolipoproteins with their respective lipoprotein particles (Vance, 2002; Sundaram & Yao, 2012) (Table 1).

Table 1: Major apolipoproteins and their functions
(Rader & Hobbs, 2008)

MAJOR APOLIPOPROTEINS			
APOLIPOPROTEIN	**PRIMARY SOURCE**	**LIPOPROTEIN ASSOCIATION**	**FUNCTION**
ApoA-I	Intestine, liver	HDL, chylomicrons	Structural protein for HDL, activates LCAT
ApoA-II	Liver	HDL, chylomicrons	Structural protein for HDL
ApoA-IV	Intestine	HDL, chylomicrons	Unknown
ApoA-V	Liver	VLDL	Unknown
ApoB-48	Intestine	Chylomicrons	Structural protein for chylomicrons
ApoB-100	Liver	VLDL, IDL, LDL, LP(a)	Structural protein for VLDL, LDL, IDL, LP(a); ligand for binding to LDL, receptor
ApoC-I	Liver	Chylomicrons VLDL, HDL	Unknown
ApoC-II	Liver	Chylomicrons VLDL, HDL	Cofactor for LPL
ApoC-III	Liver	Chylomicrons VLDL, HDL	Inhibits lipoprotein binding to receptors
ApoD	Spleen, brain, testes, adrenals	HDL	Unknown
ApoE	Liver	Chylomicron remnants, IDL, HDL	Ligand for binding to LDL receptor
ApoH	Liver	Chylomicrons VLDL, LDL, HDL	B_2 glycoprotein I
ApoJ	Liver	HDL	Unknown
ApoL	Unknown	HDL	Unknown
Apo(a)	Liver	Lp(a)	Unknown

Being responsible for the distribution of cholesterol and triglycerides, the lipoproteins play a crucial role in various lipid-related pathological conditions including obesity and diabetes, as well as Alzheimer's disease and atherosclerosis (Rader & Daugherty, 2008).

2. Classification of lipoproteins

The major plasma lipoproteins are the chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), high-density

lipoprotein (HDL) and lipoprotein A or Lp(a). The lipoproteins are classified on the basis of their density or electrophoretic mobility. The density of lipoproteins increases with protein content and decreases with lipid content, while their mobility on agarose gels depends on size and charge. Chylomicrons and VLDL are the largest and least dense lipoprotein particles, whereas HDL is the smallest and most dense lipoprotein. The majority of TG is transported in chylomicrons or VLDL, while cholesterol is primarily carried as cholesteryl esters in LDL and HDL (Table 2).

Table 2: Characteristics of plasma lipoproteins
(Rader & Hobbs, 2008)

MAJOR LIPOPROTEIN CLASSES[a]

LIPOPROTEIN	DENSITY, G/ML[b]	SIZE NM[c]	ELECTROPHORETIC MOBILITY[d]	APOLIPOPROTEINS		OTHER CONSTITUENTS
				MAJOR	OTHER	
Chylomicrons	0.930	75–1200	Origin	ApoB-48	A-I, A-IV, C-I, C-II, C-III	Retinyl esters
Chylomicron remnants	0.930–1.006	30–80	Slow pre-β	ApoB-48	E, A-I, A-IV, C-I, C-II, C-III	Retinyl esters
VLDL	0.930–1.006	30–80	Pre-β	ApoB-100	E, A-I, A-II, A-V, C-I, C-II, C-III	Vitamin E
IDL	1.006–1.019	25–35	Slow pre-β	ApoB-100	E, C-I, C-II, C-III	Vitamin E
LDL	1.019–1.063	18–25	β	ApoB-100		Vitamin E
HDL	1.063–1.210	5–12	α	ApoA-I	A-II, A-IV, E, C-III	LCAT, CETP paroxonase
Lp(a)	1.050–1.120	25	Pre-β	ApoB-100	Apo(a)	

[a]All of the lipoprotein classes contain phospholipids, esterified and unesterified cholesterol, and triglycerides to varying degrees.
[b]The density of the particle is determined by ultracentrifugation.
[c]The size of the particle is measured using gel electrophoresis.
[d]The electrophoretic mobility of the particle on agarose gel electrophoresis reflects the size and surface charge of the particle, with β being the position of LDL and α the position of HDL.
Note: VLDL, very low density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein: HDL, high-density lipoprotein; Lp(a), lipoprotein A; LCAT, lecithin-cholesterol acyltransferase; CETP, cholesteryl ester transfer protein.

2.1. Chylomicrons

After ingestion of a meal, the chylomicrons, secreted by the small intestine, mediate the transport of dietary fat into the blood circulation (Hayashi *et al.*, 1990). Chylomicrons are the largest, lipid-rich lipoprotein particles containing on average 86% TG and 3% cholesterol esters (Burnett & Barret, 2002; Davis & Wagganer, 2005). ApoB-48 is the major structural apolipoprotein in intestinal-derived chylomicrons and is essential for the assembly and secretion of chylomicrons (van Greevenbroek & de Bruin, 1998; Cartwright *et al.*, 2000; Lo *et al.*, 2008). Apart from this, chylomicrons also contain other apolipoproteins including ApoA-I, ApoA-II, ApoA-IV, ApoC-II, ApoC-III and ApoE. The ApoA-I and A-IV are synthesized in the intestine, whereas ApoA-II, ApoC-II, C-III and E are acquired as a result of transfer from HDL within circulation (Burnett & Barret, 2002; Davis & Wagganer, 2005).

2.2. VLDL

Liver synthesizes and secretes VLDL particles, which transport endogenously synthesized TG (~55%) and cholesterol esters (~12%) to the peripheral tissues (Burnett & Barret, 2002; Davis & Wagganer, 2005). The synthesis of VLDL occurs through the cotranslational lipidation of ApoB-100 in two steps (Swift, 1995; Stillemark *et al.,* 2000; Olofsson & Boren, 2005; Sundaram & Yao, 2010). The first step involves the transfer of TG and phospholipids to Apo-B100 by the action of the enzyme 'microsomal triglyceride transfer protein (MTP)' in the endoplasmic reticulum (ER), resulting in the production of nascent VLDL particles (pre-VLDL) (Rustaeus *et al.,* 1998; Olofsson *et al.,* 2000; Kulinski *et al.,* 2002). In the second step, the pre-VLDL particles undergo further lipidation to form mature VLDL in the ER and/or post-ER compartments (Alexander *et al.,* 1976; Borchardt & Davis, 1987; Rusiñol *et al.,* 1993; Valyi-Nagy *et al.,* 2002; Gusarova *et al.,* 2003). This process also involves a small GTP-binding protein ADP-ribosylation factor 1 (ARF-1), which plays a key role in intracellular vesicular trafficking (Asp *et al.,* 2005; Stillemark-Billton *et al.,* 2005). In addition to ApoB-100, other apolipoproteins in VLDL include ApoC-II, ApoC-III and ApoE, which are incorporated into the lipoprotein within the circulation as a result of transfer from HDL (Burnett & Barret, 2002; Davis & Wagganer, 2005). Following their hydrolysis by lipoprotein lipase (LPL), some VLDL particles are endocytosed by the hepatic receptors present on hepatocyte plasma membranes, while others remain in circulation as IDL (Fielding & Fielding, 2002).

2.3. IDL

IDL is derived from hydrolysis of VLDL by lipases and is comprised of about 23% TG and 29% cholesterol esters with one molecule of ApoB-100 present on the surface of each IDL particle (Burnett & Barret, 2002; Davis & Wagganer, 2005). Due to the small size of IDL compared to VLDL, the group of apolipoproteins C loses their affinity with the particle and is then exchanged with VLDL, HDL and chylomicrons. Under normal conditions, IDL particles are rarely detected in plasma in that they are either quickly removed from the circulation by liver or are subject to further lipase activity to produce LDL (Daniels *et al.,* 2009).

2.4. LDL

The hydrolysis of VLDL TGs ultimately leads to the formation of LDL particles that are the major cholesterol carriers in the plasma, containing approximately 6% TG and 42%

cholesterol esters (Burnett & Barret, 2002; Davis & Wagganer, 2005), although these proportions are variable depending upon the size of LDL particles (Davis & Wagganer, 2005). LDL contains ApoB-100 as the primary apolipoprotein with small amounts of ApoE and Apo-CIII in less-dense LDL subpopulations. The plasma concentration of ApoB-100 is directly associated with VLDL and LDL concentrations due to the presence of a single ApoB-100 molecule on each lipoprotein particle, and is directly related to the risk of coronary heart disease (Segrest *et al.,* 2001; Contois *et al.,* 2009). Moreover, the small dense LDL (sdLDL) particles have greater and rapid trans-endothelial transport and an increased susceptibility to oxidation that leads to the development and progression of atherosclerosis. This highlights the clinical significance of LDL, since the predominance of sdLDL particles has been shown to be associated with an increased risk of coronary heart disease (Austin *et al.,* 1990; Krauss, 1995; Howard *et al.,* 2000).

2.5. HDL

The hydrophobic core of HDL particles contains approximately 17% cholesterol esters and 5% TG (Burnett & Barret, 2002; Davis & Wagganer, 2005). ApoA-I is the major structural apolipoprotein constituting about 70% of HDL and is present on nearly all HDL particles. Consequently, the plasma ApoA-I concentrations correlate with plasma HDL-cholesterol (Lewis & Rader, 2005). ApoA-II is the second most abundant apolipoprotein in HDL, accounting for about 20% of total HDL protein. Additional apolipoproteins including ApoA-IV and A-V as well as the group of apolipoproteins C (ApoC-I, ApoC-II, and ApoC-III), and ApoE, are rapidly added as HDL particles grow in size. These apolipoproteins are primarily synthesized as components of VLDL and chylomicrons and transferred to HDL along with phospholipids during the lipolysis of these particles (Brown, 2007). HDL is an important lipoprotein mainly because of its atheroprotective effects, with high HDL being associated with low risks of cardiovascular diseases (CVDs) (Stamler *et al.,* 2000; Barter, 2005; Choi *et al.,* 2006), as consistently reported in several epidemiological studies (Di Angelantonio *et al.,* 2009; Khera & Rader, 2010; Brewer, 2011, Hafiane & Genest, 2013). Diverse therapeutics to raise HDL levels are in the stages of preclinical and clinical trials. However, there is a great need for the development of therapeutic strategies based on HDL functionality, rather than high plasma cholesterol concentrations, since various drugs raising HDL-cholesterol, including cholesteryl ester transfer protein (CETP) inhibitors (that inhibit the transfer of cholesterol from the HDL to

other lipoproteins) (Duffy & Rader, 2006), either exert secondary effects or do not reduce the risk of coronary heart disease events (Barkowski & Frishman, 2008; Briel *et al.*, 2009; Fazio & Linton, 2010).

2.6. Lp(a)

Lp(a) is a lipoprotein similar to LDL in lipid and protein composition but contains apolipoprotein (a). The synthesis of Apo(a) occurs in liver and it is attached to apoB-100 through disulfide bonds (Mondola & Reichl, 1982). This lipoprotein is of considerable clinical significance, as the elevated plasma Lp(a) levels are associated with an increased risk of CVDs (Nordestgaard *et al.*, 2010; Kamstrup *et al.*, 2011).

3. Transport of lipids

Three main pathways are involved in the production and transport of lipids in the body. These pathways include exogenous transport, endogenous transport, and reverse cholesterol transport.

3.1. Exogenous transport

Dietary lipids are packaged into TGs and cholesterol to form the large TG-rich, ApoB-48-containing chylomicrons which are secreted from the epithelial cells of small intestine. As these particles circulate in the blood, the TGs in chylomicrons are hydrolyzed by the action of LPL, anchored to the capillary endothelial cells via heparin sulfate-proteoglycans (HSPGs) (Lieberman *et al.*, 2006). As Apo-CII is required for the activity of LPL, chylomicrons must acquire Apo-CII by an exchange with HDL particles. Many LPL molecules interact with chylomicrons because of their large size and thus almost 50% of the TG core in chylomicrons is hydrolyzed in 10-15 min. The free fatty acids (FFA) thus produced are taken up by peripheral tissues including skeletal muscle where they are used as the main source of energy, and adipose tissue where the fatty acids are re-esterified and stored in the form of lipid droplets (Kingsbury & Bondy, 2003; Davis & Wagganer, 2005). The removal of fatty acid content from chylomicrons leads to the production of chylomicron remnants with the depletion of about 80% of the initial TGs (Fielding & Fielding, 2002). Chylomicron remnants are then removed from the circulation by receptor-mediated endocytosis which will be discussed in detail in later sections (Figure 2).

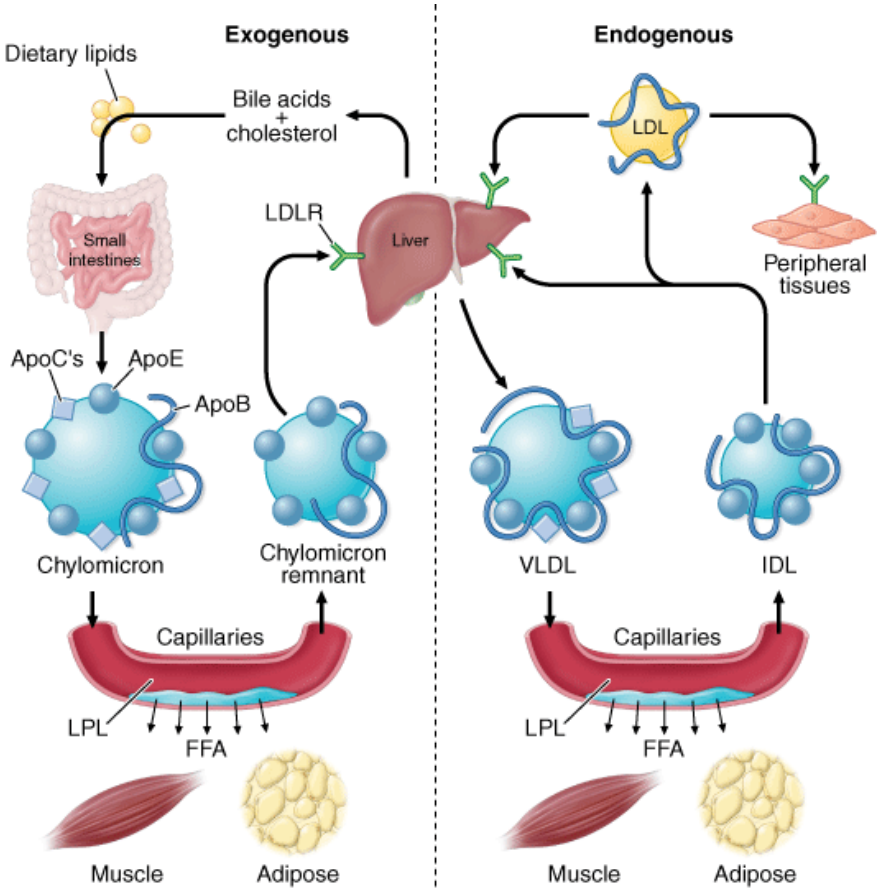


Figure 2: Exogenous and endogenous transport of lipids

In exogenous lipid transport, the intestine absorbs the dietary lipids and packages them into chylomicrons that are transported to the muscle and adipose tissues through blood. During their journey through the blood stream, chylomicrons come across lipoprotein lipase (LPL), which hydrolyzes their triglyceride (TG) content and thus leads to the production of chylomicron remnants that are subsequently taken up by the liver. In contrast, the endogenous lipid transport involves the production of VLDL by the liver and their hydrolysis by LPL to form IDL and LDL. The clearance of LDL particles from the circulation occurs through lipoprotein receptors, mainly LDL-R, present on the surface of hepatocytes. In this way, various lipoproteins and receptors continue to choreograph the lipid metabolism. (Rader & Hobbs, 2008)

3.2. Endogenous transport

Liver-derived TGs and cholesterol esters are released into the circulation in the form of the ApoB100-containing VLDL. The VLDL particles resemble chylomicrons in protein composition, but differ in that they contain apo-B100, instead of apo-B48 and have a higher ratio of cholesterol to TG (1 mg of cholesterol for every 5 mg of TG) (Rader & Hobbs, 2008). As mentioned in the previous section, VLDL particles, after LPL-mediated hydrolysis, generate other smaller and denser lipoprotein particles, called IDL. Further hydrolysis of these particles by LPL and hepatic lipase (HL) results in the removal of most of the TG content and

ApoE, thus leading to the formation of much smaller and denser lipoprotein particles, LDL. These particles are enriched in esterified cholesterol as their major lipid content and are internalized by the liver LDL receptor (LDL-R) through the recognition of ApoB-100 (Kingsbury & Bondy, 2003; Davis & Wagganer, 2005) (Figure 2).

3.3. Reverse cholesterol transport

This process involves the movement of cholesterol from peripheral tissues back to the liver for elimination via bile; HDL is the main lipoprotein carrying out this function. Nascent HDL is secreted as discoidal-shaped particles from the liver and in lesser quantities from the intestine (Rye & Barter, 2004). ApoA-I plays a pivotal role in HDL synthesis through its interaction with ATP-binding cassette protein A1 (ABCA1) on the plasma membrane of hepatocytes (Wang & Tall, 2003; Yancey *et al.*, 2003; Knight, 2004). ABCA1 transfers unesterified cholesterol and additional phospholipids from the peripheral tissues to lipid-poor ApoA-I, resulting in the formation of discoidal-shaped small pre-β-migrating HDL (pre-β-HDL) (Rye & Barter, 2004). Being unable to remove cholesterol from extra-hepatic tissues, pre-β-HDL undergoes further maturation to form spherical α-migrating HDL (α-HDL or HDL) that removes excess cholesterol from the cells with high efficiency. This maturation process requires the esterification of free cholesterol to form cholesteryl ester by the action of lecithin–cholesterol acyltransferase (LCAT), a plasma enzyme associated primarily with HDL (Jonas, 2000; Ng, 2004). In addition to LCAT, two other plasma proteins, namely phospholipid transfer protein (PLTP) and CETP are involved in the remodeling of HDL in the circulation. PLTP transfers the phospholipids from remnant ApoB-containing lipoproteins to HDL particles (Huuskonen *et al.*, 2001; Jiang *et al.*, 1999), while CETP exchanges TG from the core of remnant particles for cholesteryl ester molecules from the core of HDL (Kuivenhoven *et al.*, 1997; Barter *et al.*, 2003; Ng, 2004; Ohashi *et al.*, 2005). This facilitates the removal of cholesterol from the peripheral tissues and its subsequent delivery to the liver, thereby contributing to HDL-mediated reverse cholesterol transport. HDL also transports cholesterol directly to the liver via the scavenger receptor class BI (SR-BI) pathway, which allows the selective uptake of cholesterol, without mediating the degradation of apolipoproteins, from HDL particles (Acton *et al.*, 1996; Silver *et al.*, 2001; Brundert *et al.*, 2005). The efflux of cholesterol by nascent HDL is mediated through the action of ABCA1, while mature HDL particles also promote this efflux through ABCG1 (Zelcer & Tontonoz, 2006) (Figure 3).

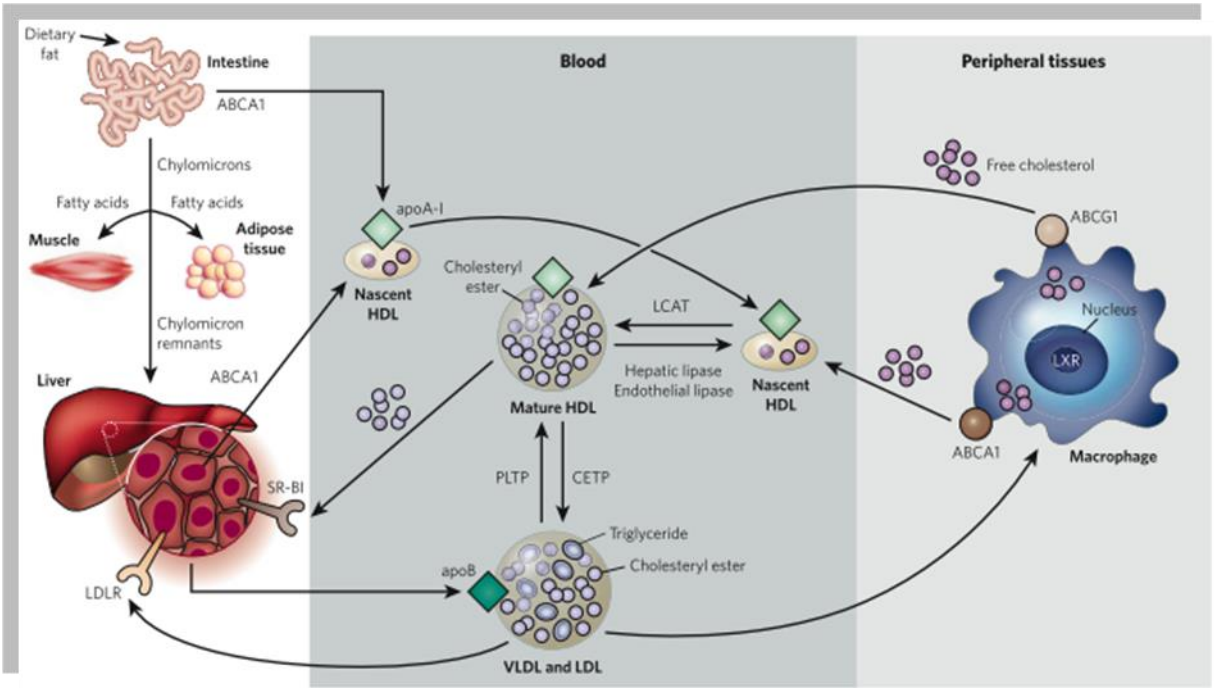


Figure 3: Schematics of the reverse cholesterol transport pathway

This pathway involves the transport of excess cholesterol from the peripheral tissues back to the liver for elimination via bile. Nascent HDL particles, secreted by intestine and liver, facilitate the efflux of cholesterol from the tissues, including macrophages, through the action of ABCA1. Mature HDL particles are produced by the esterification of their free cholesterol to cholesteryl esters by the action of LCAT enzyme and mediate the cholesterol efflux through ABCG1. The lipid content of HDL is altered by the hepatic and endothelial lipases and by the transfer proteins namely CETP and PLTP, affecting HDL catabolism. HDL cholesterol is returned to the liver directly by the selective uptake via SR-BI, and indirectly, by CETP-mediated transfer of cholesteryl esters to TG-rich lipoproteins, i.e. VLDL and chylomicrons. (Rader & Daugherty, 2008)

Elevated levels of certain plasma lipoproteins are associated with an increased risk for the development of CVDs (Lusis & Pajukanta, 2008). Therefore, the maintenance of steady-state plasma lipoprotein levels is linked to their rates of secretion into, and removal from, the circulation. This homeostasis in lipoprotein metabolism is dependent on several factors, including the presence of various lipoprotein receptors on hepatic plasma membranes that play an important role in the uptake and clearance of different lipoproteins.

4. Lipoprotein receptors

4.1. Low-density lipoprotein receptor (LDL-R)

Cellular cholesterol is obtained either from *de novo* synthesis or uptake from circulating lipoproteins. The mechanism of LDL-R-mediated endocytosis of LDL particles was

discovered by the Nobel laureates, MS Brown and JL Goldstein (Brown & Goldstein, 1986). The receptor is a transmembrane glycoprotein that is efficiently involved in the clearance of cholesterol-rich LDL particles from the plasma.

After the binding and internalization of LDL particles by LDL-R, there occurs a pH change within the early endosomes that causes a conformational change in the receptor. As a consequence, the receptor is recycled to the cell surface with the release of bound LDL particles in the endosomes and their subsequent degradation through the lysosomal pathway (Anderson *et al.,* 1977; Davis *et al.,* 1987).

The expression of *ldl-r* gene is regulated by the intracellular cholesterol levels. In the absence of cholesterol, the *ldl-r* gene is actively transcribed with the rapid uptake of LDL, while in the presence of cholesterol within the cells, the transcription of the gene is repressed and thus LDL internalization is reduced (Brown & Goldstein, 1999). The sterol regulatory element-binding protein (SREBP) plays a crucial role in the transcriptional regulation of LDL-R (Yokoyama *et al.,* 1993; Hua *et al.,* 1993). SREBPs are transcription factors synthesized as membrane-bound proteins attached to ER through an interaction with SREBP cleavage-activating protein (SCAP) and the protein encoded by the insulin-induced gene (INSIG). In cholesterol-depleted cells, SCAP undergoes a conformational change and is detached from INSIG in order to transport SREBPs from ER to Golgi complex (Hua *et al.,* 1996), where they undergo two subsequent cleavages by two Golgi-associated membrane bound proteases, namely, Site-1 protease (S1P) (Espenshade *et al.,* 1999) and Site-2 protease (S2P) (Zelenski *et al.,* 1999). This produces a transcriptionally active fragment (bHLH) that enters the nucleus and regulates the transcription of the target genes containing sterol regulatory element (SRE) in their promoter region. The target genes include LDL-R, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase) and other genes involved in cholesterol and fatty acid biosynthesis (Horton *et al.,* 2002; Goldstein *et al.,* 2006).

On the other hand, when excess cholesterol accumulates in the cells, the transport of SREBPs to the Golgi complex is blocked, resulting in the inhibition of proteolytic release of the active fragment and thus the transcription of the target genes declines (Goldstein *et al.,* 2002; Ferno *et al.,* 2011) (Figure 4).

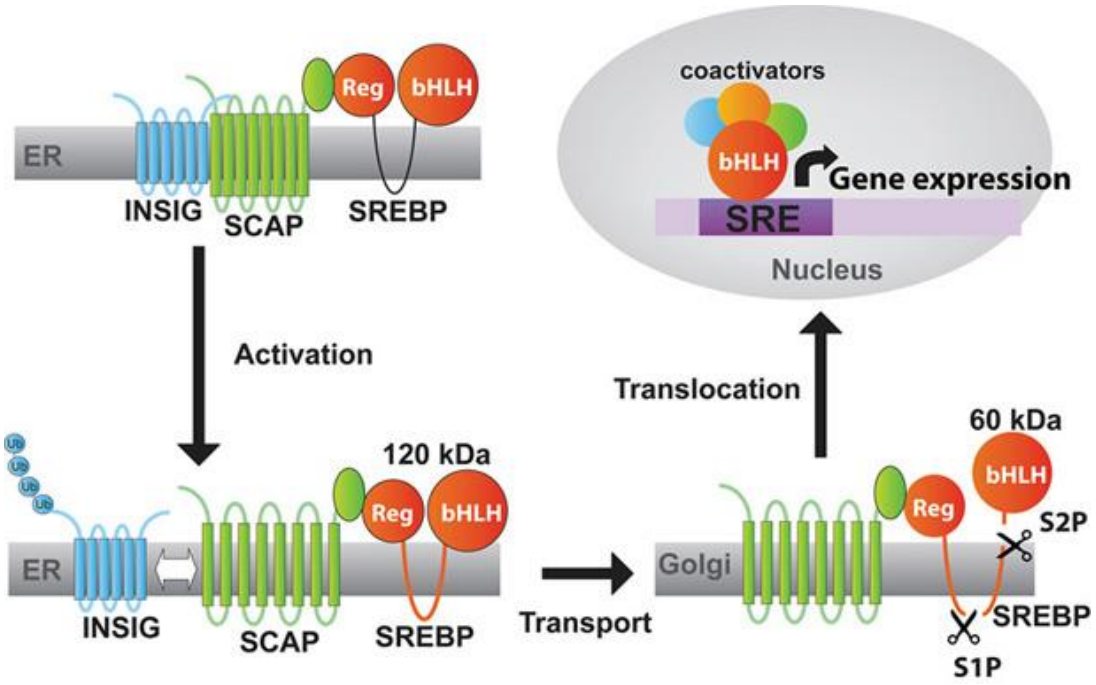


Figure 4: Proteolytic processing of SREBPs
(Ferno et al., *2011)*

LDL-R is also regulated by the transcription factors termed liver X receptors (LXRs), which are activated in response to excess cholesterol in cells (Zelcer & Tontonoz, 2006). LXRs control the expression of various genes involved in cholesterol efflux such as ABCA1 and ABCG1, thus playing an important role in reverse cholesterol transport (Repa *et al.*, 2000a; Kennedy *et al.*, 2005). Studies have shown that LXRs induce the transcription of an E3 ubiquitin ligase Idol (inducible degrader of the LDL-R), which stimulates the ubiquitination of the cytoplasmic domain of LDL-R, thus targeting it for degradation (Zelcer *et al.*, 2009; Scotti *et al.*, 2011). In this way, LXRs contribute to the feedback inhibition of LDL-R and help to maintain cholesterol homeostasis.

In humans, the mutations in LDL-R cause an autosomal dominant disorder called familial hypercholesterolemia (FH), which is characterized by the accumulation of LDL-cholesterol in the plasma (Brown & Goldstein, 1976; Rader *et al.*, 2003; van Aalst-Cohen *et al.*, 2004).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors involved in lipid and glucose metabolism, inflammation, obesity and the development of adipocyte differentiation and atherosclerosis (Fruchart, 2009). Activation of PPARα and γ by their selective ligands leads to an enhanced expression and activity of LDL-R.

PPARα-mediated increase in LDL-R expression and activity depends mainly on the activation of SREBP-2 and phosphorylation of protein kinase B (Akt) (Huang *et al.*, 2008), whereas PPARγ induces LDL-R through a mechanism associated with enhanced SREBP-2 processing (Duan *et al.*, 2012)

While LDL-R is the principal receptor for LDL, an *in vivo* study using anti-LDL-R antibody has shown that this receptor can contribute to chylomicron remnant clearance, but is not the major pathway for the removal of these TG-rich lipoproteins (de Faria *et al.*, 1996). Indeed, it has been shown that the absence of functional LDL-R has no effect on chylomicron clearance of subjects with FH in a way that the accumulation of chylomicron remnants in the circulation does not correlate with the impairment of LDL-R function. However, the chylomicron remnant clearance is greatly impaired in normolipidemic ApoE2/2 homozygotes *i.e.* the subjects showing poor recognition of the ApoE2 isoform by hepatic receptors. This indicates that the chylomicrons are largely removed from the circulation by the ApoE-recognizing receptors that are genetically distinct from LDL-R (Rubinsztein *et al.*, 1990).

4.2. LDL receptor-related protein (LRP1)

LRP1 is a multifunctional cell surface glycoprotein that was a potential candidate as receptor for chylomicron remnants because of its structural resemblance to LDL-R. The disruption of the hepatic LRP1 expression using Cre/loxP recombination system in mice did not reveal any change in the plasma lipoprotein profiles. However, the double mutant mice, lacking both functional hepatic LRP1 and LDL-R receptors show an increase in ApoB-48 concentrations. It was also observed that the liver LRP1 gene inactivation causes an upregulation of LDL-R protein levels as compared to wild type. These findings suggest that LRP1 only appears to act as a back-up receptor in absence of LDL-R (Rohlmann *et al.*, 1998). The deletion of LRP1 gene in mice results in prenatal death by impeding the embryonic development, which indicates the importance of this receptor in mediating different physiological functions due to its ability to bind various unrelated ligands (Herz *et al.*, 1992). In addition, LRP1 has been shown to be highly expressed in neurons in central nervous system (Rebeck *et al.*, 1993), where it contributes to lipoprotein metabolism, neurotransmission, synaptic plasticity and cell survival, as well as to clearance of the amyloid-β (Aβ) peptide, thus playing a critical role in the pathogenesis of AD (Herz & Bock, 2002; Lillis *et al.*, 2008; Bu, 2009). Various studies report the accumulation and aggregation of Aβ as a central event in the

pathogenesis of AD (Hardy & Selkoe, 2002; Blennow *et al.,* 2006). The sequential proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases produces two principal Aβ forms (Kang *et al.,* 1987), $A\beta_{40}$ and $A\beta_{42}$ peptides, of which the later is more toxic and has a higher tendency to form fibrils and then amyloid plaques that are considered playing a key role in the onset and/or progression of AD (Lillis *et al.,* 2008; Bu *et al.,* 2009). The finding that LRP1 is involved in the pathogenesis of AD comes from evidence that LRP1 interacts with APP. This leads to an altered APP endocytic trafficking and processing, thereby modulating the production of the Aβ (Kounnas *et al.,* 1995; Pietrzik *et al.,* 2002). Ulery *et al.,* (2000) have shown that the lack of LRP1 activity is linked to a decrease in Aβ production, whereas the presence of functional LRP1 is associated with increased production and secretion of Aβ. Moreover, LRP1 has also been shown to regulate food intake and energy homeostasis in adult CNS, mainly through the regulation of leptin-mediated signaling pathways, describing the receptor as an important therapeutic target for the treatment of obesity (Liu *et al.,* 2011).

To date, no clear data is available to describe the transcriptional regulation of LRP1. LRP1 has been shown to be down-regulated by SREBP-2, preventing an increased uptake of aggregated-LDL (agLDL), which is similar to LDL and is a potent stimulator of the accumulation of cholesteryl esters in macrophages (Khoo *et al.,* 1988; Buton *et al.,* 1999; Llorente-Cortés *et al.,* 2007). Some other studies have also demonstrated the interactions between LRP1 and PPARγ, a transcription factor involved in lipogenesis and adipose tissue differentiation (Terrand *et al.,* 2009; Tajima *et al.,* 2010). However, further experimental work is needed to investigate the nature and the mechanisms of this interaction.

4.3. Lipolysis stimulated lipoprotein receptor

In addition to other known receptors for the uptake, degradation and regulation of lipids, a receptor was identified because of its lipoprotein-binding characteristics in the presence of FFAs (Bihain & Yen, 1992). This lipoprotein receptor, referred to as the "lipolysis stimulated lipoprotein receptor" (LSR), was originally identified in fibroblasts isolated from an FH patient (Yen *et al.,* 1994). The receptor, when activated by FFAs, binds ApoE or ApoB and displays the highest affinity for TG-rich lipoproteins such as VLDL and chylomicrons (Bihain & Yen, 1992; Yen *et al.,* 1994; Yen *et al.,* 2008). The apparent number of LSR receptors on the liver plasma membranes was found to be negatively correlated with plasma

TG levels during postprandial phase in rats (Mann *et al.*, 1995), suggesting that LSR is a rate-limiting factor for the removal of chylomicron remnants.

Yen *et al.* (1994) demonstrated that LSR is genetically and biochemically distinct from LDL-R and LRP1. LSR studies were performed using FH fibroblasts that lack the ability to synthesize LDL-R. LSR affinity was reported to be higher for TG-rich lipoproteins and for lipid emulsions supplemented with recombinant ApoE than for LDL containing solely ApoB (Yen *et al.*, 1994). Also, LDL-R has been shown to be inhibited by FFAs which shows that LSR is distinct from this receptor (Bihain & Yen, 1992). That LSR is distinct from LRP1 is supported by several observations: (i) the LRP1 ligand, activated α_2-macroglobulin (α_2-MG*) does not bind to oleate-induced LDL binding site, (ii) oleate has no effects on the binding of α_2-MG* to LRP1, (iii) a 39-kDa receptor-associated protein (RAP) which inhibits LRP1 has no effects on LSR when the same concentrations of RAP were used, (iv) LSR binding of lipoproteins does not require Ca^{2+}, whereas α_2-MG* binding to LRP1 is strictly Ca^{2+}-dependent (van Dijk *et al.*, 1992), and finally, (v) ligand blotting revealed two protein bands of apparent molecular masses of 115 and 85 kDa implicated in LSR activity, which are smaller than LRP1 (600 kDa). Altogether, these observations support the existence of a chylomicron remnant receptor distinct from LDL-R and LRP1 (Yen *et al.*, 1994).

Screening of expression libraries using LSR antibodies led to the cloning of a gene, primarily expressed in the liver (Yen *et al.*, 1999). This gene was found to code for the proteins with the molecular masses similar to that of LSR α, α' and β subunits. RT-PCR and sequence analyses of the three mRNA products reveal that they are issued from the alternative splicing of a single primary transcript. Biochemical and bioinformatics analyses indicate that this receptor is a multimeric complex consisting of three subunits: α (68 kDa), α' (63.8 kDa) and β (58.3 kDa), possibly associated by disulfide bonds (Figure 5).

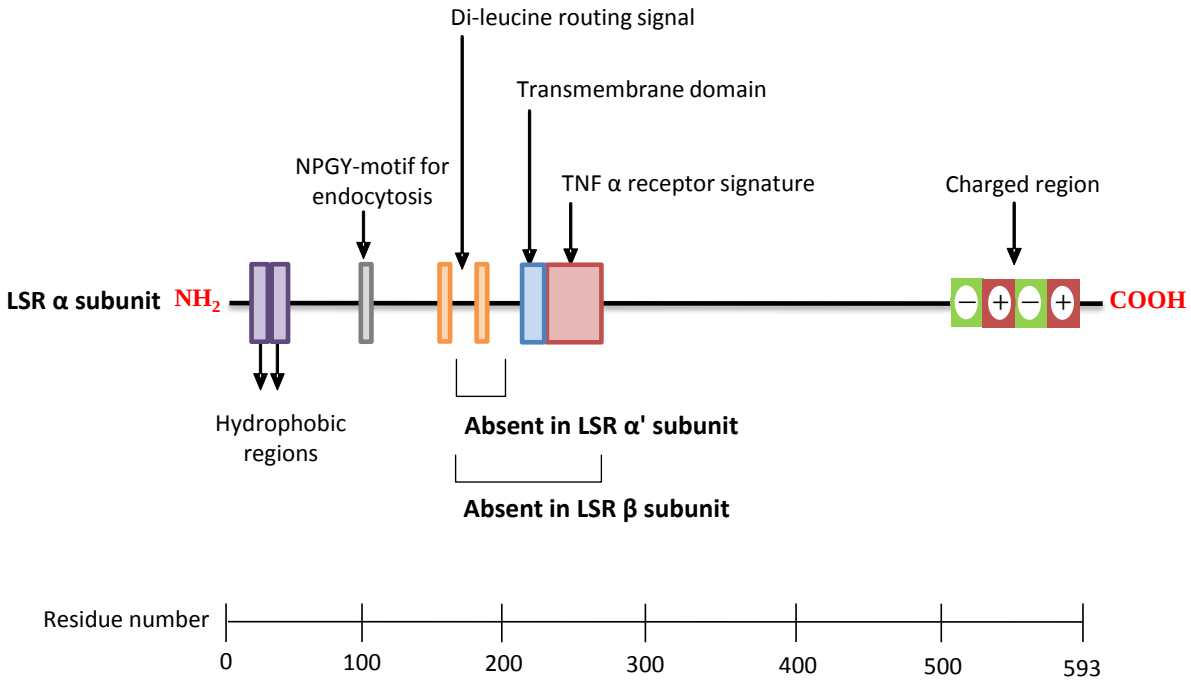


Figure 5: Schematic representation of the structure of LSR

The location of various domains in the three LSR subunits is shown. The N-terminal contains the cluster of hydrophobic residues that represents a potential site of fixation for fatty acids, and the NPGY and di-leucine routing signal correspond to the motifs involved in endocytosis. Near the transmembrane-spanning domain, there is a cysteine-rich domain representing a partial TNF-α receptor signature that provides the potential site of interaction with cytokines. The cluster of alternatively positively and negatively charged residues near the C-terminal corresponds to the potential apolipoprotein-binding site. (Yen et al., *1999)*

Northern blots revealed the presence of LSR mRNA primarily in the liver, but also in lung, intestine, kidney, ovaries and testes (Yen *et al.*, 1999). LSR mRNA and proteins were detected in substantial amounts in fetal liver at day 7 and increased at day 17 along with a liver marker called prothrombin. Irrespective of other lipoprotein receptors including (i) LDL-R, present in rat fetal liver from day 19 at 19% of the adult level, (ii) LRP1, low expression from 19 days of gestation at 6% of the adult level (Smith *et al.*, 1995) and (iii) SR-BI, not detectable in embryonic liver until day 17 (Hatzopoulos *et al.*, 1998), the early expression of LSR in fetal liver may be consistent with the critical role of this receptor in lipoprotein uptake during embryogenesis. Recent data using immunofluorescence have revealed the localization of LSR protein in specific regions in mouse brain including mouse hippocampus, cerebellum Purkinje cells, the ependymal cell interface between brain parenchyma and cerebrospinal fluid and the choroid plexus, where it plays an important role in cholesterol homeostasis (Stenger *et al.*, 2012). Furthermore, the genetic inactivation of LSR gene in mice led to embryonic lethality in homozygote embryos ($LSR^{-/-}$) between the days 12.5 and 14.5 of gestation, a period

associated with the development of specific hepatocyte functions in fetus (Morris *et al.*, 1989; Spijkers *et al.*, 2001). Also, the fetal livers were found to be much smaller than their littermates at the day 14.5, suggesting a vital role of LSR in embryonic development (Mesli *et al.*, 2004).

Studies with $LSR^{+/-}$ mice having reduced LSR expression (Yen *et al.*, 2008) revealed that these animals exhibited increased postprandial lipemia, and a 2-fold reduction in lipid clearance. Furthermore, both plasma TG and cholesterol levels were elevated when $LSR^{+/-}$ mice were placed under dietary lipid stress in the form of a high-fat/high-cholesterol diet. Plasma TG during the postprandial phase was even higher in $LSR^{+/-}$ mice lacking functional LDL-R as compared to $LSR^{+/-}$ with active LDL-R, indicating a potential functional cooperativity between the two receptors (Figure 6).

Narvekar *et al.* (2009) observed both hypertriglyceridemia and hypercholesterolemia in mice after specific hepatic LSR-knockdown using short-hairpin RNA technology. This liver-specific suppression of LSR resulted in increased levels of plasma TG, ApoE and ApoB serum levels, thus confirming the role of LSR in the removal of TG-rich lipoproteins containing ApoB and/or ApoE.

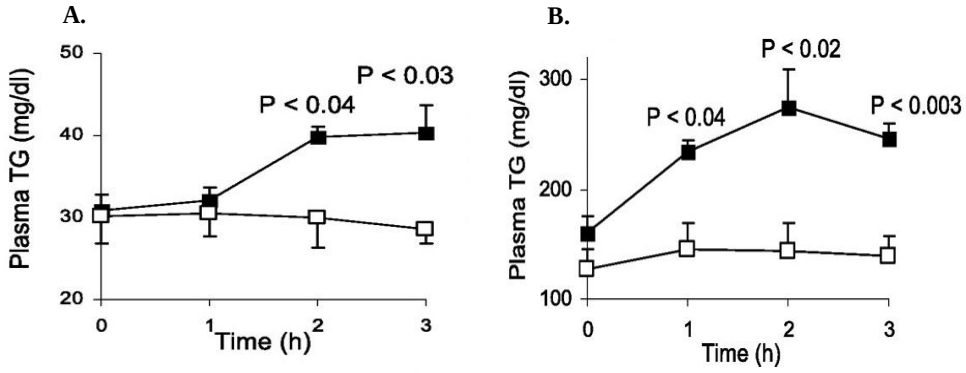


Figure 6: Determination of postprandial lipemia

(A) In overnight-fasted $LSR^{+/+}$ (□, n = 5) and $LSR^{+/-}$ (■, n = 5) and (B) LDL-$R^{-/-}$ $LSR^{+/+}$ (□, n = 6) and LDL-$R^{-/-}$ $LSR^{+/-}$ (■, n = 4) mice after gavage with olive oil. (Yen et al., *2008)*

Lactoferrin is a milk protein known to increase the postprandial TG levels in rats by inhibiting chylomicron clearance (Huettinger *et al.*, 1988). Studies have reported that lactoferrin inhibits the activity of LSR in FH fibroblasts (Yen *et al.*, 1994) and in liver membranes (Mann *et al.*, 1995). Furthermore, lactoferrin interacts with LSR only when the receptor is present in its FFA-activated form (Mann *et al.*, 1995). Moreover, ApoC-III was also shown to

inhibit LSR-mediated binding and internalization of ^{125}I-VLDL (Mann *et al.,* 1997) in primary cultures of rat hepatocytes. Owing to the inhibitory effects of ApoC-III on LPL activity, the overproduction of ApoC-III delays the clearance of TG-rich lipoproteins and is strongly associated with hypertriglyceridemia (Chan *et al.,* 2002a & b; Ooi *et al.,* 2008). Moreover, the RAP, which copurifies with LRP1 and inhibits the binding of all LRP1 ligands to their receptor (Herz *et al.,* 1991; Williams *et al.,* 1992), was also reported to inhibit LSR activity (Troussard *et al.,* 1995). Overexpression of RAP using adenovirus vector in wild-type and LDL-R$^{-/-}$ mice was associated with an accumulation of plasma cholesterol and TG, as well as apoB-48 and apoE particles (Willnow *et al.,* 1994). At similar concentrations, RAP was shown to inhibit LSR-mediated binding, uptake and degradation of ^{125}I-LDL (Troussard *et al.,* 1995), indicating that the changes in the lipid profiles in mice with RAP overexpression were not only due to the inhibition of LRP1. Moreover, other studies have also shown that LDL-R activity could also be affected at these RAP levels (Medh *et al.,* 1995; Mokuno *et al.,* 1994), which renders difficult to finally conclude about the involvement of these lipoprotein receptors in RAP-mediated changes in plasma lipid levels.

These studies therefore demonstrate that functional hepatic LSR receptor is critical in maintaining normal lipid status and therefore can act as a potential molecular link among various lipid-related disorders like hyperlipidemia, obesity and atherosclerosis (Yen *et al.,* 2008). Various factors (*e.g*., dietary or hormonal) might influence the activity of this receptor and its impaired function could lead to significant perturbations in both cholesterol and TG metabolism.

5. Leptin

Leptin is a 16-kDa polypeptide product of *ob* gene, primarily produced by adipocytes and consequently, its circulating levels are strongly correlated with adipose tissue mass (Maffei *et al.,* 1995; Considine *et al.,* 1996). Leptin serves to regulate food intake and energy expenditure as a satiety factor by providing signals to the specific regions of hypothalamus and thus playing a vital role in reducing body weight and regulating cognitive functions (Campfield *et al.,* 1995; Halaas *et al.,* 1995; Stephens *et al.,* 1995; Harvey, 2007). In the central nervous system (CNS), leptin was reported to regulate striatal regions and human eating behavior (Farooqi *et al*., 2007). In the hippocampus and cerebral cortex, this adipokine plays important roles in modulating neural development and endocrine functions. It has also

been found to exhibit neuroprotective properties both *in vivo* and *in vitro* (Maffei *et al.*, 1995; Ahima *et al.*, 1999; Tang, 2008).

5.1. Signal transduction pathways mediated by leptin

Leptin receptors (Ob-R) belong to the class-I cytokine receptor family and six isoforms (a to f) have been identified in mice (Wang *et al.*, 1996) (Figure 7).

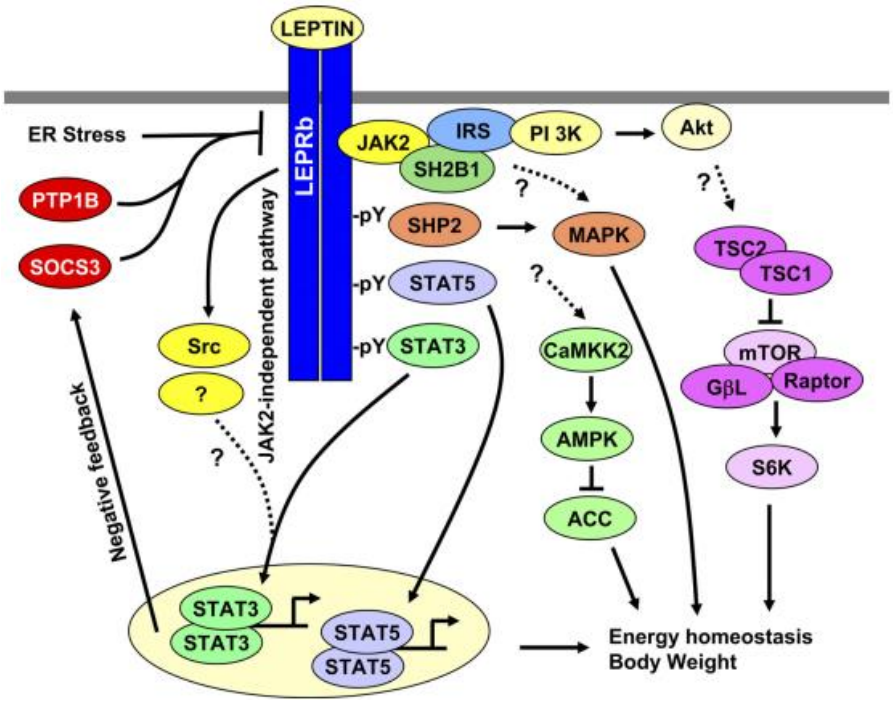


Figure 7: Leptin-mediated signaling pathways

Leptin binding to the long form of the receptor (Ob-Rb) leads to the activation of Ob-Rb-associated JAK2, which in turn phosphorylates Ob-Rb. This results in the activation of MAPK pathway through SH2-containing protein tyrosine phosphatase 2 (SHP2) that binds Ob-Rb on phosphoTyr 985. Upon activation by JAK2 through phosphorylation, STAT5 and STAT3 molecules dimerize and translocate to the nucleus to activate the transcription of their target genes including the suppressor of cytokine signaling-3 (SOCS3), which inhibits leptin signaling in a negative feedback manner. JAK2-mediated tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 results in the activation of the phosphoinositide 3-kinase (PI3K) pathway. Leptin also mediates the AMPK/acetyl-CoA carboxylase (ACC) and mTOR/ribosomal S6 kinase (S6K) pathways; however, the molecular mechanisms are not clearly understood (Morris & Rui, 2009).

Ob-R*b* is the long isoform that is widely expressed throughout the body (Tartaglia *et al.*, 1995). The genetic deficiency of functional leptin receptors results in obesity and related disorders including diabetes and CVDs (Zhang *et al.*, 1994; Clément *et al.*, 1998). Binding of leptin to the long form of receptor Ob-R*b* activates a cascade of signaling pathways that

mainly occurs through Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). Other mechanisms include the mitogen-activated protein kinase (MAPK), 5'-adenosine monophosphate-activated protein kinase (AMPK), phosphoinositide 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) (Minokoshi *et al.,* 2002; Huang *et al.,* 2004; Robertson *et al.,* 2008; Morris & Rui, 2009).

5.2. Role of leptin in peripheral tissues

In addition to the brain, leptin regulates the body fat storage by directly affecting various metabolic pathways in multiple peripheral tissues, including adipose tissue, pancreatic islets, skeletal muscle, and liver (Morris & Rui, 2009). Leptin stimulates FA oxidation in adipose tissue, mainly through the inhibition of lipogenesis by decreasing the expression of fatty acid synthase (FAS) and the activation of lipolysis by upregulating PPARα and enzymes involved in FA oxidation, namely carnitine palmitoyl transferase-I, (CPT-I), and acyl-CoA oxidase 1 (ACOX1) (Wang *et al.,* 1999). Also, leptin inhibits lipogenesis through the downregulation of the transcription factor SREBP-1 which is mainly involved in fatty acid synthesis (Soukas *et al.,* 2000). Furthermore, the overexpression of Ob-R*b* in white adipose tissue prevents diet-induced obesity (DIO), the condition reflecting elevated lipid levels, in mice mainly through the activation of STAT-3 and AMPK and increased mRNA expression of lipolytic enzymes including PPARγ-coactivator-1α, and uncoupling protein-1 and -2 (Wang *et al.,* 2005).

The induction of hyperleptinemia in normal rats by adenovirus gene transfer reduced TG content in pancreas, skeletal muscle and liver without increasing FFAs or ketones in plasma, thus promoting the intracellular FA oxidation (Shimabukuro *et al.,* 1997). Furthermore, the liver-specific overexpression of Ob-R*b* prevents hepatic steatosis in Ob-R*b* deficient Zucker diabetic fatty (fa/fa) rats (Huang *et al.,* 2004) by stimulating the lipolysis and by inhibiting lipogenesis (Lee *et al.,* 2001). However, the central leptin signaling remains dominant over the peripheral signaling, as various studies have demonstrated that liver-specific deletion of Ob-R shows no effects on energy balance and body weight, whereas the neuron-specific deletion of Ob-R induces obesity (Cohen *et al.,* 2001; Guo *et al.,* 2007). Therefore, it can be deduced that under normal conditions, leptin exerts its effects on food intake and energy expenditure by activating Ob-R*b* in the brain.

5.3. Role of leptin in LSR regulation

Leptin also functions as one of the important regulators of LSR in both *in vivo* (C57BL/6JRj mouse) and *in vitro* (Hepa1-6) experimental models. In leptin-deficient obese (*ob/ob*) and leptin-resistant diabetic (*db/db*) mice, the hepatic LSR expression was decreased. Furthermore, normalization of the lipid profiles of *ob/ob* mice was observed after restoring hepatic LSR expression in these mice following leptin replacement therapy, suggesting that leptin is intimately involved with the maintenance of hepatic LSR expression (Narvekar *et al.*, 2009). Furthermore, a recent study by Stenger et al. (2010) showed that leptin is involved in the regulation of LSR at transcriptional and translational levels in both mouse and Hepa 1-6 cells.

From these studies, we can conclude that leptin is an important regulator of energy storage and consumption and body weight by both suppressing the appetite and increasing energy expenditure. Therefore, any perturbations in leptin signaling or its transport to the brain, as in the case of leptin resistance, lead to the development of obesity and various related disorders such as CVDs and diabetes.

II. DHA and lipid metabolism

1. Polyunsaturated fatty acids

Long chain polyunsaturated fatty acids (LC-PUFAs) from the *n*-3 and *n*-6 families are derived from their respective precursors, namely, α-linolenic (ALA; C18:3) and linoleic (LA; C18:2) acids, which are considered essential fatty acids, indicating that their biosynthesis does not occur in mammals and therefore can only be obtained from the diet.

Dietary sources of *n*-3 fatty acids include fish (*e.g.*, salmon, mackerel, sardines, pollock, bluefish, and black cod), walnuts, flax seeds, linseeds and green leafy vegetables such as purslane and spinach, while *n*-6 fatty acids are mainly present in vegetable oils such as soybean, safflower, sunflower and corn oil (Benatti *et al*., 2004; Mozaffarian & Wu, 2011) (Figure 8).

Figure 8: Dietary sources of* n-*3 and* n-*6 fatty acids

1.1. Long chain *n*-3 fatty acids and their functions

Long chain *n*-3 fatty acids including eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) are mainly obtained by consuming fish or can also be produced in liver from the *n*-3 fatty acid precursor ALA.

Containing 22 carbon atoms and 6 double bonds, DHA is the longest PUFA in the structure of biological membranes. DHA is the predominant *n*-3 PUFA in the brain where its turnover is the slowest, in particular when the intake of preformed DHA or *n*-3 precursor is low. DHA constitutes about 30–40% of the phospholipids of cerebral cortex and

photoreceptor cells in the retina (Innis, 1991; Lauritzen *et al.*, 2001; SanGiovanni & Chew, 2005). Thus DHA accounts for ~17% by weight of the total fatty acids in the brain of adult rats and ~33% of the total fatty acids in the retina (Hamano *et al.*, 1996; Horrocks & Farooqui, 2004). Significant amounts of DHA are incorporated into the neuronal phospholipids, leading to increased membrane fluidity and improved synaptic transmission and signaling. DHA plays an important role in the development and maturation of brain and cognitive functions as well as those of the visual system (Salem *et al.*, 2001; Cao *et al.*, 2009). Indeed, a high intake of DHA is associated with a low risk of cognitive decline, brain aging and neurodegenerative diseases such as AD (Morris *et al*., 2003; Lim *et al*., 2005; Oster & Pillot, 2010; Bazan *et al*., 2011).

1.1.1. DHA and biological membranes

The beneficial effects of DHA are mainly attributed to its ability to be incorporated into the membrane phospholipids. DHA is mostly found in the *sn*-2 position in phospholipids with the *sn*-1 position mainly containing the saturated fatty acids, palmitic (16:0) or stearic (18:0) acid (Anderson & Sperling, 1971). The presence of DHA in the membrane leads to increased membrane thickness, fluidity, permeability, vesicle formation and fusion, lateral diffusion and lipid redistribution (Stillwell *et al*., 2005; Wassall & Stillwell, 2008; Shaikh & Teague, 2012). This ultimately influences the microenvironment of transmembrane proteins including receptors and their subsequent interactions with their ligands. It also seems to favor the organization of the membrane-signaling platforms referred to as ‘rafts’, thus affecting membrane structural and functional properties (Ma *et al.*, 2004). Lipid rafts are the sphingolipids- and cholesterol-enriched, highly specialized microdomains that compartmentalize various cellular processes (Simons & Ikonen, 1997; Brown & London, 2000; Pike, 2006). However, it is not clear whether DHA is localized inside the lipid rafts (Williams *et al.*, 2012) or outside of these structures due to its lower affinity with cholesterol (Wassall & Stillwell, 2008 & 2009) (Figure 9).

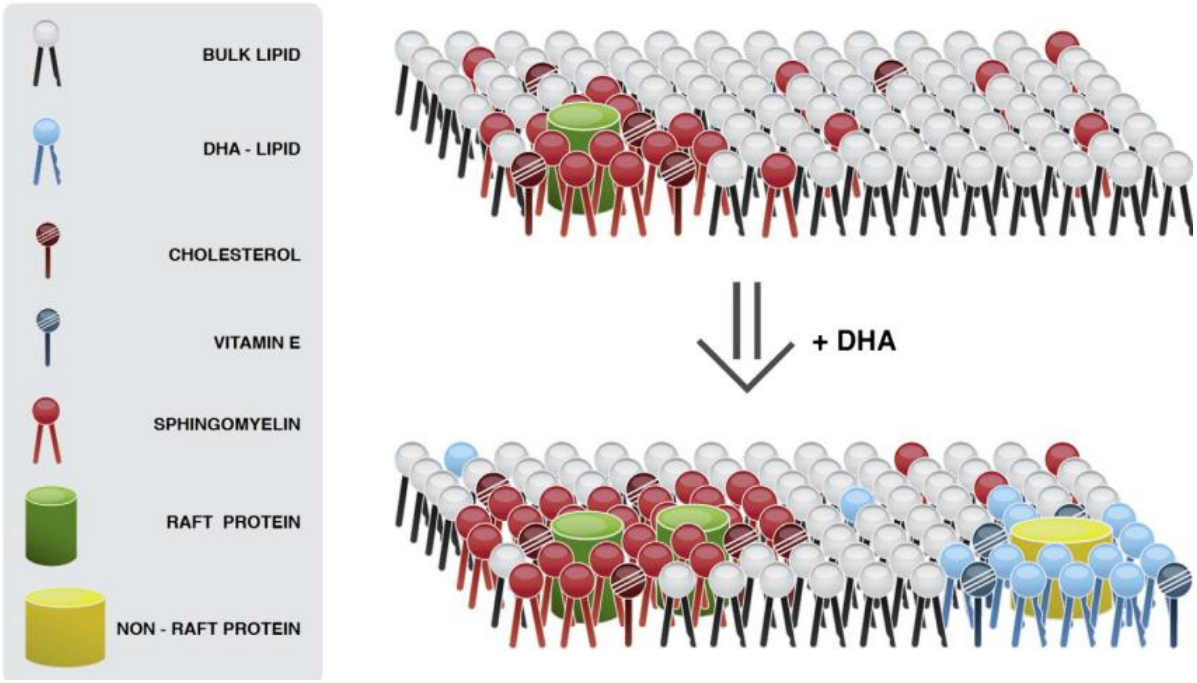


Figure 9: A proposed model for DHA incorporation into the membrane phospholipids and subsequent changes in the membrane architecture

DHA enrichment in the plasma membrane leads to the separation of highly disordered DHA-rich non-raft domain (extreme right) from the sphingolipids- and cholesterol-containing raft domain (extreme left). This disrupts the spatial distribution of various membrane-associated proteins and provides a favorable environment for their functioning (Wassall & Stillwell, 2009).

In vitro studies suggest that the lipid rafts are highly sensitive to the *n*-3 PUFA-mediated changes in the membrane lipid composition. In particular, it has been shown that DHA can change the size, distribution and composition of these (Ma *et al.*, 2004; Chapkin *et al.*, 2008). PUFA enrichment in COS-1 and Jurkat T cell lines alters the composition of rafts, which consequently leads to the suppression of signal transduction by displacement of Src family tyrosine kinases from lipid rafts (Stulnig *et al.*, 1998; Zeyda *et al.*, 2002). Likewise, the *in vivo* studies in mice have also shown that the supplementation with DHA and/or EPA leads to the alterations in the mouse T-cell raft composition and subsequent suppression of signal-transducing molecules, such as protein kinase C theta, thus affecting the downstream signaling pathways (Fan *et al.*, 2003 & 2004). This indicates the importance of DHA in influencing the plasma membrane architecture.

2. *N*-3 PUFA biosynthesis in liver

Liver acts as the principal site for the biosynthesis of DHA which involves a cascade of chain elongation and desaturation reactions (Figure 10). However, in humans, the efficiency of these processes is low (less than 0.5%) or about 5–7 mg/day based on an ALA intake of 1,000–1,500 mg/day, and is influenced by various pathological conditions (Singer *et al*., 1984 & 1986; Bourre & Piciotti 1992; Burke *et al*., 2001. Brenner, 2003; Wang *et al*., 2006; Araya *et al*., 2010). Recent data suggest that human populations may have different capacities to synthesize LC-PUFAs from plant-based medium chain PUFAs, based on the variations in the fatty acid desaturase (FADS) gene cluster in diverse populations around the world (Mathias *et al*., 2012). Thus a continuous supply of *n*-3 PUFAs in the diet is very important in order to preserve whole-body DHA content.

In liver, dietary ALA is desaturated to 18:4*n*-3 at position 6 by Δ6-fatty acid desaturase (encoded by the FADS2 gene), chain-elongated to 20:4*n*-3 by elongation of very long chain fatty acids (ELOVL) 5, with subsequent conversion into EPA by Δ5-desaturase (encoded by FADS1 gene). EPA then undergoes two successive elongation steps (ELOVL5 & 2 respectively) and a further desaturation by Δ6-desaturase to produce tetracosahexaenoic acid (24:6*n*-3) in ER. In microalgae, DHA synthesis involves an elongation step, catalysed by an elongating enzyme complex, leading to the conversion of EPA into docosapentaenoic acid (DPA; C22:5*n*-3). Subsequent desaturation of DPA through the action of Δ4-desaturase results in the conversion of DPA into DHA (Pereira *et al*., 2004). However in mammals, DHA synthesis from 24:6*n*-3 requires the removal of two carbon atoms by β-oxidation which occurs in peroxisomes through the sequential action of acyl-coenzyme A oxidases, D-bifunctional protein (DBP) and peroxisomal thiolases (Moore *et al*., 1995; Su *et al*., 2001). This metabolic pathway is referred to as 'Sprecher's shunt' (Voss *et al*., 1991). After synthesis, DHA is transported back to ER (Sprecher & Chen, 1999; Sprecher, 2000) to be incorporated into membrane phospholipids by esterification or the deacylation-reacylation reaction. The need for these translocations between ER and peroxisomes is believed to explain in part the low efficiency of DHA biosynthesis in mammals. The presence of DHA in the cell membranes has an important impact on the lipid composition, which intimately influences the physicochemical properties of the local lipid bilayer, and as a consequence, membrane fluidity, and the functionality of the membrane-associated proteins including receptors and ion channels.

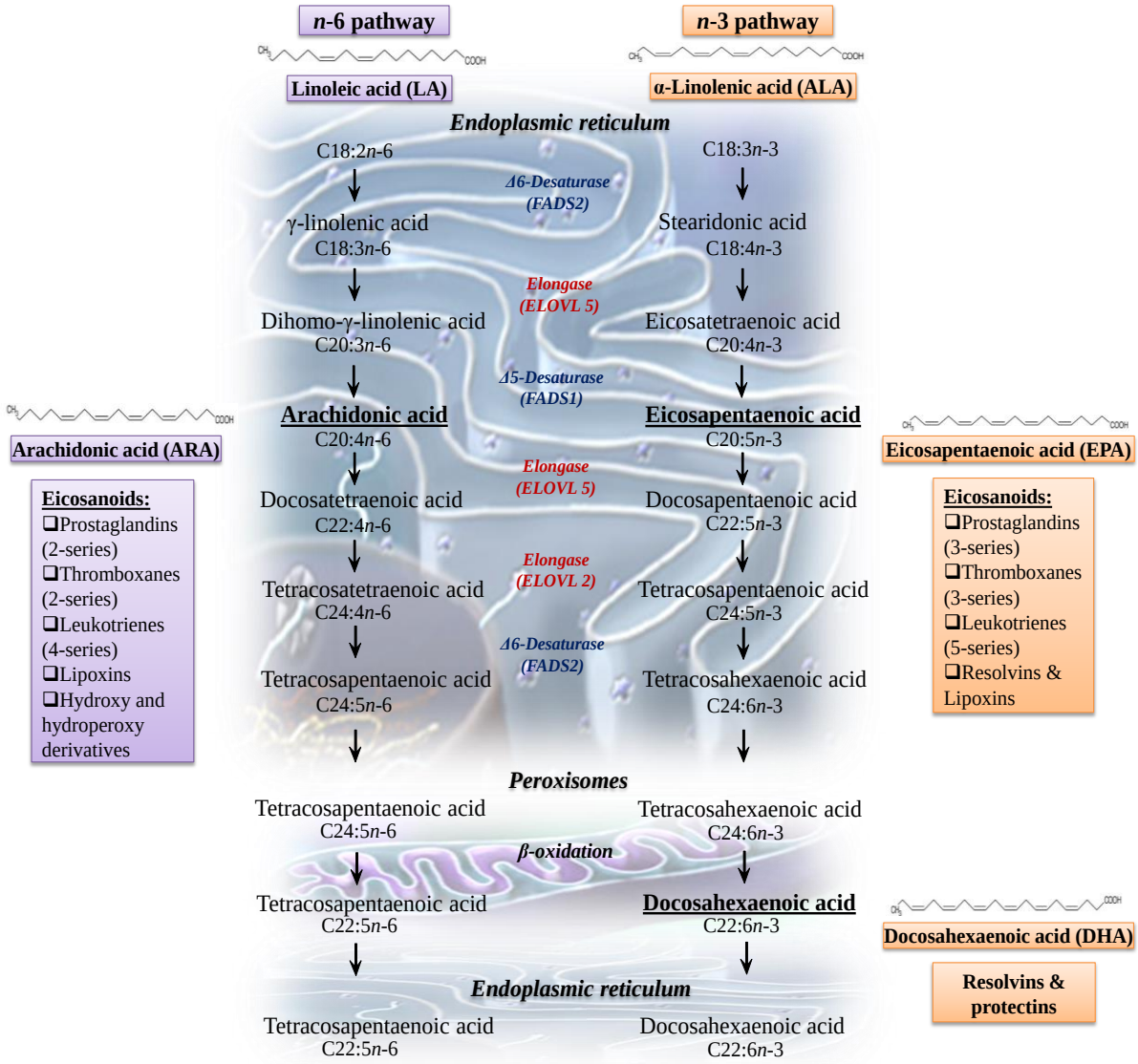


Figure 10: Biosynthesis of long-chain n-3 and n-6 fatty acids in mammals

The pathway of n-*3 and* n-*6 fatty acid biosynthesis involves a series of desaturation and elongation reactions in specific cellular compartments to produce different PUFAs. Starting from linoleic acid (LA; C18:2,* n-*6), a number of enzymatic reactions lead to the production of arachidonic acid (ARA; C20:4,* n-*6) whereas eicosapentaenoic acid (EPA; C20:5,* n-*3) and docosahexaenoic acid (DHA; C22:6,* n-*3) are derived from α-linolenic acid (ALA; C18:3,* n-*3).* n-*3 (EPA & DHA) and* n-*6 PUFAs (ARA) are further metabolized to produce signaling molecules involved in respective anti-inflammatory or pro-inflammatory cascades.*

After being synthesized in liver, DHA is secreted into the circulating plasma in the form of lipoproteins or bound to albumin and is delivered to the brain for incorporation into glycerophospholipids (Scott & Bazan, 1989; Polozova & Salem, 2007). It is important to note that low plasma DHA levels are associated with cognitive or behavioral impairments during early development or aging (Conquer *et al*., 2000). Therefore, dietary *n*-3 PUFA consumption and DHA synthesis in the liver is essential for maintaining DHA levels in the brain and thus

preserving the functions of this tissue (Rapoport *et al*., 2007). Studies have shown that among neural cells, only astrocytes are capable of producing and releasing LC-PUFAs into the extracellular fluid for their subsequent uptake by neurons (Moore *et al*., 1991; Kim, 2007).

2.1. Metabolism of long-chain *n*-6 fatty acids

The metabolic pathway for the synthesis of arachidonic acid (ARA; C20:4) from LA also involves the successive desaturation and elongation steps catalyzed by the same Δ6- (Stoffel *et al*., 2008) and Δ5-desaturases and elongases involved in *n*-3 fatty acids biosynthesis. The desaturation reaction mediated by Δ6-desaturase is a rate-limiting step in the conversion of LA to ARA. In the course of inflammatory activation, unesterified ARA can be metabolized into biologically active lipids termed as 'eicosanoids' by the action of cyclooxygenases (COX) and lipoxygenases (LOX). These include 'series 2' prostaglandins (PGs), thromboxanes (TXs) (Bergstrom *et al*., 1964; Hamberg & Samuelsson, 1974), leukotrienes (LTB4), 5-hydroxy-eicosatetraenoic acids (HETEs) and lipoxins (Samuelsson, 1981; Serhan *et al*, 1984; Pattersen *et al*., 2012), which are important mediators of inflammation, atherogenesis and prothrombosis.

In contrast, *n*-3 fatty acids can antagonize the pro-inflammatory effects of ARA through the synthesis of eicosanoids with distinct characteristics than those derived from ARA. EPA serves as a precursor for 'series 3' prostanoids and LTB5, whereas DHA is transformed into 'docosanoids' such as resolvins, docosatrienes (DT) and neuroprotectins (neuroprotectin D1), which possess anti-inflammatory, immunoregulatory, anti-oxidative and neuroprotective properties (Serhan *et al*., 2000; Serhan *et al*., 2002; Mukherjee *et al*., 2004; Arita *et al*., 2005).

Owing to these contrasting properties of *n*-3 and *n*-6 fatty acids, any modifications of lipid composition may represent an important parameter in the development of various pathophysiological conditions and disturbances related to lipid metabolism (Schmitz & Ecker, 2008).

3. *N*-3 Fatty acids and dyslipidemia

Dyslipidemia refers to the disorder of lipoprotein metabolism, mainly characterized by elevated concentrations of plasma TG (hypertriglyceridemia) and non-high density lipoprotein (HDL) cholesterol (hypercholesterolemia), low levels of HDL cholesterol (Czyżewska *et al*., 2010; Dessì *et al.,* 2013) and the predominance of sdLDL particles that are slowly metabolized and more susceptible to oxidation, thus serving as important determinants of atherogenic lipoprotein profiles (Klop *et al*., 2013). Dyslipidemia serves as one of the major risk factors for a number of pathological conditions including CVDs, diabetes, obesity and AD (Davis & Wagganer, 2005; Karim *et al*., 2013). Most notably, *n*-3 PUFAs, such as DHA and EPA, have been shown to play an important role in improving the aberrant lipid profiles of the body and lowering both fasting and post prandial plasma TG levels (Harris *et al.,* 1988; Lombardo & Chicco, 2006; Kelley *et al.,* 2007), thus reducing the risk factors for dyslipidemia.

Different studies based on animal models and clinical trials have been reported to explore the beneficial effects of *n*-3 PUFAs, EPA and DHA.

3.1. Animal studies

Numerous studies using animal models have studied the effect of *n*-3 PUFAs either in the form of fish oils that contain a mixture of both DHA and EPA or in the form of the individual PUFA. In rats, fish oil administration (containing 38% DHA and 46% EPA) by oral gavage for 30 days led to the selective incorporation of *n*-3 PUFAs in rat liver phospholipids. It also inhibited the biosynthesis of non-essential monounsaturated fatty acids (MUFAs) by decreasing the activities of stearoyl-CoA desaturase 16 (SCD16) and stearoyl-CoA desaturase 18 (SCD18) with consequent decreases in Δ6- and Δ5-desaturase activities, resulting in the inhibition of hepatic lipogenesis (Lamaziere *et al*., 2013).

In hamsters fed a high-cholesterol diet enriched with 0.4% DHA, a decrease of 29-33% in plasma total cholesterol (TC) and 29–50% in non-HDL-cholesterol was observed, compared to the control group fed with 0.4% stearic acid (Chen *et al*., 2012). Consistent with the decrease in TC and non-HDL cholesterol, there was a decline in the gene expression of hepatic transcription factor SREBP-2 and HMG-CoA reductase, both of which are important regulators in cholesterol metabolism (Horton *et al.*, 2002; Zeng *et al.*, 2004; DeBose-Boyd, 2008).

In mice, fish oil significantly lowered hepatic TG (30-50%) and ApoB (42%) synthesis, and increased LPL activity along with the rapid clearance of TG-rich lipoproteins, suggesting that these effects contribute to the TG-lowering mechanisms of *n*-3 PUFAs (Qi *et al.*, 2008). Supplementation of leptin-deficient obese *ob/ob* mice with fish oil (24.8% DHA & 1.8% EPA) for 7 days led to a decline in SREBP-1 expression, consistent with the downregulation of the enzymes involved in lipogenesis such as SCD1 and FAS. Furthermore, PPARα expression was upregulated, which indicates an increased β-oxidation (Sekiya *et al*., 2003). These results led the authors to suggest that *n*-3 PUFAs attenuate hepatic steatosis in *ob/ob* mice mainly by switching from lipid synthesis and storage to degradation.

3.2. Clinical studies

In humans, increased LDL cholesterol and TG together with low HDL cholesterol in the plasma represent risk factors reported to be strongly associated with coronary heart disease (CHD) (Sharrett *et al*., 2001; Sarwar *et al*., 2007; Liu *et al*., 2010). Since several epidemiologic studies have demonstrated the potential role of *n*-3 PUFAs in the regulation of lipid metabolism in human subjects (Zuliani *et al*., 2009), a number of investigations were undertaken to ascertain the potential beneficial effect of these dietary lipids on improving the plasma lipid and lipoprotein profile. The most consistent effect of *n*-3 PUFAs in improving plasma lipoprotein profiles is found to be a reduction in plasma TG levels (Jacobson *et al.*, 2012; Pirillo & Catapano, 2013). Fish oil supplementation (2 g EPA & 1 g DHA) in young healthy men significantly decreases TG levels and increases HDL cholesterol (Zulyniak *et al*., 2013). However, the effectiveness of *n*-3 PUFA-mediated TG-lowering could be dose-responsive as shown by Skulas-Ray *et al*. (2011) in their study using healthy subjects with moderate hypertriglyceridemia (150-500 mg/dL). Using two different doses of 0.85 g or 3.4 g EPA+DHA per day for 8 weeks, they observed that the higher dose was reduced TG by 27% while no such effects were reported for lower dose.

In a study by Grimsgaard *et al*., (1997) on healthy middle-aged men (36-56 years old), a higher TG-lowering effect was found with DHA (26%) supplementation as compared to that of EPA (21%). Moreover, a significant reduction in plasma TG levels was reported upon fish oil supplementation in subjects with combined hyperlipidemia (28% in type IIb and 41% in type IV) (Simons *et al*., 1985) and that with hypertriglyceridemia (64% in type IIb and 79% in type V) (Phillipson *et al*., 1985). In another study using hypertriglyceridemic male subjects,

DHA supplementation (3 g DHA/day) for 45 days decreases fasting TG and VLDL concentrations by 24% and 92% respectively, when compared to control group. Furthermore, the diet enriched in DHA also led to an increase in both th number (120%) and the mean diameter of large LDL particles in fasting plasma, with a 21% decrease in sdLDL particles in postprandial phase (Kelley *et al*., 2007).

Studies in healthy, 74-year (±5 years) old subjects, including both men and women, indicated the association of plasma phospholipid levels of individual fatty acids (EPA, DHA and DPA) and total *n*-3 PUFAs with lower mortality, especially in deaths from CVDs. However, the fluctuations in measurements of fatty acid levels led to the underestimation of their relationship with mortality (Mozaffarian *et al*., 2013).

Furthermore, a meta-analysis of 36 trials involving more than 500 subjects showed that supplementation with fish oil (most commonly MaxEPA) containing ~4 g/day of *n*-3 fatty acids for 7-10 weeks resulted in a 25% decrease in TG levels of normolipidemic and a 25-34% decrease in hypertriglyceridemic individuals. Total serum cholesterol concentrations remained unchanged whereas LDL-cholesterol concentrations increased in both normal (4.5%) and hypertriglyceridemic group (10.8%) (Harris 1997).

It has been demonstrated in healthy subjects that supplementation with fish oil containing 1.6 g/day EPA and DHA for three and seven weeks leads to increased selective incorporation of LC-PUFAs into plasma TG and phospholipids (Ottestad *et al*., 2012). Whether this dietary fatty acid intake-dependent alteration in plasma lipid composition has an impact on *n*-3 PUFA-mediated health benefits remains to be demonstrated.

Interestingly, a microarray analysis revealed the disparity in the gene expression profiles in normo- and dyslipidemic men upon fish oil supplementation (1.14 g DHA and 1.56 g EPA) for a period of four hours, one week and twelve weeks. A higher number of genes were regulated in dyslipidemic subjects as compared to normo-lipidemic. Also, several genes and pathways involved in the immune system, inflammation, lipid metabolism and CVDs were downregulated in dyslipidemic subjects (Schmidt *et al*., 2012).

These findings suggest that EPA and DHA appear to have beneficial TG-lowering effects. However, their effects on HDL- and LDL-cholesterol are highly variable depending on the gender and health status of the individuals studied (*e.g.*, normolipidemic *vs.*

hyperlipidemic), their genetic profile, the dose of fish oils used, the ratio of EPA and DHA, the form and the source of *n*-3 PUFAs (fish or algal oil) (Anil, 2007; Zuliani *et al*., 2009; Bernstein *et al.,* 2012). The exact mechanism for reductions in TG levels by EPA and DHA are not completely understood. However, pre-clinical clinical and experimental data provide compelling evidence for the mechanisms involving a decrease in the secretion of VLDL particles and an enhanced TG clearance from the liver, an increase in the degradation of hepatic ApoB (Davidson, 2006; Bays *et al*., 2008), inhibition of ApoC-III and upregulation of endothelial lipase activity in plasma and adipose tissue of human subjects with normal or atherogenic lipoprotein profile (Harris *et al*., 1997; Park & Harris, 2003; Khan *et al*., 2002). The regulation of gene expression by *n*-3 PUFAs also plays an important role in this regard (Clarke, 2001; Sampath & Ntambi, 2005) and is mainly mediated by various nuclear receptors.

4. TG-lowering mechanisms in liver

PUFAs act as hypolipidemic agents through controlling the balance between lipid synthesis and degradation by inhibiting hepatic lipogenesis and stimulating fatty acid oxidation in liver and skeletal muscle (Sun *et al*., 2011). In addition to exerting its effects on the localization and expression of genes through modifications in membrane composition and/or related signaling cascades, DHA also modulates the expression of various transcription factors involved in hepatic fatty acid regulation (Figure 11) such as peroxisome proliferator-activated receptors (PPARs), sterol regulatory element binding protein-1c (SREBP-1c), liver X receptor (LXR), carbohydrate regulatory element-binding protein (ChREBP)/MAX-like factor X (MLX), farnesoid X receptor (FXR), hepatocyte nuclear factor-4 alpha (Hnf-4α) and nuclear factor-kappa B (NF-κB) (Pégorier *et al*., 2004; Georgiadi & Kersten, 2012; Jump, 2013).

4.1. PPARα

PPARα, the master regulator of hepatic lipid metabolism, was the first transcription factor to be discovered to bind FAs (Göttlicher *et al*., 1992). PUFA-mediated activation of PPARα induces the expression of enzymes involved in FA oxidation (Figure 11) such as CPT-I (Brandt *et al*., 1998) and ACOX1 (Berthou *et al*., 1995). Therefore, the role of PUFAs both in the upregulation of the expression of various enzymes involved in β-oxidation in liver and skeletal tissues as well as decrease in the expression of lipogenic, glycolytic and

cholesterogenic enzymes (Jump *et al*., 1994; Xu *et al*., 1999) contributes to their lipid-lowering properties. The studies with PPARα knockout mice fed with fish oil, showed impairment in the regulation of hepatic peroxisomal and microsomal genes, indicating the implication of PPARα in the regulation PUFA-mediated gene expression. However, the decrease in plasma lipid levels (TGs, VLDL- and HDL-cholesterol) was found to be similar in PPARα-deficient mice as compared to control mice, fed on the diets containing increasing amounts of fish oil (Dallongeville *et al*., 2001), indicating that the *n*-3 PUFA effects also involved other PPARα-independent mechanisms.

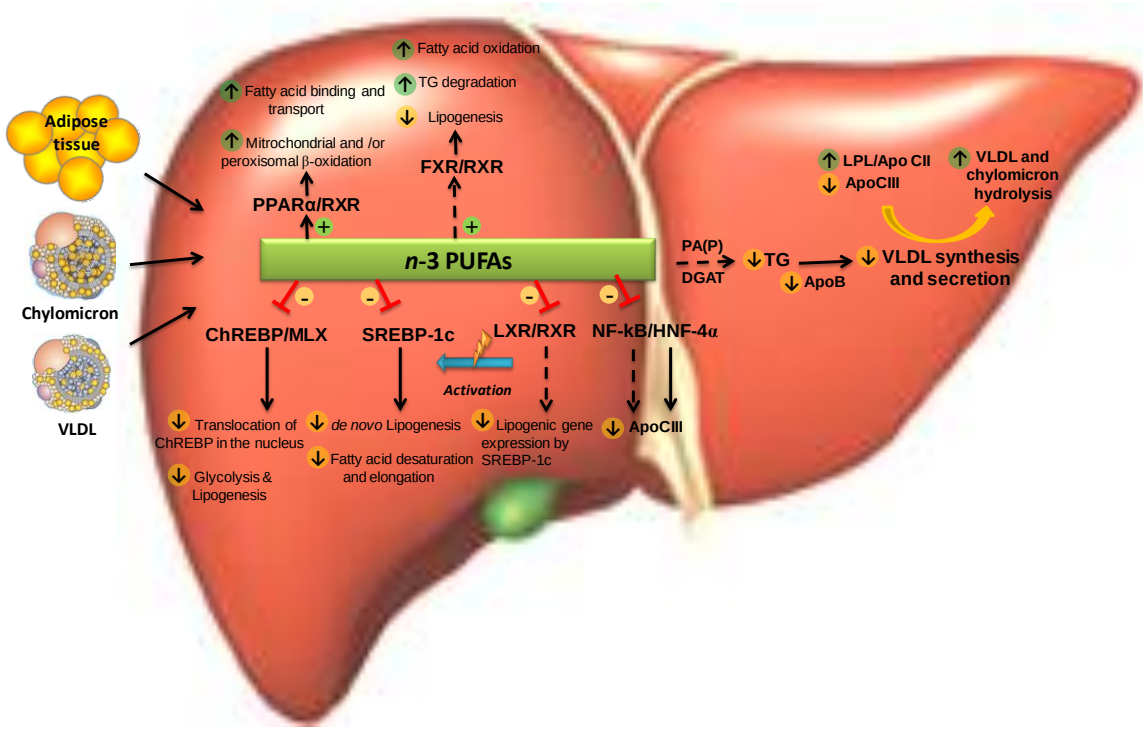


Figure 11: Potential mechanisms to regulate hepatic lipid homeostasis by EPA and DHA.

Excess fatty acid delivery from pathogenic adipose tissue, increased postprandial chylomicrons and VLDL might imbalance the hepatic lipid metabolism. Long chain n-*3 PUFAs modulate the lipid content through regulating various transcription factors that ultimately control the enzymes and proteins related to lipogenesis and lipolytic pathways, thus conserving the normal lipid metabolism in liver. This figure is not meant to localize the different processes controlled by the transcription factors and enzymes in liver. The purpose is to illustrate a global picture for the PUFA-mediated control of hepatic lipid metabolism. VLDL: very low density lipoprotein; PPARα: peroxisome proliferator-activated receptor alpha; RXR: retinoid X receptor; FXR: farnesoid X receptor; ChREBP/MLX: carbohydrate regulatory element-binding protein/MAX-like factor X; SREBP-1c: sterol regulatory element-binding ptotein-1c; LXR: liver X receptor; HNF-4α: hepatocyte nuclear factor-4 alpha; NF-κB: nuclear factor-kappa B; PAP: phosphatidic acid phosphatase; DGAT: diacylglycerol acyltransferase; TG: triglyceride; LPL: lipoprotein lipase.*

Indeed, the suppression of hepatic lipogenic (acetyl-CoA carboxylase; ACC, fatty acid synthase; FAS and S14) and glycolytic (L-pyruvate kinase; L-PK) gene expression by PUFA is controlled by PPARα-independent mechanisms (Ren *et al*., 1997; Mater *et al*., 1999; Pan *et al*., 2000). Moreover, it was reported that PUFAs inhibit the expression of Δ5- and Δ6-desaturases while PPARα agonists induce their transcription (Cho *et al*., 1999; Matsuzaka *et al*., 2002). Therefore, these findings suggest that transcription factors other than PPARα are involved in the regulatory mechanisms of PUFA-mediated expression of genes associated with lipid metabolism.

4.2. SREBPs

Several studies over the past decade demonstrate that DHA-mediated inhibition of the lipogenic pathway involves the SREBPs, the ER membrane-bound proteins from the basic-helix-loop-helix-leucine zipper family of transcription factors. Upon sterol depletion in the cells, SREBPs (SREBP-1a, SREBP-1c, & SREBP-2) are escorted from ER to Golgi by the SREBP-cleavage activating protein (SCAP). Once in the Golgi, the precursor 'pSREBP' (~125 kDa) undergoes proteolytic processing by site-1 and site-2 proteases to release the mature nuclear form 'nSREBP' (~65 kDa), which enters the nucleus and binds to the sterol-regulatory elements (SREs) in the promoter regions of the target genes. SREBP-1 (predominantly SREBP-1c) plays a pivotal role in the regulation of the expression of various genes involved in fatty acid and TG synthesis, whereas SREBP-2 controls the expression of genes related to cholesterol synthesis and uptake (Bennett *et al*., 1995; Shimomura *et al*., 1998; Horton *et al.,* 1998 & 2003).

Studies have shown that fish oil supplementation in mice selectively decreased nSREBP-1 levels mainly through inhibition of SREBP-1c gene transcription, enhanced mRNA SREBP-1 degradation and inhibition of SREBP-1 proteolytic processing. Of the *n*-3 PUFAs, DHA acts as a strong suppressor of nSREBP-1 by accelerating its degradation through the mechanisms dependent on the 26S proteasome and extracellular signal-regulated kinase (ERK) phosphorylation (Botolin *et al*., 2006). This results in decrease in the expression of genes regulated by SREBP-1, such as the low density lipoprotein receptor (LDL-R), 3-hydroxy-3-methylglutaryl (3-HMG)-CoA reductase, 3-HMG-CoA synthase, FAS, ACC, and SCD1 (Kim *et al*., 1999; Xu *et al*., 1999 & 2001; Yahagi *et al*., 1999; Teran-Garcia *et al.,* 2007).

4.3. LXRs

Besides direct regulation of SREBP-1c, *n*-3 PUFAs have also been reported to suppress SREBP-1c transcription via a mechanism that involves another nuclear hormone receptor referred to as LXR. Once activated by a variety of sterols, including oxysterols, LXR forms a heterodimer with RXR to control the expression of enzymes involved in cholesterol and bile acid synthesis and transport (Janowski *et al*, 1996; Lehmann *et al*., 1997; Repa *et al*., 2002). *In vitro* PUFA-enrichment promoted an inhibition of this LXR/RXR complex to bind with the LXR response elements (LXREs) located in the promoter region of SREBP-1c. Since LXR/RXR complex functions to activate SREPB-1c (Repa *et al.*, 2000b; Yoshikawa *et al*., 2001), this results in the downregulation of SREBP-1c expression by PUFAs, with concomitant inhibition of lipogenic pathway (Ou *et al*., 2001, Yoshikawa *et al*., 2002). Additionally, *n*-3 PUFAs also improve the hepatic steatosis mediated by LXR selective agonist (T0901317) mainly by decreasing the expression of SREBP-1c and FAS mRNA (Jung *et al*., 2011). Nevertheless, these findings remain controversial, as some other *in vivo* studies have suggested that PUFA-induced inhibition of SREBP-1c occurs through an LXR-independent mechanism (Pawar *et al.*, 2003; Takeuchi *et al.*, 2010). Takeuchi et al. (2010) reported that PUFAs decrease SREBP-1 expression primarily at the level of proteolytic cleavage, followed by reduced binding of SREBP-1 to SRE located on the promoter region of SREBP-1c, resulting in the suppression of SREBP-1c transcription, without the involvement of LXR. However, the exact mechanism for this autoregulation of SREBP-1c remains to be clarified. Therefore, further investigation is required in order to elucidate this PUFA-mediated inhibition of SREBP-1c. LXR has also been shown to regulate another transcription factor termed ChREBP. Indeed, two LXREs have been found in the promoter of the ChREBP gene (Cha & Repa, 2007).

4.4. ChREBP

The ChREBP has been identified as an important transcription factor that induces the expression of genes involved in glycolytic and lipogenic pathways (Hasegawa *et al.*, 1999). Under high glucose and insulin concentrations, the ChREBP gene transcription is stimulated along with its translocation from cytosol to the nucleus, where in the form of a heteromeric complex with MLX (Stoeckman *et al.*, 2004), it binds to the carbohydrate response element (ChoRE) on the target gene promoters. These gene include those involved in hepatic glucose transport (Glut2), glycolysis (L-PK) and lipogenesis (FAS, ACC and Spot 14) (Dentin *et al.*,

2004). PUFAs suppress the nuclear abundance of the ChREBP and MLX in the heterodimer, resulting in the downregulation of L-PK and FAS (Dentin *et al.,* 2005).

4.5. FXR

In addition, SREBP-1c and LXRs crosstalk with a bile acid receptor, FXR, a ligand-activated transcription factor which is also involved in liver cholesterol and FA homeostasis, bile acid synthesis and more importantly in TG metabolism. FXR inhibits TG synthesis by downregulating the expression of LXRs and their related target genes, SREBP-1c and FAS (Yang *et al*., 2010) in patients with non-alcoholic fatty liver disease, while it promotes the degradation of TG and FA oxidation by increasing the expression of LPL and PPARα (Pineda-Torra *et al*., 2003). FXR also contributes to plasma TG metabolism by inducing VLDL-R (Sirvent *et al.,* 2004a) and ApoC-II (Kast *et al*., 2001) expression (activator of LPL) and suppressing the expression of hepatic ApoC-III (inhibitor of LPL), FAS and HL (Claudel *et al*., 2003; Sirvent *et al.,* 2004b; Shen *et al*., 2011). FXR can bind to PUFAs including DHA, ARA and ALA, leading towards the activation of FXR target genes. This may contribute to the beneficial effects of PUFAs in lipid metabolism and needs further investigation to explore the potential mechanisms (Zhao *at al.,* 2004).

PUFAs also modulate HNF-4α, through the binding of acyl-CoA thioesters to the ligand binding domain of this transcription factor (Sladek *et al.,* 1990; Hertz *et al.,* 1998). HNF-4α controls a wide array of genes related to the synthesis (ApoA-II and -IV) and removal (ApoC-II, -III) of plasma lipoproteins. LC *n*-3 PUFAs inhibit the transcriptional activation of this nuclear receptor and thus down-regulate HNF-4α-responsive genes *e.g.,* ApoC-III promoter (Hertz *et al.,* 2001).

NF-κB is a transcription factor that controls the inflammatory pathway by regulating the expression of various genes including COX-2 and cytokines, *e.g.,* tumor necrosis factor (TNF-α) (Ben-Neriah & Karin, 2011). PUFAs inhibit ApoC-III by down-regulating NF-κB (Adkins & Kelly, 2010).

Studies have shown that PUFAs, more importantly, DHA binds directly with RXR (de Urquiza *et al.,* 2000; Lengqvist *et al.,* 2004). Since RXR forms obligate heterodimers with PPARα, it becomes challenging to determine whether DHA-mediated modulation in gene expression involves RXR or PPARα and therefore needs further investigation.

In addition to the nuclear receptors, EPA and DHA have also been shown to suppress the expression of key enzymes involved in TG synthesis, *i.e.* phosphatidic acid phosphatase (PAP) that converts phosphatidic acid to diacylglycerol (DAG) and diacylglycerol acyltransferase (DGAT) that catalyses the final step in TG synthesis (Bays *et al.*, 2008). However, these findings remain controversial and unclear (Harris & Bulchandani, 2006).

III. Role of PPARα in lipid metabolism

Peroxisome proliferator-activated receptors (PPARs) are members of the steroid hormone receptor superfamily of ligand-activated transcription factors. They mainly function as sensors and regulators of various metabolic processes including lipid, lipoprotein and glucose homeostasis, adipocyte differentiation and inflammation (Feige *et al.,* 2006; Ferré, 2004; Stienstra *et al.,* 2007; Yessoufou & Wahli, 2010; Mandard & Patsouris, 2013). PPARs exist in three subtypes, *i.e.* PPARα or NR1C1 (Nuclear Receptor subfamily 1, group C, member 1), PPARβ/δ or NR1C2 and PPARγ or NR1C3. PPARα was the first subtype to be identified in mice (Issemann & Green, 1990). The subtype PPARβ/δ is named so because it differs considerably from the frog Xenopus to mammals (PPARβ in Xenopus and PPARδ in mammals (Dreyer *et al.,* 1992; Takada *et al.,* 2000). The *PPARγ* gene generates three different transcripts: PPARγ1, PPARγ2 and PPARγ3, which are transcribed from three different promoters. The PPARγ1 and PPARγ3 transcripts translate into the identical PPARγ1 protein; however, PPARγ1 and PPARγ2 differ because of the presence of thirty additional amino acids at the *N*-terminal segment of PPARγ2 (Dreyer *et al.,* 1992; Zhu *et al.,* 1995; Fajas *et al.,* 1997 & 1998).

Encoded by three different genes, PPAR subtypes exhibit a unique tissue distribution which may account for their distinct functions.

1. Tissue distribution of PPARs

Studies in rats have shown that PPARα is mainly expressed in the tissues exhibiting high rates for mitochondrial and peroxisomal FA catabolism such as liver, heart, kidney, muscles, brown adipose tissues and intestine (Issemann & Green, 1990; Kliewer *et al.,* 1994; Braissant *et al.,* 1996; Lemberger *et al.,* 1996; Bookout *et al.,* 2006). In humans, PPARα is also expressed in heart, kidney, skeletal muscle and large intestine (Auboeuf *et al.,* 1997, Mukherjee *et al.,* 1997). However, the level of hepatic PPARα expression was reported to be significantly lower in humans as compared with mice (Palmer *et al.,* 1998). PPARα is also present in cells of the arterial wall, in monocytes/macrophages, smooth muscle cells, and endothelial cells, where it exerts anti-inflammatory and anti-thrombotic actions (Chinetti *et al.,* 1998; Staels *et al.,* 1998a; Inoue *et al.,* 1998; van Raalte *et al.,* 2004).

PPARγ is predominantly expressed in white and brown adipose tissues and macrophages (Braissant *et al.,* 1996; Bookout *et al.,* 2006). PPARγ expression has also been detected in colon intestinal mucosa and immune cells (Mansén *et al.,* 1996; Fajas *et al.,* 1997; Saez *et al.,* 1998) and at lower levels in the heart, liver and skeletal muscle (Auboeuf *et al.,* 1997; Vidal-Puig *et al.,* 1997). Of the three subtypes, PPARγ1 has a broader expression pattern that includes gut, brain, vascular cells, and specific kinds of immune and inflammatory cells (Tontonoz *et al.,* 1994a; Zhu *et al.,* 1995; Vidal-Puig *et al.,* 1997), whereas PPARγ2 is found at high levels in the different adipose tissues (Dreyer *et al.,* 1992; Chawla *et al.,* 1994; Tontonoz *et al.,* 1994a & b; Elbrecht *et al.,* 1996). Finally, the PPARγ3 expression has been shown to be restricted to adipose tissue and large intestine (Fajas *et al.,* 1998).

PPARβ/δ is ubiquitously and more abundantly expressed than PPARα and PPARγ, with its expression levels in certain tissues being dependent on the extent of cell proliferation and differentiation (Kliewer *et al.,* 1994; Braissant *et al.,* 1996; Auboeuf *et al.,* 1997; Michalik *et al.,* 2006; Wagner & Wagner, 2010). High levels of PPARβ/δ were found in the digestive tract, kidney, heart, esophagus, diaphragm and large intestine (Auboeuf *et al.,* 1997; Escher *et al.,* 2001). Various studies have indicated the important functions associated with this subtype such as enhanced FA catabolism and cholesterol efflux, improved insulin sensitivity, wound healing, anti-inflammatory actions, in different tissues including skin, placenta, skeletal muscle, adipose tissue, and brain (Braissant *et al.,* 1996; Bastie *et al.,* 1999; Peters *et al.,* 2000; Michalik *et al.,* 2001; Barak *et al.,* 2002; Peters & Gonzalez, 2009).

The diversity and specificity in their tissue distribution allows PPARs to control a multitude of biological processes, *e.g.,* PPARα and PPARβ/δ isoforms actively participate in the regulation of lipid and glucose metabolism, while PPARγ functions to regulate the expression of genes involved in adipocyte differentiation, lipid storage and regulation of inflammatory pathways. In this way, PPARs participate in maintaining the balance between energy storage and expenditure (Wang, 2010; Monsalve *et al.,* 2013).

2. Structure of PPARs

PPARs share the characteristic structural organization of most nuclear receptors (*e.g.,* steroid or thyroid hormone receptors) and contain six functional domains (Figure 12).

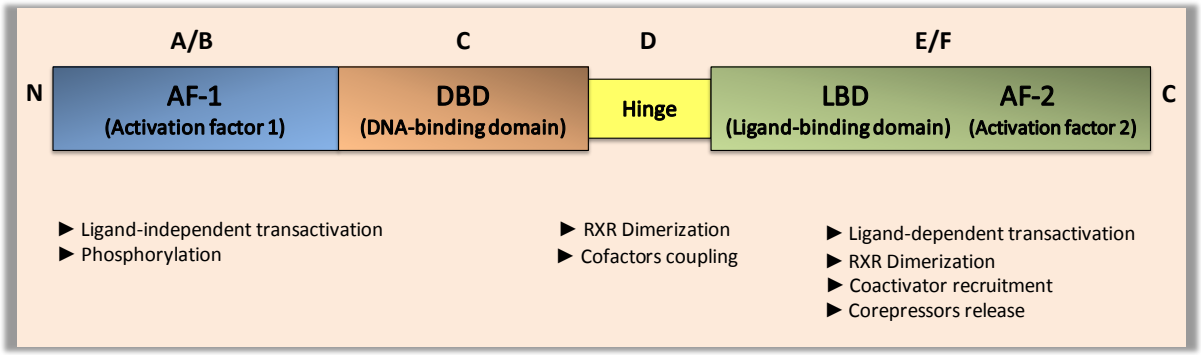


Figure 12: Schematic representation of the functional domains of PPARs
(modified from Boitier et al., *2003)*

- The *N*-terminal (A/B domain) region varies in length and sequence and consists of a ligand-independent transactivation domain, called AF-1 (activation function 1) which is responsible for the phosphorylation of PPARs (Juge-Aubry *et al.*, 1997; Werman *et al.*, 1997).
- A DNA binding domain (DBD, C domain) is comprised of two zinc fingers and is highly conserved (~80%) in the three PPAR subtypes. The DBD directs PPARs to the PPRE located in the promoter region of various target genes (Kliewer *et al.*, 1992; Desvergne & Wahli, 1999; Yoon, 2009).
- The hinge region (D domain) is highly flexible and facilitates the protein to bend or alter the conformation. It connects C and E/F domains and is involved in the protein-protein interactions, such as receptor dimerization and efficient binding of DBD to PPRE. It also acts as a site for cofactors coupling (Green & Chambon, 1988; Yoon, 2009; Monsalve *et al.*, 2013).
- The *C*-terminal ligand binding domain (LBD, E/F domain) exhibits a lower degree of similarity (~65%) among the three PPAR subtypes, which is consistent with their differential activation with various endogenous and exogenous ligands. This may ultimately account for the specific biological activity of these subtypes. This domain is involved in the receptor dimerization as well as in the ligand-dependent transcriptional regulation by AF-2 (activation function 2). AF-2 regulates ligand-dependent transactivation, recruitment of coactivators, and release of corepressors. In addition, AF-2 is also important for heterodimerization of PPARs with 9-*cis*-retinoid X receptor α (RXRα) (Dowell *et al.*, 1997; Giguère, 1999; Michalik & Wahli, 1999; Mouthiers *et al.*, 2005).

3. Mechanism of transcriptional regulation by PPARs

PPARs belong to the nuclear receptor type II family, suggesting that they predominantly reside in the nucleus, though PPARα and γ have been shown to shuttle between the nucleus and cytoplasm in a ligand-dependent manner. However, the ligands were not found to be involved in anchoring PPARs in the nucleus, which may indicate the implication of RXR in this regard (Akiyama *et al.,* 2002; Umemoto & Fujiki 2012). PPARs remain inactive in an unbound state and heterodimerize with RXR upon activation (Kliewer *et al.,* 1992; Feige *et al.,* 2005; Chan & Wells, 2009). In the absence of ligand or during antagonist treatment, the PPAR/RXR heterodimer is associated with the corepressors including the nuclear receptor corepressors (NCoR), silencing mediator for retinoid and thyroid hormone receptor (SMRT), or G-protein pathway suppressor 2 (GPS2) which are the part of multisubunit corepressor complex containing histone deacetylase (HDAC) activity. Consequently, the chromatin is maintained in condensed state due to the deacetylation of histones and the PPAR-mediated gene transcription is repressed (Chen & Evans, 1995; Hörlein *et al.,* 1995; Krogsdam *et al.,* 2002; Lazar, 2003; Fournier *et al.,* 2007; Monsalve *et al.,* 2013) (Figure 13).

On the other hand, ligand binding to either of the receptor leads to the activation of PPAR/RXR complex, although the simultaneous ligand binding to both the receptors makes the complex much more efficient for the target gene activation. The presence of ligand leads to the conformational changes in PPARs which results in the dissociation of corepressors and the subsequent recruitment of co-activators, such as steroid receptor coactivator1 (SRC1), PPAR coactivator (PGC-1), the histone acetyltransferase p300, CREB binding protein (CBP) and Ara70 (Desvergne & Wahli, 1999; Qi *et al.,* 2000; Robyr *et al.,* 2000; Boitier *et al.,* 2003; Viswakarma *et al.,* 2010). The activation of PPAR/RXR complex leads to the histone modification and altered expression of various genes by binding to the direct repeats (DR) of hexameric sequences, *5'-AGGTCANAGGTCA-3'*, separated by a single (DR1) or two (DR2) nucleotides (Zhu *et al.,* 1996 & 1997) (Figure 13). These sequences are called peroxisome proliferator responsive elements (PPREs) and are located in one or multiple copies in the promoter region of the PPAR-responsive genes. PPAR and RXR bind PPRE at the 5' and 3' half-sites respectively, and the selectivity of binding, between different PPAR isotypes, is conferred by the 5'-flanking region (DiRenzo *et al.,* 1997; IJpenberg *et al.,* 1997; Juge-Aubry *et al.,* 1997).

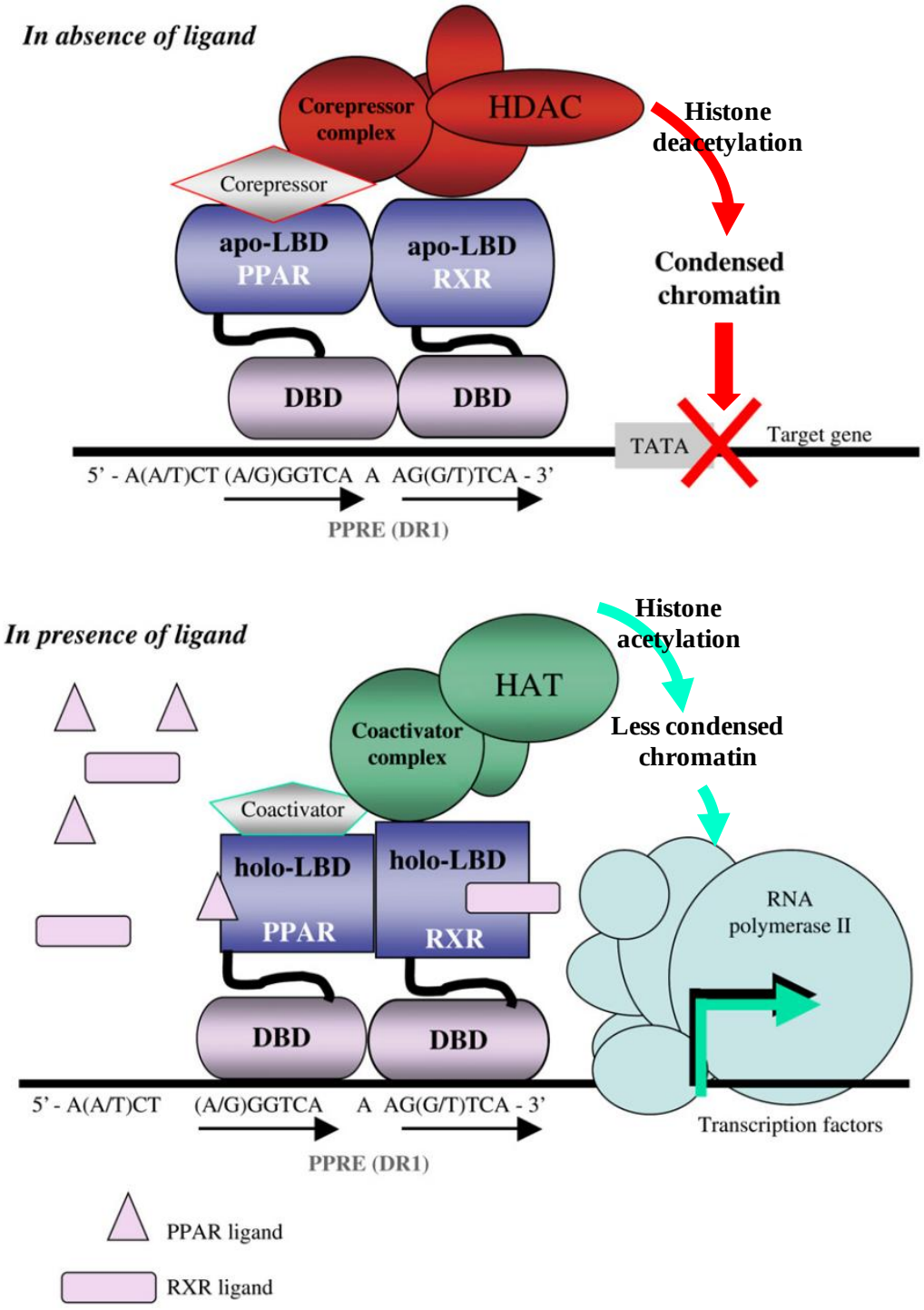


Figure 13: Schematic representation of PPAR transcriptional activation.

The transcriptional regulation by PPARs is achieved by the formation of PPAR/RXR heterodimers that bind to a PPRE located in the promoter of target genes through their DBD. In the absence of ligand (apo-LBD), PPARs associate with the multisubunit corepressor complex containing histone deacetylase (HDAC) activity, which results in the deacetylation of histones and the subsequent repression of gene transcription. However, in the presence of ligand for PPAR or RXR, the ligand-bound LBD (holo-LBD) releases the corepressors and recruits the coactivator complex containing histone acetyltransferase (HAT) activity that modifies the nucleosome structure and allows the gene transcription through the recognition of PPRE by PPAR-RXR heterodimer (Adapted from Fournier et al., *2007).*

4. PPAR ligands

PPARs were named so because they were initially shown to be activated by peroxisome proliferators. However, various specific activators and ligands for the different PPAR subtypes are now emerging. Indeed, the crystallographic data have revealed that PPARs have a broad ligand binding pocket which enables them to bind a wide variety of structurally distinct natural and synthetic ligands (Moras & Gronemeyer, 1998; Desvergne & Wahli, 1999; Willson *et al.*, 2000) (Figure 14).

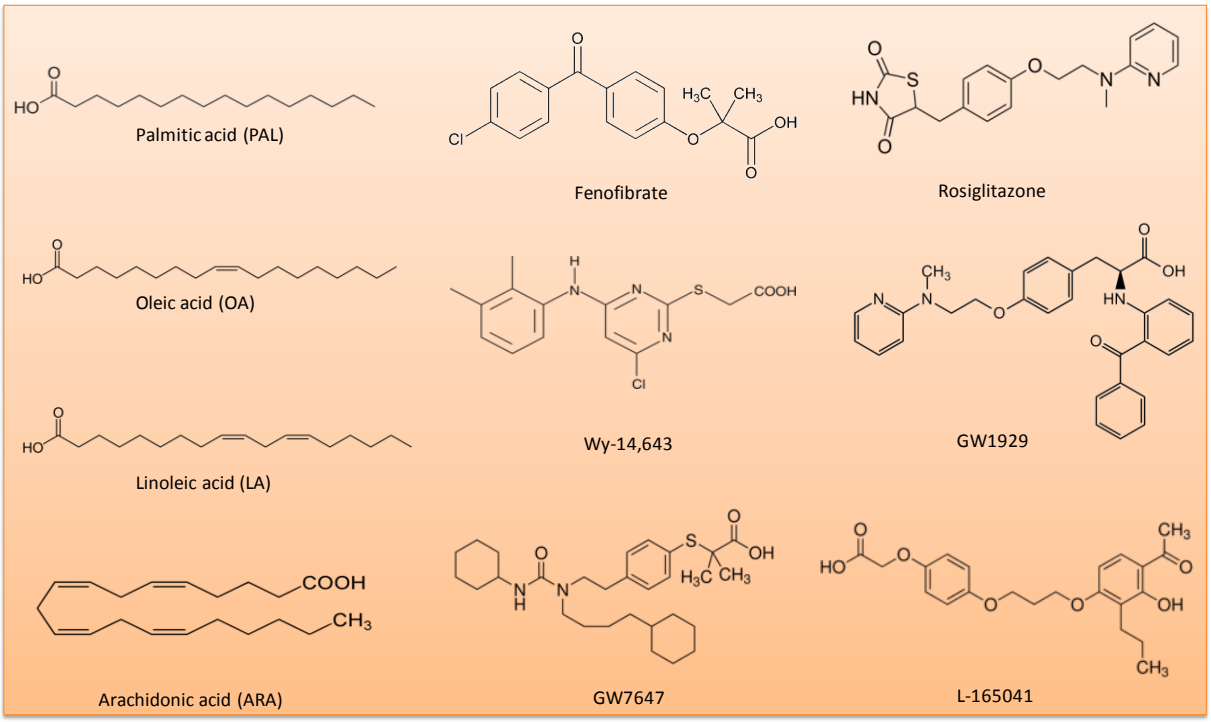


Figure 14: Chemical structure of some of the PPAR ligands

4.1. PPARα

The saturated or unsaturated fatty acids such as palmitic acid (PA), oleic acid (OA), linoleic acid (LA) and arachidonic acid (ARA) are natural ligands for PPARα (Göttlicher *et al.*, 1992 & 1993; Bocos *et al.*, 1995; Forman *et al.*, 1997). The most potent ligands were found to be the eicosanoids, which are derived from the degradation pathway of ARA and LA through the action of lipoxygenase. The unsaturated FAs bind all three PPARs, with PPARα exhibiting the highest affinity, at concentrations that correspond to their circulating blood levels. However, the saturated FAs are poor PPAR ligands in general (Desvergne & Wahli, 1999). More recent data suggest that the FAs exhibit affinities for PPARα in the nanomolar

range (Ellinghaus *et al.*, 1999; Lin *et al.*, 1999; Hostetler *et al.*, 2005 & 2006), which has been found to be consistent with their concentrations in the circulation as well as with their intracellular and intranuclear concentrations. These studies indicate that acyl-CoAs could act as better endogenous PPARα activators than the corresponding FAs (Forman *et al.*, 1997; Hostetler *et al.*, 2005 & 2006), which may account for the high expression of PPARα in metabolically active tissues.

In addition, 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE) and leukotriene B4 (LTB4), a lipid mediator of inflammation, are selective ligands for PPARα (Forman *et al.*, 1997; Kliewer *et al.*, 1997; Krey *et al.*, 1997). Moreover, 9(S)- and 13(S)-hydroxyoctadecadienoic acid (9(S)-HODE and 13(S)-HODE), produced by the action of lipoxygenase on LA and oxidized LDL, are also PPARα ligands (Delerive *et al.*, 2000). However, these ligands are not selective to PPARα and can also activate PPARγ and β/δ. N-oleoylethanolamine is another endogenous ligand of PPARα that regulates satiety and body weight (Fu *et al.*, 2003; Lo Verme *et al.*, 2005). Furthermore, it was also shown that LPL may contribute in the production of PPAR ligands through its action on circulating lipoproteins (Ziouzenkova *et al.*, 2003).

The synthetic ligands for PPARα include the fibrate drugs (bezafibrate, fenofibrate, gemfibrozil) which are extensively used in the treatment of hypertriglyceridemia (Forman *et al.*, 1997; Kliewer *et al.*, 1997; Krey *et al.*, 1997; Desvergne & Wahli, 1999). Some fibrates such as fenofibrate specifically activate PPARα, while others such as bezafibrate activate the three PPAR subtypes. These compounds require high micromolar concentrations to activate human PPARα, which may explain the high doses needed for the hypolipidemic effects observed in humans (Gaw *et al.*, 1994; Willson *et al.*, 2000). Other ligands with high specificity for PPARα include GW7647 (Brown *et al.*, 2001), GW9578 (Brown *et al.*, 1999; Willson *et al.*, 2000) and Wy-14,643 (Santilli *et al.*, 1974).

4.2. PPARβ/δ

Endogenous ligands for PPARβ/δ include eicosanoids and saturated and polyunsaturated FAs including PA, LA, ARA, ALA and EPA in the micromolar range (Forman *et al.*, 1997; Kliewer *et al.*, 1997; Krey *et al.*, 1997). Furthermore, ARA and cyclooxygenase-derived metabolite termed prostacyclin, as well as the LA-derived 15-lipoxygenase-1 product 13(S)-HODE have also been identified as PPARβ/δ specific activators (Gupta *et al.*, 2000;

Shureiqi *et al.,* 2003). Synthetic PPARβ/δ ligands include the phenoxyacetic derivatives GW501516 and GW0742, optimized from a library of hydrophobic carboxylates, L165461 or GW2433 (Brown *et al.,* 1997; Sznaidman *et al.,* 2003; Lalloyer & Staels, 2010).

4.3. PPARγ

A number of fatty acids and eicosanoid derivatives act as the natural ligands for PPARγ at micromolar concentrations. However PPARγ shows preference for PUFAs and binds LA, ALA, ARA and EPA (Kliewer *et al.,* 1997; Xu *et al.,* 1999). Furthermore, oxidized lipid components of LDL *i.e.* 9-HODE and 13-HODE and J-series of prostaglandins such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) were also identified as endogenous ligands and activators of PPARγ (Forman *et al.,* 1995; Kliewer *et al.,* 1995; Yu *et al.,* 1995; Nagy *et al.,* 1998).

The antidiabetic agents, referred to as thiazolidinediones (TZDs) or glitazones, are considered as an important class of synthetic high affinity PPARγ ligands (Lehmann *et al.,* 1995). Other PPARγ activators include GI262570, GW1929 and GW7845 (Larsen *et al.,* 2003; Lalloyer & Staels, 2010).

5. Role of PPARα in lipid metabolism

The regulation of lipid and lipoprotein metabolism is a complex process that involves a wide array of genes, whose expression is modulated in a coordinated manner through different transcription factors including PPARs. Of the three subtypes, PPARα plays a central role in the hepatic lipid homeostasis, primarily through the transcriptional control of diverse sets of genes involved in the transport and metabolism of intracellular lipids and lipoproteins (Table 3). Also, in our experimental studies, we were interested in PPARα-mediated gene regulation in liver (as discussed in Results and Discussion, chapters 2 & 3). For these reasons, we will mainly address in this section, the potential mechanisms for the regulation of hepatic lipid metabolism by PPARα subtype.

Table 3: PPARα target genes involved in lipid metabolism
(modified from Yoon, 2009)

Target genes	Gene expression
Fatty acid uptake, binding and activation	
Fatty acid transport protein (FATP)	Increase
Fatty acid translocase (FAT/CD36)	Increase
Liver fatty acid binding protein (L-FABP)	Increase
Acyl-CoA synthetase (ACS)	Increase
Carnitine palmitoyltransferase type I & II (CPT-I and CPT-II)	Increase
Mitochondrial β-oxidation	
Very long chain acyl-CoA dehydrogenase (VLCAD)	Increase
Long chain acyl-CoA dehydrogenase (LCAD)	Increase
Medium chain acyl-CoA dehydrogenase (MCAD)	Increase
Short chain acyl-CoA dehydrogenase (SCAD)	Increase
Peroxisomal β-oxidation	
Acyl-CoA oxidase (ACOX)	Increase
Bifunctional enzyme (HD)	Increase
3-ketoacyl thiolase (Thiolase)	Increase
Microsomal ω-hydroxylation	
Cytochrome P450 4A1 and 4A6 (CYP4A1 and CYP4A6)	Increase
Hydrolysis of plasma triglycerides	
Lipoprotein lipase (LPL)	Increase
Apolipoprotein C-III (ApoC-III)	Increase
Fatty acid synthesis	
Acetyl-CoA carboxylase (ACC)	Decrease
Fatty acid synthase (FAS)	Decrease
Liver ketogenesis	
HMG-CoA synthase (HMGCS)	Increase
Fatty acid interconversion	
Stearoyl-CoA desaturase (SCD)	Increase
HDL metabolism	
Apolipoprotein A-I and A-II (ApoA-I and ApoA-II)	Increase
ATP-binding cassette transporter 1 (ABCA1)	Increase
Electron transport chain	
Uncoupling protein 1, 2 and 3 (UCP1, UCP2 and UCP3)	Increase

5.1. PPARα and FA oxidation

Intracellular FA levels are largely regulated by an import/export system which is controlled in part by the two key proteins: fatty acid transport protein (FATP), which facilitates the transport of FAs across the cell membrane, and acyl-CoA synthetase (ACS), which is involved in FA esterification to prevent their efflux (Martin *et al.*, 1997; Motojima *et al.*, 1998; Chinetti-Gbaguidi *et al.*, 2005). PPARα mainly controls the peroxisomal and mitochondrial β-oxidation as well as microsomal ω-oxidation of FAs (Schoonjans *et al.*, 1996a & b; Staels *et al.*, 1998b; Fruchart *et al.*, 1999; Latruffe *et al.*, 2001). Any disturbances

in this pathway in liver are largely associated with pathological conditions such as hepatic steatosis and hepatocarcinogenesis (Cherkaoui-Malki *et al.,* 2012). PPARα activation increases FA uptake and transport across the cell membrane as well as their subsequent conversion into a metabolic form that serves as a substrate for β-oxidation. Mitochondria play an important role in β-oxidation and FAs must be modified in order to enter this organite and to be recognized by specific transporters. By increasing the expression of the enzymes that modify FAs including CPT-I and -II in the liver and muscles, PPARα promotes their entry into the mitochondria (Brandt *et al.,* 1998; Bocher *et al.,* 2002).

Furthermore, PPARα also increases the expression of enzymes involved in the degradation of FAs in mitochondria including ACOX1 (Tugwood *et al.,* 1992). Various studies have reported the involvement of PPARα in controlling the production of ketone bodies by regulating the expression of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoAS) (Rodríguez *et al.,* 1994; Ortiz *et al.,* 1999). In this way, PPARα activation increases the oxidation of FAs and thereby reduces their availability for the production and secretion of VLDL particles (Fruchart, 2009).

5.2. PPARα and plasma TG metabolism

The reduction in plasma TG levels is achieved by the induction of genes that decrease the availability of TG for hepatic VLDL secretion as well as those that promote LPL-mediated lipolysis of TG-rich plasma lipoproteins (Duval *et al.,* 2007) (Figure 15). The effects of PPARα on TG metabolism are mainly mediated by the changes in the expression of LPL and ApoC-III, the strong determinants of plasma TG levels (Yoon, 2009). Numerous studies have reported that the fibrates are involved in PPARα-dependent increase in LPL expression and therefore enhance the hydrolysis of TG-rich lipoproteins (Heller & Harvengt, 1983). PPARα activation by fibrates also increases ApoA-V expression, possibly because it accelerates the hydrolysis of TG-rich lipoproteins, by facilitating their interaction with proteoglycan-bound LPL (Prieur *et al.,* 2003; Merkel *et al.,* 2005; Fruchart & Duriez, 2006). Moreover, clinical and animal studies have indicated a positive correlation between ApoC-III levels with plasma TG concentrations (Malmendier *et al.,* 1989; Cohn *et al.,* 2004; van der Ham *et al.,* 2009). PPARα activators substantially reduce ApoC-III expression and secretion rate, thereby reducing plasma TG levels (Haubenwallner *et al.,* 1995; Staels *et al.,* 1995;

Ginsberg & Brown, 2011). PPARα also promotes the expression of cytochrome P450 4A (CYP4A), a cytochrome P450 enzyme that catalyzes the ω-hydroxylation of FAs and thereby reduces TG synthesis (Yu *et al.,* 2003; Yoon, 2009).

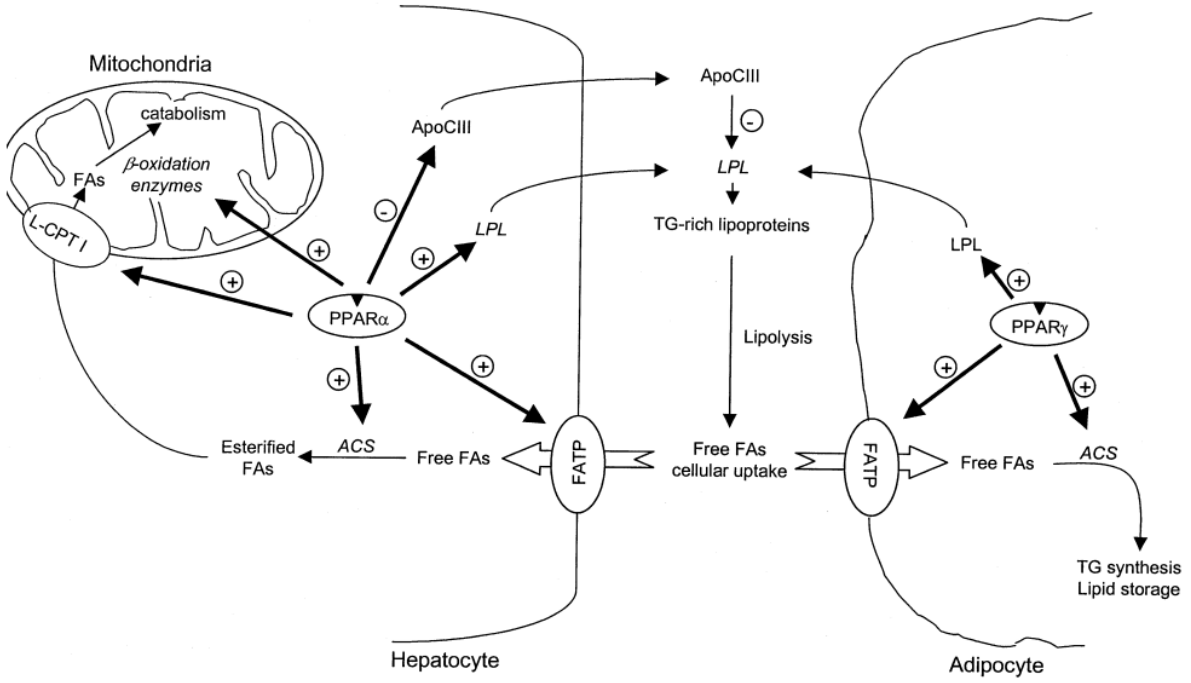


Figure 15: Role of PPARs in lipid metabolism

*In the liver, PPARα plays a central role in the regulation of the expression of a wide array of genes controlling the uptake of FAs (*i.e. *FATP), their activation to acyl-CoA esters (*i.e. *ACS), their entry into mitochondria (*i.e. *CPT-I), and their subsequent β-oxidation. Moreover, the synthesis and storage of TG in the adipose tissue is mediated by PPARγ through FATP. Both PPARα and γ promote lipolysis via enhanced induction of LPL, which ultimately leads to the hydrolysis of TG-rich lipoproteins and improves lipoprotein secretion profile of liver (Adapted from Bocher* et al., *2002).*

5.3. PPARα and lipoprotein metabolism

PPARα is involved at various steps in the metabolism of lipoproteins, most importantly, in the HDL metabolism as well as in the production and clearance of VLDL (Mandard *et al.,* 2004).

5.3.1. PPARα and HDL metabolism

Several epidemiological studies have documented an inverse relation between plasma HDL levels and the risk for the development of CVDs. Fibrates serve as the agonists for PPARα and are known as hypolipidemic drugs because of their TG-lowering and HDL-elevating properties in humans. This renders fibrates as important pharmacological

agents in the treatment of dyslipidemia and related disorders (Staels *et al.,* 1998b; Goldenberg *et al.,* 2009; Lalloyer & Staels, 2010; Tenenbaum & Fisman, 2012a & b). PPARα activation increases HDL levels in liver by stimulating the production of ApoA-I and -II, which are the major apolipoproteins of HDL and have been found to contain PPREs in their promoter regions (Vu-Dac *et al.,* 1994 & 1995; Staels *et al.,* 1998b; Yoon, 2009). On the contrary, PPARα activation in rodents resulted in a decrease in circulating HDL as well as ApoA-I and -II levels, which was mainly attributed to presence of the three differing nucleotides in the rat ApoA-I promoter, which renders it unresponsive to PPARα (Vu-Dac *et al.,* 1998; Bocher *et al.,* 2002). PPARα also facilitates HDL-mediated cholesterol efflux, termed reverse cholesterol transport, from macrophages via the induction of SR-BI and ABCA1 pathways (Chinetti *et al.,* 2000 & 2001). PPARα also inhibits the cholesteryl ester formation in the macrophages and further increases the efflux of free cholesterol through the ABCA1 pathway (Chinetti *et al.,* 2003). It is evident from the literature that the mechanisms through which the fibrates lower TG and raise HDL levels also include a reduction in VLDL synthesis and an increase in the hydrolysis of TG-rich lipoproteins (Staels *et al.,* 1998b, Mandard *et al.,* 2004).

5.3.2. PPARα and ApoB-containing lipoproteins

PPARα activation by fibrates has been found to be associated with decreased ApoB-containing lipoproteins, especially large VLDL particles, but also other ApoB-containing lipoproteins as well as total serum ApoB levels (Peters *et al.,* 1997; Fruchart *et al.,* 1999; Lindén *et al.,* 2001; Milosavljevic *et al.,* 2001). These effects are likely to be associated both with an enhanced catabolism of TG-rich lipoprotein particles and a decreased production of ApoB-containing lipoproteins including VLDL (Kesaniemi & Grundy, 1984). The possible mechanisms for the fibrate lipid-lowering effect involve an increased expression of LPL, accompanied by a decreased expression of hepatic ApoC-III, an atherogenic component of lipoproteins which inhibits LPL activity (Peters *et al.,* 1997; Staels *et al.,* 1998b; Desvergne & Wahli, 1999). Little data are available concerning the effects of fibrates on the production and secretion of VLDL in humans. However, in mice, the absence of PPARα is related with an increased production of hepatic VLDL (Tordjman *et al.,* 2001; Lindén *et al.,* 2001), whereas PPARα activation by an agonist (Wy-14,643) significantly decreases the production of VLDL.

It is also well-known that the assembly and secretion of ApoB-containing lipoproteins is mainly regulated by co- or post-translational degradation of ApoB. Studies have shown that the correct folding of ApoB and the subsequent assembly of VLDL are highly dependent on the availability of FAs and lipid biosynthesis (Ginsberg, 1995; Olofsson *et al.,* 1999; Lindén *et al.,* 2000). This may lead to an increase in the number and size of VLDL particles (Adiels *et al.,* 2006a & b, Fisher & Ginsberg, 2002). However, the positive correlation between liver TG content and VLDL assembly and secretion is not always clear according to the literature, as shown by Wiegman *et al.* (2003) in *ob/ob* mice where VLDL production remains unchanged, despite *de novo* lipogenesis.

In addition, PPARα also changes LDL profile, causing a decrease in sdLDL-cholesterol, having poor affinity for LDL-R and an increase in large LDL particles, having high affinity for this receptor (Tilly-Kiesi & Tikkanen, 1991; Yoon, 2009; Fruchart, 2009). Furthermore, PPARα activators such as fenofibrate reduce LDL-cholesterol levels by inhibiting the formation of slowly catabolized, atherogenic LDL particles and promoting the formation of rapidly catabolized LDL particles accompanying with lower plasma TG levels (Caslake *et al.,* 1993). However, in some patients with dyslipidemia or hypercholesterolemia, the response to fibrate treatment is inconsistent (Pasternak *et al.,* 1996; Scott *et al.,* 2005).

Altogether, it can be concluded that PPARs, most notably PPARα, have been the subject of extensive research because of their involvement in a number of biological processes and thereby act as valuable therapeutic targets for the management of dyslipidemia and various related disorders such as CVDs, diabetes and obesity. PPARα plays a central role by acting on hepatic lipid and lipoprotein metabolism through the modulation of the expression of a wide variety of genes. The regulatory effects of PPARα are primarily due to its versatility as a receptor to bind a number of molecules that favor or repress the particular transcriptional networks. By stimulating FA oxidation and inhibiting the synthesis of TG and ApoB-containing lipoproteins, PPARα acts to maintain hepatic lipid homeostasis. Moreover, the development of PPARα-specific activators, such as fibrates, has proved to be particularly effective in the treatment of dyslipidemia and related disorders, mainly because of their TG-lowering and HDL-elevating properties. Combination trials of fibrates with the pharmacologic agents for hypercholesterolemia termed hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) have shown further in improving the plasma lipid profiles than

each class separately. Consequently, this has proved effective in reducing the morbidity and mortality due to CVDs. However, this combination sometimes leads to the undesirable effects in patients, such as intolerable muscle symptoms like myalgias, myositis, or mitochondrial injury or liver toxicity (Kashani *et al.,* 2006; Ducobu *et al.,* 2009). This emphasizes upon the appropriate use of statins and fibrates in carefully selected patients in order to reduce vascular and coronary events in high-risk patients.

IV. Pathophysiology and management of dyslipidemia and the disorders related to lipid metabolism

1. Dyslipidemia

Dyslipidemia is highly prevalent and commonly encountered throughout the world. Research over the last decades has consistently shown that the disturbances in lipid metabolism constitute the major risk factor for the development of coronary heart disease (CHD), which is the leading cause of death worldwide. The World Health Organization (WHO) estimates that dyslipidemia is associated with more than half of global cases of ischemic heart disease and more than four million deaths per year (WHO; 2002).

Dyslipidemia is a broad term that refers to the disorders related to the disturbances in lipoprotein metabolism. It is mainly characterized by elevated total and LDL cholesterol, increased TG levels, and sometimes decreased HDL cholesterol levels in the circulation. Dyslipidemia is often associated with the production of highly atherogenic particles and is therefore a widely accepted marker for CHD.

1.1. Lipid profile assessment

The plasma lipid profile of an indivdual provides the means for identifying perturbations in lipid metabolism and the associated pathophysiological conditions. Furthermore, this profile also facilitates the selection of appropriate dietary modifications and drug treatment. The guidelines for normal lipid values have been provided by NCEP-ATP III, (2001), with defined values for normal and elevated levels of the different lipoproteins in the circulation (Table 4).

Table 4: Classification of total, LDL and HDL cholesterol and TGs
(Adapted from NCEP-ATP III, 2001)

Total cholesterol (mg/dL)	
<200	Desirable
200–239	Borderline high
≥240	High
LDL cholesterol (mg/dL)	
<100	Optimal
100–129	Near optimal/above optimal
130–159	Borderline high
160–189	High
≥190	Very high
HDL cholesterol (mg/dL)	
<40	Low
≥60	High
TGs (mg/dL)	
<150	Desirable
150–199	Borderline high
200–499	High
≥500	Very high

2. Hyperlipidemia classification

Hyperlipidemia may be primary or secondary in nature. The former is caused by the genetic defects, whereas, the latter results from acquired causes including high-fat diet, obesity, diabetes and hypothyroidism (Vogt, 1991). Fredrickson & Lees (1965) classified primary hyperlipidemia into five categories, based on the patterns of elevation in lipids and lipoproteins. *i.e.* types I to V (Table 5). In the United States, the two most common hyperlipidemias are Fredrickson type IIb (familial combined hyperlipidemia) and type IV (familial hypertriglyceridemia). Together, these two classes account for 85% of familial hyperlipidemia (Pejic & Lee, 2006).

Table 5: Classification of hyperlipidemias
(Adapted from Fredrickson & Lees, 1965; Pejic & Lee, 2006*)*

Phenotype	Elevated Lipoprotein (s)	Associated clinical disorders	Serum TC	Serum TG	Clinical features	Relative Frequency
I	Chylomicrons	LPL & ApoC-II deficiency	→	↑↑	Decreased LPL & altered ApoC-II	<1%
IIa	LDL	Familial hypercholesterolemia, Polygenic hypercholesterolemia, Nephrosis, Hypothyroidism, Familial combined hyperlipidemia	↑↑	↑	LDLR deficiency	10%
IIb	LDL & VLDL	Familial combined hypercholesterolemia	↑↑	↑	Decreased LDLR & increased ApoB	40%
III	IDL	Dysbetalipoproteinemia	↑	↑	Defects in ApoE2 synthesis	<1%
IV	VLDL	Familial hypertriglyceridemia, Familial combined hyperlipidemia, Sporadic hypertriglyceridemia, Diabetes	→↑	↑↑	Increased VLDL production & decreased clearance	45%
V	Chylomicrons & VLDL	Diabetes	↑	↑↑	Increased VLDL production & decreased LPL	5%

↑ = Increased; ↑↑ = Highly increased; → = Normal; →↑ = Normal or increased; LPL: lipoprotein lipase; VLDL: Very Low Density Lipoprotein; LDL: Low Density Lipoprotein; IDL: Intermediate Density Lipoprotein.

2.1. Type I-Hyperchylomicronemia

Hyperlipoproteinemia with phenotype I is a relatively rare disorder, associated with marked increase in the circulating levels of chylomicrons, with relatively normal plasma VLDL levels (Berger *et al.*, 1982) (Table 5). The familial deficiency in LPL or ApoC-II, an activator of LPL, leads to impairment in the clearance of chylomicrons, causing massive accumulation of TGs in the plasma. This ultimately results in the pathological conditions including xanthomatous eruption, lipemia retinalis, hepatosplenomegaly, and acute pancreatitis (Yamamura *et al.,* 1979; Brunzell *et al.,* 1983; Fojo & Brewer, 1992; Iwasaki & Tada, 1999; Sugandhan *et al.,* 2007).

2.2. Type IIa-Hypercholesterolemia

This phenotype is characterized by normal TG levels but elevated LDL due to the defects in LDL-R, or specific mutations in ApoB-100, thus resulting in familial hypercholesterolaemia (FH) (Table 5). Although the mutations in LDL-R are the most common cause of FH, recent data have also revealed that the mutations in proprotein convertase subtilisin/kexin 9 (PCSK9) and, more rarely, the autosomal recessive hypercholesterolemia (ARH) adaptor protein can also lead to the development of FH (Rader *et al.,* 2003; Allard *et al.,* 2005; Varret *et al.,* 2008; Raal & Santos, 2012; Nordestgaard *et al.,* 2013). The diagnostic hallmarks of FH are tendon xanthomas (Durrington, 2003).

2.3. Type IIb-Hypercholesterolemia

Type IIb is mixed hyperlipidemia, associated with increased levels of total cholesterol, LDL cholesterol and the TG levels (Table 5). The clinical manifestations for this type of hyperlipidemia mainly include tendon xanthomas.

2.4. Type III-dysbetalipoproteinemia

Patients with type III hyperlipoproteinemia (dysbetalipoproteinemia) have increased concentrations of both plasma cholesterol and TGs (Table 5). A biochemical characteristic of the disorder is the occurrence of β-migrating VLDL (β-VLDL) particles, which are cholesterol-enriched remnants as well as IDL, a VLDL remnant. The most common cause of this phenotype is the presence of dysfunctional form of ApoE2, though other factors remain to be identified. The clinical features of this disorder are varied and may include cutaneous xanthomas and xanthomas of the palmar creases (*xanthoma striata palmaris*) (Mahley *et al.,* 1999; Schaefer, 2009).

2.5. Type IV-familial hypertriglyceridemia

In type IV, the total cholesterol and LDL levels are typically normal but the TG level is elevated usually between 500 and 1,000 mg/dL. Patients with type IV disease are very sensitive to dietary modifications and show the symptoms of eruptive xanthomas (Martínez *et al.,* 2008; Renner *et al.,* 2008).

2.6. Type V-familial combined hypertriglyceridemia

This type is characterized by the elevated levels of both chylomicrons and VLDL in the plasma, with TG levels exceeding up to 1000 mg/dL. Since type I is quite rare, when the circulating TG levels reach above 1000 mg/dL, the most likely cause is type V hyperlipidemia, showing the main symptoms of eruptive xanthomas (Martínez *et al.*, 2008).

3. Dyslipidemia and aging

In recent decades, the rapid aging of the population together with the appearance of various age-related disorders, has led to a growing awareness of the importance of various metabolic changes associated with aging process. This is of particular importance in industrialized countries having remarkable surges in life expectancy. According to the report of World Population Aging 1950-2050 (2001), the global elderly population has been steadily increasing from 8% in 1950 to 11% in 2009, and may reach upto 22% in 2050. Globally, the number of elderly people increases by 2% per year, much faster than the overall population that is growing at an annual rate of 1.2%. In metropolitan France, the number of people aged over 60 will rise by more than 10 million. Also, the number of people aged over 75 years will increase from 5.2 million in 2007 to 11.9 million in 2060, and those aged over 85 from 1.3 to 5.4 million (Blanpain & Chardon, INSEE, 2010).

Aging is a universal process associated with the adverse changes that increase the risk of mortality and therefore depicts a disturbing picture of this last period of life. Aging of the population leads to a number of pathological conditions involving increased body fat, most often in the abdominal region, an elevation in the circulating lipid and FFA levels and a reduced oxidative capacity of different tissues. These changes ultimately lead to an atherogenic lipid profile with an increased risk of insulin resistance (IR) and CVDs (Boden *et al.*, 1994; Sial *et al.*, 1996; Shanmugasundaram *et al.*, 2010). It is well known that CVDs are involved in significant morbidity and mortality in the elderly, where they account for 80% of the deaths in patients aged over 65 (Dalal & Robbins, 2002; Shao *et al.*, 2011). To date, numerous epidemiological studies show that dyslipidemia is an independent risk factor for CVDs (Shepherd *et al.*, 2004), particularly in older adults, where it often coexists with other metabolic perturbations including type 2 diabetes mellitus (T2DM), obesity and hypertension. This renders the management of dyslipidemia a crucial step towards decreasing the risk of CVDs (Gobal & Mehta, 2010; Shao *et al.*, 2011).

It is well-established that the risk for CVDs increases with elevated LDL cholesterol and decreases with high HDL cholesterol levels (Miller & Miller, 1975; Gordon *et al.*, 1977; Goh *et al.,* 2007). After the age of 20 years, LDL cholesterol concentrations increase more rapidly in men, as compared with women and reach a plateau by the age of 50 to 60 years while in women, by the age of 60 to 70 years. This age-associated increase in LDL cholesterol may be attributed to its reduced clearance due to the decreased number of functioning hepatic LDL-R, which may contribute towards the increased incidence of CVDs (Kreisberg & Kasim, 1987; Ericsson *et al.,* 1991; Ferrara *et al.,* 1997; Shanmugasundaram *et al.,* 2010).

During puberty and early adulthood in men, serum HDL cholesterol levels decrease and thereafter remain lower than in women at all comparable ages. However, in women, the HDL cholesterol concentrations remain constant throughout their lifetime (Kreisberg & Kasim, 1987; Gobal & Mehta, 2010).

Several clinical trials have indicated that plasma TG levels can be considered as an HDL-independent risk factor for the development of CVDs (Assmann *et al.*, 1996; Hokanson & Austin, 1996; Jeppesen *et al.*, 1998; Yarnell *et al.*, 2001; Alagona, 2010). TG levels in men have been reported to increase gradually, reaching peak values between 40 and 50 years of age, and decrease slightly thereafter. However, high TG concentrations have been observed in women throughout their lifetime, most particularly during menopausal transitions (Derby *et al.*, 2009; Gobal & Mehta, 2010; Miller *et al.*, 2011).

These age-related changes in lipid metabolism can be attributed in part to the pseudo-capillarization that involves structural changes in the liver sinusoidal endothelial cells including loss of fenestrations. Since the fenestrations in the endothelium allow passage of some lipoproteins, including chylomicron remnants, age-related reduction in fenestrations impairs hepatic lipoprotein metabolism. This physiological phenomenon has been shown in elderly humans and some animals and it results in decreased endocytosis, increased leukocytes adhesion, and decreased hepatic perfusion (Le Couteur *et al.*, 2007).

Various strategies have been defined to treat dyslipidemia in the elderly population. The first line of prevention involves the modification in the lifestyle, including regular physical activity and weight control. In addition, respective lipid-lowering therapies, such as statin treatment, may be employed with special regards to the gender, diet restriction and drug side effects (Gobal & Mehta, 2010; Shanmugasundaram *et al.*, 2010).

4. Pathophysiology of dyslipidemia

The perturbations in lipoprotein profiles during dyslipidemia may be associated with other metabolic disturbances such as obesity and IR.

4.1. Obesity

Hippocrates stated "Corpulence is not only a disease itself, but the harbinger of others". Obesity has become the major worldwide health concern, with its alarmingly increasing incidence both in developed as well as in developing countries (James, 2004a & b; Haslam & James, 2005; Knight, 2011). According to WHO data, globally, there are more than 1 billion adults overweight and 300 million obese people. The prevalence of obesity is increasing substantially in the developing world with more than 115 million people suffering from obesity-related problems (Misra & Khurana, 2008; WHO, 2008). This can be explained essentially by rapid nutrition transition from a healthy traditional high-fiber, low-fat, low-calorie diet towards increased consumption of low fiber, calorie-dense foods containing refined carbohydrates, fats and red meats (Misra *et al.*, 2010).

Numerous epidemiological studies have shown that the excess of dietary fats is the main cause for the development of obesity and the associated metabolic and vascular complications. The major co-morbid conditions related to obesity include IR, obstructive sleep apnea syndrome, inflammation, thrombosis, non-alcoholic fatty liver disease (NAFLD) and dyslipidemia, all risk factors for CVDs (Vazzana *et al.*, 2011; Boden, 2011; Zalesin *et al.*, 2011; Klop *et al.*, 2013). The surrogate marker for the body fat content in populations and in clinical practice is the BMI, determined using the weight and height of an individual [weight (kg)/height $(m)^2$] (Figure 16). WHO has defined the BMI above 25 kg/m^2 as overweight, the overweight category is classified as obese when the BMI is ≥30 kg/m^2 and in the case of extreme obesity, BMI is ≥40 kg/m^2 (Kuczmarski *et al.*, 1997; Lien & Guyton, 2008).

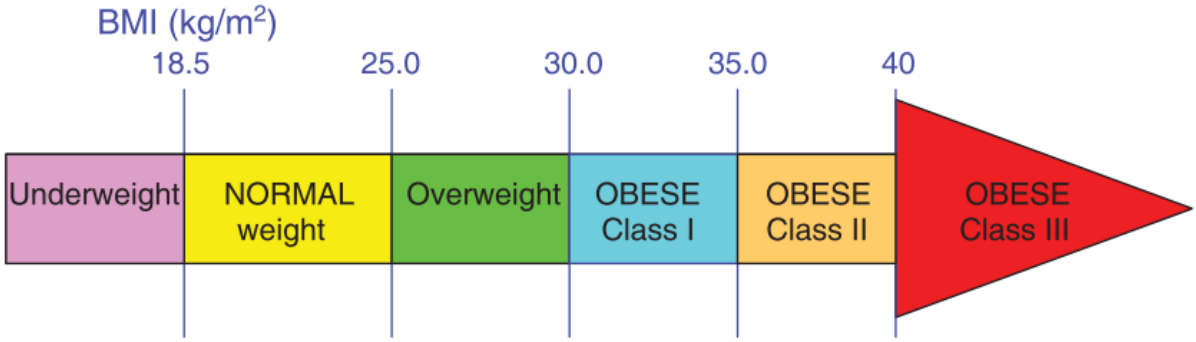


Figure 16: Body mass index (BMI)-based classification of overweight and obesity classes
(Adapted from Lien & Guyton, 2008)

Dyslipidemia is often associated with obesity and the perturbations in the processing of VLDL and chylomicrons can contribute towards aggravating this pathological condition (Figure 17). In obesity, impaired lipolysis of TG-rich lipoproteins has been observed and thought to be due to competition between the increased levels of VLDL and chylomicrons for lipolysis (Klop *et al.*, 2013). Furthermore, the hormone-sensitive lipase, a major determinant of FA mobilization in adipose and other tissues, has also been found to be associated with obesity (Large *et al.*, 1999; Holm *et al.*, 2000). In addition, the reduction in LPL mRNA levels and activity in adipose tissue and skeletal muscle respectively could also contribute to this (Clemente-Postigo *et al.*, 2011; Klop *et al.*, 2012).The increased FFA levels during post-prandial phase result in the dissociation of LPL from its binding to the endothelial surface (Peterson *et al.*, 1990; Karpe *et al.*, 1992). However, the LPL may remain associated with VLDL and IDL and contribute to the hydrolysis of their TG content. In hypertriglyceridemic state, the accumulation of TG-rich lipoproteins in particular VLDL, leads to an increased cholesteryl ester transfer protein (CETP) activity. This enzyme exchanges cholesterol esters from HDL for TG from VLDL and LDL, resulting in the production of LDL particles with increased TG content. Furthermore, the hydrolysis of the TG content in LDL by hepatic lipase results in the formation of sdLDL particles (Hokanson *et al.*, 1995; Capell *et al.*, 1996; Enkhmaa *et al.*, 2010; Klop *et al.*, 2013). Moreover, the reduction in circulating HDL cholesterol is promoted by an increased lipolysis of these TG-rich HDL particles by hepatic lipase resulting in small HDL with a reduced affinity for ApoA-I and consequently the dissociation of ApoA-I from HDL (Deeb *et al.*, 2003; Qatanani & Lazar, 2007; Subramanian & Chait, 2012). Several studies suggest that the preponderance of these slow-metabolizing sdLDL particles is associated with a 3 to 7-fold increased risk of coronary heart disease, independent of LDL cholesterol concentrations (Griffin *et al.*, 1994; Berneis & Krauss, 2002;

Packard, 2003; Enkhmaa *et al.*, 2010; Klop *et al.*, 2013). The atherogenecity associated with these particles can be attributed to their prolonged residence time (5 days) and less affinity for uptake by the hepatic LDL ApoB/ApoE recognizing receptors compared with larger LDL particles (Galeano *et al.*, 1998). Furthermore, several studies have proposed that sdLDL particles exhibit an increased propensity for transendothelial passage through the arterial wall (Björnheden *et al.*, 1996), greater binding capacity to the proteoglycans (Galeano *et al.*, 1998; Olin-Lewis *et al.*, 2002) and greater susceptibility to oxidation compared to larger LDL particles (de Graaf *et al.*, 1991; Chait *et al.*, 1993; Tribble *et al.*, 2001).

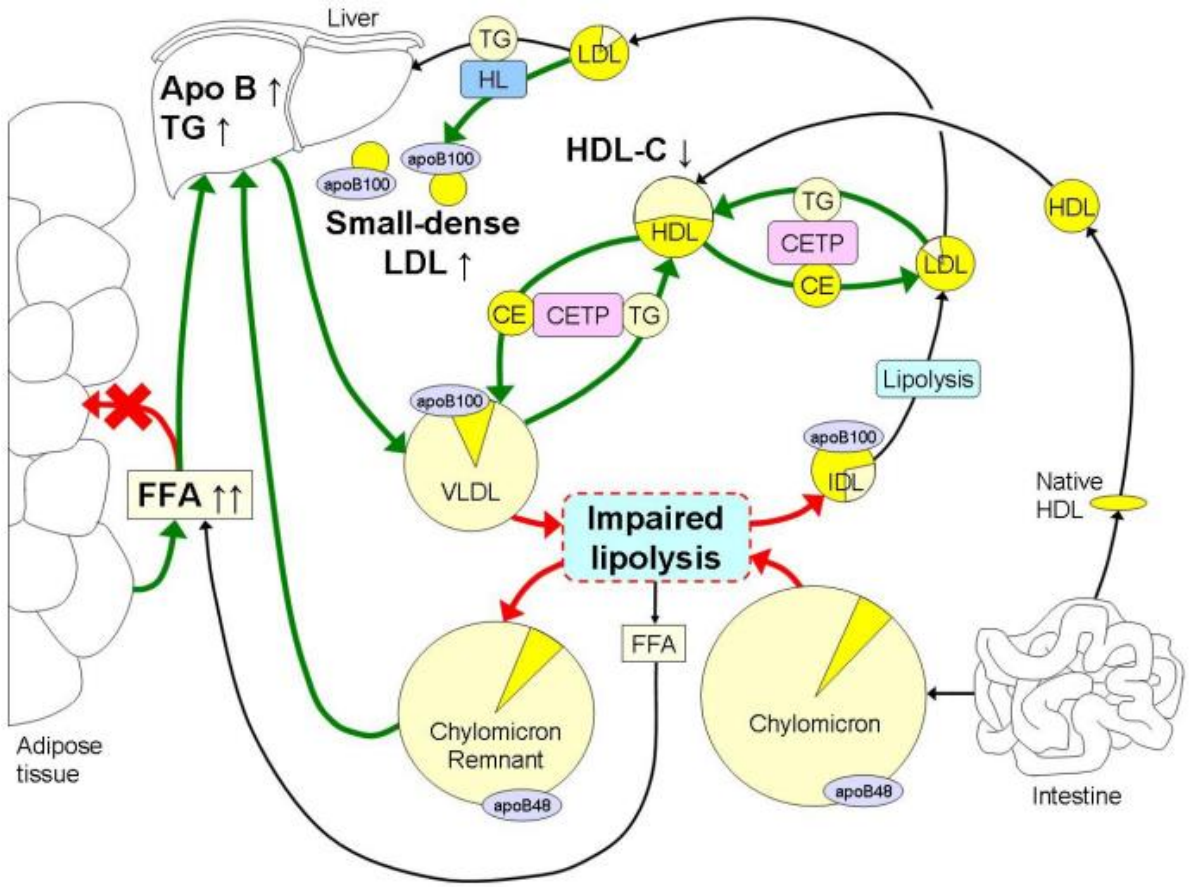


Figure 17: Pathogenesis of dyslipidemia in obesity
(*Adapted from Klop* et al., *2013)*

The dark and light yellow shadings represent cholesterol and the TG content, respectively within the different lipoproteins. Obesity-induced increases in metabolic processes are marked with green arrows, whereas the decreased specific pathways are marked with red arrows.

4.2. Insulin resistance (IR)

Insulin is produced by the pancreas in response to increased circulating glucose levels to facilitate the utilization of glucose in various tissues including skeletal muscle, liver and adipose tissue. Insulin stimulates glycogenesis and inhibits glycogenolysis in the skeletal muscle and liver. It also promotes glucose uptake in the skeletal muscle and adipose tissue, while in the liver, insulin decreases hepatic gluconeogenesis, thereby preventing an influx of more glucose into the bloodstream. Furthermore, insulin inhibits fat breakdown, or lipolysis. Insulin action therefore leads to increased glucose uptake, reduced circulating glucose levels and increased conversion of glucose into the storage molecules including glycogen or fat (Dimitriadis *et al.*, 2011).

IR is referred to a pathologic state in which the peripheral tissues do not respond appropriately to insulin, and circulating glucose levels remain elevated (Levin *et al.*, 2007; Huang, 2009). IR is commonly associated with a number of diseases, including chronic infection, obesity and T2DM (Virkamäki *et al.*, 1999; Le Roith & Zick, 2001; Miranda *et al.*, 2005). Visceral obesity, in particular, has been found to be strongly associated with IR (Gastaldelli *et al.*, 2007; Qatanani & Lazar, 2007; Jensen, 2008; Ebbert & Jensen, 2013). The adipocytes are involved in modulating the sensitivity to insulin through leptin adiponectin and resistin (Bjørbaek & Kahn, 2004; Lehrke *et al.*, 2004; Weyer *et al.*, 2001). In obese individuals, plasma FA concentrations have been found to be elevated, mainly due to an increase in FA release associated with an expansion in fat mass (Björntorp *et al.*, 1969; Jensen *et al.*, 1989; Qatanani & Lazar, 2007). These FAs are produced by the lipolysis of adipose tissue TGs. Obese patients develop IR, which prevents the suppression of adipose tissue lipolysis by insulin and generates an excess of FFAs (Figure 18). This leads to excessive influx of FAs into the muscles and liver. When transport is not sufficiently oriented towards the adipose tissue, the liver accumulates TG, potentially leading to the development of NAFLD (fatty liver).

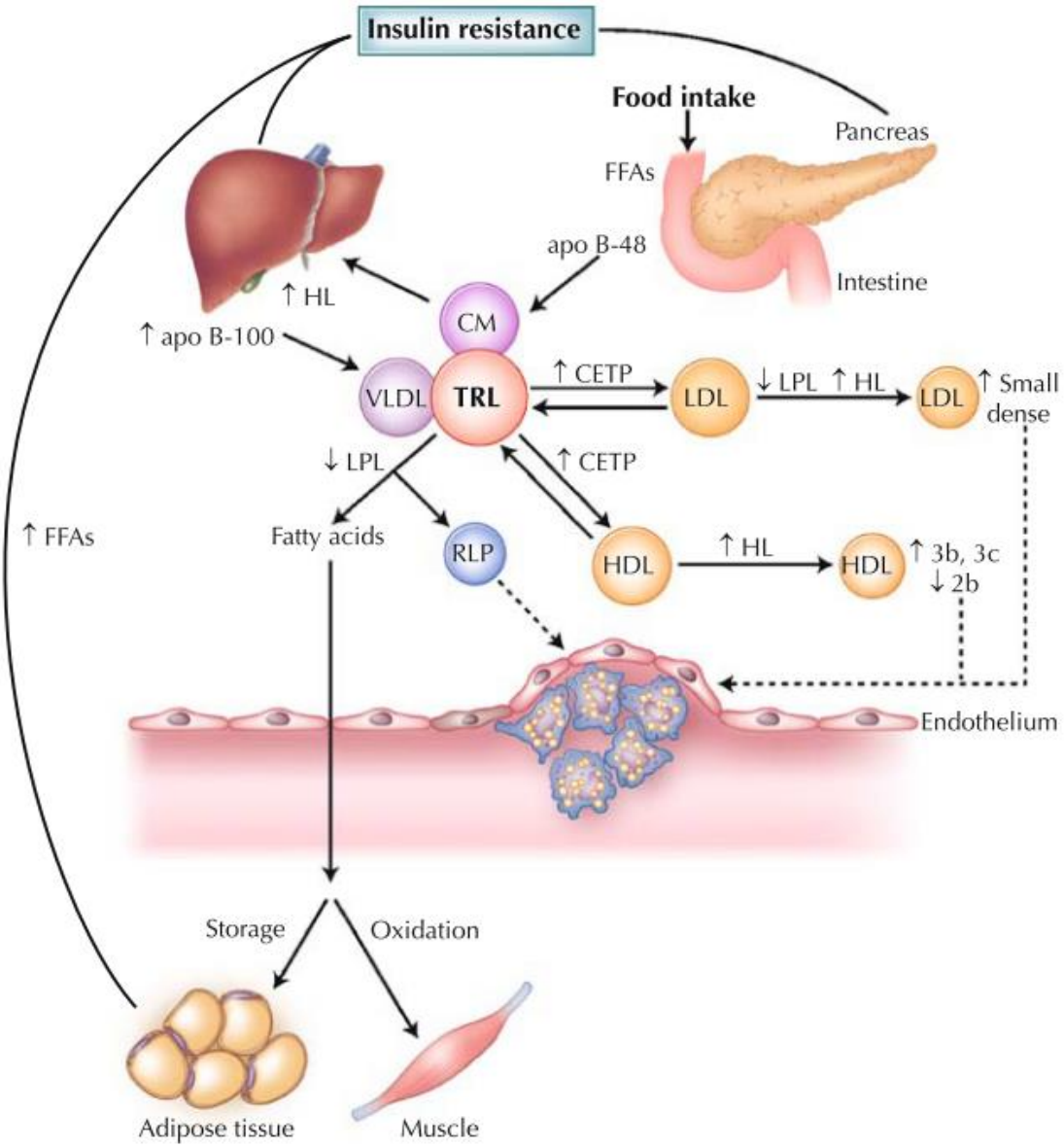


Figure 18: The hallmark of dyslipidemia in insulin resistance
(Adapted from Enkhmaa et al., *2010)*

Under normal physiological conditions, the production of hepatic VLDL and the lipolysis from adipose tissue are inhibited by insulin. However, IR promotes a dyslipidemic triad of elevated TG, low HDL and high small, dense LDL-cholesterol levels and thereby acts as a key feature in the development of diabetic dyslipidemia. In addition, the delayed clearance of TG-rich lipoproteins such as VLDL in the circulation stimulates the CETP-mediated transfer of HDL or LDL cholesteryl esters for TGs. Further hydrolysis of the TG core of LDL and HDL particles by hepatic lipase (HL) or lipoprotein lipase (LPL) results in the production of smaller and denser LDL as well as HDL particles (HDL 3b and 3c). This leads to an increase in the production of atherogenic sdLDL, accompanied with a reduction in plasma HDL levels.

It is important to note that the role of IR in metabolic syndrome (MetS) remains controversial with many experts considering IR as one of the components, but not essentially the main underlying cause of the MetS, while others refer to IR as a critical factor in the diagnosis of MetS.

5. Metabolic syndrome (MetS)

The MetS represents one of the major public-health challenges worldwide. The MetS is a multifaceted disorder characterized by a cluster of interconnected metabolic abnomalities which increase the risk of CVDs and T2DM (Eckel *et al.*, 2005; Ford, 2005; Kassi *et al.*, 2011). This disorder was first named by Reaven as 'syndrome X' (Reaven, 1988) and later renamed as MetS.

MetS ties together various interrelated risk factors including central obesity, dyslipidemia, IR, hypertension, prothrombotic and pro-inflammatory status (Grundy, 2006). Various criteria have been proposed by different health organizations in order to facilitate the diagnosis of the patients, reflecting the common pathophysiological conditions that underly MetS.

5.1. Various definitions proposed for MetS

Important definitions have been proposed by The World Health Organization (WHO), The European Group for study of Insulin Resistance (EGIR), and The National Cholesterol Education Program-Third Adult Treatment Panel (NCEP-ATPIII), American Association of Clinical Endocrinology (AACE), International Diabetes Federation (IDF), American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI), and the consensus definition combining both IDF and AHA/NHLBI (Kassi *et al.*, 2011) (Table 3).

5.1.1. The WHO definition

The first definition of MetS was proposed by a WHO consultation group (Alberti & Zimmet, 1998). This definition is based on the assumption that IR is one of the major underlying risk factor to the MetS, and features impaired glucose regulation (impaired glucose tolerance, diabetes or IR, T2DM) as the most important criteria for diagnosis. In addition, two other risk factors are also required including obesity (as measured by waist/hip ratio or BMI), hypertension, high TG levels, or low HDL and microalbuminuria (Table 6).

Table 6: Criteria for the clinical diagnosis of metabolic syndrome in adults

Criteria	WHO	EGIR	NCEP-ATPIII	AACE
Reference	Alberti & Zimmet, 1998	Balkau & Charles, 1999	2001	Einhorn *et al.*, 2003
Essential components	IR or IFG or IGT or T2DM	IR (insulin levels >75% of non-diabetic patients)		IGT
Criteria	IR already required plus two of the five following criteria	IR already required, plus two of the four following criteria	Any three of the five following criteria	IGT plus two or more of the following criteria
Abdominal obesity	BMI >30 kg/m^2			BMI ≥25 kg/m^2
Men	Waist-to-hip ratio >0.9	Waist circumference ≥94 cm	Waist circumference >102 cm	
Women	>0.85	≥80 cm	>88 cm	
Hyperglycemia (Fasting glucose levels in mg/dL)	>100	≥110	≥110	110–125
Arterial pressure (mmHg)	≥140/90	≥140/90 or taking antihypertensive drugs	≥130/85	≥130/85
Dyslipidemia				
TGs (mg/dL) and/or	≥150	≥150	≥150	≥150
HDL (mg/dL)				
Men	<40	<39	<40	<40
Women	<50	<39	<50	<50
Other criteria	***Microalbuminuria*** Urinary albumin secretion rate: ≥20 μg/min or albumin-to-creatinine ratio: ≥30 mg/g			

Table 6: (Continued).....

Criteria	AHA/NHLBI	IDF	IDF & AHA/NHLBI
Reference	Grundy *et al.*, 2004	Alberti *et al.*, 2005	Alberti *et al.*, 2009
Essential components		Central obesity	
Criteria	Any three of the following criteria	Obesity, plus two of the four following criteria	Any three of the following criteria
Abdominal obesity		BMI >30 kg/m^2	Elevated waist circumference
Men	Waist circumference >102 cm	Waist circumference-ethnicity specific	*(according to population and country-specific definitions)*
Women	>88 cm		
Hyperglycemia (Fasting glucose levels in mg/dL)	≥100	≥100	≥100
Arterial pressure (mmHg)	≥130/85	≥130/85	≥130/85
Dyslipidemia			
TGs (mg/dL) and/or **HDL (mg/dL)**	≥150	≥150	≥150
Men	<40	<40	<40
Women	<50	<50	<50

WHO: World Health Organization; EGIR: European Group for study of Insulin Resistance; NCEP-ATP III: National Cholesterol Education Program-Third Adult Treatment Panel; AACE: American Association of Clinical Endocrinology; AHA/NHLBI: American Heart Association/National Heart, Lung, and Blood Institute; IDF; International Diabetes Federation; TGs: triglycerides; HDL: High Density Cholesterol; T2DM: type 2 diabetes mellitus; IR: insulin resistance; IGT/IFG: impaired glucose tolerance/impaired fasting glucose; BMI: body mass index

5.1.2. The EGIR definition

Shortly thereafter, the EGIR proposed another definition in which microalbuminuria was excluded, and required hyperinsulinemia to be present as an essential component of the syndrome (Balkau & Charles, 1999). EGIR emphasized the use of fasting insulin levels to estimate IR and impaired fasting glucose (IFG) as an alternate for the impaired glucose tolerance (IGT) (Table 6). In addition, waist circumference instead of BMI was considered as

the main indicator to assess obesity, while introducing different cut-offs from those previously used for the other components of the syndrome.

5.1.3. The NCEP-ATP III definition

In 2001, the NCEP-ATPIII published another definition that did not include the measurement of IR as an important diagnostic component. According to this, the diagnosis relied upon the presence of three or more of the five clinically identifiable risk factors, including abdominal obesity, high fasting glucose level (IFG or T2DM), hypertension, elevated TG levels and reduced HDL cholesterol levels. Compared with the previous definitions, this definition measures obesity with a waist circumference of 102 cm (Table 6). In addition, the fasting glucose level of 110 mg/dL was included since glucose tolerance tests are not performed in the United States.

5.1.4. The AACE definition

Another definition came from AACE, who included IGT as an essential component of the syndrome, accompanyied by the presence of two or more of the risk factors, including BMI, elevated blood pressure, high TG levels and low HDL cholesterol and elevated fasting and postload (75 g/load) glucose (Einhorn *et al.*, 2003) (Table 6). Given the huge amount of evidence that suggests central obesity as a major risk factor for T2DM and CVDs, the elimination of this component from their proposed definition is surprising.

5.1.5. The IDF definition

The IDF introduced central obesity, assessed by waist circumference as a primary target for the clinical diagnosis of MetS (Alberti *et al.*, 2005). In this definition, the waist circumference values were ethnicity-specific and defined for Europeans to be >94 cm in men and >80 cm in women, and for South Asians, Chinese, and Japanese to be >90 cm in men and >80 cm in women (Table 6). For ethnic South and Central Americans, South Asian data are used, and for sub-Saharan Africans and Eastern Mediterranean and Middle East (Arab) populations, European data are used. In addition, the definition requires any two of the risk factors, including elevated blood pressure, high TG levels and low HDL cholesterol and high fasting glucose level.

5.1.6. The AHA/NHLBI definition

This definition was similar to that of IDF, with the exception of criteria for the abdominal obesity which was assessed by the waist circumference with the recommended cut-off points of 102 cm in men and 88 cm in women (Table 6). Moreover, the definition requires the risk factors identical to those already listed in IDF definition (Grundy *et al.*, 2004).

5.1.7. The IDF and AHA/NHLBI definition

In 2005, both the IDF and AHA/NHLBI attempted to reconcile the different clinical definitions for MetS proposed by different organizations (Alberti *et al.*, 2009). According to this definition, the abdominal obesity was considered as a prerequisite and included as one of the five risk factors, including high fasting glucose level, hypertension, elevated TG levels and reduced HDL cholesterol levels for diagnosis (Table 6). Thus, the presence of any three of the five risk factors may lead to the clinical diagnosis of MetS (Alberti *et al.*, 2005, Grundy *et al.*, 2005, Kassi *et al.*, 2011).

MetS represents a cluster of cardiometabolic risk factors related to dyslipidemia, IR, obesity and hypertension. It remains, however, a subject of controversy in part due the proposition of various definitions by different organizations. The overall goal of each defintiion to cover all the important pathophysiological conditions associated with MetS, including dyslipidemia, IR/T2DM, obesity and hypertension. However, investigation is actively being carried out to determine the relation and the significance of other factors such as steatohepatitis, microalbuminuria, endothelial dysfunction and inflammation, in the development of MetS.

6. Pharmacological interventions for the management of dyslipidemia

The identification and treatment of dyslipidemia is a major clinical concern in cardiovascular medicine in both the primary and secondary prevention strategies (Davidson & Toth, 2004). It is well-documented that the lowering of serum LDL cholesterol is associated with significant reduction in CVD events and deaths. This has prompted the definition of appropriate LDL cholesterol levels as the major goal to treat the disorders related to dyslipidemia. Several classes of pharmacologic agents are available for the treatment of dyslipidemia (Durrington, 2003; Hachem & Mooradian, 2006; Mooradian, 2009) (Table 7).

Table 7: Selected pharmacological agents used for the treatment of dyslipidemia
(Adapted from Mooradian, 2009)

Drug	Efficacy	Adverse effects	Specific agents and doses
Statins	LDL ↓ 18–55% HDL ↑ 5–15% TG ↓ 7–30%	Hepatotoxicity, myopathy, increased creatine kinase levels	Rosuvastatin, 5–40 mg orally once daily; fluvastatin, 20–80 mg orally nightly; simvastatin, 5–80 mg orally every evening; pravastatin, 10–80 mg orally once daily; atorvastatin, 10–80 mg orally once daily; lovastatin, 10–80 mg orally nightly; extended-release lovastatin, 10–60 mg orally nightly
Ezetimibe	LDL ↓ 15–20% HDL ↑ 1% TG ↓ 8%	No major adverse effects	10 mg orally once daily
Ezetimibe combined with simvastatin	LDL ↓ 30–60% HDL ↑ 9% TG ↓ 20%	Hepatotoxicity, myopathy, increased serum creatine kinase levels	10/10 mg to 10/80 mg orally once daily
Nicotinic acid	LDL ↓ 5–25% HDL ↑ 15–35% (ApoA-I ↑) TG ↓ 20–50% Small dense LDL ↓	Hot flashes, hyperglycemia, hyperuricemia, hepatotoxicity	Nicotinic acid, 1–2 g orally two or three times daily; extended-release nicotinic acid, 1–2 g orally nightly; sustained-release nicotinic acid, 250–750 mg orally once or twice daily
Nicotinic acid combined with statin	LDL ↓ 30–42% HDL ↑ 20–30% TG ↓ 32–44%	Hot flashes hyperglycemia, hyperuricemia, hepatotoxicity, myopathy, increased serum creatine kinase levels	Lovastatin and nicotinic acid, 20/500 mg to 20/1,000 mg orally daily; extended-release nicotinic acid and simvastatin, 500/20 mg, 750/20 mg, or 1,000/20 mg orally daily
Fibrates	LDL ↓ 5–20% HDL ↑ 10–15% TG ↓ 20–50% Small dense LDL ↓	Dyspepsia, gallstones, hepatotoxicity, myopathy	Fenofibrate, micronized, 43–130 mg orally once daily; fenofibrate, micronized, 67–200 mg orally once daily; fenofibrate, 48–145 mg orally once daily; gemfibrozil, 600 mg orally twice daily; bezafibrate, 200 mg orally twice daily; modified-release bezafibrate, 400 mg once daily
Bile-acid sequestrants	LDL ↓ 10–20% HDL ↓ 1–2% TG possible ↑	Gastrointestinal distress, constipation	Colestyramine, 4–24 g orally daily, two or three times daily, Colestipol Hydrochloride, 5–30 g orally once or twice daily; colesevelam hydrochloride, 1.875–3.75 g once or twice daily
Omega-3 fatty acid	LDL ↑ 5–10% HDL ↑ 1–3% TG ↓ 25–30%	Fishy aftertaste,[a] gastrointestinal disturbances	Omega-3-acid ethyl esters, 4 g orally daily; over-the-counter fish oil; dietary supplements containing omega-3 fatty acid, EPA and DHA, 2–4 g orally daily

[a]Refrigeration will minimize the fishy aftertaste of over-the-counter fish-oil preparations. Abbreviations: ↓, decrease; ↑, increase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, HDL cholesterol level; LDL, LDL cholesterol level; TG, triglyceride level. Reproduced from Hachem SB and Mooradian AD[8] with permission from Wolters Kluwer Health Adis (© Adis Data Information BV 2006. All rights reserved).

6.1. Statins

The use of LDL cholesterol lowering agents such as the 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins is considered to be the standard first-line therapy for the reduction of cardiovascular endpoints including myocardial infarction, stroke and death as shown in primary (Downs *et al.*, 1998; WOSCOPS, 1998) and secondary cohorts (LIPID Study Group, 1998) as well as in patients with diabetes (Colhoun *et al.*, 2004) and hypertension (Sever *et al.*, 2003). Studies have shown that the treatment of hypertriglyceridemic subjects with statins reduces LDL cholesterol levels between 15 and 60 mg/dL, depending on the type and the dose of the statins used (Bakker-Arkema *et al.*, 1996). Furthermore, the statins have also been shown to increase HDL cholesterol by 6% in individuals with below-average HDL and average LDL cholesterol levels

(Downs *et al.,* 1998). In addition, when used with moderate to high doses, statins have been shown to reduce TG levels by 7–30% (Wissler, 1994; Alaupovic *et al.*, 1997; LIPID Study Group, 1998). However, the statin-treated subjects still experience the residual risk factors because of the limited effects of statins in lowering lipid parameters other than LDL cholesterol. Therefore, strategies using various combination therapies have been developed to achieve even lower cholesterol levels (Toth, 2010; Chan & Watts, 2011; Dujovne *et al.,* 2011; Watts & Karpe, 2011).

6.2. Ezetimibe

Further trials were carried out using with ezetimibe, a drug that inhibits the intestinal cholesterol absorption. Ezetimibe, in combination with statins, results in an additional 20% lowering effect on LDL cholesterol (Bruckert *et al.,* 2003; Jackevicius *et al.,* 2008). It also decreases the TG levels by 8% and raises HDL cholesterol by 1 to 4% (Toth, 2010; Katsiki *et al.,* 2013).

6.3. Nicotinic acid

Nicotinic acid (also known as vitamin B3 or niacin) inhibits the lipolysis of adipocytes (Gille *et al.,* 2008), which results in decreased FFA levels, reduced serum VLDL and LDL cholesterol levels along with a slight increase in HDL production rate and decreased catabolism of HDL (Lamon-Fava *et al.,* 2008; Toth, 2010). These changes by niacin subsequently lead to 15%–35% decrease in TG levels and 10%–25% increase in HDL cholesterol concentrations (Chan & Watts, 2011; Klop *et al.,* 2012). However, it has been shown recently that the combination therapy using both statin and niacin in patients with a known history of CVDs and dyslipidemia was not associated with clinical benefits, despite a reduction in fasting TG and increase in HDL cholesterol levels (Boden *et al.,* 2011).

6.4. Fibrates

Several clinical trials have pointed towards the therapeutic roles of fibrates including fenofibrate and bezafibrate in the management of dyslipidemia, mainly because of their ability to reduce serum TGs and non-HDL lipoproteins (Frick *et al.,* 1987; Leaf *et al.,* 1989; Rubins *et al.,* 1999; Keating & Ormrod, 2002; McKeage & Keating, 2011). Fibrates activate the peroxisome proliferator-activated receptor α (PPARα), a transcription factor that modulates the expression of a number of genes involved in various metabolic pathways including lipid

metabolism, ultimately reducing plasma TG concentrations and enhancing HDL levels (Knopp *et al.,* 1987; Mellies *et al.,* 1987; Staels *et al.,* 1998a).Various studies have demonstrated that the combined statin/fibrate therapy is highly effective in achieving a comprehensive lipid control through the normalization of different lipid parameters such as TGs, HDL and total cholesterol concentrations (Fiévet & Staels, 2009; Tenenbaum & Fisman, 2012a & b). However, the doses of both components as well as the lipid profiles of patients on this therapy are closely monitored in view of potential adverse renal and hepatic side effects (Alagona, 2010; Geng *et al.,* 2013).

6.5. Bile acid sequestrants

Bile acid sequestrants (BAS) were traditionally considered as second-line therapy for lowering LDL cholesterol, particularly in patients who are intolerant to statin therapy (Chehade *et al.,* 2013). The BAS are anion exchange resins that bind bile acids in the gastrointestinal tract, preventing their reabsorption across the terminal ileum and thereby lowering their concentration in the enterohepatic circulation (Toth, 2010). BAS can be used in combination with ezetimibe for the patients who cannot tolerate statins. However, BAS therapy is contraindicated in patients with high baseline TG levels (>400 mg/dL), since they aggravate hypertriglyceridemia (Denke & Grundy, 1988; Shao *et al.,* 2011). Furthermore, although BAS reduce the absorption of fat-soluble vitamins, they do not, in general, have direct systemic toxicity.

6.6. *N*-3 fatty acids

The type of the dietary fat is also related with postprandial lipemia (Lopez-Miranda *et al.,* 2007). Various studies have shown that *n*-3 unsaturated FAs reduce VLDL and TGs by decreasing lipogenesis and increasing mitochondrial β-oxidation, ApoB liver degradation and LPL activity (Toth *et al.,* 2009; Zuliani *et al.,* 2009). The hypotriglyceridemic effects of EPA and DHA have been well-established. Epidemiological data have demonstrated that dietary supplementation of 3 to 5 g per day of EPA and DHA leads to about 25% reduction in fasting and postprandial TG levels in normolipidemic subjects and about 50% in hypertriglyceridemic patients (Weintraub *et al.,* 1988; Pownall *et al.,* 1999; Weber & Raederstorff, 2000; Mori & Woodman, 2006; Tenkanen *et al.,* 2006; Skulas-Ray *et al.,* 2008; Zuliani *et al.,* 2009; Vecka *et al.,* 2012; Chehade *et al.,* 2013; Pirillo & Catapano, 2013). Moreover, the combination of *n*-3 PUFAs with statin therapy appears to contribute to

normalize TG levels in patients with combined hyperlipidemia (Bays *et al.,* 2010). Thus, *n*-3 PUFAs has been proposed as second line therapy, either in addition or as an alternative to fibrates and nicotinic acid, in the treatment of severe hypertriglyceridemia (Mooradian, 2009; Zuliani *et al.,* 2009).

It is important to note that the selection of a particular lipid-lowering agent is based on the lipid profile as well as the genetic and metabolic background of the patient. Mixed forms of dyslipidemia particularly require complex combinations, comprising of two or more drugs. The American Heart Association suggests that non-HDL cholesterol (≤130 mg/dL) is a secondary target in patients with serum TG levels of 200-499 mg/dL, with statin used as a drug of first choice. For hypertriglyceridemic subjects with TG levels of 500 mg/dL or more, *n*-3 PUFAs, nicotinic acid or fibrates are primarily used as therapeutic options (Buse *et al.,* 2007; Brinton *et al.,* 2013). In subsequent follow-up, when LDL levels are over 130 mg/dL, statins should be used in the combination therapy. If serum TG levels do not exceed 200 mg/dL BAS may be selected as the pharmacological agent of choice (Hachem & Mooradian, 2006; Mooradian, 2009; Toth, 2010; Chehade *et al.,* 2013). Besides these lipid-lowering agents, the modification in an individual's lifestyle including body weight control, caloric restriction and physical activity also contribute significantly to the management of dyslipidemia and its related disorders.

In summary, dysregulation of lipoprotein metabolism, defined as dyslipidemia, is often associated with the overproduction of TG-rich lipoproteins, low HDL-cholesterol and elevated concentrations of sdLDL particles in the circulation. However, the impaired clearance of these lipoprotein particles due to the disturbances in the activity of lipoprotein receptors could also contribute significantly in the pathogenesis of dyslipidemia. These lipoprotein abnormalities are strongly correlated with obesity and IR, altogether termed as MetS, which constitutes a major risk factor for the pathogenesis of CVDs. These metabolic disorders become more severe causes of morbidity and mortality in elderly population, mainly because of the adverse changes related to the process of aging. The management of dyslipidemia is a cornerstone in the prevention of lipid-related disorders (Cornier *et al.,* 2008; Kolp *et al.,* 2013; Miller *et al.,* 2011). The use of lipid-lowering therapies has been intensively studied for the last decades, with statins emerging as first-line therapy for the patients with dyslipidemia and CVDs. Combination of statins with other lipid-regulating agents, such as ezetimibe, fibrates, niacin and *n*-3 FAs represent optimal ways for treating dyslipidemia. Furthermore, lifestyle modifications including physical activity, weight reduction and dietary

habits, may favorably alter the various risk factors related with MetS and CVDs (Durrington, 2003; Hachem & Mooradian, 2006; Mooradian, 2009; Shao *et al.*, 2011; Chehade *et al.*, 2013).

Objectives of thesis

The apparent number of LSR receptors on the rat liver membranes was found to be negatively correlated with the plasma TG levels during the postprandial phase (Mann *et al.*, 1995), which provided the first evidence that LSR could be rate-limiting for the removal of TG-rich lipoproteins from the circulation. Inactivation of a single LSR allele in mice ($LSR^{+/-}$) demonstrated that reduced expression of LSR leads to delayed clearance of high-fat meal, a 2-fold increase in plasma TG levels during the postprandial phase, and increased weight gain, as compared to $LSR^{+/+}$ control littermates (Yen *et al.*, 2008). These observations were further confirmed by siRNA-mediated suppression of hepatic LSR that caused an increase in postprandial triglyceridemia, accompanied by enhanced ApoB and ApoE levels (Narvekar *et al.*, 2009). Furthermore, obese mice exhibit increased plasma lipids as well as reduced hepatic LSR expression. Taken together, these results suggest that LSR is important in the maintenance of lipid homeostasis in the peripheral system. Interestingly, it was demonstrated that LSR is unable to efficiently bind VLDL isolated from a type III hyperlipidemic patient with the ApoE2/2 phenotype (Yen *et al.*, 1994), which may explain the increased plasma TG in this particular class of hyperlipidemia. It is therefore essential to determine the various factors that might be involved in the regulation of these LSR-mediated effects on lipid status. I participated in a study in which leptin was one of the factors recently identified in our laboratory to regulate LSR at the mRNA and protein levels (Stenger *et al.*, 2010), and decided to further pursue studies on the regulation of LSR expression and protein, focusing specifically on the *n*-3 PUFA DHA, and on the transcription factor PPARα.

In view of this previous work, the main objectives of my thesis were:

- ✓ To determine the effects of DHA supplementation on the modulation of LSR expression at transcriptional and post-transcriptional levels as well as of LSR activity and to investigate the resulting functional consequences in various *in vitro* and *in vivo* models.
- ✓ To identify the presence of putative core promoter elements and various transcription factor binding sites including the peroxisome proliferator response elements (PPREs) using an *in silico* approach to analyze the 5' regulatory regions of mouse, rat and human *lsr* gene.

- ✓ To investigate the potential role of PPARα in the regulation of LSR at the transcriptional and post-transcriptional levels using agonist and antagonist treatments in an *in vitro* cell culture model.
- ✓ To identify how pathways regulated by various transcription factors including PPARα, SREBP1 and LXR and others related to hepatic lipid metabolism, are affected *in vivo* in mice with reduced expression of LSR on a standard or a high-fat diet.

Materials and Methods

1. Animal studies

For some animal studies, wild-type C57BL/6J female or male mice were provided by Janvier Breeding (Le Genest Saint Isle, France). In other studies, $LSR^{+/+}$ and $LSR^{+/-}$ mice were used, since complete inactivation of LSR ($LSR^{-/-}$) proves to be lethal at embryonic stages in mice (Mesli *et al.*, 2004). The reproduction for these mice was carried out in the laboratory by breeding male $LSR^{+/-}$ mice with 3 to 8 months old wild-type C57BL/6J female mice. Litterpups were separated from their parents at the age of four weeks and genotyped at the age of six weeks.

The animals were housed in a pathogen-free, certified animal facility authorized to accommodate transgenic animals with a 12 h light/dark cycle, under controlled temperature of 21 ± 2°C and relative humidity of 50 ± 20%. The mice were provided standard laboratory chow diet and water *ad libitum*. Animal care was in compliance with French State Council guidelines for the use and handling of animals.

1.1. Handling of liver tissue samples

The mice were euthanized and the liver samples were harvested and rinsed in PBS. Part of the liver sample (~100 mg) was preserved in appropriate volume of RNA*later*®-RNA stabilization reagent (Qiagen) as per manufacturer's instruction, stored at 4°C overnight and then stocked at −80°C until used for RNA extraction. The rest of the liver sample was snap frozen in liquid nitrogen and stored at −80°C until used for the preparation of total membranes.

1.2. Genotyping

Mouse tail samples (0.2 cm) were excised and lysed in a buffer containing DirectPCR® Lysis Reagent (Euromedex) and proteinase K (20 mg/mL) (Biosystems). The samples were then incubated overnight at 55°C in a rotating hybridization oven for complete lysis. The crude lysates were then incubated in a water bath at 85°C for 45 min in order to inactivate proteinase K. PCR reaction was performed to amplify either the wild-type or the mutant LSR allele (Mesli *et al.*, 2004) using specific primer pairs. The wild-type LSR allele was detected using a forward (5'-CAGGACCTCAGAAGCCCCTGA-3') and a reverse primer (5'-AACAGCACTTGTCTGGGCAGC-3') located in exons 4 and 5 respectively, leading to

a 773-bp product. The mutant allele lacked this region of the *lsr* gene and was, therefore, detected by the presence of the *neo* gene. Two pairs of neo primers were used: (forward: 5'-GGCGCCCGGTTCTTTTTGTCA-3' and reverse: 5'-TTGGTGGTCGAATGGGCAGGT-3', giving a 281-bp product) and (forward: 5'-GAGGATCTCGTCGTGACCCATG-3' and reverse: 5'-GAGGAAGCGGTCAGCCCATT-3', giving a product of 179 bp). PCR reaction mixture contained 250 µM dNTPs, 1.5 µM $MgCl_2$, 0.5 µM of each primer and 5 units/µL of *Taq* polymerase. For the wild-type LSR gene, the PCR conditions included one initial denaturation step at 94ºC for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 1 min and extension at 72°C for 30 s. To amplify the *neo* gene, PCR conditions included 33 cycles at 95°C for 30 s, 68°C for 1 min, and 72°C for 30 s. In both cases, PCR cycles were preceded by an initial denaturation step at 95°C for 10 min and ended by a final extension step at 72ºC for 7 min. After PCR reaction, 20 µL of the amplified products were loaded on a 1.5% (w/v) agarose gel (Invitrogen) in 0.5X TBE (45 mM Tris base, pH 8.0, containing 45 mM boric acid, and 1 mM EDTA) after mixing with orange loading buffer (1:6), run at 100 V for approximately 30 min and photographed.

2. Cell culture studies

2.1. Hepa1-6 cells

The cell line was derived from a mouse hepatoma, BW7756, which arose in a C57/L mouse. These are adherent, epithelial-like cells, growing as monolayers with a doubling time of about 30 h (Figure 19). These cells express LSR and have been used previously as an *in vitro* cell model to determine the interactions of LSR with different molecules such as leptin and lactoferrin (Stenger *et al.,* 2010; Ahmad *et al.,* 2012). The cells also express various transcription factors involved in lipid metabolism, namely PPARα, SREBP-1 & 2 and LXRs, which makes them an interesting model for *in vitro* experiments, and this can be further extrapolated to *in vivo* experiments using mice.

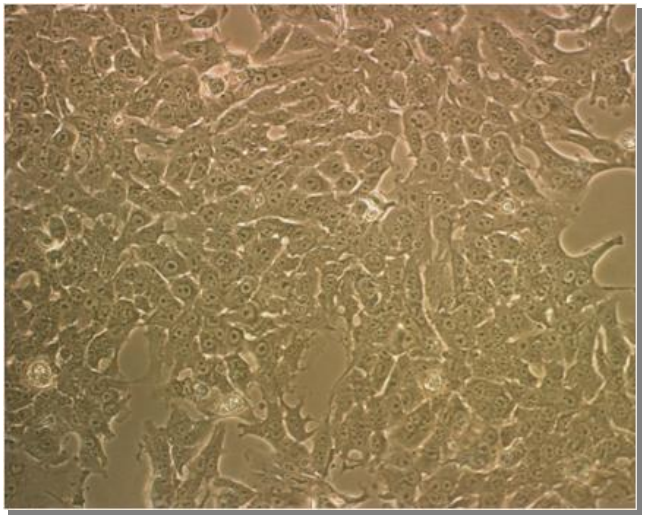


Figure 19: Morphology of Hepa1-6 cells (magnification: 100X)

2.2. HepG2 cells

Another cell line was used for some experiments in parallel with Hepa1-6. The cell line was established in 1975 from the liver tissue of a 15-year-old Argentine boy with a well-differentiated hepatocellular carcinoma. These are adherent, epithelial-like cells, growing as monolayers and in small aggregates with a doubling time of 50-60 h (Figure 20). No hepatitis B virus genome was detected in these cells. HepG2 cells synthesize and secrete various apolipoproteins A-I, A-II, B, C-II, C-III, E (Rash *et al*., 1981; Zannis *et al*., 1981; Dashti & Wolfbauer, 1987) as well as LSR, which makes them an appropriate cell model to study lipoprotein metabolism.

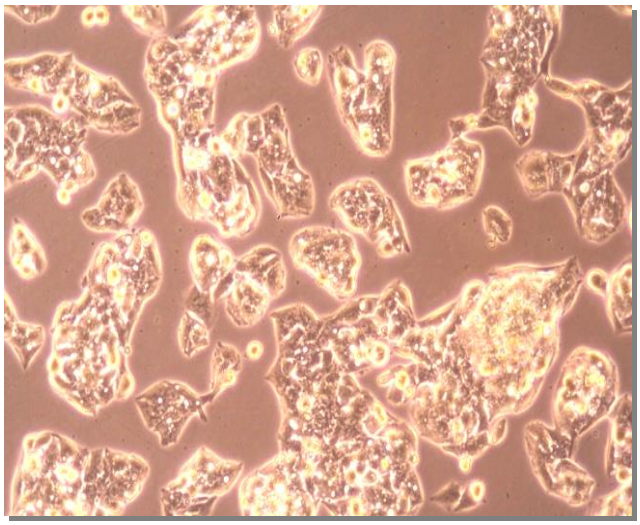


Figure 20: Morphology of HepG2 cells (magnification: 100X)

3. Cell culture maintenance

Mouse hepatoma (Hepa1-6) and human hepatoma (HepG2) cells were obtained from the cell repository Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Ref. 41965-039) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) and grown in a 95% air-5% CO_2 humidified atmosphere at 37°C. Only Hepa1-6 cells at low passages (<10) were used for the experiments. For cell culture treatments, the cells were plated at 100,000 or 200,000 cells/well in 12 or 6-well plates (BD Falcon™) for protein and RNA extraction later on. However, the cells were plated at 50,000 cells/well in 24-well plates with or without cover slide inserts for immunofluorescence analysis or cell viability assay respectively. After 48-h seeding, the cells were washed with 1X phosphate buffered saline (PBS 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and treated with different molecules according to specific procedures described in the respective chapters of Results & Discussion. DHA, WY-14,643, bezafibrate and GW6471 were purchased from Sigma-Aldrich and their stock solutions were prepared in DMSO.

3.1. Preparation of LPDS

LPDS was prepared (d1.25 g/mL) by adding 35.82 g NaBr/100 mL of FBS or fresh pooled human plasma and subjected to overnight centrifugation at 4°C, 228,850 x *g* for 24 h. The top layer containing lipoproteins (VLDL, LDL and HDL) was removed carefully and discarded, while the bottom fraction was collected and dialyzed using Spectra/Por® dialysis membrane with a molecular weight cut-off value of 3,500 Da (Spectrum laboratories) at 4°C against 1 L Buffer A (0.15 M NaCl containing 0.24 mM EDTA, pH 7.4). Dialysis buffer was changed 4 times over a 24 h period. LPDS was then filtered with 0.2 µm (Acrodisc® Syringe Filters-Pall Corporation), aliquoted and stored at −20°C.

3.2. Recovery of cell samples

3.2.1. Extraction of total proteins

For western blot analysis, the total cell proteins were extracted using RIPA buffer. The cells were placed on ice and recovered by scraping in the presence of ice-cold 1X RIPA buffer (0.5 M Tris-HCl, pH 7.4, containing 1.5 M NaCl, 2.5% (w/v) deoxycholic acid, 10%

(v/v) NP-40 and 10 mM EDTA; Millipore) supplemented with protease inhibitor cocktail (Roche; pancreas-extract 15 µg/mL; pronase 1.5 µg/mL; thermolysin 0.8 µg/mL; chymotrypsin 1.5 µg/mL; trypsin 0.2 µg/mL; pH 7.8; papain 0.001 µg/mL; pH 6.5) and completed with 10 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM sodium orthovanadate (Na_3VO_4). The cell lysates were incubated on ice for 30 min and then centrifuged at 13,000 x *g* for 30 min (4°C). The supernatant was collected and stored at −20°C. The protein concentration was determined using the BCA method.

3.2.2. Preparation of nuclear extracts

Nuclear extracts were prepared by hypotonic lysis method as described by Baer *et al.* (1998). Hepa 1-6 ($3x10^6$ cells per 100 mm Petri plate) and HepG2 ($4x10^6$ cells per 100 mm Petri plate) cells were incubated in serum-deficient medium (SDM; high-glucose DMEM only) or complete medium (CM; high-glucose DMEM supplemented with 10% (v/v) serum) for 48 h. After incubation period, the cells were scraped, washed once with PBS and resuspended in 1 mL hypotonic lysis buffer (20 mmol/L Hepes, pH 7.9, 1 mmol/L EDTA, 10 mmol/L NaCl, 1 mmol/L dithiothreitol (DTT), 0.4 mmol/L PMSF and protease inhibitor cocktail (Roche)). The cells were then incubated on ice for 20 min, lysed by pipetting and centrifuged at 14,000 x *g* for 20 s. The supernatants containing cytoplasmic extracts were stored at −80°C. Proteins were extracted from the nuclear pellet by incubation with 500 µL ice-cold hypertonic extraction buffer (420 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Hepes, pH 7.9, 25% glycerol, 1 mmol/L DTT, 0.4 mmol/L PMSF and protease inhibitor cocktail (Roche)) at 4°C, with vigorous shaking for 20 min. The extracts were then centrifuged at 14,000 x *g* for 5 min to pellet the nuclear debris and the supernatant containing nuclear proteins was collected and stored at −80°C. The protein content was measured using BCA kit.

4. Preparation of mouse liver total membranes

The liver samples frozen in liquid nitrogen were weighed and finely ground in liquid nitrogen using a mortar and pestle. A calculated amount (4.5 mL/g liver) of 0.25 M sucrose with 5 mM Tris and 1 mM $MgCl_2$, pH 7.4 (STM) containing a 1:100 dilution of protease inhibitor cocktail (Roche) and a 1:100 dilution of 0.4% (w/v) PMSF, were added to the liver samples. After additional grinding of this mixture, the material was transferred to a 15-mL tube and centrifuged for 10 min at 280 x *g* and 4°C to remove cell debris and nuclei. The

supernatant was transferred into a 50 mL conical tube and recentrifuged at 4°C (1,500 x *g*, 15 min). The supernatant (non-membrane fraction) was stored at −80ºC and the membrane pellet was resuspended with 10 mL of membrane washing buffer (20 mM Tris containing 2 mM EDTA, pH 7.4) and centrifuged at 4°C (1,500 x *g*, 15 min) as a washing step. The pellet obtained was resuspended in 1 mL of membrane washing buffer, transferred to 1.5 mL centrifuge tubes, placed on ice and sonicated for 1 min, 5 Hz at 30% cycle (Vibracell) to homogenize membrane preparations. These samples were then aliquoted and stored at −80°C. The protein concentration in each sample was determined by Lowry method.

5. Protein quantitation assays

5.1. BCA method

The total protein in cell lysates was determined using a kit based on the BCA method (Pierce®, Thermoscientific). This method involves the reduction of Cu^{+2} to Cu^{+1} by the peptide bond under alkaline conditions, followed by the chelation of two molecules of bicinchoninic acid (BCA) with one cuprous ion (Cu^{+1}) (Smith *et al.*, 1985). This results in the formation of a purple-colored complex which absorbs strongly at 562 nm. The protein concentration of the samples was calculated using bovine serum albumin (BSA) as a standard with known concentrations of 0, 100, 250, 500, 750, 1000, 1500 and 2000 µg/mL, diluted in 1X PBS. In a microplate, 25 µL of each of the standards and the protein samples were added, followed by the addition of 200 µL of the BCA working reagent, prepared as recommended by mixing 50 parts of reagent A (containing 1% (w/v) sodium bicinchoninate (BCA), 2% (w/v) sodium carbonate, 0.16% (w/v) sodium tartrate and 0.95% (w/v) sodium bicarbonate in 0.1 M sodium hydroxide) with 1 part of reagent B (containing 4% (w/v) cupric sulfate). The sample plate was then incubated at 37°C for 30 min and read at 570 nm in a microplate reader (Fluostar Galaxa; BMG Labtech France).

5.2. Lowry method

This method (Lowry *et al.*, 1951) involves the reactivity of the peptide bonds with the Cu^{+2} ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu reagent phosphomolybdic-phosphotungstic acid to heteropolymolybdenum blue by the

copper-catalyzed oxidation of aromatic acids. The colour intensity of the resulting complex is measured by absorbance at a wavelength of 750 nm.

The protein concentration of the samples was calculated (duplicate analysis) using BSA as a standard with known concentrations of 0, 5, 10, 15, 20 and 25 µg in a total volume of 100 µL 1X PBS. The samples were diluted in 1X PBS and 1 mL of reagent C, composed of 99% of reagent A [2% (w/v) Na_2CO_3, containing 0.16% (w/v) Na-tartrate, 0.2% (w/v) SDS, and 0.4% (w/v) NaOH] and 1% of reagent B [4% (w/v) $CuSO_4$-$5H_2O$], was added in each sample tube. The tubes were vortexed and incubated for 10 min at room temperature. Finally, 100 µL of reagent D (Folin Ciocalteus diluted 1:1 (v/v) in H_2O) was added to each tube while vortexing and then incubated at room temperature for 30 min. An aliquot of 200 µL of each sample was transferred to a 96-well plate and the absorbance was read at 750 nm using a microplate reader.

6. Western blotting

6.1. Electrophoresis

Proteins were separated by electrophoresis on a polyacrylamide gel under denaturing conditions. The samples were denatured at 95ºC for 5 min in Laemmli buffer (125 mM Tris-HCl, pH 8.0 containing 4% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) β-mercaptoethanol, and 0.004% (w/v) bromophenol blue). The gel with 1.5-mm thickness was prepared with the resolving gel of 8-12% polyacrylamide (depending upon the molecular mass of the protein of interest) in buffer containing 1.5 M Tris-HCl, pH 8.8, 10% (w/v) SDS, 10% APS (ammonium persulfate) and TEMED (N, N, N', N'-tetramethyl-ethylenediamine). The stacking gel of 5% was prepared in buffer containing 0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% APS and TEMED. For each Western blot, the same amount of protein (15-30 µg) was loaded into each well, in parallel with an aliquot of Precision Plus Protein^{TM} (Bio-RAD) as marker. The electrophoresis was carried out in 1X running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS) at 50 V for 15 min to allow the samples to completely migrate through the stacking gel and then at 120 V for approximately 90 min to migrate through the resolving gel.

6.2. Protein transfer onto nitrocellulose membrane

After electrophoresis, the proteins were transferred onto nitrocellulose membrane (Amersham™ Hybond™-ECL, GE Healthcare). For this, the gel and the membrane were soaked in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS and 20% (v/v) methanol) for 2-3 min. A sandwich was formed by two Whatmann™ papers pre-soaked in transfer buffer, the membrane, the gel and two other Whatmann™ papers pre-soaked in transfer buffer. The entire assembly was then placed in a vertical wet transfer system (Bio-RAD) and the electrotransfer was performed at 350 mA for 45 min at a constant voltage of 250 V (Towbin *et al.*, 1979).

6.3. Immunodetection

After electrotransfer, the efficiency of transfer and control for loading was assessed by Ponceau-S red (Sigma-Aldrich) staining. The nitrocellulose membranes were then washed with TBST buffer (20 mM Tris-HCl, pH 7.4,150 mM NaCl, 0.1% (v/v) Tween-20) to remove the dye. Nonspecific sites of the membrane were saturated in blocking buffer (5% (w/v) fat-free powdered milk or 5% (w/v) BSA in TBST buffer). The membrane was incubated with slow shaking in the presence of primary antibody diluted in blocking buffer. After three washes with TBST buffer for 5 min, the membrane was incubated for one hour at room temperature with the respective secondary antibody coupled with horseradish peroxidase (HRP-linked antibody). β-tubulin was used as loading control for cell lysates. The details of antibodies and the experimental conditions are described in Table 8. The bands were detected by chemiluminescence using the ECL-Western blotting kit (Amersham Biosciences) following the manufacturer's instructions and the images were captured using Fusion FX5 image acquisition system (Vilber Lourmat). Densitometric analysis was performed using the software *ImageJ* (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/download.html).

Table 8: Description of antibodies and western blot conditions

Antibody	Apparent mass (kDa)	Blocking conditions	Incubation conditions	Dilution used	Supplier	Catalogue number
Primary antibodies						
Anti-LSR ***(Produced in rabbit)***	56-68	5% milk in TBST	2 h at room temperature or overnight at 4°C	1:1000	Sigma-Aldrich	HPA007270
Anti-PPARα (H-98) ***(Produced in rabbit)***	55	5% BSA in TBST	Overnight at 4°C	1:500	Tebu-Bio	036SC-9000
Anti-LDL-R ***(Produced in rabbit)***	160	5% milk in TBST	Overnight at 4°C	1:500	Abcam	ab30532
Anti-β-tubulin ***(Produced in mouse)***	55	5% milk in TBST	2 h at room temperature or overnight at 4°C	1:1000	Sigma-Aldrich	T5201
Secondary antibodies						
Anti-rabbit IgG	-	5% milk/ BSA in TBST	1 h at room temperature	1:2000	Cell Signaling	7074
Anti-mouse IgG	-	5% milk in TBST	1 h at room temperature	1:2000	Cell Signaling	7076

7. Cell viability assay

The effects of DHA treatment on cell viability were evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) colorimetric assay, which detects the activity of mitochondrial dehydrogenases of living cells to reduce MTT to a water-insoluble blue formazan product (Mosmann, 1983). After 48 h, the cells were treated with various concentrations of DHA *i.e.* 5, 10, 25, 50 and 100 µM (as described in detail in Results and Discussion, section I). After 24 h incubation at 37°C in the presence of DHA, each well was incubated with 100 µL of MTT (5 mg/mL in PBS; final concentration of MTT: 2 mM) at 35°C for 10 min in a 95% air-5% CO_2 atmosphere. The medium containing MTT was removed and 450 µL of DMSO was added in each well. The cells were incubated for 10 min at room temperature with maximum shaking. Aliquots of 100 µL from each well were added to a 96-well plate with DMSO as blank and the absorbance was read at 570 nm in a microplate reader.

8. LSR activity evaluation

8.1. Preparation of human LDL

LDL fraction was isolated from pooled fresh human plasma by sequential ultracentrifugation. In order to ensure a pure fraction of LDL, which is normally between d1.019 and d1.063 g/mL, the cut-offs used for centrifugation were d1.025 and d1.055 g/mL. The density of the plasma was adjusted to 1.025 by adding 2.518 g NaBr/100 mL plasma. The d1.025 g/mL plasma was transferred to thick-walled, polycarbonate tubes (Beckman Coulter, USA) and spun at 4°C, 228,850 x *g* for 24 h in a fixed-angle MLA-80 Beckman rotor in an Optima™ MAX-E Benchtop Ultracentrifuge (Beckman Coulter, USA). The top layer containing VLDL and IDL (d1.006-1.025 g/mL) was carefully removed using a syringe with a curved needle and discarded. The density of the recovered fraction was then adjusted to 1.055 g/mL by adding 4.036 g NaBr/100 mL and centrifugation was then performed at 4°C for 24 h at 228,850 x *g* in the MLA-80 rotor. The top fraction (d1.025-1.055) containing LDL was isolated using a syringe with a curved needle, concentrated by adding 5.132 g NaBr/100 mL to the recovered fraction, and then spun again at 4°C for 24 h at 228,850 x *g*. The concentrated LDL in the form of a thick slurry was removed with a spatula and Pasteur pipette, and then transferred into Spectra/Por® dialysis membrane with a molecular weight cut-off value of 3,500 Da (Spectrum laboratories) for dialysis at 4°C against 1 L Buffer A (0.15 M NaCl containing 0.24 mM EDTA, pH 7.4). Dialysis buffer was changed 4 times over a 24 h period. After dialysis, LDL was filtered (0.2 µm), stored at 4°C in the dark and used within 4 days. Protein concentration was determined by Lowry method as described in this chapter, section 5.2.

8.2. Fluorescent labeling of LDL

LDL was labeled using DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) which is a lipophilic dye that is used to label the neutral lipid core of lipoproteins. This has been used to determine the activity of lipoprotein receptors, including the LDL-R (Stephan & Yurachek, 1993) and LSR (Yen *et al.*, 2008). The stock solution of DiI was prepared by dissolving 3 mg DiI (Sigma-Aldrich) in 1 mL DMSO. Labeling was performed by incubating 10 mg LDL with 500 µL DiI (50 µL/mg LDL protein) and 20 mL LPDS (2 mL LPDS obtained from human plasma/mg LDL protein) for 12 h at 37°C in the dark. LPDS was used as a source of lipid transfer protein *i.e.* cholesteryl ester transfer protein (CETP), which

facilitates the incorporation of DiI into the lipoproteins. The density of DiI-labeled LDL was adjusted to 1.063 g/mL by adding 7.698 g NaBr/100 mL, followed by centrifugation at 228,850 x *g* for 24 h at 4°C. The top layer containing DiI-LDL was carefully removed and then transferred into Spectra/Por® dialysis membrane with a molecular weight cut-off value of 3,500 Da for dialysis at 4°C in the dark against 1 L Buffer A (0.15 M NaCl containing 0.24 mM EDTA, pH 7.4). Dialysis buffer was changed 4 times over a 24-h period. After dialysis, LDL was filtered (0.2 µm) and used immediately. The protein content was measured by Lowry method.

8.3. Modification of LDL using cyclohexanedione

The ability of LDL to bind LDL-R was abolished by modifying the arginine residues of ApoB of the lipoprotein with cyclohexanedione (CHD; Sigma-Aldrich). For this, 5 mg DiI-labeled LDL was diluted in 1 mL buffer A, with subsequent incubation in 2 mL 0.15 M CHD in 0.2 M sodium borate (pH 8.1) at 37°C for 2 h in the dark (Shepherd & Packard, 1986). The CHD-modified DiI-LDL was then desalted to remove excess CHD by applying on a PD10 column (GE Healthcare). The column was washed in buffer A, and elution was also performed using buffer A. The different CHD-modified DiI-LDL fractions were collected and protein concentration was measured by Lowry method.

8.4. LSR activation and uptake studies using CHD-modified DiI-LDL

Hepa1-6 cells were plated at 50,000 cells/well in 24-well plates containing cover slide inserts and treated with various concentrations of DHA, as described in the section 3. After 24 h incubation at 37°C with DHA, the cells were washed twice with 1X PBS and the medium was replaced with 500 µL/well of the freshly prepared and filter-sterilized (0.45 µm; Acrodisc® Syringe Filters-Pall Corporation) DMEM containing 5 mM Hepes, 0.2% (w/v) BSA (pH 7.5) and 3.7 g/L $NaHCO_3$. A 400 mM stock solution of oleate (Sigma-Aldrich) was prepared in isopropanol. Uptake studies were performed by incubating Hepa 1-6 cells first with 0.5 mM oleate for 3 min in order to allow time for activation of LSR. Care should be taken while adding oleate, so as to inject it directly into the centre of the pool of DMEM, created by tilting the plate and then rotating the plate immediately after addition of the fatty acid. The cells were then incubated with 5 µg/mL DiI-labeled CHD-LDL (filtered using 0.2 µm) for 2 h at 37°C. The wells without oleate were also included as controls for

background and treated with the same amount of isopropanol (1% final concentration). The experiment was performed in duplicate.

8.5. Immunofluorescence

After treatments, cells were placed on ice, washed two times consecutively with ice-cold PBS/0.2% (w/v) BSA for 10 min and then once with ice-cold PBS/0.2% (w/v) BSA for 5 min followed by two consecutive washes with ice-cold PBS. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then washed repeatedly (4 times) with PBS. Blocking was performed with a solution of PBS/10% (w/v) BSA for 1 h followed by incubation with LSR antibody (1:200 dilution; Sigma-Aldrich) for 2 h at room temperature. The cells were then washed three times for 5 min with PBS and then once with PBS/1% BSA for 5 min prior to the incubation with a secondary anti-rabbit IgG antibody (1:2,000 dilution) conjugated with Alexa Fluor® 488 (A21206; Invitrogen Molecular Probes®) for 1 h at room temperature. After incubation, the cells were washed three times with PBS for 5 min and the cell nuclei were stained by incubation with 4′,6-diamidino-2-phenylindole (DAPI) (2 mg/mL; 1:200 dilution) for 15 min at room temperature. After washing three times with PBS for 5 min, the cover slips were removed and mounted on slides using Fluoromount as mounting medium (F4680; Sigma-Aldrich). After drying overnight at 4°C, the cells were observed with the confocal microscope (Fluoview FV10i, Olympus) and photographed. Images were taken from at least four different fields per slide with almost the same number of cells and analysed using *Wright Cell Imaging Facility (WCIF) ImageJ* software (University Health Network Research, Canada; http://www.uhnres.utoronto.ca/facilities/wcif/index.htm).

9. Lipid extraction and analysis

9.1. Determination of fatty acid profile in plasma and liver tissue samples

9.1.1. Sample preparation

Liver tissue samples (20-30 mg) were ground in a mortar and pestle submerged in liquid nitrogen. The ground tissues were then suspended in a 35-mL centrifuge tube containing 2 mL of 1X PBS buffer and 15 mL of extraction solvent, *i.e.* chloroform:methanol (2:1). Each tube was vortexed for exactly 1 min and then centrifuged at 863 x *g* for 10 min. The upper aqueous

layer was removed carefully along with the tissue fragments at the interface and the lower organic phase was conserved at –20°C until analysis. The organic solvent was removed from the samples by dry evaporation under nitrogen flux just before fatty acid profile analysis.

9.1.2. Analysis of fatty acid profiles in erythrocytes and liver tissue

Fatty acid profiles of various extracts were analyzed after direct transmethylation, extraction and purification of methylated derivatives prior to separation by gas chromatography (Lepage & Roy, 1986). After mixing the blood samples (plasma or erythrocytes) with a methanol:dichloromethane (3:1) solution or after resuspending the evaporated extracts in this solution, methylation was performed for 1 h at 100°C in the presence of acetylchloride. The derivatives were extracted with hexane and purified from the organic upper phase with acetonitrile. The resulting organic phase was evaporated under nitrogen flux, before the resuspension of methylated derivatives in hexane and injection into the chromatograph (Chrompack CP 9001) using a DB23 (Agilent Technologies) column (50%-cyanopropylphenyl)-methyl-polysiloxane, 30 m, 0.32 mm internal diameter, 0.25 mm film). Methylated fatty acids were detected by flam ionization. Integration of the peaks was performed by using the Millenium software (Waters).

For calibration, reference chromatograms were recorded after injection and separation of 10 fatty acid standards, *i.e.* palmitic, palmitoleic, stearic, oleic, linoleic, α-linolenic, arachidonic, eicosapentaenoic, docosatetraenoic and docosahexaenoic acids (all purchased from Sigma), methylated as described above and used at various concentrations ranging from 25 µg to 100 µg each per tube in order to define a standard curve. An internal standard ensured the reproducibility of the extraction and derivatization steps.

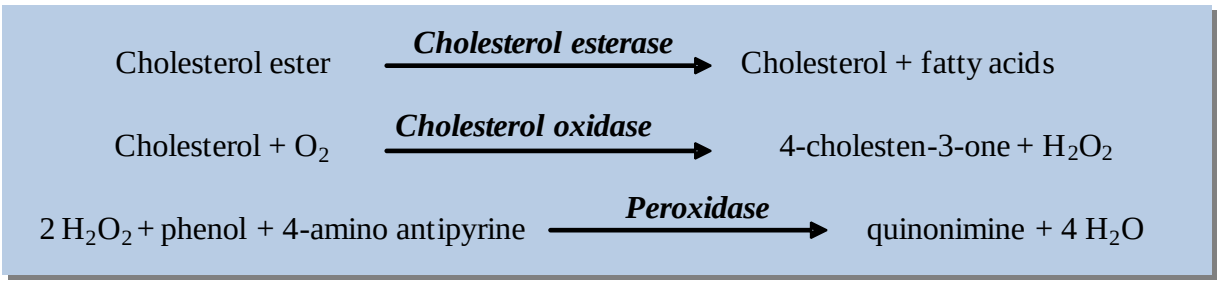
Results obtained are the amounts of each fatty acid in the tube, but were calculated and presented as the percentage of each fatty acid in the extract, assuming that the ten fatty acids followed here represent 100%.

The fatty acid analyses have been performed by M.C. Escanyé at the CHRU Nancy, Central Hospital (Service de Biochimie et Biologie Moléculaire, head: Prof. J.L Olivier).

10. Measurement of plasma cholesterol and TGs

10.1. Plasma cholesterol measurement

Cholesterol measurements were performed using an enzymatic kit according to manufacturer's instructions (BioMérieux). The reaction involves a series of enzymatic reactions, producing a colored complex, quinonimine. The intensity of coloration is measured at a wavelength of 505 nm and is proportional to the quantity of cholesterol present in the samples. The enzymatic reactions leading to the production of quinonimine are as follows:



For each measurement, calibration was done using a multi-parameter calibrator, Calimat (BioMérieux) and a serum control, Unitrol (BioMérieux). Two microliters of each sample, blank (water), Calimat and Unitrol were added to a 96-well microplate, followed by addition of 200 µL of the working reagent containing the reagents 1 and 2 (Table 9).

Table 9: Composition of reagents used in the measurement of plasma cholesterol

Reagents	Composition	
Reagent 1	Phosphate buffer	0.1 mM
	Phenol	15 mM
	Sodium cholate	3.74 mM
Reagent 2	4-aminoantipyrine	0.5 mM
	Peroxidase	≥1000 U/L
	Cholesterol oxidase	≥200 U/L
	Cholesterol esterase	≥125 U/L

The plates were incubated with shaking for 10 min at 20-25ºC and absorbance was then measured at 505 nm. The sample concentration [sample] was measured by using the following formula:

[sample] = (OD sample / OD standard) x n where n = [standard]

10.2. Plasma TG measurement

Triglyceride levels were determined using an enzymatic kit (BioMérieux, Table 10), which allows the measurement of the total glycerol in the samples. The reaction involves a series of enzymatic reactions, producing a colored complex, quinonimine, whose intensity, measured as indicated in the section 10.1, is proportional to the quantity of glycerol liberated after hydrolysis of triglycerides by lipase. The enzymatic reactions leading to the production of quinonimine are as follows:

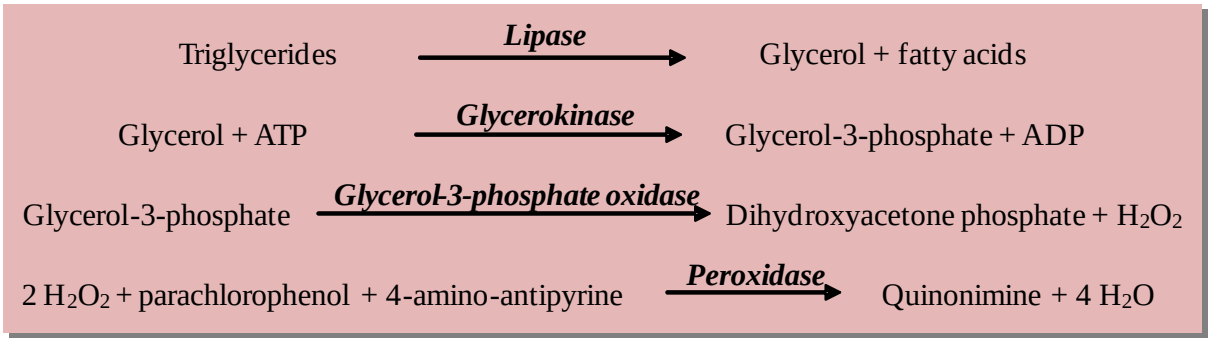


Table 10: Composition of reagents used in the measurement of plasma triglycerides

Reagents	Composition	
Reagent 1	Tris buffer (pH 7.6)	100 mM
	Parachlorophenol	2.7 mM
	Magnesium	4 mM
Reagent 2	4-aminoantipyrine	0.4 mM
	Lipase	≥1000 U/L
	Glycerokinase	≥200 U/L
	Glycerol-3-phosphate oxidase	≥2000 U/L
	Peroxidase	≥200 U/L
	Adenosine triphosphate	0.8 mM

The plates were then incubated on a gentle shaker for 10 min at 20-25ºC and absorbance was then measured at 505 nm. The sample concentration was measured by using the same formula as described for cholesterol measurement:

[sample] = (OD sample / OD standard) x n where n = [standard]

11. RNA extraction

11.1. From cell lysates

For RNA extraction, the cells were scraped in 350 µL RLT lysis buffer (Qiagen) containing β-mercaptoethanol (10 µL/mL lysis buffer) and the cell lysates were stored at −20°C. Total RNA was extracted from Hepa 1-6 cells by using RNeasy Plus Mini Kit (Qiagen) as per manufacturer's instructions. The cell lysates were vortexed for 30 s to dissociate the cells and then applied to gDNA eliminator spin columns to remove genomic DNA by centrifugation at 8,000 x *g* for 30 s. The genomic DNA remains attached to the column membrane while the solution containing RNA passes through the column and is called flow-through. A volume of 350 µL of 70% ethanol was added to the flow-through and transferred to an RNeasy spin column, followed by centrifugation at 8,000 x *g* for 15 s. The spin column membrane was then washed once with 700 µL RW1 and twice with 500 µL RPE buffer by centrifugation at 8,000 x *g* for 15 s. An additional centrifugation was performed at 13,000 x *g* for 2 min to dry the column membrane and the total RNA was eluted in 60 µL RNase-free water.

11.2. From liver samples

Frozen liver tissue samples weighing between 30 and 40 mg were homogenized each in 1 mL QIAzol Lysis Reagent (Qiagen) using an ultraturax. The probe of ultraturax, stored in 2 M NaOH, was rinsed twice in 70% ethanol and then twice in ultrapure water before grinding each tissue sample. The tissue fragments were mixed in the lysis solution until complete homogenization was achieved.

Total RNA was extracted from liver tissues using the RNeasy® lipid tissue mini kit (Qiagen). The homogenized samples were placed at room temperature for 5 min and then vortexed vigorously for 15 s after adding 200 µL of chloroform. After 2-3 min at room

temperature, the homogenates were centrifuged at 12,000 x *g* for 15 min at 4°C, resulting in the partitioning of samples in three phases: an upper colorless aqueous phase containing RNA, a white interphase containing DNA and a red lower organic phase containing protein. After transferring the upper aqueous phase to a clean tube, 600 µL of 70% ethanol was added in order to provide binding conditions needed. Up to 700 µL of the sample was loaded to an RNeasy Mini spin column, followed by centrifugation at 8,000 x *g* for 15 s at room temperature. The column was washed once with 700 µL RW1 and once with 500 µL RPE by centrifugation at 8,000 x *g* for 15 s. To avoid ethanol carry-over during RNA elution, the column was finally washed with 500 µL RPE by spinning at 8,000 x *g* for 2 min. An additional centrifugation was performed at 13,000 x *g* for 1 min to dry the column membrane and the total RNA was eluted in 60 µL RNase-free water.

11.3. RNA quantitation and gel electrophoresis

The concentration of total RNA was determined by biophotometer (Eppendorf) at 260 nm while the RNA purity was estimated by calculating the 260 to 280 nm ratio (since proteins absorb strongly at 280 nm); samples with a value greater than 1.7 were used for subsequent analyses.

The quality of RNA obtained was further verified by loading 1 µg of RNA on 1% (w/v) agarose gel (Invitrogen) in 0.5X TBE buffer. One-sixth of the final volume of loading buffer (60 mM Tris-HCl, pH 7.5 containing 60 mM EDTA, 60% (w/v) glycerol, bromophenol blue, xylene cyanol FF, and EvaGreen®; Jena Bioscience) was added to each sample and the gel was run at 100 V for approximately 1 h before analyzing the images under UV light using VersaDoc™ imaging System. The presence of two sharp bands for 28S and 18S, with 28S band twice as intense as 18S band, was indicative that the RNA was intact and suitable for gene expression studies.

12. RT-PCR

12.1. Reverse transcription (RT)

Ten micrograms of total RNA obtained after extraction were used to synthesize cDNA in a final volume of 50 µL. In a reaction mixture containing random hexamers (50 ng/µL) and deoxynucleotide triphosphates (dNTPs, 0.5 mM), RNA was denatured for 5 min at 65ºC and

then placed on ice for 1 min. The mixture was completed to 1X final concentration of RT buffer and contained 5 mM $MgCl_2$, 10 mM dithiothreitol, 2 U/µL RNase OUT inhibitor and 2 U/µL SuperScript™ II reverse transcriptase. It was incubated at 42ºC for 5 min, and then at 50ºC for 50 min, followed by a final incubation at 70ºC for 15 min. The cDNA obtained was aliquoted and stored at −80ºC. All the reagents used in these reactions were obtained from Invitrogen.

12.2. End-point PCR

Duplex PCR reaction was performed to amplify total LSR in the liver samples of DHA+ and DHA− mice obtained from long-term supplementation experiment (Results and Discussion, Chapter I, section 2.3.5). An aliquot of 2 µL of the cDNA solution obtained after reverse transcription was used for PCR as template. The reaction mixture contained 1.5 mM $MgCl_2$, 250 µM dNTPs, 0.05 and 0.5 µM of a house-keeping gene, hypoxanthine-guanine phosphoribosyl-transferase (Hprt) and LSR primers respectively and 0.1 units/µL of *Taq* polymerase. The PCR conditions were: initial denaturation at 94ºC for 1 min (or 3 min for the initial step), annealing at 56ºC for 1 min and extension at 72ºC for 1 min, followed by 25 cycles with a final extension step at 72ºC for 7 min. After PCR, the reaction was held at 4ºC until used.

12.3. Agarose gel electrophoresis

Ten µL of PCR-amplified products were loaded on a 2.0% (w/v) agarose gel in 0.5X TBE after mixing with orange loading buffer (1:6) and run at 80 V for approximately 45 min. Fragment sizes were estimated using a 50-bp DNA step ladder (Invitrogen).

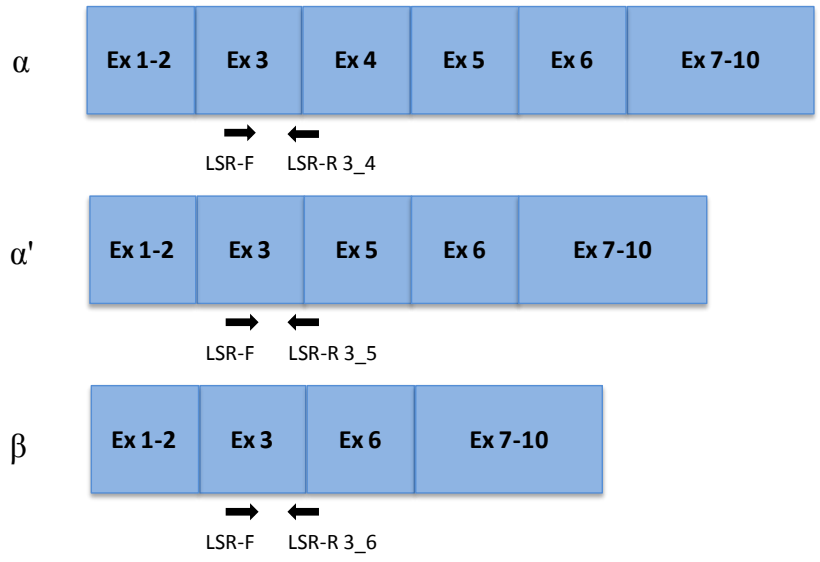
13. qPCR primer design

Quantitative real-time PCR (qPCR) primers were designed in order to amplify mouse LSR, taking Hprt as reference gene (***Table 11***). The sequences of LSR (Accession no. NM_017405.2) and Hprt (Accession no. NM_013556.2) were obtained from the NCBI nucleotide databank (http://www.ncbi.nlm.nih.gov/). The primer pairs were synthesized based on these sequences using Primer Express software (Yen *et al*., 2008). Mouse PPARα (Accession no. NM_011144.6) primer sequences were obtained from Muoio *et al*., (2002). All primer pairs were validated and then used for the SYBR Green dye-based qPCR analyses.

Table 11: Characteristics of the primers used for qPCR amplification

Gene	Primer	Sequence (5'-3')	Location	Amplicon length (bp)
LSR ***(all subunits)***	Forward	CAGGAGAATCACCATCACAGGAA	Exon 2	77
	Reverse	AGTAATACACTCCACTGTCTCCCCAG	Exon 3	
LSR-F ***(individual subunits)***	Forward	AAGATCTGGATGGGAACAACGAG	Exon 3	
LSRα-R 3_4	Reverse	CTTCTGAGGTCCTGCCAAGG	Exons 3-4	63
LSRα'-R 3_5	Reverse	CAAAGAGCCAATCAAGGACAATG	Exons 3-5	60
LSRβ-R 3_6	Reverse	CCAGCAGCATAAACAAGGACAAT	Exons 3-6	61
PPARα	Forward	ACGATGCTGTCCTCCTTGATG	Exon 7	
	Reverse	GTGTGATAAAGCCATTGCCGT	Exon 7	67
Hprt	Forward	TCAGACTGAAGAGCTACTGTAATGATCA	Exon 3	
	Reverse	AAAGTTGAGAGATCATCTCCACCAA	Exon 4	76

The qPCR primers, spanning exon junctions, were designed in order to amplify the three mouse LSR subunits, *i.e.* α, α' and β (Figure 21). Mouse *lsr* gene is comprised of ten exons (NM_017405.2) with LSRα being the largest subunit containing all the exons, LSRα' lacking exon 4 and LSRβ lacking exons 4 and 5. This information was taken into account while designing primers for SYBR Green dye-based qPCR amplification of the individual subunits. The details of the primers are provided in (Table 11).



'Ex' indicates Exon number

Figure 21: Schematic representation of primer design for mouse LSR mRNAs.

Real-time qPCR primers were designed for the amplification of the three LSR mRNAs, with the forward primer (LSR-F) always positioned at exon 3. LSRα reverse primer (LSR-R 3_4) spans the junction between exons 3 and 4, LSRα' reverse primer (LSR-R 3_5) is located at the junction between exons 3 and 5 while LSRβ (LSR-R 3_6) reverse primer spans the junction between exon 3 and exon 6.

13.1. Primer validation with a standard curve

In order to give reliable results, a real-time qPCR assay should have 100% amplification efficiency. This means that with each cycle, the amount of PCR product should double, allowing the slope of the standard curve being –3.33 (100% efficiency). Therefore, it is necessary to validate the qPCR primers before using them for the experiment by plotting standard curve with Ct values against log of ten-fold serial dilutions of nucleic acid input. For this, mouse liver samples were used to prepare five cDNA dilutions (in triplicates) ranging from 10^{-1} to 10^{-5} and the efficiency of the primers amplifying LSR α, α' and β mRNAs was calculated (Table 12).

Table 12: Validation of SYBR Green dye-based qPCR primers for the amplification of LSR mRNAs

LSR subunits	Efficiency (%)	R^2	Slope
LSRα	99.6	0.927	−3.331
LSRα'	100.16	0.998	−3.318
LSRβ	101.8	0.983	−3.278

13.2. TaqMan gene expression assay

Real-time PCR was performed in the StepOnePlus™ Real-time PCR system (Applied Biosystems) with 500 ng of cDNA and TaqMan® Gene Expression Master Mix, according to Applied Biosystems' procedures. The master mix contained highly purified AmpliTaq Gold® DNA polymerase, Uracil-DNA glycosylase, dNTPs with dUTP, ROX™ dye as passive reference along with optimized buffer components. The assay consisted of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ (6-carboxyfluorescein) dye label as fluorophore on the 5' end and minor groove binder (MGB) nonfluorescent quencher (NFQ) on the 3' end. TaqMan primers were used to determine the expression of mouse acyl-CoA oxidase 1 (ACOX1; Mm00443579_m1) using Hprt (Mm00446968_m1) as endogenous control. The primers were purchased from Applied Biosystems.

13.3. Real-time qPCR using PowerSYBR Green

Quantitative PCR was performed in StepOnePlus™ Real-time PCR system (Applied Biosystems) with 500 ng of cDNA and the *Power*SYBR Green PCR Master Mix (according to Applied Biosystems' procedures). The qPCR reaction mixture contained highly purified AmpliTaq Gold® DNA polymerase, dNTPs, passive reference dye (ROX), SYBR® Green I

dye and optimized buffer components. After RT, cDNA samples were diluted 20 times in order to avoid the inhibition by RT reaction mixture and 5 µL of the samples was added in each well. Amplification parameters were 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s and 60ºC for 1 min. Each sample was analyzed in triplicate. Since SYBR Green binds non-specifically to any double-stranded DNA, the specificity of the experiment is determined by melt curves. In melt curve analysis, the temperature of the target DNA is gradually increased typically in 0.3°C increments from about 50°C to 95°C, while the fluorescence values are continually recorded. As SYBR Green dye is only fluorescent when bound to the double stranded DNA, a drop in the fluorescence is observed as the temperature is gradually increased, with subsequent denaturation of the target double-stranded DNA. These melt curves are then used to derive the melting temperature (Tm) by plotting the derivative of the fluorescence of the amplified target as a function of temperature (−dF/dT). PCR products melt at different temperatures, depending upon their lengths and sequences and thus producing distinct peaks. However, a fully optimized qPCR reaction will produce single peak, representing the amplification of the desired product and thus the specificity of the assay is ensured.

In our experiment, Hprt was selected as a reference gene in every analysis to correct for differences in inter-assay amplification efficiency. Ct (cycle threshold) values were determined as the number of PCR cycles required for the fluorescent signal, accumulating from the amplification of the target sequence, to raise above the background level. Quantitation was performed by the 2-ΔΔCt method (Livak & Schmittgen, 2001). The obtained results were tested for statistical significance ($p<0.05$) using the Relative Expression Software Tool 2009 (REST, Version 2.0.13). The fold changes of mRNA levels in all samples were expressed relative to the corresponding control conditions.

14. *In silico* prediction of potential LSR regulatory regions and transcription factor binding sites

The 5'-upstream regions of mouse (NC_000073.6), rat (NC_005100.3) and human (NC_000019.9) *lsr* genes were scanned for the presence of potential regulatory sequences using promoter prediction software Gene2Promoter (Genomatix, Munich, Germany; http://www.genomatix. de/online_help/help_eldorado/Gene2Promoter_Intro.html). The identification of the putative promoter sequence is based on the detection of RNA Polymerase II binding site upstream of the transcription start site (TSS). The sequences were then

analysed for the presence of potential transcription factor binding sites (TFBS) using MatInspector (Genomatix, Germany; http://www.genomatix.de/matinspector.html), that contains library of weight matrices representing TFBS (Cartharius *et al*., 2005). The 5'-upstream sequences were scanned for TFBS by selecting two matrices *i.e.* vertebrates and general core promoter elements. The sequence similarities between mouse, rat and human *lsr* putative regulatory regions were determined using DiAlign TF (Genomatix, Germany; http://www.genomatix.de/online_help/help_dialign/dialign_TF.html).

Results and Discussion

I. Role of DHA in the regulation of hepatic LSR

1. Introduction

For decades, dyslipidemia has emerged as an important worldwide health concern that is mainly caused by abnormalities in lipid and lipoprotein metabolism. Therefore, it acts as a powerful risk factor for the development of various lipid-related disorders such as obesity and insulin resistance, altogether referred as the metabolic syndrome (Halpern *et al.,* 2010; Klop *et al.,* 2013). The factors characterizing dyslipidemia include the elevated plasma triglycerides (TGs) and low-density lipoprotein (LDL)-cholesterol along with decreased high-density lipoprotein (HDL)-cholesterol concentrations. Various physiological, dietary and hormonal factors are involved in the regulation of dyslipidemia. The lipolysis stimulated lipoprotein receptor (LSR) is one such important factor, mainly because of its role in the uptake and subsequent clearance of ApoB- and/or ApoE-containing TG-rich lipoproteins during the postprandial phase (Bihain & Yen, 1992; Yen *et al.,* 1994; Yen *et al.,* 2008). The receptor is activated in the presence of FFAs, released after hydrolysis of the TG core of lipoprotein particles by lipoprotein lipase (LPL). Upon activation, LSR undergoes a conformational change which reveals the binding site for the ApoB or ApoE moiety of the lipoprotein particle. Inactivation of both LSR alleles is embryonic lethal (Mesli *et al.,* 2004) whereas the absence of a single LSR allele ($LSR^{+/-}$) in mice leads to the delayed clearance of intravenously injected lipid emulsions and as a consequence, elevated postprandial lipemia (Yen *et al.,* 2008). Liver-specific LSR knockdown results in increased postprandial hypertriglyceridemia accompanying an increase in the levels of both ApoE and ApoB (Narvekar *et al.* 2009). In both mouse and Hepa 1-6 cell models, leptin treatment increases LSR mRNA and protein levels through an ERK-dependent mechanism, suggesting that leptin acts as an important regulator of LSR expression (Stenger *et al.,* 2010). Furthermore, mouse models of obesity (*ob/ob*) and type II diabetes (*db/db*) exhibit decreased LSR expression that can be restored by leptin replacement (Narvekar *et al.,* 2009). Moreover, the reduced LSR levels lead to hyperlipidemia, due to the accumulation of atherogenic ApoB-containing particles in the plasma which may in turn contribute to the development of obesity and atherosclerosis (Yen *et al.,* 2008).

Among the various methods for the treatment of dyslipidemia, fish oil or *n*-3 PUFA supplementation in the diet is an effective strategy that consistently reduces both fasting and postprandial TG levels (Harris *et al.,* 1988; Anil, 2007; Kelley *et al.,* 2007; Zuliani *et al.,*

2009; Zhang *et al.*, 2010) in normal and hypertriglyceridemic human subjects, as well as in animal models (Harris, 1996; Eslick *et al.*, 2009; Bernstein *et al.*, 2012). Fish oil feeding in mice decreases TG and total cholesterol (TC) levels and improves insulin sensitivity in obese mice, thus reducing hepatic steatosis (Saraswathi *et al.*, 2009). Various epidemiological studies have shown that individual FAs such as DHA and EPA have TG-lowering effects through a myriad of molecular mechanisms including reduced secretion of VLDL and enhanced TG clearance by the liver, an increase in the degradation of hepatic ApoB (Davidson, 2006; Bays *et al*., 2008; Jacobson *et al.*, 2012), inhibition of ApoC-III and upregulation of endothelial lipase activity in plasma and adipose tissue of human subjects with normal or atherogenic lipoprotein profiles (Harris *et al*., 1997; Park & Harris, 2003; Khan *et al*., 2002; Pirillo & Catapano, 2013). In addition, *n*-3 PUFAs inhibit hepatic lipogenesis and stimulate mitochondrial FA oxidation in liver and skeletal muscles (Halvorsen *et al.*, 2001; Sun *et al*., 2011) by regulating the expression of various transcription factors involved in lipid metabolism such as PPARs, SREBPs, LXR, ChREBP/MLX, FXR, HNF-4α and NF-κB (Sekiya *et al*., 2003; Pégorier *et al*., 2004; Jump, 2013). *N*-3 PUFAs, in particular DHA, exert their effects on various cellular processes and signaling pathways by altering the physical and chemical properties of membrane microdomains and the modulation of membrane receptors and ion channels (Kitajka *et al.*, 2002; Barceló-Coblijn *et al.*, 2003). Studies have shown that *n*-3 PUFAs also decrease plasma LDL-cholesterol concentrations by stimulating LDL-R activity in rats (Spady, 1993). Indeed, cell culture studies using fibroblasts and HepG2 cells indicated an increase in LDL-R protein levels upon DHA supplementation in the cell media (Yu-Poth *et al.*, 2005).

In view of these findings, as well as the requirement of LSR for FAs to bind lipoproteins, the objective of this study was to elucidate the role of DHA in the regulation of LSR. Different *in vitro* and *in vivo* models were employed to determine the effects of DHA supplementation on LSR expression and activity.

2. Results and discussion

2.1. *In vitro* experimental data

In the previous studies conducted in our laboratory, we have established that DHA enrichment induces changes in the neuronal plasma membrane properties that could protect neurons from apoptosis induced by soluble Aβ oligomers, the neurotoxic peptide found as the component of amyloid plaques in Alzheimer's disease (Florent *et al.,* 2006). In the view of these findings, the objective of this study was to determine the effects of DHA enrichment on the lipid metabolism in liver. We initiated this work with cell culture studies with the aim to investigate the role of DHA in the regulation of LSR expression and activity, using Hepa 1-6 cells as cell model.

2.1.1. Effects of DHA on cell viability

The initial step for the DHA treatments in Hepa 1-6 cells was to determine the concentrations of DHA that are not toxic to the cells. For this purpose, the viability of Hepa 1-6 cells was determined using MTT assay, which determines mitochondrial activity and thereby reflects the overall cellular metabolic activity (Mosmann, 1983; van Meerloo *et al.,* 2011). The cells were cultured in DMEM and seeded in 24-well plates, as described in Materials & Methods, section 3. After 48 h seeding, the cells were washed with 1X PBS and the medium was changed to DMEM containing 10 mM Hepes, 1% BSA (pH 7.5) and 3.7 g/L $NaHCO_3$. The cells were then treated with various concentrations of DHA *i.e.* 5, 10, 25, 50, 100 and 500 µM for 24 h. As DHA stock solution was prepared in DMSO, identical volumes of DMSO (0.25% (v/v)) were added to a separate set of cells as controls. The cell survival was measured by using the MTT reduction assay (as described in Materials & Methods, section 7). DHA significantly increased the mitochondrial activity, which was correlated with an increase in cell proliferation at the concentrations of 5, 10, 25 and 50 µM. However, DHA showed cytotoxic effects at higher concentrations, with cell viability reduced to 28% and 95% at 100 and 500 µM DHA respectively, when compared to control condition treated with DMSO (Figure 22).

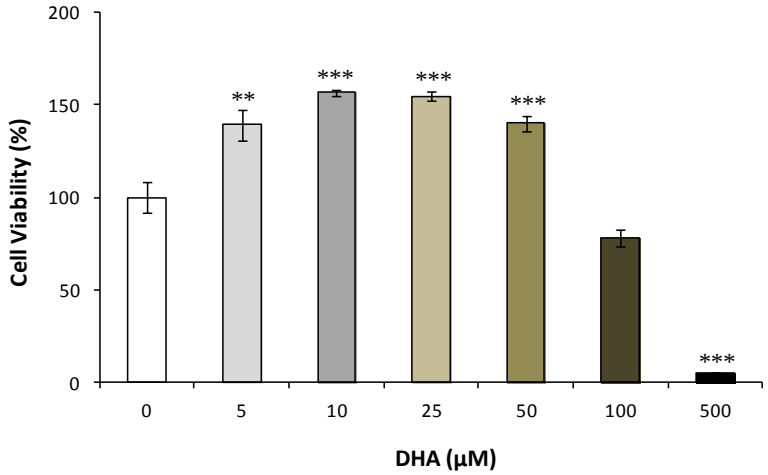


Figure 22: Effects of DHA treatment on viability of Hepa 1-6 cells

Cells were incubated with indicated concentrations of DHA (stock solution prepared in DMSO) for 24 h and the cell survival was assessed by measuring MTT reduction activity. The control cells were treated with the vehicle and their absorbance values were normalized to 100%. Results are represented as means ± SEM (quadruplicate determinations) and p*-values are indicated where significant compared to the control condition,* ** p*<0.01,* *** p*<0.001.*

On the basis of these observations, DHA concentrations were used in the range of 0-25 µM in the following treatments of Hepa 1-6 cells.

2.1.2. Effects of DHA on LSR expression

Since DHA modulates lipid metabolism by controlling the expression of various enzymes and receptors involved in lipid synthesis and/or degradation, the effects of DHA on the protein and mRNA levels of total LSR were determined. Hepa 1-6 cells were incubated with various concentrations of DHA for 24 h at 37°C (as described in Materials & Methods, section 3 and the section 2.1.1 in this chapter). After incubation, the cells were washed three times with 1X PBS containing 2% (w/v) BSA and recovered for protein and RNA analyses (as described in Materials & Methods, sections 3.2 & 11.1). Western blot analysis revealed a significant increase in LSR protein levels at 5 and 10 µM DHA followed by a decrease at 25 µM DHA concentration, compared to the control cells that received no treatment (Figure 23A). Real-time qPCR analysis revealed no changes in LSR mRNA levels with DHA treatment (Figure 23B).

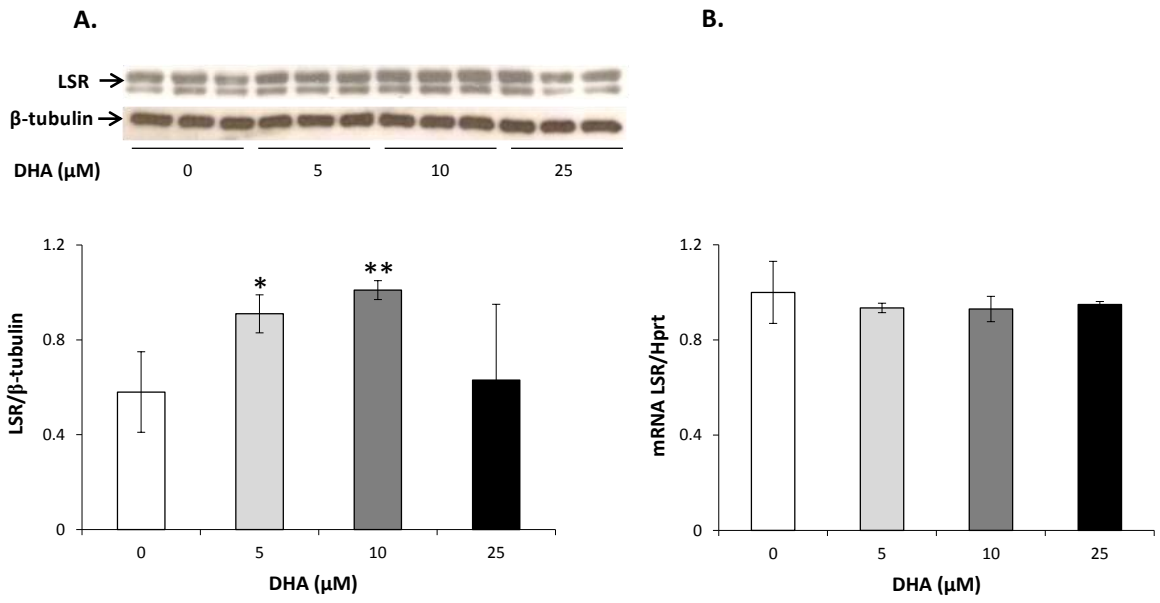


Figure 23: Effects of DHA on LSR mRNA and protein levels in Hepa 1-6 cells

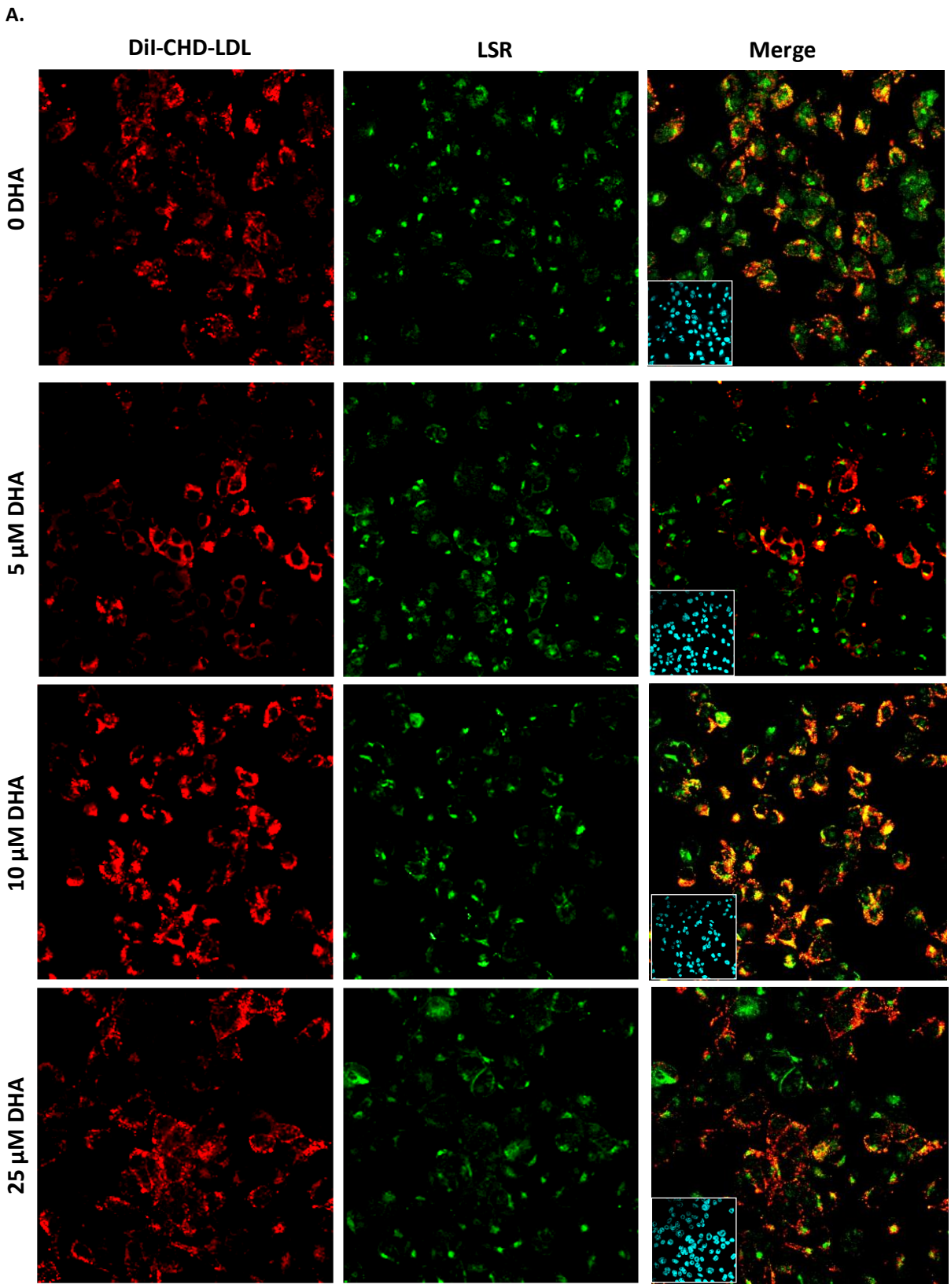
Hepa 1-6 cells were incubated with indicated concentrations of DHA for 24 h. (A) Western blot analysis was performed to determine the changes in total LSR protein levels using whole cell lysates. β-tubulin was used as loading control. The blot is shown in the upper panel, the densitometric analysis was performed and the results are shown in the lower panel as means ± SD. (B) Real-time qPCR experiment was performed to determine total LSR expression taking Hprt as reference gene. Cells treated with vehicle alone (DMSO) were used as controls. Results are represented as means ± SD, n=3. The p*-values are indicated where significant compared to the control condition, * p<0.05, ** p<0.01.*

DHA is known to exert its impact on lipoprotein metabolism also by regulating the expression of genes involved in lipid metabolism. A similar effect of DHA was observed on the expression of the lipoprotein receptor LDL-R, using fibroblasts and HepG2 cells (Yu-Poth *et al.,* 2005). According to this study, DHA increased LDL-R protein levels in both of these cell lines but had no effects on mRNA levels, suggesting that DHA did not modulate the LDL-R expression at transcriptional levels. In addition, these effects were shown to be independent of acyl-CoA: cholesterol acyltransferase (ACAT) and SREBP-1, and thus the exact mechanisms involved in this DHA-mediated effect on LDL-R remain to be investigated. Since DHA treatment only increased LSR protein levels, this suggests that DHA affects LSR expression primarily at translational and/or post-translational levels, without changing LSR mRNA levels.

2.1.3. Effects of DHA on LSR activity in Hepa 1-6 cells

In order to determine whether the DHA-mediated increase in LSR protein levels also led to increased LSR activity as a lipoprotein receptor, binding and uptake studies were

performed using LDL particles, that were labeled with a hydrophobic dye 'DiI' and treated with CHD that modifies the arginine residues on LDL particles and thus rendering them unable to bind with LDL-R (as descibed in Materials & Methods, section 8). Hence, the binding and uptake of DiI-LDL to LSR was determined in the presence of oleate that activates the receptor. For this experiment, Hepa 1-6 cells grown in serum-deficient medium (SDM), were treated with various concentrations of DHA for 24 h and then with 0.5 mM oleate and 5 µg/mL CHD-DiI-LDL for 2 h, as described previously (Yen *et al.*, 2008). After treatment, the cells were fixed and labeled with LSR antibody to determine the co-labeling of LSR with DiI-CHD-LDL. Immunofluorescence analysis revealed an increase in DiI-CHD-LDL uptake at 5 and 10 µM DHA followed by a decrease at 25 µM DHA, when compared with control condition (0.5 mM oleate + 5 µg/mL LDL). The quantitative analysis of the results using *WCIF-ImageJ* revealed a significant increase in co-labeling of CHD-DiI-LDL and LSR at 5 and 10 µM DHA (Figure 24), which indicates LSR binding to LDL and therefore would reflect the activity of LSR.


A.
DiI-CHD-LDL
LSR
Merge
0 DHA
5 µM DHA
10 µM DHA
25 µM DHA

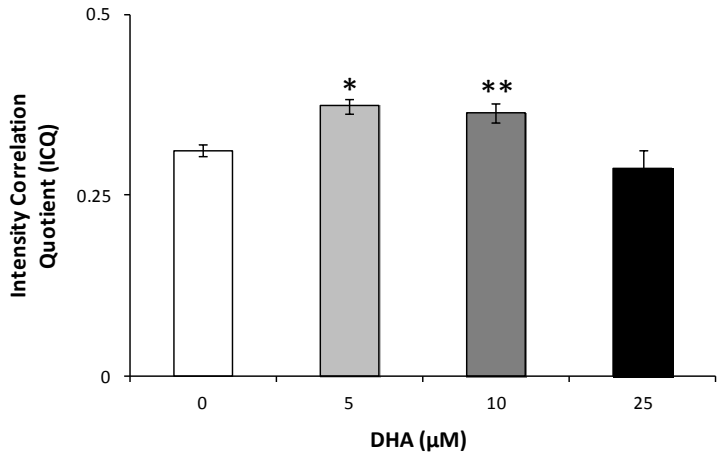


Figure 24: Effects of DHA on DiI-CHD-LDL binding in Hepa 1-6 cells

Hepa 1-6 cells plated on cover slips were incubated with the indicated concentrations of DHA for 24 h. The cells were then treated with 0.5 mM oleate for 2-3 min and incubated with 5 µg/mL CHD-DiI-LDL for 2 h. The cells were fixed with 4% paraformaldehyde and labeling was performed on non-permeabilized cells using anti-LSR antibody. (A) Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue color). Immunofluorescent images demonstrate the co-localization (orange color) of CHD-DiI-LDL (red color) with LSR (green color). The experiment was repeated twice and the images were taken from at least four different random fields (n=4) per slide having almost the same cell density. (B) The images were quantitatively analyzed using WCIF-ImageJ and the results are shown as means ± SEM. The p*-values are indicated where significant compared to the control condition,* * p*<0.05,* ** p*<0.01.*

Biochemical and bioinformatics data indicate that LSR is expressed on the surface of hepatocytes as a multimeric complex of α and α' subunits present on plasma membrane and β subunit(s) attached to them on intra- or extra-cellular side (Yen *et al.*, 1999). The receptor is activated in the presence of FFA that binds to the receptor, causing changes in its conformation which exposes the binding site for ApoB/E containing TG-rich lipoproteins. However, the length of the FA chain and its degree of saturation play an important role in determining the extent of LSR activation, with oleate being the most efficient (Bihain & Yen, 1992; Mann *et al.*, 1995). Furthermore, MaxEPA (fish oil) treatment led to an increase in the activity of LSR in animal models such as rats and Watanabe heritable hyperlipidemic (WHHL) rabbits, with concomitant decrease in TG levels (Bihain *et al.*, 1995). It is well-known that dietary *n*-3 PUFAs, notably DHA, have a particular structure that allows these molecules to incorporate into the membrane phospholipids. By modifying the FA composition of the lipid bilayer, DHA affects physicochemical properties of biological membranes by increasing their fluidity and altering the microenvironment (Spector & Yorek; 1985; Kuo *et al.*, 1989; Spady *et al.*, 1995; Wassall & Stillwell, 2009; Shaikh & Teague, 2012). This may lead to the conformational changes in different proteins associated with the plasma

membrane, *e.g.*, the receptors, which in turn, may alter the affinity of the receptor for its ligands. Other mechanisms may include the changes in the recycling rate or the localization of the receptor which leads to altered interactions with other membrane components, the lipids as well as the proteins. In this way, DHA likely modulates the expression and activity and thus the functions of various membrane-associated proteins including receptors, transporters and enzymes such as those involved in lipid metabolism.

Studies have shown that *n*-3 LC-PUFAs including DHA regulate the activity and expression of LDL-R (Rumsey *et al.*, 1995; Yu-Poth *et al.*, 2005). Indeed, in some studies, the dietary FA-induced changes in LDL-R activity have been found to be associated with the alteration in the plasma membrane composition and physical properties (Kuo *et al.*, 1989). Our results also indicate that despite no modifications in mRNA levels, DHA increases LSR-mediated uptake of LDL. Furthermore, the results obtained in Figure 23A are in correspondence with the Figure 24. This indicates that the increase in LSR protein levels by DHA treatment may be associated with the increased LSR activity. However, further experiments are required to link this DHA-mediated increase in LSR activity and protein levels with the plasma lipid profiles in Hepa 1-6 cells.

2.2. *In vivo* experimental data

The *in vitro* data prompted us to determine the effects of DHA supplementation in the regulation of plasma lipid profiles and LSR expression. Three different supplementation experiments were performed in mice, primarily differing from one another with respect to the age of mice at the start of the experiment, the chemical forms of DHA used, and the study duration (Table 13). The details of these treatments are provided in their respective sections.

Table 13: Summary of the different treatments performed in male C57Bl/6J mice

Experiment	Age (months)	Duration (months)	Form of DHA	Percentage of DHA in the diet
Short-term DHA supplementation	4	0.5-1	Ethyl ester (EE)	0.24%
Long-term DHA supplementation	9	3	Ethyl ester	0.24%
Very-long-term FO supplementation	9	6	TG in fish oil	0.20%*

* *DHA was added by supplementing diet with fish oil that also provided 0.04% EPA.*

2.2.1. Short-term DHA supplementation

The results from our *in vitro* data revealed that DHA is involved in the regulation of LSR, mainly by increasing LSR levels and activity. We next sought to determine if these effects of DHA may be extended to the *in vivo* settings. For this purpose, we started with a short-term DHA supplementation experiment in mice.

2.2.1.1. DHA-ethyl ester

During this DHA short-term supplementation experiment, 15-week old C57Bl/6J male mice were placed on a standard diet, Harlan 2018S, or the diet supplemented with 0.24% (w/w) DHA in the form of ethyl ester (EE) for 15 and 30 days. The DHA-EE form results from the synthetic transesterification of FA with one ethanol molecule. Various studies have evaluated the absorption and bioavailability of *n*-3 LC-PUFA in the form of EE. Most of these studies have compared the amount of EPA and DHA in blood plasma after ingestion of FAs in the form of TGs or EE (or incorporated into phospholipids (Tang *et al*., 2012)). Although some studies have demonstrated a similar rate of absorption for both of these forms (Krokan *et al.,* 1993), most suggest that TGs are better absorbed (Dyerberg *et al.,* 2010; Neubronner *et al.,* 2011). EPA incorporation into plasma lipids was reported to be significantly lower and slower when administered as EE (el Boustani *et al.,* 1987). Plasma lipid concentrations of EPA and DHA were consistently and significantly higher after ingestion of servings of salmon compared to three capsules of fish oil with EE (Visioli *et al.,* 2003). In rats, DHA supplementation in the form of TG resulted in higher concentrations of DHA in erythrocytes and plasma as compared with DHA-EE (Valenzuela *et al.,* 2005). This difference in bioavailability between the two forms studied could be explained by the fact that FA-EE offers greater resistance to digestive enzymes as the compared with FA-TG (Yang *et al.,* 1990).

Taking into account all these observations, we first sought to determine the bioavailability of DHA in mice fed with DHA-EE.

2.2.2. DHA enrichment in erythrocytes

Blood samples were collected at the end of the study to determine the bioavailability of DHA in mice. Fatty acid profiles were determined in erythrocyte samples from the mice placed on standard or DHA-enriched diet. Compared to the mice on standard diet, DHA supplementation yielded ~3-fold increase in total *n*-3 PUFA content of the erythrocyte membrane already after 15 days of treatment (Figure 25). Furthermore, a 2.7-fold increase

was observed in erythrocyte DHA levels following 15 days of treatment. No further modification occurred in these values after 30 days as compared to levels after 15 days.

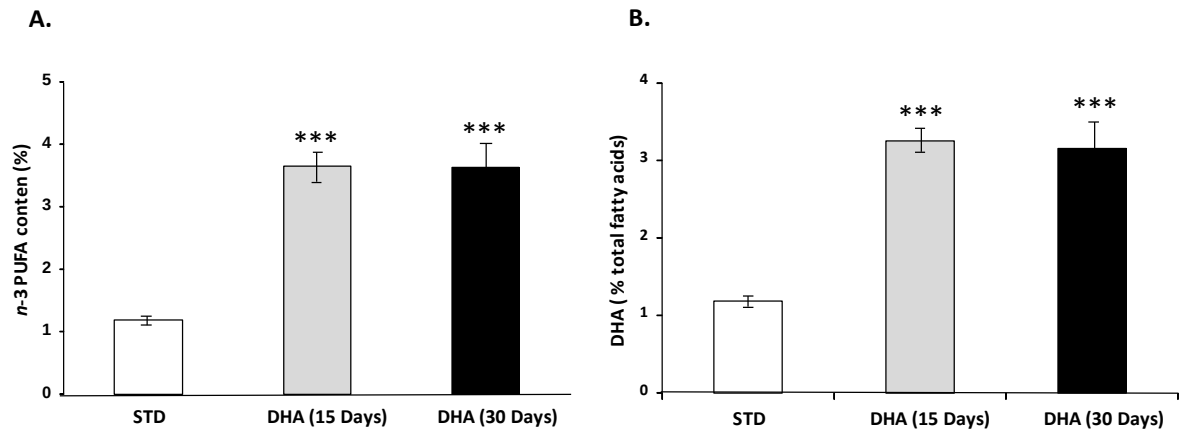


Figure 25: The* n-3 *PUFA and DHA levels in erythrocytes

Fatty acid profiles of erythrocytes were determined in 15-week old C57Bl/6J mice placed on either standard diet or the diet enriched with 0.24% DHA-EE for 15 and 30 days. (A) The n-*3 PUFA content was determined at the beginning as well as at the end of study. (B) DHA enrichment is shown here as % of total fatty acids. Results are shown as mean ± SEM and* p-*values are indicated where significant compared to the control condition.* *** p*<0.001.*

The results indicate that the 15- and 30-day supplementation of mice with DHA-EE significantly increased erythrocyte *n*-3 PUFA as well as DHA levels, which may act as an indicator of the bioavailability of this form of DHA. An increase in *n*-3 PUFAs in mice on a DHA-supplemented diet as compared to animals on a standard diet may yield erythrocytes that are less prone towards inflammation (Calder, 2009; Chapkin *et al.*, 2009).

2.2.3. Determination of LSR mRNA levels

After the 30 days of treatment with DHA, mice were sacrificed and livers were removed from standard (n=3) and DHA-supplemented (n=3) mice, from which total RNA was extracted. Real-time qPCR analyses were performed to determine the expression of total LSR and LSR subunits α, α' and β. No differences were observed in LSR expression levels in both groups (Figure 26).

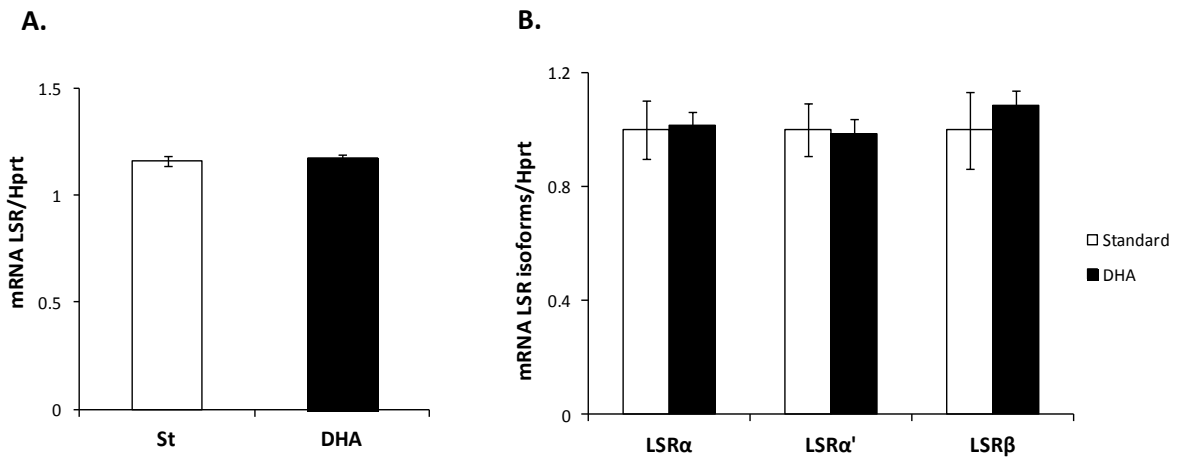


Figure 26: Determination of LSR expression in liver samples

(A) LSR expression was determined in liver samples from the standard (n=3) and DHA-supplemented mice (n=3). (B) The expression of LSR isoforms, i.e. α, α' and β, was determined in the same liver samples (triplicate determination). Hprt was used as a reference gene. Results are expressed as means ± SD.

These results indicate that supplementation of mice with DHA-EE caused no changes in the expression of total LSR as well as that of the three LSR subunits, suggesting that DHA did not regulate LSR at the transcriptional level. Similar results have been obtained by Yu-Poth *et al.* (2005) on LDL-R expression, where no changes were observed in LDL-R mRNA levels in fibroblasts and HepG2 with DHA supplementation. This indicates that in our experimental conditions, DHA may regulate LSR at translational and/or post-translational levels.

2.3. Long-term DHA supplementation

Taking into account the results obtained from the short-term supplementation experiment, where no change in LSR mRNA levels were observed, we next designed two long-term supplementation experiments for 3 and 6 months.

2.3.1. Supplementation with DHA-deficient or DHA-enriched diets

The nutritional protocol was defined by feeding two groups of nine-month old C57Bl/6J male mice with two different diets, for 12 weeks. One group was fed a diet containing DHA (DHA+) under the chemical form of fatty acid-ethyl ester (EE), while the other group was fed a diet that did not contain any DHA (DHA–). The diet composition was provided by the manufacturer as requested by us (Figure 27)

The nutritional ingredients were incorporated into the diet directly during its preparation by Safe Company (Augy, F). The *n*-3 LC-PUFA supplementation was performed by adding DHA-EE (KD-Pharma, Bexbach, Germany) into the DHA+ diet to achieve a final concentration of PUFAs in the diet of 0.3% (w/w). The determination of the FA composition confirmed the actual presence of 0.24% DHA in DHA+ diet. The DHA− diet did not contain any traces of DHA and absence of DHA was adjusted with sunflower oil, thereby providing similar caloric density. The endogenous DHA synthesis from its precursor was also prevented in both diets by adding the dietary components in a proportion that would not supply ALA in significant quantities.

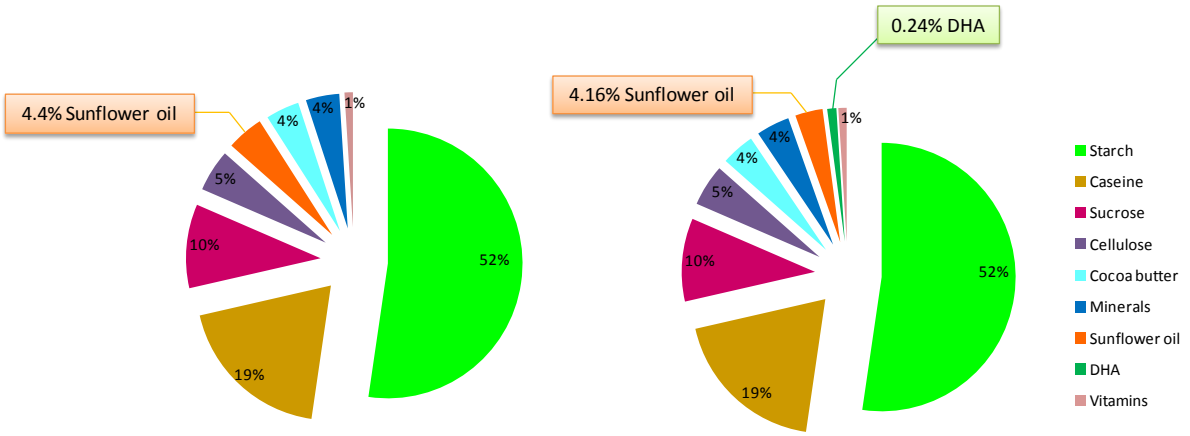


Figure 27: Composition of DHA-deficient and DHA-supplemented diets

2.3.2. Changes in body weight

Under the influence of both dietary treatments, food intake showed no significant difference between the mice fed on DHA+ or DHA− diets (data not shown). However, the body weight was observed to evolve distinctly between the two groups (Figure 28). Indeed, mice of DHA− group experienced a regular increase in their body weight which may be associated with the age factor, related to the consumption of a DHA-poor diet. Furthermore, the comparison between the two groups reveals a two-fold less weight gain in DHA+ mice between the 2nd and 12th weeks of treatment, when compared to the DHA− group. Although the statistical significance of this difference could not be confirmed, this trend, however, suggests that the long-term DHA supplementation improves the weight gain related to aging, without significant changes in energy intake.

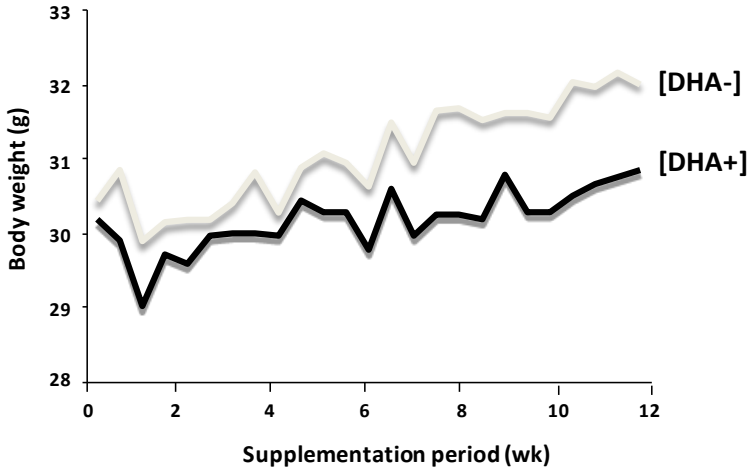


Figure 28: Evolution of body weight in mice supplemented with DHA− or DHA+ diets

2.3.3. Effects of DHA supplementation on plasma TG and TC levels

We next measured the plasma lipid levels at three time points during the study, *i.e.* before (0), and after (6 and 12 weeks of treatment), in order to determine the metabolic effects related to dietary supplementation. No significant differences were observed in TG levels in both the groups when compared with the same time points (Figure 29A). However, the plasma TG levels were found to be decreased over time in both groups, showing that the decrease appeared independent of DHA supplementation. This indicates that we did not observe the hypotriglyceridemic effect of DHA which has been widely reported in the literature. However, the plasma TC levels were significantly lower in DHA+ mice as compared to DHA– animals 6 weeks after the start of the study. Interestingly, this was no longer observed after 12 weeks (Figure 29B).

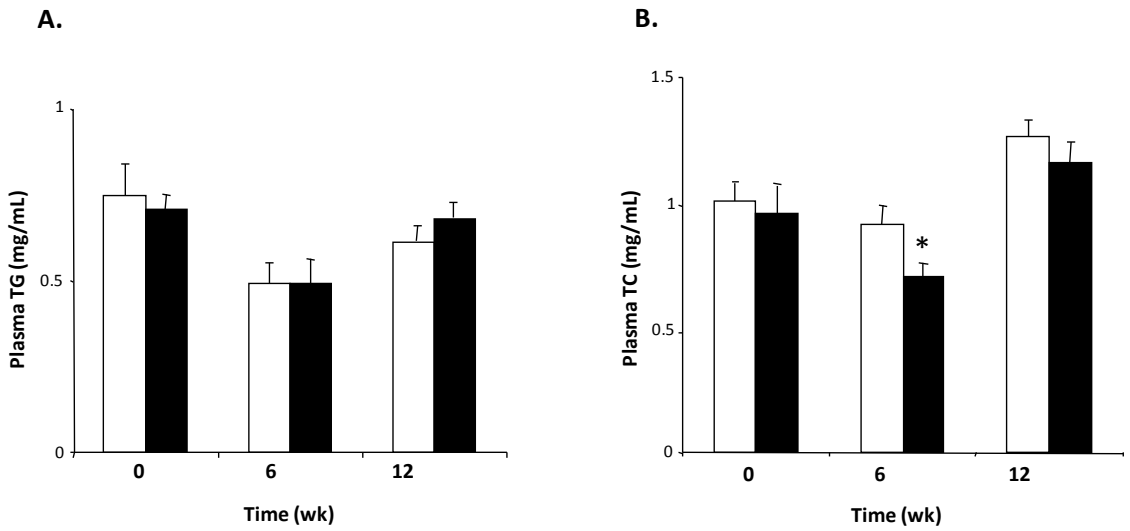


Figure 29: Plasma lipid levels in mice supplemented with DHA− or DHA+ diets

Nine-month old C57Bl/6J mice were placed on either DHA– (open bars) or DHA+ (closed bars) diets for 12 weeks. Blood samples were collected at 0, 6 and 12 weeks after the experiment was begun. (A) Plasma total cholesterol (TC) (B) and triglycerides (TGs) were analyzed. Results are shown as mean ± SEM; p*-values are indicated where significant comparing DHA– and DHA+ values from the same time point,* * p*<0.05.*

The results obtained are in line with several studies that have demonstrated a negative correlation between DHA and TC, thus confirming the hypocholesterolemic effects of DHA (Holub, 2009; Jacobson *et al.*, 2012; Tang *et al.*, 2012). One such study in rats by Spady (1993) has elucidated that the positive effects of DHA (EPA as well), used at 4% weight in the diet, were associated with reduced rate of LDL formation and stimulated activity of hepatic LDL-R, thus leading to a decreased plasma LDL-cholesterol.

2.3.4. DHA enrichment in erythrocytes and liver

In order to determine the DHA enrichment in liver and erythrocyte membranes, blood samples were collected after a 3-h fasting period at 0, 6 and 12 weeks after the start of the dietary study. Fatty acid profiles were determined in erythrocyte and liver samples from the mice fed DHA– or DHA+ diets (Figure 30). The erythrocyte membrane DHA levels were found to be increased up to 1.7-fold after 6 weeks and 2.3-fold after 12 weeks of supplementation in DHA+ mice as compared to DHA– mice. Similarly, a six fold increase in DHA content was observed in the liver of DHA+ mice as compared to the other group.

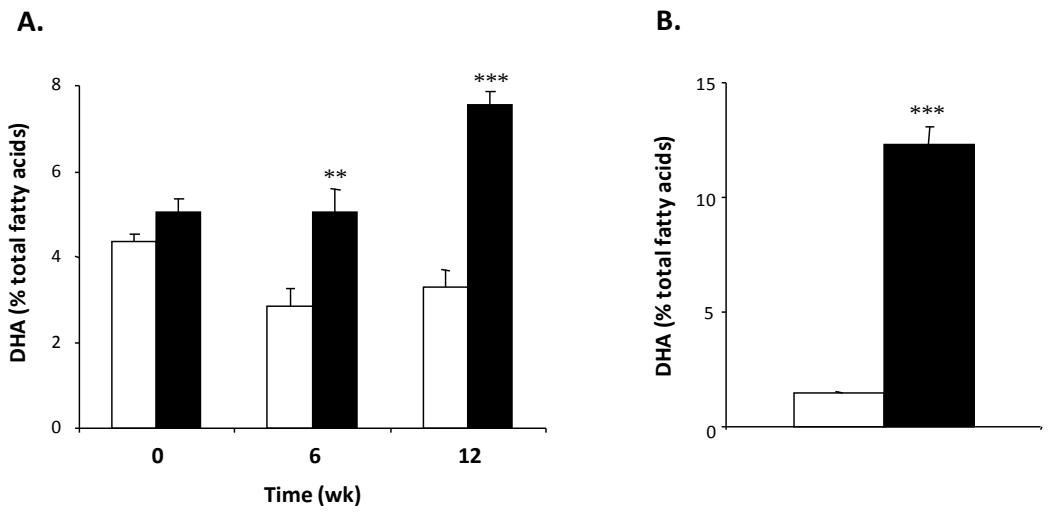


Figure 30: DHA levels in erythrocytes and liver

Nine-month old C57Bl/6J mice were placed on either DHA– (open bars) or DHA+ (closed bars) diets for 12 weeks. (A) Blood samples were collected at 0, 6 and 12 weeks after the experiment was begun. Erythrocytes were isolated for FA profile analysis. (B) Liver FA profile was also determined at the end of the study (as described in Materials & Methods, section 9). DHA enrichment is shown here as % of total FAs. Results are shown as mean ± SEM and p*-values are indicated where significant comparing the DHA– and DHA+ groups at the same time point.* ** p*<0.01,* *** p*<0.001.*

It is interesting to find such an enrichment of this *n*-3 fatty acid in the erythrocytes. Indeed, this measure is often used to assess the periphery as well as the central FA status of the subjects (Nelson *et al.*, 1997). Since erythrocyte membranes reflect cardiac membrane *n*-3 PUFA content, the amount of EPA+DHA in erythrocyte membranes, referred as the Omega-3 Index, is considered as an important biomarker for the risk of CHD (Harris & Von Schacky, 2004; Harris, 2007; Pottala *et al.*, 2010). More generally, the Omega-3 index is considered a useful marker for the bioavailability of DHA and EPA upon supplementation (Katan *et al.*, 1997; Poppitt *et al.*, 2005; Lucas *et al.*, 2009; O'Sullivan *et al.*, 2011). It was also suggested to reflect the central *n*-3 PUFA levels, brain DHA metabolism and neurotransmission, and was therefore proposed, though still controversial, a biomarker for the risk of brain aging and AD (Rapoport *et al.*, 2011; Tan *et al.*, 2012).

In our experiment, this enrichment demonstrates the efficiency of the EE form of DHA to be utilized after dietary ingestion. DHA is known to be found primarily in the phospholipids in brain as well as other excitatory membranes (*e.g.*, retina). These cells therefore could serve as a form of storage and/or transport for DHA in the periphery and for the central nervous system (CNS). A significant correlation (p = 0.01) was also observed between liver and erythrocyte DHA content, showing that the amount of DHA in liver and

erythrocyte membranes were strongly correlated together (Figure 31). Furthermore, it has been shown in obese patients that the changes in liver phospholipid PUFA composition is correlated with that of erythrocytes and therefore act as an indicator for the perturbations in liver lipid metabolism (Elizondo *et al.*, 2007).

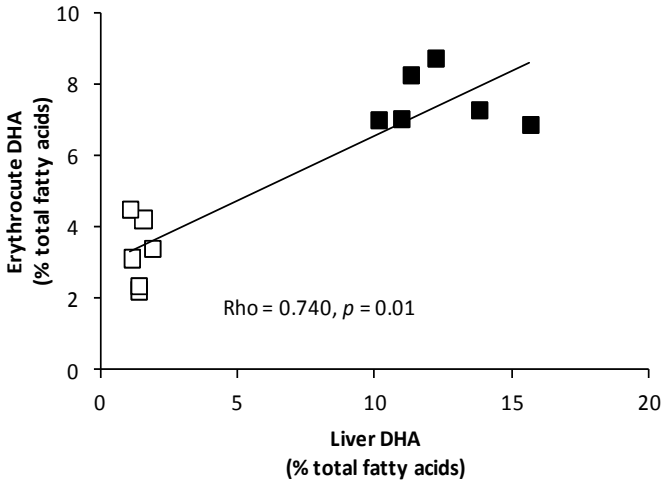


Figure 31: Correlation between liver and erythrocyte DHA levels

The liver DHA content (measured as % total fatty acids) was plotted against erythrocytes DHA content, after 12 weeks of experiment in DHA– (open square, n=6) or DHA+ (closed square, n=6) mice. The correlation was obtained using Spearman's rank correlation coefficient.

From these results, it can be inferred that DHA supplementation can lead to enrichment of this FA into membranes of a variety of cells because of its ability to be incorporated efficiently into the membrane phospholipids (Cao *et al.*, 2006). Owing to this incorporation, DHA affects the localization, recruitment and functions of various proteins in the plasma membrane, including enzymes and receptors, and also controls the expression of genes involved in different biological processes (Hsu *et al.*, 2004; Harris, 2007; Shaikh, 2012).

2.3.5. Determination of hepatic LSR mRNA levels

We next sought to determine the possible effects of DHA supplementation on LSR expression. Total RNA was extracted from liver of mice of each group, *i.e.* DHA– and DHA+, and used for reverse transcription. Duplex PCR was the performed to amplify total LSR as described in Materials & Methods, section 12.2. No significant variation in LSR expression was detected in both groups (Figure 32).

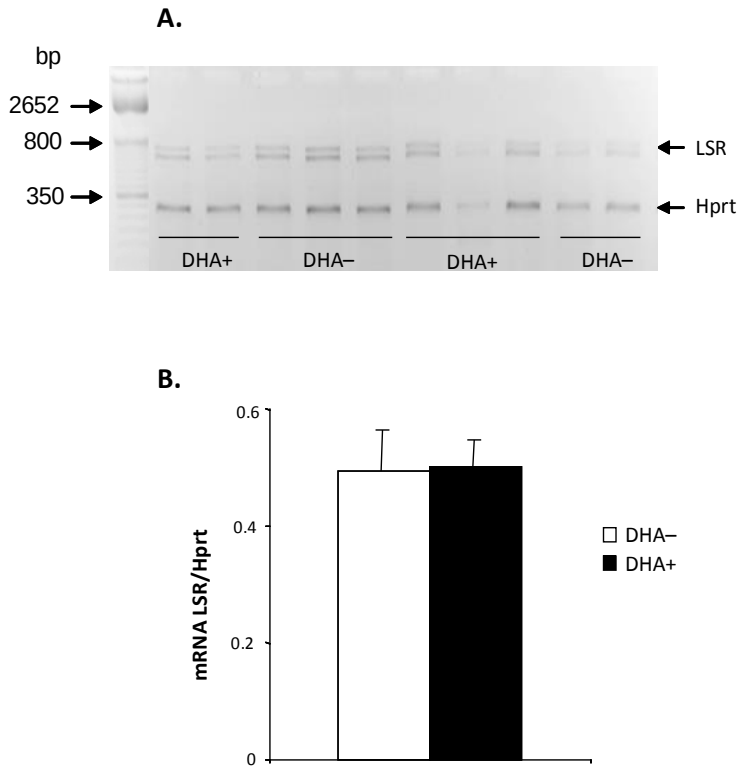


Figure 32: PCR amplification and quantification of LSR in liver samples

LSR expression was determined in liver samples from mice placed on diets with (n=5) or without DHA (n=5) supplementation (see Figure 30). (A) Duplex PCR was performed using specific primers to amplify LSR (600-700 bp) and Hprt (300 bp) (as indicated). Hprt was used as a reference gene. (B) Comparison of LSR expression was done by calculating LSR to Hprt ratio for each group. Results are represented as means ± SEM.

These data appear to indicate that the hepatic LSR mRNA levels were not different between the DHA– and DHA+ groups, suggesting that DHA did not regulate LSR at the transcriptional level. Interestingly, similar results were obtained previously (Figure 26), where one-month DHA-EE supplementation, containing similar levels of DHA, did not modify LSR mRNA levels. This confirms that DHA-supplementation in the diet has no effects on LSR transcription.

2.3.6. Determination of hepatic LSR protein levels

A Western blot was performed to determine LSR protein levels in liver total membranes of mice fed the DHA– or DHA+ diets. Densitometric analysis of LSR bands for each group revealed a 1.8-fold increase in LSR expression in mice on DHA+ diet as compared to those on a DHA– diet (Figure 33).

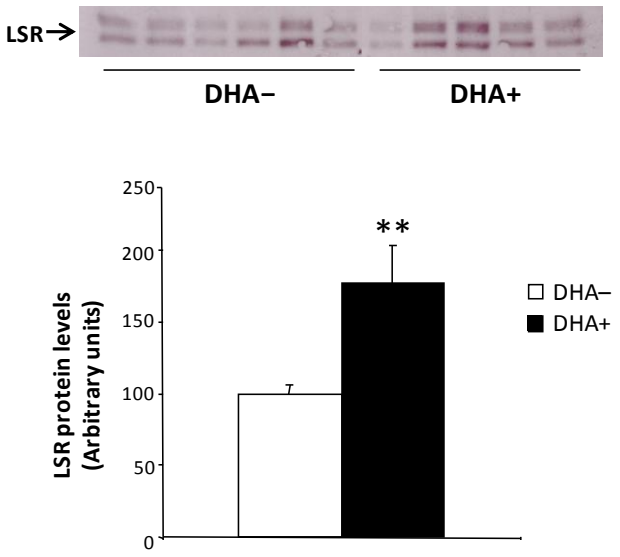


Figure 33: LSR protein levels in the liver samples

Western blot was performed for liver total membrane proteins from DHA– (open bar, n=6) and DHA+ (closed bar, n=5) mice using anti-LSR antibody. A representative blot is shown in the upper panel, and densitometric analysis was performed using ImageJ. The results are shown in the lower panel as means ± SEM. ** $p<0.01$.

Previous studies indicate that LSR is primarily involved in the clearance of TG-rich lipoproteins including VLDL and chylomicrons. Several studies suggested the involvement of DHA in the reduction of VLDL and TG (Jacobson, 2008; Pirillo & Catapano, 2013). The potential mechanisms include a decrease in TG production and VLDL assembly and secretion through the inhibition of diacyl-glycerol acetyl transferase (DGAT) and phosphatidic acid phosphohydrolase (PAP), an increase in LPL activity to accelerate TG removal, an inhibition of lipogenesis through decreased SREBP-1c expression and an increased FA oxidation, mainly through the activation of PPARα (Dreyer *et al.,* 1993; Kim *et al.,* 1999; Bays *et al.,* 2008; Poudyal *et al.,* 2011; Jump *et al.,* 2013). Various studies provide the evidence that the control of hepatic lipid metabolism occurs by the DHA-mediated regulation of the protein and/or mRNA levels and/or activity of various enzymes and receptors involved. These findings are further supported by Yu-Poth *et al.* (2005), showing an increase in LDL-R protein levels with DHA supplementation with no changes in LDL-R mRNA levels. Our results also indicate that LSR protein levels are significantly increased by DHA supplementation in mice, while PCR data suggested no transcriptional regulation on *lsr* gene expression by DHA. Since LSR is a multimeric complex comprising of α, α' and β subunits (Yen *et al.,* 1999), differences at the protein level may have a consequence on the activity of this receptor. The significant DHA enrichment (6-fold) in the liver membranes of DHA+ mice may lead to changes in LSR microenvironment that could affect its membrane targeting

and/or anchorage at the cell surface. This could optimize the multimeric conformation of LSR and promote its function. Indeed, there is a large difference in the liver composition of DHA− and DHA+ mice (Figure 30B), which very likely influences the plasma membrane architecture and the functions of associated proteins.

We next correlated LSR protein (Figure 33) with plasma TG and TC levels (Figure 29) in order to determine the relationship between these parameters.

2.3.7. Correlation of LSR protein and plasma TC and TG

The plasma TC and TG levels measured for the DHA– and DHA+ were plotted against LSR protein from the corresponding mice. No significant correlation was observed between LSR protein levels and plasma TC or TG when considering both DHA− and DHA+ groups. Interestingly, significant negative correlations between LSR protein on the one hand and plasma TC or fasting plasma TG on the other hand were identified only in mice on the DHA+ diet (Figure 34).

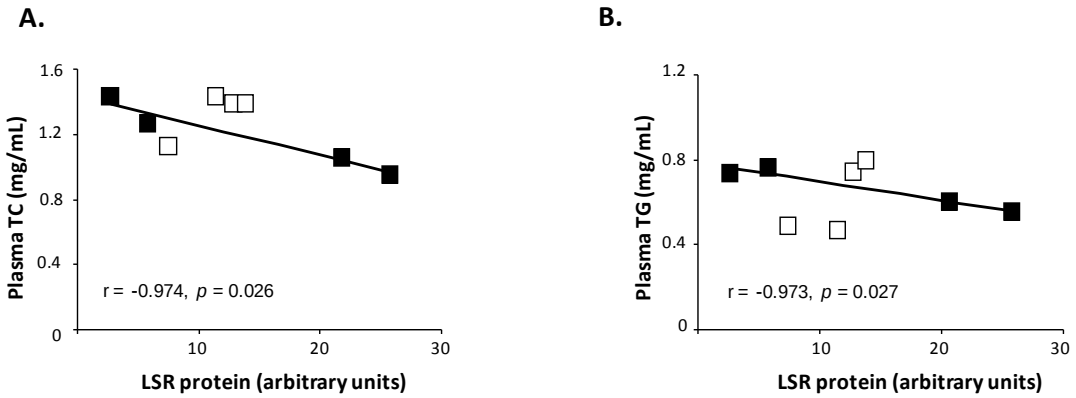


Figure 34: Correlation of LSR protein expression with plasma TC and TG in DHA+ mice

After 12 weeks of supplementation in DHA– (open square, n=4) or DHA+ (closed square, n=4) mice, the values of plasma (A) TC and (B) TG were plotted against LSR protein levels.

As discussed previously, DHA possesses hypocholesterolemic and hypotriglyceridemic properties through mechanisms that involve the regulation of various enzymes and receptors at transcriptional and/or translational levels (Holub, 2009; Jacobson, 2008; Tang *et al.*, 2012; Pirillo & Catapano, 2013). Furthermore, the significant DHA enrichment in liver (Figure 30B) may lead to the increased LSR protein levels and activity. The apparent number of LSR receptors on the surface of rat hepatocytes was also found to be negatively correlated with the postprandial TG levels (Mann *et al.*, 1995), suggesting that DHA enrichment in liver

membranes may lead to increase in LSR and consequently, to lower lipid levels. This in turn could reveal animals that are more or less efficient in clearing plasma TC and TG through this pathway.

2.4. Very long-term FO supplementation

This next study enabled us to determine the effects of a diet enriched with *n*-3 LC-PUFAs, *i.e.* DHA and EPA, in the form of TGs in fish oil. This study was even more completed by considering two types of diet that differed by dietary fat concentration and caloric density. Indeed, a high-fat diet was designed with the objective to induce moderate alterations in the lipid status. These normal- and high-fat diets were supplemented with fish oil in order to determine whether supplying *n*-3 PUFAs could prevent the metabolic disturbances caused by this high-fat and cholesterol-containing diet.

During this study, four groups (n=20 each) of 9-month old C57Bl/6J male mice were fed one of the four different diets for 6 months. The composition of these diets is described below:

- **STD:** Standard diet, containing 5% (w/w) lipids (12% kcal from fat).
- **FO:** STD diet containing *n*-3 LC-PUFAs (0.2% w/w DHA and 0.03% w/w EPA) in the form of TGs in fish oil.
- **HF:** High-fat Western-type diet, containing 22% (w/w) lipids (42% kcal from fat, primarily from lard) and 0.15% (w/w) cholesterol.
- **HF+FO:** HF diet, containing *n*-3 LC-PUFAs (0.2% DHA, 0.03% EPA) in the form of TGs in fish oil.

The composition of the diets was determined on the basis of the literature and experience from previous studies (Table 14). The nutritional ingredients were incorporated into the diet directly during its preparation (Safe, Augy, F).

Table 14: General composition of STD and HF diets

Components	STD % (w/w)	HF % (w/w)
Rice starch	34.1	28.0
Caseine	32.1	26.0
Lard	1.0	17.0
Sucrose	12.0	10.0
Minerals	8.0	7.0
Cellulose	7.0	6.0
Oil	4.0	4.5
Vitamins	1.2	1.0
L-Cystine	0.5	0.4
Cholesterol	–	0.15

The treatment with Western-type diet was meant to induce moderate dyslipidemia and weight gain and was designed to provide an excess of lipids (~ 22%, w/w) in the form of lard and 0.15% (w/w) cholesterol in HF and HF+FO diets, rendering them as hypercaloric, as compared to the normocaloric STD and FO diets. Furthermore, for *n*-3 LC-PUFA supplementation, the fish oil was added to the diet, in order to attain a final concentration of 0.2% (w/w) DHA in FO and HF+FO diets.

During the nutritional program, food intake was measured twice a week that allowed us to determine whether the animals adapted well to their diets. Their food consumption was stabilized 2 weeks after the start of the study and stayed constant throughout the program. However, we calculated that mice on high-fat diets ingested 12% less food than mice on normal-fat diets (in average, 3.2 g/d *vs.* 3.6 g/d, $p<0.001$). Interestingly, this difference indicated that the mice adjusted their nutrient intake by caloric intake, which is a known characteristic of their feeding behavior. Indeed, since high-fat diets provided higher caloric intake, mice on HF or HF+FO diets received approximately 14.5 kcal/d, which was 7.5% higher ($p < 0.001$) than mice on STD or FO diets (13.5 kcal/d) (Data obtained from Ahmad ALLOUCHE, PhD thesis, Université de Lorraine, 2012).

2.4.1. Changes in body weight

During the six-month treatment, the body weight was measured in all the groups of mice placed on the different diets (Figure 35). At the beginning of the experiment, all mice had similar body weight, *i.e.* approximately 31 g. This allowed us to conclude that compared to T0, mice placed on FO diet had lost up to 2.8 g until the 11th week, without showing any

diseased condition or deprivation (Data obtained from Marine HANSE, PhD thesis, Université de Lorraine, 2011). Furthermore, the HF mice gained more weight (*i.e.* about 2 g on the average) after 3 months, when compared to the weight gain in these mice at the end of the study. Also, a decrease in body weight was observed in FO and HF+FO mice, compared to their respective control groups on STD and HF diets only after three months of *n*-3 LC-PUFA supplementation. The effects on weight gain observed during fish oil supplementation were consistent with various studies in the literature, which indicate an anti-adipogenic effect of EPA/DHA during development of obesity and suggest that EPA/DHA could reduce accumulation of body fat by limiting both hypertrophy and hyperplasia of fat cells (Raclot *et al.,* 1997; Ruzickova *et al.,* 2004; Arai *et al.,* 2009; Hassanali *et al.,* 2010).

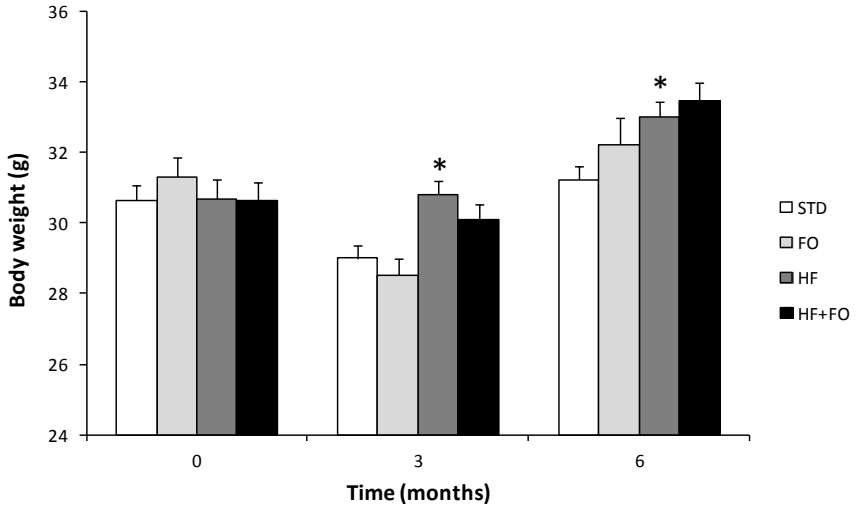


Figure 35: Body weight of mice placed on different dietary conditions

Four groups of 9-month old C57Bl/6J male mice were fed on standard (STD), fish oil (FO), high-fat (HF) or HF+FO diets for 6 months. Body weight changes were measured on the first day of feeding and after three and six months of the treatment. The results are represented as means ± SEM and p-*values are indicated where significant comparing with the STD diet at the same time point,* *p*<0.05.*

However, these beneficial effects on body weight disappeared after 6 months of treatment. Since food consumption was remarkably stable over the period from 2 to 24 weeks, this effect could possibly be explained in terms of metabolic adaptation or modification in mice after three months of treatment. This observation should prompt caution and additional experiments to know more about the health beneficial conditions of *n*-3 PUFAs prior to following high-dose and/or long-term DHA or fish oil supplementation treatments.

2.4.2. Effects of different dietary conditions on plasma TG and TC levels

The plasma TG levels showed no significant differences between the different groups during the course of treatment (Figure 36). However, it was interesting to note that up to the 12th week of the treatment, the HF mice showed a tendency towards higher TG levels than HF+FO mice, although the difference was not significant. It has been shown in the literature that HF diet does not always cause an increase in plasma TG levels in C57Bl/6J mice, possibly due to increased TG clearance (Biddinger *et al.*, 2005). Surprisingly, compared to the STD mice, the mice on FO diet showed higher TG levels in the beginning of the experiment, which then returned to levels similar to the STD mice at the end of the study.

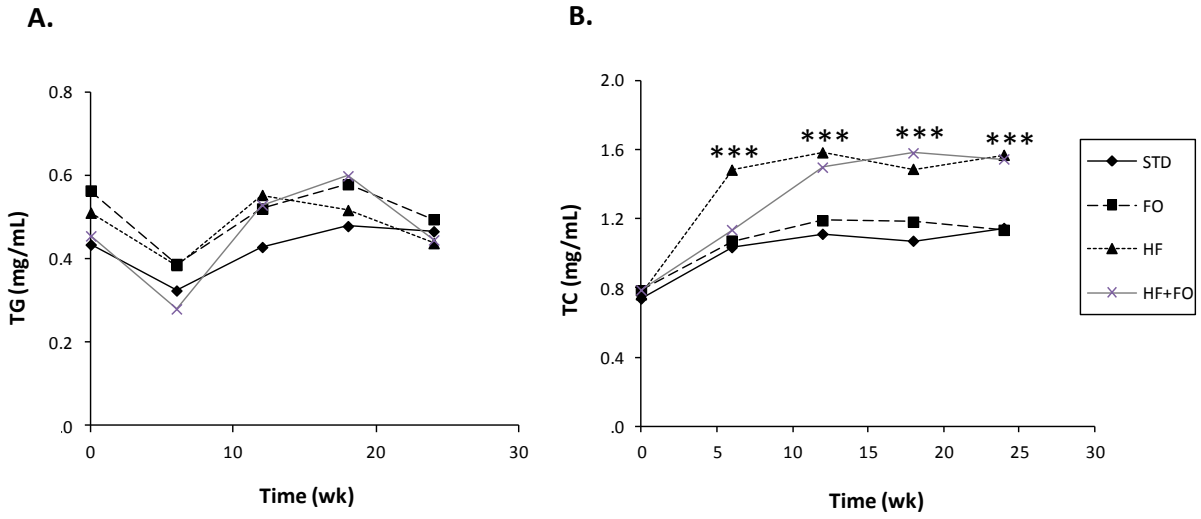


Figure 36: Plasma lipid levels in mice placed on different dietary conditions

Blood samples were collected from the four groups of mice, placed on different dietary conditions (see Figure 35) and plasma (A) TG and (B) TC levels were measured after every six weeks during the treatment. Results are shown as mean ± SEM and p*-values are indicated where significant, compared to the standard diet at the same time point. ****p*<0.001.*

Plasma TC levels measured at 6 weeks increased significantly in HF mice, compared with STD mice (Figure 36B), most likely due to the 0.15% cholesterol in HF and HF+FO. Similarly, a significant increase was observed in plasma TC levels in HF+FO mice, but only after 12 weeks of treatment, compared to the mice on FO diet containing no cholesterol. This suggests that the presence of *n*-3 LC-PUFAs delays the development of hypercholesterolemia in mice fed an HF diet. During the entire study period, plasma TC levels were lower for the STD and FO mice as compared with HF and HF+FO mice. Plasma TC levels remained unchanged in all four groups after 12th week of experiment till the end of the study.

These observations are consistent with the well-known cholesterol-lowering properties of *n*-3 LC-PUFAs, (Holub, 2009; Zhang *et al.*, 2010; Tang *et al.*, 2012; Pirillo & Catapano, 2013). These properties have been found to be associated with the beneficial effects of DHA in improving dyslipidemia and various related diseases such as CVDs, diabetes and AD (Sadovsky & Kris-Etherton, 2009; Zuliani *et al.*, 2009; Skulas-Ray *et al.*, 2011).

2.4.3. Effects of different dietary conditions on LSR protein and mRNA levels

In view of the potential beneficial effects regarding lower weight-gain and hypolipidemic effects of *n*-3 LC-PUFA supplementation, we next sought to determine whether these changes in body weight and lipid profiles reflected the *n*-3 LC-PUFA-mediated changes in the expression of LSR.

Western blot analyses performed on total liver membranes showed a 2.4-fold decrease in LSR protein levels in mice placed on HF diet for 6 months, compared to those placed on STD diet (Figure 37A) (Data obtained from Marine HANSE, PhD thesis, Université de Lorraine, 2011). Furthermore, real-time qPCR analyses were performed to determine LSR expression using mRNA samples extracted from the liver of mice placed on these four dietary conditions. Consistent with the results from Western blot analysis, LSR expression in HF mice was decreased upto 1.8-fold, as compared to the STD mice (Figure 37B).

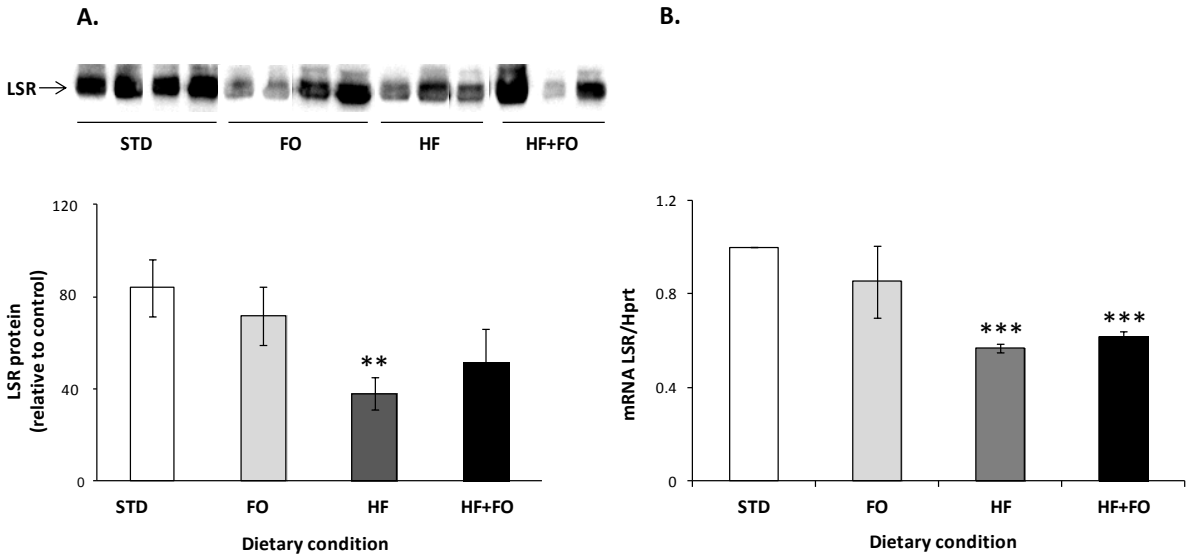


Figure 37: Effects of dietary conditions on LSR protein and mRNA levels in mouse liver

(A) Western blot analysis determined LSR protein levels in liver total membranes from mice (n=8 per group) treated with different dietary conditions for six months (see Figure 35). A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SEM. (B) Total RNA was extracted from these mice (n=6 for STD and FO; n=3 for HF and HF+FO) and real-time qPCR was performed to determine LSR expression using Hprt as reference gene. Values are represented as means ± SEM. The p*-values are indicated where significant,* **p*<0.01,* ***p*<0.001*.*

The decrease in LSR expression with HF diet supplementation in mice is in agreement with previous studies which have reported reduced expression of LSR in the mice supplemented with HF diet as well as in the mouse models of obesity lacking either leptin (*ob/ob*) or leptin receptor (*db/db*) (Narvekar *et al.*, 2009). On the other hand, no significant difference was observed in the expression of LSR in HF+FO mice, as compared with HF mice. Interestingly, the FO diet seemed to have little effect on the expression of LSR, but when the HF diet was combined with FO, the LSR protein levels were found to be slightly higher than that observed in mice placed on HF diet alone. Although these results are not significant, they suggest a possible action of DHA to reduce the HF diet-mediated hyperlipidemia and weight gain which is in accordance with the role of *n*-3 PUFA, as shown by various studies using different animal models (Lombardo *et al.*, 2007; Kasbi *et al.*, 2011; Lu *et al.*, 2011). Also, in view of these results, we can observe that the effects of different dietary treatments were more obvious after 3-month treatment. This further indicates that this duration could be sufficient to obtain the direct effects of the tested dietary components and to avoid the dietary adaptation due to long-duration nutritional programs.

2.4.4. Determination of LDL-R protein and mRNA levels

On the other hand, supplementation of HF diet with fish oil significantly increased the LDL-R mRNA and protein levels in liver total membranes of mice, as compared to those of mice fed on HF diet alone. Interestingly, when compared with the STD diet, no significant changes in LDL-R protein levels were observed in the FO and HF mice, whereas, a significant decrease was observed in LDL-R mRNA levels under these conditions (Figure 38).

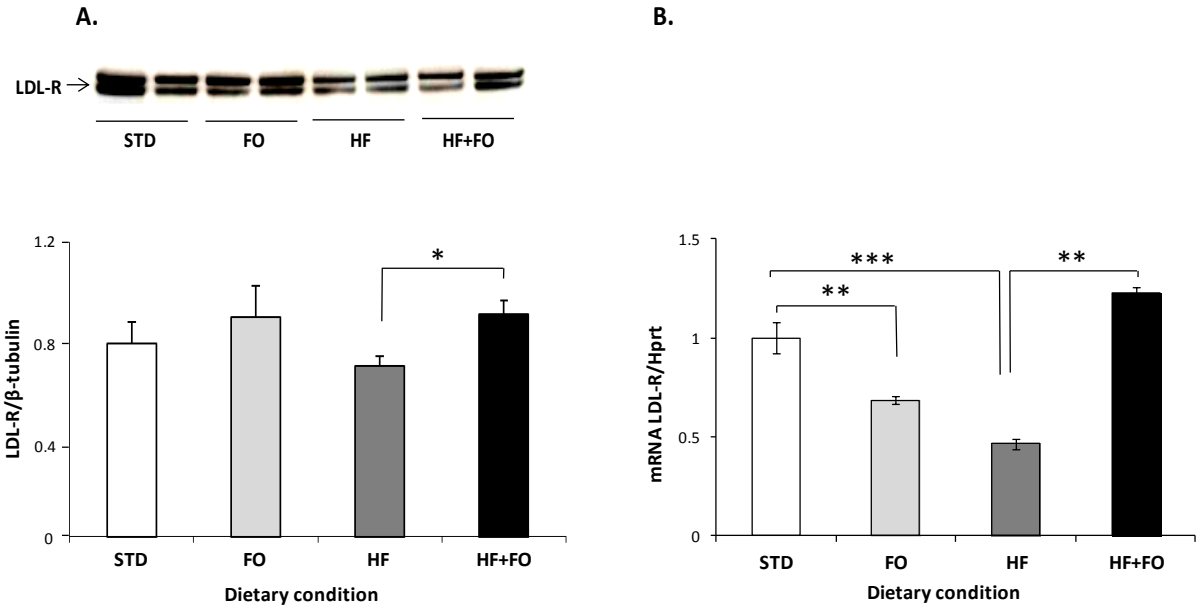


Figure 38: Effects of dietary conditions on LDL-R protein and mRNA levels in mouse liver

(A) Western blot analysis determined LDL-R protein levels in liver total membranes from mice (n=8 per group) treated with different dietary conditions for 6 months (see Figure 35). β-tubulin was used as loading control to normalize LDL-R protein levels. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SEM. (B) Total RNA was extracted from these mice (n=3 per group) and real-time qPCR was performed to determine LDL-R expression using Hprt as reference gene. Results are represented as means ± SD. The p*-values are indicated where significant,* *p<*0.05*,* **p<*0.01*,* ***p<*0.001*.

It has been well-established that elevated LDL-cholesterol in the plasma represents a risk factor for the development of CVD (Werner & Pearson, 1998; Baigent *et al.,* 2005; Jacobson *et al.,* 2012). Plasma LDL levels are mainly regulated by LDL-R through receptor-mediated endocytosis that accounts for 70-80% of LDL turnover (Bilheimer *et al.,* 1982; Pittman *et al.,* 1982; Goldstein & Brown, 1987). Studies have shown that the HFD, containing cholesterol, leads to suppression of LDL-R activity as well as to reduced LDL-R protein and mRNA levels (Rudling, 1992; Horton *et al.,* 1993).

Our results indicate that fish oil-mediated increase in LDL-R expression in HF+FO mice may likely to be associated with the clearance of cholesterol-rich lipoproteins (LDL) in order to reduce their presence in the circulation and ultimately the distribution of lipids to peripheral organs. However, this increase in LDL-R expression seems insufficient to lower plasma TC levels observed in HF+FO mice. This may be caused by increased LDL levels observed in these mice during the course of experiment (Figure 39). Therefore, we can speculate that the age-related changes and/or the duration of the experiment may lead to the high plasma TC levels which may reflect impairment in lipid metabolism (Data obtained from Ahmad ALLOUCHE, PhD thesis, Université de Lorraine, 2012).

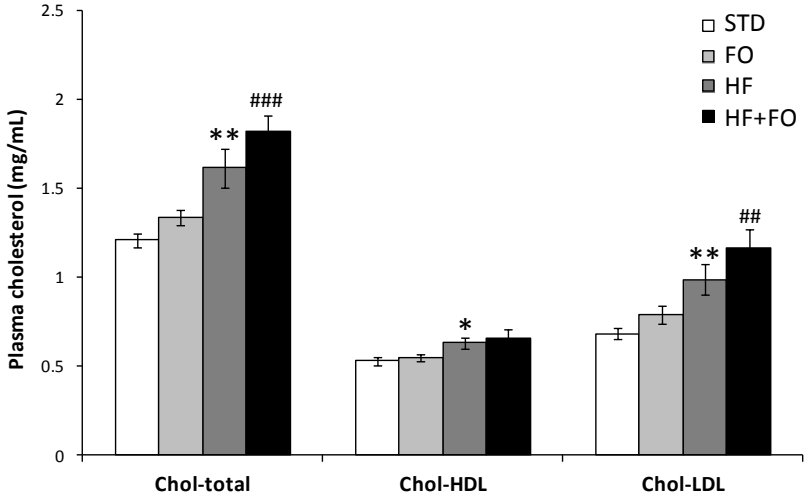


Figure 39: Changes in the distribution of cholesterol in plasma lipoproteins

The plasma concentrations of total, HDL and LDL cholesterol were assessed for the mice treated with different dietary conditions for 6 months (see Figure 35). Results are represented as means ± SEM and the p*-values are indicated where significant, *p<0.05 and **p<0.01 represent the significance relative to the mice on STD diet, while ##p<0.01 and ###p<0.001 represent the significance relative to the mice on FO diet*

Moreover, the serum cholesterol values measured at 24 weeks in mice fed on HF diet showed a significant increase in the plasma concentration of LDL-cholesterol relative to mice on STD diet, with small but significant increase in HDL-cholesterol. This led to an overall effect of raising the proportion of LDL-cholesterol with the proportion of HDL-cholesterol reduced to about 40% in these mice. This change in lipoprotein profile as a result of a dietary cholesterol intake in rodents is already known for a long time (Kushwaha *et al.,* 1978; Tsuda *et al.,* 1983). But it should be noted that this reversal of the distribution of cholesterol between HDL and LDL, though non-significant, was also observed in STD and FO mice suggesting

that a change in the lipid metabolism may have occurred in these mice due to age and/or the duration of the supplementation experiment.

3. Conclusions

Mounting evidence supports the notion that dietary *n*-3 LC-PUFAs (EPA and DHA) exert hypolipidemic properties and are, therefore, involved in the prevention of various disorders related to lipid metabolism, including obesity, diabetes and CVDs. One of the most important mechanisms through which DHA improves plasma lipid profiles involves its influence on the expression of various enzymes and receptors related to lipid metabolism and the modification in the physicochemical properties of plasma membrane (Sun *et al.,* 2011; Zuliani *et al*., 2009; Zhang *et al.,* 2010; Shaikh & Teague, 2012).

Plasma lipid levels are influenced by the amount and type of dietary FA. One of the mechanisms involved in the maintenance of lipid metabolism includes the clearance of TG-rich lipoproteins by LSR. During this study, different *in vivo* and *in vitro* experiments were designed to find out whether DHA is involved in LSR regulation. Our *in vitro* findings using Hepa 1-6 as cell models revealed an increase in LSR activity and protein levels in the presence of DHA. However, no changes were observed in LSR mRNA levels. Although the exact mechanisms for DHA-mediated regulation of LSR remain to be explored, it can be speculated that these effects might be related to the changes in the plasma membrane composition. This could lead to the localization of the receptor in a favorable environment, in order to optimize its activity.

Furthermore, three *in vivo* studies were carried out in order to investigate the effect of dietary intake of DHA on LSR expression using mice as experimental models. All these diets contained the same quantity of DHA. However, there were differences in the age of mice at the start of the experiment, in treatment duration and in the chemical form of DHA (EE or TG). The 4-month old mice fed on a diet supplemented with DHA-EE for one month showed no changes in LSR mRNA levels. Similar effects on LSR mRNA levels were observed when 9-month old mice were placed on the same diet for three months, whereas LSR protein levels were significantly increased under these conditions. Moreover, in the view of DHA enrichment in the liver during this study, we can speculate that DHA may play an important role in LSR regulation at translational and/or post-translational levels, as has been explained earlier in this chapter. On the other hand, the dietary supplementation of DHA in the form of

fish oil in 9-month old mice for 6 months did not significantly alter LSR mRNA and protein levels. Such very long-term supplementation experiments often lead to complicated interpretations due to the involvement of the age factor, which has been shown in the literature to aggravate the pathological conditions. In addition, an incomplete data is available for some studies that make comparison hardly possible. Therefore, further investigation is needed to explore the mechanisms involved in LSR regulation by DHA. Finally, as DHA is known to act as an activator for various transcription factors involved in hepatic lipid metabolism including PPARs, SREBPs and LXRs (Jump *et al.*, 2008), the possible involvement of these TFs in mediating DHA effects on LSR should also be considered.

II. *In silico* analysis of *lsr* gene potential regulatory regions and transcription factor binding sites

1. Introduction

In eukaryotes, gene transcription is an intricate process that determines the expression of a gene depending upon various factors including the type of cells and the developmental stages. This process is accomplished by a number of enzymes and other proteins in the nucleus that transcribe DNA into mRNA (Orphanides & Reinberg, 2002; Zhang, 2002). The protein synthesis occurs in the cytoplasm through the process of translation in which mRNA binds to the ribosomal subunits to form a polypeptide, which then folds into an active protein (Figure 40).

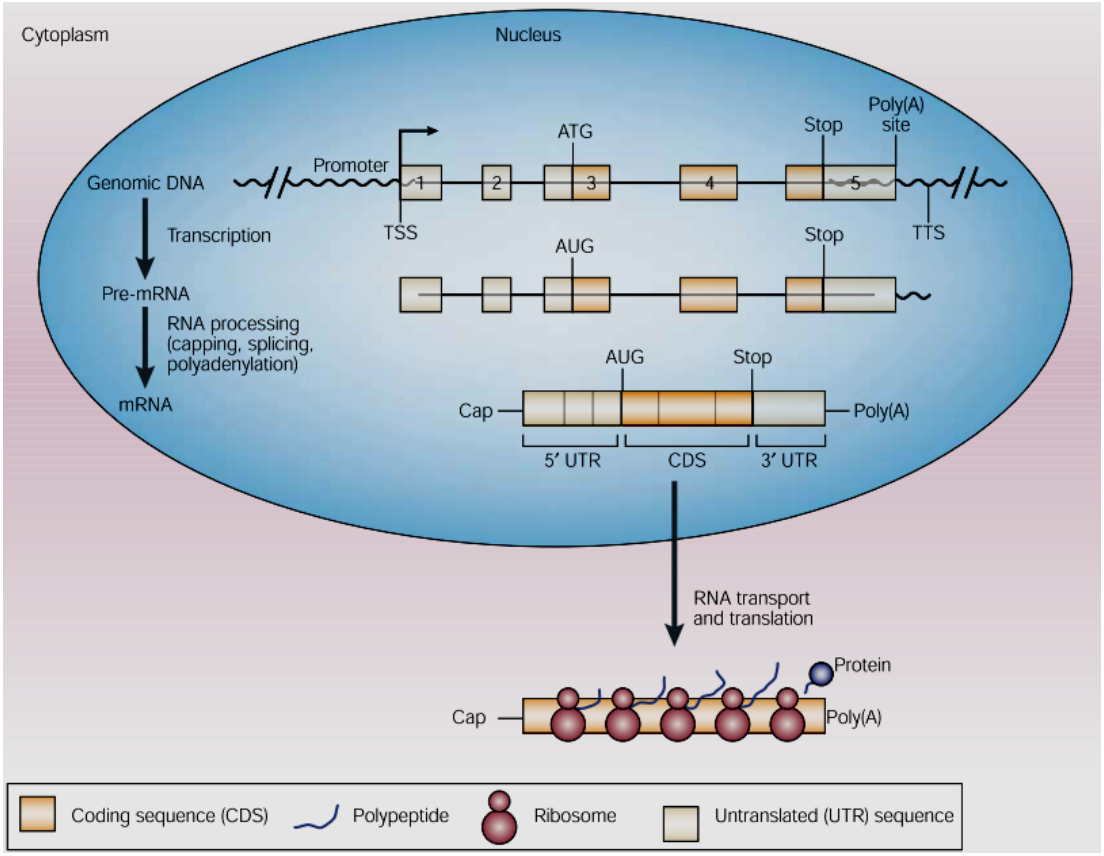


Figure 40: The central dogma of eukaryotic gene expression

The typical process of eukaryotic gene expression involves the complex process of transcription which takes place by the binding and construction of transcriptional machinery with the promoter region of a gene, leading to the formation of an mRNA precursor molecule (pre-mRNA or primary transcript) in the nucleus. Pre-mRNA is stabilized by certain post-transcriptional modifications of the molecule, including capping in 5' and polyadenylation in 3' as well as intron removal and exon splicing. The mature mRNA is then transported from the nucleus to the cytoplasm to participate in the process of translation. TSS: transcription start site; TTS: transcription termination site. (Zhang, 2002)

1.1. Core promoter elements

The regulation of gene expression in eukaryotes is exerted at different levels in order to attain controlled expression to meet cellular demands. This regulation occurs primarily at the level of transcription initiation through the involvement of the core promoter of a gene which controls the recruitment and functioning of the relevant RNA polymerase (Strachan & Read, 2003; Juven-Gershon & Kadonaga, 2010). Transcription of protein-encoding genes is dependent on the assembly of pre-initiation complex (PIC) which comprises of template DNA and RNA polymerase II, an enzyme that catalyzes DNA-dependent synthesis of mRNA. Besides this, the binding of TATA-binding protein (TBP) to the TATA box appears to be a pivotal intermediary step in transcriptional activation and deactivation. TBP is recruited to the TATA box along with six general transcription factors (GTFs) including TFIIA, B, D, E, F and H which facilitate the recognition of target promoters by RNA polymerase II (Orphanides *et al.,* 1996; Roeder, 1996; Pugh, 2000; Woychik & Hampsey, 2002). Here, we will particularly discuss about the different sequence elements found in the promoter regions and various tools and procedures that we employed to identify the potential regulatory regions in *lsr* gene.

A typical eukaryotic gene promoter contains basal or core promoter elements, promoter proximal elements, and distal enhancer elements. Core promoter is defined as the minimal DNA sequence that is sufficient to direct the accurate transcription initiation by RNA polymerase II (Roeder, 1996; Smale, 2001; Butler & Kadonaga, 2002). The core promoter usually encompasses the transcriptional start site (TSS) and extends either upstream or downstream for about 35 nucleotides (nt), whereas a proximal promoter of about 500 bp is located immediately upstream of the core promoter. The core promoter contains various core promoter elements which play an important role in specifying the TSS. It is noteworthy that the core promoters generally contain some, not all of these core promoter elements (Butler & Kadonaga, 2002). The most common elements found in the core promoter region include TATA box, a pyrimidine-rich initiator element (Inr), TFIIB recognition element (BRE), the downstream core promoter element (DPE) and the motif ten element (MTE), downstream core promoter element (DCE) and X core promoter elements 1 and 2 (XCPE1 and XCPE2) (Figure 41). Among these, TATA box and Inr are considered to be especially important because only these motifs are directly recognized by the GTFs (Roeder 1996; Smale 1997). GC box and CAAT box are also thought to be important promoter elements besides TATA box and Inr.

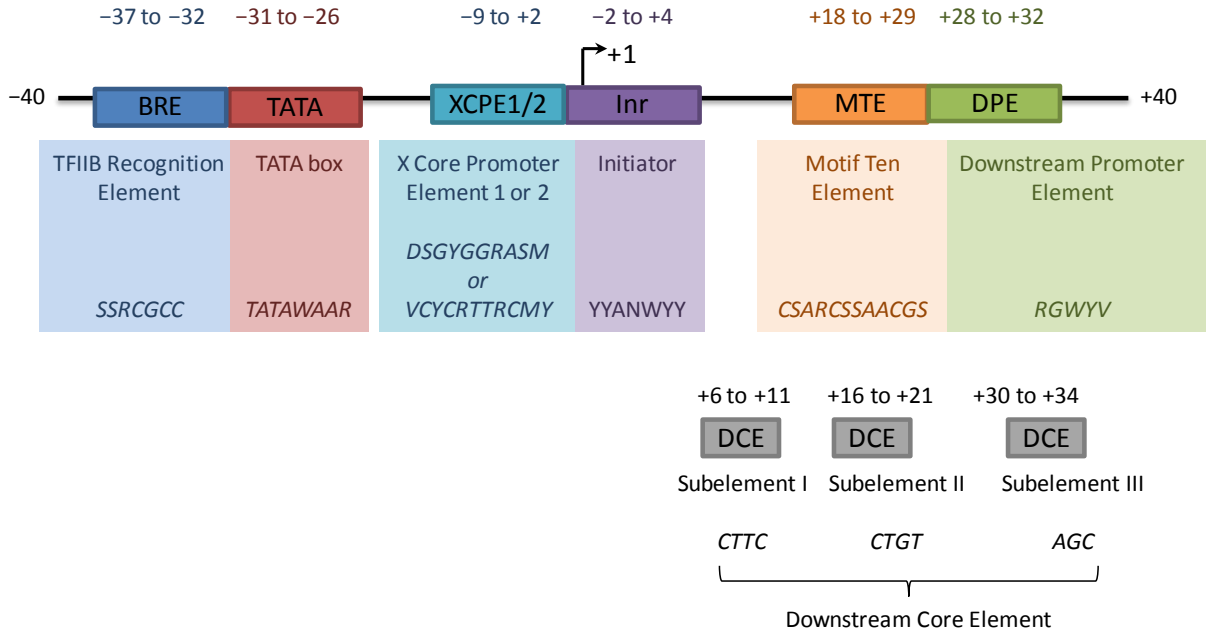


Figure 41: General representation of the core promoter elements

Various upstream and downstream core promoter elements, that play an important role in RNA polymerase II-mediated transcription, are shown. Any specific core promoter may contain some, all, or none of these motifs. The consensus sequence and the location of each element are depicted and numbered relative to the TSS (+1). S= C/G, R= A/G, W= A/T, D= A/G/T, Y= C/T, M= A/C, N= A/T/G/C, V= A/C/G. (modified from Anish et al., *2009; Juven-Gershon & Kadonaga, 2010)*

1.1.1. The TATA box

The TATA box is primarily located ~26–31 nt upstream of the TSS and is the first core promoter element to be identified (Goldberg, 1979, Breathnach & Chambon, 1981; Butler & Kadonaga, 2002), showing a consensus sequence of TATW(A/T)AAR(A/G), which may be variable by one or two mismatches and yet functional *in vivo* (Singer *et al.*, 1990). However the presence of TATA box is not the only important factor for the recruitment of RNA polymerase II, since ~80% of human core promoters lack TATA box (Suzuki *et al.*, 2001; Yang *et al.*, 2007; Anish *et al.*, 2009).

1.1.2. The initiator

On the other hand, the Inr is a conserved sequence that encompasses the TSS and directs accurate transcription initiation either by itself or in conjunction with TATA-box or DPE (Smale & Baltimore, 1989; Yang & Elnitski, 2008). However, Inr and TATA-box work synergistically when present at a distance of 25-30 bp from each other and independently when separated by more than 30 bp (O'Shea-Greenfield & Smale, 1992). Inr has a consensus

sequence of Y(C/T)-Y(C/T)-A(+1)-N(A/G/C/T)-W(A/T)-Y(C/T)-Y(C/T) in humans, where the A (+1) nt is typically referred to the +1 position of the core promoter (Smale & Kadonaga, 2003).

1.1.3. The TFIIB recognition element

The eukaryotic BRE is a GC-region with a consensus sequence of S(G/C)-S(G/C)-R(G/A)-C-G-C-C. It serves as a binding site for TFIIB and is located immediately upstream of some TATA box elements (Lagrange *et al.,* 1998; Butler & Kadonaga, 2002).

1.1.4. The downstream core promoter element

The DPE is most commonly found in TATA-less promoters located precisely at +28 to +32 relative to the A+1 position in the Inr. The consensus sequence for the DPE has been defined to be R(A/G)(+28)-G-W(A/T)-Y(C/T)-V(G/A/C), with a minor preference for G at +24 position. DPE functions in association with the Inr to bind TFIID in order to direct accurate and efficient initiation of transcription in TATA-less promoters (Burke & Kadonaga, 1997; Kutach and Kadonaga, 2000).

1.1.5. The motif ten element

In addition, the MTE has been identified as a distinct core promoter element which is located +18 to +29 relative to A+1 position in the Inr element, with the sequences from +17 to +22 being essential for MTE-induced transcriptional activity. It has a consensus sequence of C-S(C/G)-A-R(A/G)-C-S(C/G)-S(C/G)-A-A-C-G-S(C/G), and has the ability to promote transcription by RNA polymerase II in combination with Inr and independently of TATA box and DPE elements. Moreover, the addition of MTE also compensates for the loss of transcriptional activity caused by the mutation of a TATA box or DPE (Ohler *et al.,* 2002; Lim *et al.,* 2004; Jin *et al.,* 2006).

1.1.6. Downstream core element

The DCE is another core promoter found in a large number of promoters, but it is preferentially present in promoters containing the TATA box. The DCE was initially discovered in human β-globin and shows a marked difference in terms of sequence and factor requirements from that of the DPE. The DCE is composed of three subelements, and each is distinct from the DPE sequence: SI is CTTC and is located from +6 to +11, SII is CTGT and is positioned from +16 to +21, and SIII is AGC and it resides from +30 to +34 and it can

function independently of the other two subelements. The DCE-driven transcriptional activity is dependent on TFIID (Lewis *et al.*, 2000; Lee *et al.*, 2005).

1.1.7. X Core Promoter Element 1

XCPE1 is located between nt −8 and +2 relative to TSS and has a consensus sequence of D(G/A/T)-S(G/C)-G-Y(T/C)-G-G-R(G/A)-A-S(G/C)(+1)-M(A/C). XCPE1 exerts significant promoter activity only in the presence of activator binding sites and/or in the absence of TFIID, so that it could use either free TBP or the whole TFIID complex to mediate the transcriptional activity. It is interesting to note that about 1% of the TATA-less human genes contain XCPE1 in their promoter region (Tokusumi *et al.*, 2007).

1.1.8. X Core Promoter Element 2

XCPE2 is a novel core promoter element found at the second start site of the hepatitis B virus (HBV) X mRNA. It has also been shown to be present in human TATA-less promoters, where they drive transcriptional activation at one of the start sites present within the promoters containing multiple TSS. The location of XCPE2 is similar to XCPE1, *i.e.* between nt −9 and +2. However, there exist some differences between the two core promoter elements. First, the XCPE2 consensus sequence, V(A/C/G)-C-Y(C/T)-C-R(G/A)-T-T-R(G/A)-C-M(C/A)(+1)-Y(C/T), is different from that of XCPE1, and secondly, unlike XCPE1, it can show significant transcriptional levels by itself. Nevertheless, XCPE2 is mainly dependent on RNA polymerase II, TFIIB, a mediator protein and either a free form of TBP or TFIID to drive the transcriptional activation (Anish *et al.*, 2009).

In addition to core promoter elements, the promoter proximal elements are located between 50 and 200 bp upstream of the TSS and regulate transcription through their interactions with transcriptional activators. Finally, distal enhancer elements act as important binding sites for the factors regulating RNA polymerase II activity and can be present far from the transcription initiation site in either direction or orientation (Nikolov & Burley, 1997).

The identification and characterization of promoter regions is crucial as a first step towards determining the transcriptional regulatory mechanisms involved. The promoter sequence initiates and regulates the gene transcription and is usually located proximal to or overlapping the TSS (Juven-Gershon & Kadonaga, 2010). With regards to LSR, though the cloning of rat *lsr* gene has already been reported (Yen *et al.*, 1999), the promoter region and various regulatory elements, however, remain uninvestigated. Therefore, this *in silico* analysis

was performed with the objective to identify and to compare the potential regulatory regions in the 5' upstream sequences of the mouse, rat and human *lsr* gene.

2. *In silico* analysis of the potential regulatory regions

The potential regulatory regions located upstream of the mouse, rat and human *lsr* genes were analysed using various bioinformatics tools available online. The genomic sequences of the mouse (NC_000073.6), rat (NC_005100.3) and human (NC_000019.9) *lsr* genes were retrieved from NCBI. The mouse, rat and human *lsr* genes are located on the chromosomes 7, 1 and 19 respectively. Sequence comparison using Clustal w2 online available bioinformatics tool (Larkin *et al.,* 2007; http://www.ebi.ac.uk/Tools/msa/clustalw2/) showed 54% sequence identity of mouse and rat *lsr* gene with human, and 84% identity between mouse and rat *lsr* gene full-length sequences. Initially, the putative TSS in the mouse, rat and human *lsr* gene sequences was predicted by mapping the full length mRNA/cDNA sequences (including the complete 5' UTR) shared in common by the three LSR subunits (α, α' and β) to the genomic sequences and taking the immediate 5' sequence as the potential promoter region. The identification of the putative promoter regions was confirmed using online available Genomatix (http://www.genomatix.de/) tools, namely Gen2promoter and PromoterInspector (Scherf *et al.,* 2000; Genomatix). These tools predict the probable promoter sequences with high specificity depending on the presence of eukaryotic RNA polymerase II regions.

2.1. *In silico* identification of regulatory elements

The putative regulatory sequences were analysed using MatInspector (Genomatix) in order to identify putative transcription factor binding sites (TFBSs) within these sequences (Cartharius *et al*., 2005). The analysis identifies the 'core' region of the binding site, *i.e.* the region with the highest nucleotide conservation (in capital letters) as well as a 'matrix' similarity score which also takes into account the nucleotides present on either side of the core region. Scores greater than 0.90/0.75 for core/matrix values generally represent 'good' matches to the consensus sequences and are considered to be potentially high-affinity sites. It should be noted that all sites identified in *lsr* gene potential promoters meet these minimum requirements for potentially high affinity matrices.

2.1.1. Potential core promoter elements in mouse *lsr* gene

The analysis of the mouse *lsr* gene 5'-flanking regulatory region (750 bp) predicted the core promoter elements including one site for each of RNA polymerase II TFIIB, Inr and MTE, for the activator-, mediator- and TBP-dependent core promoter element (XCPE1) as well as four sites for Sp1/GC box elements and two sites for CCAAT/enhancer binding protein (Table 15).

Table 15: Core promoter elements within the putative promoter of mouse* lsr *gene
(The capital letters in the sequence indicate core region of the binding site)

Core promoter elements	Position	Core similarity	Matrix similarity	Sequence
RNA polymerase II TFIIB	−713/−707	1	1	ccgCGCC
Core promoter initiator elements	−181/−171	1	0.949	gtTCAGtcctt
Core promoter motif ten elements	−593/−573	1	0.842	atacccgAGCGggaggtggaa
Activator-, mediator- and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters	−587/−577	1	0.825	gaGCGGgaggt
Sp1/GC box elements	−33/−17	1	0.98	caaggcccGCCCcgcc
	−238/−222	1	0.924	aaggcccgGCCCctctc
	−24/−8	1	0.921	cccccgccGCCCacccc
	−301/−285	1	0.882	cacctaGGCGggtccat
CCAAT/enhancer binding protein	−563/−549	1	0.973	caagttagGAAAtaa
	−329/−315	1	0.834	gctctgcTTGGcacc

It is evident from this analysis that no consensus TATA box was found in the mouse sequence. One RNA polymerase II binding site has been identified, but it is located very- and certainly too- far from the TSS (−713/−707). The MTE and Inr sequences are also located at a distant position from one another as well as from the TSS. Furthermore, this sequence appears to possess the characteristics of a TATA-less promoter, containing several GC-rich sequences and CCAAT binding sites. Similar observations have also been obtained in some other gene promoters, including the mouse and human PPARα (Gearing *et al.,* 1994; Pineda-Torra *et al.,* 2002). However, the functional analysis of this sequence is required in order to confirm its potential role as a promoter sequence.

2.1.2. Putative regulatory elements in mouse *lsr* gene

MatInspector analysis for the detection of potential TFBSs in this putative promoter predicted the presence of a large number of regulatory elements including the ones involved

in cell growth, development and differentiation, cell cycle regulation and response to extra- and intracellular signals. Among these, we selected two classes of TFs, *i.e.* PPARs, because they are actively involved in lipid metabolism and the signal transducer and activator of transcription (STAT), because of their implication in several signaling pathways as well as their presence in high frequency and with strong affinity within the putative promoter region (Table 16).

Table 16: Transcription factor binding sites within the putative promoter of mouse* lsr *gene
(The capital letters in the sequence indicate core region of the binding site)

Potential regulatory elements	Position	Core similarity	Matrix similarity	Sequence
PPAR	−688/−666	1	0.831	tgggaacCTTTgatccgaaacag
	−873/−851	1	0.766	atgtgACCTgacaccatttacaa
STAT	−2133/−2115	1	0.96	gccgtgcccAGAAaccttt
	−1554/−1572	1	0.953	cagaagggccaGGAAatgg
	−893/−875	1	0.947	gcaaagTTCTgagcagaag
	−56/−38	1	0.94	ccacTTCCgggggagggagg
	−447/−429	1	0.938	cattgaTTCCtcgaatcat
	−790/−772	1	0.931	cagtctcccAGAAtgcgtt
	−422/−404	1	0.93	gtggagcccAGAAagaccg
	−445/427−	1	0.905	ttgaTTCCtcgaatcattc
	−58/−40	1	0.846	gcccacTTCCggggaggga

PPARs regulate the gene transcription by binding to the PPAR response elements (PPREs), located in the promoter sequence of the genes regulated by them. PPRE consists of two more or less conserved AGGTCA hexamers separated by a single nt (referred as DR1 elements), with PPAR occupying the 5'-motif (Dreyer *et al.,* 1992; IJpenberg *et al.,* 1997). PPREs have been identified in the promoters of numerous genes including ACOX1 (−1918 to −1906, Varanasi *et al.,* 1996), Stearoyl coA desaturase 1 (−664 to −642, Miller & Ntambi, 1996) and malic enzyme (−340 to −328, IJpenberg *et al.,* 1997), which shows no positional bias for PPREs.

Studies using *in vitro* and *in vivo* functional validation assays on 30 putative PPREs in eight validated PPAR target genes indicated the presence of at least one functional and strong or multiple medium PPREs. These PPREs were present in the promoter regions without positional preference relative to the TSS and the PPRE patterns also show limited conservation in both human and mouse species (Heinäniemi *et al.,* 2007). The sequence analysis of whole 5'-upstream region of mouse *lsr* gene using MatInspector identified two

sites for PPARs, whose functional significance remains to be defined. Interestingly, our results obtained in Hepa 1-6 cells have shown an increase in LSR expression using PPARα-selective agonists and a decrease in LSR with antagonist treatment (as described in Results and Discussion, section III). This may indicate that the presence of PPREs in mouse *lsr* putative regulatory region confers this PPARα responsiveness to LSR.

A number of studies have reported the implication of STAT factors in the regulation of various cellular processes such as growth, survival, differentiation and immune system (Bromberg & Darnell 2000; Ihle, 2001). Interestingly, multiple STAT binding sites were identified throughout the putative regulatory region of mouse *lsr* gene, which may indicate an involvement of STAT factors in LSR functional regulation.

2.1.3. Potential core promoter elements in rat *lsr* gene

The analysis of the rat *lsr* gene 5'-flanking regulatory region (750 bp) indicates lesser core promoter elements than those identified in mouse. These elements include five sites for Sp1/GC-box elements and two for vertebrate TBP (Table 17).

Table 17: Core promoter elements within the putative promoter of rat* lsr *gene
(The capital letters in the sequence indicate core region of the binding site)

Core promoter elements	Position	Core similarity	Matrix similarity	Sequence
Sp1/GC box elements	−114/−98	1	0.98	caaggcccGCCCcgcc
	−105/−89	1	0.921	cccccgccGCCCacccc
	−319/−303	1	0.915	aggtcccgGCCCctctc
	−88/−72	1	0.915	caagcccaGCCCcgggg
	−382/−366	1	0.882	cacctaGGCGgtccat
TATA binding protein factor	−594/−578	1	0.876	cttttTAAAaggcgctg
	−599/−583	1	0.854	tggaacttTTTAaaagg

In this region, no binding site was identified for RNA polymerase II, which may be because of the strict sequence matching requirements by MatInspector to identify a sequence stretch as putative RNA polymerase II binding site. The studies have reported the presence of at least 5 out of 7 matches with the consensus sequence in 12% of a collection of 315 TATA-containing promoters (Lagrange *et al.*, 1998). However, this sequence requires to be further investigated through experimental work.

2.1.4. Putative regulatory elements in rat *lsr* gene

Similar to that of mouse, the rat potential regulatory elements include four sites for PPARs, twelve sites for STAT and one site for lactotransferrin motif (Table 18).

Table 18: Potential regulatory elements within the putative promoter region of rat* lsr *gene
(The capital letters in the sequence indicate core region of the binding site)

Potential regulatory elements	Position	Core similarity	Matrix similarity	Sequence
PPAR	−254/−232	1	0.913	ctttcaaCTTTggccagagcagc
	−949/−927	1	0.835	ctgacacCTTTgtacaaaacaga
	−956/−934	1	0.771	atgtgACCTgacacctttgtaca
	−767/−745	1	0.76	tgggaacCTTTgaaccgaaacag
STAT	872/−854	1	0.98	cataatTTCCcagaatgag
	−870/−852	1	0.975	taatTTCCcagaatgagtt
	−1263/−1245	1	0.961	caatTTCCtggacttaagc
	−2237/−2219	1	0.96	gccgtgcccAGAAaccttt
	−1215/−1197	1	0.957	agaattTTCTgagatgcct
	−45/−27	1	0.952	tccaTTCCcgggagtgtgc
	−137/−119	1	0.94	ccacTTCCggggagggaag
	−527/−509	1	0.938	cattgaTTCCtcgaatcat
	−525/−507	1	0.905	ttgaTTCCtcgaatcattc
	−1265/−1247	1	0.899	ggcaatTTCCtggacttaa
	−47/−29	1	0.862	gttccaTTCCcgggagtgt
	−139/−121	1	0.846	gcccacTTCCggggaggga
Lactotransferrin motif	−886/−878	1	0.942	gGCACtggc

The presence of high affinity matrices and highly frequent PPARs and especially STAT binding sites may prove to be important regulatory sites for rat *lsr* gene. Interestingly, a single putative binding site has been identified for lactoferrin. Lactoferrin is a milk protein that has been shown to inhibit LSR activity in FH fibroblasts (Yen *et al.,* 1994) and in liver membranes (Mann *et al.,* 1995). Various studies have shown that the exogenous lactoferrin is internalized by the human carcinoma cells and ultimately transported to the nucleus, where it can affect the transcription of various genes (Fleet, 1995; He & Furmanski, 1995). Once validated experimentally, the presence of this element in the rat *lsr* gene putative regulatory region may play an important role in understanding the molecular mechanisms of interaction between LSR and lactoferrin.

2.1.5. Potential core promoter elements in human *lsr* gene

The analysis of the human *lsr* gene 5'-flanking regulatory region (750 bp) predicted the core promoter elements including three sites for RNA polymerase II TFIIB, five for core promoter motif ten elements, three for XCPE1, one for vertebrate TBP and seven for Sp1/GC-box elements (Table 19).

Table 19: Core promoter elements within the putative promoter of human* lsr *gene
(The capital letters in the sequence indicate core region of the binding site)

Core promoter elements	Position	Core similarity	Matrix similarity	Sequence
RNA polymerase II transcription factor II B	−674/−668	1	1	ccgCGCC
	−669/−663	1	1	ccgCGCC
	−601/−595	1	1	ccgCGCC
Core promoter motif ten elements	−696/−676	1	0.894	ccAGCGctgcgcaggctgatc
	−121/−101	1	0.887	gatctcggtgCGCTtggtttg
	−582/−602	1	0.868	cccgcgccttCGCTcggcccc
	−56/−36	1	0.781	ccctcccAGCGggccagtcac
	−701/−681	1	0.771	cacgcccAGCGctgcgcaggc
Activator-, mediator- and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters	−175/−165	1	0.877	gggacCCGCcc
	−179/−169	1	0.877	cgGCGGgaccc
	−666/−656	1	0.855	cgcccCCGCcc
TATA binding protein factor	−412/−396	1	0.864	ccggggacTTTAgaggg
Sp1/GC box elements	−651/−667	1	0.963	gcgcccccGCCCtcggt
	−176/−160	1	0.936	cgggacccGCCCtatct
	−673/−657	1	0.921	cgcgccgCGCCcccgcc
	−361/−345	1	0.919	cccgaagCGCCcccccct
	−706/−670	1	0.895	agagtcaCGCCcagcgc
	−240/−224	1	0.882	cacctaGGCGggtccat
	−184/−168	1	0.849	gtgcgcggcgGGACccg

Analysis of the human *lsr* gene putative regulatory elements indicates the presence of large number of core promoter elements including multiple binding sites for RNA polymerase II, which are also present at distant positions (−674/−668, −669/−663 and −601/−595) relative to the putative TSS. These observations are comparable to the RNA polymerase II binding sites found in mouse *lsr* regulatory region (−713/−707).

Similarly, multiple MTE sites are also found in this sequence, whereas no Inr elements were detected. As MTE functions in conjunction with Inr, the functionality of these predicted elements can be doubtful. However, the presence of single or multiple XCPE1 elements has been described in 1% of human protein-coding genes with TATA-less promoters. It was also

shown in this study that some activator elements such as Sp1/GC box, nuclear factor 1 (NF1)/CCAAT boxes, and nuclear respiratory factor 1 (NRF1), are essentially present in XCPE1-containing promoters (Tokusumi *et al.*, 2007). Some of these elements such as Sp1/GC boxes were also found in our sequence in various dispersed locations along with NRF1 sequences (−69/−52; −670/−653), which may indicate that this whole assembly of factors may play an important role in the regulation of human *lsr* gene. Taking these observations into account for human *lsr* gene potential regulatory region, the presence of these activators for XCPE1 was also investigated in mouse *lsr* upstream region. Multiple sites for Sp1/GC and CCAT boxes as well as for NF1 (−325/−304; −650/−629) were also identified, which may also point towards the importance of these elements in mouse *lsr* gene regulation. In addition, one TATA-binding protein factor was also observed, though very far from the putative TSS and therefore needs experimental work to identify its importance.

2.1.6. Putative regulatory elements in human *lsr* gene

MatInspector analysis of mouse *lsr* gene 5'-upstream region identified six PPREs and eight STAT binding sites, which are located throughout the putative proximal and distal regulatory regions. The potential TBFSs include six sites for PPARs and eight sites for STAT (Table 20).

Table 20: Potential regulatory elements within the putative promoter region of human lsr gene

(The capital letters in the sequence indicate core region of the binding site)

Potential regulatory elements	Position	Core similarity	Matrix similarity	Sequence
PPAR	−2766/−2744	1	0.908	cacttaatgggaAAAGgacagtg
	−2183/−2161	1	0.885	cttttgtgcaccAAAGgacacta
	−1769/−1747	1	0.839	ggcagttcctcaAAAGgttaaaa
	−934/−912	1	0.825	agataagagggggaAGGTcagtt
	−1378/−1356	1	0.778	attactaggggcacAGGTagcag
	−200/−178	1	0.771	ggcagggagggaacAGGTgcgcg
STAT	−641/−623	1	1	ctcgttccgGGAAttccta
	−643/−625	1	0.969	ggctcgttccgGGAAttcc
	−466/−449	1	0.957	acttTTCCtggccctgatt
	−418/−400	1	0.95	tcccTTCCggggactttag
	−1975/−1957	1	0.937	aataaattcgtGGAAatat
	−1770/−1752	1	0.882	tggcagTTCCtcaaaaggt
	−420/−402	1	0.819	cttcccTTCCggggacttt
	−1070/−1052	1	0.787	acatctgcgGGAAgggtgt

The TFBSs found in this region do not match with the mouse and rat binding sites with respect to their location relative to TSS. This may lead to differential regulation of LSR expression by PPARs and STATs in these three species.

3. Similarities between mouse, rat and human *lsr* regulatory sequences

The regulatory regions identified by Gen2promoter in mouse, rat and human *lsr* gene 5'-flanking sequences were aligned using DiAlign TF (Genomatix) to determine the presence of common TFBSs. This tool performs an alignment for multiple sequences and identifies the TFBS matches in the aligned regions using MatInspector. TFBSs are indicated only if they are 85% common to the aligned sequences and are located at the same positions. The analysis showed 52% identity between mouse and human, 50% identity between rat and human and 87% identity between mouse and rat putative regulatory sequences. The common sites present in these well-conserved sequences of *lsr* gene in the three species include the regulatory elements for PPAR/RXR heterodimers and STAT.

This bioinformatics analysis allowed us to determine the presence and location of potential regulatory elements in *lsr* gene along with the identification of TFBSs. This analysis, of course, does not eliminate other potential TFBSs that can occur elsewhere in the genome and that are not constrained to the regulatory sequences.

4. Conclusions

This study was an initial step towards the *in silico* identification of LSR putative promoter regions in three different species, *i.e.* mouse, rat and human. Although we did find some core promoter elements in the putative promoter regions, they are not located exactly at the same position, relevant to the TSS, as described in the literature. This may point towards several possibilities regarding this analysis. First, the TTS is located at some different upstream position in the sequence, or there exist multiple TSS. Second, as *lsr* gene undergoes alternative splicing to produce three different subunits (Yen *et al.,* 1999), an RNAse-mediated cleavage/maturation may occur in the precursor mRNA, prior to the splicing, at the last 5' nt of LSR mRNAs from the three subunits, ensuring the same TSS for them. Third, there is the possibility for the presence of core promoter elements, but with the nucleotide sequences

somewhat different, if not entirely, from the elements discovered so far, though the possibility for the presence of entirely newer elements cannot be ignored as well.

While concluding the results obtained from this analysis, we cannot ignore the limitations of such study. Although various computational prediction tools and approaches are available to determine the TSS, putative promoter regions and the various regulatory motifs associated with a particular promoter, however, the accuracy of prediction remains doubtful. In the case of LSR, as no experimental data is available so far about the position of TSS, it becomes even more complicated to locate the core promoter elements. Though Gen2promoter provides information about the presence of certain TSS(s) with the predicted sequences, none of these sites contain the core promoter elements at a position and with the sequence evident from the literature. This points towards the need to conduct the experimental work using PCR techniques and specific expression vectors in order to confirm the location of TSS and the potential promoters in different species.

Many online tools are available to identify the potential TFBSs, however, the possibility of getting false positive matches, in spite of using high affinity matrices, cannot be neglected. It is evident that these bioinformatics tools cannot identify the functional status of a TFBS in the promoter. This emphasizes upon the need of wet lab experiments using site-directed mutagenesis, mobility shift assays, reporter gene experiments, etc. in order to confirm the presence, position and the functional strength of the TFBSs. Once confirmed *in vitro*, further studies could be carried out to determine the functional significance of these elements *in vivo*. Nevertheless, the study of core promoter elements remains an interesting area of investigation and continues to expand and contribute to our understanding of promoter-specific transcriptional regulation.

III. Role of Peroxisome Proliferator-Activated Receptor alpha in the regulation of hepatic LSR

1. Introduction

Liver acts as a key site for the maintenance of lipid homeostasis due to the presence of various receptors and enzymes involved in the regulation of lipogenesis and lipolysis. The lipolysis stimulated lipoprotein receptor (LSR) modulates hepatic lipoprotein metabolism by mediating the clearance of TG-rich lipoproteins including chylomicrons and VLDL (Bihain & Yen, 1992; Yen *et al.*, 1994; Yen *et al.*, 2008). The free fatty acids (FFAs), produced through the hydrolysis of dietary fat incorporated in the chylomicrons, activate LSR by inducing a conformational change in the receptor (Bihain & Yen, 1992; Yen *et al.*, 1994), revealing a binding site for the lipoproteins containing either ApoE or ApoB or both. Biochemical and bioinformatics analyses indicate that the receptor is a heterotrimer or tetramer complex enclosing two or more of the three subunits, α, α' and β, produced from alternative splicing of a single *lsr* gene (Yen *et al.*, 1999). The amount of LSR on the liver plasma membranes was found to be associated with reduced postprandial TG levels in rats (Mann *et al.*, 1995). Furthermore, the removal of a single LSR allele in mice, leading to 50%-reduced expression of LSR, enhanced plasma TG and cholesterol and reduced dietary lipid clearance, thus suggesting this receptor as a rate-limiting factor in the removal of lipoproteins during the postprandial phase (Yen *et al.*, 2008). In our previous work, Stenger *et al.* (2010) identified leptin as an important regulator of hepatic LSR at transcriptional and translational levels in both *in vivo* and *in vitro* models. Since we have shown that leptin treatment leads to an increase in LSR expression, we were further interested in determining as to which other regulatory factors, including transcription factors, could modulate LSR expression in liver.

Among various players in peripheral lipid and energy homeostasis, peroxisome proliferator-activated receptors (PPARs) exhibit paramount importance. PPARs are the ligand-activated transcription factors that are involved in the regulation of lipid, glucose and amino acid metabolism, adipogenesis, inflammation and atherosclerosis by transcriptional activation of various target genes (Brown & Plutzky, 2007). Three PPAR subtypes, *i.e.* PPARα, PPARβ/δ and PPARγ, have been identified with their tissue-specific expression and functions (Kliewer *et al.*, 1994). PPARα is the key transcription factor that governs the modulation of the expression of a number of genes regulating hepatic lipid and lipoprotein metabolism. PPARα is predominantly expressed in the tissues with active FA oxidation such as liver, kidney, heart, brown adipose tissue, muscle, small and large intestine (Auboeuf *et al.*,

1997; Braissant *et al.*, 1996; Escher *et al.*, 2001; Bookout *et al.*, 2006). Studies have shown that PPARα knockout mice (PPARα$^{-/-}$) are viable and fertile (Lee *et al.*, 1995), but show an impairment in the expression of various target genes as well as an increase in cholesterol and TGs (Costet *et al.*, 1998). Upon activation by endogenous ligands such as FAs and their derivatives or exogenous ligands such as fibrates or Wy-14,643 (Krey *et al.*, 1997), PPARα heterodimerizes with the retinoic X receptor (RXR) and enters into the nucleus, where it binds to the PPAR response elements (PPREs), located in the promoter region of the target genes (Forman *et al.*, 1997; IJpenberg *et al.*, 1997). Owing to the implication of PPARα synthetic agonists in lowering plasma TGs and raising plasma HDL levels, this transcription factor has gained importance in the improvement of lipid-related disorders such as obesity and dyslipidemia (Fu *et al.*, 2003; Mori *et al.*, 2007; Kimura *et al.*, 2011).

Since hepatic LSR appears to be important in the maintenance of normal lipid status (Yen *et al.*, 2008, Narvekar *et al.*, 2009), this study was undertaken to determine the potential regulation of LSR by PPARs, and more specifically by PPARα.

2. Results and discussion

2.1. Effects of cell culture conditions on PPARα expression in Hepa 1-6 and HepG2 cell lines

With this objective in mind, we first sought to determine the endogenous PPARα levels in mouse hepatoma cell line, Hepa 1-6. The cells were cultured for 24 h in either serum-deficient medium (SDM; high-glucose DMEM only), complete medium (CM; high-glucose DMEM supplemented with 10% (v/v) serum), or lipoprotein-deficient serum (high-glucose DMEM supplemented with 5% (v/v) LPDS equivalent to 10% (v/v) serum) as described in Materials & Methods, section 3.

Cell lysates were recovered either in hypotonic lysis buffer to extract cytoplasmic and nuclear fractions or in RIPA buffer to prepare total cell lysates (as described in Materials & Methods, section 3.2) and used for western blot analysis using anti-PPARα. The results indicated the presence of PPARα in cells cultured in CM. Interestingly, PPARα levels significantly (p = 0.011) decreased to about 50% in cells cultured in LPDS, and were completely undetectable in cells cultured in SDM (Figure 42A). Similar results were observed

for the cytoplasmic and nuclear extracts from the cells incubated with CM and SDM (Figure 42B), although the signal was much lower, particularly in the nuclear fraction.

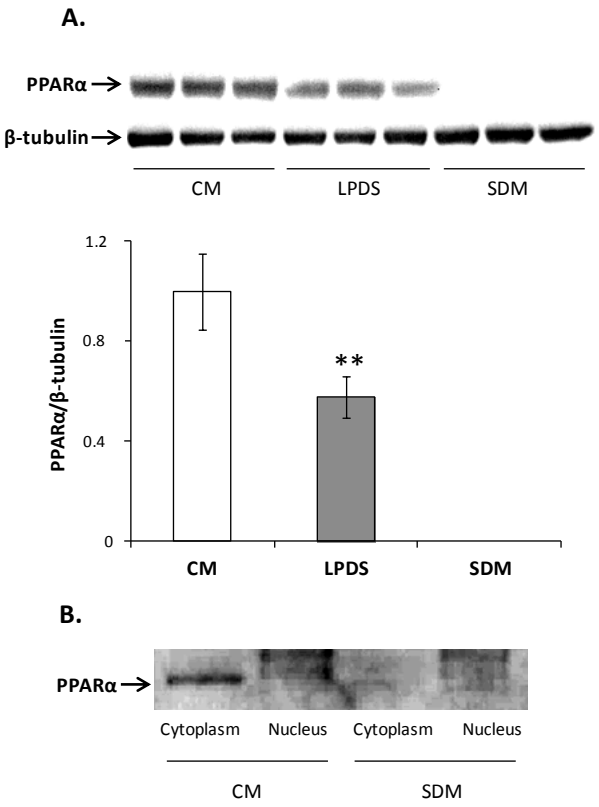


Figure 42: Effects of different medium conditions on PPARα protein levels in Hepa 1-6 cells

(A) Western blot analysis was performed to detect PPARα protein using total cell lysates from Hepa 1-6 cells cultured at 37°C for 24 h in CM, LPDS or SDM. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SEM, N=2; n=3. The p*-value is indicated where significant compared to CM as control condition, ** p<0.01. (B) Cytoplasmic and nuclear fractions were prepared from Hepa 1-6 cells cultured in CM or SDM, in order to determine PPARα protein levels. Results are represented as means ± SEM, N=2; n=3.*

In order to find out if this was species-specific, same treatments were performed on human hepatoma cell line, HepG2 (as shown in the Figure 42A & B). PPARα protein levels were determined by western blot using total cell lysates (Figure 43A) and cytoplasmic and nuclear fractions (Figure 43B). Interestingly, the results obtained were in parallel to what was observed in Hepa 1-6 under these culture medium conditions.

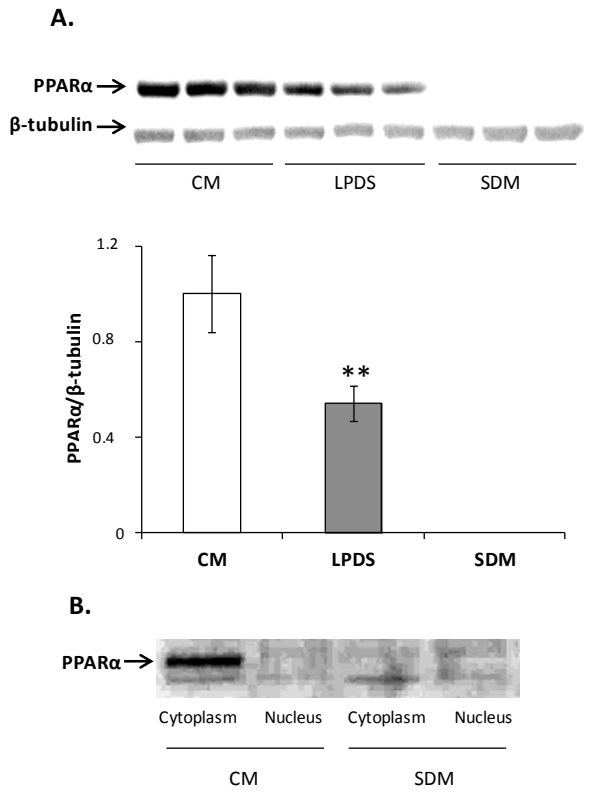


Figure 43: Effects of different medium conditions on PPARα protein levels in HepG2 cells

(A) Western blot analysis was performed to detect PPARα protein using total cell lysates from HepG2 cells cultured at 37°C for 24 h in CM, LPDS or SDM. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SEM, N=2; n=3. The p*-value is indicated where significant compared to CM as control condition, ** p<0.01. (B) Cytoplasmic and nuclear fractions were prepared from HepG2 cells cultured in CM or SDM, in order to determine PPARα protein levels. Results are represented as means ± SEM, N=2; n=3.*

Studies using various cell lines including rat hepatoma cell line (Fao), monkey kidney (COS-7) and HepG2 cells demonstrate that PPARα activation is more pronounced in the cells incubated with low serum (0.5% FBS) or SDM (Rodriguez *et al.*, 2001; Grau *et al.*, 2006; König & Eder, 2006; König *et al.*, 2007; Huang *et al.*, 2008). However, our results indicate the opposite effects in both Hepa 1-6 and HepG2 cell lines with the undetectable PPARα protein levels in SDM and high PPARα protein levels in the presence of CM.

On the contrary, real-time qPCR analysis revealed no changes in PPARα mRNA expression in Hepa 1-6 cells incubated in these different medium conditions. In order to confirm that PPARα was active, the expression of a well-known PPARα-responsive gene, acyl-CoA oxidase 1 (ACOX1) (Tugwood *et al.*, 1992) was also determined. This enzyme catalyzes the first step of peroxisomal β-oxidation of FAs and has been reported to contain

PPREs in the promoter region (Varanasi *et al.*, 1996). Similar to PPARα, no modifications were observed in ACOX1 mRNA levels in all conditions (Figure 44).

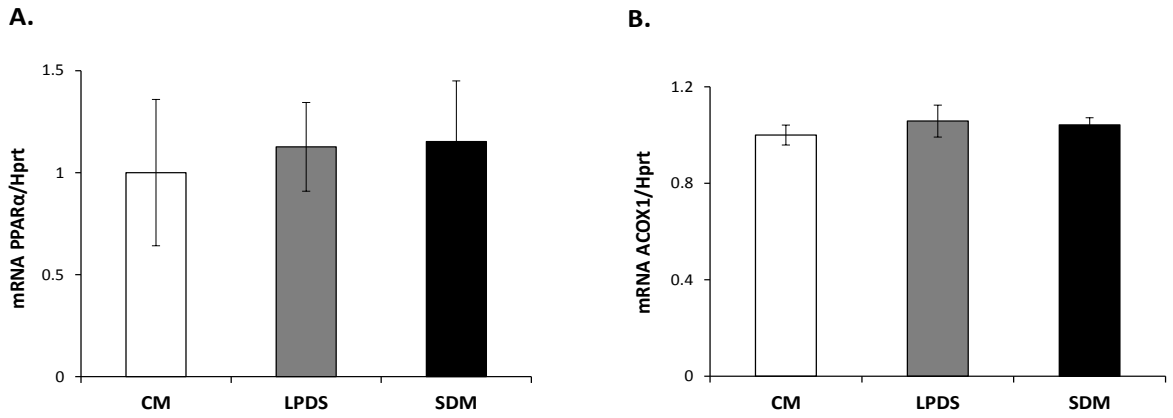


Figure 44: Effects of different medium conditions on PPARα and ACOX1 expression in Hepa 1-6 cells

Total RNA was extracted from the cells cultured in CM, LPDS and SDM and real-time RT-qPCR was performed to determine the expression of (A) PPARα and (B) ACOX1 using Hprt as reference gene. Values are represented as means ± SD, n=3.

Various *in vitro* studies using COS-7, human breast cancer (MCF-7) and human cervical cancer (Hela) cell lines have shown that PPARs are predominantly localized in the nucleus in the absence or presence of ligands, but constantly undergo nuclear and cytoplasmic shuttling in response to ligand activation. However, it is interesting to note that significant quantities of PPARs can also be located in the cytosol of some cells (Hager *et al.*, 2000; Akiyama *et al.*, 2002; Feige *et al.*, 2005; Patel *et al.*, 2005; Umemoto & Fujiki, 2012), thus allowing PPARs to regulate gene expression by influencing both transcriptional and post-transcriptional events (Thuillier *et al.*, 1998; Patel *et al.*, 2005).

Our findings suggest that in the absence of exogenous ligands, PPARα protein is mainly found in the cytoplasm when Hepa 1-6 and HepG2 cells were cultured in CM. However, no PPARα protein was detected in nuclear and cytoplasmic fractions under SDM conditions. Similar results were obtained using total cell lysates from both cell lines. However, no changes in the expression of PPARα and ACOX1 were observed at transcriptional level in Hepa 1-6 cells. This suggests that in the absence of ligand activation, the cell culture medium-dependent changes were observed in PPARα protein levels, whereas the mRNA levels remained unchanged.

Thus, from these results, we can conclude that while the amount of serum in the cell culture medium influences PPARα protein levels, there was no effect on mRNA levels of this transcription factor. It was therefore decided to use CM as cell culture medium in which PPARα protein was detectable.

2.2. Effects of cell culture conditions on LSR expression in Hepa 1-6 and HepG2 cell lines

In our previous studies, we have described the effects of various cell culture treatments on LSR expression in Hepa 1-6 cells maintained under standard culture conditions, *i.e.* the cells grown in CM (Stenger *et al.,* 2010; Ahmad *et al.,* 2012). Similar to PPARα, LSR expression was also determined in Hepa 1-6 cells cultured in CM, LPDS and SDM for 24 h (see Figure 43). Western blot analysis using anti-LSR revealed a significant increase in LSR protein levels in the cells cultured in LPDS or SDM, as compared with CM (Figure 45A). However, densitometric analysis of the upper (indicating both LSRα and α' subunits) and the lower (indicating LSRβ) LSR bands revealed no changes in their expression (data not shown). Similar results were obtained when HepG2 cells were cultured in these medium conditions (Figure 45B).

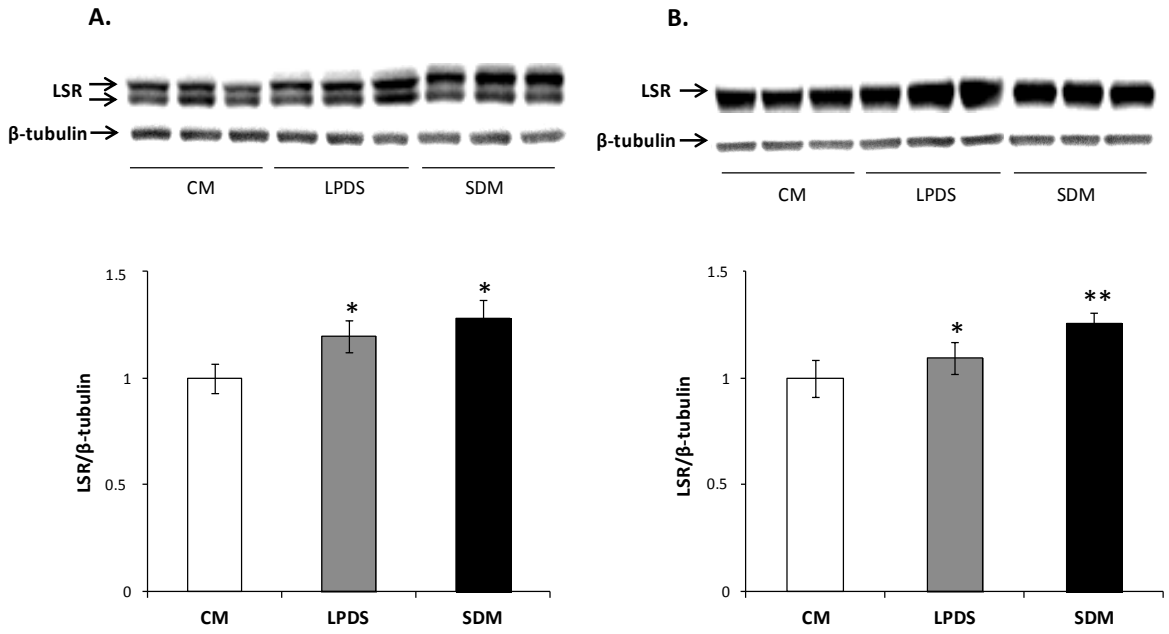


Figure 45: Effects of different medium conditions on LSR protein levels

Western blot analysis was performed to detect LSR protein in total cell lysates from (A) Hepa 1-6 and (B) HepG2 cells cultured at 37°C for 24 h in CM, LPDS or SDM. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SEM, N=2; n=5. The p*-values are indicated where significant compared to CM as control condition, ** p<0.01, * p<0.05.*

Real-time qPCR was performed to determine LSR expression in cells under different culture medium conditions using primers for the amplification of all mRNAs derived from mouse *lsr* gene (total LSR). In addition, specific primers were designed to amplify the mRNAs from the individual LSR subunits. Consistent with the findings from western blot analysis, a significant increase in total LSR mRNA expression was observed in Hepa 1-6 cells incubated with LPDS and SDM, as compared to those incubated in the presence of CM. Interestingly, the expression of LSRα and α' subunits was also increased significantly in cells cultured in SDM *vs.* CM, while no changes were observed in mRNA levels for LSRβ subunit (Figure 46).

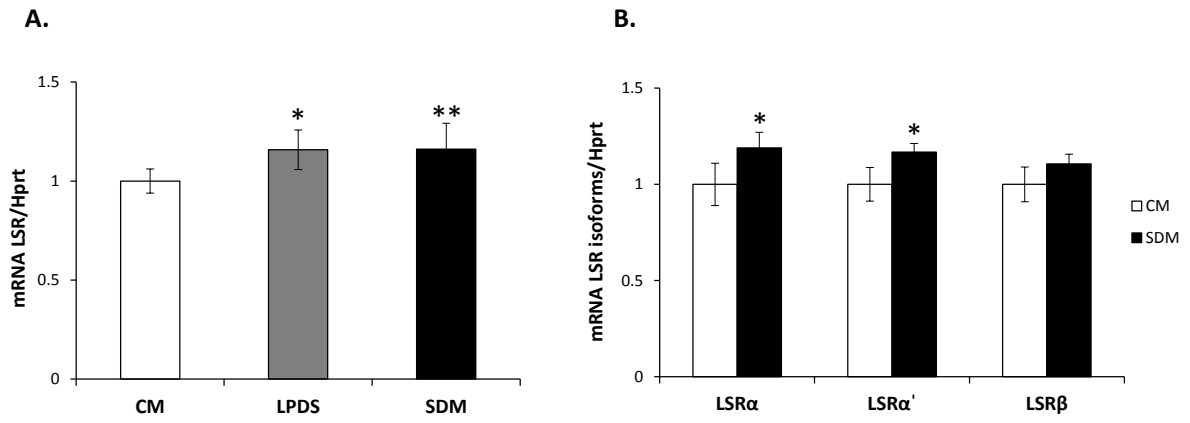


Figure 46: Effects of different medium conditions on LSR mRNA levels in Hepa 1-6 cells

Total RNA was extracted from the cells cultured in CM, LPDS and SDM and real-time RT-qPCR was performed to determine the expression of (A) total LSR and (B) LSR α, α' and β subunits, using Hprt as reference gene. Results are shown as means ± SD, n=3 and p*-values are indicated where significant compared to the control condition, ** p<0.01.*

These findings suggest that the absence of serum or lipoproteins in the medium results in an increase in LSR protein and mRNA levels in Hepa 1-6 cells. Moreover, an increase in the expression of LSR mRNA encoding subunits α and α' was also observed whereas the expression of LSRβ mRNA was not modified significantly. This indicates that some factors in the serum were likely to be responsible for this decrease in LSR expression in CM. Since the serum contains very high levels of lipids including cholesterol (Whitford & Manwaring, 2004), we next measured the cholesterol content in the serum and LPDS samples that were used to culture the cells. Because of the removal of lipoproteins from the serum during the preparation of LPDS, its cholesterol content was already lower as compared with the serum sample (Figure 47).

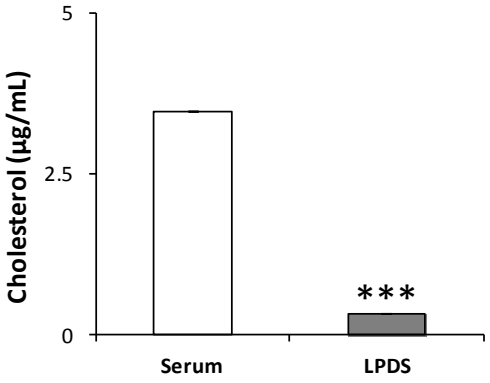


Figure 47: Determination of cholesterol content in serum and LPDS
The p*-value is indicated relative to serum as control condition, *** p<0.001*

From these observations, it can be concluded that the presence of cholesterol in serum may account for the decrease in LSR expression in Hepa 1-6 cells cultured in CM. Such a regulation by cholesterol has been well-documented for LDL-R in the literature. It has been shown that in the absence of cholesterol, the *ldl-r* gene is actively transcribed with the rapid uptake of LDL, while in the presence of cholesterol within the cells, the transcription of the gene is repressed and thus LDL internalization is reduced (Brown & Goldstein, 1999). Furthermore, SREBPs play a crucial role in the transcriptional regulation of LDL-R (Hua *et al.,* 1993; Yokoyama *et al.,* 1993) and these transcription factors, in turn, also depend on the cholesterol content to acquire their mature and active form. Also, LPDS is widely used to upregulate LDL-R expression in various cell culture studies (Goldstein *et al.,* 1983), as it increases LDL-R activity as well as protein and mRNA levels (Srivastava *et al.,* 1995). Such a cholesterol-sensitive pathway may also be hypothesized for LSR with the involvement of cholesterol-responsive transcription factors such as SREBPs and LXRs. For this purpose, additional experiments are required, such as those concerned with cholesterol enrichment and depletion.

Yen *et al.* (1999) have shown that *lsr* gene undergoes alternative splicing to produce α, α' and β subunits. They further reported that the receptor exists as a multimeric complex, consisting of a transmembrane spanning domain found in α or α' subunits, but not in the β subunit, which may be localized extra- or intracellularly (Bihain and Yen, 1998) (Figure 48). Compared to LSRα and α' subunits, the LSRβ subunit lacks the putative lysosomal targeting signals, the transmembrane-spanning domain, and the cysteine-rich domain, but contains the putative FFA and ligand binding domains (Yen *et al.,* 1999). Taking into account the possible functions of LSR subunits, it can be concluded that the absence of lipoproteins in both LPDS and SDM preferentially increases the mRNA expression of LSRα and α' subunits and therefore may be involved in regulating the appropriate splicing of the primary transcript.

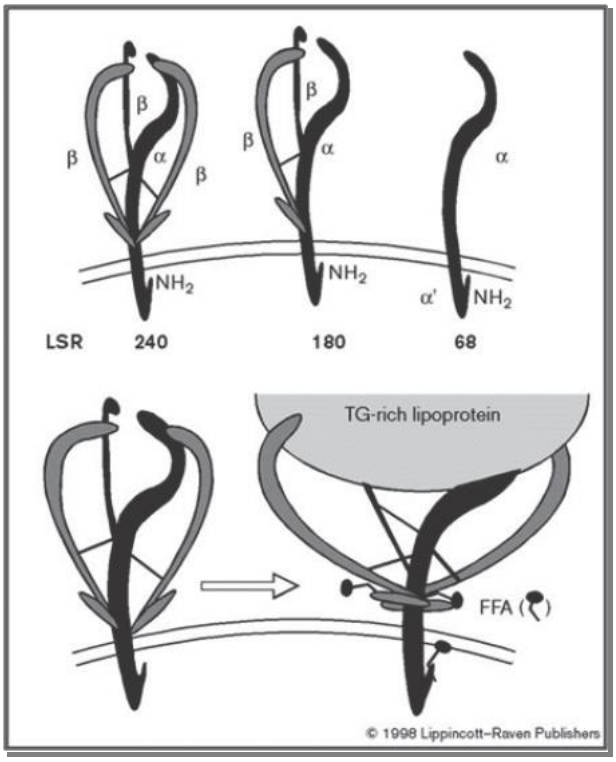


Figure 48: Model for the structural organization of LSR and its activation

Biochemical data suggests that LSR could exist as a multimeric complex of one α or α' (solid) subunit containing the transmembrane domain associated by disulfide bridges with two or three β (gray) subunits, located extracellularly. (Bottom panel) Upon FFA binding to the hydrophobic regions, LSR undergoes a conformational change that exposes a binding site for the recognition of ApoE of a TG-rich lipoprotein, leading to its binding, internalization and degradation. (Bihain & Yen, 1998)

To summarize, we can conclude that the presence of cholesterol in the medium (*i.e.* culturing in the presence of CM) decreases LSR protein and mRNA levels. On the other hand, the removal of extracellular sources of cholesterol (*i.e.* culturing in LPDS or SDM) increases the expression of total LSR as well as LSR subunits. Indeed, both LPDS and SDM media are deprived of lipoproteins such as chylomicrons, VLDL and LDL which serve as principal transporters of lipids into the cells. This led us to speculate that among different possible factors, the presence of various lipoproteins in the medium could be suspected to be responsible for the decrease in LSR expression in CM. At the same time, the possible effects of growth factors present in the serum cannot be ignored.

2.3. LDL-loading experiments

In order to determine the potential effects of lipoproteins on LSR expression, Hepa 1-6 cells were incubated with LPDS or SDM in absence or presence of purified human LDL.

2.3.1. In LPDS

Hepa 1-6 cells were cultured at 37°C for 24 h in LPDS containing 0, 10, 20 or 50 µg/mL LDL and then recovered for protein extraction. Western blots were performed using anti-LSR and anti-LDL-R antibodies to determine the effects of LDL treatment on the expression of these two lipoprotein receptors (Figure 49).

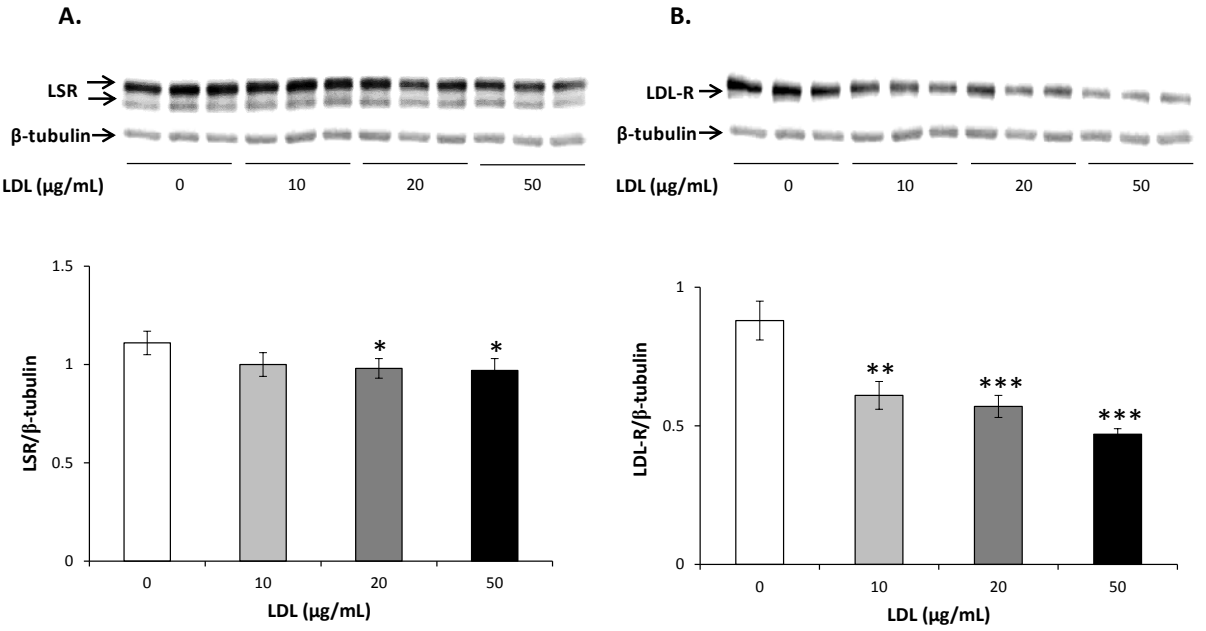


Figure 49: Effects of LDL supplementation on LSR and LDL-R protein levels in Hepa1-6 cells

Western blot analysis was performed to detect (A) LSR and (B) LDL-R protein levels using total cell lysates from Hepa 1-6 cells treated with the indicated concentrations of LDL in LPDS medium for 24 h at 37°C. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SD, n=3. The p*-values are indicated where significant compared to the control condition, * p<0.05, ** p<0.01, *** p<0.001.*

LDL supplementation of LPDS medium led to a small, yet significant (p = 0.04) decrease in LSR protein levels with 20 and 50 µg/mL LDL as compared to the control. However, a more significant and dose-dependent decrease was observed in LDL-R protein for all the LDL concentrations used in the experiment, which is consistent with previous studies that used LPDS to upregulate the LDL-R (Goldstein *et al.*, 1983).

Interestingly, the densitometric analysis of the LSR subunits, from the western blot shown in Figure 49A, revealed a significant decrease at protein levels for all the LDL concentrations tested (Figure 50). However, the ratio between the LSR subunits α+α' and β remained unchanged.

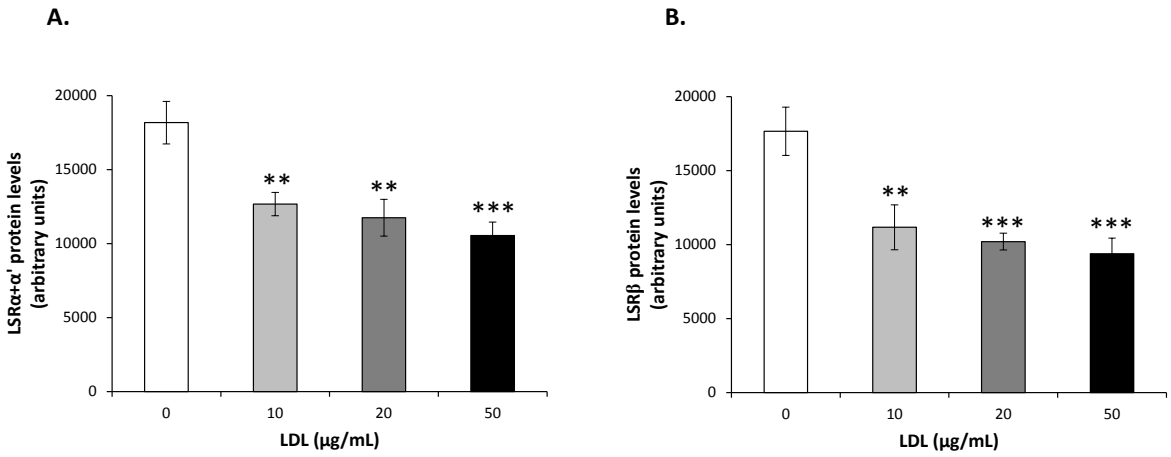


Figure 50: Effects of LDL supplementation on LSR subunits in Hepa1-6 cells

Densitometric analysis was performed for the results of western blots shown in Figure 49A, to determine the protein levels of the individual LSR (A) α+α' and (B) β subunits. The results are represented as means ± SD and the p*-values are indicated where significant compared to the control condition,* ** p*<0.01,* *** p*<0.001.*

Our results indicate that LDL cholesterol could play an important role in the regulation of LSR subunits. The effects were much more pronounced for the individual LSR subunits than for the total LSR.

2.3.2. In the absence of serum

Hepa 1-6 cells were cultured at 37°C for 24 h in SDM supplemented with 0, 10, 20 and 50 µg/mL LDL in the absence of serum. Western blot analyses revealed LSR and LDL-R protein levels (Figure 51).

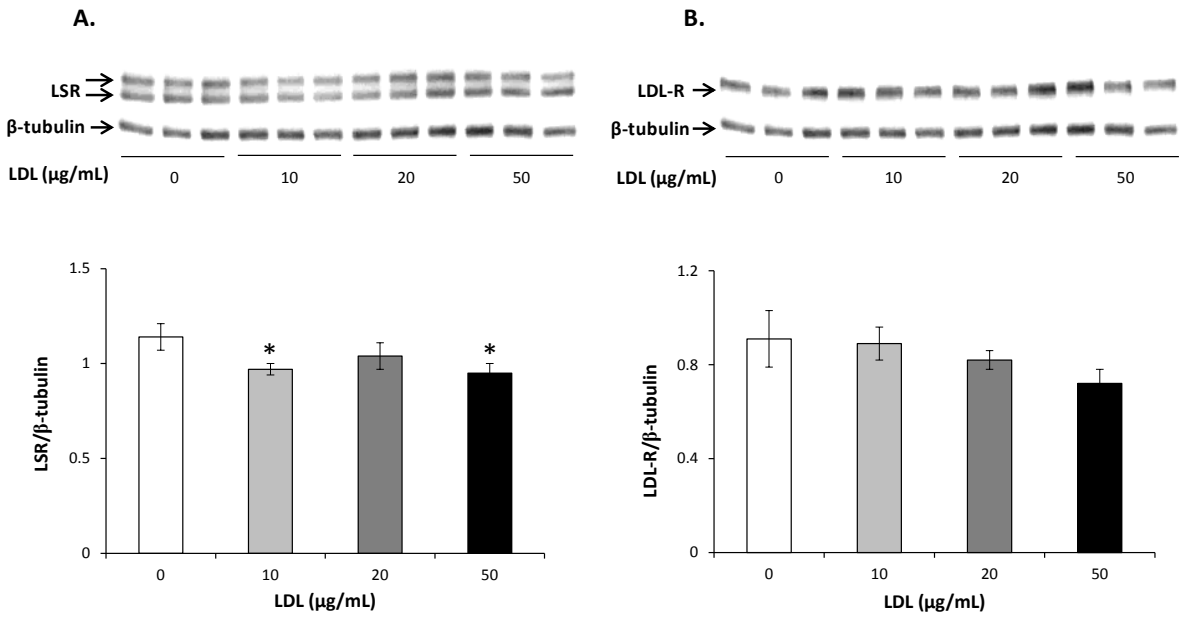


Figure 51: Effects of LDL supplementation on LSR and LDL-R protein levels in Hepa1 6 cells

Western blot analysis was performed to detect (A) LSR and (B) LDL-R protein levels in total cell lysates from Hepa 1-6 cells treated with the indicated concentrations of LDL in SDM for 24 h at 37°C. β-tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SD, n=3. The p*-values are indicated where significant compared to the control condition,** $p<0.05$.

The results demonstrate a significant decrease in LSR protein levels at 10 and 50 µg/mL LDL concentrations as compared to the control condition. However, unlike the results obtained using LPDS, no significant changes were observed in LDL-R protein levels under these conditions, although a trend towards dose-dependent decrease in LDL-R could be noticed.

Furthermore, the measurement of band intensities from the western blot shown in Figure 51A revealed a significant decrease in protein levels of LSR α+α' and β subunits at all LDL concentrations as compared with controls (Figure 52). However, the ratio between the subunits α+α' and β remained unchanged. These results were in accordance with the results obtained using LPDS (Figure 50).

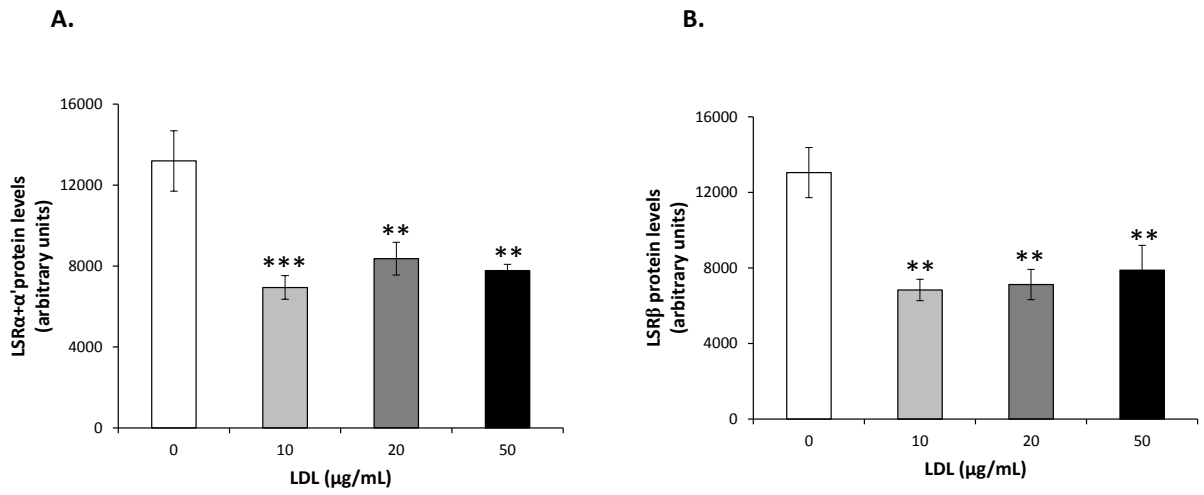


Figure 52: Effects of LDL supplementation on LSR subunits in Hepa1-6 cells

Densitometric analysis was performed for the results of western blots shown in Figure 51A, to determine the protein levels of the individual LSR (A) α+α' and (B) β subunits. The results are represented as means ± SD and the p*-values are indicated where significant compared to the control condition,* ** p*<0.01,* *** p*<0.001.*

These results, taken together, suggest that the presence of LDL results in a decrease in LSR and LDL-R protein levels. The decrease in LDL-R was much more pronounced with LDL treatment in LPDS than in SDM, which is consistent with the well-established mechanisms of LDL-R regulation by cholesterol and cholesterol-responsive transcription factors, SREBPs (Yokoyama *et al.,* 1993; Hua *et al.,* 1993; Brown & Goldstein, 1999).

It has already been shown that LDL is not the preferred ligand for LSR under physiological conditions. This is mostly due to the presence of cholesterol as the main LDL component, which renders LDL incapable to generate enough FFAs in order to induce changes in receptor conformation that, in turn, unmasks the lipoprotein binding site (Mann *et al.,* 1995). However, in the absence of functional LDL-R which consequently raises the plasma LDL concentrations, a significant part of LDL is cleared by hepatocytes through LSR (Attie *et al.,* 1982; Bihain & Yen, 1992). In this experiment, the decrease in the protein levels of total LSR and more importantly LSR subunits, both in the presence of LPDS or SDM, might represent a feedback mechanism in order to avoid the accumulation of excess cholesterol in the cells. Further investigation is required in order to determine the transcription factors involved in this feedback regulation.

2.4. VLDL-loading experiments

It has been well-documented that LSR is involved in the clearance of TG-rich lipoproteins, preferentially VLDL. Binding of VLDL to LSR occurs only when LSR is present in its active conformation, which requires FFAs. Oleate has been shown to be the most efficient activator of LSR-mediated lipoprotein elimination pathway (Bihain & Yen, 1992). Therefore, taking these observations into account, the experiment was conducted to determine the changes in LSR protein levels by incubating Hepa 1-6 and HepG2 cell lines with VLDL in the presence or absence of oleate. Moreover, the cells were incubated in LPDS based on the observations from the previous experiment (Figure 49), leading to the pronounced effects on LSR and LDL-R protein levels.

2.4.1. In Hepa 1-6 cells

Hepa 1-6 cells were incubated in the presence or absence of 0.8 mM oleate in LPDS containing 10, 20 and 50 µg/mL VLDL at 37°C for 24 h. Western blot analysis was performed to determine LSR (Figure 53) protein levels.

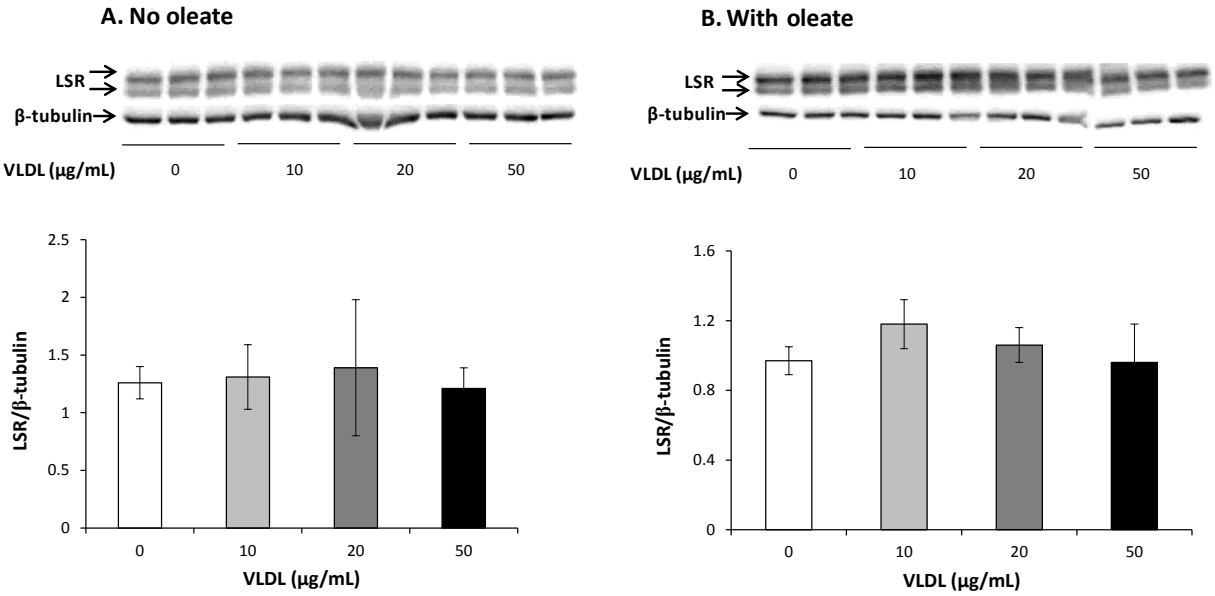


Figure 53: Effects of VLDL treatment on LSR protein levels in Hepa 1-6 cells

Western blot analysis was performed to detect LSR protein in total cell lysates from Hepa 1-6 cells pretreated (A) without or (B) with 0.8 mM oleate for 5 min and then incubated at 37°C for 24 h with the indicated concentrations of VLDL in LPDS. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SD, n=3.

In the absence of oleate, no significant changes were observed in LSR protein levels at all the concentrations of VLDL tested. However, a trend towards an increase in LSR protein was observed for 10 µg/mL VLDL, only in the presence of oleate. Furthermore, no significant differences were observed in the protein levels of individual LSR subunits (data not shown).

Besides the clearance of most of the hepatic LDL, LDL-R may also contribute to the removal of both ApoB-100- and ApoE-containing lipoprotein. Thus, we next measured LDL-R protein levels under similar conditions (as described in Figure 53). Similar to LSR, no significant changes were observed in LDL-R protein levels in the presence or absence of oleate in Hepa 1-6 cells (Figure 54).

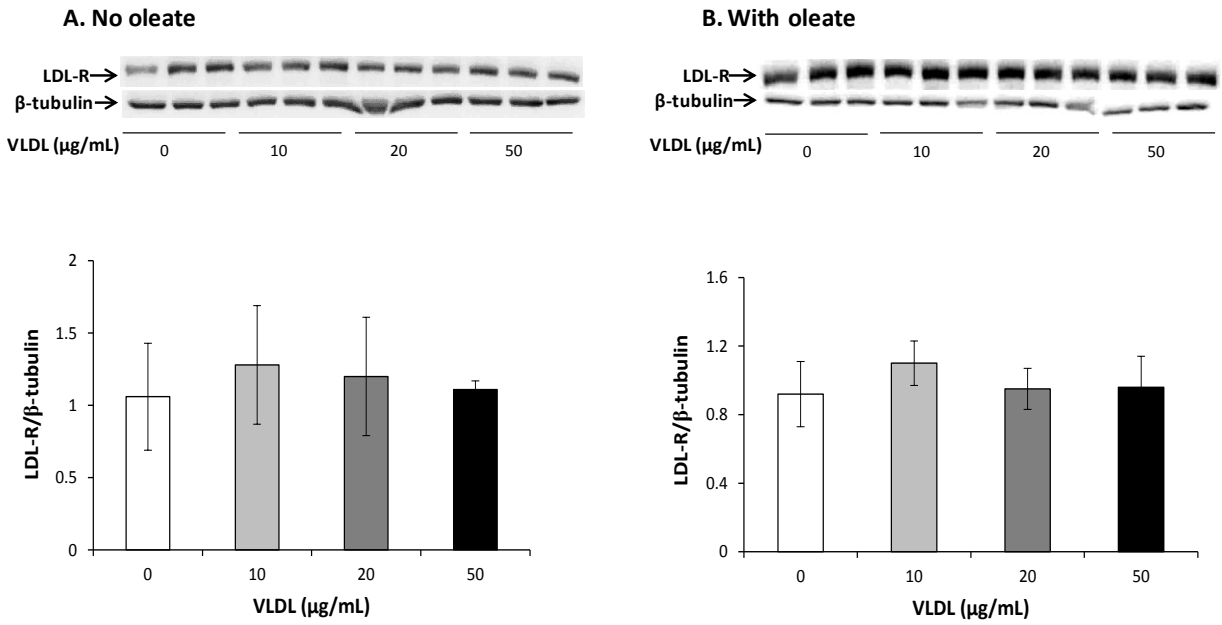


Figure 54: Effects of VLDL treatment on LDL-R protein levels in Hepa 1-6 cells

Western blot analysis was performed to detect LDL-R protein in total cell lysates from Hepa 1-6 cells pretreated (A) without or (B) with 0.8 mM oleate for 5 min and then incubated at 37°C for 24 h with the indicated concentrations of VLDL in LPDS. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SD, n=3.

2.4.2. In HepG2 cells

The same experiment described above for Hepa 1-6 cells was performed for HepG2 cells with the results shown for LSR and LDL-R in Figure 55 & Figure 56 respectively.

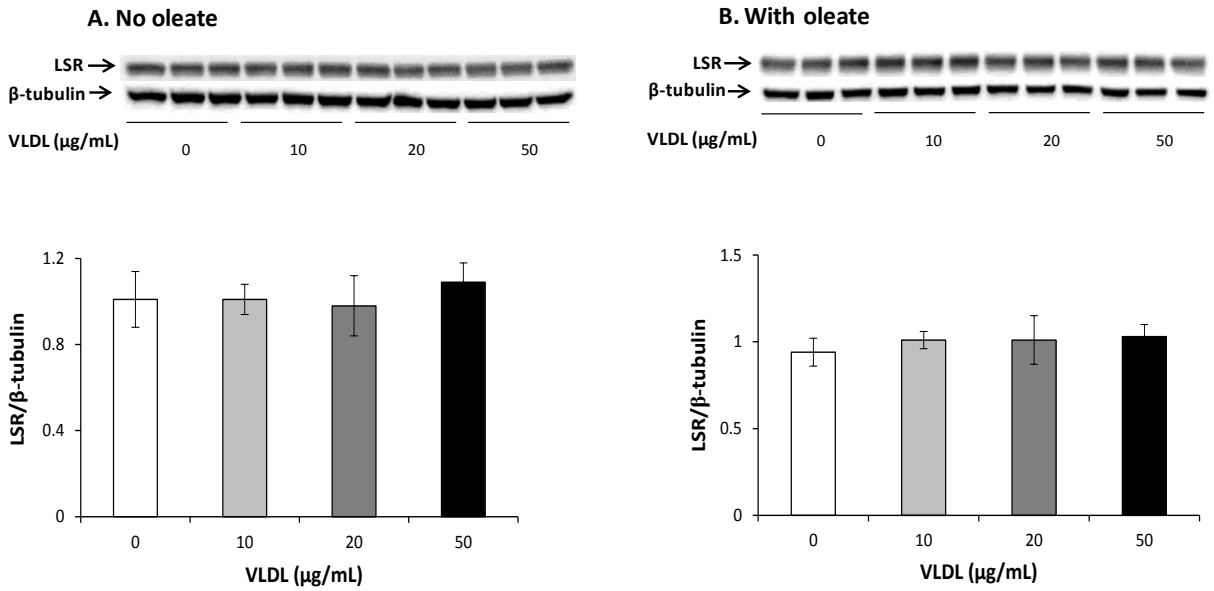


Figure 55: Effects of VLDL treatment on LSR protein levels in HepG2 cells

Western blot analysis was performed to detect LSR protein in total cell lysates from HepG2 cells pretreated (A) without or (B) with 0.8 mM oleate for 5 min and then incubated at 37°C for 24 h with the indicated concentrations of VLDL in LPDS. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SD, n=3.

Similar to Hepa 1-6 cells, no changes in LSR protein levels were observed with VLDL treatment in HepG2 cells, both in the absence or presence of oleate.

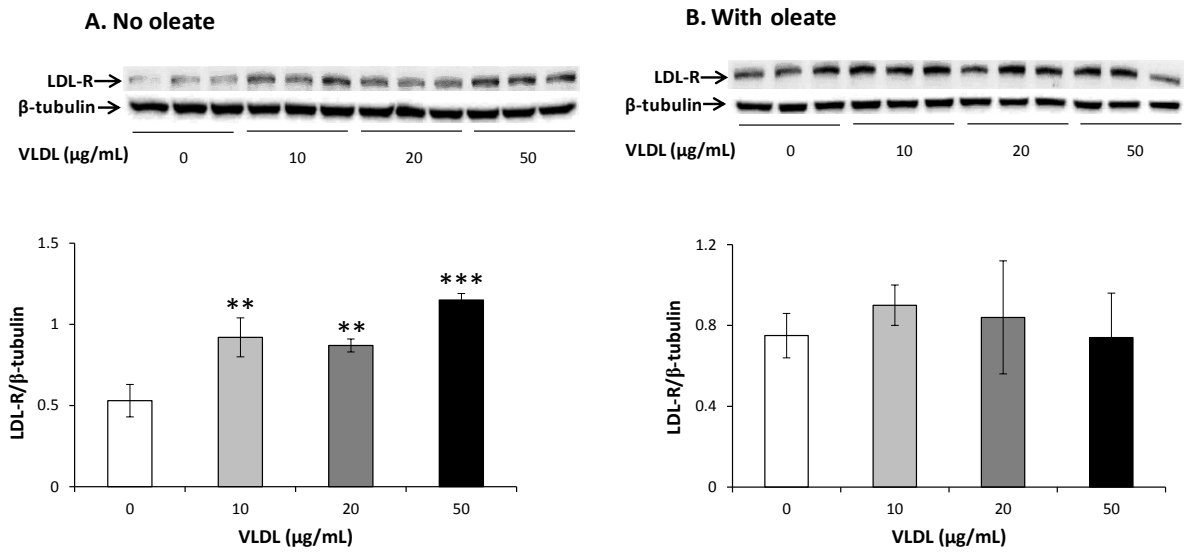


Figure 56: Effects of VLDL treatment on LDL-R protein levels in HepG2 cells

Western blot analysis was performed to detect LDL-R protein in total cell lysates from HepG2 cells pretreated (A) without or (B) with 0.8 mM oleate for 5 min and then incubated at 37°C for 24 h with the indicated concentrations of VLDL in LPDS. β–tubulin was used as loading control. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SD, n=3. The p*-values are indicated where significant compared to the control condition, ** p<0.01, *** p<0.001.*

Interestingly, a significant increase was observed in LDL-R protein levels at all the concentrations of VLDL in the absence of oleate. However, this effect was not observed in the presence of oleate.

Taken together all these results, we sought to determine the potential factors that might be involved in the regulation of LSR and PPARα, taking LDL-R as an indicator for the possible effects under different conditions. These factors included different medium conditions and the loading of cells with lipoproteins including LDL and VLDL as summarized in Table 21, Table 22 and Table 23. The study has allowed us to get an idea about distinct pattern of expression of LSR and PPARα in mouse and human hepatoma cell lines.

Table 21: Effects of different medium conditions on the regulation of LSR and PPARα in Hepa 1-6 and HepG2 cell lines

Media conditions	Hepa 1-6		HepG2	
	LSR	**PPARα**	**LSR**	**PPARα**
CM/LPDS/SDM	↑ protein and mRNA levels in SDM and LPDS, compared with CM	↓ protein levels in LPDS and no detection in SDM, compared with CM, while mRNA levels remain unchanged	↑ protein levels in SDM and LPDS, compared with CM	↓ protein levels in LPDS and no detection in SDM, compared with CM

Table 22: Effects of LDL supplementation on LSR and LDL-R protein levels in Hepa 1-6 cells

LDL	Hepa 1-6	
	LSR	**LDL-R**
In LPDS medium	↓ protein levels at 20 and 50 µg/mL in the presence of LPDS	↓ protein levels at all concentrations in the presence of LPDS
In absence of serum (in SDM)	↓ protein levels at 10 and 50 µg/mL in the absence of serum	No changes in protein levels in the absence of serum

Table 23: Effects of VLDL supplementation on LSR and LDL-R protein levels in Hepa 1-6 and HepG2 cell lines

VLDL	Hepa 1-6		HepG2	
	LSR	**LDL-R**	**LSR**	**LDL-R**
In the absence of oleate	No changes at protein levels	No changes at protein levels	No changes at protein levels	↑ protein levels at all VLDL concentrations
In the presenceof oleate	No changes at protein levels	No changes at protein levels	No changes at protein levels	No changes at protein levels

The initial study culturing both mouse and human hepatoma cells in different media conditions indicated an increase in LSR protein levels in SDM and LPDS, when compared with CM, *i.e.* the typical cell culture condition for the maintenance of these cells (Table 21). This was also accompanied by an increase in the mRNA levels of total LSR and the LSR subunits in Hepa 1-6 cells grown in SDM. Conversely, a decrease was observed in PPARα protein levels in both cell lines in LPDS relative to CM, while no protein was detected in SDM. Similar results were obtained for cytoplasmic and nuclear fractions, suggesting that there are some factors in the serum that affect PPARα at post-transcriptional levels and this is

why we observed no changes in PPARα mRNA levels in Hepa 1-6 cells under these conditions. The high levels of LSR protein in SDM, relative to CM, may also indicate that in the absence of exogenous sources of cholesterol, the cells may upregulate LSR in order to internalize the available lipids in the medium. Moreover, it can also be speculated that LSR upregulation might be involved in providing the cells with sufficient cholesterol levels. In contrast, the relatively low levels of LSR in CM may also be explained by the presence of cholesterol-carrying lipoproteins in CM, thus downregulating LSR expression as a feedback mechanism. This also points towards the sensitivity of the receptor in the handling of nutritional changes.

Since LPDS and SDM do not contain lipoproteins and since we found an increase in LSR expression in cells cultured in these two media, it could be speculated that the lipoproteins in the serum play an important role in LSR regulation. For this, further experiments were designed to determine the effects of LDL supplementation on LSR protein levels in Hepa 1-6 cells (Table 22). The results showed a significant decrease in LSR at 20 and 50 µg/mL LDL in the presence of LPDS and also at 10 and 50 µg/mL LDL in the absence of serum. Interestingly, significant decrease was observed in the protein levels of all LSR subunits with LDL treatment, both in the presence of LPDS and in SDM. Since most cholesterol is transported through LDL, this supports the notion that the cellular cholesterol content could regulate LSR expression. It can be further concluded that, similar to LDL-R, there may exist a transcriptional control for LSR by cholesterol-responsive transcription factors such as SREBPs and LXRs, which requires further investigation.

The effects of VLDL (a preferred ligand for LSR) supplementation on LSR protein levels were next determined in Hepa 1-6 and HepG2 cell lines in the presence or absence of oleate (Table 23). It was observed that the absence of oleate resulted in significant increase in LDL-R protein levels at all the concentration of VLDL only in HepG2 cells. No significant changes were observed in LSR and LDL-R protein levels for all other conditions in both cell lines. This suggests that VLDL may not be responsible for the decreased LSR expression in the cells incubated with CM.

These data provided us the clue about the potential regulators of LSR and PPARα expression in growing cells. These experiments also helped us to speculate about the culture conditions to be employed during our experimental studies for the determination of the possible effects of PPARα in the regulation of LSR. According to our results, since both

PPARα and LSR were found to be present in the cells incubated with CM, we next used these conditions in our following experiments to investigate the effects of PPARα activators on LSR expression.

2.5. Effects of PPARα activation by bezafibrate on LSR expression

PPARα is a ligand-activated transcription factor. Various natural and synthetic ligands have been identified, which may either selectively activate PPARα or act as pan-activators (Desvergne & Wahli, 1999; Willson *et al.*, 2000). Of the wide variety of synthetic ligands developed in the last decades, fibrates have emerged as valuable therapeutic agents because of their ability to reduce TG and elevate HDL-cholesterol levels, mainly by lowering the hepatic ApoC-III levels and by increasing LPL expression. This favors the use of fibrates as hypolipidemic drugs for the treatment of dyslipidemia (Haubenwallner *et al.*, 1995; Staels *et al.*, 1998a; de Man *et al.*, 2000; Tenenbaum & Fisman, 2012a; Ewang-Emukowhate & Wierzbicki, 2013; Klop *et al.*, 2013; Rizzo *et al.*, 2013). All the fibrates are PPARα-selective agonists, except bezafibrate (Figure 57) that activates the three PPAR subtypes (Peters *et al.*, 2003; Tenenbaum & Fisman, 2012b). Furthermore, a PPARα-specific fenofibrate has been shown to induce the expression of LDL-R in the mouse hepatoma cell line, AML12 (Huang *et al.*, 2008).

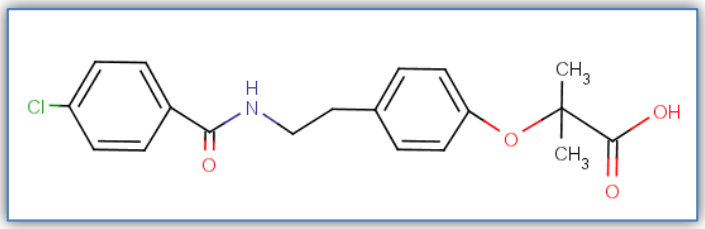


Figure 57: Chemical structure of bezafibrate

In view of the potential PPREs identified in the 5' region of the *lsr* gene (as decribed in Results and Discussion, Chapter II), this study was initiated to determine if bezafibrate treatment led to changes in LSR expression in Hepa 1-6 cells. Cell-based transactivation assays have reported the activation of PPARs by bezafibrate at higher concentrations relative to their specific ligands, showing an EC_{50} of 90 µM for murine PPARα and 50 µM for human PPARα (Henke *et al.*, 1998; Willson *et al.*, 2000). Cell culture studies have also pointed towards the effectiveness of bezafibrate at higher doses (Casarin *et al.*, 2012). In line with these observations, Hepa 1-6 cells were incubated with various concentrations of bezafibrate,

i.e. 100, 200, 400 and 600 µM in CM for 24 h (as described in Materials & Methods, section 3). No toxic effects were observed on the cells at these concentrations. Moreover, a significant increase was observed in total LSR mRNA levels at 200, 400 and 600 µM bezafibrate, along with an increase in expression of ACOX1, used as positive control (Figure 58).

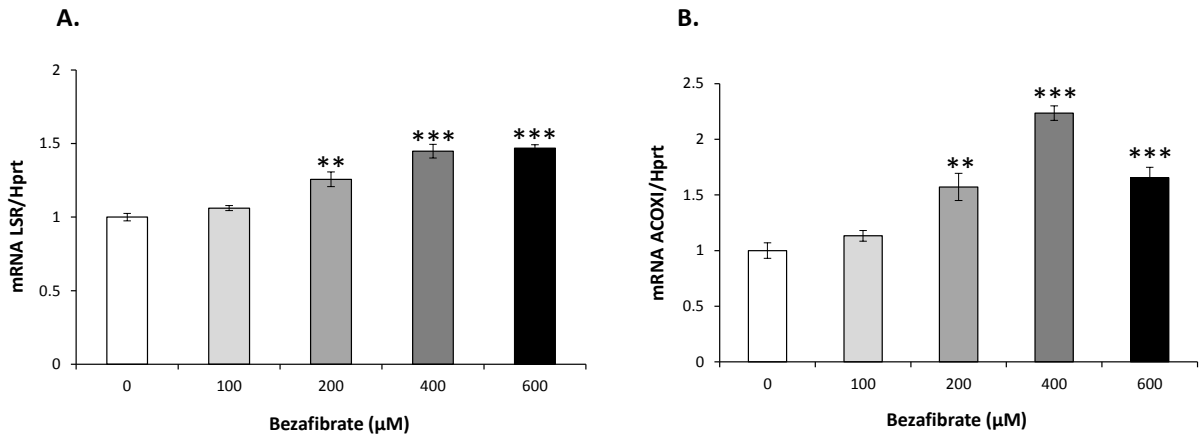


Figure 58: Effects of bezafibrate on LSR and ACOX1 mRNA levels

Hepa 1-6 cells were incubated with indicated concentrations of bezafibrate in CM for 24 h at 37°C and recovered for RNA extraction. The relative changes in (A) LSR and (B) ACOX1 mRNA levels were determined by real-time qPCR experiment using Hprt as reference gene. Cells treated with vehicle alone (DMSO) were used as controls. The results are shown as means ± SD, n=3 and p*-values are indicated where significant compared to the control condition, ** p<0.01, *** p<0.001.*

This indicates that bezafibrate increases LSR expression in a manner dependent upon the activation of PPARs as deduced from ACOX1 upregulation observed in parallel. Though bezafibrate is an agonist for the three PPARs, various studies support the role of bezafibrate as a dual ligand for both PPARα (predominantly expressed in liver) and β/δ (ubiquitously expressed). Furthermore, the TG-lowering properties of bezafibrate depend mainly on an increased lipolysis of TG-rich lipoproteins such as VLDL and a decrease in VLDL secretion from the liver, the effects mediated by the activation of PPARα (Staels *et al.*, 1998a; Pineda-Torra *et al.*, 1999; Nakajima *et al.*, 2009). In knockout studies using PPARα- and β/δ-null mice, a fibrate analog Wy-14,643, also referred to as pirinixic acid (Santilli *et al.*, 1974; Kliewer *et al.*, 1994; Figure 59), has been shown to be a more potent activator of PPARα than bezafibrate (Peters *et al.*, 2003). Also, recent crystallographic data suggest that two WY-14,643 molecules bind to the ligand-binding domain of human PPARα, which may account for their role in maintaining PPARα functional activity (Bernardes *et al.*, 2013).

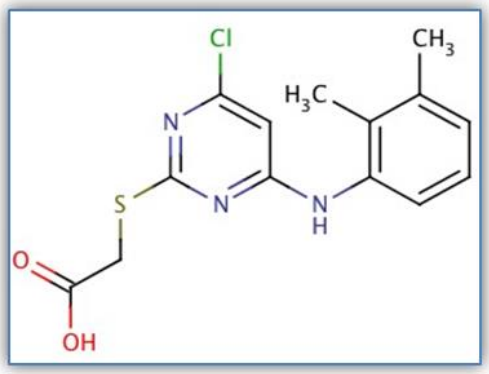


Figure 59: Chemical structure of Wy-14,643

In order to be more precise in our identification of PPAR subtype involved in the regulation of LSR, we next tested the effect of this PPARα–specific synthetic agonist on LSR expression in Hepa1-6 cells.

2.6. Effects of PPARα activation by Wy-14,643 on LSR expression

Various studies have demonstrated that the potency of Wy-14,643 as an activator of PPARα is species-specific with the receptor activation occurring at concentrations as low as 0.1 µM in the mouse compared to 10 µM in *Xenopus* (Keller *et al.*, 1997; Willson *et al.*, 2000; Brown *et al.*, 2001). Using a cell-based transactivation assay, it was established that Wy-14,643 is a strong activator of murine PPARα at as low concentrations as an EC_{50} value of 0.63 µM (Issemann & Green, 1990; Henke *et al.*, 1998; Willson *et al.*, 2000).

Taking these findings into account, we next studied the effects of PPARα in the regulation of LSR expression by incubating Hepa 1-6 cells with 10 and 25 µM of Wy-14,643 in CM at 37°C for 24 h. Total RNA was recovered and LSR mRNA levels were determined using real-time qPCR. We observed a significant induction in LSR mRNA levels at 10 and 25 µM Wy-14,643. As Wy-14,643 can also exert its effects on gene expression independent of PPARα (Sanderson *et al.*, 2009; He *et al.*, 2011), we verified the activation of PPARα by measuring also the mRNA levels of ACOX1, which is known to be specifically activated by PPARα. ACOX1 mRNA expression was also increased in cells treated with both 10 and 25 µM Wy-14,463, indicating that the increase in LSR expression was most likely due to the Wy-14,643-induced activation of PPARα (Figure 60).

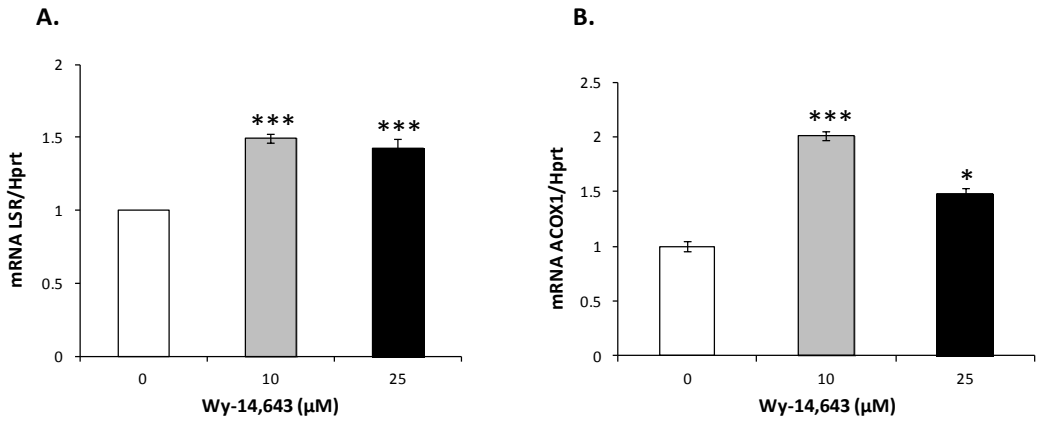


Figure 60: Effects of Wy-14,643 on LSR and ACOX1 mRNA levels in Hepa1-6 cells

Hepa 1-6 cells were incubated at 37°C for 24 h with Wy-14,643, a PPARα agonist, at the indicated concentrations in CM. Real-time qPCR experiment was performed to determine (A) LSR (N=4; n=11) and (B) ACOXI (N=1; n=3) expression using Hprt as reference gene. Cells treated with vehicle (DMSO) alone were used as controls. The results are represented as means ± SEM and p*-values are indicated where significant compared to the control condition, ** p*<0.05, **** p*<0.001.*

Results from western blots also revealed an increase in LSR protein levels, consistent with changes in mRNA levels, at both Wy-14,643 concentrations (Figure 61).

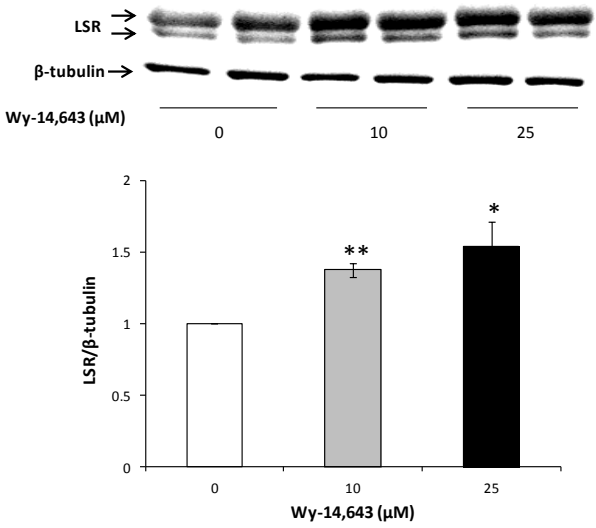


Figure 61: Effects of Wy-14,643 on LSR protein levels in Hepa1-6 cells

Western blot analysis was performed to detect LSR protein in total cell lysates from Hepa 1-6 cells incubated at 37°C for 24 h in CM with indicated concentrations of Wy-14,643, a PPARα agonist. β-tubulin was used as loading control. Cells treated with vehicle (DMSO) alone were used as controls. A representative blot is shown in the upper panel. Densitometric analysis was performed and results are shown in the lower panel as means ± SEM, N=3; n=5. The p*-values are indicated where significant compared to the control condition, ** p*<0.05, *** p*<0.01.*

A large body of evidence reveals that PPARα is implicated in the regulation of genes involved in diverse functions. However, it possesses special significance in the modulation of genes related to lipid and lipoprotein metabolism, particularly in liver. Furthermore, PPARα activation by agonists such as Wy-14,643 has been found to be associated with lower circulating TG levels and reduction of lipid storage in liver, muscle, and adipose tissue (Chou *et al.*, 2002), leading to an improved insuline sensitivity (Guerre-Millo *et al.*, 2000; Kim *et al.*, 2003). Similarly, other agonists including the fibrates (bezafibrate) are widely used in clinical studies for the treatment of dyslipidemias and type-II diabetes (Wagner & Wagner, 2010; Tenenbaum & Fisman, 2012b). PPARα mediates these beneficial effects on lipid homeostasis, mainly through the regulation of various target genes implicated in gluconeogenesis, FA oxidation and transport, and also by modulating the lipoprotein metabolism in liver (Gulick *et al.*, 1994; Fruchart *et al.*, 1999; Kersten *et al.*, 1999). Among these genes, we proposed LSR as one of the PPARα potential target genes, mainly because of the implication of LSR in the metabolism of TG-rich lipoproteins in liver. The apparent number of LSR binding sites expressed at the surface of hepatocytes have been found to be correlated negatively with plasma TG levels measured in the postprandial phase (Mann *et al.*, 1995). In addition, the presence of putative PPREs in the 5' regulatory region of mouse, human and rat *lsr* genes together with the experimenatl data (as decribed in Results and Discussion, Chapter II) indicates that LSR may represent a potential PPARα target gene. In this study, we have described for the first time that LSR expression is upregulated by PPARα. Using both pan-activator and PPARα-specific agonist, LSR expression was increased with concomitant increase in ACOX1 expression, showing the effective activation of PPARα.

2.7. Effects of PPARα inhibition on LSR expression

In order to confirm whether the induction of LSR expression by Wy-14,643 was dependent on the activation of PPARα, we next sought to determine the effects of PPARα activity inhibition in the regulation of LSR expression. For this, a highly potent and specific PPARα antagonist, GW6471 (Figure 62), was used which exhibits an inhibitory concentration (IC50) of 0.24 µM in a cell-based reporter assay (Xu *et al.*, 2002).

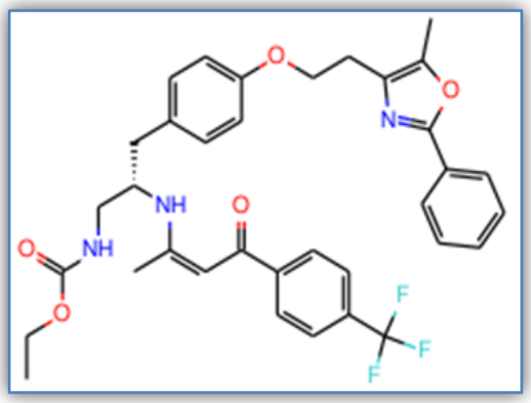


Figure 62: Chemical structure of GW6471

Binding of an antagonist such as GW6471 to PPARα leads to the destabilization of receptor-coactivator complex with the instantaneous recruitment of corepressors such as the Nuclear-CoRepressor (N-CoR) and the silencing mediator for retinoid and thyroid hormone receptors (SMRT) (Hu & Lazar, 2000; Xu & Lambert, 2003), which ultimately results in the suppression of PPARα target gene expression.

Hepa 1-6 cells were incubated with 1 and 5 µM GW6471 in CM for 24 h (as described in Materials & Methods, section 3). A significant decrease in LSR was observed in cells treated with 5 µM GW6471. A similar decrease in ACOX1 expression was also observed in cells treated with the same concentration of GW6471 (Figure 63).

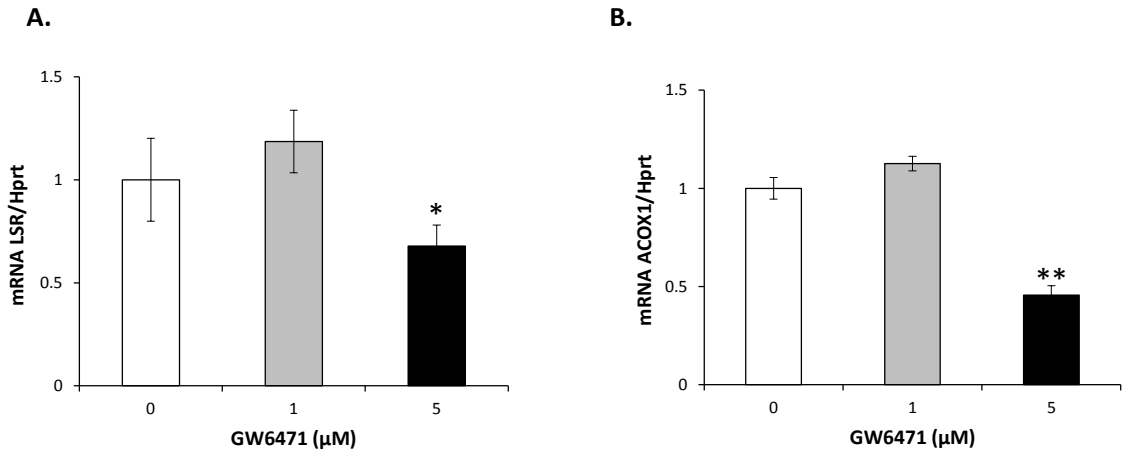


Figure 63: Effects of GW6471 on LSR and ACOX1 mRNA levels in Hepa1-6 cells

Hepa 1-6 cells were incubated at 37°C for 24 h with the indicated concentrations of GW6471, a PPARα antagonist, in CM. Real-time qPCR was performed to determine (A) LSR and (B) ACOX1 expression, using Hprt as reference gene. Cells treated with vehicle (DMSO) alone were used as controls. The results are shown as means ± SD; N=1; n=3 and p*-values are indicated where significant compared to the control condition, * p<0.05, ** p<0.01.*

The results indicate that the inhibition of PPARα activity by GW6471 decreases LSR expression, thus leading us to conclude that LSR is a potential target gene for this transcription factor.

2.8. Determination of LSR expression in the presence of PPARα antagonist and agonists

The *in vitro* results obtained so far from the treatment of agonists or antagonist alone point towards the involvement of PPARα in the regulation of LSR. Further experiments were conducted by incubating Hepa 1-6 cells with both antagonist and agonist simultaneously. For this, the cells were pretreated at 37°C for 6 h with 5 µM GW6471 in CM in order to block the activity of PPARα, and then incubated at 37°C for 48 h with 10 µM Wy-14,643 and 400 µM bezafibrate. The cells were then harvested for the extraction of total RNA and LSR and ACOX1 expression levels were determined using real-time qPCR analysis (Figure 64).

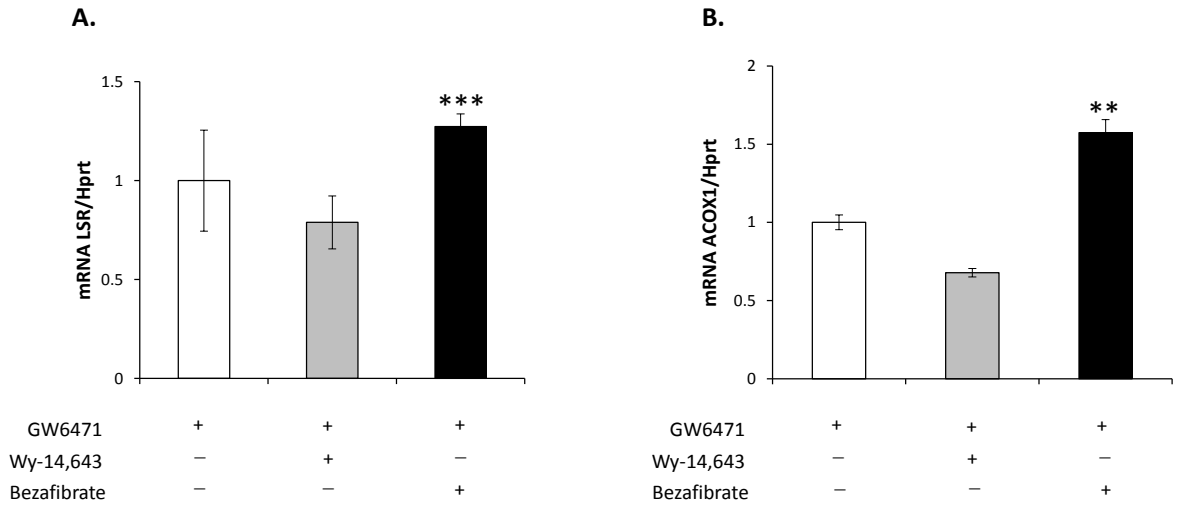


Figure 64: Effects of PPARα antagonist and agonists on LSR and ACOX1 mRNA levels

Hepa 1-6 cells were incubated at 37°C in CM with GW6471 (5 µM) for 6 h and for the following 48 h with Wy-14,643 (10 µM) and bezafibrate (400 µM) in the presence of GW6471. Real-time qPCR experiment was performed to determine (A) LSR and (B) ACOX1 mRNA expression using Hprt as reference gene. Cells treated with vehicle (DMSO) alone were used as controls. The results are shown as means ± SD; n=3 and p*-values are indicated where significant compared to the wells treated with GW6471 alone as the control condition, ** p<0.01, *** p<0.001.*

The results demonstrate a trend towards decrease in LSR and ACOX1 mRNA levels in the presence of both GW6471 and Wy-14,643, when compared to the control cells treated with GW6471 alone. Considering the fact that Wy-14,643 and GW6471 are highly selective

PPARα agonist and antagonist, respectively, it can be concluded that the pretreatment of cells with GW6471 prevented the Wy-14,643-mediated activation of PPARα. This, in turn, may explain the absence of increase in LSR expression that could be expected upon Wy-14,643 treatment as shown in Figure 60.

In contrast, the bezafibrate-induced expression of LSR, albeit lower, was still present despite pretreatment of Hepa 1-6 cells with the PPARα antagonist. Indeed, instead of increasing 44% as calculated from the results depicted in Figure 58, bezafibrate-induced LSR expression was only 27% in cells pretreated with GW6471. Since bezafibrate is also able to act as an activator for PPARβ/δ and PPARγ (Peters *et al.,* 2003; Tenenbaum & Fisman, 2012a), it is possible that these PPAR subtypes may also regulate LSR expression. Also, additional factors, such as the existence of cross-talk among different PPARs, the competition among them for the same heterodimer partner (RXR) and their interactions with other nuclear receptors including LXRs and SREBPs, could also play an important role in directing their actions on gene expression and this adds to the complexity in understanding various PPAR-mediated biological processes (Shi *et al.,* 2002; Ide *et al.,* 2003; Yoshikawa *et al.,* 2003; Matsusue *et al.,* 2006; Gustafsson *et al.,* 2009; Moreno *et al.,* 2010). Hence, taking into account the tissue-specific distribution of PPARs, further investigation is required to highlight the implication of PPARs in the modulation of LSR expression and the consequent significance of this regulation for the maintenance of lipid status.

3. Conclusions

Previous studies have demonstrated the role of leptin as a positive regulator in the transcriptional regulation of LSR in both mouse and Hepa 1-6 cell line models (Stenger *et al.,* 2010). To date, no data are available on the transcriptional regulation of LSR. In this study, we focused on the regulatory effects of PPARα on LSR expression, mainly due to the presence of PPREs in the 5' regulatory regions of mouse, rat and human *lsr* genes. Moreover, PPARα is highly expressed in liver and it is considered as a master regulator of hepatic lipoprotein metabolism. Indeed, LSR is abundantly expressed in liver and it also shows a valuable contribution in the clearance of TG-rich lipoproteins (Bihain & Yen, 1992; Yen *et al.,* 1994; Mesli *et al.,* 2004). This study allowed us to identify PPARα as a modulator of LSR expression in Hepa 1-6 cells. The results indicate a significant increase in LSR protein and mRNA levels in Hepa 1-6 cells treated with PPARα-selective activator, Wy-14,643. This

increase in LSR expression was accompanied by an increase in the expression of a well-known PPARα-responsive gene, *i.e.* ACOX1, thus suggesting LSR as a potential PPARα target gene. Furthermore, the decrease in LSR and ACOX1 expression upon treatment with PPARα-specific antagonist (GW6471) also led to the same conclusions. Interestingly, a significant increase in LSR mRNA was observed with both GW6471 and bezafibrate treatment, which may be consistent with the role of bezafibrate as a pan-activator and the possible involvement of other PPAR subtypes in the regulation of LSR expression. This also instigates further investigation in order to determine the physiological roles and functional consequences of this PPARα-mediated LSR regulation using PPARα knockout or RNAi studies in mice.

IV. Effects of dietary conditions on the expression of genes related to hepatic lipid metabolism in $LSR^{+/-}$ mice

1. Introduction

The liver plays a central role in many aspects of lipid and lipoprotein metabolism. Hepatic lipid metabolism is a highly coordinated process, involving several inter-dependent and cross-regulated pathways. Any perturbations in hepatic lipid homeostasis can cause serious metabolic consequences including dyslipidemia, obesity and diabetes, which may ultimately lead to the development of CVDs. Lipid accumulation in the liver can occur through the impairment in the processes involved in fatty acid (FA) uptake (either through dietary sources or from adipose tissue), synthesis, oxidation or export of lipids from the liver (Anstee & Goldin, 2006). In addition, an increased circulating FA pool in the liver through both dietary uptake and lipolysis in adipose tissue can contribute to the pathogenesis of non-alcoholic fatty liver disease (NAFLD), which represents a group of liver diseases including steatosis, non-alcoholic steatohepatitis (NASH) and cirrhosis without alcohol abuse (Vuppalanchi & Chalasani, 2009; Krawczyk *et al.,* 2010). Numerous epidemiological studies have revealed a strong relation between NAFLD and various parameters of metabolic syndrome, including dyslipidemia (Toledo *et al.,* 2006; Chatrath *et al.,* 2012), obesity (Ruhl & Everhart, 2004; Gastaldelli *et al.,* 2007), IR (Adams & Lindor, 2007; Cankurtaran *et al.,* 2007) and T2DM (Toledo *et al.,* 2006; Gaggini *et al.,* 2013). Indeed, NAFLD is considered as the hepatic component of metabolic syndrome and has been found to be strongly associated with the risk of CVDs (Ioannou *et al.,* 2006; Arslan *et al.,* 2007; Sun & Lü, 2011; Bhatia *et al.,* 2012; Brea & Puzo, 2013).

IR is regarded as an important factor in the pathogenesis of NAFLD, mainly by increasing FFA flux into the liver and the subsequent cellular insults including oxidative stress, lipid oxidation, and inflammation (Day & James, 1998). The dyslipidemic profile observed in NAFLD is mainly characterized by large VLDL, small dense LDL, and decreased large HDL and is strongly correlated with the intrahepatic lipid content. However, elevated VLDL is considered as an important metabolic disturbance related to obesity and metabolic syndrome (Cali *et al.,* 2007; Tacer & Rozman, 2011). Various receptors and enzymes function in cooperation with one another in order to regulate the hepatic lipid and lipoprotein homeostasis. Among these, LSR has emerged as an important lipoprotein receptor due to its involvement in the clearance of ApoB/ApoE-containing TG-rich lipoproteins such as VLDL and chylomicrons (Bihain & Yen, 1992; Mann *et al.,* 1995; Yen *et al.,* 2004). Studies have shown that the complete inactivation of LSR gene proves to be lethal in mice at embryonic

stage (Mesli *et al.*, 2004), suggesting that LSR plays a vital role in liver and embryonic development. However, the inactivation of a single functional allele of LSR yields a viable mouse, and leads to increased plasma TG levels as well as increased weight gain when mice are placed on a Western-type diet containing high fat (HF) and cholesterol, as compared to littermate LSR$^{+/+}$ controls (Yen *et al.*, 2008). Furthermore, the expression of hepatic LSR has been found to be impaired in mouse models of obesity and T2DM. Also, the knockdown of hepatic LSR using adenovirus-mediated shRNA resulted in increased postprandial TG levels, along with increased levels of both ApoB and ApoE (Narvekar *et al.*, 2009). Our recent data demonstrates leptin to be an important factor involved in the upregulation of LSR in both mouse and cell models (Stenger *et al.*, 2010). LSR$^{+/-}$ mice placed on HF diet showed an increased body fat mass accompanied by an increase in plasma leptin levels. However, hepatic protein levels of leptin receptor, Ob-R and the downstream p-ERK signaling protein were found to be reduced in these mice, indicating that the canonical signaling pathway for leptin was considerably reduced. This could contribute to the decreased LSR and elevated postprandial lipemia observed in obese mouse models (Stenger *et al.*, 2010). Taken together, these observations suggest that LSR is intimately involved in lipid homeostasis, and we therefore sought to determine the global changes in the expression of various genes involved in hepatic lipid metabolism in LSR$^{+/-}$ mice, with or without HF diet challenge.

2. Experimental procedures

2.1. High-fat diet supplementation

In order to study the effects of LSR genotype and high-fat (HF) diet on the expression of the genes involved in hepatic lipid metabolism, 6-month old female LSR$^{+/+}$ and LSR$^{+/-}$ mice (n=7 for each group) were placed on standard (STD) or HF diet, containing 60% fat kcal from lard (Dietex, Saint Gratien, France) for 6 weeks (Table 24). Our previous studies have shown that female LSR$^{+/-}$ were more responsive to HF diet, as compared to males, and therefore we used female mice in this experiment. The methods for the production and genotyping of LSR$^{+/+}$ and LSR$^{+/-}$ mice have been described in the Materials & Methods, section 1.

Table 24: Composition and energy value (% kcal) of each component in high-fat diet

Composition	% (w/w)	kcal/g	% kcal
Lipids	34.3	3.09	60
Proteins	25.9	1.04	20
Carbohydrates	25.2	1.01	20
Fibers	5	/	/
Others	4.5	/	/
Total		**5.13**	**100**

Components	% (w/w)
Caseine	29.94
Bitartrate choline	0.33
L-cystine	0.45
Lard	29.25
Rice starch	10.08
Cellulose	6.96
Soybean oil	4.86
Sucrose	11.84
Minerals	4.88
Vitamins	1.39

During the experimental period, body weight and food intake of the animals were monitored. Plasma samples were collected at the beginning and the end of the experiment in order to measure plasma TG and TC levels. Liver TG and TC levels were also determined in these groups.

2.2. Pathway-focused qPCR array analysis

Total RNA (1 µg) from the liver samples was reverse-transcribed using the RT² First Strand Kit (SABiosciences; as per manufacturer's instruction). Changes in gene expression were determined by a pathway-focused Fatty liver qPCR array (PAMM_157Z; SABiosciences). The cDNA from individual animals was used as a template for the PCR array according to the array instructions using SYBR green chemistry on StepOnePlus™ (Applied Biosystems). Data were analyzed using SABiosciences RT² Profiler PCR Data Analysis software at (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) and were considered significant at >1.3 fold change and $p<0.05$. Relative quantitation for each gene was performed using average cycle threshold (Ct) of a panel of five housekeeping genes

including β-actin, (Actb), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-2-microglobulin (B2M), β-glucuronidase (Gusb) and heat-shock protein 90 alpha (cytosolic) class B member 1 (Hsp90αb1) present on the array.

3. Results and discussion

In this study, no significant differences in body weight were observed between LSR$^{+/+}$ and LSR$^{+/-}$ mice on STD diet. However, the body weight in LSR$^{+/+}$ mice on HF diet was increased upto 27% relative to LSR$^{+/+}$ mice on STD diet. In addition, LSR$^{+/-}$ mice on HF diet also showed 40% increase in body weight as compared with LSR$^{+/-}$ mice on STD diet. Furthermore, an increase of 10% in body weight was observed in LSR$^{+/-}$ mice on HF diet, when compared with LSR$^{+/+}$ mice on HF diet. No differences in food intake were observed in all the groups. Although the plasma TC levels remained unchanged in all the groups, the liver TC levels increased to about 20% in LSR$^{+/+}$ mice on HF diet as compared with LSR$^{+/+}$ mice on STD diet. Also, an increase of about 27% was observed in LSR$^{+/-}$ mice on HF diet as compared with LSR$^{+/-}$ mice on STD diet. Moreover, the liver TG levels were found to be increased up to 12% in LSR$^{+/-}$ mice on HF diet, as compared with LSR$^{+/-}$ mice on STD diet (Unpublished laboratory data).

3.1. Determination of LSR expression

Real-time RT-qPCR analysis was performed to determine the expression of hepatic LSR in these mice. The results indicated a 58% decrease in LSR expression in mice with single functional LSR allele, relative to LSR$^{+/+}$ mice with the same genetic background, when both were placed on STD diet (Figure 65A), which was to be expected. Furthermore, a 50% decrease in LSR expression was obtained in LSR$^{+/-}$ mice relative to LSR$^{+/+}$ mice, when both groups were placed on HF diet (Figure 65A). Moreover, a non-significant reduction of 17% in LSR mRNA was observed when LSR$^{+/+}$ mice were fed with HF diet, as compared with the mice on STD diet (Figure 65B). However, no differences in LSR expression were observed when LSR$^{+/-}$ mice were placed on HF diet as compared with LSR$^{+/-}$ mice on STD diet (Figure 65B).

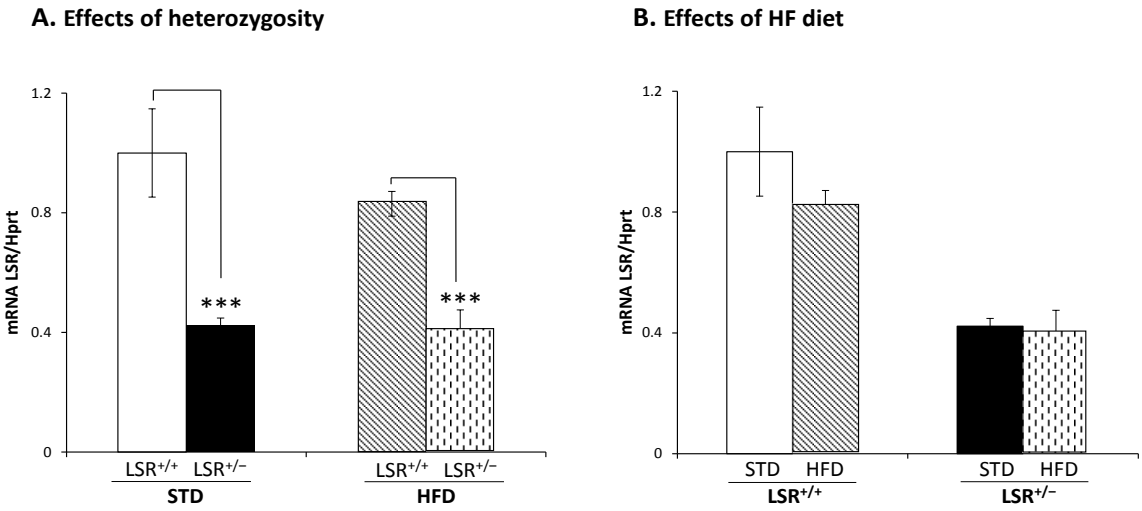


Figure 65: Effects of heterozygosity and high-fat diet on LSR expression

Six-month old female LSR$^{+/+}$ and LSR$^{+/-}$ mice were placed on standard (STD) or high-fat (HF) diet for 6 weeks. The effects of (A) heterozygosity and (B) HF diet on the expression of LSR were determined in the liver samples (n = 3 per group). Hprt was used as a reference gene. The expression values obtained for all the conditions were normalized relative to LSR$^{+/+}$ on STD diet as control. Results are expressed as means ± SD and p*-values are indicated where significant,* *** p*<0.001.*

It has previously been shown that the inactivation of both the alleles of LSR proves to be lethal at embryonic stages in mice (Mesli *et al.,* 2004). However, the lack of single LSR allele has been demonstrated to decrease LSR expression by more than 50% accompanied by a significant increase in both plasma TC and TG levels during postprandial phase. This, together with the reduced clearance of lipids after a dietary lipid challenge, suggests that LSR plays a crucial role in the transport and distribution of lipoproteins (Yen *et al.,* 2008). In this experiment, our data suggests that LSR$^{+/-}$ represents a true heterozygous genotype with the expression of LSR reduced to half in these mice, compared with LSR$^{+/+}$ mice. Furthermore, we observed a non-significant decrease in LSR$^{+/+}$ mice on HF diet as compared with LSR$^{+/+}$ mice on STD diet. Taking these observations into account, our next goal was to determine whether these genotype and/or HF diet-induced changes in LSR expression are associated with the genes related to lipid metabolism, included in qPCR array.

4. Mouse fatty liver qPCR array

The qPCR array technique was employed to determine the expression of 84 key genes identified to be associated with pathologies including NAFLD, which is often linked with obesity, as well as IR (Table 25). This array was chosen primarily because it contained the genes including the transcription factors (TFs) of interest, as well as other genes that

participate in lipid homeostasis. The array provides five housekeeping genes including Actb, B2M, GAPDH, Gusb and Hsp90ab1 for normalization. In our experiment, B2M was not used for normalization, since its expression appeared significantly modified. The variations were considered significant at >1.3-fold change and *p*-value <0.05.

Table 25: A complete list of genes involved in "fatty liver" qPCR array showing their distribution in various pathways

The common names for some genes are indicated in the parenthesis.

Pathways	Genes included in qPCR array
Insulin signaling pathway	*Akt1*, *Foxa2* (Hnf3b), *Gsk3b*, *Igf1*, *Igfbp1*, *Insr*, *Irs1*, *Mapk1* (Erk2), *Mapk8* (Jnk1), *Mtor*, *Pik3ca* (p110a), *Pik3r1* (Pi3k p85α), *Pklr*, *Ppargc1a* (Pgc 1α), *Prkaa1*, *Ptpn1*, *Slc2a4* (Glut4), *Socs3*, *Srebf1* (SREBP-1a & -1c).
Adipokine signaling pathway	*Adipor1*, *Adipor2*, *Akt1*, *Cd36*, *Irs1*, *Lepr* (Ob-R), *Mapk8* (Jnk1), *Mtor*, *Nfkb1*, *Ppara*, *Ppargc1a* (Pgc-1α), *Prkaa1*, *Rxra*, *Serpine1* (Pai-1), *Slc2a1*, *Slc2a4* (Glut4), *Socs3*, *Stat3*, *TNF-α.*
Genes involved in T2DM	*Gck*, *Insr*, *Irs1*, *Mapk1* (Erk2), *Mapk8* (Jnk1), *Mtor*, *Pik3ca* (p110A), *Pik3r1* (Pi3k p85α), *Pklr*, *Slc2a2*, *Slc2a4* (Glut4), *Socs3*, *TNF-α*, *Xbp1*.
Carbohydrate metabolism	*Acly*, *G6pc*, *G6pdx*, *Gck*, *Gsk3b*, *Mlxipl*, *Pck2*, *Pdk4*, *Pklr*, *Rbp4*.
β-Oxidation	*Acadl*, *Acox1*, *Akt1*, *Cpt1a*, *Cpt2*, *Fabp1*, *Irs1*, *Mtor*, *Ppara*.
Cholesterol metabolism/transport	*Abca1*, *Abcg1*, *Apoa1*, *Apob*, *Apoc3*, *Apoe*, *Cd36*, *Cnbp*, *Cyp2e1*, *Cyp7a1*, *Hmgcr*, *Ldlr*, *Lepr* (Ob-R), *Nr1h2* (LXRβ), *Nr1h3* (LXRα), *Nr1h4* (FXR), *Ppara*, *Ppard*, *Pparg*, *Prkaa1*, *Rxra*, *Srebf1*, *Srebf2*.
Other lipid metabolism/transport	*Acaca*, *Acsl5*, *Acsm3*, *Dgat2*, *Fabp3*, *Fabp5*, *Fasn*, *Gyk*, *HNF-4α*, *Lpl*, *Ppa1*, *Scd1*, *Slc27a5*.
Oxidative phosphorylation	*Atp5c1*, *Ndufb6*, *Ppa1*.
Inflammatory response	*Cebpb*, *Fas* (Tnfrsf6), *Ifng*, *Il10*, *Il1b*, *Il6*, *Nfkb1*, *Rxra*, *Tnf*.
Apoptosis	*Akt1*, *Casp3*, *Cebpb*, *Fas* (Tnfrsf6), *Mapk1* (Erk2), *Mapk8* (Jnk1), *Nfkb1*, *Pik3ca* (p110a), *Pparg*, *Prkaa1*, *Rxra*, *Serpine1* (Pai-1), *Socs3*, *TNF-α.*

4.1. Functional categorization of differentially-expressed genes

Using this pathway-specific qPCR array, the expression of genes involved in hepatic lipid metabolism was assessed in the liver of LSR$^{+/+}$ and LSR$^{+/-}$ mice, on STD or HF diets. Out of 84 genes included in the array, 32 genes were significantly downregulated in LSR$^{+/-}$ mice as compared with LSR$^{+/+}$ mice placed on STD diet. These genes were mainly related to insulin and adipokine signaling and lipid metabolism (Figure 66A). This suggests that the absence of one LSR allele can lead to the downregulation of a number of genes related to

various aspects of hepatic lipid metabolism, indicating the potential of LSR in the maintenance of these cellular metabolic processes. Furthermore, 11 genes were downregulated and 2 genes were upregulated in LSR$^{+/-}$ mice relative to LSR$^{+/+}$ mice, when both were fed with HF diet (Figure 66B). In LSR$^{+/+}$ mice on HF diet, 37 genes were downregulated and 2 genes were upregulated, compared with LSR$^{+/+}$ on STD diet (Figure 66C). However, in LSR$^{+/-}$ mice on HF diet, 16 genes were downregulated and 8 genes were upregulated, as compared with LSR$^{+/-}$ mice on STD diet (Figure 66D). Taken together, these results suggest that the number of genes that are downregulated in Figure 66B are much less as compared to Figure 66C, which may show a propensity of LSR$^{+/-}$ (as indicated in Figure 66A) to be more susceptible to diet-induced obesity. Furthermore, the HF treatment in both LSR$^{+/+}$ and LSR$^{+/-}$ mice (Figure 66B) indicates less differences in terms of gene expression in both these groups.

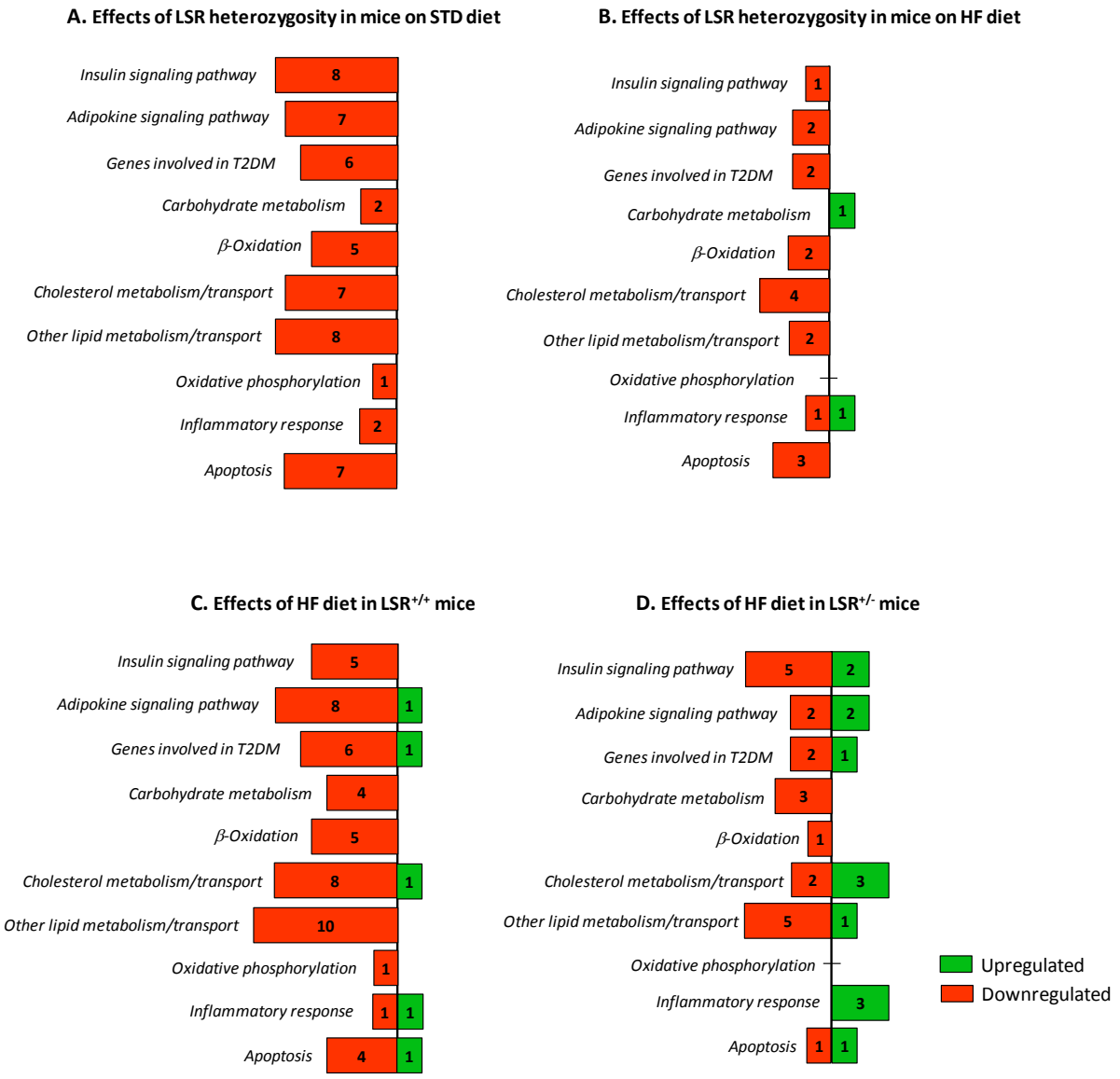


Figure 66: Global gene expression in functional categories

The changes in gene expression in the four groups of mice are represented in the form of colored bars showing the number of genes up- or down-regulated. The distribution of genes is indicated in different groups. Only the genes with a fold change of >1.3 and p*-value <0.05 are represented. It should be noted that the number of genes indicated in this figure is higher than that indicated in the text for this figure, mainly because of the occurence of some genes in more than one pathway and therefore being counted more than once (see table 25).*

4.2. Regulation of PPARα and target genes

Hepatic lipid metabolism is mainly controlled by PPARα in many respects, including FA uptake, binding and activation, their intracellular trafficking and β-oxidation, ketogenesis, TG storage and lipolysis (Yoon, 2009; Rakhshandehroo *et al.*, 2010). This qPCR array contained a set of genes involved in hepatic lipid metabolism and the transcription factors (TFs) regulating these genes. These TFs include *PPARα*, *Srebf1* (SREBP-1), *Nr1h2* (LXRβ),

Nr1h3 (LXRα), *Nr1h4* (FXR), *NF-κB*, and *HNF-4α*. In addition, the crosstalk of PPARα is also well-known with other transcription factors including SREBP-1 and LXRs (Yoshikawa *et al.*, 2003; Ide *et al.*, 2003). In this study, apart from LSR$^{+/-}$ mice on HF diet relative to LSR$^{+/-}$ mice on STD diet, a large number of the genes modified in all the groups are the PPARα target genes, as reported in the literature (Rakhshandehroo *et al.*, 2010). A comparison in the gene expression profile of LSR$^{+/+}$ mice on HF diet compared with LSR$^{+/+}$ mice on STD diet indicates the central position of PPARα in this gene network (Figure 67). This is consistent with the role of this TF in controlling multiple sets of genes and various inter-connected metabolic pathways.

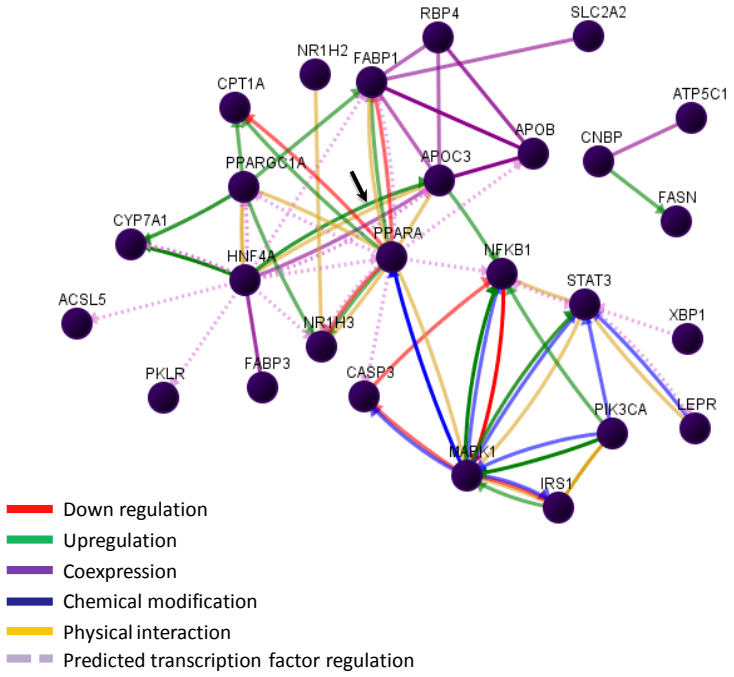


Figure 67: An overall representation of the interactions among various genes and the involvement of PPARα in multiple pathways

The relationships among the groups of genes are represented in LSR$^{+/+}$ mice on HF diet relative to LSR$^{+/+}$ mice on STD diet. The central location of PPARα (PPARA) in the whole gene network is depicted by an arrow. Only the genes that were significantly upregulated or downregulated are shown; fold change >1.3, and p*-value <0.05.*

In our experiment, a significant decrease of 1.5 fold was observed in PPARα in LSR$^{+/-}$ mice, as compared with LSR$^{+/+}$ mice, on STD diet. Furthermore, a significant decrease of 1.4 fold in PPARα expression was also observed in LSR$^{+/-}$ mice, relative to LSR$^{+/+}$ mice, on HF diet. Also, a decrease of 1.3 fold was observed in LSR$^{+/+}$ mice on HF diet, as compared with LSR$^{+/+}$ mice on STD diet. In each case, the decrease in PPARα expression was accompanied by a decrease in the genes regulated by PPARα (Table 26).

Table 26: The list of PPARα target genes in mice groups under different conditions

Conditions	PPARα expression	PPARα target genes	
		Decreased expression	**Increased expression**
Effects of heterozygosity in mice on STD diet	↓	*Acsl5, Acsm3, Adipor2, Cpt2, Fabp1, Fabp3, Fabp5, Fasn, Lpl, Nfkb1, Nr1h2, Nr1h3, Scd1, Srebf1*	---
Effects of heterozygosity in mice on HF diet	↓	*Fabp3, Scd1*	*G6PC*
Effects of HF diet in LSR$^{+/+}$ mice	↓	*Acaca, Acsl5, Acsm3, Adipor2, ApoC3, Cpt1a, Cpt2, Fabp1, Fabp3, Fabp5, Fasn, Lepr, Nfkb1, Nr1h2, Nr1h3, Pdk4, Scd1, Stat3*	*TNF-α, Cyp7a1*

↓ = *decreased gene expression*

4.3. Gene expression differences between LSR$^{+/-}$ mice on STD diet and LSR$^{+/+}$ mice on HF diet

The data obtained by comparing the gene expression changes in LSR$^{+/-}$ mice with LSR$^{+/+}$ mice, both on STD diet, would suggest that LSR$^{+/-}$ mice may already have a tendency towards a "high-fat"-like profile (Table 27). We further questioned whether the profile of LSR$^{+/-}$ on the STD diet was similar to that of LSR$^{+/+}$ mice on a HF diet. For this, the gene expression profile in LSR$^{+/+}$ mice on STD diet was compared with that of LSR$^{+/-}$ mice on STD diet (Table 27) or LSR$^{+/+}$ mice on HF diet (Table 28). About 70% of the genes were found to be common in both groups of mice, indicating that a similar set of genes is regulated in response to the absence of one LSR allele or in response to the HF diet in mice having both alleles. In other words, similar metabolic pathways including adipokine signaling and lipid metabolism and transport are affected in both the cases.

Table 27: Changes in gene expression profile in LSR+/- mice relative to LSR+/+ mice, both groups were placed on STD diet

(PPARα target genes are underlined. The fold changes are indicated in descending order and only the genes with a fold change of >1.3 and p*-value of <0.05 are included in the table)*

Gene	Gene description	Fold change in LSR$^{+/-}$	*p*-value
Insulin signaling pathway			
Srebf1	Sterol regulatory element binding transcription factor 1	-2.6	0.00041
Ptpn1	Protein tyrosine phosphatase, non-receptor type 1	-1.7	0.015
Irs1	Insulin receptor substrate 1	-1.7	0.0014
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	-1.4	0.0037
Akt1	Thymoma viral proto-oncogene 1	-1.4	0.035
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	-1.4	0.019
Mapk1	Mitogen-activated protein kinase 1	-1.4	0.028
Gsk3b	Glycogen synthase kinase 3 beta	-1.3	0.026
Adipokine signaling pathway			
Irs1	Insulin receptor substrate 1	-1.7	0.001
Slc2a1	Solute carrier family 2 (facilitated glucose transporter), member 1	-1.6	0.027
Adipor2	Adiponectin receptor 2	-1.6	0.023
Ppara	Peroxisome proliferator activated receptor alpha	-1.5	0.009
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	-1.5	0.034
Akt1	Thymoma viral proto-oncogene 1	-1.4	0.035
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	-1.4	0.019
Genes involved in T2DM			
Slc2a2	Solute carrier family 2 (facilitated glucose transporter), member 2	-2.8	0.008
Irs1	Insulin receptor substrate 1	-1.7	0.001
Gck	Glucokinase	-1.7	0.037
Xbp1	X-box binding protein 1	-1.6	0.007
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	-1.4	0.003
Mapk1	Mitogen-activated protein kinase 1	-1.4	0.028
Carbohydrate metabolism			
Gck	Glucokinase	-1.7	0.037
Gsk3b	Glycogen synthase kinase 3 beta	-1.3	0.026
β-Oxidation			
Fabp1	Fatty acid binding protein 1, liver	-1.7	0.014
Irs1	Insulin receptor substrate 1	-1.7	0.001
Ppara	Peroxisome proliferator activated receptor alpha	-1.5	0.009
Cpt2	Carnitine palmitoyltransferase 2	-1.4	0.023
Akt1	Thymoma viral proto-oncogene 1	-1.4	0.035

Table 27: Continued.....

Cholesterol metabolism/transport			
Srebf1	Sterol regulatory element binding transcription factor 1	-2.6	0.0004
Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	-1.7	0.009
Ppara	Peroxisome proliferator activated receptor alpha	-1.5	0.009
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	-1.4	0.019
Nr1h2	Nuclear receptor subfamily 1, group H, member 2	-1.4	0.021
Apob	Apolipoprotein B	-1.3	0.041
Nr1h3	Nuclear receptor subfamily 1, group H, member 3	-1.3	0.010
Other lipid metabolism/transport			
Scd1	Stearoyl-Coenzyme A desaturase 1	-3.0	0.003
Slc27a5	Solute carrier family 27 (fatty acid transporter), member 5	-2.7	0.001
Fabp5	Fatty acid binding protein 5, epidermal	-2.3	0.025
Fasn	Fatty acid synthase	-2.2	0.003
Lpl	Lipoprotein lipase	-1.9	0.009
Fabp3	Fatty acid binding protein 3, muscle and heart	-1.9	0.035
Acsm3	Acyl-CoA synthetase medium-chain family member 3	-1.7	0.012
Acsl5	Acyl-CoA synthetase long-chain family member 5	-1.6	0.031
Oxidative phosphorylation			
Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1.4	0.018
Inflammatory response			
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	-1.5	0.034
Fas	Fas (TNF-α receptor superfamily member 6)	-1.4	0.036
Apoptosis			
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	-1.5	0.034
Casp3	Caspase 3	-1.4	0.008
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	-1.4	0.003
Fas	Fas (TNF-α receptor superfamily member 6)	-1.4	0.036
Akt1	Thymoma viral proto-oncogene 1	-1.4	0.035
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	-1.4	0.019
Mapk1	Mitogen-activated protein kinase 1	-1.4	0.028

Note: *Some genes are involved in more than one pathway, see Table 25.*

Table 28: Changes in gene expression profile in LSR+/+ mice on HF diet relative to LSR+/+ mice on STD diet

(PPARα target genes are underlined. The fold changes are indicated in descending order and only the genes with a fold change of >1.3 and p*-value of <0.05 are included in the table)*

Gene	Gene description	Fold change in HF diet	*p*-value
Insulin signaling pathway			
Pklr	Pyruvate kinase liver and red blood cell	-4.1	0.000556
Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	-2.1	0.008744
Irs1	Insulin receptor substrate 1	-1.5	0.000624
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	-1.4	0.001067
Mapk1	Mitogen-activated protein kinase 1	-1.4	0.01941
Adipokine signaling pathway			
Lepr	Leptin receptor	-7.3	0.002
Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	-2.1	0.008
Adipor2	Adiponectin receptor 2	-1.7	0.015
Slc2a1	Solute carrier family 2 (facilitated glucose transporter), member 1	-1.7	0.005
Stat3	Signal transducer and activator of transcription 3	-1.5	0.025
Irs1	Insulin receptor substrate 1	-1.5	0.0006
Ppara	Peroxisome proliferator activated receptor alpha	-1.3	0.023
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	-1.3	0.030
TNF-α	Tumor necrosis factor	1.7	0.002
Genes involved in T2DM			
Pklr	Pyruvate kinase liver and red blood cell	-4.1	0.0005
Slc2a2	Solute carrier family 2 (facilitated glucose transporter), member 2	-1.9	0.028
Xbp1	X-box binding protein 1	-1.6	0.018
Irs1	Insulin receptor substrate 1	-1.5	0.0006
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	-1.4	0.001
Mapk1	Mitogen-activated protein kinase 1	-1.4	0.019
TNF-α	Tumor necrosis factor	1.7	0.002
Carbohydrate metabolism			
Pklr	Pyruvate kinase liver and red blood cell	-4.1	0.0005
G6pdx	Glucose-6-phosphate dehydrogenase X-linked	-2.4	0.001
Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4	-1.8	0.025
Rbp4	Retinol binding protein 4, plasma	-1.5	0.016

Table 28: Continued....

β-Oxidation			
Fabp1	Fatty acid binding protein 1, liver	-1.8	0.006
Irs1	Insulin receptor substrate 1	-1.5	0.0006
Cpt2	Carnitine palmitoyltransferase 2	-1.5	0.013
Cpt1a	Carnitine palmitoyltransferase 1a, liver	-1.4	0.032
Pparα	Peroxisome proliferator activated receptor alpha	-1.3	0.023
Cholesterol metabolism/transport			
Lepr	Leptin receptor	-7.3	0.002
Cnbp	Cellular nucleic acid binding protein	-1.4	0.042
Nr1h2	Nuclear receptor subfamily 1, group H, member 2	-1.4	0.015
Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	-1.4	0.028
Apob	Apolipoprotein B	-1.4	0.021
Nr1h3	Nuclear receptor subfamily 1, group H, member 3	-1.3	0.013
Ppara	Peroxisome proliferator activated receptor alpha	-1.3	0.023
Apoc3	Apolipoprotein C-III	-1.3	0.026
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	3.9	0.019
Other lipid metabolism/transport			
Fabp5	Fatty acid binding protein 5, epidermal	-7.8	0.006
Scd1	Stearoyl-Coenzyme A desaturase 1	-7.7	0.0009
Acsl5	Acyl-CoA synthetase long-chain family member 5	-2.5	0.006
Acaca	Acetyl-Coenzyme A carboxylase alpha	-2.4	0.0001
Fasn	Fatty acid synthase	-2.4	0.001
Acsm3	Acyl-CoA synthetase medium-chain family member 3	-2.2	0.005
Dgat2	Diacylglycerol O-acyltransferase 2	-1.6	0.034
Slc27a5	Solute carrier family 27 (fatty acid transporter), member 5	-1.5	0.007
Fabp3	Fatty acid binding protein 3, muscle and heart	-1.4	0.033
HNF-4α	Hepatic nuclear factor 4, alpha	-1.3	0.038
Oxidative phosphorylation			
Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1.5	0.009
Inflammatory response			
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	-1.3	0.03
TNF-α	Tumor necrosis factor	1.7	0.002
Apoptosis			
Casp3	Caspase 3	-1.5	0.004
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	-1.4	0.001
Mapk1	Mitogen-activated protein kinase 1	-1.4	0.019
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	-1.3	0.03
TNF-α	Tumor necrosis factor	1.7	0.002

Note: *Some genes are involved in more than one pathway, see Table 25.*

4.4. Gene expression differences between LSR$^{+/-}$ mice on HF diet and LSR$^{+/+}$ mice on HF diet

On the other hand, a greater difference (~66%) was observed in the gene expression profiles while comparing LSR$^{+/-}$ mice on HF diet (Table 29) with LSR$^{+/+}$ mice on HF diet (Table 28). Interestingly, LSR$^{+/-}$ mice on HF diet showed a significant upregulation of the two genes related to inflammatory pathway, interleukin 1-β (*Il-1β*) (1.8 fold) and interleukin 10 (*Il-10*) (3.1 fold), suggesting that perhaps these mice are "pre-programmed" in terms of their metabolic processes for an increased response to inflammation. Indeed, expression of these 2 genes was increased in LSR$^{+/-}$ on STD diet versus LSR$^{+/+}$ on the same diet, but only borderline, and did not achieve statistical significance.

Interestingly, these LSR$^{+/-}$ mice on HF diet also exhibit a significant increase (1.9 fold) in protein tyrosine phosphatase, non-receptor type 1 (*Ptpn1*), commonly known as protein tyrosine phosphatase 1B (PTP1B). This protein acts as a negative regulator of insulin and leptin signaling (Koren & Fantus, 2007) and its inhibition may lead to increased hepatic insulin sensitivity (González-Rodríguez *et al.*, 2010; Ma *et al.*, 2011). This suggests that the HF diet treatment in LSR$^{+/-}$ mice could render them more susceptible to an impaired hepatic insulin signaling pathway (Table 29) relative to LSR$^{+/+}$ mice on HF diet. However, the physiological consequences of these modifications in gene expression remain to be investigated.

Table 29: Changes in gene expression profile in LSR+/- mice on HF diet relative to LSR+/- mice on STD diet

(The fold changes are indicated in descending order and only the genes with a fold change of >1.3 and p-*value of <0.05 are included in the table)*

Gene	Gene description	Fold change in HF diet	*p*-value
Insulin signaling pathway			
Pklr	Pyruvate kinase liver and red blood cell	-2.6	0.009
Igfbp1	Insulin-like growth factor binding protein 1	-2.0	0.029
Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	-2.0	0.005
Insr	Insulin receptor	-1.4	0.001
Foxa2	Forkhead box A2	-1.4	0.024
Srebf1	Sterol regulatory element binding transcription factor 1	1.9	0.04
Ptpn1	Protein tyrosine phosphatase, non-receptor type 1	1.9	0.025
Adipokine signaling pathway			
Lepr	Leptin receptor	-7.5	0.0002
Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	-2.0	0.005
CD36	CD36 antigen	1.6	0.001
TNF-α	Tumor necrosis factor	2.7	0.005
Genes involved in T2DM			
Pklr	Pyruvate kinase liver and red blood cell	-2.6	0.009
Insr	Insulin receptor	-1.4	0.001
TNF-α	Tumor necrosis factor	2.7	0.005
Carbohydrate metabolism			
G6pdx	Glucose-6-phosphate dehydrogenase X-linked	-2.7	0.002
Pklr	Pyruvate kinase liver and red blood cell	-2.6	0.009
Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4	-1.7	0.026
β-Oxidation			
Cpt1a	Carnitine palmitoyltransferase 1a, liver	-1.5	0.001
Cholesterol metabolism/transport			
Lepr	Leptin receptor	-7.5	0.0002
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-1.9	0.0005
CD36	CD36 antigen	1.6	0.001
Srebf1	Sterol regulatory element binding transcription factor 1	1.9	0.04
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	2.8	0.009
Other lipid metabolism/transport			
Scd1	Stearoyl-Coenzyme A desaturase 1	-8.8	0.002
Fabp5	Fatty acid binding protein 5, epidermal	-7.5	0.00003
Acaca	Acetyl-Coenzyme A carboxylase alpha	-3.2	0.00004
Acsl5	Acyl-CoA synthetase long-chain family member 5	-1.7	0.006
Acsm3	Acyl-CoA synthetase medium-chain family member 3	-1.4	0.003
Lpl	Lipoprotein lipase	1.9	0.0005

Table 29: Continued....

Inflammatory response			
Il1b	Interleukin 1 beta	1.8	0.003
TNF-α	Tumor necrosis factor	2.7	0.005
Il10	Interleukin 10	3.1	0.004
Apoptosis			
Casp3	Caspase 3	-1.3	0.016
TNF-α	Tumor necrosis factor	2.7	0.005

Note: *Some genes are involved in more than one pathway, see Table 25.*

Interestingly, the leptin receptor, *Lepr* (Ob-R) was decreased to 7.3 fold in LSR$^{+/+}$ mice placed on HF diet, relative to LSR$^{+/+}$ mice on STD diet (Table 28). *Lepr* was also decreased 7.5 fold in LSR$^{+/-}$ mice on HF diet as compared with LSR$^{+/-}$ mice on STD diet (Table 29). However, no significant changes were observed in leptin levels for all the groups (A. Pinçon *et al*., unpublished data). Since leptin and insulin signaling pathways interact with each other, the downregulation of *Lepr* may consequently lead to the perturbations in both these pathways (Szanto & Kahn, 2000). On the other hand, no differences in *Lepr* gene expression were observed in LSR$^{+/-}$ mice as compared with LSR$^{+/+}$ mice, when both groups were placed on STD diet or HF diet.

Furthermore, it is to be noted that lesser genes were modified by HF diet treatment in LSR$^{+/-}$ mice as compared with LSR$^{+/+}$ mice (Table 30). Interestingly, as already shown (Figure 65B), we did not observe significant changes in LSR mRNA levels in LSR$^{+/-}$ mice on HF diet, relative to LSR$^{+/-}$ mice on STD diet. Also, a non significant decrease was observed in LSR$^{+/+}$ on HD diet, compared with LSR$^{+/+}$ mice on STD diet (Figure 65B). These results may indicate that heterozygosity and HF diet treatment could affect the similar genes and/or associated pathways. Moreover, under these conditions, the regulatory mechanisms would likely function to protect the animals from additive harmful effects in order to maintain the minimal expression levels.

Table 30: Changes in gene expression profile in LSR+/- mice relative to LSR+/+ mice, both on HF diet

(PPARα target genes are underlined. The fold changes are indicated in descending order and only the genes with a fold change of >1.3 and p*-value of <0.05 are included in the table)*

Gene	Gene description	Fold change in LSR$^{+/-}$	*p*-value
Insulin signaling pathway			
Mtor	Mechanistic target of rapamycin (serine/threonine kinase)	-1.3	0.024
Adipokine signaling pathway			
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	-2.4	0.044
Pparα	Peroxisome proliferator activated receptor alpha	-1.4	0.01
Genes involved in T2DM			
Slc2a2	Solute carrier family 2 (facilitated glucose transporter), member 2	-1.6	0.033998
Mtor	Mechanistic target of rapamycin (serine/threonine kinase)	-1.3	0.024
Carbohydrate metabolism			
G6pc	Glucose-6-phosphatase, catalytic	2.8	0.00049
β-Oxidation			
Pparα	Peroxisome proliferator activated receptor alpha	-1.4	0.01
Mtor	Mechanistic target of rapamycin (serine/threonine kinase)	-1.3	0.024
Cholesterol metabolism/transport			
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-1.8	0.042
Pparγ	Peroxisome proliferator activated receptor gamma	-1.6	0.023
Pparα	Peroxisome proliferator activated receptor alpha	-1.4	0.01
Pparδ	Peroxisome proliferator activator receptor delta	-1.3	0.016
Other lipid metabolism/transport			
Scd1	Stearoyl-Coenzyme A desaturase 1	-3.4	0.00035
Fabp3	Fatty acid binding protein 3, muscle and heart	-1.9	0.0057
Inflammatory response			
Ifng	Interferon gamma	-2.6	0.000094
Il10	Interleukin 10	1.9	0.007
Apoptosis			
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	-2.4	0.044
Pparγ	Peroxisome proliferator activated receptor gamma	-1.6	0.023
Casp3	Caspase 3	-1.3	0.008

Note: *Some genes are involved in more than one pathway, see Table 25.*

5. Conclusions

In conclusion, using a qPCR array-based approach, we have identified the changes occurring in a number of genes involved in lipid metabolism when one functional LSR allele is absent. This emphasizes upon the importance of LSR not only in the regulation of hepatic

lipid metabolism, but also suggests the therapeutic potential of LSR as target for treating various related metabolic disorders including insulin and adipokine signaling and carbohydrate metabolism. A wide array of genes, related to these pathways, was downregulated in the absence of one LSR allele in LSR$^{+/-}$ mice on STD diet, suggesting that LSR plays an important role in the regulation of important signaling pathways. Furthermore, the results also indicate that reduced LSR may also lead to increased expression of genes related to inflammation, particularly in LSR$^{+/-}$ mice on HF diet. However, with regards to hepatic lipid metabolism, we observed a significant downregulation in the expression of genes involved in cholesterol and FA metabolism. As this array specifically included the genes and pathways related to NAFLD, the gene expression profiles from the mice under HF diet treatment or lacking one LSR allele led us to conclude that LSR may represent an important target in the treatment of this disease.

Additional measurements including plasma TGs are currently underway to complete the phenotype of the different groups studied. This study broadens the therapeutic application of LSR beyond the hepatic lipid metabolism by revealing its effects on the regulation of other metabolic pathways in liver.

General Conclusions and Perspectives

The maintenance of lipid homeostasis is primarily controlled by liver which houses various metabolic pathways involving a number of enzymes and receptors working in coordination with one another. The lipolysis stimulated lipoprotein receptor (LSR) is one of the crucial factors involved in the clearance of ApoB and/or ApoE containing TG-rich lipoproteins during postprandial phase (Bihain & Yen, 1992; Mann *et al.,* 1995; Yen *et al.,* 2004). The absence of this receptor ($LSR^{-/-}$) in mice has been shown to be lethal at embryonic stages. However, the heterozygosity ($LSR^{+/-}$) leads to dyslipidemia, which becomes associated with excess weight gain in aged mice and in those placed on high-fat diet (Yen *et al.,* 2008). Furthermore, the expression of hepatic LSR has been found to be considerably reduced in mouse models of obesity and T2DM. Also, the knockdown of hepatic LSR using adenovirus-mediated shRNA resulted in increased postprandial TG levels, along with increased levels of both ApoB and ApoE (Narvekar *et al.,* 2009). We have recently identified leptin as an important regulator of hepatic LSR expression (Stenger *et al.,* 2010). The purpose of this research work was to pursue the investigation regarding the mechanisms involved in the regulation of LSR expression in the liver.

It is well-established that the nutritional modulation in the form of dietary fat intake can lead to the remodeling of the cellular membrane lipids, which consequently affects the physicochemical properties of membranes as well the functionality of the membrane-associated proteins. DHA is an important component of the membrane phospholipids and is well known for its hypotriglyceridemic properties, by controlling the expression of a number of enzymes and receptors involved in lipid metabolism (Zuliani *et al.,* 2009; Jacobson *et al.,* 2012; Pirillo & Catapano, 2013). It is, therefore, considered as an important dietary element for the prevention of various disorders related to lipid metabolism such as dyslipidemia, obesity, diabetes, cardiovascular and neurodegenerative diseases. As LSR actively participates in hepatic lipid metabolism upon activation by free fatty acids, we were interested to know whether DHA affects LSR expression and/or activity using *in vitro* and *in vivo* models. DHA treatment in Hepa 1-6 cells leads to an increase in LSR protein levels with no changes in LSR mRNA levels. Furthermore, an increase in LSR activity was observed with DHA treatment in these cells. The increase in protein levels accompanied with increased LSR activity suggests that DHA could possibly be involved in the modulation of receptor function. This, in turn may be associated with providing a conducive microenvironment for the organization of the receptor on the membrane surface. Indeed, *in vivo* experiments indicate that DHA supplementation in mice results in the enrichment of liver and erythrocyte membranes.

Moreover, it was shown that LSR protein levels increased with 3-month DHA ethyl ester treatment in mice, whereas no changes were observed in LSR mRNA levels in this study. This suggests that DHA could play an important role in the translational and/or post-transcriptional regulation of LSR. In addition, in short-term DHA supplementation for one month, we observed no changes in LSR mRNA levels. However, further work should be carried out to determine LSR protein levels as well as activity in hepatic plasma membrane in these samples. Indeed, the biochemical data suggests that LSR could exist as a multimeric complex of one α or α' subunit containing the transmembrane domain associated by disulfide bridges with two or three β subunits, located extracellularly. Binding of free fatty acids causes the conformational changes in LSR that expose a binding site for the recognition of ApoE of a TG-rich lipoprotein, leading to its binding, internalization and degradation. Studies to correlate the activity of LSR in DHA-fed mice with the plasma TG and cholesterol levels could provide further information about the physiological significance of this DHA-mediated regulation.

It is well-established that DHA enrichment in the plasma membranes leads to the alterations in its properties as well as the localization of various membrane-associated proteins (Ma *et al.,* 2004; Chapkin *et al.,* 2008). It can, therefore, be speculated that DHA could affect the localization of LSR receptor in or out the various microdomains of the hepatic membrane. Further work should be carried out focusing on raft markers in order to determine the mechanism of LSR regulation by DHA.

Another possible mechanism by which DHA elicits its regulatory effects involves the transcription factors related to lipid metabolism, particularly PPARα and SREBPs (Göttlicher *et al*., 1992; Pégorier *et al*., 2004; Jump, 2013). Taking this into consideration, we carried out studies to determine whether the expression of PPARα is modified with DHA treatment. Interestingly, we observed no changes in PPARα mRNA levels in both *in vivo* and *in vitro* models, which is consistent with the lack of changes in LSR mRNA levels with DHA supplementation as obtained in the different models tested. This suggests that PPARα is not involved in DHA-mediated effects on LSR. There exists contradicting data on the involvement of PPARα in DHA-mediated effects, where some studies demonstrated an increased PPARα expression with DHA supplementation (Keller *et al.,* 1993; Zúñiga *et al.,* 2011), while others showed that the fish oil-mediated effects are not dependent on PPARα (Dallongeville *et al.,* 2001). In addition, some studies have shown that ability of FAs to

activate PPARα is much lower than other PPARα activators such as fibrates (Forman *et al.,* 1997; Krey *et al.,* 1997). Further studies should focus on whether PPARα is involved in DHA-mediated regulation of LSR expression. For this, PPARα-specific antagonist or siRNA could be used to repress PPARα activity, with subsequent DHA treatment under *in vitro* conditions.

PPARα is a ligand-activated transcription factor that functions mainly to control the expression of a wide array of genes related to hepatic lipid metabolism (Gervois *et al.,* 2000; Duval *et al.,* 2007). An important class of therapeutic agents used in the treatment of dyslipidemia includes fibrates, which serve as ligands for PPARα. This prompted us to study the role of PPARα in the regulation of hepatic LSR expression. First, an *in silico* analysis was performed to identify the putative core promoter elements and the transcription factor binding sites, including PPAR response elements (PPREs) in the 5' regulatory region of mouse, rat and human *lsr* genes. Interestingly, high affinity binding matrices for PPARs were found, with two PPREs in mouse, three in rat and six in human 5' regulatory region of *lsr* gene. Since PPARα is highly expressed in the liver where it controls numerous genes and enzymes related to lipid metabolism, we sought to determine whether LSR represents a potential target gene for this transcription factor. For this, Hepa 1-6 cells were treated with a pan-activator (bezafibrate) and PPARα-selective agonist (Wy-14,643) as well as antagonist (GW6471). The significant increase in LSR expression upon treatment with agonists and the decrease with antagonist treatment occurred through PPARα-dependent mechanisms, since concomitant changes in the expression levels of a well known PPARα-responsive gene, ACOX1, were observed. These results suggest that PPARα plays an important role in the regulation of LSR expression in liver. However, the treatment with both GW6471 and bezafibrate led to an increase in LSR expression in Hepa 1-6 cells which may be consistent with the role of bezafibrate as a pan-activator and the possible involvement of other PPAR subtypes in the regulation of LSR expression. These findings lead us to the following perspectives.

- The presence, position and functional strength of the putative PPREs identified in mouse/rat *lsr* gene 5' regulatory region should be confirmed using site-directed mutagenesis, mobility shift assays and reporter gene experiments.
- Further experiments should be conducted using PPARα knockout mice or RNA interference to determine the physiological roles and functional consequences of this PPARα-mediated LSR regulation.

- The role of other PPARs such as PPARγ and PPARβ/δ in LSR regulation should be studied by using specific pharmacological antagonists and agonists or siRNAs.

Furthermore, while optimizing the cell culture conditions for PPARα experiments, we found an interesting observation regarding the differential regulation of PPARα and LSR in different media conditions. In these experiments, PPARα protein levels were detected only in complete and lipoprotein-deficient medium in Hepa 1-6 cells, whereas no signals for PPARα protein were detected in serum-deficient medium. In contrast, we observed a significant increase in LSR protein and mRNA levels in cells cultured in serum-deficient and lipoprotein-deficient media, relative to complete medium. Further experiments to determine whether the decrease in LSR expression in complete medium is dependent upon the presence of lipoproteins in the serum revealed a decrease in LSR protein levels when cells were incubated in the presence of media containing LDL. This indicates that LSR could respond to the changes in the cellular cholesterol levels, which further leads us to conclude that LSR expression may be sensitive to cholesterol-responsive factors such as SREBPs and LXRs. However, further investigation is required to address this question.

In order to investigate the pathways regulated by various transcription factors including PPARα, SREBPs and LXR as well as others related to hepatic lipid metabolism, the qPCR array approach led us to identify a number of genes, including PPARα, which were downregulated in the absence of one functional LSR allele in $LSR^{+/-}$ mice placed on the standard diet. These genes were found to be associated with various interconnected pathways, including hepatic lipid and carbohydrate metabolism, insulin and adipokine signaling and inflammation. Interestingly, most of these genes were target genes for PPARα. In addition, the results also indicate that reduced LSR levels may also lead to inflammation as deduced from increased expression of related genes. This was particularly evident in $LSR^{+/-}$ mice on high-fat diet. This suggests that LSR plays an important role in the maintenance of various metabolic processes in mouse liver, and any dysfunctions in LSR expression, as reflected in $LSR^{+/-}$ mice, could lead to "high-fat like" changes in key pathways and eventually increased risk for the pathological consequences. This study opens the door for further investigation of the involvement of LSR in the regulation of pathways other than hepatic lipid metabolism, such as insulin signaling, β-oxidation, inflammation and apoptosis. In the view of these observations, further work should focus on *in vivo* experiments using a combination of PPARα agonist and high-fat diet treatments in mice. This could be useful to investigate

whether the decrease in LSR expression by high-fat diet is restored by a treatment with PPARα agonist and the consequent beneficial effects on the hepatic lipid status.

These findings provide an important step towards understanding LSR involvement in the maintenance of hepatic lipid homeostasis. Furthermore, these results enabled us to identify different factors, including diet, involved in the regulation of hepatic LSR expression and provided the first evidence confirming the involvement of a transcription factor that is crucial in regulating hepatic lipid metabolism.

Présentation synthétique de la thèse

L'homéostasie lipidique peut être l'objet de fréquentes perturbations qui se manifestent par différentes formes de dyslipidémies, dont l'hypertriglycéridémie et l'hypercholestérolémie. Ces perturbations ont été identifiées comme des facteurs de risque communs à diverses pathologies associées au vieillissement parmi lesquelles se trouvent l'obésité et le diabète de type 2, ainsi que les maladies cardiovasculaires et neurodégénératives comme les démences de type Alzheimer, des pathologies qui posent un problème majeur de santé publique dans tous les pays. Il est donc important de comprendre les mécanismes sur lesquels repose la régulation du statut lipidique. C'est dans cet objectif qu'a été préparée la présente thèse dans l'équipe BFLA, Biodisponibilité et Fonctionnalités des Lipides Alimentaires, du laboratoire UR AFPA, Unité de recherche Animal & Fonctionnalités des Produits Animaux. Depuis de nombreuses années, l'équipe BFLA consacre ces efforts à identifier et caractériser les mécanismes qui affectent la disponibilité et les fonctions des lipides alimentaires et qui contribuent ainsi à promouvoir et accélérer les désordres métaboliques et cellulaires liés au vieillissement.

Le statut lipidique et la lipémie sont maintenus à des niveaux physiologiques grâce à un dispositif très complexe composé de récepteurs de lipoprotéines, d'enzymes et de protéines de transport interagissant de façon cohérente les uns avec les autres. Caractérisé dans notre laboratoire, le récepteur LSR (*lipolysis stimulated lipoprotein receptor*) est impliqué de façon essentielle dans la régulation de la distribution des lipides entre les différents tissus. Il participe activement en effet à la clairance des lipoprotéines circulantes riches en triglycérides (TG) durant la période postprandiale (Bihain & Yen, 1992; Yen *et al*, 2008; Stenger *et al*, 2012). La diminution de l'expression du récepteur LSR est associée à une prise de poids (Stenger *et al*, 2010) et a été observée dans des modèles de souris obèses montrant une adiposité accrue (Narvekar *et al*, 2009). De plus, le LSR est activé en présence d'acides gras libres et est sous le contrôle de la leptine, une adipokine agissant comme un facteur de satiété et produite par le tissu adipeux (Stenger *et al*, 2010). Au vu de l'importance du LSR dans l'homéostasie lipidique, l'objectif principal de ce travail a été d'identifier des facteurs qui peuvent réguler les niveaux d'expression et d'activité du récepteur. Notre attention s'est focalisée sur le DHA, ou acide docosahexaénoïque, un acide gras polyinsaturé (AGPI) *n*-3, et nous nous sommes intéressés en particulier à l'implication du facteur de transcription PPARα (*peroxisome proliferator-activated receptor alpha*) dans des modèles cellulaires et animaux ainsi qu'au moyen d'une étude bioinformatique.

Le manuscrit de cette thèse a été construit selon l'articulation présentée ci-après.

Introduction : Ce chapitre propose une synthèse bibliographique présentée en quatre sections.

- ✓ La première section décrit les mécanismes de transport des lipides circulants et le métabolisme des lipoprotéines, incluant notamment les données structurales et les clés de classification des lipoprotéines. Elle fait également le point sur les récepteurs des lipoprotéines, LDL-R, LRP1 and LSR, et sur les mécanismes transcriptionnels qui contrôlent leurs niveaux d'expression.
- ✓ L'objet de la deuxième section est le métabolisme des AGPI à longue chaîne, détaillant plus particulièrement la propriété des acides gras de type *n*-3, dont le DHA, d'améliorer les dyslipidémies et surtout l'hypertriglycéridémie.
- ✓ La troisième section traite des récepteurs PPAR, en abordant les aspects de distribution tissulaire, de spécificité de ligand et de mode d'activation transcriptionnelle, ainsi que de leurs fonctions biologiques. Les propriétés du sous-type PPARα ont été décrites de façon plus détaillée en raison de son implication comme régulateur principal du métabolisme lipidique au niveau hépatique.
- ✓ Enfin, la quatrième section évoque les conséquences des désordres du métabolisme lipidique en lien avec les questions majeures de santé publique que posent désormais la dyslipidémie, l'obésité, la résistance à l'insuline et le syndrome métabolique. Cette section inclut aussi les pistes et les stratégies thérapeutiques à l'étude.

Objectifs : Ce chapitre résume le contexte scientifique général dans lequel cette thèse a été développée et énonce les différents objectifs visés.

Matériels et Méthodes : Ce chapitre détaille les diverses approches expérimentales suivies et les techniques analytiques utilisées lors des études menées dans le cadre de cette thèse.

Résultats et Discussion : Ce chapitre se compose de quatre sections séparées correspondant chacune à une étude particulière. Chaque étude est décrite dans son contexte scientifique par ses objectifs particuliers, ses procédures expérimentales spécifiques, les résultats obtenus et leurs commentaires, ainsi que la discussion et la conclusion propres auxquelles ils ont mené.

- ✓ Le DHA possède la capacité bien connue d'améliorer le statut lipidique en contrôlant l'expression, la localisation et/ou l'activité de divers récepteurs et enzymes. Le LSR nécessitant une activation préalable par des acides gras libres pour exercer sa fonction essentielle dans le métabolisme des lipoprotéines, la première section est consacrée à la détermination des effets d'une supplémentation en DHA sur l'expression et l'activité du LSR dans des modèles cellulaires et animaux.
- ✓ Les propriétés hypotriglycéridémiantes du DHA reposent notamment sur des mécanismes transcriptionnels qui contrôlent le métabolisme lipidique. Nous avons voulu définir alors les facteurs de transcription qui peuvent réguler l'expression du gène *lsr* dont les séquences promotrices sont toujours inconnues. Pour ce faire, nous avons mené une analyse *in silico* des régions 5' régulatrices du gène *lsr* chez la souris, le rat et l'homme afin d'identifier les différents éléments présents. La seconde section décrit donc l'identification de possibles éléments promoteurs et sites de réponse à des facteurs de transcription comme les PPAR en particulier.
- ✓ La troisième section décrit l'étude menée à la suite de l'analyse *in silico* et cherchant à déterminer dans des modèles *in vitro* si le facteur PPARα est effectivement impliqué dans la régulation transcriptionnelle du LSR.
- ✓ La quatrième section rapporte une étude transcriptomique spécifique grâce à laquelle nous avons étudié l'impact de régimes riches en lipides sur l'expression hépatique de facteurs de transcription, enzymes et protéines de transport impliqués dans le métabolisme lipidique chez la souris. Des analyses comparatives nous ont permis d'évaluer l'influence du statut hétérozygote sur les dérégulations induites par les lipides alimentaires en excès.

Conclusions et perspectives : Ce dernier chapitre récapitule les principaux éléments des sections du chapitre Résultats et Discussion et propose une discussion globale conduisant aux conclusions et aux perspectives auxquelles le travail expérimental de cette thèse a mené.

1. Objectifs de la thèse

Il a été montré chez le rat que dans les membranes hépatiques, le nombre apparent de récepteurs LSR est inversement corrélé avec les niveaux de TG plasmatiques durant la période postprandiale (Mann *et al*, 1995). Cette observation a fourni la première preuve que le

LSR peut être le facteur limitant de la clairance des lipoprotéines circulantes riches en TG. Dans ce même ordre d'idées, l'inactivation d'un allèle LSR et en conséquence l'activité réduite du récepteur conduisent, chez la souris $LSR^{+/-}$ sous régime riche en lipides, à une clairance retardée et une triglycéridémie deux fois plus élevée durant la période postprandiale, ainsi qu'à un gain de poids supérieur à celui des souris contrôle $LSR^{+/+}$ membres de la même portée (Yen *et al*, 2008). Ces observations ont été confirmées par des études d'interférence par l'ARN qui ont montré que la suppression du LSR hépatique par des siRNA augmente la triglycéridémie postprandiale ainsi que les niveaux d'apoprotéine (Apo) B et E (Narvekar *et al*, 2009). De plus, une association a été montrée entre l'hyperlipidémie et l'expression réduite du LSR dans le foie de modèles de souris obèses. Pris dans leur globalité, ces résultats suggèrent donc que le récepteur LSR est important dans le maintien de l'homéostasie lipidique dans le système périphérique. De façon intéressante, LSR est incapable de fixer efficacement les VLDL isolées à partir d'un patient hyperlipidémique de type III avec un génotype *ApoE2/2* (Yen *et al*, 1994), ce qui explique probablement l'hypertriglycéridémie typique de cette classe particulière de patients. Il est donc essentiel de déterminer les différents facteurs qui participent à la régulation du LSR et de ses effets sur le statut lipidique. À la suite d'une étude à laquelle j'ai participé montrant l'implication de la leptine dans la régulation du LSR tant au niveau de la protéine que du messager (Stenger *et al*, 2010), j'ai décidé de poursuivre mes travaux sur la régulation du LSR en étudiant plus spécifiquement si les propriétés correctrices de la dyslipidémie démontrées par le DHA pouvaient impliquer le LSR et si la régulation transcriptionnelle de l'expression du récepteur était susceptible de concerner le facteur de transcription PPARα.

Les objectifs visés au cours de ma thèse ont été les suivants.

- ✓ Déterminer les effets d'une supplémentation en DHA sur les niveaux d'expression et d'activité de LSR et évaluer les conséquences fonctionnelles de cette modulation dans différents modèles *in vitro* et *in vivo*.
- ✓ Rechercher la présence de possibles éléments promoteurs et sites de fixation de divers facteurs de transcription comme les éléments PPRE de réponse aux PPAR, par une analyse *in silico* des régions 5' régulatrices du gène *lsr* chez la souris, le rat et l'homme.
- ✓ Définir le rôle potentiel du facteur PPARα dans la régulation du récepteur LSR par une approche pharmacologique menée sur un modèle cellulaire *in vitro*.

- ✓ Appréhender par une étude transcriptomique si et comment une activité LSR réduite peut affecter l'expression hépatique de facteurs de transcription comme PPARα, SREBP1, LXR et d'autres acteurs associés au métabolisme lipidique chez la souris sous régime standard ou enrichi en lipides.

2. Effets du DHA sur le récepteur LSR

Des preuves de plus en plus nombreuses indiquent que les AGPI *n*-3 à longue chaîne comme le DHA possèdent des propriétés hypolipémiantes susceptibles d'apporter un bénéfice préventif contre le développement de maladies associées à des désordres du métabolisme lipidique. Parmi les mécanismes les plus importants sur lesquels reposent ces capacités du DHA, il faut considérer la modulation de l'expression de divers récepteurs et enzymes ainsi que la modification des propriétés physicochimiques et structurales des membranes cellulaires (Sun *et al*, 2011; Zuliani *et al*, 2009; Zhang *et al*, 2010; Shaikh & Teague, 2012).

Les niveaux plasmatiques de lipides sont influencés par les quantités et les types d'acides gras ingérés. La clairance par le LSR des lipoprotéines riches en TG est un élément de l'homéostasie lipidique. Dans cette étude, diverses expérimentations ont été menées *in vivo* et *in vitro* afin de savoir si le DHA peut participer à la régulation du LSR. Une augmentation de l'activité et des niveaux protéiques de LSR a été démontrée dans les cellules de la lignée d'hépatome murin Hepa 1-6 cultivées en présence de DHA, mais sans aucun changement au niveau des ARNm. L'influence favorable du DHA sur le LSR reste donc à expliciter, mais il est raisonnable de penser qu'elle pourrait résulter de changements de composition de la membrane plasmique. Ceci pourrait alors contribuer à localiser le récepteur dans un environnement favorable susceptible d'en optimiser l'activité.

Par ailleurs, nous avons mené trois études *in vivo* pour investiguer les effets du DHA alimentaire sur l'expression du LSR chez la souris. Les régimes testés étaient supplémentés par les mêmes proportions de DHA, mais ces études se distinguaient par l'âge des souris, le temps de traitement et la forme chimique, ester éthylique ou TG, du DHA. Les souris âgées de quatre mois sous régime supplémenté en ester éthylique de DHA durant un mois ont montré des niveaux d'ARNm hépatiques de LSR inchangés. Il en a été de même chez les souris âgées de neuf mois et supplémentées ainsi durant trois mois, alors que les niveaux de protéine LSR étaient significativement supérieurs dans cette condition. Tenant compte de l'enrichissement en DHA mesuré dans le foie, le DHA pourrait jouer un rôle important en régulant le LSR au

niveau traductionnel et ultérieurement. Dans la dernière étude, la supplémentation alimentaire en DHA sous forme de TG dans l'huile de poisson pendant six mois chez des souris de neuf mois au départ n'a induit aucun changement concernant LSR, ni au niveau protéique, ni à celui des messagers. Il faut néanmoins souligner que les études de supplémentation aussi longues mènent souvent à des interprétations difficiles, notamment en raison de l'influence parallèle du facteur âge que la littérature a bel et bien identifié comme un facteur aggravant des conditions pathologiques. De plus, les comparaisons avec les résultats publiés sont particulièrement difficiles en raison du fait que les données rapportées sont souvent incomplètes. Des expérimentations complémentaires devront donc explorer et préciser les mécanismes impliqués par le DHA dans la régulation du LSR. Mais comme le DHA est aussi connu pour activer divers facteurs de transcription comme les PPAR, SREBP et LXR impliqués dans le métabolisme lipidique hépatique (Jump *et al,* 2008), il nous faut considérer la participation possible de ces facteurs dans les effets du DHA sur le LSR et l'homéostasie lipidique.

3. Analyse *in silico* des régions 5' régulatrices du gène *lsr*

Cette étude représentait l'étape initiale vers l'identification *in silico* de possibles régions promotrices et régulatrices dans le gène lsr de trois espèces, *i.e.* souris, rat et homme. Nous avons bien identifié des éléments promoteurs basiques, mais leur localisation par rapport au point de départ (site « +1 ») nous laisse sceptiques tant elle se distingue de ce qui est communément rapporté dans la littérature. Différentes explications possibles peuvent être avancées. Premièrement, le site +1 effectif pourrait être distinct et en amont du site considéré jusqu'à présent, à moins qu'il n'en existe d'autres en plus. Deuxièmement, le gène *lsr* étant capable de coder trois sous-unités différentes grâce à un épissage alternatif du transcrit primaire (Yen *et al,* 1999), le site +1 considéré à ce jour et commun aux trois ARNm de LSR pourrait avoir été déduit par erreur de l'extrémité 5' unique générée par un mécanisme de maturation par clivage survenant avant l'épissage. Troisièmement, des éléments promoteurs basiques existants auraient pu ne pas être identifiés en raison de séquences nucléotidiques éloignées des séquences consensus, voire même encore inconnues.

L'analyse des résultats obtenus rappelle les limites d'une telle étude. En dépit de la diversité des outils et approches disponibles pour la prédiction et l'analyse de séquences, leur précision reste souvent imparfaite. Dans le cas du LSR, la localisation des éléments promoteurs basiques est d'autant plus compliquée qu'aucune donnée expérimentale n'a

confirmé la position du +1. Ceci nécessite la conduite d'une étude expérimentale dédiée à ce point et permettant d'identifier la localisation du site +1 et des éléments promoteurs potentiels dans le gène *lsr*.

Plusieurs programmes sont disponibles en ligne pour identifier les sites de fixation putatifs des facteurs de transcription, mais à nouveau, la possibilité d'identifier de fausses pistes ne peut être éliminée. Il va de soi que ces outils de bioinformatique ne peuvent identifier la fonctionnalité réelle de chacun de ces sites proposés sur la base d'analyses de séquences. Des approches expérimentales basées notamment sur la mutagenèse dirigée, le retard sur gel et la construction de vecteurs hybrides avec des gènes rapporteurs devront donc être envisagées pour localiser et confirmer les sites suggérés par l'analyse *in silico* qui disposent effectivement de la capacité fonctionnelle qui a conduit à leur identification.

4. Implication du facteur PPARα dans la régulation du récepteur LSR

Des études préalables nous avaient permis de démontrer le rôle de la leptine comme régulateur positif dans la régulation transcriptionnelle du LSR chez la souris comme dans les cellules de la lignée Hepa 1-6 (Stenger *et al,* 2010). Aucune donnée sur ce niveau de régulation du LSR n'étant disponible à ce jour, nous avons voulu en étudier l'un des aspects en focalisant notre attention sur l'implication éventuelle du facteur PPARα. Ce choix se justifiait par le rôle déterminant bien connu de ce facteur dans la régulation d'acteurs hépatiques du métabolisme lipidique et par son expression élevée dans le foie, tout comme le LSR. Il se justifiait aussi par la présence, suggérée lors de notre analyse *in silico*, de sites PPRE potentiels dans les régions 5' régulatrices du gène *lsr* humain et de ses homologues de souris et de rat.

L'étude que nous avons menée nous a permis d'identifier le facteur PPARα comme l'un des régulateurs de l'expression du LSR expression dans les cellules Hepa 1-6. En effet, l'approche pharmacologique développée nous a conduits à observer une augmentation significative des niveaux de messager et de protéine LSR dans ces cellules d'hépatome de souris traitées par un ligand agoniste sélectif du facteur PPARα, la molécule Wy-14,643. En parallèle, une activation transcriptionnelle comparable a aussi été constatée pour l'expression du gène de la protéine peroxysomale ACOX1, l'acyl-coenzyme A oxydase 1, la première enzyme de la voie de β-oxydation des acides gras. Le gène *ACOX1* étant bien connu pour être

régulé par le facteur PPARα, il est logique de considérer que le gène *lsr* est lui aussi l'un des gènes cibles dont la transcription est régulée par le facteur PPARα. Nous avons pu conforter cette hypothèse par l'observation d'une diminution coordonnée de l'expression du LSR et d'ACOX1 en réponse à un traitement par un antagoniste spécifique du facteur PPARα, la molécule GW6471. De plus, nous avons constaté qu'un traitement simultané par la molécule GW6471 et le bézafibrate, un activateur de l'ensemble des facteurs PPAR, a conduit à une augmentation significative de l'ARNm du LSR, ce qui suggère que l'expression du gène *lsr* puisse être régulée aussi par d'autres sous-types de PPAR. Ces résultats doivent nous inciter à poursuivre ces travaux afin de déterminer la signification physiologique et les conséquences fonctionnelles de cette régulation du LSR par les facteurs PPAR, notamment à l'aide de souris KO ou de siRNA permettant d'éteindre sélectivement l'expression du facteur PPARα.

5. Effets de l'hétérozygotie liée au LSR sur le métabolisme lipidique hépatique

Après avoir démontré l'implication essentielle du LSR dans l'homéostasie lipidique et conclu à sa régulation par au moins un facteur PPAR, nous avons voulu évaluer la place qu'occupe le LSR dans le système très complexe chargé de l'homéostasie lipidique. Une étude transcriptomique a donc été entreprise à l'aide de la technique de *qPCR array* regroupant sur une même plaque 84 gènes codant des protéines – récepteurs, enzymes ou transporteurs – impliquées dans le métabolisme lipidique. En comparant le transcriptome du foie de souris $LSR^{+/-}$ à celui des souris contrôles, nous avons constaté que l'inactivation d'un allèle *lsr* induit des perturbations qui se répercutent sur de nombreux gènes dont l'expression est significativement diminuée en conséquence, en particulier ceux impliqués dans le métabolisme du cholestérol et des acides gras. Ceci souligne bien l'importance du LSR non seulement dans la régulation du métabolisme lipidique hépatique, mais suggère aussi que le LSR peut être une cible thérapeutique potentielle pour le traitement de maladies métaboliques comme celles liées à l'altération des voies de signalisation de l'insuline et des adipokines ou du métabolisme glucidique. En effet, plusieurs gènes associées à ces voies sont apparus significativement sous-exprimés dans les souris $LSR^{+/-}$, ce qui suggère une influence large du LSR, y compris d'ailleurs en augmentant l'expression de gènes associés à l'inflammation, en particulier dans les souris $LSR^{+/-}$ sous régime hyperlipidique. La plaque de *qPCR array* ciblant spécifiquement les gènes et les voies associées à la stéatose hépatique non alcoolique

(NAFLD) et à la résistance à l'insuline, les profils d'expression génique des souris sous un tel régime sont apparus proches de ceux des souris LSR hétérozygotes. Ceci nous a conduits à considérer que ces dernières présentaient un profil de type « hyperlipidique », suggérant que le LSR peut représenter une cible d'intérêt pour le traitement de cette stéatose. Des analyses complémentaires sont en cours, avec notamment des mesures de triglycéridémie, afin de compléter le phénotype des différents groupes de souris constitués pour ce travail.

Cette étude a surtout permis d'élargie le spectre et le potentiel des applications thérapeutiques du LSR au-delà du seul métabolisme lipidique en révélant pour la première les effets dépendants de son activité sur la régulation d'autres voies métaboliques dans le foie.

6. Conclusions et perspectives

Le maintien de l'homéostasie lipidique est majoritairement assuré par le foie où sont exprimés les nombreux enzymes et récepteurs impliqués de façon coordonnée dans les diverses voies métaboliques concernées. Le récepteur LSR est l'un de ces acteurs cruciaux, impliqué dans la clairance des lipoprotéines à ApoB et/ou ApoE riches en triglycérides durant la période postprandiale (Bihain & Yen, 1992; Mann *et al*, 1995; Yen *et al*, 2004). L'absence de ce récepteur chez la souris $LSR^{-/-}$ est apparue létale dès les stades embryonnaires. Cependant, le statut hétérozygote $LSR^{+/-}$ lui-même conduit à une dyslipidémie associée à une prise de poids excessive chez la souris âgée ou sous régime hyperlipidique (Yen *et al*, 2008). De même, l'expression hépatique du LSR est considérablement réduite dans des souris modèles d'obésité et de diabète de type 2. Enfin, l'extinction du LSR hépatique à l'aide de shRNA entraîne une augmentation des niveaux postprandiaux de TG, ainsi que d'ApoB et ApoE (Narvekar *et al*, 2009). Après avoir identifié la leptine comme un régulateur important de l'expression hépatique du LSR (Stenger *et al*, 2010), nous avons mené les travaux de cette thèse afin de poursuivre la recherche des mécanismes impliqués dans la régulation de l'expression du LSR dans le foie.

Le DHA est un AGPI essentiel des phospholipides membranaires. Ses propriétés hypo-triglycéridémiantes sont bien documentées, reposant notamment sur des mécanismes affectant l'expression de plusieurs enzymes et récepteurs impliqués dans le métabolisme lipidique (Zuliani *et al*, 2009; Jacobson *et al*, 2012; Pirillo & Catapano, 2013). Le DHA est donc considéré comme un nutriment important pour la prévention de diverses pathologies associées au métabolisme lipidique telles que les dyslipidémies, l'obésité et le diabète, ainsi que les

maladies cardiovasculaires et neurodégénératives. Puisque le LSR participe activement au métabolisme lipidique hépatique après activation par les acides gras libérés par la lipolyse, il est intéressant de savoir si le DHA peut influer sur l'expression et/ou l'activité du LSR. Le traitement de cellules Hepa 1-6 par le DHA a conduit à l'augmentation des niveaux de protéine LSR, mais sans modifier les taux d'ARNm. En toute cohérence, une augmentation parallèle de l'activité LSR a aussi été mesurée dans ces mêmes cellules traitées par le DHA. Le DHA libre pourrait donc participer à la fonction du récepteur LSR, notamment en l'activant. Mais ceci pourrait aussi résulter de l'enrichissement membranaire en DHA, susceptible de réorganiser la bicouche de façon à promouvoir un microenvironnement optimal et propice à l'activité du récepteur. La supplémentation nutritionnelle en DHA chez la souris indique que l'acide gras alimentaire offre une bonne biodisponibilité qui se reflète par l'enrichissement des membranes, en particulier dans les érythrocytes ainsi qu'aux niveaux hépatique et neuronal. Dans les souris ayant bénéficié d'une supplémentation en DHA sous forme d'ester éthylique durant un ou trois mois, l'enrichissement en l'AGPI est associé à une augmentation des niveaux de protéines LSR, mais sans changement des niveaux d'ARNm, tout comme dans les cellules Hepa 1-6. Ceci semble donc écarter la possibilité que le DHA régule la transcription du gène *lsr*, suggérant au contraire qu'il joue un rôle important au niveau traductionnel et/ou post-traductionnel. Cet aspect semble en effet particulièrement important pour le LSR que des analyses biochimiques ont présenté sous la forme d'un complexe multimérique comprenant une sous-unité α ou α' permettant l'ancrage par un domaine transmembranaire, associée par des ponts disulfures à deux ou trois sous-unités β extracellulaires. La fixation d'acides gras libres a été proposée comme capable de modifier la conformation du LSR et d'exposer en conséquence le site reconnaissant l'ApoE d'une lipoprotéine riche en TG, permettant sa capture, son internalisation et sa dégradation.

Des études complémentaires devraient pouvoir expliciter les mécanismes ayant conduit à l'augmentation de l'activité LSR constatée en réponse au DHA. La mesure parallèle des taux plasmatiques de TG et de cholestérol apportera sans doute des indices permettant d'élucider la signification physiologique de l'effet régulateur exercé par le DHA. L'analyse des différents domaines membranaires devrait aussi évaluer l'impact de l'enrichissement en DHA sur la localisation fine du récepteur LSR et sa capacité à constituer un complexe pleinement fonctionnel. Enfin, il faudra évaluer l'importance relative des effets régulateurs qu'exerce le DHA au niveau transcriptionnel sur le métabolisme lipidique, en particulier par

l'intermédiaire des facteurs PPAR et SREBP (Göttlicher *et al*, 1992; Pégorier *et al*, 2004; Jump, 2013).

Ce dernier point a été pris en considération pour justifier l'étude détaillée que nous avons entreprise sur le rôle du facteur PPARα, visant notamment à savoir si son expression est modifiée par le DHA. De façon intéressante, nous n'avons observé aucun changement des niveaux de messagers du PPARα, que ce soit dans des modèles *in vivo* et *in vitro*, ce qui est cohérent avec le fait que nous n'avons jamais pu constater d'effet transcriptionnel du DHA sur le gène *lsr*, quel que soit le modèle cellulaire ou animal considéré. Ceci suggère que le facteur PPARα ne contribue pas aux effets du DHA sur le LSR. Des contradictions persistent néanmoins sur cette possible contribution, puisque certains auteurs ont démontré une augmentation de l'expression du facteur PPARα en réponse à une supplémentation en DHA (Keller *et al*, 1993; Zúñiga *et al*, 2011), tandis que d'autres ont rapporté que les effets induits par l'huile de poisson sont indépendants de ce facteur (Dallongeville *et al*, 2001). D'autres études ont aussi montré que les acides gras sont capables d'activer le facteur PPARα de façon bien moins efficace que ne le font d'autres activateurs comme les fibrates (Forman *et al*, 1997; Krey *et al*, 1997). De prochaines études devraient tenter de clarifier cette situation controversée et définir si l'effet du DHA sur le LSR n'inclue réellement aucun élément régulateur transcriptionnel et en tout cas s'il est réellement indépendant du facteur PPARα. Des expériences *in vitro* menées à l'aide d'antagonistes ou de siRNA spécifiques de ce dernier pourraient ainsi permettre de réprimer son activité et de vérifier la persistance des effets du DHA sur le LSR.

Le facteur PPARα appartient à la catégorie des facteurs de transcription activés par un ligand et s'implique alors de façon déterminante dans le contrôle de l'expression d'un grand nombre de gènes notamment ceux liés au métabolisme lipidique hépatique (Gervois *et al*, 2000; Duval *et al*, 2007). Parmi les ligands possibles de ce facteur figurent les fibrates, une classe importante d'agents thérapeutiques agents utilisés pour le traitement des dyslipidémies. Ceci nous a conduits à étudier plus spécifiquement le rôle du facteur PPARα dans la régulation de l'expression hépatique du LSR. L'analyse *in silico* des séquences des régions 5' régulatrices du gène *lsr* de souris, de rat et d'homme nous a suggéré la présence potentielle d'éléments du promoteur basique et de sites de fixation spécifiques de facteurs de transcription. De possibles éléments PPRE de réponse aux PPAR ont été identifiés au nombre de 2 chez la souris, 3 chez le rat et 6 chez l'homme. Le facteur PPARα étant hautement

exprimé dans le foie où il contrôle plusieurs gènes associés au métabolisme lipidique, il nous fallait alors savoir si le gène *lsr* pouvait être une cible de ce facteur. La modulation de l'activité de ce facteur dans des cellules Hepa 1-6 traitées par des agonistes et des antagonistes et les conséquences sur l'expression du LSR comme sur celle du témoin positif ACOX1 nous permet clairement de conclure que l'expression du LSR dans le foie est sous le contrôle du facteur PPARα, mais aussi d'autres PPAR. Ces observations ouvrent un certain nombre de perspectives.

- La présence effective de PPRE doit être confirmée afin de corroborer l'implication du facteur PPARα dans le contrôle transcriptionnel du LSR. Il faudra en déterminer le nombre, la position et la fonctionnalité dans les régions 5' régulatrices du gène *lsr* de souris.
- La signification physiologique et les conséquences fonctionnelles de cette régulation doivent être définies dans des modèles permettant d'éteindre l'expression du facteur PPARα.
- L'implication parallèle d'autres PPAR comme PPARγ et PPARβ/δ dans la régulation du LSR devra être établie au moyen de ligands ou de siRNA spécifiques et leur signification fonctionnelle dans le contexte de l'homéostasie lipidique devra être élucidée.

Un autre résultat intéressant nous semble devoir être mentionné. Lors de l'optimisation des conditions de culture, nous avons observé une régulation du PPARα et du LSR en fonction du milieu tout à fait différente de celle décrite ci-dessus. La protéine PPARα n'était détectable que dans les cellules Hepa 1-6 cultivées dans le milieu complet ou celui sans lipoprotéines, mais pas dans le milieu sans sérum. À l'inverse, les niveaux de protéine et d'ARNm de LSR étaient significativement augmentés dans les cellules cultivées dans les milieux sans lipoprotéines et sans sérum par rapport à celles cultivées dans le milieu complet. Ces observations tendent vers une toute autre conclusion que celle avancée précédemment, suggérant cette fois que l'expression du gène *lsr* puisse être indépendante de celle du facteur PPARα. Nous avons également observé que l'expression du LSR est moindre dans les cellules cultivées en présence de LDL dans le milieu. Le LSR semble donc sensible aux variations de niveaux cellulaires de cholestérol, ce qui nous conduit à supposer que l'expression du LSR puisse être aussi régulée par des facteurs de transcription comme les

SREBP et LXR répondant au cholestérol. De nouvelles études devront explorer ces voies de régulation et évaluer le rôle joué par les lipoprotéines du sérum et le cholestérol cellulaire.

L'analyse transcriptomique menée par qPCR *array* a permis d'identifier de nombreux gènes hépatiques dont PPARα qui sont sous-exprimés en conséquence de la suppression d'un allèle *lsr* chez la souris $LSR^{+/-}$. Les gènes affectés participent à plusieurs voies métaboliques interconnectées, dont les métabolismes hépatique et glucidique, la signalisation par l'insuline ou les adipokines, et l'inflammation. Or, tous ces gènes sont des cibles du facteur PPARα. Il faut souligner par ailleurs que la baisse de l'activité LSR liée au statut hétérozygote semble aussi promouvoir une inflammation comme en témoigne l'induction transcriptionnelle des gènes concernés, en particulier chez la souris $LSR^{+/-}$ sous régime hyperlipidique. Ceci suggère que le LSR joue un rôle important dans le maintien de divers processus métaboliques dans le foie de souris. Par extension, toute altération du niveau d'activité LSR semble pouvoir altérer les principales voies métaboliques de façon analogue à ce que peut induire un régime hyperlipidique, conduisant à accroître les risques pathologiques en conséquence. Cette hypothèse ouvre la porte pour de futures recherches investiguant l'implication du LSR dans la régulation parallèle de voies distinctes du métabolisme lipidique hépatique comme la signalisation de l'insuline, la β-oxydation, l'inflammation et l'apoptose. Dans ce contexte, de nouvelles expériences *in vivo* devront évaluer en détail l'étendue des dégâts occasionnés par des régimes hyperlipidiques chez des souris traitées par des antagonistes du facteur PPARα. Ceci pourrait contribuer à définir si la diminution de l'activité LSR consécutive à l'administration d'un régime riche en lipides peut être restaurée ou compensée par un traitement visant à activer le facteur PPARα et à améliorer en conséquence le statut lipidique hépatique.

Dans leur ensemble, les résultats de cette thèse constituent des éléments importants pour comprendre l'implication du LSR dans le maintien de l'homéostasie lipidique hépatique. Ils ont permis de montrer que des critères nutritionnels comme la supplémentation en DHA ou l'excès de lipides participent à la régulation du LSR hépatique, apportant aussi les premiers indices sur la nature des facteurs de transcription régulant le métabolisme lipidique hépatique.

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Annex

Up-regulation of hepatic lipolysis stimulated lipoprotein receptor by leptin: a potential lever for controlling lipid clearance during the postprandial phase

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ABSTRACT **As a hepatic receptor for triglyceride-rich lipoproteins, the lipolysis-stimulated lipoprotein receptor (LSR) may be involved in the dynamics of lipid distribution between the liver and peripheral tissues. Here, we explore the potential role of leptin in regulating LSR. At physiological concentrations (1–10 ng/ml), leptin increased LSR protein and mRNA levels in Hepa1–6 cells through an ERK1/2-dependent and α-amanitin-sensitive pathway. *In vivo*, leptin treatment of C57BL6/Rj mice (1 μg 2×/d, 8 d) led to a significant increase in hepatic LSR mRNA and protein, decreased liver triglycerides and increased VLDL secretion as compared to controls. $LSR^{+/-}$ mice with elevated postprandial lipemia placed on a high-fat (60% kcal) diet exhibited accelerated weight gain and increased fat mass as compared to controls. While plasma leptin levels were increased 3-fold, hepatic leptin receptor protein levels and phosphorylation of ERK1/2 were significantly reduced. Therefore, leptin is an important regulator of LSR protein levels providing the means for the control of hepatic uptake of lipids during the postprandial phase. However, this may no longer be functional in $LSR^{+/-}$ mice placed under a chronic dietary fat load, suggesting that this animal model could be useful for the study of molecular mechanisms involved in peripheral leptin resistance.—Stenger, C., Hanse, M., Pratte, D., Mbala, M.-L., Akbar, S., Koziel, V., Escanyé, M.-C., Kriem, B., Malaplate-Armand, C., Olivier, J.-L., Oster, T., Pillot, T., Yen, F. T. Up-regulation of hepatic lipolysis stimulated lipoprotein receptor by leptin: a potential lever for controlling lipid clearance during the postprandial phase. *FASEB J.* 24, 4218–4228 (2010). www.fasebj.org**

Key Words: leptin receptor · liver · adipose tissue · peripheral leptin resistance

Postprandial lipemia represents the transitory increase in plasma lipids, primarily triglycerides (TGs), due to the processing of dietary fats following ingestion of a meal. The rapid removal of these exogenously derived lipids is critical in order to avoid prolonged accumulation in the circulation. Indeed, elevated postprandial lipemia is a risk factor that is associated with a number of disorders. It has been reported to be related to risk of atherosclerosis (1–3) and is often associated with metabolic diseases such as obesity (4–7). The regulation of postprandial lipemia has been the subject of numerous studies. A number of factors influence the degree of lipemia during the postprandial phase, among which includes the hydrolysis of TG by the lipolytic system in the periphery, as well as in the space of Disse (for review, see refs. 8, 9). Indeed, reduced sulfation of heparan proteoglycans in the liver leads to a significant increase in postprandial lipemia (10). The final destination of the dietary lipids in the form of chylomicron residual particles nevertheless remains the hepatocyte *via* receptor-mediated endocytosis (11).

We have recently demonstrated that the hepatic lipolysis stimulated lipoprotein receptor (LSR) was actively implicated in the regulation of postprandial lipemia (12). Indeed, in mice with reduced expression of LSR ($LSR^{+/-}$), lipoprotein clearance was impaired, leading to increased TG and cholesterol levels due to the accumulation of atherogenic apoB-containing particles in the plasma (12). As a hepatic receptor for the removal of lipoproteins during the postprandial phase, LSR could be involved in determining the availability of lipids for the peripheral tissues. Indeed, we observed that when mice with reduced LSR expression ($LSR^{+/-}$) were placed on a Western-type cholesterol-containing diet, the increase in body weight was directly correlated with the increase in plasma cholesterol and TG, unlike

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their control littermates (12), suggesting that the delayed clearance of apoB-containing lipoproteins due to decreased LSR could lead to increased influx into the circulation and therefore nonhepatic peripheral tissues.

Among the peripheral tissues, the adipose tissue is the site for energy storage due to its unlimited capacity to accumulate TG. It has been recognized to play an active role as an endocrine organ, sending signals to other tissues through the secretion of adipokines into the circulation. Leptin is the adipokine that provides the link between food intake and energy storage in the adipose tissue, through its action as satiety factor at the level of the arcuate nucleus of the hypothalamus (13, 14), the feeding control center in the brain. Binding of leptin to the long form of its receptor (Ob-Rb) leads to activation of the JAK/STAT pathway (15) and extracellular signal-regulated kinase (ERK) (16), which, in turn results in enhanced transcription of genes coding for anorectic hormones (17, 18). The absence of leptin or inhibition of leptin signaling is sufficient to lead to increased food intake, and as a consequence, increased fat mass, which is associated with glucose intolerance, as demonstrated in *ob/ob* and *db/db* mouse models, which lack either the protein or the signaling-competent form of its receptor (13). Leptin has now been recognized to exert peripheral effects, including the regulation of insulin production in pancreatic β-cells (19, 20).

During our studies of the $LSR^{+/-}$ mice, we observed significant changes in tissue lipid distribution in aging $LSR^{+/-}$ mice, most notably in the adipose tissue, which led us to investigate the potential role of leptin as regulator of this lipoprotein receptor in the liver.

MATERIALS AND METHODS

Materials and antibodies

All chemicals and solvents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) unless otherwise indicated. Cell culture media and supplements were obtained from Invitrogen (Cergy Pontoise, France). Anti-LSR and anti-β-tubulin antibodies were purchased from Sigma-Aldrich and anti-Ob-R from Abcam (Paris, France) (for cell lysates) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) (for liver membranes). ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), AkT, phosphorylated AkT (p-AkT), ATP citrate lyase (ACL) antibodies, and PD98059 were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-fatty acid synthase (FAS) antibodies were purchased from BD Biosciences (Le Pont de Claix, France). Mouse recombinant leptin was purchased from Merck Chemicals (Fontenay-sous-Bois, France). Secondary anti-mouse and anti-rabbit HRP-conjugated antibodies were acquired from Santa Cruz Biotechnology and Cell Signaling Technology, respectively.

Animal studies

The production and genotyping of LSR heterozygote mice on a C57BL6/Rj background have been described earlier (12). All animals were provided a normal rodent chow diet and water *ad libitum* in a room maintained on a 12-h light-dark cycle with a mean temperature of 21–22°C and relative humidity of 50 ± 20%. Animals were handled in accordance with French State Council guidelines for the use and care of laboratory animals.

For the leptin treatment study, 10-wk-old C57BL6/Rj male mice (Janvier Breeding, Le Genest St. Isle, France) were injected i.p. with PBS or leptin (1 μg/injection) in PBS 2×/d at 9:00 AM and 6:00 PM for 7 d, during which the body mass and food intake were monitored. To measure food intake, mice were housed individually in cages. The wire lid containing the same amount of food pellets was measured before at 9:00 AM, and then 24 h later to determine the food intake. Care was taken to take into account any pellets that may have fallen into the cage. On d 8, the last injection was performed at 9:00 AM, food was removed from the cages, and the animals were sacrificed at noon. This experiment was performed in 2 separate experiments using 2 different leptin lots and on a total of 7 mice injected with PBS and 8 mice injected with leptin. Similar results in food intake and weight change were observed. Livers were perfused with PBS, followed by preparation of homogenates and total liver membranes, as described previously (12). Membrane-free cytosolic fractions from the homogenates were used for detection of ERK1/2 and its phosphorylated form. Liver samples (30–100 mg) were also snap frozen in liquid nitrogen for determination of TG content after lipid extraction or for total RNA extraction.

In a group of animals from the leptin treatment study, VLDL secretion was measured using the Tyloxapol (Triton WR1339) method to block degradation of lipoproteins. After the last injection on d 8, the mice were deprived of food for 3 h, and then injected i.v. (tail vein) with 15% Tyloxapol (Sigma-Aldrich) in physiological saline (500 mg/kg body weight). Blood samples were taken before (0), 30, 60, and 120 min after injection, by retro-orbital bleeding after isoflurane anesthesia. Plasma (EDTA) was immediately recovered by centrifugation and stored at −20°C for later analysis of plasma TG content. Because of the Tyloxapol injection, the livers of these animals were not used in any of the biochemical or expression analyses.

In the high-fat-diet study, 10-wk-old $LSR^{+/-}$ and $LSR^{+/+}$ were placed on a high-fat diet (60% kcal primarily from lard; SDS Dietex, Saint Gratien, France) provided *ad libitum*. Mice were weighed on a regular basis, and food intake was measured weekly. For analyses of leptin, adiponectin, and insulin, blood was obtained from unfed (3 h) animals. Plasma was stored at −20°C for later analysis. Body fat mass was measured using the EM-Scan (21). Near the end of the study (wk 9), oxygen consumption (Vo_2) was measured in the animals using indirect calorimetry (Panlab Oxylet; Bioseb, Paris, France). After a 24-h adaptation period, measurements of Vo_2 were taken every 45 min for 24 h, starting from 9:00 AM. The animals were not anesthetized, nor were blood samples taken the week before the calorimetric measurements. Average energy expenditure over the time period was calculated using the conversion factors 4.8 kcal/L O_2 and 4.19 kJ/kcal.

Postprandial lipemia after gavage with olive oil and clearance of Intralipid was measured in 14-mo-old female $LSR^{+/+}$ and $LSR^{+/-}$ mice as described previously (12).

Cell culture studies

The mouse Hepa1–6 liver cell line was obtained from LGC Promochem (Molsheim, France). Cells were maintained in high-glucose DMEM containing 10% heat-inactivated FBS, 100 mM glutamine, 100 mM penicillin and streptomycin, as described previously (12), and used only at low passages (<10). Cells were seeded either in 6-well plates (3×10^5 cells/well) or in 24-well plates on cover slides, and then grown for 48 h to achieve 80% confluence before use in experi-

ments. On the day of the experiment, cells were washed in PBS, and then incubated at 37°C for 1 h with fresh growth medium containing the indicated concentrations of leptin. In experiments using the inhibitors α-amanitin or PD98059, cells were preincubated for 3 h with these agents. Cells were then incubated 1 h at 37°C with fresh medium containing leptin and inhibitor. Cells were then placed on ice and washed 2 times with ice-cold PBS. For Western blot analysis, cell lysates were prepared using ice-cold RIPA buffer, followed by centrifugation at 13,000 *g* for 30 min at 4°C. Protein was measured in the recovered supernatants using the Lowry assay. For immunofluorescence studies, cells were fixed 15 min at room temperature in 4% paraformaldehyde in PBS. For real-time PCR analysis, cell pellets were prepared, washed with PBS, and snap-frozen for total RNA extraction.

Western blot analysis

Identical amounts of protein (20–30 μg) were applied to Novex 4–12% (Invitrogen) gradient or 10% SDS-PAGE gels. After electrophoresis, separated proteins were transferred onto nitrocellulose membranes for Western blot analysis, as described previously (12). Loading was systematically verified using Red Ponceau staining. Bands were revealed by chemiluminescence (GE Healthcare, Orsay, France) using a peroxidase-conjugated secondary antibody and a chemiluminescence kit (GE Healthcare). Densitometric analysis of the autoradiographs was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/download.html).

Immunofluorescence studies

Fixed cells were permeabilized using 0.1% TritonX-100 in PBS containing 0.1% (w/v) BSA. After washing in PBS, cells were incubated with anti-LSR (1:100 dilution) for 1 h at room temperature. For detection, cells were then incubated with anti-rabbit IgG conjugated with AlexaFluor 488 (Invitrogen). Cells were washed and mounted on slides using Fluormount (Sigma-Aldrich). Cells were visualized using a Nikon microscope (Nikon, Tokyo, Japan), and digitized images were taken using identical exposure times of ≥3 different areas. Analysis was performed on individual cells using ImageJ software, as described previously (12).

Real-time PCR analysis of LSR mRNA levels

Frozen liver samples (30 mg) or cell pellets (1–2×10^6 cells) were homogenized in QIAzol Lysis reagent (Qiagen, Courtaboeuf, France), according to manufacturer's instructions. Total RNA was extracted using RNeasy lipid tissue minikit (Qiagen); the integrity of the RNA was verified by the presence of 28S and 18S bands on agarose gels. Ten micrograms of total RNA was used for RT from which 500 ng was used for real-time PCR, as described previously (12). Reactions were prepared using the Applied Biosystems (Foster City, CA, USA) SYBR Green PCR Master Mix and then performed on the StepOnePlus real-time PCR system (Applied Biosystems). Hypoxanthine guanine phosphoribosyltransferase (HPRT) was used as the reference housekeeping gene. Relative expression calculations and statistical analyses were performed using the Relative Expression Software Tool (REST) 2009.

Biochemical analyses

Plasma TG was measured using an enzymatic kit (Biomerieux, Craponne, France), according to manufacturer's instructions. Leptin, adiponectin, and insulin were measured by ELISA (R&D Systems, Lille, France).

Statistical analysis

All results are shown as the means ± SE, unless otherwise indicated. Statistical analysis was performed using Student's *t* test; significance was considered as $P < 0.05$.

RESULTS

Our first goal in this study was to determine the effect of leptin on hepatic LSR *in vitro* using the mouse hepatoma cell line Hepa1–6, which has previously been demonstrated to exhibit LSR activity and expression (12). Hepa1–6 cells were incubated for 1 h with increasing concentrations of leptin typically found in the physiological range (1–10 ng/ml). Western blots revealed a significant increase in LSR protein induced by leptin concentrations, even at levels as low as 1 ng/ml (**Fig. 1*A***, top panel). Leptin signaling through interaction with its receptor, Ob-Rb is known to be mediated through several pathways, one of which is the phosphorylation of mitogen-activated protein kinase (MAPK) family member ERK (16–18, 22, 23). On the same nitrocellulose membrane, an increase in p-ERK1/2 was detected (Fig. 1*A*, middle panel); there was no detectable change in ERK1/2 (Fig. 1*A*, bottom panel). The ratio between the LSR and ERK1/2 signal was increased by 38–44% in cells treated with leptin as compared to that measured for cells incubated in absence of leptin. We verified that these cells express Ob-R, whose levels did not significantly change in cells incubated with increasing concentrations of leptin (data not shown). Leptin interaction with Ob-R is also known to activate phosphorylation of AkT. However, we were unable to detect p-AkT at these concentrations of leptin (data not shown). Studies using the mitogen-activated protein kinase (MEK) inhibitor PD98059 showed that when ERK1/2 phosphorylation was inhibited before treating cells with leptin (Fig. 1*B*, middle panel), the increase in LSR protein expression in response to leptin was no longer observed (Fig. 1*B*, top panel). These results, therefore, indicate that the observed increase of LSR protein is most likely mediated by leptin through its canonical downstream signaling form, p-ERK1/2.

Immunofluorescence studies were next performed in which staining with anti-LSR followed by detection with a fluorescent secondary antibody revealed both intracellular and surface staining of this receptor (Fig. 1*C*, top left panel). Image analysis revealed that the fluorescence signal of this staining was significantly increased in cells incubated with 10 ng/ml leptin as compared to control cells (Fig. 1*C*, top right panel and bar graph). These results were, therefore, in agreement with those of the immunoblots in Fig. 1*A*. To determine whether *de novo* transcription of LSR was induced by leptin, as suggested by the phosphorylation of ERK1/2, Hepa1–6 cells were preincubated 3 h at 37°C with

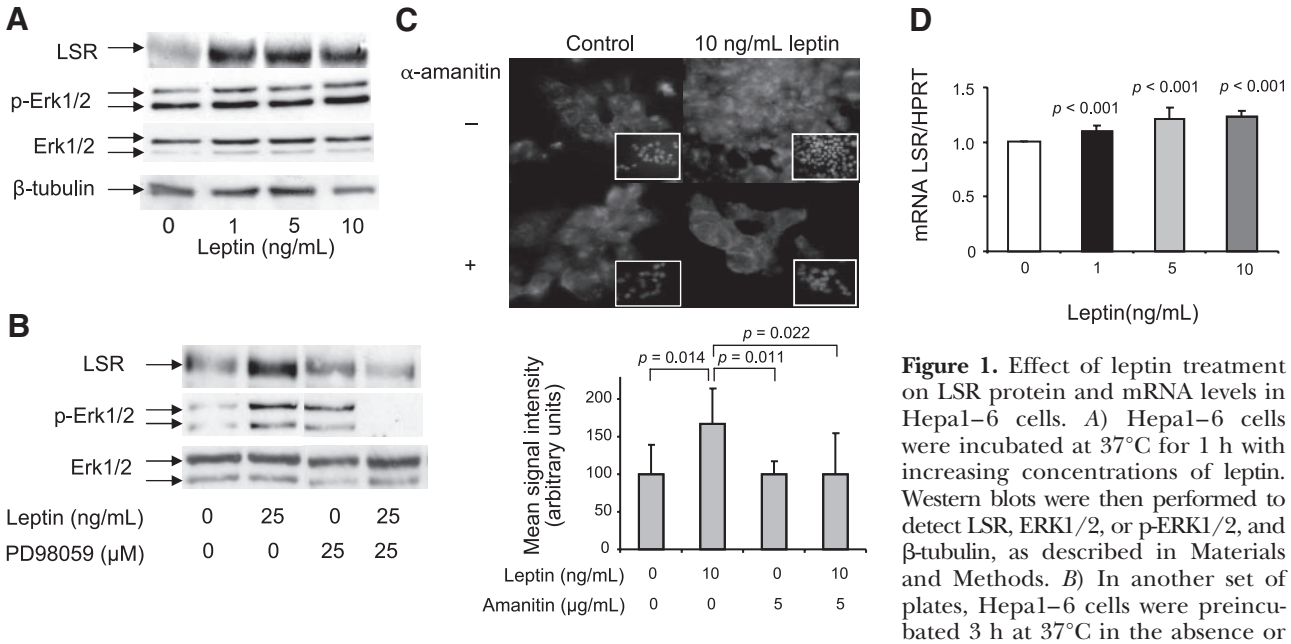

Figure 1. Effect of leptin treatment on LSR protein and mRNA levels in Hepa1–6 cells. *A*) Hepa1–6 cells were incubated at 37°C for 1 h with increasing concentrations of leptin. Western blots were then performed to detect LSR, ERK1/2, or p-ERK1/2, and β-tubulin, as described in Materials and Methods. *B*) In another set of plates, Hepa1–6 cells were preincubated 3 h at 37°C in the absence or presence of 25 μM PD98059 before addition of 25 ng/ml leptin. Cells were further incubated at 37°C for 1 h in fresh medium containing the inhibitor. Western blots were performed to detect LSR, ERK1/2, and p-ERK1/2 as indicated; the same nitrocellulose membrane was used to detect the 3 proteins. Representative blots are shown from experiments using 3 different cell preparations. *C*) Top panels: representative images of LSR staining in Hepa1–6 cells incubated at 37°C for 1 h with 0 or 10 ng/ml leptin. Insets: corresponding DAPI staining of nuclei. Bottom panels: images of cells preincubated with 5 μg/ml α-amanitin for 3 h before leptin treatment, as described in Materials and Methods. Bar graph summarizes mean ± SD signal intensity after image analysis. *D*) mRNA levels of LSR were determined in cells incubated with the indicated concentrations of leptin using real-time PCR, as described in Materials and Methods. Results are shown as means ± SD (triplicate determinations) of LSR mRNA expression relative to HPRT, used as reference housekeeping gene. Only *P* values for significant differences are indicated; for *D*, *P* values are for tests comparing PBS-treated cells to leptin-treated cells.

α-amanitin and then incubated 1 h with 10 ng/ml leptin in fresh medium containing α-amanitin. Treatment with this RNA polymerase inhibitor abolished the leptin-mediated increase in LSR protein levels (Fig. 1*C*, bottom panels and bar graph), suggesting, therefore, that this leptin effect was mediated at least in part through increased transcription. This was further supported by results from real-time PCR, which revealed that LSR mRNA expression relative to that of the reference housekeeping gene HPRT was significantly increased in cells incubated in the presence of the same concentrations of leptin used that led to an increase in LSR protein (Fig. 1*A*, *D*).

We next sought to determine whether we could observe a similar leptin-mediated increase of LSR *in vivo*. C57BL6/Rj mice were injected i.p. 2×/d at 9:00 AM and 6:00 PM with 1 μg mouse leptin/injection. Daily monitoring of the mice revealed no detectable change in food intake (data not shown). However, body weight decreased continuously during the treatment with leptin and reached significance between d 4 and 6 (**Fig. 2*A***). After 7 d of treatment, mice were sacrificed on d 8, and total liver membranes and extracts were prepared. In mice treated with leptin, a significant increase in hepatic LSR in membranes was observed as compared to mice treated with PBS (Fig. 2*B*, top panel). Immunoblot detection revealed no detectable difference in liver membrane Ob-R between mice injected with PBS and those injected with leptin (Fig. 2*B*, bottom panel). Analysis of LSR expression relative to that of Ob-R showed that LSR was significantly increased by more than 60% in leptin-treated mice as compared to controls (Fig. 2*B*, bar graph). Further, real-time PCR analysis indicated that LSR mRNA expression was increased almost 2-fold in mice that had been treated with leptin (Fig. 2*C*). These *in vivo* results, therefore, are in keeping with our cell culture studies, which show that leptin can modulate hepatic LSR protein and mRNA levels. Interestingly, TG content in liver samples was measured and found to be slightly, but significantly, decreased in the animals that were treated with leptin (Fig. 2*D*). A previous study reported that treatment of leptin could lead to decreased lipogenesis by reducing expression of key enzymes involved in TG synthesis, including ACL and FAS. Even though our study used 20-fold less leptin, we sought to determine whether the expression of these two enzymes was affected. Immunoblots revealed only a very small decrease in expression of ACL and no change in FAS protein levels (Fig. 2*E*). We next measured TG output from the liver using the Tyloxapol method to inhibit lipoprotein removal from the circulation. Results revealed that in the initial 30 min after i.v. injection of Tyloxapol, a greater amount of TG was released from the liver in leptin-treated mice as compared to controls (Fig. 2*F*). This could contribute to the lower hepatic TG content observed in animals injected with very low doses of leptin.

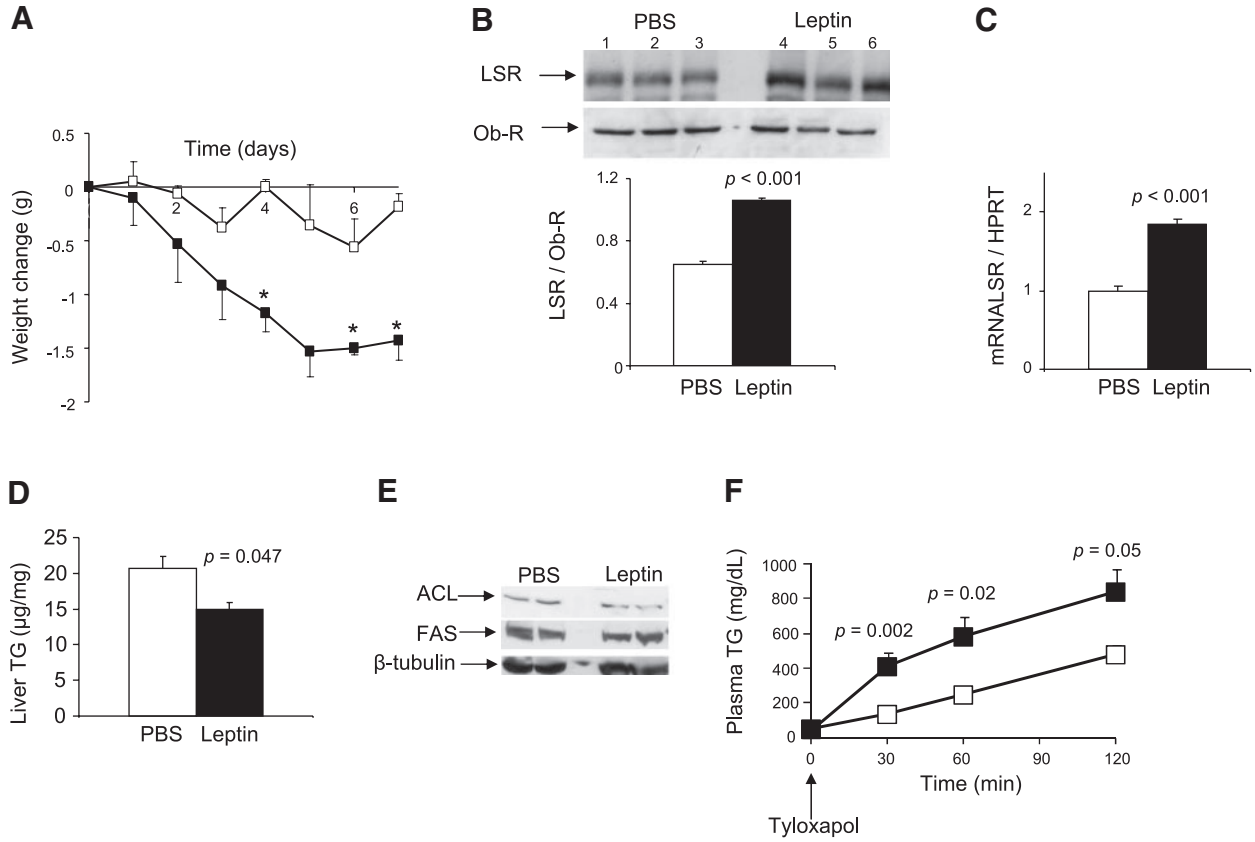

Figure 2. Body weight changes, hepatic LSR, Ob-R levels, and liver TG metabolism in mice treated with leptin. *A*) C57BL6/Rj male mice were i.p. injected 2×/d with PBS (□, n=5) or leptin (1 μg/injection, 2 μg/d) (■, n=5) for 8 d as described in Materials and Methods, during which changes in body weight were monitored. *$P < 0.05$ *vs.* PBS-injected mice. On d 8, animals were sacrificed, and livers were removed. *B*) Top panel: total membranes were isolated and immunoblots were performed to detect hepatic LSR and Ob-R as indicated. Each lane represents a different animal. Bottom panel: LSR expression relative to that of Ob-R (n=3/group). *C*) LSR mRNA expression was determined as described in Materials and Methods and is shown relative to HPRT as mean ± SD (PBS, n=2; leptin n=3, triplicate determinations). *D*) TG content of liver samples (n=3/group) was determined as described in Materials and Methods. *E*) Expression of two enzymes involved in TG synthesis, ACL and FAS, shown in representative Western blots. *F*) In a separate group of PBS- and leptin-treated animals (n=5/group), TG secretion was determined on d 8 as described in Materials and Methods. Mice were deprived of food for 3 h, and then injected i.v. with Tyloxapol. Blood samples were obtained at the times indicated to determine plasma TG levels. Mean ± SE values are indicated, with *P* values showing significant differences between PBS- and leptin-treated mice.

We had previously shown that reduced LSR expression in $LSR^{+/-}$ mice led to increased postprandial lipemia, and in view of leptin's influence on LSR protein levels, our next goal in this study was to determine the impact of low LSR on lipid homeostasis in the form of weight gain or changes in fat mass. Monitoring of aging $LSR^{+/-}$ mice maintained on a standard laboratory chow diet revealed that the body weights of 14-mo-old female $LSR^{+/-}$ mice were 1.5-fold higher as compared to controls (**Fig. 3*A***), despite no detectable difference in food intake (Fig. 3*B*). The higher body weight was accompanied by an increase in body fat mass (Fig. 3*C*), and a large 4-fold increase in plasma leptin concentrations (Fig. 3*D*). Unfed plasma glucose and insulin levels and glucose tolerance curves did not differ in the 2 groups of mice, indicating that there was no glucose intolerance in these aging $LSR^{+/-}$ mice (data not shown). We verified in these animals that postprandial lipemia was elevated following the gavage of a high-fat meal (**Fig. 4*A***) and that their ability to clear Intralipid was significantly reduced (Fig. 4*B*), as has been previously reported in younger $LSR^{+/-}$ mice (12).

We next sought to determine whether a chronic dietary lipid challenge could accelerate this process in younger animals. $LSR^{+/+}$ and $LSR^{+/-}$ 10-wk-old female mice were placed on a very high fat diet (60% kcal) for a period of 10 wk. In **Fig. 5*A***, the slope of the weight gain curve was much more pronounced in $LSR^{+/-}$ mice as compared to their control littermates. This difference was observed as early as 10 d after being placed on the diet. Despite this difference in weight gain, food intake was not significantly different with the exception of the last point measured before the end of the study (Fig. 5*B*). At t = 10 wk, the final body weights of $LSR^{+/+}$ and $LSR^{+/-}$ mice were 25.5 ± 0.84 and 30.1 ± 2.0 g (P=0.034), respectively. As shown in Fig. 5*C*, concomitant to this increased body weight, fat mass was observed to be significantly increased by 1.3-fold. To estimate energy expenditure, oxygen consumption

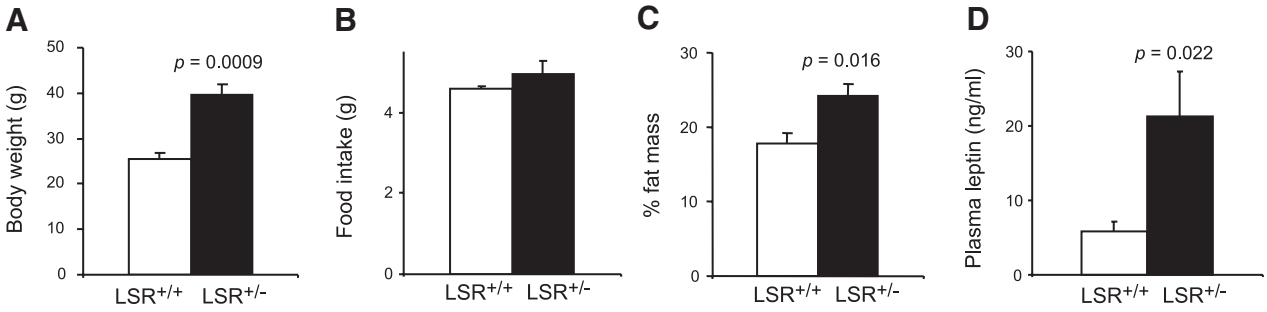

Figure 3. Body weight, fat mass, and leptin levels in 14-mo-old $LSR^{+/-}$ mice. *A–C*) Body weight (*A*), food intake (*B*) and fat mass (*C*) were measured in 14-mo-old female $LSR^{+/+}$ (open bars) and $LSR^{+/-}$ (solid bars) mice, as described in Materials and Methods. *D*) Plasma leptin values were also measured from blood samples obtained after a 3-h food-deprivation period. Mean ± SE values (n=5/group) are shown, and P values are indicated where significant comparing $LSR^{+/+}$ to $LSR^{+/-}$ groups.

was measured over a 24-h period in these animals using indirect calorimetry, and results revealed that the average energy expenditure over the 24-h period was significantly higher in $LSR^{+/-}$ mice as compared to $LSR^{+/+}$ animals.

Animals were sacrificed 10 wk after the start of the

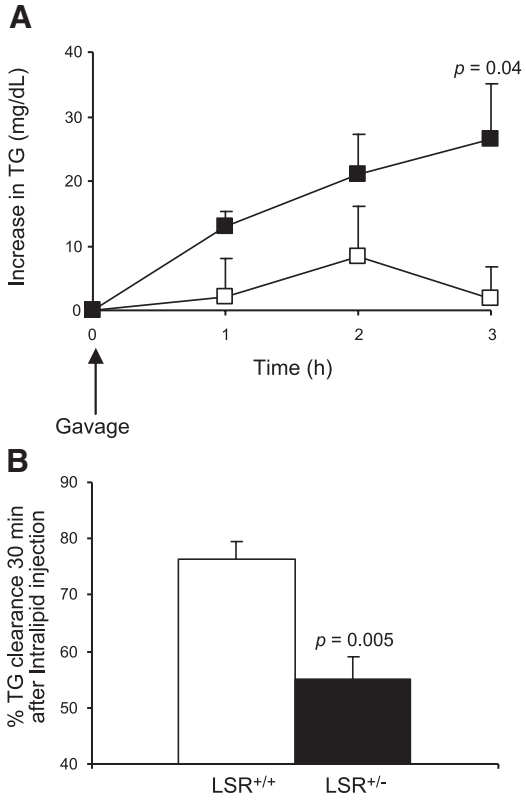

Figure 4. Elevated postprandial lipemia and decreased Intralipid clearance in 14-mo-old $LSR^{+/-}$ mice. *A*) Plasma TG levels were measured in $LSR^{+/+}$ (□) and $LSR^{+/-}$ (■) mice after gavage of olive oil. *B*) After i.v. injection of Intralipid into $LSR^{+/+}$ (open bar) and $LSR^{+/-}$ (solid bar) mice, blood samples were taken at 2 and 30 min, and plasma TG was determined, as described in Materials and Methods. Results are shown as percentage TG cleared in 30 min, using plasma TG levels at 2 min as the 100% value. Mean ± SE values (n=5/group) are shown, and P values are indicated where significant comparing $LSR^{+/+}$ to $LSR^{+/-}$.

diet, and lipid content was analyzed in the liver, skeletal muscle, and adipose tissue (Supplemental Table 1). TG content expressed relative to the PL content was found to be increased almost 2-fold in adipose tissue of $LSR^{+/-}$ mice fed the high-fat diet ($LSR^{+/+}$, 1.79±0.36; $LSR^{+/-}$, 3.46±0.58; P=0.038). Plasma leptin levels were 3-fold higher in $LSR^{+/-}$ mice as compared to control animals (Fig. 5*E*) and strongly correlated to fat mass in both groups ($LSR^{+/+}$, r=0.86, P=0.028; $LSR^{+/-}$, r=0.97, P=0.033), as has been previously reported (24, 25). However, neither adiponectin nor insulin levels were found to be different in these mice (Fig. 5*F*, *G*, respectively). Therefore, under conditions of a high-fat chronic dietary challenge, $LSR^{+/-}$ mice were more susceptible as compared to their wild-type littermates toward weight gain and increased fat mass associated with increased plasma leptin levels.

Total liver membranes were prepared from these mice and used for immunoblots to obtain a relative measure of LSR protein levels in the $LSR^{+/+}$ and $LSR^{+/-}$ groups that were fed the high-fat diet. Immunoblot analysis confirmed the reduced hepatic LSR protein levels in $LSR^{+/-}$ mice (**Fig. 6*A***). Interestingly, linear regression analysis of all data points revealed a significant and negative correlation between the relative degree of hepatic LSR protein expression and plasma leptin levels (Fig. 6*B*).

Protein levels of Ob-R in hepatic membranes were analyzed in these same animals by immunoblotting. **Figure 7*A*** shows that Ob-R was significantly decreased in $LSR^{+/-}$ mice as compared to $LSR^{+/+}$ mice fed the high-fat diet. Furthermore, a positive correlation was observed between hepatic Ob-R protein and plasma leptin levels only in $LSR^{+/+}$ (r=0.86, P=0.03) and not in $LSR^{+/-}$ mice (r=−0.14, ns) fed the high-fat diet (data not shown). Ob-R levels were measured in age-matched $LSR^{+/-}$ and $LSR^{+/+}$ mice fed a standard diet and found to be similar in both groups (Fig. 7*B*). In $LSR^{+/-}$ mice fed the high-fat diet, the relative amount of phosphorylated form of ERK1/2 (p-ERK1/2), as compared to ERK1/2 was significantly lower as compared to controls fed the same diet (Fig. 7*C*). This was

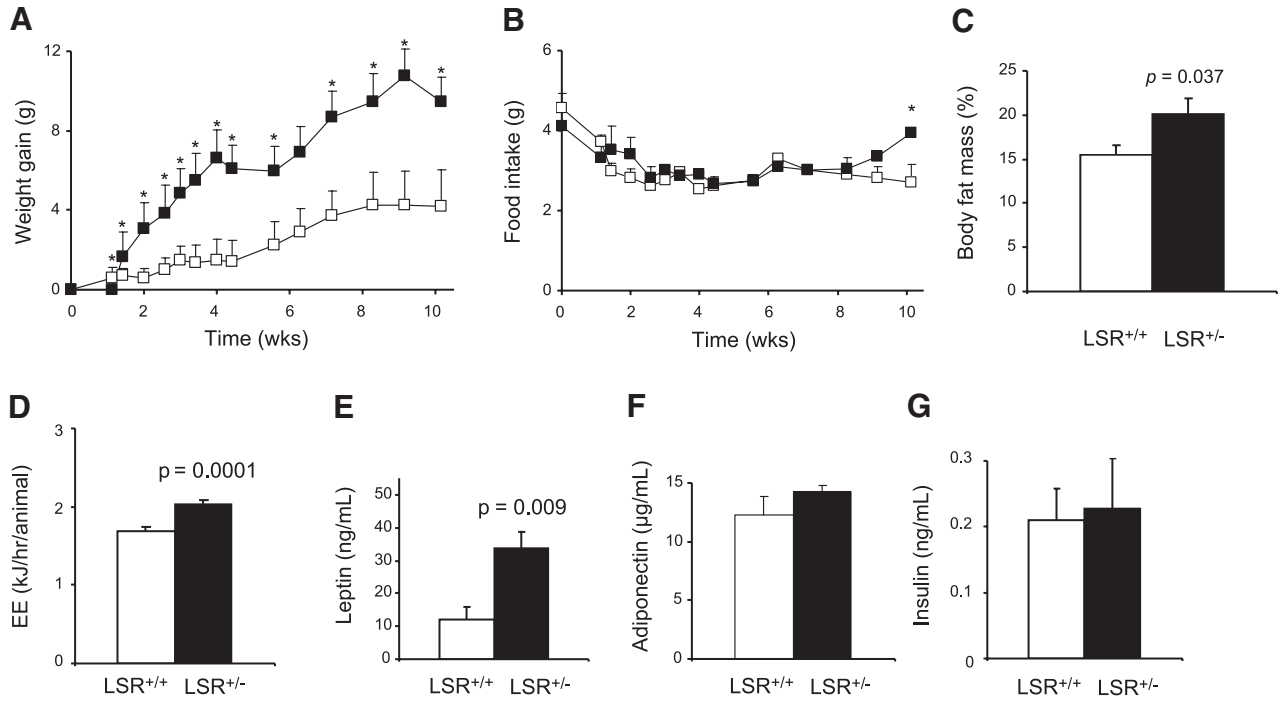

Figure 5. Weight gain, food intake, fat mass, energy expenditure, leptin, adiponectin, and insulin levels in $LSR^{+/-}$ mice following a 10-wk high-fat diet. $LSR^{+/+}$ (n=6, open bars or □) and $LSR^{+/-}$ female mice (n=5, solid bars or ■) were placed on a high-fat (60% kcal) diet for 10 wk, as described in Materials and Methods. *A*, *B*) Weight gain (*A*) and food intake (*B*) were monitored during the study. *$P < 0.05$ *vs.* $LSR^{+/+}$ group. *C*) Body fat mass was measured as described in Materials and Methods. *D*) Oxygen consumption was measured in mice near the end (wk 9) of the dietary study using indirect calorimetry, as described in Materials and Methods. Energy expenditure (EE) is expressed as the mean ± SE (kJ/h/animal). *E–G*) Plasma leptin (*E*), adiponectin (*F*), and insulin (*G*) levels were measured in plasma collected at the end of the study after a 3-h food-deprivation period. Mean ± SE values are shown, with *P* values indicating significance after comparing $LSR^{+/+}$ with $LSR^{+/-}$ groups.

not the case in $LSR^{+/-}$ mice fed a standard diet. Indeed, in these mice, p-ERK1/2 was detected in both $LSR^{+/-}$ and $LSR^{+/+}$ mice at similar levels (Fig. 7*D*). Taken together, these data indicate that reduced LSR expression in mice led to accelerated weight gain and increased leptin. Furthermore, a 10-wk chronic stress of dietary lipid led to decreased leptin receptor levels in the liver, as well as lower levels of the downstream signaling form of the ERK1/2 pathway, but only in $LSR^{+/-}$ animals.

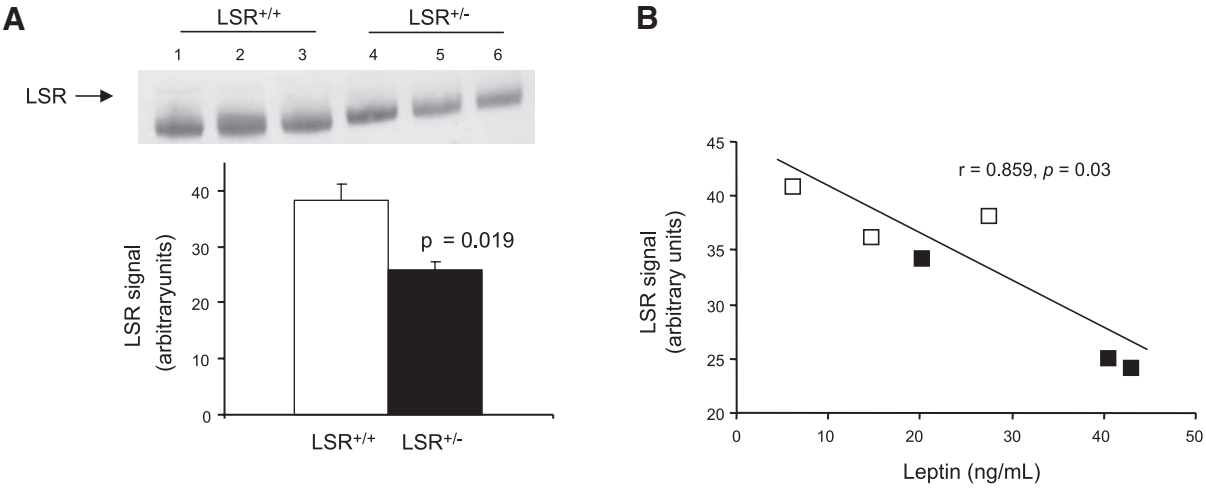

Figure 6. Relationship between hepatic LSR protein and plasma leptin levels in $LSR^{+/-}$ mice after 10 wk on a high-fat diet. *A*) Immunoblots were performed to detect the level of expression of LSR in liver total membrane preparations obtained from $LSR^{+/+}$ (open bar) and $LSR^{+/-}$ (closed bar) mice fed on a high-fat diet for 10 wk (see Fig. 5). Each lane represents a different animal. Mean ± SE values are shown with *P* value indicating significance after comparing $LSR^{+/+}$ with $LSR^{+/-}$ groups. *B*) Linear regression analysis of LSR signal intensity on the immunoblot and plasma leptin levels from high-fat fed animals (n=3/group, $LSR^{+/+}$, □; $LSR^{+/-}$, ■), with the correlation and *P* value indicated.

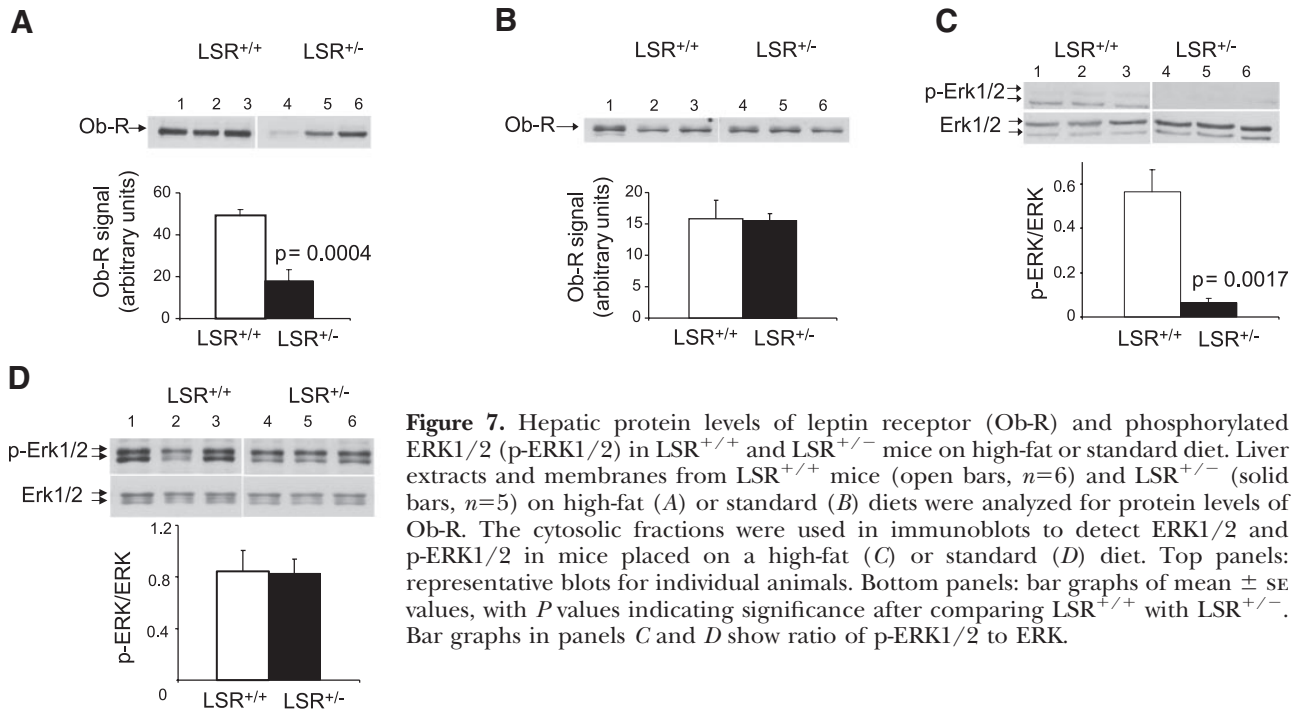

Figure 7. Hepatic protein levels of leptin receptor (Ob-R) and phosphorylated ERK1/2 (p-ERK1/2) in $LSR^{+/+}$ and $LSR^{+/-}$ mice on high-fat or standard diet. Liver extracts and membranes from $LSR^{+/+}$ mice (open bars, $n=6$) and $LSR^{+/-}$ (solid bars, $n=5$) on high-fat (*A*) or standard (*B*) diets were analyzed for protein levels of Ob-R. The cytosolic fractions were used in immunoblots to detect ERK1/2 and p-ERK1/2 in mice placed on a high-fat (*C*) or standard (*D*) diet. Top panels: representative blots for individual animals. Bottom panels: bar graphs of mean ± SE values, with *P* values indicating significance after comparing $LSR^{+/+}$ with $LSR^{+/-}$. Bar graphs in panels *C* and *D* show ratio of p-ERK1/2 to ERK.

DISCUSSION

In this study, we provide *in vitro* and *in vivo* evidence for a new peripheral role of leptin in the modulation of the lipoprotein receptor LSR that is expressed in the liver.

In vitro data using immunoblotting and immunofluorescence revealed that LSR protein levels were increased in Hepa1–6 cells incubated 1 h with leptin at levels in the physiological range. PD98059-mediated inhibition of phosphorylation of ERK1/2 completely suppressed leptin-mediated increase of LSR protein levels, in keeping with previous studies showing that leptin interaction with Ob-Rb leads to phosphorylation of ERK1/2 (16). Leptin has also been shown to activate STAT3 (15), but we were unable to detect phosphorylation in these cells in the presence of leptin (data not shown). Indeed, leptin-mediated signaling in hepatoma cell lines has previously been shown to be independent of STAT3 activation (26). A weak band of the phosphorylated form on residue Ser-473 of Akt in cells incubated with 10–25 ng/ml of leptin was detected (data not shown). Nevertheless, the data using the inhibitor PD98059 indicated that ERK1/2 phosphorylation represented the principal downstream signaling form by which leptin mediated its effect on LSR protein. The phosphorylated form of ERK is known to translocate into the nucleus and activate transcription factors. Inhibition of the leptin effect in cells preincubated with α-amanitin indicates that a part of leptin's effect may be due to increased transcription of the *lsr* gene (**Fig. 8*A***), which was confirmed by real-time PCR analysis. Both the increase in mRNA and protein levels of LSR in such a brief time illustrates the potent action of physiological concentrations of leptin in the regulation of LSR.

In vivo studies confirmed the activating effect of leptin that was observed *in vitro*. Following 7 d of 2×/d administration of leptin, liver membrane-associated protein levels of LSR in wild-type C57BL6/Rj mice were significantly increased. Interestingly, this was accompanied by a small, but significant, decrease in liver TG content. Leptin has been shown to suppress the expression of lipogenic enzymes, leading to decreased TG in the liver (27). However, in our study, ACL expression was only slightly decreased, while that of FAS was not significantly different in mice treated with leptin as compared to controls. The dose of leptin used in the previous report (27) was 20-fold higher than that used in this study, which could explain the lack of effect on these lipogenic enzymes. Instead, we observed an increase in VLDL measured as TG appearance in the plasma after i.v. injection with Tyloxapol. Tyloxapol has been previously shown to coat the surface of lipoprotein particles, thus inhibiting their removal from the circulation (28). Therefore, after the 3-h food-deprivation period, the TG that is measured represents that being secreted from the liver in the form of VLDL. In view of the fact that these mice are losing weight, it is possible that this may reflect increased energy substrate demand by the peripheral tissues, most notably the skeletal muscle. Indeed, a recent paper demonstrated that in *ob/ob* mice treated with leptin, lipoprotein lipase activity in only skeletal muscle and not adipose tissue was increased (29). This would be consistent with increasing availability of free fatty acids to muscle. Another potential explanation for the decreased hepatic TG content may be increased TG metabolism as a result of increased LSR activity. Indeed, adenovirus-mediated overexpression of LSR in *ob/ob* mice led to an increase in TG input in the liver that was compensated

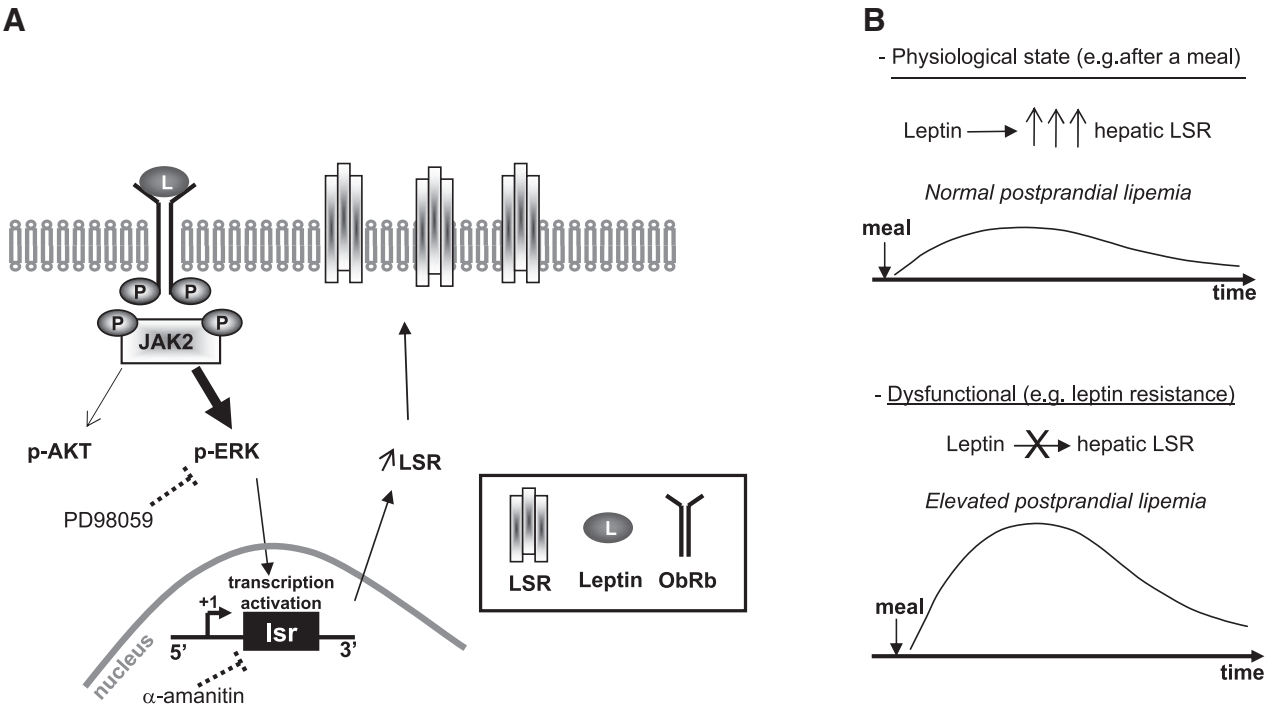

Figure 8. Schematic diagram for leptin-mediated up-regulation of LSR and potential consequences on postprandial lipemia. *A*) We propose that the canonical leptin-induced signaling pathway involving phosphorylation of ERK leads to increased transcription of the *lsr* gene and increased protein levels of LSR at the surface of hepatocyte membranes. *B*) Under normal conditions, physiological levels of leptin are sufficient to maintain an optimal amount of LSR at the cell surface, permitting clearance of TG-rich lipoproteins during the postprandial phase. However, when leptin interaction with its receptor in the liver is impaired, such as in leptin resistance, LSR protein levels at the cell surface may no longer be optimal, leading to decreased efficiency in removing lipids from the cell surface, resulting in elevated postprandial lipemia.

for by increased fatty acid oxidation (30). Further investigation is required to delineate the mechanisms underlying leptin's regulation of lipid metabolism in the liver.

The leptin injections led to a significant decrease in animal body weight, without any evident change in food intake. This was surprising, because if the degree of weight change observed (1.5 g) was directly due to a modified dietary input, it should have been detectable. The weight loss itself does not seem to be the cause for the changes in LSR levels, since food restriction alone does not lead to increased LSR. We measured LSR protein levels in mice that were placed on a food restriction diet for 1 wk. Even with a significant average body weight loss of 4 g, no detectable difference was observed in hepatic levels of LSR (data not shown). Therefore, the increase in LSR reported in Fig. 2 is directly related to the leptin injection, rather than a consequence of reduced body weight.

The majority of the papers studying leptin peripheral effects has used supraphysiological doses, which clearly will involve leptin signaling at the central nervous system level, as evidenced by decreased food intake (27, 31). We cannot completely rule out the possibility of leptin activity at the level of the central nervous system. Nevertheless, using lower leptin doses allowed us to avoid or at least to minimize the added influence of the hypothalamus-mediated response to leptin. This raises a very interesting point in demonstrating the specific and significant peripheral effect of leptin on LSR expression *in vivo*. From a therapeutic point of view, we could speculate that by virtue of its effect on LSR, such doses of leptin could help the liver to increase LSR and thus reduce postprandial lipemia without affecting other physiological and vital functions in the liver.

In view of these data, we propose that the leptin-mediated increase in LSR may provide a means to increase removal of lipids during the postprandial phase, which would contribute to the dynamics of lipid distribution and utilization among the different tissues.

Indeed, our data indicate that reduced LSR expression in the $LSR^{+/-}$ mouse, shown previously to exhibit elevated postprandial lipemia and decreased lipid clearance (12, 30), is also associated with increased body fat mass and increased energy expenditure, two hallmarks of obesity (32). This is observed in aged $LSR^{+/-}$ mice on a standard diet, and can be accelerated in younger $LSR^{+/-}$ mice by exposure to a very high fat diet. The weight gain is significant but does not approach massive obesity. Furthermore, another marker for obesity, adiponectin, was not modified in these mice. Also, neither glucose intolerance nor hyperinsulinemia were detected in these mice, which would indicate the absence of any defect in glucose metabolism or problem in insulin resistance.

In these $LSR^{+/-}$ mice, we also observed an increase in leptin, which would be expected due to the higher

fat mass. However, further investigation revealed that Ob-R was significantly decreased in the $LSR^{+/-}$ mice fed the high-fat diet. In addition, the phosphorylated form of ERK1/2 was largely reduced in these mice. A previous study has reported a high-fat diet-induced decrease in Ob-R/STAT-mediated signaling in T lymphocytes (33). Furthermore, exercise training appears to decrease both plasma leptin and hepatic Ob-R levels (34), suggesting a close link between metabolic state and the regulation of leptin and Ob-R levels in the liver. We also observed a strong positive correlation between hepatic membrane Ob-R levels and leptin levels in the plasma in wild-type mice. This is in keeping with a study by Cohen *et al.* (35), who reported an association between plasma leptin and hepatic Ob-R levels. Strikingly, this correlation was no longer detected in the $LSR^{+/-}$ mice fed the high-fat diet, reinforcing the notion that the signaling is somehow impaired. These data, combined with the reduced levels of Ob-R and impaired phosphorylation of ERK1/2 in the $LSR^{+/-}$ mouse fed the high-fat diet lead us to propose this as a new model for the study of mechanisms underlying the development of leptin resistance in the periphery. If these mice placed on a high-fat diet develop such signaling impairments in liver, it would also be interesting to investigate the status of leptin signaling capacity in other compartments such as the central nervous system in this mouse model.

This study demonstrates a new peripheral role for leptin in the regulation of a hepatic receptor in the periphery involved in the clearance of lipoproteins following a meal (Fig. 8*A*). We would propose that the presence of leptin is important to maintain LSR protein at optimal levels to ensure efficient lipid clearance during the postprandial phase (Fig. 8*A*, *B*), which would be complementary to its action as a satiety factor. However, if leptin signaling in the liver is defective, this could lead to events, including suboptimal LSR protein expression, which would contribute to elevated postprandial lipemia (Fig. 8*B*). This promotes increased distribution of lipids in the periphery, mainly the adipose tissue, leading to increased fat mass, and provides a novel explanation for the delayed postprandial lipemia that is often associated with obese subjects. The role of leptin in the control of postprandial lipemia *via* LSR provides, therefore, a new lever for the regulation of the removal of lipids during the postprandial phase, an important risk factor associated with cardiovascular and metabolic diseases. FJ

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Lactoferrin and its hydrolysate bind directly to the oleate-activated form of the lipolysis stimulated lipoprotein receptor

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The hepatic removal of triglyceride-rich chylomicrons during the postprandial phase represents an important step towards determining the bioavailability of dietary lipids amongst the peripheral tissues. Indeed, elevated postprandial lipemia is often associated with obesity and increased risk of coronary heart disease. The milk protein, lactoferrin, has been shown to inhibit hepatic chylomicron remnant removal by the liver, resulting in increased postprandial lipemia. Despite numerous studies on potential targets for lactoferrin, the molecular mechanisms underlying the effect of lactoferrin remain unclear. We recently demonstrated that the lipolysis stimulated lipoprotein receptor (LSR) contributes to the removal of triglyceride-rich lipoproteins during the postprandial phase. Here, we report that while lactoferrin does not have any significant effect on LSR protein levels in mouse Hepa1–6 cells, this protein colocalizes with LSR in cells but only in the presence of oleate, which is needed to obtain LSR in its active form as lipoprotein receptor. Ligand blotting using purified LSR revealed that lactoferrin binds directly to the receptor in the presence of oleate and prevents the binding of triglyceride-rich lipoproteins. Both C- and N-lobes of lactoferrin as well as a mixture of peptides derived from its hydrolysis retained the ability to bind LSR in its active form. We propose then that the elevated postprandial lipemia observed upon lactoferrin treatment *in vivo* is mediated in part by its direct interaction with free fatty acid activated LSR, thus preventing clearance of chylomicrons and their remnants through the LSR pathway.

Structured digital abstract

- Lsr and Lsr bind by comigration in gel electrophoresis (View interaction)
- Lsr binds to Lf by filter binding (View interaction)
- Lf and Lsr colocalize by fluorescence microscopy (View interaction)
- Lsr physically interacts with VLDL by filter binding (View interaction)

Abbreviations

apoE, apolipoprotein E; FFA, free fatty acid; HPRT, hypoxanthine guanine phosphoribosyltransferase; LDL, low-density lipoprotein; LDL-R, LDL receptor; Lf, lactoferrin; LRP, LDL-R-related protein; LSR, lipolysis stimulated lipoprotein receptor; TG, triglyceride; VLDL, very-low-density lipoprotein.

Introduction

During the postprandial phase after ingestion of a meal, triglycerides (TGs) circulate in the plasma in the form of intestinally derived chylomicrons. These TG-rich lipoproteins distribute fatty acids to different tissues via the lipoprotein lipase system and are ultimately removed from the circulation through receptor-mediated processes in the liver [1]. Elevated postprandial lipemia is often associated with obesity [2] and has been reported in patients with cardiovascular disease [3]. Lactoferrin (Lf) is a milk protein that when injected intravenously has been demonstrated to increase postprandial lipemia by inhibiting chylomicron remnant removal from the liver [4].

Lf is a non-heme iron-binding glycoprotein belonging to the transferrin class. This monomeric protein of 689 residues folds into two domains of high sequence similarity, called the N- and C-lobes [5], each of which binds one ferric ion [6].

On the N-lobe, Lf contains an Arg-rich region similar to that of apolipoprotein E (apoE), which is the ligand for the uptake of chylomicron remnants [7]. Studies on parenchymal liver cells demonstrated that Lf inhibits the association of apoE-containing lipoproteins to a receptor which is distinct from the low-density lipoprotein (LDL) receptor (LDL-R) and LDL-R-related protein (LRP) but was unidentified [8].

It was at this time that Bihain and Yen [9] reported that fibroblasts from a patient deficient in LDL-R were able to internalize significant amounts of LDL via a previously unidentified receptor. It was demonstrated that this new pathway involves a receptor that mediates the binding of apoB- and apoE-containing lipoproteins, leading to their subsequent internalization and degradation [10]. This receptor displays highest affinity for TG-rich lipoproteins [chylomicrons and very-low-density lipoprotein (VLDL)] and is referred to as the lipolysis stimulated lipoprotein receptor (LSR) since it can only bind and internalize lipoproteins in the presence of free fatty acids (FFAs). Since LSR is activated by these lipolytic products, it is thus primarily active during the postprandial phase when the influx of chylomicrons into the circulation is high, which leads to increased lipase-mediated hydrolysis of the chylomicron TGs, leading to high FFA levels in the space of Disse of the liver [10–12]. LSR was identified as a multimeric complex consisting of three subunits derived by alternative splicing from the same gene [13]. *In vivo* data now demonstrate that LSR plays an important role in the clearance of TG-rich lipoproteins during the postprandial phase [14]. It was observed that Lf inhibited oleate-induced lipoprotein binding to rat hepatocyte plasma membranes [11,12]. This inhibition was shown to occur without a significant decrease of the amount of membrane-associated oleate and thus differs from the inhibition caused by albumin that is achieved through oleate sequestration [11], suggesting that LSR is the molecular target of Lf. Even though this hypothesis was based on experiments done on liver membranes under conditions optimal for LSR activity, interaction between LSR and Lf remained to be demonstrated.

The aim of this study was to determine if the inhibition of LSR by Lf is due to direct interaction of these two proteins. Here, we show that bovine Lf and its hydrolysis products display the ability to bind to the FFA-activated form of the purified receptor and compete for binding of VLDL to LSR.

Results

In order to study the direct interaction of LSR with Lf, it was necessary to use Lf with high purity. Since SDS/gel analysis revealed that commercially available Lf contained a number of contaminating proteins, including albumin (Fig. 1A), we purified Lf from raw bovine milk to > 95% purity (Fig. 1A). Since Lf inhibits postprandial lipemia when injected intravenously into rats [4], we next verified that the purified Lf exhibited this same biological effect. C57BL/6RJ male mice were injected intravenously with Lf and then immediately received olive oil by gavage. Plasma TGs measured 1 h after the gavage were found to be significantly increased ($P = 0.04$) by a factor of 4.3 in the group of mice that received purified Lf (Fig. 1B), while the 1.9-fold increase in plasma TGs in the group of mice that received commercial Lf only tended towards significance ($P = 0.08$). We verified that Lf did not have any effect on free glycerol levels (increase in free glycerol 1 h after gavage: controls 16 ± 4.5 $\mu g \cdot mL^{-1}$ and Lf group 18.8 ± 2.3 $\mu g \cdot mL^{-1}$). The difference between purified and commercial Lf could be due to impurities detected in the latter, including albumin and immunoglobulin (Fig. 1A). These results therefore indicate that pure Lf retains its *in vivo* effect on postprandial lipemia, confirming that the effect derives purely from Lf and not from other contaminants such as albumin.

As Lf has been shown to enter the nucleus and induce gene expression [15,16], we first sought to determine whether Lf demonstrated any effect on LSR mRNA or protein levels in the mouse hepatoma cell line, Hepa1–6. Since LSR activity as a lipoprotein

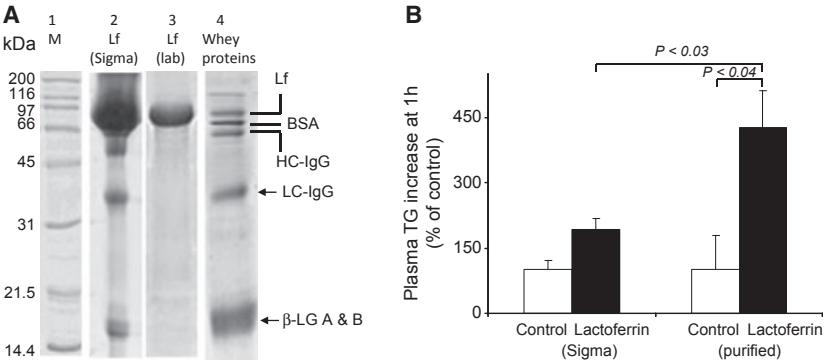

Fig. 1. Effect of Lf on plasma TG level during the postprandial phase. (A) SDS/PAGE showing the Lf used for this study. Lane 1, molecular weight marker (M); lane 2, bovine Lf from Sigma; lane 3, bovine Lf purified from whey proteins (Lf lab); lane 4, whey proteins obtained after casein precipitation. LC-IgG, light chain immunoglobulin; HC-IgG, heavy chain immunoglobulin; LG, lactoglobulin. (B) Plasma TG increase 1 h after Lf injection. C57BL6/RJ male mice were injected intravenously (tail vein) with 100 μL NaCl/P_i (open bars) or NaCl/P_i containing 2 mg bovine Lf (closed bars) obtained from Sigma or purified from raw milk. After gavage with 300 μL olive oil, blood samples were removed and plasma TG levels were measured as described in Materials and methods. Results are shown as mean ± SEM (n = 3 or 4 per condition) of the increase in plasma TG relative to control. Statistical significance is indicated by P values (compared to the corresponding NaCl/P_i group).

receptor is dependent upon the presence of FFAs, we incubated cells for 3 h at 37 °C in the absence or presence of oleate and Lf. The concentrations of Lf used were those shown previously to inhibit lipoprotein binding to liver membranes in the presence of oleate [11]. Incubation of cells in the presence of Lf appeared to slightly decrease cell LSR protein levels relative to actin as a loading control (Fig. 2A). However, because of the variations in expression, this difference was not statistically significant. Parallel experiments were conducted to determine whether LSR mRNA levels were affected. Only a small decrease was observed in cells incubated in the presence of 0.8 mM oleate and Lf compared with 0.8 mM oleate alone (Fig. 2B). No significant changes were observed when cells were incubated with or without Lf in the absence or presence of 0.4 mM oleate (Fig. 2B). Therefore, at least in short-term experiments, Lf did not have any detectable effect on LSR protein levels but did have a small inhibitory effect on LSR mRNA levels in the presence of oleate. We then initiated a study on the interaction between LSR and Lf both at the cellular and molecular levels. Intact cells were used, in which Alexafluor-488-labelled Lf was incubated with Hepa1–6 cells with or without oleate. Figure 3 shows strong labeling of permeabilized cells with both Lf and LSR, with little apparent colocalization in the merge picture in the absence of oleate (Fig. 3, top panels). However, when cells were incubated in the presence of oleate, colocalization of Lf and LSR labeling increased dramatically, as evidenced by the increased orange intensity in the merged picture (Fig. 3, lower right panel). This suggests that Lf interacts directly with LSR but only in

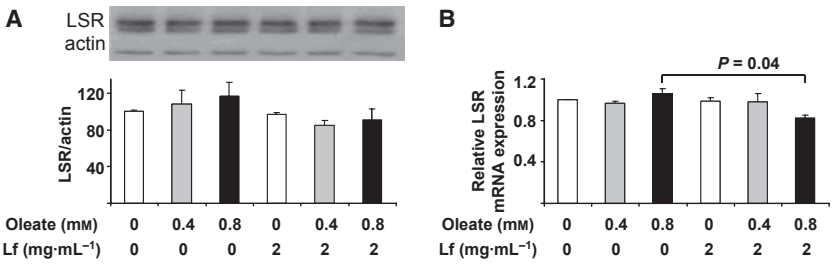

Fig. 2. Effect of lactoferrin on LSR mRNA and protein levels in Hepa1–6 cells. Hepa1–6 cells were incubated for 3 h with 2 mg·mL^{-1} Lf with increasing concentrations of oleate as indicated. (A) Cell lysates were then prepared from which immunoblots were performed as described in Materials and methods to detect LSR. Actin served as loading control. A representative blot is shown and an analysis of three experiments with three different preparations of cells is shown in the bar graph as mean ± SEM ratio of LSR/actin. The values of LSR/actin for cells incubated in the absence of oleate and Lf were normalized to 100%, allowing us to compare the variations between different experiments. (B) Experiments were conducted in which mRNA was directly extracted from cells and then used in qPCR analysis. Data are shown as the relative expression of LSR versus the housekeeping gene, HPRT (mean ± SEM, n = 3).

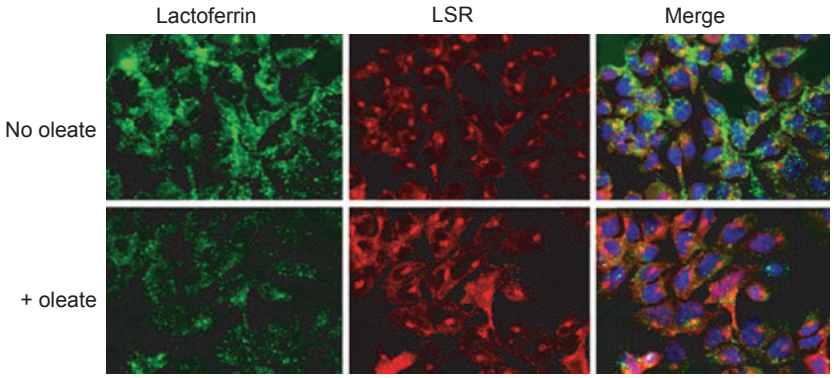

Fig. 3. Colocalization of Lf and LSR in Hepa1–6 cells. Hepa1–6 cells were incubated with 2 mg·mL^{-1} Lf labeled with Alexafluor 488 in the absence (top panels) or presence (bottom panels) of oleate for 20 min at 37 °C. The cells were then washed, fixed, permeabilized and labeled with anti-LSR IgG as described in Materials and methods. Visualization of Lf is shown in the left panels and LSR in the middle panels. Merged pictures are shown in the right panels.

the presence of oleate, thus when LSR is in its active conformation.

In order to confirm this possible interaction, ligand blot experiments were performed using purified LSR and Lf. LSR was purified from rat hepatocyte plasma membranes using anion exchange chromatography. The protein appeared to be at least 95% pure, as judged by SDS/PAGE analysis (Fig. 4A). Three bands were obtained under reducing conditions, with apparent molecular mass ranging from 54 to 70 kDa, which is consistent with the masses of the subunits that compose the receptor. Under non-reducing conditions, one single band was observed at an apparent molecular weight of ~ 240 kDa, representing the LSR complex (Fig. 4A) [13]. The identity of LSR was confirmed by immunoblot analysis (Fig. 4B).

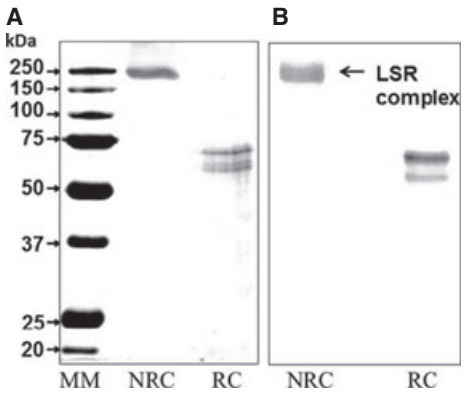

Fig. 4. SDS/PAGE and western blot of purified LSR. The purified LSR was loaded on 10% SDS/PAGE under non-reducing (NRC) or reducing (RC) conditions. (A) Silver staining. (B) Western blot. Revelation was made as described in Materials and methods. MM, molecular weight marker.

Ligand blots were then carried out in which LSR was migrated on SDS/PAGE under non-reducing conditions and, after transfer on nitrocellulose, incubated with the pure Lf. It was previously shown that FFAs were necessary for LSR binding to LDL [11,12]. In view of the results of the colocalization experiments, the assay was conducted both in the absence and in the presence of oleate. As shown in Fig. 5A, lanes 1 and 2, Lf was revealed to bind to a band corresponding to the LSR complex (apparent molecular mass 240 kDa) [13] but only when oleate was present (Fig. 5A, lane 2), suggesting that FFAs are also required for Lf binding to this receptor. Control experiments in the absence of Lf demonstrate that this is not due to binding of anti-Lf IgG to LSR (data not shown).

We have previously reported that Lf can inhibit LSR activity as a lipoprotein receptor in liver cell and membrane studies as well as ligand blots using membrane extracts [11,12]. Ligand blots were performed by incubating immobilized purified LSR with TG-rich VLDL in the presence of oleate and increasing concentrations of Lf. VLDL binding was revealed by detection of apoB on the 240-kDa LSR complex (Fig. 5B). This binding was decreased in intensity with increasing concentrations of Lf, thus demonstrating that direct binding of Lf to LSR can prevent binding of its lipoprotein ligand.

In order to determine which part of Lf is recognized by LSR, Lf was hydrolyzed by trypsin under mild conditions to obtain the N- and C-lobes separately. Four fragments with apparent molecular masses of 24, 34, 44 and 51 kDa were obtained on SDS/PAGE profile (data not shown). N-terminal sequencing and mass spectrometry analysis (Table 1) allowed the identification

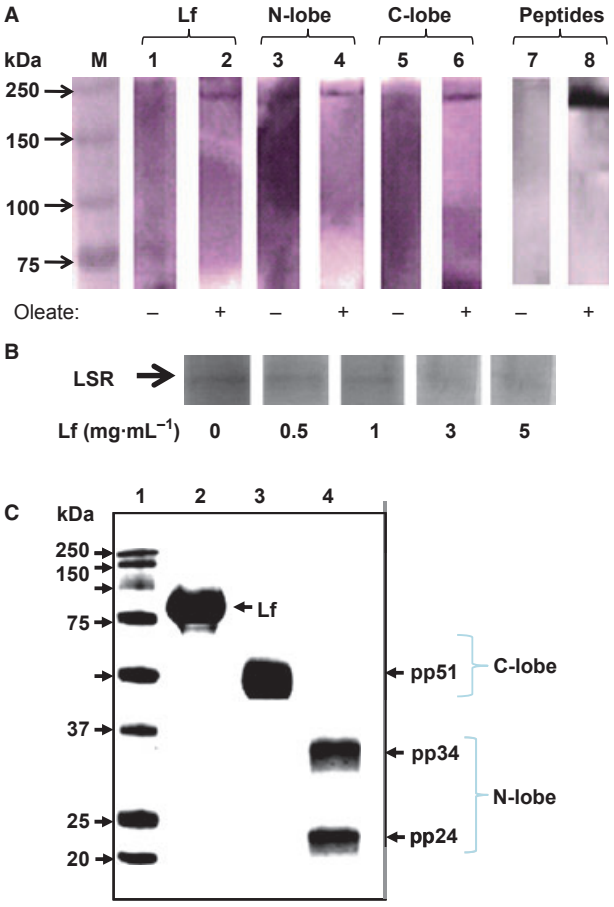

Fig. 5. Ligand blots showing the interaction between LSR and bovine Lf and its hydrolysis products. (A) Ligand blots of LSR with entire Lf or its hydrolysis products. LSR was loaded on SDS/PAGE under non-reducing conditions, and then transferred onto a nitrocellulose membrane and incubated with full-length Lf (2 mg·mL^{-1}, lanes 1 and 2), its N-terminal lobe (2 mg·mL^{-1}, lanes 3 and 4) and C-terminal lobe (2 mg·mL^{-1}, lanes 5 and 6), and peptides derived from tryptic/chymotryptic hydrolysis (2 mg·mL^{-1}, lanes 7 and 8). Strips were incubated without oleate (lanes 1, 3, 5 and 7) or with 0.8 mM oleate prior to incubation with Lf (lanes 2, 4, 6 and 8). Lf was then detected by immunoblotting with anti-Lf IgG as described in Materials and methods. (B) Ligand blots of LSR with VLDL in the presence of increasing concentrations of Lf. Purified LSR on nitrocellulose membranes was incubated with 0.8 mM oleate and 20 μg·mL^{-1} VLDL in the presence of the indicated concentrations of Lf. The VLDL was detected with anti-apoB IgG as described in Materials and methods. (C) SDS/PAGE of Lf and its mild hydrolysis products used for the ligand blot shown in (A). lane 1, M, molecular weight marker; lane 2, pure bovine Lf; lane 3, peptide representative of the C-domain; lane 4, peptides of the N-domain.

of the first two fragments as deriving from the N-terminal lobe and the second two fragments as belonging to the C-lobe (with or without the peptide connecting the two lobes of Lf). Precise identification of the C-terminus of the peptides corresponding to the N-lobe (a tryptic fragment) was difficult to obtain because of the presence of two Lys residues (at positions 280 and 282) and one Arg residue (at position 284) in this region. Indeed, it has been reported that two N-lobe fragments of apparent mass 34 kDa correspond either to the 1–284 Lf region [17] or to the 1–280 region [18], showing that identification of the C-terminal extremity remains difficult to determine. Moreover, determination of the mass of the entire N- and C-lobe peptides did not allow precise identification of the C-terminus because of the microheterogeneity of the glycan structures of Lf.

After isolation of the fragments accounting for each lobe by liquid chromatography (Fig. 5C), ligand blots were performed once again in the same conditions as above except that incubation was performed with fragments of the N-lobe (mixture of PP24 and PP34) or of the C-lobe (P51) rather than with the whole Lf molecule. As shown in Fig. 5A (lanes 3–6), both lobes of Lf are able to bind to LSR but, again, only if oleate is present. This also suggests that there are structural motifs or sequences located not only at the N-lobe but also the C-lobe of Lf that are recognized by LSR.

We pursued this line of thought by subjecting the trypsin hydrolysate of Lf to further hydrolysis by chymotrypsin in order to determine whether small peptides derived from Lf hydrolysis possess the ability to bind to the receptor. Non-digested N- or C-lobes or intermediate peptides having apparent molecular masses > 10 kDa were removed by ultrafiltration. Mass spectrometry analysis confirmed that peptides contained in the ultrafiltrate were small (Table 2), except for four peptides displaying masses of 2726, 2564, 2402 and 2240 Da. Fragmentation by tandem mass spectrometry showed that these peptides actually correspond to four glycopeptides with the same aglycoform VKNDTVW(543–549) produced by chymotrypsin action on the C-lobe. These glycopeptides carry on the Asn545 residue an oligomannosidic-type glycan structure (data not shown). The mass spectrometry analysis showed that the glycan moiety possesses a variable number of mannose residues, up to nine units, and this result is in accordance with the N-glycan structures of Lf reported by Spik *et al.* [19] and Coddeville *et al.* [20]. Furthermore, the experimental molecular mass of the deglycosylated peptide chain produced by MS2 fragmentation was 860 ± 1 Da, which is very close to the theoretical mass of the apoheptapeptide of 860.44 Da. The hydrolysate does not contain any other *N*-glycopeptide, as all the other molecular masses found in this fraction are lower than the usual masses of *N*-acetyllactosamine-type and

Table 1. Identification of the peptides obtained after mild tryptic hydrolysis of Lf. X corresponds to a residue that was not identified by Edman sequencing.

Fragment name	N-ter sequence (Edman)	Peptide (MS-MS)	Domain
PP51	SFQLFGXPP-	Ser285-Arg689	C-domain
PP44	VVWCAVGPEE-	Val335-Arg689	C domain
PP34	APRKNV-	Ala1-Lys280/282/Arg284[a]	N-domain
PP24	SAGXIIPMG-	Ser122-Lys280/282/Arg284[a]	N-domain

[a] Uncertainties remain for the identification of the C-terminal end of the fragment.

Table 2. Identification of the peptides obtained after hydrolysis of Lf by trypsin and chymotrypsin. The M_r values correspond to monoisotopic masses. nd, not determined; Man, mannose; GlcNAc, *N*-acetylglucosamine; RT, retention time.

RT (min)	M_r (experimental)	M_r (calculated)	Sequence	Residues
13.52[a]	959.5 (+57)	959.427 (+57)	CAVGPEEQK (+57)	348–356
15.19	721.5	721.33	RPTEGY	428–433
15.30	649.4	649.32	GGRPTY	652–657
15.35	344.2	344.14	YY	523–524
15.80	585.6	585.37	AVAVVK	435–440
17.70	204.2	204.09	W	nd
17.89	702.5	702.34	KGEADAL	386–392
18.12	581.4	581.27	LGTEY	661–665
18.48	637.3	637.27	NLDGGY	393–398
18.89	2726.0	860.44 (aglycoform)	VKNDTVW (9 Man + 2 GlcNAc)	543–549
19.00	2564.0	860.44 (aglycoform)	VKNDTVW (8 Man + 2 GlcNAc)	543–549
19.23	800.4	800.32	PTYEEY	655–660
19.27	2402.0	860.44 (aglycoform)	VKNDTVW (7 Man + 2 GlcNAc)	543–549
19.49	2240.0	860.44 (aglycoform)	VKNDTVW (6 Man + 2 GlcNAc)	543–550
20.38	419.2	419.18	SAGW	122–125
20.79	746.5	746.40	APVDAFK	237–243
20.80	1222.5	1222.47	ENTNGESTADW	550–560
21.57	666.4	666.32	VDSALY	314–319
22.73	674.3	674.43	RMKKL	25–29
23.30	878.4	878.38	QDGAGDVAF	200–208
23.68	595.3	595.28	ETTVF	211–215
25.13	787.4	787.40	TESLEPL	139–145
26.73	402.3	402.23	VVW	345–347
27.12	404.2	404.19	LSW	136–138
27.50	802.5	802.45	LSKAQEK	271–277
28.28	779.3	779.37	DQYELL	225–230
31.41	1060.4	1060.51	SWTESLEPL	137–145
32.90	674.3	674.33	EDLIW	264–268

[a] Peptide identified as a Cys-containing peptide after reduction and alkylation. It was also observed in its protonated alkylated form $[M + 57 + H]^+$.

oligomannosidic-type structures. Finally, the glycopeptides were discarded by cold ethanol precipitation in order to determine whether the interaction between the mixture of Lf peptides and LSR was due to the amino acid sequences rather than to the glycan moiety. Ligand blots performed on the mixture of these peptides (Fig. 5A) or on the mixture of peptides after elimination of the glycosylated fractions (data not shown) highlighted their ability to bind to LSR in the presence of oleate.

Discussion

Elevated postprandial lipemia following intravenous injection of Lf was shown to be due to accumulation of chylomicron remnants in the circulation and reduced clearance of these particles by the liver [4]. This led to numerous investigations of the molecular mechanisms involved with the objective of identifying the Lf-sensitive liver receptor(s) for these TG-rich lipoproteins. However, Lf has many other biological

activities such as in host defense against infection or immunomodulation and, as a result, displays various affinities for a number of proteins on the cell surface [21,22], which rendered the task of identifying the liver receptor difficult. Indeed, at the hepatic level, there have been studies reporting association of Lf with LRP1, the asialoglycoprotein receptor, as well as heparan sulfate proteoglycans [23,24]. Of these different proteins, LRP1 was a potential candidate as chylomicron remnant receptor, but it was later demonstrated to play a back-up role in clearance of these particles in the absence of the LDL-R [25]. Indeed, the presence of a third pathway other than LRP1 and LDL-R for chylomicron remnants was postulated [25,26]. Furthermore, GST-RAP, a specific inhibitor of LRP, did not appear to modify Lf's effect on β-VLDL binding to liver cells, leading the authors to conclude that the Lf effect was not mediated through LRP nor through the LDL-R [27]. While Lf has been shown to inhibit the binding and uptake of apoE containing lipoproteins, including chylomicron remnants, in the liver [28,29], these studies used cell-based models or tissue extracts without identifying the actual target receptor or performing direct binding studies of Lf with the receptor in a purified state.

We identified LSR as an apoB,E receptor that plays an important role in the removal of TG-rich lipoproteins during the postprandial phase [14]. It serves as lipoprotein receptor only in the presence of FFA, which induces a conformational change to expose a binding site on the receptor for apoB or apoE [11]. We have previously shown that Lf can prevent the binding of lipoproteins to FFA-activated LSR [11]. In this study, we now demonstrate that Lf colocalizes with LSR in Hepa1–6 cells and that Lf binds to purified LSR but, in both cases, only in the presence of oleate and thus only when LSR is in its active form. Furthermore, Lf is able to directly compete with the TG-rich VLDL for binding to LSR. Taken together, this suggests that the previously observed inhibitory effect of Lf on oleate-induced binding of lipoproteins to hepatocytes or hepatic membranes is mediated by the direct interaction of Lf with the receptor. Since during the postprandial phase chylomicron influx into the liver is high leading to increased FFA production by lipolysis, LSR would be in its active form. We would propose then that the elevated postprandial lipemia observed upon Lf treatment *in vivo* is mediated in part by its direct interaction with FFA-activated LSR, thus preventing clearance of chylomicrons and their remnants through the LSR pathway.

We tested whether Lf demonstrated any effect on LSR mRNA or protein levels. Although protein levels did not change significantly, we did observe a small but significant decrease in LSR mRNA in cells incubated with Lf and oleate. Lf has previously been reported to enter the nucleus and induce gene expression via the transcription factor AP-1 [30]. Bioinformatics analysis revealed potential AP-1 response elements in the sequence upstream of the mouse *lsr* gene. Taking into account that AP-1 is involved in the control of genes related to cell survival and apoptosis [31,32] and that a previous study has also reported that LSR expression can be induced by P53 [33], it may be that LSR is in some manner involved in the regulation of the cell cycle. Further investigation is required to examine this question. Nevertheless, because of the rapid effect of Lf on chylomicron remnant clearance, it seems unlikely that this was due to Lf's effect on LSR mRNA or protein levels.

Ligand blot studies using purified LSR revealed that both the C- and N-lobes of Lf were able to bind directly to LSR in the presence of oleate. Furthermore, a peptide mixture resulting from further hydrolysis of both lobes retained the capacity for recognizing the active form of LSR. Since the removal of glycopeptides did not affect the ability of Lf to interact with activated LSR, this suggests that the recognition is based purely on the amino acid sequence. The original hypothesis of the Lf effect was based on the fact that Lf possesses a number of Arg and Arg-Lys clusters on its N-terminal side that resemble the binding site of apoE, which is considered the ligand for the chylomicron remnant receptor. On the basis of chemical modification experiments, previous studies using liver parenchymal cells proposed that Arg residues were crucial for the recognition of Lf and its inhibitory effects on lipoprotein remnants [28,34]. These studies were carried out on human Lf, which possesses a cluster of four Arg residues (positions 2–5) and an Arg/Lys-rich sequence at positions 25–31 (R-X-X-R-K-X-R), similar to that of apoE [4]. However, conflicting results were reported when Huettinger *et al.* [28] observed that digestion of Lf by trypsin that cleaved through the Arg (positions 2–5) cluster but left the helical region near position 35 intact suppressed the inhibitory effect of Lf on the internalization of remnants. On the other hand, Ziere *et al.* [29] demonstrated that truncated Lf lacking the first 14 residues and thus the Arg (positions 2–5) cluster was a more effective competitor than Lf for liver uptake of lipoprotein remnants. Therefore, the importance of the four Arg cluster in Lf's effect on chylomicron remnant clearance remains unclear.

Here, we use purified bovine Lf which, when injected into mice, leads to elevated postprandial

plasma TG levels (Fig. 1B). This is in agreement with previous reports using bovine Lf that have demonstrated inhibition of chylomicron remnant clearance during the postprandial phase [35]. Interestingly, bioinformatics analysis revealed that bovine Lf possesses only an RK sequence instead of the Arg (2–5) cluster described in human Lf, thus clearly excluding a predominant role of this cluster in the binding to LSR. Moreover, the Arg/Lys-rich sequence at positions 25–31 is divergent in the bovine sequence (WRMKK LG) compared with that of human. This observation, along with the fact that both N- and C-terminal lobes of bovine Lf bind to the receptor, suggests that at least two sequences or structural motifs are recognized. Furthermore, given the size of the peptides present in the tryptic/chymotryptic hydrolysate that retain the ability to bind to LSR, our results suggest that this receptor recognizes one or several sequences on Lf rather than conformational features. For the N-lobe, the RMKKL sequence at positions 25–29 is found in the peptide mixture isolated from the tryptic/chymotryptic hydrolysate together with another basic peptide IVKLLSKAQ EKFGK at positions 268–280, while no peptide possessing more than one basic residue and belonging to the C-lobe has been recovered in the hydrolysate, suggesting that determinants of the interaction might involve other features than basic sequences.

In conclusion, this study clearly demonstrates a direct interaction of Lf with the FFA-activated form of hepatic LSR, which prevents the binding of TG-rich lipoproteins to this receptor and therefore could explain the elevated postprandial lipemia and reduced chylomicron remnant clearance observed in rodents treated with Lf. Further experiments are needed to identify the specific peptides of Lf that bind to LSR and to determine whether these peptides, when isolated, have the ability to modulate postprandial lipemia.

Materials and methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich Co. (St Quentin Fallavier, France) unless otherwise indicated. Cell culture media and supplements, Oregon Green 488 dye and ProLong Gold antifade fluormount reagents were obtained from Invitrogen (Alfortville, France). Mouse recombinant leptin was purchased from Merck Chemicals (Darmstadt, Germany). Secondary anti-rabbit horseradish peroxide (HRP) conjugated IgG were acquired from Cell Signaling Technology (Boston, MA, USA).

Purification of proteins

Purification of Lf

Bovine Lf was purified from raw skim milk. First, whey proteins were separated from caseins according to the procedure described in Egito *et al.* [36]. Lf was then purified from the whey proteins by ÄKTA-FPLC chromatography (GE Healthcare, Uppsala, Sweden) by passing sequentially through three Hitrap CM-FF 5/5 cation exchange columns (1.5 × 2.5 cm) equilibrated in 50 mM Tris/HCl buffer, pH 8.0. A linear gradient of NaCl in the same buffer was applied (1 $mL \cdot min^{-1}$) for the elution. For the colocalization experiments, Lf was conjugated with fluorescent probe Alexafluor 488 according to the manufacturer's instructions (Pierce, Thermo Fisher, Illkirch, France). The Lf : dye ratio was 1 : 16 for the reaction at room temperature for approximately 1 h in the dark. The mixture was dialyzed against $NaCl/P_i$ buffer for 48 h and concentrated. Coupling efficiencies were determined to check the amount of fluorescent dye linked to protein (3.17 mol of dye per mol of protein).

Purification of LSR

LSR-enriched solubilized protein fractions were first prepared from rat hepatocyte plasma membrane according to Mann *et al.* [11]. Membrane proteins were solubilized in 20 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 2 mM EDTA, 13 mM Chaps and inhibitor cocktail and fractionated by ÄKTA-FPLC on a Mono-Q HR 5/5 anion exchange column (GE Healthcare). The column was washed with 4 mL Tris/HCl buffer and proteins were eluted with a 13-mL linear gradient of 150–900 mM NaCl in the same buffer (flow rate 0.5 $mL \cdot min^{-1}$). Fractions were collected and placed on ice; the presence of LSR in fractions was verified by immunoblots. The purity of eluted fractions was analyzed by SDS/PAGE. Protein concentrations were determined using a Bradford reagent assay.

Animal studies – effect of Lf on plasma TG level during the postprandial phase

Adult male C57BL/6RJ mice (Janvier Breeding, Le Genest Saint Isle, France) were housed in certified animal facilities on a 12-h light/dark cycle with a mean temperature of 21–22 °C and relative humidity of 50 ± 20%, and were provided with rodent chow diet and water *ad libitum*. Animals were handled in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the use and care of laboratory animals. Mice 14 weeks old were divided into two groups. Lf (2 mg) in $NaCl/P_i$ was injected intravenously (tail vein) to one group of animals while $NaCl/P_i$ was injected to the other group as a control. Immediately after injection of Lf ($t = 0$), the mice were given 300 μL of olive oil by gavage, and samples

of blood from the retro-orbital cavity were collected at $t = 0$ and 1 h into paraoxon-treated tubes to inhibit lipase activity [37]. Blood samples were immediately centrifuged (10 000 ***g***, 5 min at 4 °C) and the plasma was stored at −20 °C. Plasma TG concentrations were analyzed using a colorimetric enzymatic kit (Biomérieux, Craponne, France) according to the manufacturer's instructions.

Cell studies

The mouse hepatoma cell line (Hepa1–6) was obtained from DSMZ (Braunschweig, Germany) and was maintained in DMEM containing 10% fetal bovine serum and 1 mM glutamine as previously described [14]. Cells were seeded in six-well plates and used after 48 h (80–90% confluence). The cells were washed in NaCl/P_i, incubated for 1 h with 10 ng·mL^{-1} leptin in complete DMEM to optimize LSR expression [38] and then treated with 0.8 mM oleate, followed by incubation with Lf in DMEM at 37 °C in a 95% air, 5% CO_2 environment. Cells were placed on ice and washed twice with NaCl/P_i containing 0.2% BSA, followed by two washes with NaCl/P_i.

qPCR analysis

Hepa1–6 cells treated with Lf were scraped in NaCl/P_i, collected and centrifuged to recover the cell pellets and stored at −80 °C. Cell pellets were homogenized in QIAzol lysis reagent according to the manufacturer's instructions. RNA extraction and qPCR experiments were conducted as described by Stenger *et al.* [38]. The housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) was used as a reference. Calculations of relative expression and statistical analysis were performed using RELATIVE EXPRESSION SOFTWARE TOOL (REST) 2009. The results are expressed as the means of three independent experiments.

Western blot analysis

After incubation with Lf, the washed cells were recovered in radioimmunoprecipitation (RIPA) lysate buffer as described previously [14,38]. Protein samples (25 μg) were separated by SDS/PAGE and transferred onto nitrocellulose membrane. Immunoblots were performed using anti-LSR (Sigma, 1/1000 dilution) followed by HRP-conjugated secondary IgG. The bands were revealed by chemiluminescence (GE Healthcare). Actin was used as loading control. Densitometric analysis of autoradiographs was performed using the software IMAGEJ (US National Institute of Health, Bethesda, MD, USA: http://rsbweb.nih.gov/ij/download.html).

Colocalization experiments

Hepa1–6 plated onto coverslips was incubated with recombinant mouse leptin (10 ng·mL^{-1}) for 1 h at 37 °C to optimize LSR expression and then incubated with Alexafluor-488-labelled Lf (2 mg·mL^{-1}) in the presence of 0.8 mM oleate for 20 min at 37 °C. The cells were fixed with 4% paraformaldehyde at room temperature for 15 min and then permeabilized with NaCl/P_i containing 0.1% BSA and 0.1% Triton X-100. The presence of LSR was detected with rabbit anti-LSR IgG (1/100 dilution), followed by incubation with an IgG conjugated with Alexafluor 555 recognizing rabbit IgG (1/2000 dilution; Invitrogen). Finally, the nuclei of cells were labelled by incubating with DAPI (4′,6-diamidino-2-phenylindole, 1/500 dilution). The cells were then mounted onto slides using ProLong Gold antifade fluormount reagent (Invitrogen) and images were taken using a confocal microscope (FV10i Fluoview; Olympus Biotech, Lyon, France).

Ligand blots

Binding of bovine Lf and its hydrolysates to LSR

Purified LSR (20 μg·well^{-1}) was subjected to electrophoresis on a 10% SDS/polyacrylamide gel under non-reducing conditions and transferred to nitrocellulose membrane. After blocking with 3% BSA, nitrocellulose strips were washed and then incubated for 5 min in the presence or absence of 0.8 mM oleate in NaCl/P_i at 37 °C. The strips were incubated for 1 h at 37 °C with Lf or its hydrolysates (2 mg·mL^{-1}). The nitrocellulose strips were incubated with rabbit anti-Lf IgG PA1-40280 (Thermoscientific, Brebieres, France; 1/500 dilution) for 2 h at 37 °C. The LSR–Lf complex was detected after incubation with anti-rabbit IgG HRP-linked IgG (1/2000 dilution), and the protein bands were revealed by chemiluminescence.

Binding of VLDL to LSR in the presence of Lf

VLDL ($d < 1.006$ g·mL^{-1}) was prepared from fresh human plasma as described previously [9]. Purified LSR on nitrocellulose was prepared as described above. After blocking with 3% BSA, the strips were incubated for 30 min at 37 °C with 0.8 mM oleate in the presence of 0.1 M phosphate buffer, 350 mM NaCl and 2 mM EDTA (pH 8.0) as described previously for optimal binding of lipoprotein to LSR [11]. The nitrocellulose strips were then incubated for 1 h at 37 °C with 20 μg·mL^{-1} VLDL protein, in the presence of increasing concentrations of Lf. Following washes with NaCl/P_i containing 0.5% Triton X-100, strips were incubated with rabbit anti-apoB IgG (Santa-Cruz Biotechnology, Heidelberg, Germany) to detect VLDL binding to LSR. Protein bands were detected as described above for the Lf ligand blot. Control experiments revealed no bands in the absence of oleate (data not shown).

Isolation of the N- and C-terminal lobes of Lf

Lyophilized Lf was solubilized in 100 mM Tris/HCl buffer, pH 8.2, containing 25 mM $CaCl_2$, and subjected to mild

hydrolysis by trypsin (EC 3.4.21.4) in the following conditions: 37 °C, 4 h, enzyme/peptide molar ratio 1 : 50. The reaction was stopped by addition of HCl until a pH of 3.0 was achieved. The N-terminal lobe was purified using a procedure adapted from Takayama *et al.* [39]. The Lf hydrolysate (2 mL, 8 mg·mL^{-1}) was loaded onto a TSK SP-5PW column (75 mm × 7.5 mm; Interchim, Montluçon, France) equilibrated with a 50 mM sodium phosphate buffer, pH 7.0. Elution was carried out with a linear gradient of NaCl (0–0.5 M) and 250 mM NH_4OH in the same buffer (flow rate 1 mL·min^{-1}). The C-terminal lobe was purified on a Nucleosil C4 column (150 × 2 mm inner diameter, 5 μm particle size, 10 nm pore size; Cluzeau, Sainte-Foy-la-Grande, France) connected to an Alliance 2690 HPLC system (Waters, Milford, MA, USA). The hydrolysate (0.1 mg·mL^{-1}) was solubilized in water containing 5% acetonitrile and 0.1% trifluoroacetic acid. Elution was carried out by a linear gradient of acetonitrile in water, in the presence of 0.1% trifluoroacetic acid. The fractions were separated by SDS/PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The protein bands were stained with 0.2% Ponceau S Red, excised from the PVDF membrane and stored in 1% acetic acid. Before enzymatic digestion, the band proteins on PVDF membrane were destained with ultra-pure H_2O along with salts and detergents that passed through the PVDF membrane while the protein retained on the PVDF membrane were drawn through the PVDF by absorption forces (ProSorbTM system; Applied Biosystems, Foster City, CA, USA). The protein was digested by trypsin and peptides were submitted to microsequencing by Edman degradation [40] (Service Commun de Séquence des Protéines, Université de Lorraine). Identification of the corresponding protein bands was performed using the BLAST program for protein sequencing http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Mass spectrometry analysis of the N- and C-lobes

Mass spectrometry analysis of the N- and C-lobes was performed as previously described [41]. Electrospray ionization with Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS) analysis was performed in the Laboratoire de Spectrométrie de Masse Biologique et Protéomique of ESPCI-ParisTech (École Supérieure de Physique et de Chimie Industrielles de la Ville de Paris, France). The Lf tryptic hydrolysate was separated by SDS/PAGE and revealed by 0.1% Coomassie Blue R-250 in 2% acetic acid and 50% ethanol. The bands corresponding to the N-lobe and C-lobe were cut and conserved in 1% acetic acid at −20 °C. The excised bands corresponding to C-lobe and N-lobe were digested with trypsin and chymotrypsin, respectively. The generated peptides were applied to an UltiMate® 3000 Nano LC system (Dionex, Amsterdam, Netherlands) coupled to a hybrid linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT; Thermofisher, San Jose, CA, USA). The sample (8 μL injected) was desalted and preconcentrated using a PepMap C18 pre-column (5 × 0.3 mm inner diameter, 3 μm particle size, 10 nm pore size; Dionex) before eluting and separating on a reversed phase analytical column (PepMap C18, 150 × 0.075 mm inner diameter, 3 μm particle size, 10 nm pore size; Dionex). The peptides were eluted with 1% solvent B (90% acetonitrile and 0.1% formic acid in water) in solvent A (2% acetonitrile and 0.1% formic acid in water) for 5 min at a flow rate of 15 μL·min^{-1} in the first step, 5% solvent B in solvent A for 3 min at a flow rate of 300 nL·min^{-1} in the second step and then a linear gradient of 5–25% of solvent B in solvent A for 32 min at a flow rate of 220 nL·min^{-1}. Data were acquired by the LTQ-FT with automatic alternation between MS and MS/MS modes according to Hesse *et al.* [42], with a modified m/z range of 400–2000. Mass accuracy tolerance was set to 10 p.p.m. in MS mode and 0.1 Da in MS/MS mode. Data were processed using MYPROMS 2.7 (Unit 900 INSERM-Mines ParisTech, Paris, France). Two missed cleavages for trypsin, chymotrypsin and fixed modifications such as carbamidomethylation of cysteine residues, oxidation of methionine residues were allowed.

Hydrolysis of the N- and C-terminal lobes of Lf by chymotrypsin: production of Lf smaller fragments

The peptides obtained following tryptic hydrolysis of Lf and accounting for the N- and C-terminal lobes were incubated with chymotrypsin (EC 3.4.21.1) for 15 h at 37 °C (enzyme : peptides = 1 : 200). Reaction was stopped by addition of HCl. Amicon centrifuge filters (Millipore, St-Quentin-en-Yvelines, France) were successively used to isolate peptides of apparent molecular mass lower than 10 and 3 kDa. The samples were then loaded onto a PD-10 column for desalting and lyophilized. To eliminate glycopeptides, the sample was dissolved in ultra-pure water and the glycopeptides were extracted by precipitation with nine volumes of cold ethanol (0 °C).

Reduction and alkylation of peptides containing disulfide bonds

After tryptic and chymotryptic hydrolysis, reduction of disulfide bonds and alkylation of cysteine residues were carried out on a fraction of peptide hydrolysate. A volume of 10 μL of 100 mM NH_4CO_3 was added to 15 μL of hydrolysate solution followed by addition of Tris/HCl buffer (pH 8) to reach a pH of 8.5. Reduction was then achieved by adding 100 mM DL-dithiothreitol (100-fold molar excess over the cysteine content) to the peptide solution and incubated at 37 °C for 1 h. Alkylation was performed by adding 200 mM iodoacetamide (2.5-fold molar excess over

dithiothreitol) and incubating at room temperature in the dark for 45 min.

Mass spectrometry analysis of peptides

Liquid chromatography coupled to mass spectrometry (LC-MS) was performed using a system (ThermoFisher Scientific, San Jose, CA, USA) equipped with an LTQ ion trap as mass analyzer. Chromatographic separation was performed on a C18 Alltima reverse phase column (150 × 2.1 mm inner diameter, 5 μm porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5 × 2.1 mm inner diameter, 5 μm porosity – Grace/Alltech) at 25 °C and with mobile phases consisting of water modified with trifluoroacetic acid (0.1%) for A and acetonitrile modified with trifluoroacetic acid (0.1%) for B. The peptides were eluted using a linear gradient from 5% to 60% of B phase for 60 min at a flow rate of 0.2 $mL{\cdot}min^{-1}$. A double photodiode array and mass detection was performed during the entire run. The mass spectrometric conditions were as follows: electrospray positive ionization mode was used; source gases were set (in arbitrary units per minute) for sheath gas, auxiliary gas and sweep gas at 20, 5 and 5, respectively; capillary temperature was set at 250 °C; capillary voltage at 26 V; and tube lens, split lens and front lens voltages at 90 V, −42 V and −5.75 V, respectively. The ion optics parameters were optimized by automatic tuning using a standard solution of the dipeptide Val-Trp at 0.1 $g{\cdot}L^{-1}$ infused in mobile phase (A/B 50 : 50) at a flow rate of 5 $\mu L{\cdot}min^{-1}$. Full scan MS spectra were repeatedly performed from 100 to 2000 m/z during the time of the run and, also, specific MS2 scans were realized in order to obtain structural information for peptides of interest. First, LC-MS analysis was carried out on tryptic and chymotryptic hydrolysate which allows the characterization of peptides without disulfide bonds. In this case, main peptides were searched on the basis of a UV 215 nm chromatogram and were also observed on MS spectra as their protonated form $[M + H]^+$. In a second step, LC-MS analysis focused on the reduced and alkylated peptide hydrolysate fraction and allowed the characterization of peptides containing disulfide bridges. In this case, alkylated peptides were searched by comparison between UV 215 nm chromatograms of non-treated hydrolysate and reduced/alkylated hydrolysate. Peptides were also observed on MS spectra as their protonated alkylated form $[M + 57 + H]^+$, the additional mass of 57 amu corresponding to the acetamide group carried on the sulfur atom of cysteine.

Statistical analysis

Results are shown as mean ± SD or SEM as indicated in the figure captions. The non-parametric Mann–Whitney U test was used for Fig. 1B, and Student's t test for Fig. 2A. Statistical analysis of the qPCR results was performed using the REST software as described above.

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List of Communications

Publications

1. Stenger C., Hanse M., Pratte D., Mbala M.L., **Akbar S**., Koziel V., Escanyé M.C., Kriem B., Malaplate-Armand C., Olivier J.L., Oster T., Pillot T., Yen F.T. (2010). Upregulation of hepatic lipolysis stimulated lipoprotein receptor by leptin: a potential lever for controlling lipid clearance during the postprandial phase, **FASEB J, 24:4218-4228.**

2. Ahmad N., Girardet J.M., **Akbar S.,** Lanhers M.C., Paris C., Yen F.T., Corbier C. (2012). Lactoferrin and its hydrolysate bind directly to the oleate-activated form of the lipolysis stimulated lipoprotein receptor. **FEBS J, 279:4361-73.**

3. Allouche A., Royer L., **Akbar S.,** Youssef I., Hanse M., Escanyé M.C., Koziel1 V., Olivier J.L., Malaplate-Armand C., Dhaussy A., Huertas A., Pillot T., Yen F.T., Oster T. Long-term dietary fish-oil supplementation prevents amyloid stress in Aβ-injected mice fed a high-fat diet. (Submitted in **J. Nutr.**)

4. Layeghkhavidaki H., Lanhers M.C., **Akbar S.,** Gregory-Pauron L., Oster T., Feidt C., Corbier C., Yen F.T. Inhibitory action of benzo[a]pyrene on hepatic lipoprotein receptors associated with dyslipidemia and weight gain in mice. (Submitted in **Plos One**).

Poster presentations

1. Hanse M., Stenger C., **Akbar S**., Malaplate-Armand C., Olivier J.L., Oster T., Thierry Pillot T., Yen F.T. Peripheral action of leptin on hepatic lipid metabolism during the post-prandial phase: up regulation of hepatic Lipolysis Stimulated Lipoprotein Receptor. 7ème Congrés de Lipidomique (GERLI), 3-6 Octobre 2010, ANGLET-BIARRITZ, France. (*Best poster prize*)
2. Stenger C., Hanse M., **Akbar S**., Yen F.T. Peripheral regulation of hepatic Lipolysis Stimulated Lipoprotein Receptor by leptin. 36th FEBS congress « Biochemistry for tomorrow's medicine », 25-30 Juin 2011, Torino, Italy.
3. **Akbar S**., Stenger C., Hanse M., Oster T., Yen F.T. Effet régulateur de la leptine sur l'expression du *Lipolysis Stimulated Receptor* (LSR). RP2E Seminar, 19 Janvier 2012, Nancy, France.
4. Hanse M., Stenger C., **Akbar S**., Pinçon A., Yen F.T. Link between the Lipolysis Stimulated Receptor and lipogenesis in the liver. "Atherosclerosis, Thrombosis and Vascular Biology", 18-20 Avril 2012, Chicago.
5. Layeghkhavidaki H., Lanhers M.-C., **Akbar S.**, Grova N., Appenzeller B., Feidt C., Corbier C., Yen F.T. Significant weight gain in mice exposed to the pollutant benzo[a]pyrene is associated with changes in hepatic lipoprotein metabolism. 81st European Atherosclerosis Society (EAS) Congress, 02-05 June 2013, Lyon, France.

Résumé : Le maintien de l'homéostasie lipidique est devenu un enjeu majeur de santé publique dans tous les pays, des perturbations du métabolisme des lipoprotéines étant associées à plusieurs affections comme les dyslipidémies, l'obésité et le diabète de type 2 qui peuvent favoriser le développement de maladies cardiovasculaires. De ce fait, il est essentiel d'identifier les mécanismes impliqués dans la régulation du statut lipidique. Le récepteur LSR (*lipolysis stimulated lipoprotein receptor*) est un acteur important du métabolisme hépatique, puisqu'il joue un rôle clé dans la clairance des lipoprotéines à ApoB/ApoE riches en triglycérides durant la période postprandiale. L'objectif de cette étude a donc été de définir les conditions dans lesquelles l'activité hépatique du LSR est régulée. Nous avons tout d'abord montré qu'un traitement *in vitro* par l'acide docosahexaénoïque (DHA) peut augmenter les niveaux de protéine et d'activité LSR dans les cellules d'hépatome de souris Hepa 1-6. En toute cohérence, un régime supplémenté en DHA a conduit chez la souris à enrichir les membranes des cellules sanguines et du foie en cet acide gras, ainsi qu'à élever les niveaux de protéine LSR hépatique. Mais aucune de ces deux études n'a montré de changement au niveau des ARNm. Ceci suggère que les propriétés physicochimiques des membranes aient été modifiées par l'enrichissement en DHA, influant positivement sur le microenvironnement de LSR et son ancrage à la surface de la cellule. Nous avons ensuite cherché à déterminer le rôle du récepteur PPARα activé par les proliférateurs de peroxysomes comme facteur de transcription dans la régulation du gène *lsr*. Une analyse *in silico* nous a permis d'identifier des éléments PPRE de réponse spécifiques aux PPAR dans la région 5' régulatrice du gène humain et de ses homologues de souris et de rat. Des traitements pharmacologiques par des agoniste et antagoniste spécifiques de PPARα nous ont permis de confirmer *in vitro* que ce récepteur est impliqué dans la régulation transcriptionnelle de l'expression du LSR dans les cellules Hepa 1-6. Enfin, une analyse transcriptomique a révélé une diminution de l'expression de PPARα et d'autres gènes impliqués dans le métabolisme lipidique hépatique chez la souris $LSR^{+/-}$ sous régime standard ou riche en graisses, ainsi qu'un profil inflammatoire marqué chez la souris $LSR^{+/-}$ sous régime hyperlipidique. En conclusion, toutes ces études indiquent que l'activité LSR hépatique est sous le contrôle de facteurs nutritionnels capables d'activer divers mécanismes de régulation, faisant du LSR une cible d'intérêt potentiel pour des stratégies nutritionnelles ou thérapeutiques destinées à prévenir ou traiter les dyslipidémies.

Mots-clés: acide docosahexaénoïque, dyslipidémie, métabolisme lipidique hépatique, homéostasie lipidique, récepteur des lipoprotéines activé par la lipolyse (LSR), métabolisme des lipoprotéines, obésité, récepteur activé par les proliférateurs de peroxysomes (PPAR), éléments de réponse aux proliférateurs de peroxysomes (PPRE)

Abstract: The maintenance of lipid homeostasis is considered an important health concern worldwide. Any disturbances in the lipoprotein metabolism can manifest into a number of metabolic disorders including dyslipidemia, obesity and type 2 diabetes mellitus, which ultimately lead to the development of a number of pathologies including cardiovascular and neurodegenerative diseases. Therefore, it is crucial to identify the mechanisms involved in the regulation of lipid status. One of the critical factors implicated in hepatic lipid and lipoprotein metabolism is the lipolysis stimulated lipoprotein receptor (LSR) which plays an important role in the clearance of ApoB/ApoE containing triglyceride-rich lipoproteins during the postprandial phase. In this study, our goal was to identify factors involved in the regulation of hepatic LSR expression. Firstly, we demonstrated that *in vitro* treatment of Hepa 1-6 hepatoma cells with docosahexaenoic acid (DHA) led to an increase in LSR protein levels as well as its activity. Furthermore, the mice placed on the diet supplemented with DHA showed DHA enrichment in liver and erythrocyte membranes, accompanied by an increase in hepatic LSR protein. However, the mRNA levels remained unchanged in both *in vitro* and *in vivo* studies, suggesting that alterations in membrane physicochemical properties due to increased DHA may result in changes in LSR microenvironment that could affect its anchorage at the surface of cell membrane. Specific peroxisome proliferator response elements were identified in the upstream region of human, mouse and rat *lsr* gene by *in silico* analysis. We therefore sought to determine the role of the transcription factor, peroxisome proliferator-activated receptor (PPARα), in LSR regulation. *In vitro* pharmacological studies using PPARα-selective agonist and antagonist agents demonstrated that PPARα is indeed involved in the transcriptional regulation of LSR expression. Furthermore, qPCR array analysis revealed the downregulation of PPARα and various genes involved in hepatic lipid metabolism in $LSR^{+/-}$ mice on standard and high-fat diets together with an increased inflammatory profile in $LSR^{+/-}$ mice on high-fat diet. In conclusion, these studies show that the hepatic LSR activity is controlled by dietary factors that can activate various pathways involved in regulating lipid homeostasis, therefore representing LSR as a potential target for either nutritional or therapeutic strategies towards the prevention or treatment of dyslipidemia.

Key words: docosahexaenoic acid, dyslipidemia, hepatic lipid metabolism, lipid homeostasis, lipolysis stimulated lipoprotein receptor, lipoprotein metabolism, obesity, peroxisome proliferator-activated receptor, peroxisome proliferator response elements