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Examining the diverse contributions of EL to systemic lipid metabolism

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Abstract

English version

Endothelial Lipase (EL) is the most recently identified member of the plasma triglyceride lipase family. EL has a higher specificity for Phospholipids (PL) compared to triglycerides (TG) and has been shown to be a major regulator of HDL-C levels both *in vitro* and *in vivo*. More recent data showed that EL also promotes the catabolism of apoB-containing lipoproteins. In humans, genetic variants for EL are associated with increased HDL-C levels but these changes do not translate into a reduction in cardiovascular disease risk. Early clinical studies demonstrated a strong correlation between EL mass and several traits of metabolic syndrome such as body mass index (BMI), visceral adiposity, inflammation, insulin resistance and atherogenic lipoprotein levels. The causal relationship linking EL to one of multiple of these traits is still unknown. To date, whether increased EL levels may be causal to the observed phenotype or a secondary outcome has not been established.

The aim of this thesis is to assess the role of EL in TG-rich lipoprotein (TRL) metabolism and to investigate the relationship between EL activity, hepatic lipid metabolism and the development of key traits of metabolic syndrome *in vivo*. In order to compare the onset and severity of metabolic syndrome traits in the presence or absence of EL activity, WT and EL deficient mice (EL-KO) were chronically fed a high fat diet. Results demonstrated that EL deficient mice gained significantly more weight and they developed a more severe glucose intolerance and meta-inflammatory state. The appearance of these traits was accompanied by a severe dyslipidemia that was once again more pronounced in EL-KO mice, compared to WT. The most evident difference between EL-deficient mice and control group was the disproportioned diet-induced increase in TG. This phenotype originated from accumulation of post-prandial TG-rich lipoproteins. *In vivo* kinetic experiments demonstrated that the lipolytic clearance of TG-labeled TRL was significantly impaired in EL-KO mice without any effect on TG secretion. The *in vitro* determination of EL activity towards radiolabeled-TRLs suggested that EL may function as a Lipoprotein Lipase (LPL) activity enhancer. As a consequence, EL may have

a major role in reducing TG levels during postprandial hyperlipemia and this effect may be mediated by both direct lipolysis and facilitation of LPL.

Finally, the assessment of liver function revealed that the dietary treatment induced severe steatosis in EL-KO mice. Consistent with reduced lipolysis, the TG-derived FA uptake was impaired in EL-KO mice and this was associated with the upregulation of lipogenic genes and the development of abnormalities in the FA composition.

In conclusion, data from this thesis support a model by which EL is required for efficient triglyceride-rich lipoprotein clearance and the absence of this function impairs hepatic fat intake and results in compensatory *de novo* lipogenesis, which may contribute to systemic obesity and insulin resistance.

Abstract

Versione in Lingua Italiana

La Lipasi Endoteliale (EL) è l'ultimo membro identificato della famiglia delle lipasi plasmatiche dei trigliceridi (plasma triglyceride lipase). EL ha una maggior specificità per i fosfolipidi (PL) che trigliceridi (TG) ed è un enzima chiave nella regolazione dei livelli di HDL sia *in vitro* che *in vivo*. Studi più recenti hanno indicato che EL è in anche grado di promuovere il catabolismo delle lipoproteine contenenti apoB. Nell'uomo, varianti nel gene che codifica per EL, sono state associate a più elevati livelli di colesterolo HDL ma sorprendentemente ciò non si traduce in un ridotto rischio cardiovascolare. Studi clinici hanno evidenziato che la concentrazione e attività di EL sono positivamente associate alla severità dei tratti tipici della sindrome metabolica come indice di massa corporea, obesità centrale, infiammazione, insulino resistenza e profilo lipoproteico più aterogenico. Quale sia il nesso causale di questa associazione non è noto e ad oggi non è possibile stabilire se elevati livelli di EL siano alla base dell'eziologia del profilo osservato o se, al contrario, siano un tratto secondario.

Scopo di questa tesi è di stabilire quale sia il ruolo di EL nel metabolismo delle lipoproteine ricche in trigliceridi (TRL) e di investigare la relazione tra attività di EL, metabolismo lipidico epatico e sviluppo dei tratti caratteristici della sindrome metabolica *in vivo*. Al fine di poter comparare non solo la severità ma anche il tempo di insorgenza della sindrome metabolica, in presenza o assenza di EL, sono stati utilizzati topi controllo (*wild type*, WT) e topi con deficit di EL (EL-KO), sottoposti ad una dieta ricca di grassi a lungo termine. I risultati hanno dimostrato che i topi EL-KO accumulano significativamente più peso rispetto ai WT, sviluppano una più severa intolleranza al glucosio, e uno stato metainfiammatorio. La comparsa di questi tratti è accompagnata da una severa dislipidemia, ancora una volta più marcata nei topi EL-KO rispetto ai WT. La differenza più significativa, tuttavia, è lo sproporzionato aumento dei livelli di TG in risposta alla dieta. All'origine di questo fenotipo vi è l'accumulo di lipoproteine post-prandiali ricche di TG. Esperimenti cinetici *in vivo* hanno dimostrato che la clearance di TRL radiomarcate è molto ridotta nei topi EL-KO e questo fenomeno è dovuto a

una ridotta lipolisi, mentre la secrezione epatica di TG non è affetta. Quando l'attività di EL nei confronti delle TRL marcate è stata testata *in vitro*, è emerso che EL potrebbe fungere da facilitatore dell'attività della lipasi lipoproteica (LPL). Di conseguenza, EL potrebbe rivestire un ruolo importante nel ridurre I livelli di TG nel contesto dell'iperlipemia postprandiale e questo effetto potrebbe essere mediato sia da un'attività lipolitica diretta, sia dalla facilitazione dell'attività lipolitica di LPL. Infine, la valutazione della funzionalità epatica ha rivelato che i topi EL-KO sviluppano una franca steatosi. La capacità del fegato di captare gli acidi grassi derivanti dai TG, è ridotta nei topi con deficit di EL ed è stato osservato un parallelo aumento dei livelli di espressione di geni coinvolti nella lipogenesi e una drammatica alterazione del contenuto di acidi grassi.

In conclusione, i dati raccolti in questa tesi supportano l'ipotesi che EL sia essenziale per un'efficiente clearance delle TRL e, in sua assenza, via sia un ridotto flusso di acidi grassi al fegato. Ciò indurrebbe un aumento compensatorio della lipogenesi e potrebbe ulteriormente contribuire all'insorgenza di obesità e insulino resistenza.

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Introduction

1 Plasma Lipases: at the crossroads between lipoprotein and tissue lipid metabolism

The Triglyceride lipase gene family includes several genes that share a high degree of homology (*LIPC,LIPG, LPL, PS-PLA1, LIPH, PLRP-2, PL, PLRP-1)* [1]. Although the cell expression pattern and the substrate specificity highly differ among the different members, all the enzymes are able to hydrolyze ester bonds of hydrophobic lipids [1]. The function of each member and impact on the overall physiology is highly dependent on the site of expression. Three of the cited genes*, LIPC, LIPG* and *LPL*, encode enzymes that function in the plasma (Hepatic Lipase, Endothelial Lipase and Lipoprotein lipase, respectively) and catalyze the hydrolysis of lipids on circulating lipoproteins [2-4]. The released fatty acids are then available for uptake by peripheral tissues where they can exert trophic or modulatory functions. As a result, plasma lipase activities affect not only the lipoprotein metabolism but also determine the availability of Free Fatty Acids (FFA) in peripheral tissues thus influencing systemic lipid homeostasis.

1.1 Overview of Plasma Lipoprotein Metabolism

Lipoproteins are heterogeneous lipid-protein complexes. They exert several functions including the transport of hydrophobic lipids through the circulation [5]. The two more clinically relevant non-polar lipids are cholesterol and Triglycerides (TG) [6]. Epidemiological evidence, in fact, has associated the levels of different plasma lipids and lipoproteins with the risk of developing Cardiovascular Disease (CVD)[5].

Physiologically, cholesterol is an essential component of lipid membranes, and plays a major role in the synthesis of steroid hormones, bile acids, and vitamin D [7]. The cholesterol membrane content affects the properties of cell membranes including fluidity and depolarization capacity [8]. The major sources of cholesterol are endogenous synthesis and exogenous uptake from the diet. The first pathway is quantitatively more relevant (~80% of total circulating cholesterol) [9, 10].

TG are macromolecules containing three fatty acid (FA) molecules esterified

to a glycerol backbone. TG represents the main source of energy for peripheral tissues and the main energy storage form for adipose tissue [9].

Plasma lipoprotein classification

After *ex novo* synthesis or absorption from dietary source, cholesterol and TG are incorporated in macromolecular complexes named lipoproteins. Lipoproteins are composed of highly hydrophobic lipid cores containing non-polar lipids (mainly TG and esterified cholesterol, CE) and an outer layer containing polar phospholipids, non-esterified cholesterol (UC) and several apolipoproteins. Lipoproteins represent the vehicle through which different lipid species can be delivered to and taken up by target tissues. Lipoproteins are extremely heterogeneous in size, shape, lipid and apolipoprotein composition. Several classification methods have been suggested, that highlight the different peculiarities of these macromolecules. The clinically most accepted classification methods distinguish lipoproteins based on their surface charge (electrophoretic mobility) and density. Based on these criteria lipoproteins can be classified into five categories: chylomicrons (CM), very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) low-density lipoproteins (LDL) and high-density lipoprotein (HDL) (Table I-1) [11].

Lipoprotein	Density (g/mL)	Size (nm)	Electrophoretic mobility
Chylomicrons (CM)	<0.93	75-1200	origin
VLDL	0.93-1.006	30-80	pre-β
IDL	1.006-1.019	25-35	β
LDL	1.019-1.063	18-25	β
HDL	1.063-1.210	5-12	α

Table I-1 Classification of the major human plasma lipoproteins

Plasma lipoprotein composition

Plasma lipoproteins are characterized by a very different lipid and protein composition, that affects their physical and chemical properties and influences their ability to interact with specific receptors (table I-2).

Chylomicrons

Chylomicrons are large, TG-rich lipoproteins secreted by the intestine. Given their large size and high TG content, CM are characterized by a very low density (<0.93 g/mL). They are responsible for the transport of diet-derived cholesterol and TG to peripheral tissues. CM contain a variety of apolipoproteins (A-I, A-II, A-IV, A-V, B48, C-II, C-III, E) [12]. The structurally more relevant apolipoprotein is apoB-48, which is secreted by enterocytes at a ratio of 1:1 molecule of CM [12]. After being released in the circulation CM undergo an extensive remodeling by peripheral cells. The interaction between apoC-II and lipolytic enzymes such as Lipoprotein Lipase (LPL) leads to the hydrolysis of TG and release of free fatty acids [2]. FFA can then be taken up by peripheral cells through membrane transporters. The removal of TG from CM results in the formation of smaller particles, that progressively acquire Cholesterol Esters and apoE from circulating HDL. The resulting lipoproteins are characterized by a wide compositional and density range and they are collectively named Chylomicron Remnants.

Very-Low Density Lipoproteins

VLDL are TG rich lipoproteins produced by the liver. They are characterized by very low density (table I-1) and a relatively low protein content (8-10%). Their main protein constituent is apoB-100 but they also contain apoE and C-peptides. VLDL are synthesized by the liver through the activity of microsomal triglyceride transfer protein (MTP) [12, 13]. MTP catalyzes the assembling of apoB-100 with endogenous TG. This initial core can be further enriched with apoE and C-peptides [13]. The main function of VLDL is to transport endogenous TG from the liver to peripheral cells, where they can be depleted of TG and remodeled into Intermediate Density Lipoproteins (IDL).

Intermediate-Density lipoproteins

IDL are lipoproteins with a density range of 1.006-1.1019 g/mL. They have a very short half-life and in healthy subjects their concentration is normally very low. They result from the partial catabolism of VLDL. They undergo further remodeling, that leads to the formation of LDL. This process is associated with the activity of Hepatic Lipase (HL) and hepatic triglyceride hydrolase (H-TGL) [3]. IDL can be taken up by the liver through the LDL receptor-related-protein (LRP) or LDL receptor (LDL-R).

Low Density Lipoproteins

LDL are lipoproteins with a hydrated density of 1.019-1.063 g/mL and a particle size of 20-30 nm. They contain ~20% and 80 % of protein and lipids, respectively. The lipid core is enriched in Cholesterol esters whereas the main protein component is apoB-100 (1:1 molecule:particle). ApoB-100 is the ligand for the LDL-R, a receptor highly expressed on the membrane of hepatocytes and other cell types [13].

High Density Lipoproteins

HDL are an heterogeneous class of lipoproteins. They are believed to have several functions including antioxidant, ant-inflammatory and anti-thrombotic properties [14]. Traditionally they are believed to play a key role in the Reverse Cholesterol Transport, the process by which excess cholesterol is transported from peripheral cells to the liver for excretion in the bile [15]. HDL particles are enriched with cholesterol and phospholipids. They contain several different apolipoproteins including apoA-I, A-II, A-IV, C-I, C-II, C-III and apo-E. ApoA-I is responsible for the structural stabilization of the core and it is secreted by liver and intestine in the very early phases of HDL generation [16].

Lipoprotein	CM	VLDL	IDL	LDL	HDL
Triglycerides (%)	80-95	45-65	15-35	4-8	2-7
Phospholipids (%)	3-6	15-20	25-30	18-24	26-32
Free Cholesterol (%)	1-3	4-8	6-8	6-8	3-5
Esterified Cholesterol (%)	2-4	16-22	18-30	45-50	15-20
Proteins (%)	1-2	6-10	15-20	18-22	45-55
Major apolipoproteins	A-I, A-IV, B, C-I, C-III, E	B, E, C-I, C-II, C-III	B, E	B	A-I, A-II, E

Table I-2 Lipid and apolipoprotein composition of major plasma lipoproteins

Pathways of plasma lipoprotein metabolism

Lipoprotein metabolism is traditionally described as an exogenous pathway, endogenous pathway and reverse cholesterol transport. The exogenous pathway is the process by which lipids from dietary source are transported from the intestine to the liver. The endogenous pathway is the pathway of transport of *de-novo* synthesized lipids from hepatocytes to peripheral cells. Finally, the reverse cholesterol transport is the retrograde transport of cholesterol in excess from peripheral cells to the liver (Figure I-1).

The exogenous pathway

In the fed state, lipids from the diet are absorbed by the intestinal cells. Dietary TG are hydrolyzed by pancreatic and intestinal lipases in the gastrointestinal tract so that enterocytes mainly absorb them in the form of FFA and monoacylglycerol. Cholesterol, in turn is absorbed in the esterified form and hydrolyzed into UC and FFA in the enterocyte. FFA, MAG and UC are re-esterified into TG and assembled with apoB-48. This process is

catalyzed by the microsomal triglyceride transfer protein (MTTP) and it is the key step in CM formation [17]. CM are released in the lymphatic system and from there to plasma. As soon as they reach the vascular compartment CM interact with lipases facing the vascular lumen. Lipases, in particular Lipoprotein Lipase (LPL), extensively hydrolyze TG and release FFA that can be taken up by peripheral tissues such as muscle and adipose tissue via FA transporters (eg Fatty acid transport proteins, FATPs and CD36) [2, 18]. Intracellularly FFA can undergo oxidation or re-esterification. As a consequence of TG hydrolysis, CM become smaller in size, progressively acquire CE and apoE and, in turn they transfer apoA and C peptides to HDL. The resulting particles are named CM remnants and they can be cleared by the liver via LDL-R, LRP and syndecan-4 receptor pathways. The binding is mediated by apoE and leads to the internalization of the whole remnant particle [19, 20].

The endogenous pathway

The first step in the endogenous pathway is the formation of VLDL particles. Hepatocytes assemble TG into VLDL on apoB-100 backbone and then secrete them into plasma [13]. This process is mediated by MTP and it is highly dependent on the intracellular TG availability. After secretion, VLDL particles are transported to peripheral tissues where, similar to CM, the TG is hydrolyzed by vascular lipases, in particular LPL. This process leads to the formation of IDL [2]. IDL are relatively more enriched in apoE and CE and can be partially taken up by the liver. The majority of IDL, anyway undergo further metabolism, leading to the formation of LDL. Hepatic lipase (HL) progressively hydrolyzes TG in IDL and this process is accompanied by the transfer of most of the exchangeable apolipoproteins to other particles [21]. The result is the generation of LDL particles, characterized by a CE core and mainly containing apoB-100.

LDL metabolism is highly dependent on intracellular cholesterol trafficking and plasma transport. These two components determine the overall balance between LDL formation from TG-rich lipoproteins and LDL clearance. Approximatively 70 % of the LDL particles are cleared by LDL-R in the liver

[22]. LDL-R is activated by apoB-100 and apoE and promotes the endocytosis of LDL. The levels of active LDL receptor are regulated by the intracellular cholesterol content through different pathways at a transcriptional and post-transcriptional level [22]. One mechanism involves the activation of SREBP (Sterol regulatory Element Binding Protein) transcription factors. When cholesterol levels are low, SREBs are cleaved and translocated from the endoplasmic reticulum to the nucleus, where they actively promote the transcription of LDL-R and HMG-CoA reductase, the key enzyme in de-novo cholesterol synthesis [23]. On the other hand, when cholesterol levels are high SREBPs are inactive and oxidized products of cholesterol metabolism stimulate the activation of Liver X Receptors (LXRs). LXRs promote the expression of IDOL (inducible degrader of the LDLR), a E3-ubiquitin ligase that induces ubiquitination and degradation of the LDLR [24]. Finally, a recently discovered post-transcriptional regulatory mechanism involves PCSK9 (proprotein convertase subtilisin/kexin type 9). PCSK9 is a secreted protein that binds to the extracellular domain of LDL-R and triggers its translocation to the lysosomes [25].

Reverse Cholesterol transport

The HDL-mediated removal of cholesterol from peripheral cells and its transport to the liver for excretion through the bile is named reverse cholesterol transport (RCT). Since the accumulation of cholesterol in the arterial wall and arterial macrophages is believed to be a key step in the development of atherosclerosis, RCT is considered an anti-atherogenic process [26].

Nascent discoidal HDL are secreted by liver and intestine in an ABCA1-dependent fashion. This process consists of the synthesis of apoA-I that is readily lipidated with phospholipids and free cholesterol. These lipid-poor particles can acquire free cholesterol from other peripheral cells through a process named cell cholesterol efflux [27]. After secretion, small HDL particles are further remodeled by several enzymes and transfer proteins.

A key enzyme in HDL maturation is Lecithin:Cholesterol Acyl Transferase

(LCAT), which is responsible for the esterification of cholesterol in plasma. Its preferred substrate is HDL and it uses apoA-I as cofactor [28, 29]. As result LCAT promotes the transition of HDL from small poorly lipidated particles to spherical CE enriched lipoproteins. Mature HDL can interact with Cholesteryl Ester Transfer Protein (CETP), that promotes the exchange of CE and TG from HDL and TG-rich lipoproteins, respectively [29].

Furthermore, Phospholipid Transfer Protein (PLTP) promotes the exchange of surface components (phospholipids, cholesterol and apolipoproteins) from triglyceride-rich lipoproteins to HDL [29].

The TG-rich HDL particles can be hydrolyzed by lipases (LPL, HL and endothelial lipase, EL) in the vascular compartment. This process destabilizes the HDL structure, leading to conversion of mature particles into small particles [16]. During this process, the apolipoprotein component, and notably apoA-I, can be released in plasma in a very poorly lipidated or free form. These small particles are then cleared by the kidney. CE-rich HDL, finally can be taken up by hepatocytes via Scavenger receptor class B member 1 (SR-BI) [29].

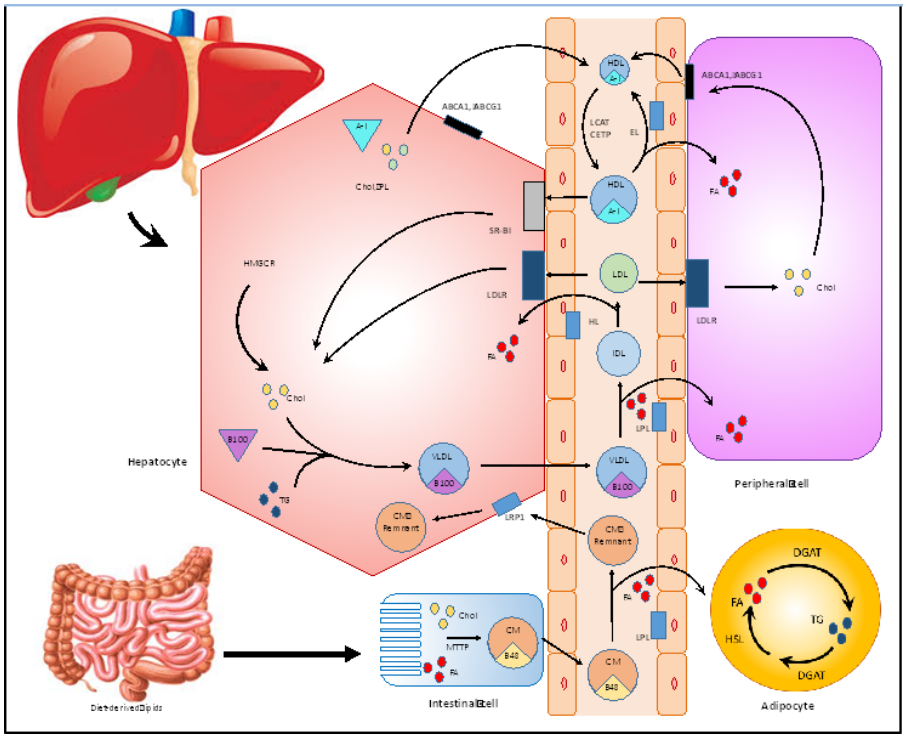

Figure I-1 Schematic of plasma lipoprotein metabolism

Lipids from dietary source are absorbed by the intestine and hydrolyzed into FA and cholesterol. They are then re-esterified into TG and CE and packed into chylomicrons with apoB48 by MTTP. Chylomicrons are firstly secreted into the lymph and then released into the blood stream. CM-associated TG are hydrolyzed by LPL thus providing FFA for peripheral cells. In adipocytes, TG are re-synthesized by acyl CoA:diacylglycerol acyltransferase (DGAT) for energy storage. During fasting TG can be hydrolyzed by adipose TG lipase (ATGL) and hormone sensitive lipase (HSL). Chylomicron remnants (CMRs) are internalized into the liver by LRP1. In the liver triglycerides and cholesterol (either taken up from the circulation or synthesized ex-novo) are combined with apoB100 to form VLDL particles. VLDL are secreted into the circulation where their TG can be hydrolyzed by LPL. This process is accompanied by the remodeling of VLDL into IDL. IDL are hydrolyzed by hepatic lipase (HL) thus generating LDL particles. LDL deliver cholesterol to the peripheral tissues and they are finally cleared from the circulation via LDLR pathway. The retrograde transport of cholesterol from peripheral cells to the liver is mediated by HDL. HDL are generated in the liver in an ABCA-dependent fashion. HDL interact with ATP-binding cassette A1 (ABCA1) and ABCG1 transporters located at the membrane of peripheral cells, thus promoting the efflux of cholesterol. HDL are further remodeled by LCAT and CETP. This process leads to the formation of mature CE enriched particles that can interact with EL and SR-BI. EL promotes the hydrolysis of HDL-PL thus generating smaller particles that can further acquire lipids whereas SR-BI catalyzes the selective uptake of HDL-CE.

1.2 Tissue Fatty acid metabolism

Peripheral tissues use lipoprotein-associated TG as source of FA for β-oxidation and for long term energy-storage. These two processes are highly regulated and their relative utilization depends on the features of the tissue and on the metabolic state (fasted vs fed conditions)[30]. Three organs are capable of storing TG and hydrolyzing them to release FA: adipose tissue, skeletal muscle and the liver. In fed conditions, dietary TG is absorbed by the intestine and released in the circulation in CMs [30]. CM deliver TG to peripheral tissues where it can be hydrolyzed into FFA. The quantitatively more relevant hydrolysis process happens in tissues that express Lipoprotein Lipase (LPL) [31]. In the postprandial state, there is a high availability of nutrients from the diet that exceeds the tissue energetic needs. In this phase, LPL expression is highly increased in the adipose tissue [2]. LPL hydrolyzes TG and releases FA that are mainly taken up by adipocytes, where they can be re-esterified and stored in the form of TG. In these conditions hepatocytes respond to the insulin stimulation by down-regulating the production of VLDL.

During fasting conditions, on contrary, the adipose tissue actively hydrolyses TG and release FFAs that are transported in complex with albumin and serve as substrates for β-oxidation in skeletal and heart muscles [30]. At the same time, the liver synthesizes VLDL that can provide an additional source of TG and FA to peripheral tissues.

Liver

The healthy liver contains typically 1-10% of fat by weight. The lipid sources in the liver are TG from CM remnants and FFA derived from peripheral lipolysis (mainly occurring in the adipose tissue during the fasting state). In physiological conditions, the adult liver does not express LPL but expresses high levels of Hepatic lipase (HL) [32]. HL has higher affinity for smaller particles (such as IDL, LDL and HDL) and shows a mixed TG and Phospholipid lipase activity [33].

Mechanisms of FA uptake

The uptake of released FFA is mediated by membrane transporters. The main FFA transporters are: FA Transport Protein (FATP), CD36, Caveolins, and FA-Binding Protein (FABP) [34].

FATP

FATP exists in 6 different isoforms [35]. All of the different variants share a common motif and fatty acylCoA synthase function. The two most abundant isoforms in the liver are FATP2 and FATP5, which are responsible for the translocation and activation of FA [36, 37]. The result of FATP activity is the activation of incoming FA through the conversion to CoA thioesters. These derivatives are thus available for further metabolism.

CD36

FAT/CD36 (fatty acid translocase / cluster of differentiation 36), is an integral membrane protein that binds different ligands including lipoproteins phospholipids and long chain fatty acids. CD36 has been isolated in several cell types including macrophages, adipocytes, myocytes, enterocytes, and hepatocytes. CD36 is one of the best characterized FA transporters. The regulation of CD36 activity is this dependent on its translocation from intracellular compartments to the plasma membrane [38]. This process is promoted by insulin signaling and by the activation of other transcription factors such as Forkhead box protein O1 (FoxO1) [31]. Several transcription factors have been directly associated with increased CD36 expression (liver X receptor, LXR, pregnane X receptor, and PPARγ) [31].

Caveolins

The Caveolin family comprises three membrane integral proteins named Caveolin 1, 2, and 3 [39]. They are located in the caveolae, membrane regions characterized by a particular lipid composition and enriched in certainproteins. Caveolins have been associated with receptor-independent endocytosis of several molecules [39]. Through this process caveolins are believed to promote the uptake of TG and FA from the circulation. Although the mechanism has not been fully clarified, Caveolin 1 knockout mice

exhibited a reduced TG accumulation in the liver [40].

FABPs

FABPs are an heterogeneous group of proteins that bind lipophilic ligands including FA [41]. Among the different isoforms FABP4 and 5 are highly expressed in adipocytes and macrophages and to a lesser extent hepatocytes [41]. FABPs are located in cellular membranes where they promote the transport of FA to specific cellular organelles (nucleus, mitochondrion, endoplasmic reticulum and lipid droplets). Depending on the localization FABPs can promote either β-oxidation (e.g. mitochondrion) or lipid storage (e.g lipid droplets) [42, 43]. The physiological relevance of FABP4 in the liver is unclear but it has been reported a positive association between FABP4 levels and Non-Alcoholic Fatty Liver disease (NAFLD) [44]. The expression of FABPs is under the control of of PPARγ and it is positively regulated by insulin signaling.

De novo Lipogenesis

In addition to the ability to take up circulating TG-rich lipoproteins, hepatic cells can synthetize FA *ex novo* from glucose and other carbohydrate precursors [45, 46]. This process, named *de novo* Lipogenesis (DNL) consists of the synthesis of FA from acetyl-CoA subunits. The resulting FA can then be esterified to the glycerol backbone thus generating TG [47](Figure I-2).

The first step in the DNL is the production of acetyl-CoA from citrate. This step is catalyzed by ATP-citrate lyase (ACL) [48]. AcCo-A is then converted to malonyl-CoA by acetyl CoA carboxylase (ACC) [49].

The newly formed malonyl-CoA can be condensated with acetyl-coA in a reaction catalyzed by fatty acid synthase (FAS). This multifunctional cytosolic protein homodimerizes into the active form and catalyzes the elongation of the nascent FA by multiples of two carbons. The final product of FAS activity is typically palmitate (C16:0) [50].

Fatty acids derived from the FAS reaction can be further elongated and desaturated by other enzymes all located in the endoplasmic reticulum. Fatty

acid elongation is catalyzed by Elovl family members (elongation of very long-chain fatty acid) [51]. There are 7 Elovl isoforms, with different tissue and substrate specificity [51]. Elovl6 is believed to be the main player in the hepatic elongation of fatty acids. The most common substrates are fatty acids consisting of 12, 14 or 16 carbons that derive from the FAS activity or are absorbed from the diet. The further desaturation of FA is mediated by Stearoyl-CoA desaturases (SCDs) [52]. Their activity introduces a single desaturation in Δ9 position of saturated fatty acids. Although seven isoforms of SCDs have been identified, SCD-1 is the most abundant in liver and adipose tissue [52].

Regulation of De novo Lipogenesis

De novo lipogenesis is a process regulated at multiple levels. In the postprandial phase, higher levels of insulin activate the transcription factors LXR (liver X Receptor), and SREBP1c (sterol regulatory element-binding protein 1c) [23, 46]. At the same time, increasing intracellular levels of glucose activate the carbohydrate-responsive element- binding protein (ChREBP) [46]. These transcription factors altogether are responsible for the promotion of glycolyisis, activation of Krebs cycle, and lipogenesis.

Another family of transcriptions factors highly involved in the regulation of insulin metabolism and FA utilization is peroxisome proliferator-activated receptors (PPARs). PPARγ is the master regulator of de novo lipogenesis whereas PPARα exerts opposite functions, inducing and increase in FA utilization via β-oxidation [53-55].

LXR

The liver X receptors (LXRα and LXR β) are ligand-activated nuclear transcription factors. LXRα is mainly expressed in the liver, adipose tissue, and intestine, whereas LXRβ is ubiquitous [56, 57].

Their natural ligands are oxysterols and other cholesterol metabolites. Physiologically LXRs act as sensors for cholesterol overload and promote the transcription of genes involved in intestinal cholesterol absorption, reverse

cholesterol transport, bile acids synthesis and glucose metabolism [56]. LXR is able to increase the expression of genes involved in HDL lipidation and reverse cholesterol transport such as ABCA1, ABCG1, ABCG5, ABCG8, apoE and PLTP [56,59]. On the other hand, LXR activation upregulates the expression of SREBP1c, leading to an increase in fatty acid synthesis and TG plasma secretion [58, 59].

SREBPs

SREBPs are a family of transcription factors that promote the activation of enzymes required for the synthesis of cholesterol, FA, TG and phospholipids [23]. SREBPs are firstly synthesized in the Endoplasmic reticulum as precursors [23]. When activated, SREBPs are progressively cleaved and the nuclear form of SREBP is translocated into the nucleus where it promotes the transcription of its target genes [60-64]. Three different SREBP proteins have been isolated in humans. SREBP-1a and 1c (encoded by the SREBF-1 gene) and SREBP-2 (encoded by SREBF-2). SREBP-1c isoform is highly expressed in liver, adipose tissue and skeletal muscle, whereas SREBP-1a is predominant in intestinal cells [65]. Different SREBP family members are able to activate the same genes, indicating a high degree of redundancy in the function of these transcription factors. Nevertheless, SREBP-1a seems to be more selective in inducing the expression of genes involved in cholesterol and FA synthesis (HMG-CoA synthase, HMG-CoA reductase, ACC, FAS, SCD-1) whereas SREBP-1c seems to be more selective for lipogenic genes [23]. At the transcriptional level, SREBP1c transcription is induced by insulin and LXRα [23]. When activated, SREBP1c promotes transcription of selected target genes that include ACC, FAS, and SCD1. The overall results of SREBP1c activation is thus *de novo* lipogenesis.

ChREBP

The carbohydrate-responsive element- binding protein (ChREBP) is a transcription factor activated by increasing intracellular glucose levels [66]. Several glycolysis intermediates are able to promote ChREBP activation. Activated ChREBP translocates into the nucleus where it binds Carbohydrate response elements (ChoRE) and promotes the transcription of selected genes

including pyruvate kinase, FAS, ACC and SCD1 [66, 67].

PPARs

PPARs are ligand-inducible transcription factors [68]. Three PPARs have been isolated in mammalian cells: PPARα , PPARδ and PPARγ [68]. PPARs are mainly activated by binding with dietary fatty acids. Upon activation, PPARs heterodimerize with retinoid X receptor (RXR) and bind to PPAR-responsive regulatory elements. These events trigger the expression of genes involved in adipogenesis and lipid metabolism.

Different PPAR isoforms have specific functions and their activation results in different metabolic processes. PPARα, the first PPAR to be identified, is expressed predominantly in the liver, heart and brown adipose tissue (BAT) and is the main promoter of fatty acid oxidation [69]. PPARδ is ubiquitously expressed. Its function is similar to that of PPARα but its higher abundance in the skeletal muscle, liver and heart confers it a primary role in the metabolic homeostasis of those tissues [70]. PPARγ is highly expressed in white adipose tissue (WAT) and BAT, where it is a master regulator of adipogenesis as well as a potent modulator of whole-body lipid metabolism and insulin sensitivity [71, 72].

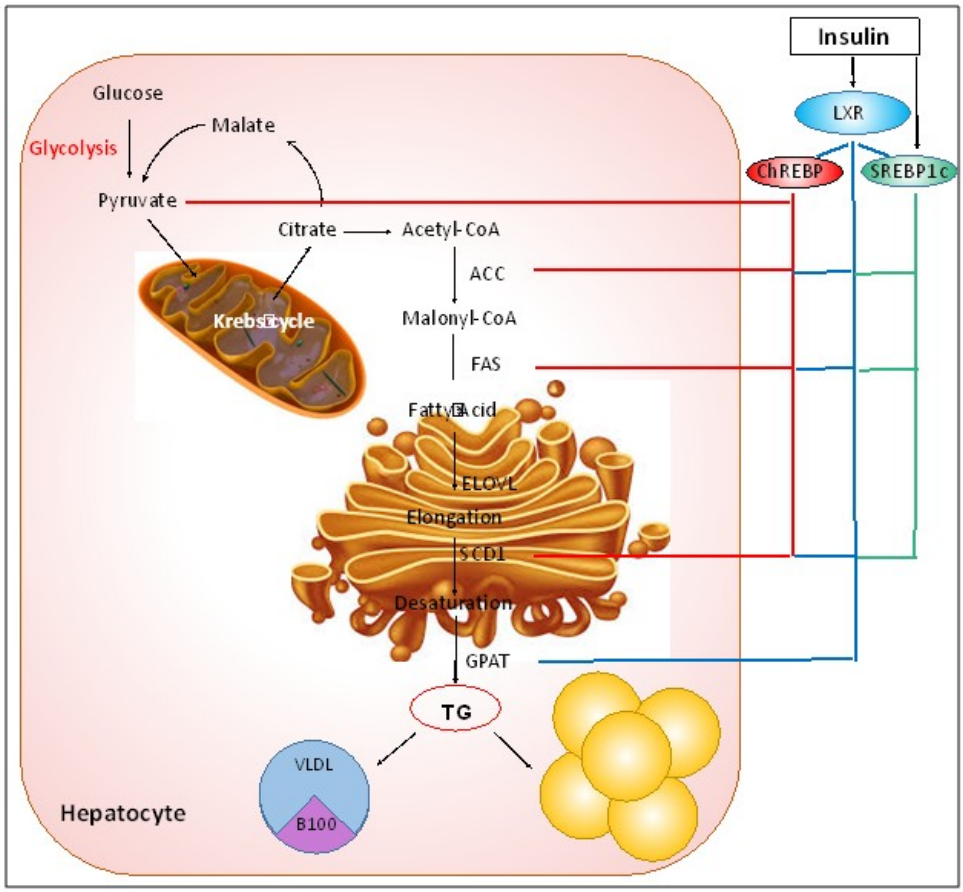

Figure I-2 Regulation of de novo lipogenesis in the liver by ChREBP, SREBP1c and LXR.

In the postprandial state, insulin plasma levels increase. Insulin binding to its receptor promotes the translocation and activation of liver X receptors (LXR). LXR induces the transcription of lipogenic genes including sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate-responsive element-binding protein (ChREBP). The activation of these regulatory genes promotes the transcription of lipogenic genes including acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), elongation of long-chain fatty acids family member 6 (ELOVL6) stearoyl-CoA desaturase 1 (SCD1). In addition to these genes, ChREBP induces the expression of pyruvate kinase, a key gene in the shift of glycolysis products into the Krebs cycle.

Adipose Tissue

The adipose tissue represents the main TG storage compartment in the human body. On average, 30% and 20% of the body weight in women and men respectively is fat [30]. The source of fat in adipocytes is mainly the uptake of dietary-derived TG while only a minor fraction of TG is derived from *de novo* lipogenesis. In the fed state LPL is synthesized in the adipocyte and it is translocated to the luminal side of endothelial cells [2]. Secreted LPL is bound to proteoglycans at the endothelial cell surface and represents the active fraction of total LPL [73]. A consistent fraction of synthesized LPL doesn't enter the secretory pathway but gets degraded intracellularly [73, 74]. The balance between degraded and secreted LPL varies with the metabolic state and represents one of the main regulatory mechanisms of TG utilization in the adipose tissue [31, 73]. The FA derived from LPL hydrolysis reach the adipocyte through a mechanism involving specific transporters such as Fatty Acid Translocases (mainly CD36) and Fatty Acid Binding Proteins (mainly FABP1) [31].

The mobilization of TG from adipose tissue storage is a tightly regulated mechanism. During starvation and stress the levels of circulating catecholamines increase thus activating β-adrenoreceptors located on adipocyte cell membrane [30]. This triggers a cascade of events that lead to the activation of three key lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and Monoacylglycerol lipase (MGL)[75]. The concerted activity of these three enzymes promotes the complete hydrolysis of TG into glycerol and three 3 FA chains. ATGL is the first enzyme in this process and it is responsible for the conversion of most TG into DAG in human adipocytes. ATGL requires the presence of its coactivator, CGI-58 (comparative gene identification-58)[75]. The second enzyme is HSL. The regulation of HSL activity is dependent on its phosphorylation status. β-adrenergic stimulation induces a sudden and marked increase in cAMP [76]. This in turn activates Protein Kinase A that actively phosphorylates HSL and promotes the translocation of HSL to the surface of the lipid droplets. Here HSL actively interacts with phosphorylated perilipin-1 [75, 76]. In the fully

phosphorylated status HSL is able to hydrolyze TG and DAG into MAG. The final step of TG hydrolysis is the conversion of MAG into Glycerol and FA, catalyzed by MGL [76].

Skeletal muscle

Skeletal muscle is one of the metabolically more active compartments of the body. Skeletal muscle requires a huge amount of FA for β-oxidation. Physiologically, myocytes synthesize and store a limited amount of TG. The quantitatively more relevant source of FA is the uptake of non-esterified FA and lipoprotein-associated TG from the circulation [77]. The mechanisms responsible for this process are similar to those described for adipose tissue. Extracellular lipolysis is believed to be mediated mainly by LPL whereas the released FA are taken up via facilitated transport (CD36 and FATP1) [78].

The first important step in FA oxidation is the formation of FA–CoA, mediated by ACC. When intracellular glucose concentration is low, such as in fasted conditions, ex novo lipogenesis is inhibited [77]. This leads to reduced levels of Malonyl-CoA, the main regulator of FA translocation to the mitochondrion. Malonyl-CoA, in fact, constitutively inhibits the carnitine palmitoyltransferase I transporter (CPT-I). CPT-I promotes the transport of FA into the mitochondrion where they enter the β-oxidative pathway, resulting in acetyl-CoA production. Energy produced in the β-oxidation of fatty acids and the oxidation of acetyl–CoA in the Krebs cycle is used to generate ATP via oxidative phosphorylation [77].

2 Structure, function and physiology of the plasma lipase family members

The pancreatic lipase gene family comprises a group of genes that are widely conserved across species [79]. To date, ten different members have been identified in humans: phosphatidylserine-specific phospholipase A1 (PLA1A), membrane-associated phosphatidic acid selective phospholipase A1a (LIPH), membrane-associated phosphatidic acid-selective phospholipase A1b (LIPI), endothelial lipase (LIPG), human lipoprotein lipase (LPL), hepatic lipase (LIPC), pancreatic lipase (PL), pancreatic lipase-related protein 1 (*PLRP1*), pancreatic lipase-related protein 2 (PLRP2) and pancreatic lipase-related 3 (PLRP3) (Figure I-3) [79]. Five of these enzymes (PL, LPL, HL, EL and PLRP2) exhibit substrate specificity for TG and catalyze the hydrolysis of FA. These proteins are thus collectively named TG lipases [1, 79, 80]. The five members are characterized by very different functional roles, with PL and PLRP2 being produced in the pancreas and secreted in the gastrointestinal tract and LPL, HL and EL being secreted in the vascular lumen. This latter subpopulation of lipases displays a high affinity for circulating lipoproteins. Their activity thus plays an essential role in both lipoprotein and tissue metabolism. Hydrolysis of phospholipid and TG in fact, not only remodels the circulating lipoprotein particles, thus modulating their properties and affecting their catabolism, but also generates free FA. Released FA can then reach the intracellular compartment and act as energy supply or signaling molecules.

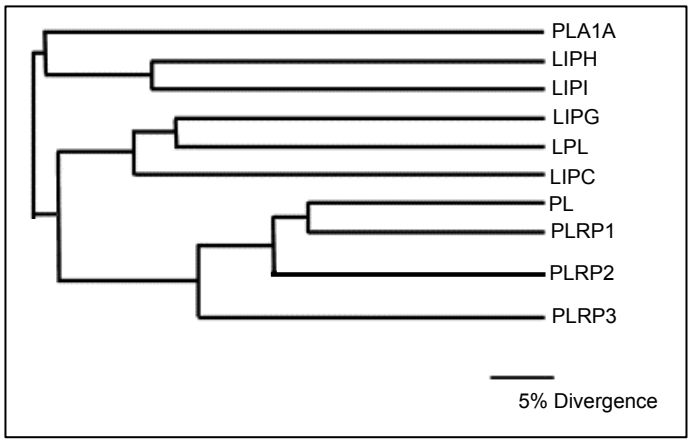

Figure I-3: Phylogenetic relationships of members of the pancreatic lipase gene family.
Phosphatidylserine-specific phospholipase A1 (*PLA1A*), membrane-associated phosphatidic acidselective phospholipase A1a (*LIPH*), membrane-associated phosphatidic acid-selective phospholipase A1b (*LIPI)*, endothelial lipase (*LIPG*), human lipoprotein lipase (*LPL*), hepatic lipase (*LIPC*), pancreatic lipase (*PL*), pancreatic lipase-related protein 1 (*PLRP1*), pancreatic lipase-related protein 2 (*PLRP2*), pancreatic lipase-related 3 (*PLRP3*). LIPG, LPL and LIPC display a high degree of homology, possibly suggesting an origin from the same precursor.

2.1 Structure

Lipoprotein Lipase

The LPL gene is located on chromosome 8p21.3 and encodes for a protein of 448 amino acids. The gene includes 10 exons spanning ~30 kb [81]. The first exon encodes the 5'-untranslated sequence (containing most of the regulatory elements), the coding signal peptide sequence and the first two amino acids of the mature protein [81]. Exons 2-9 contain the remaining 1339 nucleotides that encode for the other 446 amino acids and include the translation stop codon. The 10^{th} exon encodes for the long 3'-untranslated region of LPL mRNA (1948 nucleotides) (Figure I-4 A, table I-3) [82].

The amino acid sequence is extremely conserved among species [1]. Although the crystal structure of LPL has not been resolved yet, the high similarity to other lipases such as PL permits one to perform comparative modeling analyses [83]. The structure of LPL can be schematically divided into two big domains: amino terminal and C terminal. The amino terminal

domain contains the catalytic syte (Ser^{132}, Asp^{156}, His^{241}) responsible for the lipolytic activity, and the lid domain that protects the enzymatic active site (Figure I-4B) [82]. The N-terminal domain of LPL also contains eleven amino acid residues (residues 65–68 and 73–79) that act cooperatively to bind apoC-II, the main activator of LPL. This same region has been proposed as the key interaction site between LPL and its inhibitors apoC-I and apoC-III) [82, 84]. The binding mechanism has not been elucidated but it seems to involve a destabilization of LPL interaction with lipid substrates. The C domain consists of a shorter amino acidic sequence and contains the Heparin-binding domain. This domain is believed to play an essential role in determining the substrate specificity across plasma lipases [84]. In native conditions, LPL spontaneously dimerizes to form head-to-tail LPL homodimers. This association is stabilized by the presence of disulfide bonds. Several studies demonstrated the importance of the dimer conformation in determining the activation status of LPL [85, 86]. The dimer in fact exist in two limit conformations: open, where the substrate has full access to the catalytic site, and closed, where the lid blocks any access to the catalytic pocket [83, 85, 86].

Hepatic Lipase

Hepatic Lipase is encoded by the LIPC gene, located on the chromosome 15 (15 q 21.3) [87]. *LIPC* includes eight introns and nine exons, spanning ~69 Kb. The gene map reveals a structure that is very similar to that of LPL and it is characterized by exons of comparable length (~200 bp) (Figure I-4A, table I-3) [21]. The 5' non translated region contains the binding site for positive transcription factors, such as HNF4α1 and 2, Coup (chicken ovalbumin upstream promoter) and PPARγ1 and 2 [21].

The HL protein is composed by 499 amino acids. After synthesis HL pro-protein loses the 22 amino acids leader peptide [88]. The resulting mature protein consists of 477 amino acids [88]. Similar to LPL, the enzyme can be divided in to two major regions: the short carboxy-terminal domain and the wide amino-terminal domain. The C domain contains the highly conserved PLAT (Polycystin-1, Lipoxygenase, Alpha-Toxin) domain. The amino terminal

domain contains the catalytic triad (Ser146, Asp 172, and His257) and the lid domain, covering the active site [83]. At the center of the protein there are ten cysteine residues that are likely involved in the formation of dysulphide bonds. Finally, HL contains four heparin-binding sites, responsible for the protein binding to heparan-sulphate residues at the cell surface [21].

Endothelial Lipase

The LIPG gene, coding for Endothelial Lipase is located on chromosome 18 (18q21.1) (Figure I-4 A, table I-3). It is composed of 10 exons and 9 introns, spanning ~10.4 Kb. LIPG was first cloned in 1999 by two different research groups [89, 90]. Hirata and colleagues isolated *LIPG* in human umbilical endothelial cells (HUVEC), through a subtraction hybridization methodology that was aimed at characterizing genes preferentially expressed in differentiating cells [90]. This approach led to the identification of a ~500 amino acids fragment that displayed a high degree of structural similarity to LPL (44%) and HL (41%)[90, 91].

Jaye and colleagues, the same year, identified EL following a completely different approach [89]. This group was in fact screening genes that were differentially upregulated in macrophage-like cells (THP-1) upon stimulation with oxidized LDL. Nucleotide sequencing of the amplified genes that were specifically expressed in THP-1 exposed to oxidized LDL, demonstrated the presence of a clone with a unique open reading frame of 1500 nucleotides. The analysis of the deduced primary structure revealed features that were consistent with the two already known plasma lipases [89]. The N-terminal region includes an 18-amino acid signal peptide that mediates the enzyme secretion. Analogously to what reported for HL and LPL, the primary structure of endothelial lipase exhibit ten cysteine residues that are likely responsible for the formation of disulfide bonds. This feature is highly conserved among lipases and determines the tertiary structure of the protein [83]. The catalytic triad (Ser151, Asp175 and His 256) is located in the N-terminal domain and Ser 151 is included in the characteristic ‘GXSXG’ lipase motif. The active site is protected by the presence of lid domain consisting of 19 amino acids [83]. This region likely contributes to the specificity for triglyceride and

phospholipids. Notably, homology models of HL, LPL, and EL demonstrated that the binding pockets of the three lipases display very different properties. EL binding pocket seems to be more flexible and its conformation changes during the catalysis process [83]. Based on different modeling strategies four possible heparin binding sites (14- KLHKPK-19, 282-RFDKK-285, 292-RKNR-295, 304-KKMRNKR-310 and 427-RRIRVK-432) and five glycosylation sites have been proposed. The comparison of EL sequence with that of PL suggests that the PLAT domain, a region that is shared among all the Triglyceride lipases, is probably located in the region 347-482 (Prosite Prorule annotation)[92, 93]. The predicted molecular weight of mature EL is 56794 Da [94]. When assessed by western blot, human EL exists in at least two different forms, characterized by an apparent molecular weight of 40 and 68 KDa [94, 95]. The 68 KDa variant probably represents the fully glycosylated protein, whereas the 40KDa is probably an alternative splicing or post translational cleavage product. Human *LIPG* is a heparin binding protein, behaves as a homodimer with a proposed head-to-tail conformation [94] and is subject to proprotein convertase cleavage at a site in the 'hinge' region separating the N- and C-terminal enzyme domains [96].

Gene	Chromosome	Exons	Transcript length	Translation length	Panel
LPL	Chr8 (19,901,717-19,967,258)	10	3846	475	Figure I-4A
LIPC	Chr15 (58,410,569-58,569,843)	9	2751	499	Figure I-4B
LIPG	Chr18 (49,560,699-49,599,182)	10	10441	500	Figure I-4C

Table I-3 Summary of LPL, LIPC and LIPG gene characteristics

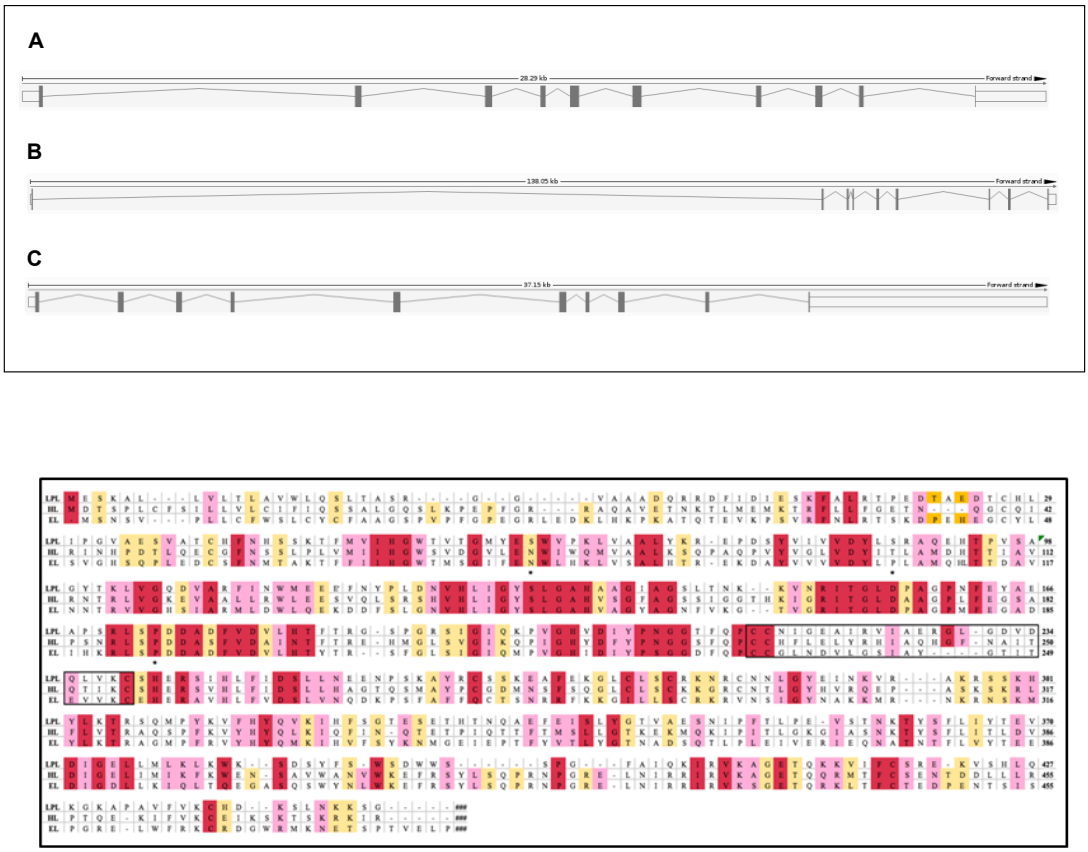

Figure I-4. LPL, HL and EL gene and protein structure.

Panel A-B-C: LPL, HL and EL gene structure. The schematic illustrates exons (full boxes), intronic regions (thin line) and untranslated regions (empty boxes) (Ensembl genome browser [97]). Panel D: Sequence alignment of LPL, HL and EL amino acid sequence. Amino acid similarity decreases from red (identical)>pink>yellow>white. The amino acids of the catalytic triad are indicated with stars. The amino acids included in the lid region are marked with a black box.

2.2 Post-transcriptional modifications

LPL, HL, and EL are all associated with the luminal side of capillaries where they are anchored to proteoglycans exposed on the endothelial cell membrane. After synthesis lipases undergo several post-transcriptional steps that end up with the translocation of the mature dimeric enzymes in the vascular lumen. The dimerization and glycosylation are early events in lipases maturation and happen in the ER. This step is crucial and seems to require a transmembrane protein named lipase maturation factor 1 (*LMF1*). LOF mutations in this gene have been associated with combined lipase deficiency (*cld*) [98]. Homozygous *cld* mice develop severe hypertriglyceridemia and die after birth [99]. A comparable lipid phenotype has been reported in humans carrying loss-of-function mutations Y439X and W464X in the human orthologue gene. Lack of LMF1 leads to lipases misfolding and aggregation-accumulation of immature precursors in the ER [98, 100]. Although the exact mechanism of action of LMF1 has not been elucidated, its activity seems to be specific for a subclass of lipases including LPL, HL, and EL but not the highly homologue PL [98, 101].

After glycosylation and folding into mature form, a fraction of the mature lipases is translocated to the Golgi apparatus for further processing whereas a large pool is retained in the ER as inactive precursor. Lipases are finally secreted into cytoplasm via secretory vesicles. Part of the vesicles are then sorted to the lysosomes for degradation and part are delivered to the intracellular side of the plasma membrane where they interact with heparan sulphate proteoglycans (secretatogue) and are delivered to the vascular lumen.

2.3 Tissue expression

Lipoprotein Lipase

Lipoprotein lipase is synthesized in several parenchymal cells. After synthesis, it is secreted in the extracellular compartment and transported to the surface of endothelial cells. Here it anchors to the cell membrane through binding to heparan sulphate proteoglycans [74]. The exact mechanism underlying this process has not been fully clarified. LPL mRNA has been isolated in different tissues. It is very abundant in heart, skeletal muscle and adipose tissue, where it is believed to exert trophic functions [31, 102, 103]. Its role in other tissues is less clear. In the kidney, LPL mRNA is found in tubular and glomerular parenchymal cells whereas the protein seems to be localized at the endothelial level [104, 105]. In the mammary gland LPL is highly expressed only during lactation [106]. It seems to be synthesized by adipocytes and secreted in the milk in association with lipid droplets [107]. Finally, in some adult tissues LPL is expressed in infiltrating phagocytic cells (macrophages in lung and spleen, Kupffer cells in the liver). The role of LPL in the liver is still controversial. In the early phases of embryo development and in the first 3 weeks after birth LPL expression is high and it progressively decreases with development [108]. In the adult liver it is normally detectable in endothelial cells lining the hepatic sinusoid but not hepatocytes [109]. However, in certain conditions LPL expression can be rescued [110]. Inflammation and fibrates decrease LPL expression in macrophages but increase the expression in the liver [69, 111, 112]. When LPL is selectively overexpressed in the liver LPL-/- mice develop hypertriglyceridemia, insulin resistance and steatosis [108, 113].

Hepatic Lipase

Hepatic lipase is synthesized and secreted mainly in the liver and binds to heparan sulfate proteoglycans (HSPG) on the cell surface of hepatocytes and endothelial cells [114, 115]. It has also been isolated in steroidogenic tissues

such as adrenals and ovaries and macrophages infiltrating the atherosclerotic plaque [116-119].

Endothelial Lipase

The EL expression pattern is unique compared to other plasma lipases. Endothelial lipase is expressed in endothelial cells both in *in vivo* and *in vitro*. Immunohistochemistry studies have revealed that the expression of EL varies in different vessels. Intense staining has been reported in large vessels such as aorta and major arteries in the kidneys, spleen and lung [120]. EL seems to be abundant in the microvessels in the spleen and lung whereas it seems to be poorly expressed in other capillary bed [90]. EL mRNA and protein have been detected in several other tissues. *In vivo* data indicate that EL may play an important role in reproduction. EL mRNA has been detected in mouse embryos (E8.5 to E11.5), but not later in development [121]. In adult mice, EL mRNA expression is high in ovaries during pregnancy and testes [121]. In-situ hybridization studies have demonstrated that placenta is one of the sites with highest expression of EL [89, 90]. Both LPL and EL protein have been detected in the placental villus where they are likely involved in the hydrolysis of TG and PL-derived FA that can be thus delivered to the fetus. EL is mainly synthesized in endothelial cells and then transported to the syncytiotrophoblasts cell surface [122, 123]. EL mRNA has been detected in lungs, where it has been suggested to play a role in the metabolism of the surfactant fluid [90]. Finally, EL is abundant in thyroid glands in the epithelial cells that line the follicles [124]. Its functional significance in the thyroid is not known. It has been demonstrated that HL is regulated by the thyroid hormone and its activity it's reduced in severe hypothyroidism leading to increased levels of HDL cholesterol [125]. It is thus possible that EL levels may be similarly regulated by the same hormonal axes.

2.4 Activity, physiology and regulation of plasma lipases

Lipoprotein Lipase

Lipolytic activity

LPL affects plasma levels of all the lipoprotein subclasses. Its preferential substrates are chylomicrons and VLDL, where it is able to hydrolyze TG and to a lesser extent PL (Figure I-5 B). Although its role in TG-rich lipoprotein metabolism had been first identified in 1943, many aspects of LPL reaction and regulation have not been clarified [126].

The interaction between lipoproteins and LPL happens in the vascular compartment. Several factors such as the diameter of the vessel, the blood flow rate, LPL density at the vascular wall, and the diameter of the lipoprotein itself can affect this initial step. The sequestration of large particles such as chylomicrons in small vessels facilitates LPL-lipoprotein interaction and maximizes the lipolysis rate [2]. This scenario typically occurs in the postprandial phase. The LPL-lipoprotein interaction seems to follow a competitive kinetic. Different lipoprotein subclasses such as VLDL and chylomicrons can compete for the binding to LPL. The relative concentration of lipoprotein subclasses can thus influence the lipolytic rate and plasma FFA concentration.

LPL substrate specificity is higher for TG than to PL. Among TG, LPL is able to hydrolyze any FA but it displays a higher specificity for unsaturated FA such as oleate. Furthermore, FA in the 1 position of glycerol and Phosphatidylcholine backbone (sn-1) are hydrolyzed more efficiently than those in the 2 position [2].

Once the substrate reaches the catalytic site it is hydrolyzed into FA and ester derivative (Figure I- 5B). The overall mechanism of reaction is shared among all the plasma lipases and it's typical of the α/β hydrolase family. The active site of EL, LPL, and HL consists of a highly conserved catalytic triad. The substrate hydrolysis starts with the nucleophilic addition of the hydroxyl group

of the catalytic serine residue to the carbonyl carbon atom of the glycerol-FA ester bond (Figure I- 5A). This generates a tetrahedral intermediate that is stabilized by two hydrogen bonds between the negatively charged carbonyl oxygen atom and the amino groups of two amino-acid residues of the active site. The further transfer of a proton to the oxygen atom induces the cleavage of the ester bond, the release of the alcohol product, and the formation of the serine-ester. The last step is the deacylation, in which a water molecule hydrolyzes the covalent bond between serine and acyl derivatives [127, 128].

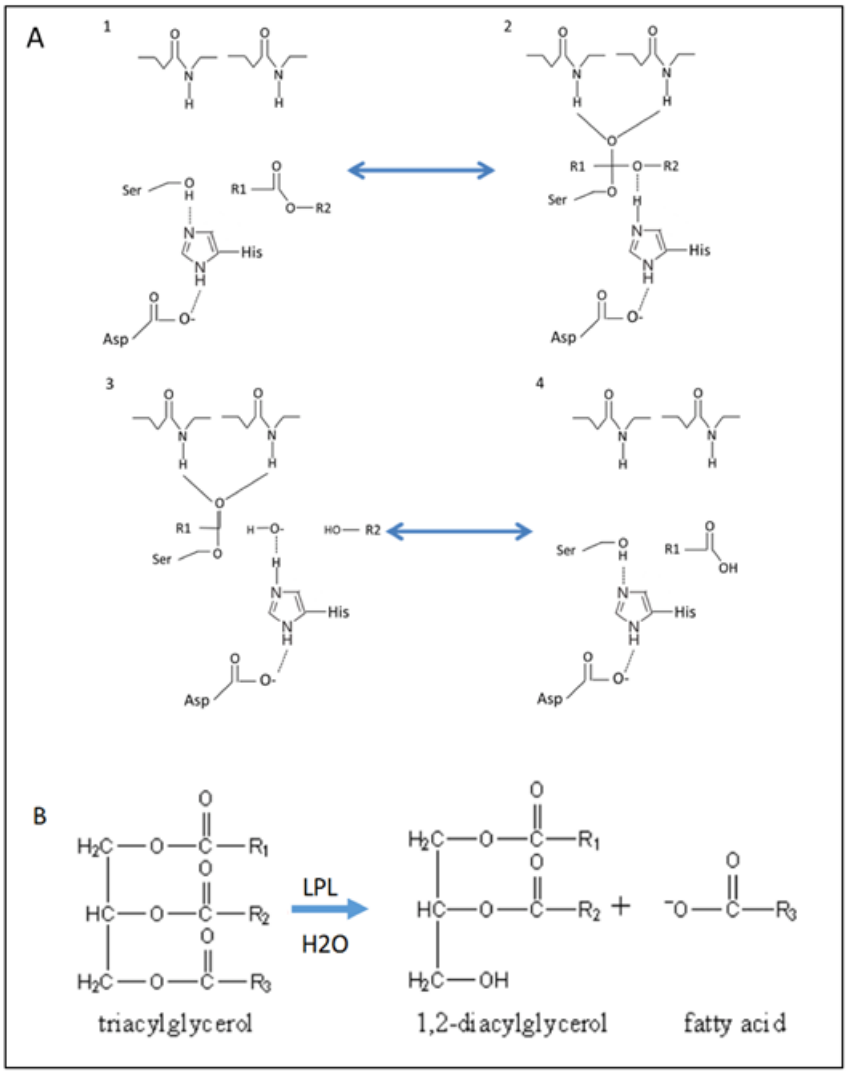

Figure I- 5. The lipase-mediated TG hydrolysis reaction.

Panel A Mechanism of the lipase reaction. (1) Nucleophilic addition of the serine OH to the ester bond; (2) tetrahedral intermediate; (3) acyl-enzyme and released alcohol (4) regenerated enzyme and released acyl product). Panel B LPL- induced hydrolysis of triglycerides

Non-lipolytic activity

LPL can mediate receptor-independent uptake of the apoB-containing lipoproteins [129-131]. This is partially explained by the fact that LPL-derived VLDL remnants and IDL have higher affinity for LDL-R located on peripheral cells such as macrophages. Furthermore, LPL activity increases the binding affinity of apoE-containing lipoproteins, for three LRP, LRP-2, and VLDLR [132-134].

Regulation of lipoprotein lipase activation and expression

Full activation of lipoprotein lipase requires the presence of a specific cofactor, apolipoprotein C-II. This is a unique feature of LPL, since no activator seems to be required for HL and EL activity. The binding site for this apolipoprotein has been identified in the amino terminal region of LPL. It has been suggested that apoC-II promotes LPL activity by facilitating the interaction of LPL with the substrate. apoC-II is present in both HDL and TG-rich lipoproteins. Genetic defects that lead to decreased apoC-II levels are associated with hypertriglyceridemia [135-137]. Interestingly overexpression of apoC-II leads to a similar phenotype. A possible explanation is that the increased content of surficial lipoproteins can negatively affect the initial interaction between lipoprotein and cell surface lipases [138].

The ability of LPL to bind both proteoglycans and lipoproteins is in fact essential for a fully efficient activity. Virtually any factor that decreases this bridging interaction can negatively impact the lipolysis rate.

A recently identified protein that promotes LPL-mediated TG-rich lipoprotein lipolysis is GPIHBP1 (Glycosylphosphatidylinositol Anchored High Density Lipoprotein Binding Protein 1) [139]. This is a glycosylphosphatidylinositol-anchored protein that plays a major role in transporting lipoprotein lipase (LPL) from the subendothelial spaces to the capillary lumen. Once in the vascular lumen GPIHBP1 is able to bind positively charged apolipoproteins and LPL. It has been proposed that this molecule may stabilize LPL binding to the endothelial cell membrane and promote its interaction with chylomicrons. The exact mechanism is still unknown but missense mutations in GPIHBP1

gene have been associated with hyperchylomicronemia [140-142]

ApoC-III is a small apolipoprotein that circulates on both TG-rich lipoproteins and HDL. Apo C-III affects the TG-rich lipoprotein metabolism at different levels [143]. One of the proposed mechanisms involves the negative regulation of LPL activity [144]. *In vivo* studies in *apoc3* transgenic and KO mice corroborated this hypothesis and showed that apoC-III delays TG hydrolysis [145-147]. The molecular mechanisms underlying apoC-III-mediated inhibition are still under investigation. One proposed mechanism is that apoC-III displaces LPL from lipid emulsion particles and expose LPL to additional inactivating factors such as ANGPTL3 and 4 [148].

apoA-V has emerged as a potent modulator of plasma TG [149, 150]. Contrary to apoC-III, it is thought to exert its effect through different mechanisms [143]. The effect on LPL activity seems to be secondary to an apoA-V mediated increase in VLDL and Chylomicrons availability at the endothelial wall. apoA-V is in fact able to promote the binding of TG-rich lipoproteins to proteoglycans. This stabilization of the substrate maximizes the LPL-lipoprotein interaction and results in increased lipolysis. *In vivo* studies in Apoa*5* KO and transgenic mice have demonstrated that apoA-V plasma levels are inversely correlated with triglyceridemia [151, 152].

ANGPTL3 and *4* genes are expressed predominantly in the liver, and encode for secreted proteins that belong to the family of angiopoietins. Their structure comprises an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain [153, 154]. ANGPTL3 and 4 play and important role in glucose and lipid metabolism [155, 156]. Loss of function mutations in *ANGPTL3* are associated with betahypolipoproteinemia [157]. *In vivo* studies in KO and transgenic mice have demonstrated that Angptl3 and 4 levels are inversely correlated with LPL activity and positively correlated with TG plasma levels [154, 158, 159]. In addition to their effect on LPL, Angptl3 and 4 are also able to inhibit HL and EL. The mechanism by which Angptl3 and 4 inhibit LPL appears to be different. ANGPTL3 inhibition can be overcome by heparin and translates into a net decrease in LPL catalytic activity [160]. It has been hypothesized that this effect may be mediated by the recruitment of proprotein

convertases PCSK6 and FURIN cleavage and dissociation of LPL from the cell surface [154, 160, 161]. ANGPTL4 Is believed to inhibit LPL by promoting the monomerization and unfolding of LPL.

LPL is expressed in several tissues and its synthesis is regulated by different nutritional and hormonal pathways. Fasting and refeeding regulates LPL expression in a tissue-specific manner [73, 162]. In the postprandial state, LPL synthesis is high in adipocytes and low in heart and skeletal muscle [73, 163]. In the adipose tissue this effect seems to be mediated by insulin and by glucose level itself through distinct pathways [162]. Short-term high carbohydrate diet is in fact sufficient to increase LPL without affecting insulin sensitivity [163]. Insulin on the other hand has a direct positive effect on LPL transcription and glycosylation [164-166]. Glycosylated LPL is more efficiently secreted into the vascular compartment and seems to be more catalytically active. During fasting, on contrary, LPL levels in the adipose tissue fall drastically [167]. This latter effect seems to be mediated by an increased fraction of LPL that is converted to the inactive form rather than by decreased LPL synthesis [164-167].

LPL expression in the heart responds to fasting and refeeding through a post-transcriptional regulatory mechanism. During fasting, heparin-hydrolysable LPL that represents the active enzyme pool, is increased [168, 169].

Finally, LPL expression can be modulated by other hormonal axes. It has been observed that catecholamines reduce adipose tissue LPL, thyroid hormones increases LPL activity in rodents and growth and sex hormones inhibit LPL in adipose tissue while increasing its activity in skeletal muscle and heart [170].

Hepatic Lipase

Lipolytic activity

Hepatic lipase is mainly synthesized by hepatocytes and hydrolyzes TG and PL in chylomicron remnants, IDL and HDL [33, 171]. HL active site is characterized by the presence of the α/β catalytic triad and the hydrolysis reaction is thought to follow the same general mechanism (Figure I- 5A). Despite the evident high degree of homology with LPL, HL displays a different substrate specificity and the biochemistry of HL reaction is differently regulated [172]. The first striking difference is that HL does not require apoC-II as activator and its reaction cannot be inhibited by NaCl [173]. This biochemical peculiarity is the basis of most of the currently used activity assays. HL post heparin plasma activity indeed is defined by subtracting NaCl-sensitive LPL activity from total post heparin lipase activity [174, 175]. Structure-function studies on HL/LPL chimeric proteins suggested that the different catalytic properties may be due to differences in the amino-terminus domain [84, 176]. The substrate specificity and the different heparin-binding properties may instead be explained by the sequence differences in the C-terminal domain [93]. In this regard, the lid region seems to be responsible for the different PL/TG lipase activity ratio. In fact, the lid region changes its conformation when it binds a substrate and the flexibility of the lid can determine which substrates ultimately reach the active site [93, 177]. Notably the degree of a sequence homology between the lids of HL, LPL and EL is low. The LPL lid permits a high specificity for TG with very low PL activity [93, 177]. HL, on the contrary, is less specific. The absolute (in bulk) substrate specificity is comparable for TG and PL [178]. When the substrates are diluted in the surface of micelles HL seems to prefer TG rather than PL (TG>DAG>PE>PA>PC) [177, 178].

Furthermore, HL seems to be less stereo-selective than LPL and shows a comparable affinity for FA in sn1 and sn3 on the glycerol backbone [178]. The relevance of these observations *in vivo* is not clear. The relative magnitude of TG and PL lipase activity may be determined by the substrate availability rather than by the different specificity.

The impact of HL activity on plasma lipoprotein metabolism is complex and reflects the different effects on single lipoprotein subclasses. TG-rich lipoproteins are firstly hydrolyzed by LPL, that is quantitatively more abundant in peripheral tissues. HL on the other hand converts VLDL and CM remnants into LDL by promoting the hydrolysis of TG and PL [179, 180]. The result is the formation of small, dense LDL particle. In a similar fashion HL converts large, TG-rich HDL2 into small HDL3 [181, 182]. Consistently, subjects with HL deficiency display elevated plasma concentrations of intermediate density lipoproteins and HDL [179, 180].

The very first event in HL reaction is the interaction between HL and lipoproteins. This step is not clear and different models have been proposed. In the early models, HL was described by analogy with LPL. After heparin injection LPL is massively released in the circulation [183]. Although LPL activity in the post heparin plasma is extremely high, the enzyme is less stable and is more prone to cleavage into inactive monomers [148]. This observation corroborated the importance of proteoglycans in stabilizing LPL and mediating the interaction with lipoproteins. To what extent bound and unbound forms contribute to the overall plasma lipolytic activity in physiological conditions is not known. Recent studies suggest that HL may be inactive when it is bound to the cell surface and only after it is released in the circulation it is converted to the active form [171]. According to this model, the liver may function as a reservoir of inactive EL that can be initially taken up by HDL and then released into the circulation in the active form [171, 184]. HL activation may thus require a two-step process involving an HDL-mediated displacement from endothelial cell proteoglycans and a second displacement from HDL particles [184, 185]. Some studies suggest that HDL protein and lipid composition may in fact modulate this process. The presence of apoE on HDL particles appears to reduce the initial binding of HDL to HL and translates into a reduced HL activity [185]. During the postprandial phase, apoE is exchanged with TG-rich lipoproteins and this results in a lower HDL-apoE content. Interestingly in this condition HDL's ability to activate HL is higher. ApoA-II particle content seems to be one of the main negative regulators of HL activation rate. ApoA-II in fact enhances HL affinity to HDL. This increases

the displacement of inactive HL from endothelial cells but decreases the release of HDL-bound HL into the circulation [186, 187].

Non-lipolytic activity

In addition to the lipolytic activity, HL plays a role in the uptake of HDL and apoB-containing lipoprotein remnants by cell receptors. For instance, it has been demonstrated that hepatocyte uptake of plasma lipoproteins is increased by HL. The mechanism does not require catalytically active HL and seems to involve the binding of HL to LRP, LDLr and proteoglycans [75, 188, 189]. Overexpression of catalytically active human HL in HL KO mice overexpressing apoB-48 or apoB-100 was sufficient to induce a significant reduction in apoB-containing lipoprotein plasma levels [190].

Regulation of hepatic lipase activation and expression

The HL activation status seems to be highly dependent on the ratio between proteoglycan-bound (inactive) and released (active) form. The equilibrium between these two pools of HL is probably influenced by the lipoprotein composition. ApoE and apoA-II are the two main inhibitors of HL activity and their mechanism of action seems to be related to their ability to stabilize HL in its proteoglycan or HDL-bound form [185].

Several hypothetic regulatory elements have been described in the murine *Lipc* promoter [21, 191]. *In vivo* and *in vitro* studies showed a strong association between sterol intracellular levels and HL expression. In HepG2 cells, the pharmacological inhibition of the cholesterol biosynthetic pathway increases the expression of HL mRNA and protein [192]. In studies in rats, high cholesterol diet downregulates HL protein expression [193, 194]. Finally, in humans, the treatment with bile acid resins increase HL plasma levels [195].

HL activity in post heparin plasma is affected by sex hormones. Estrogens decrease HL activity whereas progestinic and androgenic hormones increase

HL activity [196, 197]. These observations have been widely supported by human studies either in physiological conditions (pregnancy, postmenopausal status) or upon pharmacological supplementation [197-200]. At a physiological level, HL expression seems to be gender dependent. Women in fact seem to have larger, less dense LDL and lower hepatic lipase activity [201-203] . Men, on contrary, display higher HL activity that is accompanied by the formation of denser and smaller LDL [204].

Whether HL is responsive to insulin is still controversial. Insulin stimulation of HL transcription is required during embryogenesis [191]. In adult humans the association is not clear. There is a positive association between insulin levels and HL activity in control subjects ad insulin-responsive diabetic patients [205] whereas type-I diabetic subjects display a significantly reduced HL activity [206, 207].

Catecholamines, finally have been associated with a reduced secretion of HL. This downregulation of HL protein seems to happen both at a transcriptional and post-transcriptional level [208-210].

Endothelial Lipase

Lipolytic activity

Endothelial lipase displays the highest PL activity among the plasma lipases. Although in an initial study no TG lipase activity was reported, further investigations demonstrated that EL is capable of hydrolyzing triacylglycerol [89, 90]. Unlike LPL, EL does not require apoC-II or other activators [90, 211]. Biochemical studies on EL lipolysis demonstrated that this enzyme is inhibited by the addition of serum and, similar to LPL but not HL, it is sensitive to NaCl [211]. Its activation/inhibition profile is thus intermediate between LPL and HL. The specific factor responsible for dose-dependent serum inhibition is not known but its effect extends to both the TG and PL activity.

McCoy and colleagues tested the relative affinity of LPL, HL, and EL for TG and PL *in vitro* [211]. In these analyses they used a lipid emulsion containing TG and PL. This substrate allows one to determine the activity independently

of potential lipoprotein-dependent factors such as the protein composition. The results of this study indicated that the TG:PL activity ratio was 140 for LPL, 24 for HL and 0.65 for EL. In a second set of experiments the ability of lipases to hydrolyze isolated lipoproteins was tested. The authors showed that when incubated with HDL, EL displayed the highest catalytic activity (measured as total released FA). LPL on contrary displayed the highest affinity for TG-rich lipoproteins and HL seemed to have an intermediate phenotype (fig 6). EL has been shown to have a positional specificity for fatty acids in position 1 (sn-1) of Phosphatidylcholine [212, 213] Furthermore, the phospholipid FA composition seems to affect EL affinity to the substrate. Incubation of EL with synthetic reconstituted HDL showed that the presence of a long chain FA in position 2 increased the EL activity [214]. Finally, among the natural PL, EL preferentially hydrolyzes phosphatidylethanolamine (relative specificity: phosphatidylethanolamine > Phosphatidylcholine > phosphatidylserine > Phosphatidic Acid) [215]. The molecular explanation for this peculiar substrate specificity is probably the lid region sequence. This region is the less conserved among the plasma lipases [89, 90]. In a recent study the structure of EL, LPL, and HL has been modeled by analogy with pancreatic lipase. The comparative study of their structure and the computational analysis of their predicted binding to known inhibitors revealed that EL lid region is more flexible and the conformation of EL active site changes more during the catalytic process [83].

The physiological role of EL in lipoprotein metabolism has not been fully elucidated. Since it was firstly cloned in 1999, EL has been identified as a major regulator of HDL-C *in vivo* [89]. Although its activity against HDL is extremely high recent studies have highlighted a substantial hydrolytic activity towards apoB-containing particles. Early studies showed that, in mice, overexpression of human EL in the liver induces a dramatic decrease in circulating HDL and apoA-I levels [89, 213]. The effect on apoB-containing lipoproteins was more modest and overexpression of hEL in LDLr KO mice lead to a 50% reduction of non-HDL cholesterol [89].

In a more recent study instead, overexpression of EL in strains with high

levels of apoB-containing lipoproteins lead to significant decrease in VLDL, LDL, PL, TG, total cholesterol and apoB levels [216]. This dramatic change was due to an impaired apoB-containing particles catabolism. Overexpression of a catalytically inactive EL (ELS149A) was not only ineffective but also impaired the endogenous EL activity [216]. Taken altogether these data indicate that EL enzymatic activity may contribute to apo-B containing lipoprotein metabolism.

Studies on EL-deficient animals proved the role of EL in HDL metabolism. Jin and colleagues took advantage of antibody-mediated inhibition of murine EL (mEL). In female wildtype mice, EL deficiency caused a significant increase in both HDL-cholesterol and PL (+29.5 and 27% at 24 hrs, respectively) [217]. The same experiment was performed in human apoA-I transgenic mice and HL-deficient mice. In apoA-I transgenic mice, 24 hours after injection of anti-mEL antibody plasma levels of total cholesterol, HDL-C, and phospholipid were significantly increased (+15, 20 and 23% respectively) [217]. The authors demonstrated that these changes were due a slower HDL-phospholipid catabolic rate that translated into a net increase in the number of HDL particles. The study of HL deficient mice provided interesting results. Similar to what observed in the other two strains, EL inhibition raised HDL-C, total cholesterol and PL. In addition to that, HL–/– mice had larger HDL and they displayed an increase in triglyceride levels [217]. This suggests that EL and other lipases may have concerted activity on plasma lipoprotein remodeling and the effects of EL on triglyceride-rich lipoprotein metabolism may also be magnified in the absence of HL.

EL KO mice have been developed by two different groups. The characterization of these models showed very contradictory results. In both models, a gene dose-dependent increase in HDL-C was observed but the analysis of HDL composition showed no difference in the HDL particle number and composition or an increase in HDL particle size [213, 218]. However, very different effects were observed on apoB-containing lipoprotein levels. Ma and colleagues didn't observe any change in both LDL and VLDL plasma levels either on chow or high fat diet [219, 220]. On the contrary Ishida and

colleagues observed a dramatic increase in LDL-C levels (+90%)[213].

Non-lipolytic activity

In vitro and *in vivo* data demonstrated that, in addition to its enzymatic activity, EL modulates lipoprotein metabolism by promoting lipoprotein uptake.

Strauss and colleagues firstly observed this effect in HepG2 cells. In the study, cells were infected with adenovirus encoding EL, LPL or LacZ. Cells were then incubated with dual-labeled HDL (^{3}H-Cholesterol esters and I-125 total protein) and binding, holoparticle uptake and CE-selective uptake were measured [221]. Results indicated that both HDL particle and HDL-CE uptake were significantly increased in EL and LPL expressing cells whereas HDL binding was significantly increased in EL-overexpressing cells only. In order to test whether this effect was secondary to EL lipase activity, the same experiment was repeated in presence of the EL inhibitor tetrahydrolipstatin (THL) or using a mutant catalytically-inactive EL. Paradoxically the presence of inactive or inhibited EL lead to a further increase of all the measured parameters. When the authors analyzed the density of EL protein at the cell membrane, they observed that in absence of any catalytic activity the amount of EL was higher. They concluded that fatty acids that are liberated from phospholipids release EDL from the cell surface and thus impair its non-catalytic activity [221].

In similar experiments Fuki and colleagues confirmed the ability of EL to bridge lipoproteins to the cell surface [222]. Consistent with the results from Strauss, no difference was noticed in cell uptake between cells expressing WT EL or the EL-S149A catalytically-inactive mutant. Furthermore, in this study the authors demonstrated that EL binding to heparan sulfate proteoglycan (HSPG) is required to this activity and that the bridging capacity is not HDL-selective. Interestingly cells expressing EL, LPL, and HL all displayed a high bridging capacity towards VLDL and LDL compared to control cells whereas the activity towards HDL was moderate but significantly higher for EL and HL compared to LPL. The absolute bridging capacity (total I-125 protein counts), represents the sum of different processes namely

binding, uptake and degradation. The authors showed that incubation with either LDL or HDL in fact leads to a substantial increase in lipoprotein binding to the cell surface but notably, after binding, almost all LDL particles were internalized and degraded, whereas HDL was mainly released in the medium. The observed effect is probably a result of two distinct binding kinetics. Fuki and colleagues speculate that VLDL and LDL interact with EL through two different binding sites. The lipid component binds to EL with low affinity and high capacity whereas apoB binds to EL with high affinity. Surprisingly the HDL binding kinetics seems to be described by a low affinity high capacity interaction and this process is significantly magnified by increasing HDL-clustering. These findings support the idea that EL alone can promote the CE uptake from HDL particles. The relevance of this process to apo-B containing lipoprotein metabolism is less clear. Data from this study indicate that most of LDL and VLDL are internalized and degraded and authors conclude that EL alone is probably not sufficient to promote a significant CE uptake from these lipoproteins. Nevertheless *in vivo* EL coexist with LPL and HL in the endothelial wall and it is possible that its high capacity lipid binding may facilitate VLDL and LDL metabolism through a mechanism that involves the combined activity of multiple lipases.

In an *in vivo* study, Broedl and colleagues tried to address this question by inducing the expression of catalytically inactive EL-S149A in WT, apoA-I transgenic, and HL-deficient mice. In contrast to what observed *in vitro*, catalytically inactive ELS149A didn't induce any reduction of HDL-C or apoA-I plasma levels in wild-type and apoA-I transgenic mice, suggesting that PL-hydrolysis may be a key step in HDL catabolism. A moderate reduction of total and HDL-C was observed in HL-KO mice [223].

Regulation of endothelial lipase activation and expression

EL was first isolated in endothelial cells and macrophage-like cells. Hirata and colleagues identified EL through an approached aimed to discriminate genes that were selectively expressed in activated endothelial cells [90]. Given this

premise, many efforts have been focused on investigating whether factors known to stimulate endothelial cell activation were able to modulate LIPG expression. In cultured HUVEC, several pro-inflammatory stimuli such as cytokines, shear stress and cyclic stress positively regulated EL mRNA [95]. HUVEC and HCAEC stimulated with IL-1β and TNF-α in fact displayed a 6.5 and 4 fold increased EL mRNA [95]. An independent study confirmed that both EL mRNA and protein expression are upregulated upon stimulation with IL-1β and TNF-α in a dose-dependent fashion [224]. The protein is in the active form and the increased expression translates in increased phospholipase and TG lipase activity in the cell medium. The mechanism underlying the positive regulation of EL expression is not completely clear but it seems to involve NF-kB pathway [224-227]. In obese subjects, plasma inflammatory cytokine levels are directly correlated with plasma EL concentration. Furthermore, LPS-induced endotoxemia significantly increases plasma EL levels [228].

In vitro studies showed that in brain capillary endothelial cells EL expression was reduced by treatment with LXR and PPAR agonists whereas in mice the same effect was observed after treatment with SREBP inhibitors [229, 230].

Similar to LPL and HL, EL activity is also regulated at the post-transcriptional level. The intracellular maturation of EL is affected by LMF- 1 activity [98, 100].

After secretion, EL activity may be regulated through the modulation of its binding to heparan sulphate proteoglycans, though cleavage-inactivation (Proprotein Convertases such as PC5/6) or through the binding to lipase inhibitors (ANGPTL3 and ANGPTL4) [96, 231-234].

3 Lipases and metabolic disorders

Lipases play a major role in both plasma lipoprotein metabolism and tissue FA utilization. Primary and secondary defects in lipase activity have been associated with several traits of metabolic disorders including dyslipidemia, atherosclerosis, obesity, and diabetes. Given the very different substrate specificity and role in lipoprotein and tissue metabolism, factors that selectively impair EL, HL, and LPL activity translate in different phenotypes.

Lipoprotein Lipase

The current knowledge about the role of LPL in the development of hypertriglyceridemia derives largely from the observation of the clinical phenotype of human carriers of genetic mutations.

Complete LPL deficiency in mice is not compatible with life. LPL-/- mice survive during gestation but die within three days after birth. Necropsy reveals that pups have no TG storage in the tissues and display a massive hypertriglyceridemia (>20000 mg/dL) and hypoglycemia [235]. Heterozygous mice have hyperinsulinemia and tend to have fat mass increase with aging. [236]. This phenotype seems to be rescued when LPL is reconstituted in heart and liver [108, 237].

The first report of a genetic case of lipase deficiency in humans was in 1932 [238]. The patient was a young boy who displayed eruptive xanthomas, hepatosplenomegaly and milky plasma. All the symptoms were reverted with fat free diet and the phenotype was not secondary to any other known pathology. Since his parents were first cousins, the disease was classified as “idiopatic familial lipemia” [239]. Later studies highlighted the cause of the similar diseases as a defect in post-heparin plasma activity and this phenotype was re-classified by Fredrickson as “type I hyperlipoproteinemia” [240]. The clinical traits include TG plasma levels >1000 mg/dL, very low or absent lipase activity, eruptive xanthomas, lipemia retinalis and pancreatitis. To date, two genetic diseases are associated with this phenotype: familial lipoprotein lipase deficiency (MIM 238600) and familial apolipoprotein C-II deficiency (MIM 207750) [173].

Familial LPL deficiency is a rare inherited disease (1:100,000 subjects) [173]. To date about sixty different mutations have been identified [241, 242]. Most of the loss of function mutations are in the exons 4,5 and 6 and they lead to major structural defects in LPL protein [33]. Mutant proteins are either degraded intracellularly or result in truncated forms that can be secreted but display no catalytic activity. The plasma lipid profile in carriers is consistent with Type I dyslipidemia [33, 243]. The severity of hypertriglyceridemia varies with the dietary fat intake. Although chylomicron accumulation is the main feature of LPL deficiency, several studies have shown that VLDL clearance is also impaired and contributes to the overall phenotype [244]. Hepatomegaly and xanthomas are typical traits and the latter are a consequence of lipid-loaded macrophages accumulation [245]. Fat accumulation is not altered in these subjects but the FA composition is abnormal and shows increased content of monounsaturated FA [246, 247]. The composition is consistent with increased de novo lipogenesis, probably a compensatory response to reduced hydrolysis of diet-derived TG [247].

In addition to complete LOF mutations, some mutations lead to reduced activity and translate in milder clinical phenotype. The common mutation N291S in exon 6 induces a partial loss of TG activity (~50%) [248]. It has been associated with decreased HDL-C and increased TG in subjects with familial combined hyperlipidemia (FCHL)[248] and with increased TG levels, non-HDL cholesterol and decreased HDL-C levels in subjects with familial hypercholesterolemia heterozygotes (FH) [249].

The −93G promoter allele has been identified in different populations (Americans, Africans, Caucasians) [250]. It is associated with ~ 40 % reduction in LPL activity [250, 251]. In Caucasians T-93G is in linkage disequilibrium (LD) with another common variant, D9N. Subjects who carry the −93G/9N haplotype show decreased HDL-C, increased TC and TG and have increased risk of CVD, obesity and type II diabetes [252, 253]. This observation was not confirmed in another study in African carriers, where T-93G was associated with mildly decreased TG levels [254].

Several common polymorphisms in LPL gene have been identified. The most

studied are HindIII and S447X that have been associated with TG and HDL-C concentrations [252, 255-258]. The directionality of the association is not clear and it has not been confirmed in all the populations investigated [259, 260] . Finally, *HindIII* has been positively associated with body mass index in obese subjects [261, 262].

In the general population, LPL levels in pre-heparin plasma have been proposed as a marker of metabolic syndrome [263-267]. Metabolic syndrome is a cluster of conditions including high blood pressure, hyperglycemia, increased body mass index (BMI) and central obesity, and dyslipidemia. LPL mass in plasma do not vary with age and gender but can be affected by other metabolic factors. Ex vivo analysis of human plasma has shown that TG is normally associated with TG-rich lipoproteins in the postprandial space and promotes their catabolism [268]. Reduced levels of LPL thus impair postprandial lipoproteins clearance and are inversely correlated with the concentration of TG [269]. Human studies demonstrated that LPL levels are also inversely correlated with, oxidative stress and severity of metabolic syndrome [263, 266]. In diabetic subjects, LPL levels are decreased [265]. This is partially explained by the fact that LPL has been associated with insulin sensitivity [264].

Hepatic Lipase

The role of HL in metabolic disease is controversial. Genetic HL deficiency is strongly associated with cardiovascular disease, that represents the main cause of mortality in this population [195]. However, the relationship between HL activity and CVD is not clear and different studies have supported a potential pro- or anti-atherogenic potential. At the physiological level, HL activity promotes the catabolism of mature, TG-rich HDL. This leads to a net reduction of circulating HDL-C levels and to the parallel regeneration of small HDL particles. In addition, HL is also responsible for the conversion of VLDL into IDL and LDL and the density of lipoproteins increases with this process.

The combined reduction of HDL-C levels and increase of dense LDL particles are known risk factors for CVD. As a consequence, HL activity has been

expected to increase the risk for atherosclerosis [270-272].

Surprisingly, data from animal studies indicated that HL-deficient mice do not have increased atherosclerosis [273] and in apoE-KO background it seems to be protective [274]. Furthermore, HL expression ameliorates the lipid profile and aortic cholesterol accumulation in mice on high cholesterol diet [192, 275] [119]. HL deficient mice seem to be less susceptible to diet-induced obesity [276] and this effect seems to be directly mediated by its lipolytic activity [277].

In humans, HL deficiency is a very rare recessive disease. To date twelve patients from six families have been detected [152, 278-281]. The first patient with genetic HL deficiency was identified in 1982 in a Canadian family. Its clinical phenotype was characterized by hypertriglyceridemia, eruptive xanthomata and pancreatitis [278, 279, 282]. This phenotype is not very consistent across different mutations and even in the same family seems to be very variable. Plasma TG levels are normal to high and pancreatitis does not always occur. The very common feature of HL is an abnormal TG enrichment in LDL and HDL particles [282]. As a consequence of abnormal VLDL metabolism, these particles display abnormal migration when separated by charge on agarose gel and VLDL are retained in β position [282]. HL deficient lipoproteins display a Cholesterol:Triglycerides ratio of 1:1 [282-284] and when analyzed by FPLC they display an abnormal size pattern. ApoB and HDL-C plasma levels are often increased and HDL-C can represent up to 50% of the total cholesterol pool [282]. The overall phenotype is a result of impaired hydrolysis of triglyceride (and phospholipid) in lipoproteins and reduced uptake of remnants by the liver [283].

Heterozygous subjects have a 50 %reduction in post-heparin plasma hepatic lipase activity. The lipid profile is variable, some carriers have a normal lipoprotein composition whereas others display TG enrichment [282, 284, 285].

HL deficiency is inversely correlated with HDL-C levels both in the general population and in carriers of HL mutations [286, 287]. Women display a lower plasma HL activity compared to men and this has been proposed as one of

the possible explanations for gender-dependent differences in HDL-C levels [288, 289]. The sibling-pair linkage analysis of different populations of normotriglyceridemic Caucasians revealed that variants in HL locus accounted for 25% of the total interindividual variation in plasma HDL-C levels [290, 291]. Sequencing analysis of selected individuals from this population allowed identification of four polymorphisms in HL promoter region (−250 G-to-A, −514 C-to-T, −710 T-to-C, and −763 A-to-G)[291]. Association analysis of the plasma lipid and lipoprotein levels revealed a small effect of the -514 CT genotype on plasma HDL-C and apolipoprotein AI levels among men but not in women. Men carrying the TT genotype had very high plasma HDL-C and apolipoprotein AI levels. No effect on total cholesterol or triglyceride concentrations was observed [291]. In the San Antonio Family Heart Study, maximum-likelihood complex segregation approach lead to the identification of a major locus effect on low HDL-C allele, and heritability for plasma HDL-C levels. Linkage analyses however excluded tight linkage between the detected major locus and markers for HL locus [290]. In two further studies on male coronary artery disease (CAD) patients, the polymorphism in HL promoter was highly associated with lower HL activity (-25% in Heterozygotes and 50% in homozygotes) [292, 293]. The variants were more common in individuals with CAD than controls [294]. However this finding has not been confirmed in other studies [295]. The rare haplotype of the hepatic lipase gene promoter was observed to be more common among African Americans and Japanese Americans than among Caucasians [295-298]. C-514T polymorphism has been associated with increased obesity in Chinese boys but not girls [299].

Multiple studies demonstrated a positive correlation between HL activity, diabetes and central obesity [300-304]. In obese subjects, the fraction of smaller, denser LDL, and mature HDL is decreased [286,249, 301, 305] and this lipoprotein profile can be normalized by weight loss [83]. Interestingly, data from the look AHEAD GWAS study indicated that in diabetic obese subjects LIPC (-105 A-to-T) single-nucleotide polymorphism was significantly associated with HDL-C and triglyceride changes with intensive lifestyle intervention [302].

Endothelial Lipase

Since it was cloned in 1999, EL has been recognized as major regulator of HDL levels in animal studies. The first evidence of a relationship between EL activity and HDL levels in humans was observed in 2002 [306]. In this study 20 unrelated subjects with HDL-C levels greater than the 90th percentile were recruited and the EL gene was sequenced. 17 polymorphic sites in EL were found and 6 potentially functional variants were identified (four amino acid changes Gly26Ser, Thr111Ile, Thr298Ser, and Asn396Ser, and 2 in the promoter 303A/C and 410C/G). The occurrence of these variants was then tested in 176 black controls, 165 white controls, and 123 whites with high HDL-C. Results indicated that Thr111Ile variant was the most common and was not directly associated with HDL-C levels. Interestingly, Gly26Ser, Thr298Ser, and 303A/C were found in the black and high HDL-C white cohorts but were absent in the control white group, thus indicating a possible functional association for these SNPs [306].

Further studies lead to the identification of several other variants in LIPG (table I-4 and HGMD database [314]) and GWAS studies supported the association between SNPs in the LIPG locus and HDL-C levels [295, 307-314]. Although while for some of the cited variants results from different populations are not consistent, the overall association between EL gene and HDL-C levels seems established. Several epidemiologic studies have clearly shown an inverse correlation between HDL-C levels and CVD risk [315, 316]. This association seems to be independent of other risk factors and it has been consistently reported in different populations [317-319]. Given this evidence, LOF mutations in LIPG were expected to induce a reduction in CVD risk. Surprisingly, studies aimed to determine the association between CVD and LIPG genetic variants did not give clear results. In the Lipoprotein and Coronary Atherosclerosis Study, (LCAS) patients with the missense variant c.111 C-to-T displayed increased HDL-C, apoC-III and higher HDL-C/LDL-C ratio. However, no significant association between the SNP and progression of coronary lesions was observed during the 2.5-year follow-up period [218]. A similar result has been observed in a study in 1138 CAD cases and 2237 matched controls from the EPIC-Norfolk cohort [320] and in a Danish study on

three different populations of patients with CHD (988 subjects from the Diet, Cancer, and Health study, 241 subjects from the Nurses' Health Study, and 262 subjects from the Health Professionals Follow-up Study) [321]. In both these two latter studies, no association between *LIPG* variants and CVD was reported.

A study on 265 Chinese patients with CAD and matched control healthy subjects, the Thr111Ile variant was found to be associated with decreased risk of CAD [322]. In contrary, three studies on independent cohorts and one study on Japanese subjects showed a positive correlation between LIPG variants and incidence of CVD and stroke, respectively [323- 326].

Furthermore, results from a Mendelian randomization study indicated that a single nucleotide polymorphism in *LIPG* (Asn396Ser) selectively increased HDL-C but these changes did not result in any reduction in the risk of myocardial infarction [327].

It has to be noted that most of the studies tested the association of CVD markers with known coding mutations in *LIPG*. Furthermore, all studies identified a high percentage of carriers of the relative common Thr111Ile variant. Interestingly, Khetarpal and colleagues in 2011 observed that the 229T-to-G regulatory variant in the untranslated 5' region induced a decrease in EL promoter activity. This variant was in LD with the more common Thr111Ile variant. Since Thr111Ile variant *in vitro* does not seem to affect EL enzymatic activity, it is possible that the association between this variant and high HDL-C levels may be mediated by the presence of 229T-to-G regulatory variant. This haplotype may partially explain the contradictory results observed in the previous studies [328].

The relationship between EL protein mass and HDL-C levels has been corroborated by several animal studies [213, 217, 329]. The first study that showed that correlation in humans was conducted by Badellino and colleagues in 2006 [330]. In this study, 858 asymptomatic subjects with a family history of premature CAD were recruited from the Study of the Inherited Risk of Atherosclerosis (SIRCA) population. Authors demonstrated that EL

concentrations in pre- and post-heparin plasma were negatively associated with HDL-cholesterol levels [330]. Furthermore, EL mass was positively correlated with small HDL3 concentrations and negatively correlated with large, mature HDL particles. No association was observed between EL and apoA-I levels [330]. Interestingly, in this study, EL mass was found to be associated with markers of metabolic syndrome and subclinical atherosclerosis such as the coronary artery calcification index [330].

This observation along with strong data from *in vitro* and *in vivo* studies supports a positive association between EL and features of metabolic syndrome such as inflammation, lead to further investigations.

Very early studies on EL structure and regulation already highlighted that LIPG mRNA levels were upregulated by inflammatory cytokines such as TNF-α and IL-β [95, 224]. The physiological meaning of EL upregulation is still not clear. It is well known that the other two plasma lipases (HL and LPL) are downregulated in the inflammatory state and it has been proposed that the concerted activity of all lipases may contribute to fulfill the cellular needs for HDL-derived FA during the inflammation [331, 332]. EL overexpression during inflammation likely explains the decrease in HDL-C levels observed in acute inflammation [333]. In a study on 74 moderately obese men, EL plasma mass was positively correlated with obesity, fasting plasma insulin, and plasma CRP, IL-6, and sPLA(2)-IIA concentrations. Interestingly, EL mass association with inflammation was independent of fat accumulation, LPL-activity or lipoprotein plasma levels [334].

Another study in healthy subjects from the SIRCA population demonstrated a strong positive correlation with C-reactive protein, interleukin-6, soluble TNF receptor II, soluble intercellular adhesion molecule 1, and leptin and an inverse correlation with adiponectin. Furthermore, experimentally-induced endotoxemia directly induced an increase in EL concentrations [228]. Finally, in a study on 80 sedentary men and 14 women post-heparin plasma EL concentration has been demonstrated to be positively associated with BMI, visceral adipose tissue, plasma cholesterol and TG. In this study, fat samples were collected from women during hysterectomy surgery. The analysis of

mRNA levels in visceral and subcutaneous adipose tissue revealed no difference in EL expression in these two tissues and demonstrated a relative 10^3 higher expression of LPL.

Interestingly, variants in EL gene have been associated with interindividual variability in HDL-C levels in response to physical exercise. It is known that exercise training can increase HDL-C levels, whereas inactivity promotes a pro-atherogenic lipid profile. In two studies on carriers of common LIPG variants, it has been demonstrated that EL genotype negatively affects exercise-induced increase in HDL-C levels [335, 336].

It has been proposed that EL may be associated with the development of insulin sensitivity. In humans, EL is positively associated with several markers of insulin resistance [330, 334] and different studies showed that EL plasma levels are increased in diabetic patients [337-339]. *In vitro* studies gave controversial results. In an early study in rat hepatocytes, insulin had no effect on endothelial lipase synthesis [340], whereas in a more recent study on human aortic endothelial cells, insulin significantly reduced EL mRNA and protein. Consistently, in the latter study, diabetic patients treated with insulin but not oral anti-diabetic drugs, displayed a significant reduction in EL mRNA and protein [341].

Taken altogether results from individuals with metabolic syndrome indicate a strong association of EL with several markers of the disease. The correlation between plasma levels of EL and cytokines is strong and independent of other traits such as lipoprotein profile both in obese subjects and healthy individuals. This suggests that inflammation alone may be sufficient to induce EL increased expression. However, although inflammation is a condition commonly associated with obesity and metabolic syndrome, it is still unclear if that is an early event in the pathogenesis of the disease. As a consequence, even if EL expression may be sustained by inflammation, other factors may contribute for the EL upregulation in subjects with metabolic syndrome.

Missense mutations

dbSNP number	Nucleotide change	Amino acid change	Effect on function	Phenotype	Reference
rs9963243	c.76G>A	p.G26S	↓ Lipase activity	↑ HDL-C	[306, 342, 343]
NA	c.82G>A	p.E28K	=Lipase activity (predicted)	NA	[343]
rs200103565	c.115A>C	p.K39Q	=Lipase activity (predicted)	↓ HDL-C	[344]
rs375653841	c.155A>G	p.N52S	↓ Lipase activity	NA	[343]
rs61729804	c.160C>T	p.R54C	↓ Lipase activity	NA	[343]
rs61729804	c.218C>T	p.P73L	↓ Lipase activity	NA	[343]
rs2000813	c.332C>T	p.T111I	= Lipase activity	= HDL-C =↑↓ CAD ↑Stroke ↑diabetic retinopathy	[218, 306, 320-326, 328, 338, 343, 345, 346]
rs111384586	c.346G>A	p.A116T	↓ Lipase activity (predicted)	↑ HDL-C	[346]
NA	c.388C>T	p.L130F	↓ Lipase activity (predicted)	↑ HDL-C	[347]
NA	c.526G>A	p.G176R	↓ Lipase activity (predicted)	= ↑ HDL-C	[346]
NA	c.586G>A	p.G196R	↓ Lipase activity	↑ HDL-C	[347, 348]
NA	c.660C>G	p.H220Q	↓ Lipase activity	↑ HDL-C	[347, 348]
NA	c.716T>C	p.I239T	↓ Lipase activity (predicted)	= ↑ HDL-C	[346]
NA	c.817G>T	p.E273	↓ Lipase activity (predicted)	↑ HDL-C	[349]
NA	c.1012A>C	p.T338P	↓ Lipase activity	NA	[343]
NA	c.1024A>G	p.M342V	↓ Lipase activity (predicted)	= ↑ HDL-C	[346]
NA	c.1162G>T	p.E388*	↓ Lipase activity	↑ HDL-C	[347]

rs181279169	c.1166G>A	p.R389Q	↓ Lipase activity	NA	[343]
rs138438163	c.1171G>A	p.E391K	↓ Lipase activity	↑ HDL-C	[344]
rs77960347	c.1187A>G	p.N396S	↓ Lipase activity	↑ HDL-C	[348]
rs117623631	c.1486A>G	p.R476W	↓ Lipase activity	= ↑ HDL-C	[348]
NA	c.1486A>G	p.T496A	↓ Lipase activity (predicted)	↑ TC	[350]
rs113235863	c.1501T>C	p.501R	↓ Lipase activity (predicted)	↑ HDL-C	[346]

Regulatory/intronic mutations

dbSNP number	Mutation Type	Codon number	Codon change	Phenotype	Reference
rs34474737	Regulatory	-24 rel to initiation codon	T-G	Decreased promoter activity. LD with 111C-T	[328]
rs3744841	Regulatory	+482 relative to termination codon	G-A	↑HDL-C	[351]
rs3744841	Regulatory	-384 relative to transcription initiation site	A-C	HDL-C ↑CAD	[320, 325, 352, 353]
NA	Intronic	+ 2864 relative to initiation codon	T-C	HDL-C	[354]
NA	Intronic	+ 42 relative to initiation codon	C-T	HDL-C	[354]

Deletions

Nucleotide deletion	Phenotype	Reference
c.342delA	↑ HDL-C	[346]
c.388delC	↑ HDL-C	[355]

Table I-4 Summary of genetic variants identified in LIPG.
Data were obtained from specific references and HGMD database [314].

Aim

Plasma triglyceride lipases, lipoprotein lipase (LPL), hepatic lipase (HL) and endothelial lipase (EL) are secreted enzymes that bind to the endothelial surface and catalyze the hydrolysis of lipids on circulating lipoproteins [1]. The released fatty acids are then available for uptake by peripheral tissue where they can exert trophic or modulatory functions. As a consequence, the concerted activity of the three lipases has profound effects on plasma and tissue lipid metabolism.

The specificity and physiological activity of LPL and HL have been extensively explored *in vivo* and *in vitro*. LPL preferentially hydrolyzes triglyceride (TG) in chylomicrons and VLDL, whereas HL hydrolyzes TG and phospholipid (PL) in HDL and LDL [2, 33]. The study of human carriers of LPL and HL deficiency highlight the importance of these lipases on lipoprotein and tissue metabolism [173]. Furthermore, clinical studies have shown that lipase activity is tightly linked to the pathogenesis of metabolic disorders such as dyslipidemia, diabetes and obesity [263-267, 300-304].

EL is the most recently identified member of the plasma triglyceride lipase family [89, 90]. Analysis of EL substrate specificity revealed that EL hydrolyzes both PL and triglycerides TG on lipoproteins and has a relatively higher specificity for PL compared to TG [89, 90, 211]. Since it was cloned in 1999, EL has been shown to be a major regulator of HDL-C levels both *in vitro* and *in vivo* [89, 90, 218]. Consistent with its biochemical profile, in humans, genetic variants for EL are associated with increased HDL-C levels but these changes do not translate into a reduction in CVD risk (Table I-4). Given the established inverse correlation between HDL-C and cardiovascular disease, early human studies have focused on better understanding the relationship between EL activity and a pro-atherogenic lipid profile. These studies consistently demonstrated a strong correlation between EL mass and several traits of metabolic syndrome such as body mass index (BMI), visceral adiposity, inflammation, insulin resistance and atherogenic lipoprotein levels [228, 229, 330]. However, metabolic syndrome is by definition a heterogeneous condition, and the causal relationship linking EL to one of multiple traits is still unknown.

Furthermore, very little is known about the role of EL in apoB-containing lipoproteins and tissue lipids. In *vitro* data indicated that EL can hydrolyze PL and TG in apoB containing lipoproteins [211, 222]. EL overexpression promotes the clearance of LDL particles and translates in significantly reduced LDL, VLDL, apoB, total cholesterol, TG and PL levels [216]. In contrast, EL deficiency is associated with increased levels of non-HDL cholesterol and apoB-containing lipoproteins [356]. The physiological significance of EL-mediated hydrolysis of TG-rich lipoproteins is not known. LPL is thought to be the main enzyme responsible for the catabolism of TG-rich lipoproteins [2]. However, LPL deficiency in humans is not associated with reduced adiposity and it has been proposed that EL could provide an alternative pathway for free fatty acid uptake into adipose tissue [173, 357].

The Aim of this thesis is to assess the role of EL in TG-rich lipoprotein metabolism and to investigate the relationship between EL activity, hepatic function and the development of key traits of metabolic syndrome *in vivo*.

This general aim will be pursued through three specific aims

- Specific Aim 1: *Establishing the effect of EL deficiency on the onset of clinical hallmarks of metabolic syndrome in vivo.*
  Metabolic syndrome is a cluster of modifiable/ risk factors including obesity, hypertriglyceridemia, and hyperglycemia. These conditions can be efficiently induced in mice by feeding a long-term high-fat diet. We took advantage of the availability of a mouse model of EL deficiency to compare the onset and severity of metabolic syndrome traits in the presence or absence of EL activity. The ultimate goal of this experimental set was to clarify the directionality of the association between increased EL levels and metabolic syndrome.

- Specific Aim 2: *Defining the role of EL in plasma TG-rich lipoprotein metabolism.*
  Using a similar experimental design, the plasma lipoprotein profile of WT and EL-deficient mice on regular chow diet and in response to a high fat diet was extensively characterized. To understand the basis of

EL-mediated TG hydrolysis in the setting of a high fat diet, hepatic TG production, clearance and tissue fatty acid (FA) uptake have been studied.

Finally, EL and LPL have distinct, but complementary tissue distribution and lipid substrates. *In vitro* experiments have been specifically designed to assess whether there is a cooperative effect of both lipases on TG-rich lipoprotein metabolism.

- Specific Aim 3: *Defining the impact of EL deficiency on liver lipid metabolism.*

  Hydrolysis of TG-rich lipoproteins not only affects plasma lipoprotein metabolism but determines the availability of FA in peripheral tissues and contribute to the regulation of *de novo* lipogenesis. Alterations in FA utilization by the liver are associated with the development of fatty liver disease [221]. EL, but not LPL, is expressed in adult liver and may contribute to hepatic lipid sensing and regulation of hepatic lipid storage. To test this hypothesis, the hepatic lipid accumulation in EL deficient and WT mice was evaluated and the expression levels of key genes involved in the regulation liver lipid metabolism was determined. This specific aim is focused on understanding the effect of EL deficiency on liver lipid homeostasis.

Methods

1 *In vivo* procedures

Animal care and diet treatment

All the animal experiments were performed at the University of Pennsylvania. All the procedures were performed according to the regulations and with the prior approval of the Institutional Animal Care and Use Committees of the University of Pennsylvania (IACUC, protocol number 803056).

Male C57BL/6 (or wild-type, WT) were obtained from Jackson. Male endothelial lipase (EL)-KO mice on a C57BL/6 background were previously described [213]. All mice were housed in a pathogen-free animal facility with a daylight cycle from 07.00 AM to 7.00 PM. Animals had access to food and water. For the characterization of the baseline phenotype, mice were fed a regular chow diet (Lab Diet). For experiments that required the induction of the metabolic syndrome phenotype, 10 weeks old mice were fed a high fat diet (D12451- Open Source Diets- 45% Kcal from fat) for 12 weeks.

Non-fasted body weights were measured weekly, beginning at the time of weaning. During the diet treatment, the leftover food in each cage was weighted weekly. The calculation of the daily food consumption was done by dividing the weight of the consumed food in each cage by the number of animals in the cage.

Blood was collected either via retro-orbital bleeding or tail vein bleeding (see single protocol) under isoflurane anesthesia, using EDTA containing- capillary tubes.

When euthanasia was required by the experimental design, mice were sacrificed by cervical dislocation under deep isoflurane anesthesia.

Metabolic phenotyping and activity measurements

Body composition (lean and fat mass) was measured by DEXA (dual-energy X-ray absorptiometry) scan and magnetic resonance imaging by the Mouse Phenotyping, Physiology and Metabolism Core at the University Of Pennsylvania School Of Medicine, according to the method described by

Varela and colleagues [358]. Metabolic rates were measured by indirect calorimetry in open-circuit oxymax chambers, using the the Comprehensive Lab Animal Monitoring System, CLAMS (Columbus Instruments, Columbus, OH, USA). Mice were housed singly in sealed, open flow chambers and maintained at 24 °C under a 12-h light–dark cycle (light: 07.00 AM-7.00 PM). Food and water were available *ad libitum*. All the mice were acclimated to monitoring cages for 24 h before the initiation of experiment. To calculate oxygen consumption (VO2), carbon dioxide production (VCO2) and RER (ratio of VCO2 to VO2), the gas concentrations were measured in the incurrent and excurrent gas flow. VCO2, VO2 and calculated heat were normalized to lean mass, for each animal.At 12 weeks mice were sacrificed and the different fat depot (inguinal, subcutaneous and brown fat) were collected and weighted.

Glucose Phenotyping

Glucose Tolerance Test

The glucose tolerance test (GTT) is a standardized method to measure the glucose clearance from the circulation, after an acute loading. This test is used in both mice and humans to determine potentially sub-pathological traits that may predispose to frank insulin resistance. Mice were fasted overnight, weighted and bled via tail vein to measure the baseline glycaemia. Each mouse received an intraperitoneal bolus of sucrose (10 uL/g of body weight of a 20% sucrose solution in PBS). Blood glucose was measured at 15, 30, 60, 90 and 120 min after injection, via the tail vein with the BREEZE 2 glucometer (Bayer).

Insulin Tolerance Test

The insulin tolerance test (ITT) is a standardized method to measure the body tissue responsivity to insulin. Mice were fasted for 4 hours, weighted and bled via tail vein to measure the baseline glycaemia. Each mouse received an intraperitoneal bolus of insulin (Humalin-R, 0.75 U/Kg body weight). Blood glucose was measured at 15, 30, 60 and 120 min after injection, via the tail vein with the BREEZE 2 glucometer (Bayer).

Determination of systemic inflammatory response

In order to evaluate the inflammatory response in WT and EL-KO mice, a model of experimentally-induced low-dose endotoxemia was used. Lipopolysaccharide (LPS) injections are used in mice and humans in the literature as a model for low level inflammation as seen in obesity, atherosclerosis, and other inflammatory diseases [359, 360]. This procedure induces a transient mild inflammatory response without causing infection and it's thus typically well tolerated by the animals. WT and EL-KO mice were maintained at 24 °C under a normal 12 h light–dark cycle. Food and water were available ad libitum. In order to permit the determination of baseline cytokine levels, prior to LPS injection blood was collected via tail vein bleeding. Mice were then intraperitoneally injected with LPS solution in PBS at a dose of 0.5 mg/kg (Sigma Aldrich). Mice were monitored every hour to check their well-being. Blood was collected via tail vein bleeding at one and three hours after injection, using EDTA-containing capillaries. Mice were then euthanized as described previously. Blood was spun and plasma was collected for cytokines determination. TNF-α plasma concentration was determined using the mouse TNF-alpha Quantikine ELISA Kit (R&D systems).

Oral Fat Tolerance Test

In order to determine post-prandial TG clearance in mice the Oral Fat Tolerance Test was performed. WT and EL-KO mice on high fat diet or chow (n=6 per group) were fasted overnight. During this period were maintained at 24 °C under a normal 12 h light–dark cycle and had access to water. Prior to gavage blood was collected to determine the baseline TG plasma levels. Mice were then weighted and gavaged with 10 uL/g of body weight of olive oil (Sigma Aldrich). After gavage, blood was collected at 1, 3, 5 and 7 hours via retro-orbital bleeding, using EDTA-containing capillaries. Blood was spun and plasma was collected for TG determination, FPLC and agarose gel electrophoresis analysis. The TG content was measured by colorimetric enzymatic assays in a 96 wells plate (Infinity Triglycerides reagents, Thermo Fisher).

In vivo clearance kinetic experiments

Isolation and labeling of human TG-rich lipoproteins

TG-rich lipoproteins (TRL) were purified by sequential ultracentrifugation of the plasma of non-fasted anonymous healthy volunteers. Plasma was isolated from 200 ml of blood. It was then transferred to ultracentrifugation tubes and a layer of KBr solution (density=1.006 g/L) was added on top. The tubes were sealed and ultracentrifugated in a Beckman XL590 Ultracentrifuge (Beckman Coulter) using a 70.1 Ti rotor (Beckman Coulter) at a speed of 40,000 RPM for 18 hours. At the end of the run, TG-rich lipoproteins, including VLDL, CM and remnants, that are characterized by a density <1.006 g/L floated on the top of the tube. Lipoproteins were then collected and the total protein content was measured by BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific) and further processed for radioactive labeling.

^{125}I protein labeling: Dialyzed human TG-rich lipoproteins were labeled with ^{125}I by the iodine monochloride method [361]. Lipoproteins were then dialyzed against PBS to remove excess of non- incorporated ^{125}I. For direct ^{125}I labeling, 1.5 mg of of TRL in PBS were iodinated with 1 mCi of ^{125}I, 300 ul of 1 M glycine, and 150 uL of 1.84 M NaCl / 2.84 uM ICl solution, vortexed and applied to a PG510 desalting column (Amersham Biosciences) that was pre-equilibrated with 0.15 M NaCl / 1 mM EDTA solution. Iodinated proteins were eluted in NaCl/EDTA solution to a final volume of 2 mL. They were then dialyzed against PBS and protein concentration was assessed by BCA. ^{125}I activity was determined by gamma counting (Packard Cobra II Auto5 Gamma counter).

3H-TO-labeling: TRL were labeled with [3H] Triolein (TO), using cholesteryl ester transfer protein to facilitate the incorporation [362]. Briefly, 3 mg of TRL protein was used for labeling with 0.5 mCi of 3H-TO (Perkin Elmer). 0.5 mCi of 3H-TO in toluene was dried down under a nitrogen and resolubilized in 150 uL of ethanol. 3H-TO solution and lipoprotein depleted human plasma (100 mg protein / 3 mg TRL protein) were added to TRL and the resulting mixture was incubated at 37 °C overnight. Labeled TRL were then subjected to a “rinse” ultracentrifugation in the same conditions described above. This step

permits the re-floctation of TRL and removal of excess of non-incorporated 3H-TO. TRL were then collected, dialyzed against PBS, and fractionated by FPLC to measure TG and ^{3}H activity in the VLDL-CM fractions relative to unincorporated ^{3}H activity. Activity was determined by scintillation counting (Beckman LS 6500 Scintillation System).

In vivo clearance determination

In vivo experiments were designed to determine the plasma lipolysis rate of TRL-associated TG and particle clearance from the circulation [361, 363]. Prior to the initiation of the experiment mice were fasted 4 hours and bled to determine the baseline lipids. They were then injected with dialyzed ^{3}H or ^{125}I labeled TRL were administered by intravenous tail vein injection into WT and EL-KO mice. Mice were bled at 1, 3, 5, 10 and 15 minutes after TRL administration. Mice were euthanized at 15 minutes after injection as previously described and tissues collected.

Plasma ^{3}H and ^{125}I activity at each timepoint were determined using scintillation counting and gamma counting, respectively (Beckman LS 6500 Scintillation System and Packard Cobra II Auto5 Gamma counter). The relative ^{3}H activity remaining the in circulation was calculated by normalizing the activity from each timepoint by that of the 1 minute timepoint for each mouse. The fractional catabolic rates were calculated with the SAAM II program (SAAM Institute) by fitting a biexponential curve to the [^{125}I] and [^{3}H] counts normalized to the 1-minute time point. The differential uptake of labeled ^{3-}H FA in each tissue was determined by scintillation counting of ~150 ug of homogenized tissue in PBS. The results were expressed as activity/ mg of tissue and normalized to the 1-minute time point.

In vivo reconstituted HDL clearance

rHDLs containing 18:1-12:0 Top Fluor PC, UC and apoA-I were prepared according to the cholate dialysis method. Human HDL were isolated sequential ultracentrifugation of the plasma (d<1.21 g/L) and apoA-I was purified using the cold ethanol precipitation method (~95% purity by SDS-PAGE) [364]. The apoA-I was combined with palmitoyl-2-oleoyl-sn-glycero-3-

phosphocholine (POPC, Sigma Aldrich), non-esterified cholesterol (FC, Avanti polar Lipids), esterified cholesterol (CE, Avanti Polar Lipids) and Top fluor PC (1-palmitoyl-2-(dipyrromethene borondifluoride)undecanoyl-sn-glycero-3-phosphocholine, Avanti polar Lipids), using the sodium cholate dialysis method (weight ratio = 10.5: 0.3:0.3:1.5:1.2 mg for POPC:FC:CE:apoA-I: top FluorPC)[365]. After the additions, sodium cholate was removed by dialysis (dialysis tubes with molecular weight cut-off =12-14 kDa, spectra/Por, spectrum Lab). 0.2 mg of Top-Fluor PC were intravenously injected to each mouse and tissues were collected after 6 hours. 100 ug of tissue were homogenized and lipids were extracted according to the Folch method [366]. Lipids were then dried down and resuspended in 5% Triton X solution in PBS. Fluorescence was determined using a microplate reader (Synergy MX, BioTech).

In vivo TG secretion

Hepatic VLDL secretion was measured in WT and EL-KO mice (n=8) [367]. Briefly, mice were fasted for four hours and then administered the surfactant Pluronic acid (25 mg in 0.4 ml PBS) by intraperitoneal injection to inhibit peripheral lipolysis. Mice were bled at 0, 60, 120, and 240 minutes after Pluronic acid administration for plasma collection. Plasma TGs were measured at each timepoint with the Infinity Triglycerides reagent (Thermo Scientific) in 96 wells plates. 60 and 240 minute plasma samples were used for calculating the TG secretion rate by linear regression of the curved describing the measured concentration kinetic.

Post-heparin plasma collection

Post heparin plasma was collected from WT and EL-KO mice in order to measure the lipase activity against emulsion substrate (see section 3). Mice were weighted and then fasted for 4 hours. They were injected heparin solution via tail vein (300 units heparin/kg body weight). 5 minutes after injection blood was collected and centrifuged at 10,000 g for 10 min at 4°C to separate plasma.

2 *Ex vivo* analyses

Histology

Hematoxilin and eosin staining

Hematoxylin and Eosin (H & E) staining is the most common staining technique in histopathology. This uses a combination of two dyes, Hematoxylin and Eosin that allow one to easily visualize nucleus and cytoplasmic inclusions in tissue sections. Immediately after euthanasia, tissues (liver, fat, skeletal muscle) from WT and EL-KO mice (n=6 per group) were collected, sectioned and fixed in a 4% paraformaldehyde solution at 4 °C overnight. Tissues were then dehydrated by equilibration with increasing concentrations of ethanol solution, and infiltrated with wax. The infiltrated tissues were embedded into paraffin wax blocks (Paraplast X-tra) and sectioned using a microtome (6 μM thickness) at the Histology and Gene expression Core of the University of Pennsylvania. Prior to proceed to H&E staining, sections were deparaffinized in xylene, (10min X2) and rehydrated with distilled water. They were then stained with Hematoxylin (CAT Hematoxylin) for 3-5 min, rinsed with water and differentiated with acid alcohol (1% HCl in 70% alcohol). After washing slides were stained with Eosin (Edgar Degas Eosin) for 4 min. Slides were then dehydrated and differentiated with 95% alcohol. Slides were finally cleared by immersion in in xylene and mounted with mounting media (Permount).

Adipocytes size and number characterization

Images were acquired with a Leica DM4000B upright scope paired with a Spot RT/SE slider camera. For the adipocyte analysis, a total of 6 tissue specimens per group were processed. For each specimen, 3 images/section and 5 different sections were analyzed (15 images/specimen, 6 specimens per group). Images were acquired at a 200X magnification and then analyzed with Adiposoft software [368].

Oil red O staining

Oil red O staining specifically marks lipid inclusions. The staining is based on the lipophily of the dye that permits a high affinity for neutral lipids. As a result,

stained lipids are red and the further counterstaining with hematoxylin confers nuclea a blue appearance. To this purpose liver tissues from WT and EL-KO mice (n=6 per group) were collected immediately after sacrifice, fixed in 4% PFA for 1 hour and then cryopreserved by immersion in 30% sucrose solution over-night. Tissues were then embedded in OCT cryo-medium and frozen in liquid nitrogen. The further processing of the specimen was performed at the Pathology Core of the Children's Hospital of Philadelphia. 8 μM thickness sections were obtained using the cryotom (Thermo Scientific Microm HM 560). Sections were air dried, rinsed with 60% isopropanol, stained with Oil Red O solution for 15 mins and rinsed again with 60% isopropanol. Nuclea were then counterstained with alum haematoxylin, slides were rinsed and mounted with acqueous glycerine for analysis.

Images were acquired with a Leica DM4000B upright scope paired with a Spot RT/SE slider camera.

Plasma and Tissue Lipid analysis

Plasma levels of total cholesterol, HDL cholesterol, phospholipid, triglyceride and Non-Esterified Fatty acids were determined enzymatically by colorimetric and immunoturbidrimetric assays using Axcel clinical analyzer, unless indicated otherwise in the specific procedure. Non HDL cholesterol levels were calculated by subtraction of HDL-C levels to total Cholesterol levels.

Tissue TG and cholesterol content was determined as follows. Frozen mouse liver samples were weighted and homogenized in PBS using a high speed mechanical homogenizer (TissueLyser II, Quiagen). Homogenized sample was diluted 1:50 with a solution containing 1% or 0.25% deoxycholate for assaying TG and cholesterol, respectively. Hepatic concentrations of cholesterol and triglycerides were then measured using commercial kits (Infinity Triglycerides and Infinity Cholesterol reagents, Thermo Fisher) and normalized per gram liver weight.

FPLC fractionation of plasma lipoproteins

Plasma lipoproteins were separated by Fast Protein Liquid Chromatography

(FPLC) with size exclusion method that separate lipoproteins according to their size. Columns were equilibrated with buffer before loading sample. Equal volumes of plasma from WT and EL-KO mice were mixed into two separate pools and injected into the column (GE AKTA Purifier 100, mounting 2 Superose 6 Increase 10/300 GL columns).

Phosphate buffer containing 0.15 M NaCl, 0,03% EDTA and 0,02% NaN3 was used as mobile phase. Once in the column, the lipoprotein classes segregate by size, based on their ability to be retained in the gel pores. This principle determines the differential elution time of different lipoprotein subclasses. The elution profile was monitored by UV absorption at 280 nm. FPLC was performed at 0.3 mL/min constant flow rate for 84 minutes at room temperature. Fortysix fractions of 0.5 ml each were collected in 96 wells-plate and total cholesterol and TG content was measured in each fraction by colorimetric enzymatic assays (Infinity Cholesterol and Infinity Triglycerides reagents, Thermo Fisher). The apolipoprotein content of 150 uL of each fraction was determined by western blot (see specific procedure).

Agarose gel Electrophoresis of Plasma lipoproteins

Agarose gel electrophoresis is a well-established technique routinely used in clinical laboratories for screening lipoprotein abnormalities in plasma. It is based on the principles of zone electrophoresis and allows lipoproteins to separate based on charge and size. 2 uL of plasma from the OFTT experiment (pre, 3 and 7 hours time-points) was loaded onto agarose gel (Titan Lipoprotein gels, Helena) and run in Barbital buffer (pH=8.5) at constant voltage for 50 minutes. At the end of the run, gels were dried in ventilated oven, stained with Fat-Red-methanol solution and images were acquired using ChemiDoc Imaging System (Biorad). Electrophoretograms are evaluated visually for the presence of quantitatively or qualitatively abnormal protein bands.

Protein expression analysis

Protein expression was analyzed by Western Blot. Depending on their nature, samples were pre-treated in different ways. For FPLC fractions 150 uL of

each fraction was dried down to a final volume of 20 uL using a personal evaporator at 37 °C (Genevac, SP Scientific). For liver protein analysis, frozen tissue was homogenized in RIPA buffer containing a complete set of protease inhibitors (Roche), using a high speed mechanical homogenizer (TissueLyser II, Quiagen). The homogenate was spun at 15000 rpm. The surnatant, was collected, protein content was determined by BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific) and 50 ug of total protein were loaded into the gel. Finally, for total plasma protein content, 1uL of sample was utilized. All samples were combined with NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent, according to the manufactures specifications. Samples were heated at 95 °C for 10 minutes to help denaturation and then run into gradient gels. All runs were conducted in presence of a pre-stained protein standard to verify the expected protein molecular weight (SeeBlue Plus2, Invitrogen). For apoB48 and apoB100 determination precast 3-8% Tris-Acetate gels and tris-acetate buffer were used (Novex 3-8% Tris-Acetate Midi-Gels and Tris-Acetate SDS Running Buffer, Invitrogen). For other proteins, 4-12 % gradient was used and the run has been conducted using 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (NuPAGE Novex Bis-Tris Gels and NuPAGE MOPS SDS Running Buffer, Invitrogen). Gels were run in the XCell SureLock apparatus and transferred to nitrocellulose membrane (Biorad) using the XCell II Blot Module with SDS and methanol containing transfer buffer (NuPAGE Transfer Buffer with 20% methanol, Invitrogen).

The membranes were blocked over-night in 5% fat free milk (Thermo Fisher) and then immuno-blotted against target proteins. For this purpose the following antibodies were used.

Primary antibodies:

- Rabbit anti Apolipoprotein B48/100 (K23300R), Meridian Life Science,
- Rabbit anti Apolipoprotein E (NB100-2040), Novus Biologicals
- Mouse anti B-Actin, clone AC-15 (A5441), Sigma Aldrich
- Rabbit anti LPL, H-53 (sc-32885), Santa Cruz
- Rabbit anti HL, H-70 (sc-21007), Santa Cruz
- Goat anti SCD1, E-15 (sc-14720), Santa Cruz

- Rabbit anti human EL, was generated in the Rader lab against a peptide in the N-terminal region of EL [89].

Secondary antibodies:

- Goat anti rabbit IgG-HRP (sc-2030), Santa Cruz
- Rabbit anti mouse IgG-HRP (sc-358914), Santa Cruz
- Rabbit anti goat IgG-HRP (1721034), Biorad

The detection was performed by incubation of immunoblotted membranes with Luminata Crescendo Western HRP substrate (EMD Millipore) and the image was obtained using ChemiDoc Touch Gel and Western Blot Imaging System and analyzed using Image Lab software (Biorad).

Gene expression analysis

Immediately after euthanasia tissues were collected and snap frozen in liquid nitrogen to preserve nucleic acids. Total RNA was extracted from tissues using Trizol (Thermo fisher). Tissues were homogenized in Trizol with a high speed mechanical homogenizer (TissueLyser II, Quiagen) and RNA was extracted with chlorophorm/isopropanol method according to the manufacturer specifications (Trizol, Thermo fisher). RNA was quantified by spectrophotometric absorbance determination at 260 nm (Take3 Micro-Volume Plates. BioTek). Absorbance at 230 and 280 nm was also measured to determine nucleic acid purity. 0.1 µg of RNA was retrotranscripted in cDNA using High-capacity cDNA Reverse Transcription Kit (Applied Biosystem). The gene expression was evaluated through quantitative Real Time PCR, using pre-optimized reagent and QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). Briefly, the reaction mixture included a mix of unlabeled PCR primers and a TaqMan probe with FAM dye label (Applied Biosystems- TaqMan Gene Expression Assays), and TaqMan Fast Advanced Master Mix (Applied Biosystems). Reactions were run in 384-well plate under uniform cycling conditions. The cDNA templates were mixed with the reaction mix and equal volumes (10 µl) was loaded to each well. Cycling conditions were as follows: Uracil N-glycosylase (UNG) activation (50°C, 2 min), polymerase activation (95°C for 20 sec), 40 PCR cycles (denaturation,

95°C for 1 sec and an annealing step performed at 60°C for 20 sec). Data analysis was based on the ΔΔCT method with normalization of the raw data to an housekeeping gene (actin). Relative quantification is used to compare the gene expression levels between different groups of mice and results were expressed as fold-changes (assuming control group average expression as unit).

Hepatic fatty acid composition

The analysis of liver FA composition was conducted by Dr Papasani V Subbaiah, at the Department of Medicine, Section of Endocrinology, Diabetes, and Metabolism, University of Illinois at Chicago. Levels of hepatic FA subspecies were measured using gas chromatography/mass spectrometry (GC/MS) as previously described [366].

3 *In vitro* Lipase activity assays

Generation of EL and LPL conditioned media

EL and LPL were generated using recombinant adenoviruses. Recombinant adenoviruses encoding either human EL (AdhEL) and human LPL (AdhLPL) [211]. Subconfluent COS cells were infected with recombinant adenoviruses in serum-free medium (3,000 particles/cell). After 48 h, heparin was added to a final concentration of 10 U/ml to detach lipases from the cells surface. The plates were incubated for an additional 30 min. The media were then harvested, aliquoted and frozen at −80°C. Expression of enzymes was confirmed by Western blotting.

In vitro *activity against a large lipid emulsion*

Triglyceride lipase activity was measured according to a modification of the method of Nilsson-Ehle and Schotz [369]. Conditioned medium containing LPL and EL was used as the enzyme source whereas a glycerol-stabilized large lipid emulsion has been used as substrate. Briefly, 300 mg triolein (Sigma Aldrich), 18mg egg phosphatidylcholine (Sigma Aldrich) and 3H-Triolein (Perkin Elmer 99 µg per 300 mg nonradioactive triolein) were dried under nitrogen. 5 mL of glycerol (Fisher Scientific) were added and the mixture was sonicated using a Branson 450 microtip sonicator for five minutes. Concentrated emulsions were allowed to clear overnight. 15 µL of this concentrated emulsion was combined with 90 µL distilled water, 15 µl of 1.0 M Tris pH 8.0, 15 µl of 15% BSA solution, and 15 µl of 3.0 M NaCl to give a final volume of 150 µl of working emulsions.

Working emulsion substrate (150 µl) was combined with different dilutions of EL and LPL conditioned media (150 µl) or post-heparin plasma (20 µl, diluted 1:8) were incubated at 37 °C for 30 minutes. Heat-inactivated serum was added to provide apoC-II for LPL reaction (0.5% of final volume). The reaction was terminated by the addition of 3.25 ml of methanol:chloroform:heptane solvent (1.41:1.25:1.00). 1.05 ml of pH 10.0 Buffer (Thermo Fisher Scientific) was added and tubes were spun at 2,000 RPM for 20 minutes. The upper

phase containing liberated fatty acids was used for scintillation counting (0.5 ml per sample) [370]. The relative amount of hydrolysis of ^{3}H-Triolein to ^{3}H-oleic acid was calculated for each sample.

In vitro *activity against labeled human TRL*

In this assay, increasing volumes of EL and LPL conditioned media were incubated with ^{3}H-TO labeled TRL. TRL were isolated and labeled as previously described (see section 1). 50 uL of this substrate was incubated with 70 uL of enzyme containing media. For the combination assay, different volumes of each medium were mixed and brought to a final volume of 70 uL. 2% weight/volume BSA was added to prevent product inhibition of the reaction. Mixtures wre incubated for 4 hours at 37 °C. The reaction was stopped by adding 1 mL of ice cold ethanol. Tubes were immediately placed in dry ice and kept at -20 °C overnight. Samples were then spun 13500 rpm/20 min to permit protein precipitation. The surnatatant, containing all lipid species, was transferred in glass tubes and dried down under nitrogen. Lipids were resuspended in 60 uL of chlorophorm and separated into the different species by Thin Layer Chromatography, TLC (mobile phase: Exane: diethyl ether: Acetic Acid (170:30:1), stationary phase: glass microfiber silica paper, Agilent Technologies). Samples were run together with lipid standards (cold Triolein, Oleate and Diacylglycerol) to identify the migration point of selected lipid species. The three bands were cut and counted for each condition using a scintillation counter. Each sample was run at least in triplicate. TG lipase activity was expressed as percentage of counts of the FA band compared to total counts (sum of activity in Triolein, Oleate and Diacylglycerol bands).

4. Statistical Analysis

All data are represented as mean and standard deviation, with error bars showing standard deviation. Statistical comparisons between two groups were performed using a two-tailed Student's t-test. When required, correction for multiple comparison has been applied. Statistical significance was defined as $P < 0.05$ for all analyses. All the tests were run using Graph Pad Prism software.

Results

Specific aim 1: Establishing the effect of EL deficiency on the onset of clinical hallmarks of metabolic syndrome

1.1 EL deficiency alters adipocyte phenotype, fat distribution and exacerbates diet-induced obesity

In order to induce the essential traits of metabolic syndrome, three independent cohorts of WT and EL-KO mice (n=8) were put on high fat diet (45% calories from fat). Body weight and food consumption were recorded weekly for 12 weeks. As expected, in both groups the diet induced a significant increase in body mass (Figure R-1.1). EL-KO mice in all the three replicate experiments, displayed a significantly higher weight increase compared to WT (Figure R-1.1 and R1.2A) and this change was not accompanied by increased food intake (Figure R-1.2B).

The NMR analysis of body composition revealed that weight increase was due to increased fat mass (36.98 ± 9.14g in WT and 42.46 ± 9.65g in EL-KO, P=0.0180) whereas the lean tissue was decreased in EL-KO mice (63.09 ± 7.03 in WT and 56.14 ± 4.39 in KO, P=0.0415) (Figure R-1.3A).

Traditionally, adipose tissue has been classified into white fat, mainly involved in lipid storage, and brown fat, that constitutively express high levels of thermogenic genes. Given the different metabolic and clinical significance of these two fat depots, the body fat composition and distribution in the two groups was assessed. Fat pads from WT and EL-KO mice were collected after 12 weeks of High Fat diet and weighted (Figure R-1.3B). No difference was observed in brown fat mass between WT and EL-KO mice (0.631 $\pm$ 0.043 and 0.648 $\pm$ 0.052 g for WT and KO, respectively) whereas white fat mass was significantly increased in EL-KO mice compared to WT and this difference was particularly striking for the inguinal depot (subcutaneous fat- WT: 3.05 $\pm$ 0.803 KO: 3.71 $\pm$ 0.151g, inguinal fat- WT: 4.014 $\pm$ 0.819, KO: 4.9 $\pm$ 0.925 g) (Figure R-1.3B). The mass and function of visceral fat depot has been positively associated with the risk of developing metabolic disease. This

raised the hypothesis that the increased deposition of visceral fat observed in EL-KO mice may be accompanied by differences in the adipocyte metabolic and morphologic profile. To address this question, histological analysis of inguinal fat was performed. Samples of ~50 mg of tissue were fixed in PFA, paraffin-embedded, sectioned and stained with H&E (Figure R-1.4A). The adipose cell size and number of cell per analyzed area were determined using Adiposoft analysis software. The cell-size range in the collected samples was 1000-19000 μm^2 (FigureR-1.5). The average adipocyte area was significantly higher in EL-KO mice compared to WT (WT: 175.33 $\pm$ 10.83 KO: 294.31 $\pm$ 35.67 μm^2), indicating that visceral fat is not only hyperplasic but also hypertrophic in these mice (Figure R-1.4B-C).

Given the high heterogeneity among adipose cells size, the cell-size distribution in the two groups was calculated. Figure R-1.5 shows the relative frequency of each subpopulation. Results indicate that EL-KO mice exhibit a reduction in small adipocyte and a parallel increase in large hypertrophic cells.

Increase in both adipose cell size and number is a crucial process in obesity development and reflects changes in the adipocyte energy metabolism. In order to evaluate the impact of EL deficiency on the adipose tissue function the mRNA levels of key genes involved in fat lipid homeostasis was analyzed (Figure R-1.6).

Results indicated that EL-deficiency alters the expression levels of genes involved in FA utilization (Figure R-1.6). *Lpl*, the main responsible for the hydrolysis of dietary-derived TG and *Fabp4* a cytoplasmic chaperone involved in the trafficking of released FA are both significantly upregulated. Consistent with the increase adipocyte size, there is a trend to increase expression of genes responsible for lipogenesis and lipid storage and *Fasn* is significantly more expressed in EL-KO mice compared to WT.

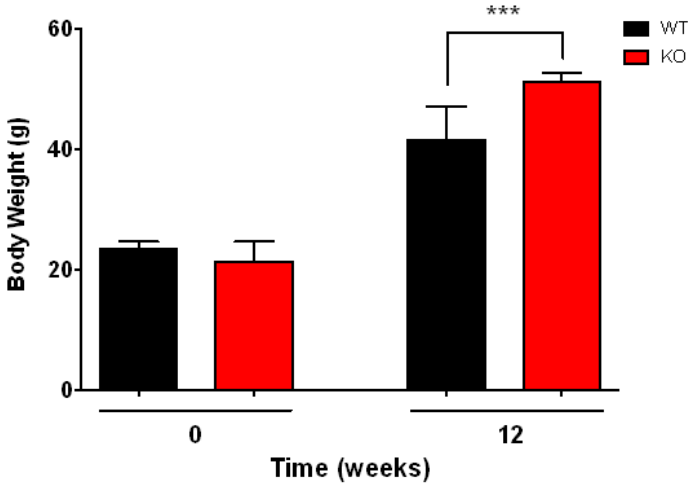

Figure R-1.1 Body weight of WT and EL-KO mice pre-and after long-term high-fat diet.

Body weights of EL WT vs. KO male mice (8 per group) prior and 12 weeks after the initiation of high-fat diet. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, A-B: Student's unpaired t-test.

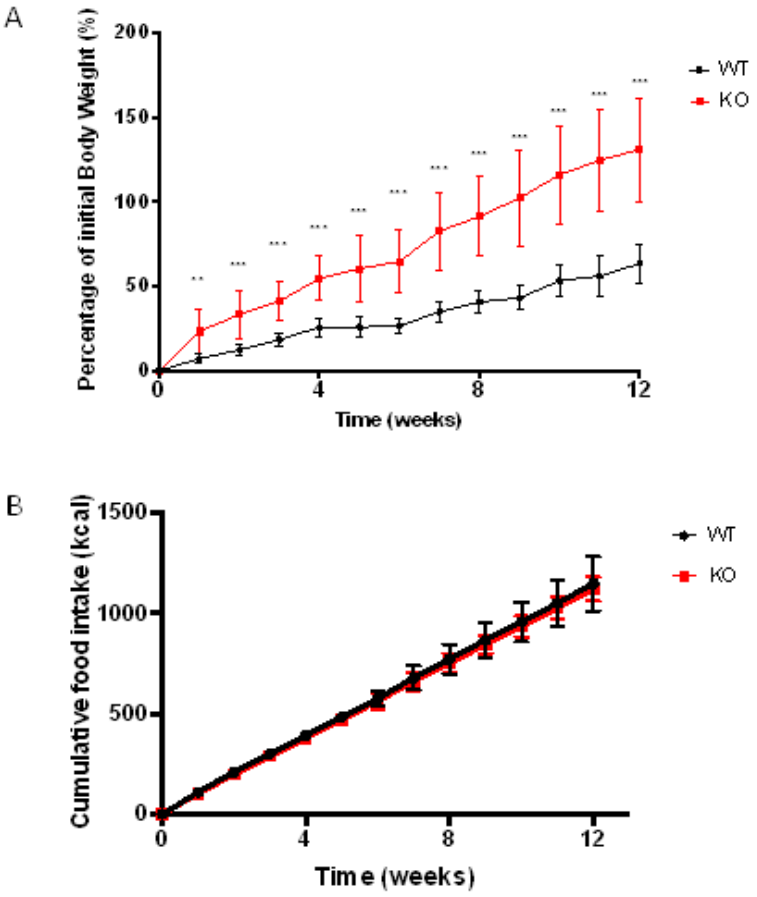

Figure R-1.2. Body weight increase in WT and EL-KO mice in response to high fat feeding.

Panel A: Weight increase in WT and KO mice. Body weights were measured weekly starting at the week of initiation of the high-fat diet (week 0). Data are expressed as percentage of initial body weight. Panel B: Food weights from cages of EL WT vs. KO mice fed a 45% high-fat diet. Data represents the total food weight per cage, divided by the number of mice per cage. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, C-D: Student's unpaired t-test with correction for multiple comparison.

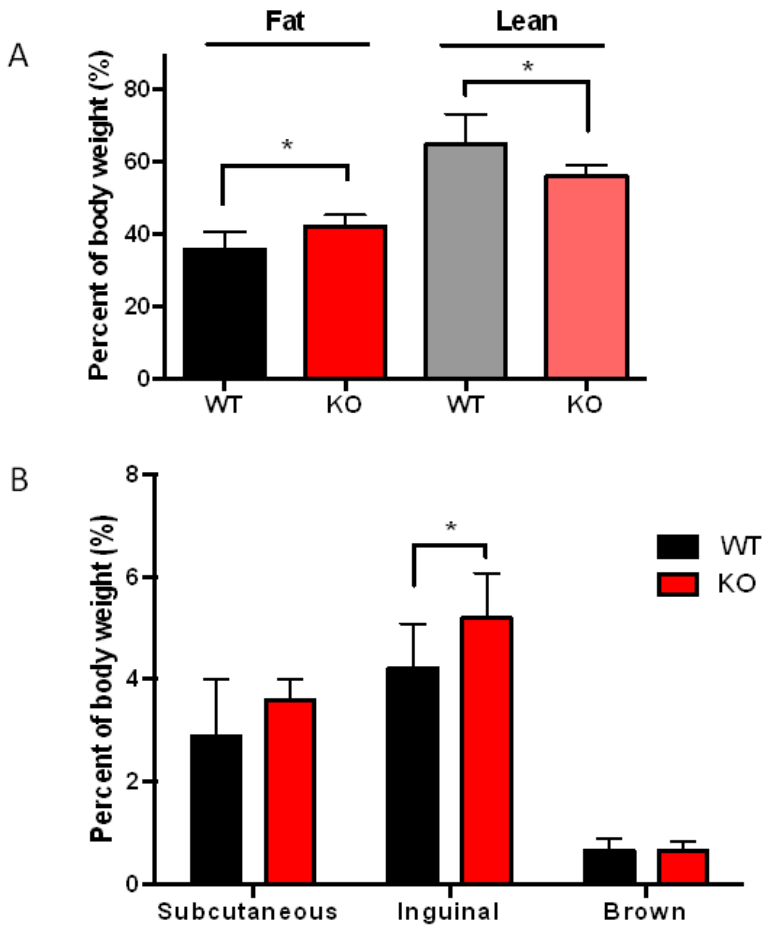

Figure R-1.3: Body composition and fat distribution in WT vs EL-KO mice.

Panel A: Fat and lean mass measured by NMR in mice fed a high-fat diet for 12 weeks. Masses are expressed as percentages of total body weight for each mouse. Panel B: Adipose depot mass from mice after sacrifice at 12 weeks of feeding high-fat diet. Data are expressed as mean ± SD. *P<0.05, Student's unpaired t-test.

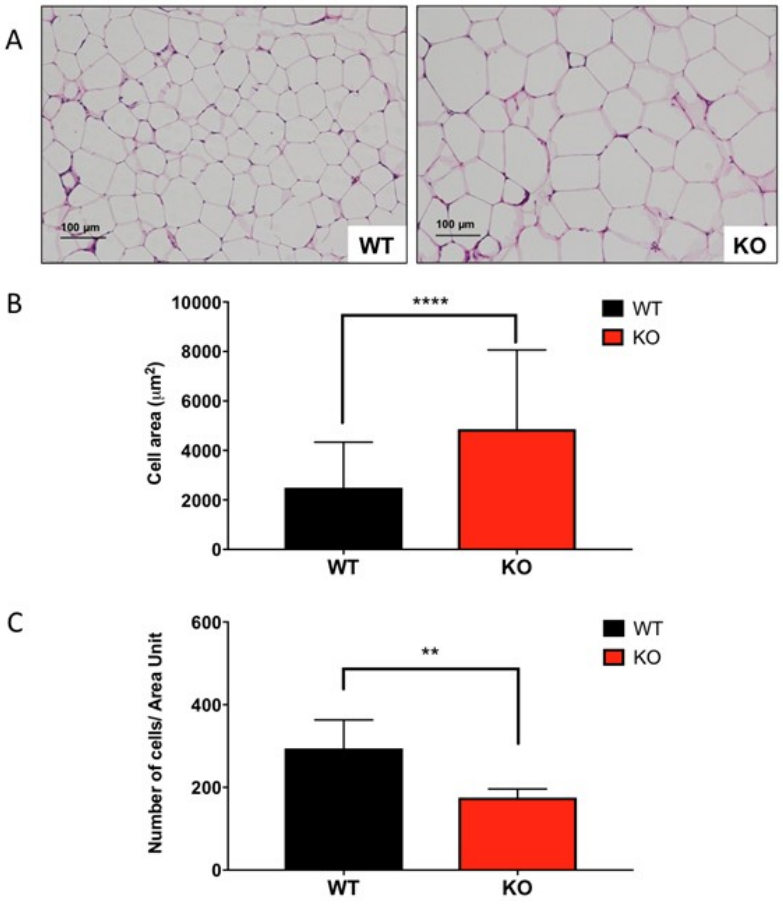

Figure R-1.4: Histological analysis of adipocyte morphology and cell size

Panel A: Representative H&E staining of inguinal fat from WT and EL-KO mice fed a high-fat diet for 12 weeks. Average adipocyte area (μm^2) (Panel B) and number of cells per area unit (Panel C) were calculated using Adiposoft software. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.001 Student's unpaired t-test

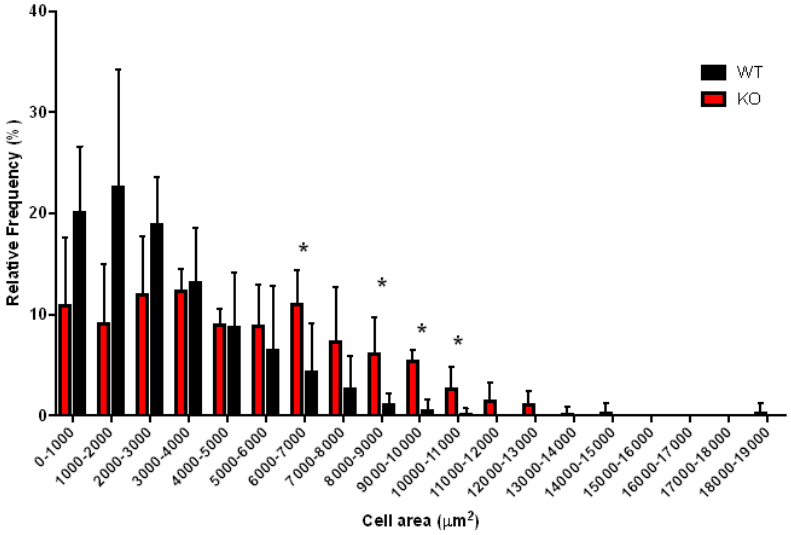

Figure R-1.5: Size distribution of inguinal adipocytes from WT and EL-KO mice on high-fat diet

Adipose tissue sections from WT and KO mice were stained with H&E and the cell areas were determined using the Adiposoft image software. The x axis shows the size range of different cells subpopulations and the y axis shows the relative frequency of each subpopulation (mean ± SD). *P<0.05, Student's unpaired t-test

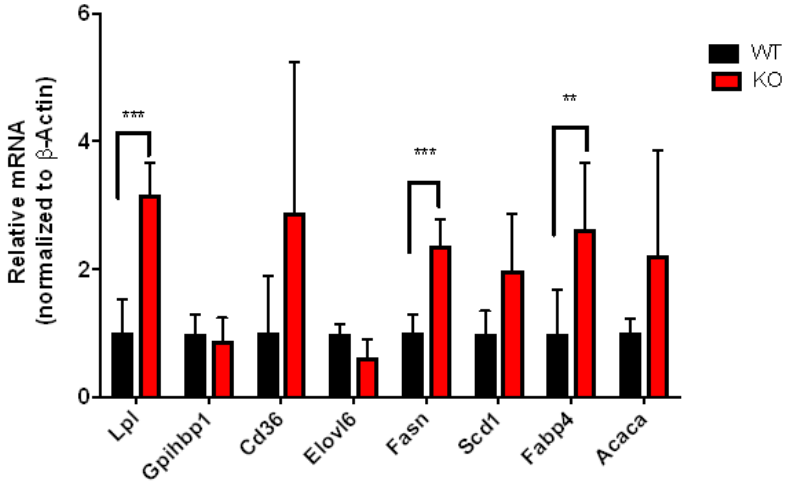

Figure R-1.6: Adipocyte gene expression pattern of WT and EL-KO mice fed a high-fat diet for 12 weeks.

Relative mRNA levels of WT and EL-KO adipocytes isolated from inguinal fat. Gene expression was normalized to expression of actin. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, Student's unpaired t-test.

1.2 EL-deficiency worsen diet-induced glucose intolerance

In order to assess the role of EL in the modulation of glucose metabolism, a Glucose Tolerance Test (GTT) was performed. WT and EL-KO mice were fasted overnight. Fasted blood glucose levels were determined and then 10 µl/g body weight of a 20% (w/v) glucose solution was administered intraperitoneally. Subsequently, the blood glucose level at 15, 30, 60, 90 and 120 min was measured. When fed a regular chow diet, WT and EL-KO mice displayed a normal response to glucose load. The hematic glucose concentration reached the peak at 30 min after injection and progressively normalized. No differences were observed between the two groups in this setting (Figure R-1.7A). As expected, upon exposure to high fat diet, both groups of mice developed progressive glucose intolerance (Figure R-1.7B-C). However, in EL-KO mice the blood glucose concentration remained persistently high even in the later time points (Figure R-1.7B-C). These results indicate that EL deficiency may be associated with the development of an earlier and more pronounced glucose intolerance and may predispose to frank insulin resistance. To directly test the tissues response to insulin an intraperitoneal insulin tolerance test was performed. In this experiment, mice were challenged by injecting a bolus of human insulin (0.75 U/Kg of body weight) and blood glucose was monitored over time. Results indicated a modest elevation of glucose levels in EL-KO mice compared to WT, thus indicating that despite the progression of glucose intolerance the peripheral response to insulin is still preserved (Figure R-1.8).

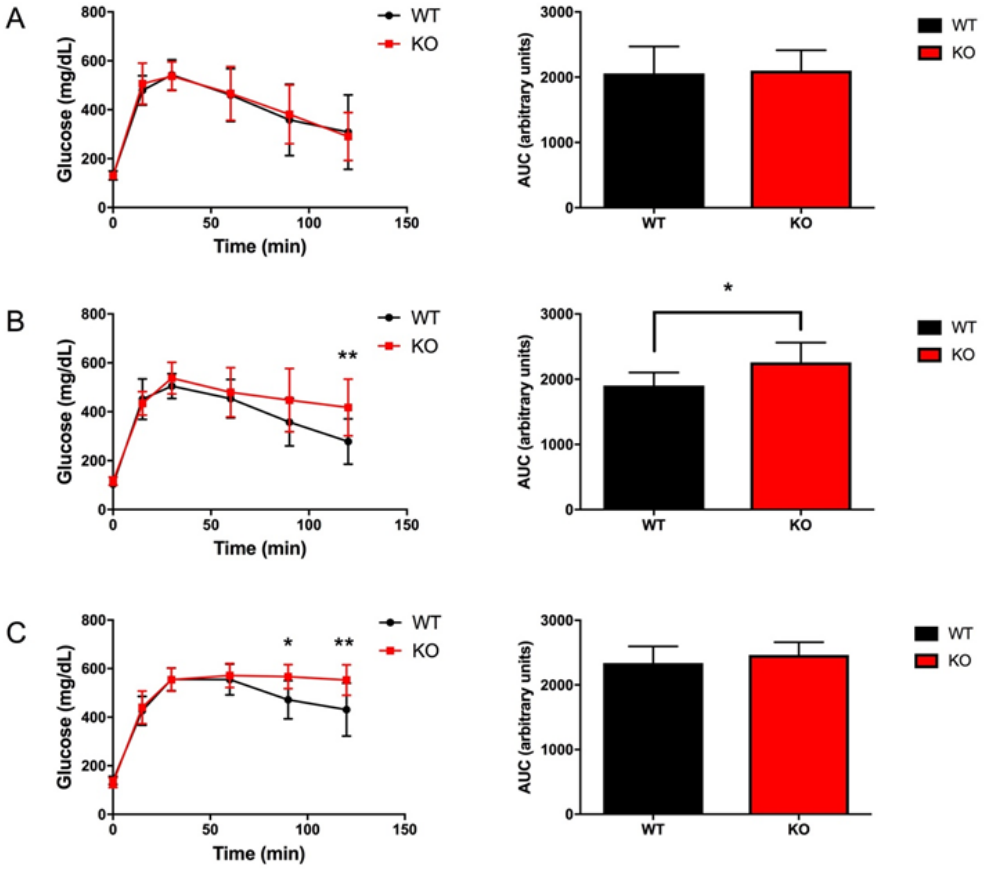

Figure R-1.7: Progression of glucose intolerance in mice on high-fat diet

Mice (n=10 per group) were fasted overnight and administered 10 µl/g body weight of 20% glucose solution intraperitoneally. Glucose was measured from tail blood before the beginning of experiment and at 15, 30, 60, 90 and 120 min after injection. Panel A: EL WT vs. KO mice on chow diet. Panel B: EL WT vs. KO mice after 6 weeks of high-fat diet. Panel C: EL WT vs. KO mice after 12 weeks of high-fat diet. For each panel, results were expressed as glucose concentration at the indicated time-points (curve on the left) and respective Area Under the Curve (AUC) (bar graph on the right). Data are expressed as mean ± SD *P<0.05, **P<0.01, Student's unpaired t-test.

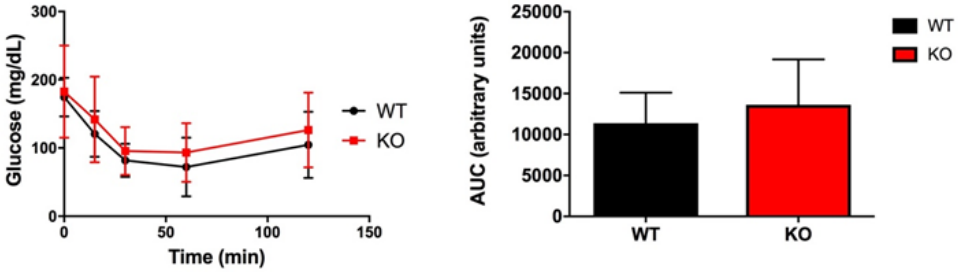

Figure R-1.8: Insulin Tolerance Tests in EL WT vs. KO mice fed a high-fat diet for 12 weeks.

Intraperitoneal Insulin tolerance test (ITT) was performed by injection of human insulin (Humalin-R, 0.75 U/kg body weight) into WT and EL-KO mice (n=6) after 4 h fast. Blood samples were collected immediately before and at 15, 30, 60, and 120 min after injection. Results were expressed as glucose concentration at the indicated time-points (curve on the left) and respective Area Under the Curve (AUC) (bar graph on the right). Data are expressed as mean ± SD.

1.3 Effect of EL deficiency on systemic inflammatory response

Endothelial lipase plasma levels have been associated with several markers of inflammation in humans [228, 334] and *in vitro* experiments have demonstrated that pro-inflammatory stimuli are able to increase EL gene and protein expression in different cell types [224, 371].

In order to determine whether absence of EL may directly or indirectly impact the inflammatory state associated with obesity, the inflammatory response to experimentally induced endotoxemia in WT and EL-KO mice was determined. Briefly, a low dose of Lipopolysaccharide (LPS) (0.5 mg/Kg body weight) was injected in both mice cohorts and the inflammatory response was monitored by measuring TNFα plasma concentration at different time points (0, 1 and 3 hours after injection). EL deficiency induced a marked increase in cytokine levels at 3 hrs (1616.47 ± 719.86 and 6812.60 ± 3382.14 pg/mL TNFα in WT vs. EL-KO, respectively) (Figure R-1.9). This data indicated that lack of EL is associated with a more intense inflammatory response and this may be at least partially due to a more severe obese phenotype.

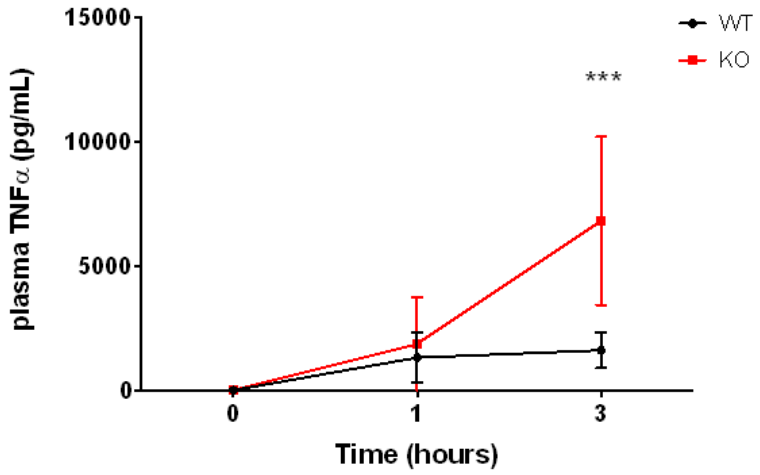

Figure R-1.9: Response to experimentally induced endotoxemia

Endotoxemia was induced by intraperitoneal injection of low-dose LPS (0.5 mg/Kg body weight). Plasma was collected prior to injection and at 1 and 3 hrs post-administration. TNFα concentration was measured by ELISA.
Data are expressed as mean ± SD ***P<0.001, Student's unpaired t-test.

1.4 Energy expenditure and muscle FA utilization are not affected by EL deficiency

EL-KO mice might gain more weight compared to WT as a result of impaired exergy expenditure. In order to test this hypothesis, basic metabolic parameters in WT and EL-KO mice after 8 weeks of high-fat diet, were measured using the comprehensive lab animal monitoring systems (CLAMS) at the Mouse Phenotyping, Physiology and Metabolism Core (University of Pennsylvania). The following parameters were then measured for 24 hours: oxygen consumption (VO2), carbon dioxide production (VCO2), respiratory exchange ratio (RER), caloric heat production (Heat) and physical activity level (Total movement). No difference in any of the measured parameters was observed between WT and EL KO mice (Figure R-1.10).

To better explore the energy utilization phenotype in these mice, the total mRNA levels of key genes involved in FA uptake and *de novo* lipogenesis in heart and skeletal muscle were measured (Figure R-1.11B-C). Furthermore, H&E staining of skeletal muscle sections was performed to highlight potential histological abnormalities and determine the presence of fat deposition (Figure R-1.11A). Results indicated no difference in the gene expression levels of myocytes and cardiomyocytes between WT and EL-KO mice. Consistent with this gene expression pattern, the histological findings from skeletal muscle samples were comparable (Figure R-1.11).

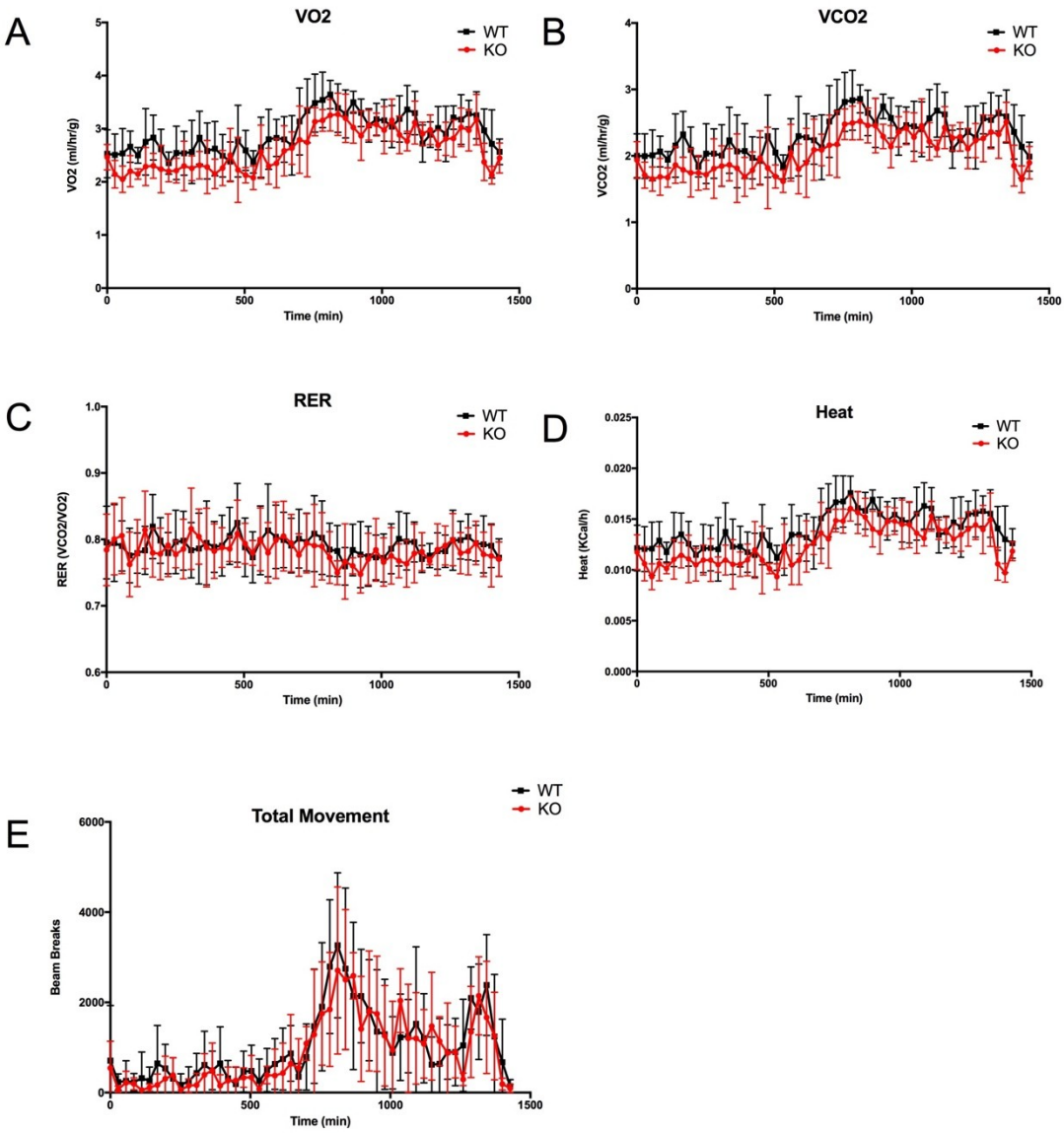

Figure R-1.10: Metabolic phenotyping of WT and EL KO mice

WT and EL-KO mice were monitored for basic metabolic parameters using the Comprehensive Lab Animal Monitoring System (CLAMS) at the Mouse Phenotyping, Physiology and Metabolism Core (University of Pennsylvania). The following parameters were measured for 24 hours: oxygen consumption (VO2, Panel A), carbon dioxide production (VCO2, panel B), respiratory exchange ratio (RER, Panel C), caloric heat production (Heat, Panel D) and activity level (Total movement, Panel E).

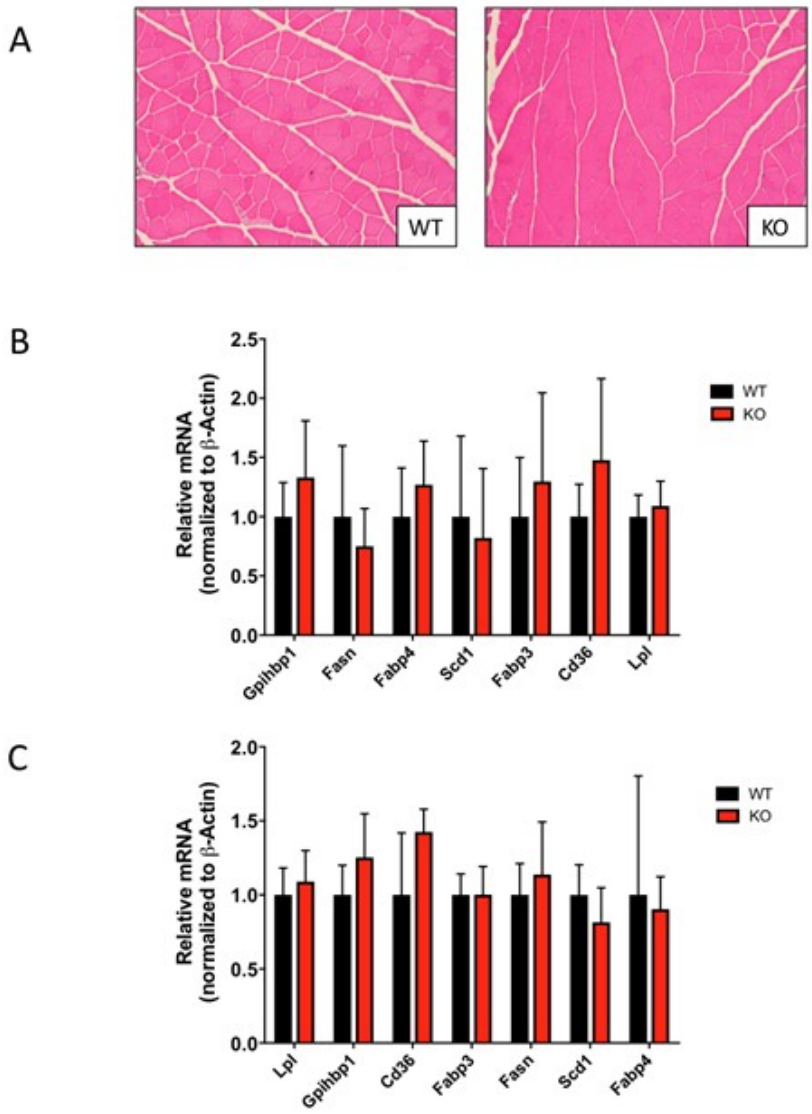

Figure R-1.11: Skeletal muscle and heart gene expression pattern

Panel A: Representative H&E staining of skeletal muscle from WT and EL-KO mice fed with high-fat diet for 12 weeks. Panel B: Relative mRNA levels of WT and EL-KO myocytes (n=6 per group). Panel C: Relative mRNA levels of WT and EL-KO cardiomyocytes. Gene expression was normalized to expression of actin. Data are expressed as mean ± SD. Student's unpaired t-test.

Specific Aim 2: Defining the role of EL in plasma TG-rich lipoprotein metabolism

2.1 EL-KO mice display increased TC, PL, HDL and non-HDL-C on low-fat diet

In order to study the role of EL in TG-rich lipoprotein metabolism, an extensive characterization of the plasma lipoprotein profile of WT and EL-deficient mice in response to a high fat diet was performed. Fasted plasma lipids from adult WT and EL-KO mice (n=8) on regular chow diet were measured before the initiation of the diet. Consistent with previously published data, absence of EL induced a significant increase in total cholesterol and phospholipids and this was due to an increase in both HDL-C and non-HDL-C plasma levels (Figure R-2.1A and Table R-1)[213, 216]. No differences in TG and non-esterified fatty acids were observed between WT and EL-KO mice in these conditions (Figure R-2.1A-B and Table R-1).

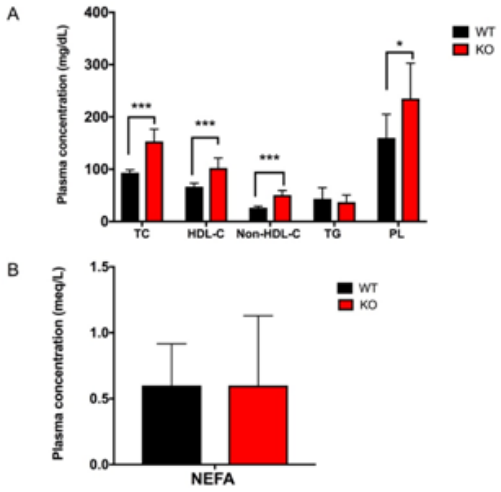

Figure R-2.1: Plasma lipid profile of WT and EL-KO mice on regular chow diet

Panel A: Plasma lipids concentrations Panel B: Plasma Non-Esterified Fatty Acids (NEFA). Plasma from adult male mice (n=8) was collected after 4 hours fasting. All measurements were performed using an autoanalyzer. Data are expressed as mean ± SD *P<0.05, ***P<0.001, Student's unpaired t-test.

2.2 EL-deficiency is associated with a more severe diet-induced dyslipidemia and markedly affects TG plasma levels

In order to assess whether EL deficiency may affect the development of diet-induced dyslipidemia, serial measurements of plasma lipids during the high-fat diet treatment were done. In both strains all the measured parameters progressively increased with time on diet and they were overall higher in EL-KO mice (Table R-1 and Figure R-2.2A-F). However, the calculation of the percentage of change compared to baseline levels for each parameter indicated that non-HDL cholesterol, NEFA and phospholipids increased at the same rate in WT and EL-KO mice (Table R-1 and Figure R-2.2B, E-G). On contrary, TC levels increased at a lower rate in EL-KO mice and that was explained by a significant reduction in HDL-C (Table R-1 and Figure R-2.2A, B, G). Finally, TG concentration, that was comparable between the two groups at the baseline, was dramatically higher in EL-KO mice at 12 weeks (Table R-1 and Figure R-2.2D, G).

		Chow diet		4 weeks on high-fat diet		12 weeks on high-fat diet	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
TC (mg/dL)	WT	93.2	5.7	149.4	19.7	168.7	27.0
	EL-KO	153.1	23.6	201.5	43.6	220.2	43.1
HDL-C (mg/dL)	WT	66.8	6.5	102.9	18.0	126.3	16.4
	EL-KO	102.3	19.2	138.9	29.3	146.7	27.6
Non-HDL-C (mg/dL)	WT	26.4	2.9	46.6	11.2	42.4	13.1
	EL-KO	50.8	8.6	63.5	16.0	76.7	16.3
TG (mg/dL)	WT	43.4	21.5	35.7	9.8	90.2	56.6
	EL-KO	37.0	13.7	38.7	9.1	146.8	90.0
NEFA (meq/L)	WT	0.6	0.1	0.7	0.2	0.8	0.2
	EL-KO	0.6	0.2	0.8	0.2	0.9	0.1
PL (mg/dL)	WT	160.0	45.1	266.5	25.6	289.4	35.4
	EL-KO	235.3	67.7	343.9	54.7	335.8	48.8

Table R-1: Plasma lipid profile of WT and EL-KO before and after 4 and 12 weeks on high-fat diet.

Results and statistically different findings are shown in figure R-2.2.

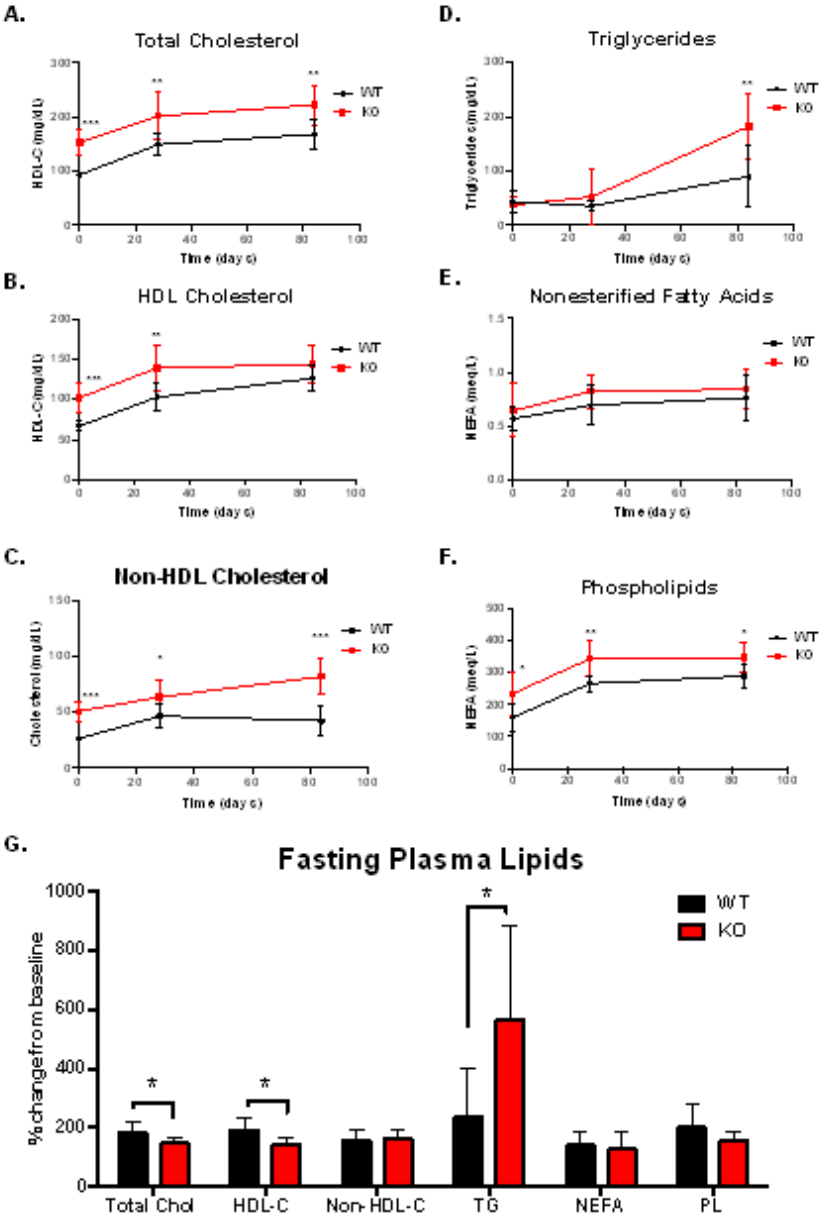

Figure R-2.2: Plasma lipid profile of WT and EL-KO mice before and after 4 and 12 weeks on high-fat diet.

Panels A-F: Plasma lipids were measured on day 0, at 4, and 12 weeks of initiation of the high-fat diet. Each lipid measurement was performed on plasma collected after a 4 hour fasting, using an autoanalyzer. Panel A: Total Cholesterol. Panel B: HDL-Cholesterol. Panel C: Non-HDL Cholesterol. Panel D: Triglycerides. Panel E: Non-Esterified Fatty Acids (NEFA). Panel F: Phospholipids.
Panel G: Relative change in 4 hour fasting plasma lipids on week 12 vs. baseline (Day 0) of the high fat diet. Data are expressed as mean ± SD *P<0.05, **P<0.01, ***P<0.001, Student's unpaired t-test.

2.3 The analysis of fasted and fed plasma lipid profile in EL-KO mice reveals diet-induced abnormalities in lipoprotein remodeling

The initial characterization of plasma lipids demonstrated that the dietary intervention was able to exacerbate the dyslipidemic phenotype in EL-deficient mice. To further investigate the nature of this association, we evaluated the plasma lipoprotein profile in the fasted versus fed state. To this purpose, the same pool of WT or EL-KO mice was bled twice (both the collections were performed at week 8 on diet). The first collection was performed after 4 hour fasting. Mice were then re-fed and the second collection was performed the following day. During this time, mice were fed *ad libitum*. Plasma from each group of mice was pooled and lipoproteins were fractionated using Fast Protein Liquid Chromatography (FPLC). Eluted fractions were then tested for cholesterol and triglyceride content. The apolipoprotein expression in selected fractions was assessed by western blot.

Long-term high-fat feeding profoundly affected cholesterol and TG profiles in both WT and EL-KO mice (figures R-2.3-5). Nevertheless, the two strains responded very differently to fasting-refeeding. In fasting conditions WT mice displayed very little TG content compared to EL-KO mice and it was almost equally eluted in VLDL-CM and LDL regions (peak concentration= 6.67 μg/mL and 4.80 μg/mL, respectively, figure R-2.3A). Upon re-feeding, TG levels increased dramatically in both peaks (15.40 μg/mL and 14.21μg/mL in VLDL-CM and LDL peaks, respectively figure R-2.4A). TG-enrichment in the CM peak was consistent with post-prandial lipid loading and the coexistence of a TG-rich peak in the LDL region indicated a further remodeling of CMs into IDL-LDL.

In EL-KO mice, the fasted plasma TG content was extremely high in both peaks (17.42 μg/mL and 22.82 μg/mL in VLDL-CM and LDL peaks, respectively, figure R-2.3A). Notably, the VLDL-CM peak concentration in KO mice in fasted condition was comparable to what observed in WT mice in the fed state (figure R-2.5). In the postprandial state this peak was further enriched in TG (24.92 μg/mL), whereas the IDL-LDL peak did not change

(22.50 μg/mL, figure R-2.4A). WB analyses of apolipoproteins in these fractions revealed a relatively higher apoB-48 content in EL-KO mice both in fasted and fed conditions (figure R- 2.3 and 2.4A).

The analysis of the cholesterol profile in fasted WT mice was consistent with the effects of a long-term high-fat diet and revealed the presence of major cholesterol-enriched HDL peak and a smaller LDL shoulder (figure R-2.3B). This profile was only minimally affected by re-feeding (figure R-2.4B).

In fasted EL-KO mice, the amount of cholesterol eluted in the LDL region was significantly higher than in WT and it became even higher upon refeeding. Interestingly, lipoproteins eluted in this region were significantly more enriched in apoE and apoB-100 (figure R-2.3B and 2.4B).

Taken altogether these data are supportive of an impaired remodeling of TG-rich lipoproteins in EL-KO mice defect seems to be exacerbated by feeding.

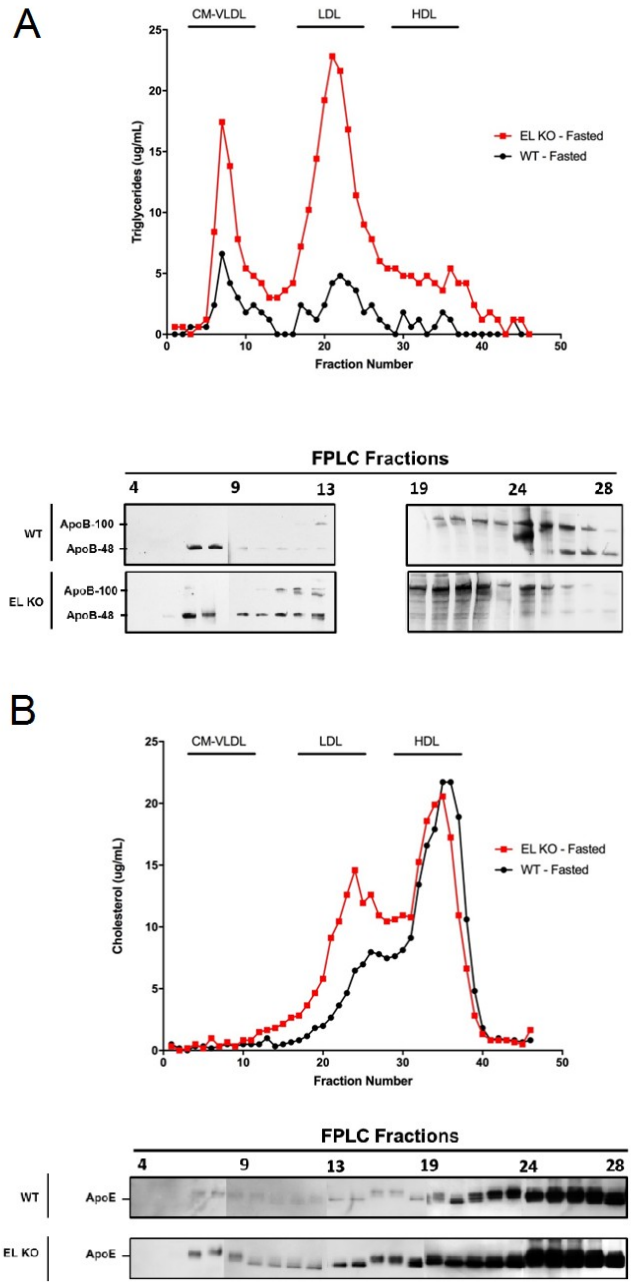

Figure R-2.3: FPLC analysis of fasted plasma lipoproteins from WT and EL-KO mice after 8 weeks of high-fat diet

Triglycerides (Panel A) and Cholesterol (Panel B) from pool of 4 hours fasted plasma were fractionated by fast-protein liquid chromatography (FPLC) to separate lipoproteins. Triglycerides and cholesterol were measured by colorimetric plate assays. 100 µl of plasma per pool were used for FPLC fractionation. Selected fractions were then analyzed by SDS-page electrophoresis to separate proteins and immunoblotted against apoB (panel A) and apoE (Panel B).

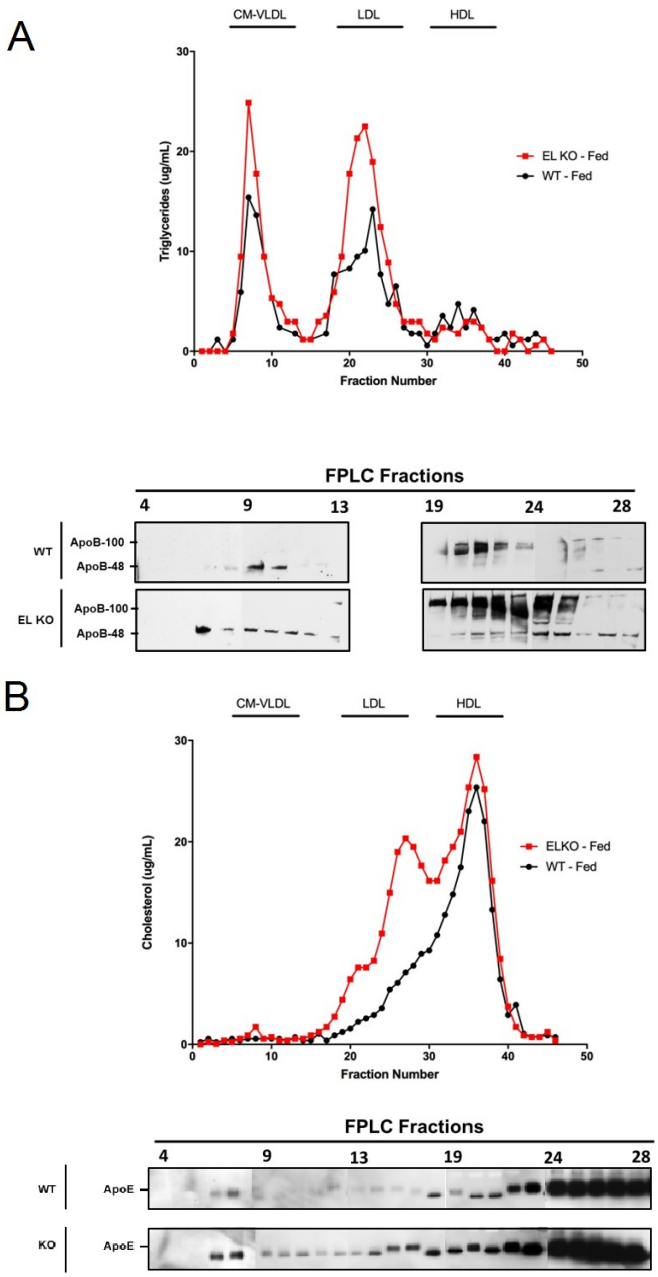

Figure R-2.4: FPLC analysis of fed plasma lipoproteins from WT and EL-KO mice after 8 weeks of high-fat diet

Triglycerides (Panel A) and Cholesterol (Panel B) from pool of 4 plasma from mice fed *ad libitum* were fractionated by fast-protein liquid chromatography (FPLC) to separate lipoproteins. Triglycerides and cholesterol were measured by colorimetric plate assays. 100 µl of plasma per pool were used for FPLC fractionation. Selected fractions were then analyzed by SDS-page electrophoresis to separate proteins and immunoblotted against apoB (panel A) and apoE (Panel B).

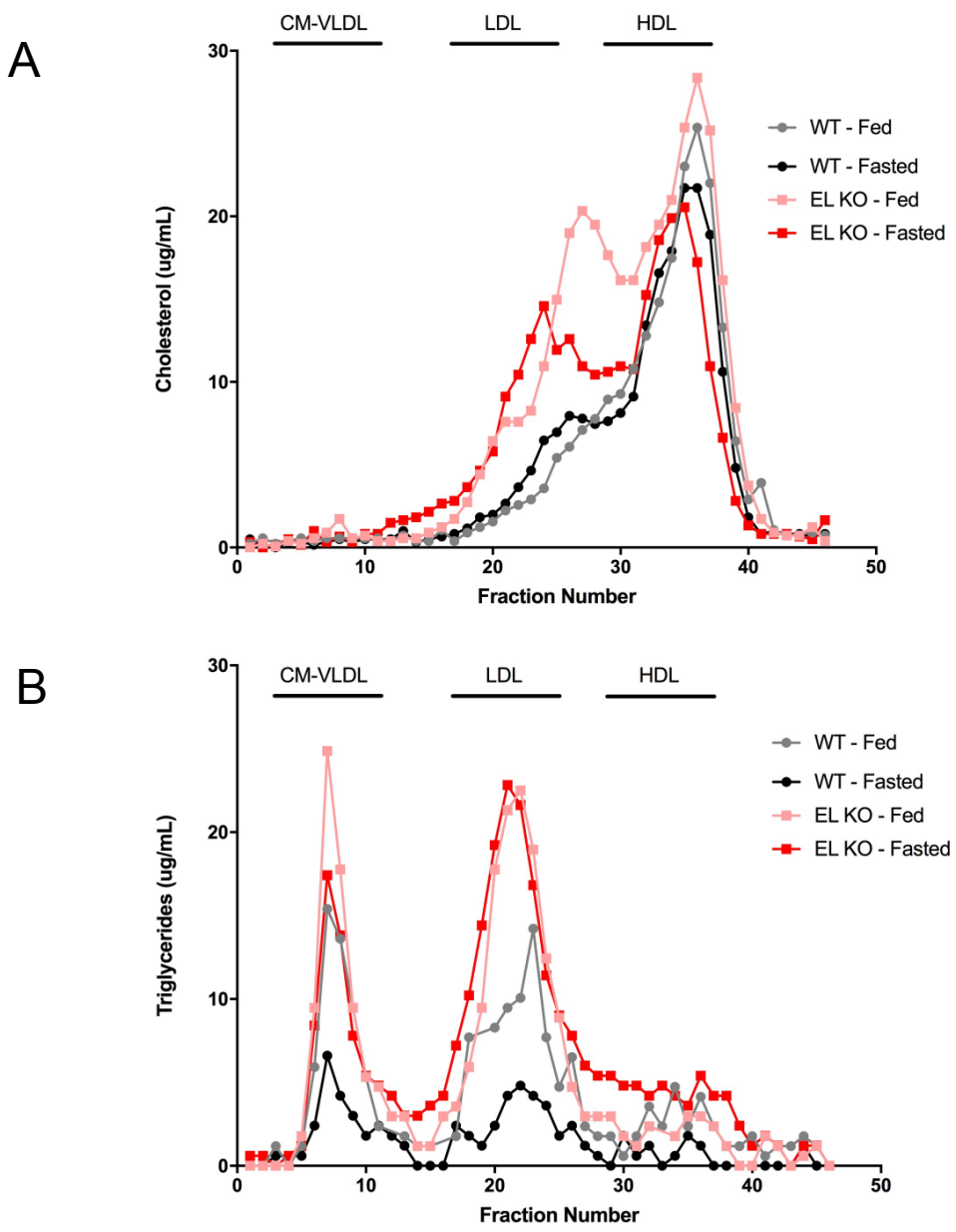

Figure R-2.5: Overlapping FPLC profile of fed and fasted plasma lipoproteins from WT and EL-KO mice after 8 weeks of high-fat diet

Cholesterol (Panel A) and Triglycerides (Panel B) FPLC profiles shown in Figures R2.3 and R2.4 were plotted together in order to better highlight the changes occurring in the postprandial state.

2.4 EL deficiency delays postprandial TG-rich lipoprotein clearance

The observation that EL-KO mice displayed an abnormal lipid and lipoprotein profile and that this phenotype became more severe in the fed state, prompted to the direct investigation of the effect of EL deficiency on postprandial TG-rich lipoprotein clearance. To this purpose, an Oral Fat Tolerance Test (OFTT) was performed on two separate cohorts of WT and EL-KO mice on regular low and high-fat diet. Briefly, WT and EL KO mice were gavaged with olive oil (10 ul/g of body weight) and plasma was collected at different time points to permit TG measurements (0-1-3-5 and 7 hours post gavage). Results from this experiment are summarized in Table R-2. WT mice displayed a normal TG clearance profile in either low or high-fat feeding conditions (figure R-2.6A,B and C, Table R-2). The TG concentration reached the maximum peak at 3 hours post-gavage and it completely normalized at 7 hours (figure R-2.6A,B and C, Table R-2). In contrast, we observed that loss of EL markedly affected TG clearance. In EL-KO mice on chow diet, TG plasma levels significantly increased upon olive oil gavage at 1, 3, 5 and 7 hours and they were still double the baseline at this latter time-point (Figure R-2.6A). This alteration was even more dramatic after 10 weeks of high fat diet. In these conditions, EL-KO mice displayed persistently high triglyceridemia with virtually no clearance by 7 hours (Figure R-2.6 B and C).

These results were supportive of a markedly impaired post-prandial TG-rich lipoprotein clearance that resulted in severe plasma TG accumulation. In order to discern the nature of the accumulated TG-rich lipoproteins, two different qualitative analyses were performed on plasma from the OFTT experiment. WT and EL-KO samples from the 7 hours time-point were pooled and an FPLC fractionation of plasma lipoproteins was performed. TG, cholesterol and apoB content of eluted fractions was determined using colorimetric assay and WB. In WT mice, 7 hours after gavage the lipoprotein profile was completely normalized and showed very little residual TG in the CM-VLDL region and non-detectable apoB (Figure R-2.7A and C). The cholesterol profile indicated a major HDL peak accompanied by a smaller LDL

shoulder (Figure R-2.7B). Conversely, the analysis of TG profile in EL-KO mice revealed a massive accumulation of TG in the CM-VLDL region (+223% of WT) and a minor peak in the LDL region (Figure R-2.7A). The analysis of cholesterol profile confirmed this data and showed a substantial TC enrichment of lipoproteins eluted in the CM-VLDL and LDL regions, accompanied by a parallel reduction of cholesterol in HDL fractions (Figure R-2.7B). When analyzed by WB, the CM-VLDL fractions from EL-KO mice appeared to be enriched in apoB-100 and apoB-48. Notably, the densitometric analysis of the bands indicated that the apoB-48: apoB-100 ratio was on average 3.1. Although in mice apoB-48 can be synthetized by both liver and intestine, the relatively higher abundance of this protein compared to apoB100 was suggestive of the presence of CM remnants (Figure R-2.7C).

In addition, plasma samples collected at different time-points of the OFTT experiment were analyzed by agarose-gel electrophoretic analysis. This technique separates plasma lipoproteins by charge/size and permit to discriminate between CM (non-migrating) and other apoB-containing lipoproteins (β and pre-β migrating). Results from this experiment are shown in figure R-2.8. Before the beginning of the experiment, mice were fasted overnight. As expected, in this condition no chylomicrons were detected in WT and EL-KO mice (Figure R-2.8, O hrs). 3 hours post-gavage the TG concentration reached the maximum peak (figure R-2.6C). This was evidently represented in the EF analysis and translated in the presence of a major CM band at the origin of the gel (Figure R-2.8, 3 hrs). Notably, the CM band was significantly more intense in EL-KO mice compared to WT and it was still persistent at 7 hours after gavage (Figure R-2.8, 3 and 7 hrs).

In summary, loss of EL markedly delayed chylomicron and VLDL-TG clearance and resulted in high levels of circulating triglyceride-rich particles.

Time-point (hrs)	Chow diet TG (mg/dL)				High-fat diet TG (mg/dL)			
	WT		KO		WT		KO	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	112.31	21.22	109.27	57.45	129.17	37.86	181.45	57.78
1	144.39	61.34	252.00	63.49	319.47	140.61	294.75	118.22
3	293.66	101.21	906.62	718.60	415.20	214.46	1445.26	569.49
5	269.11	175.55	863.20	740.02	267.25	140.35	1078.08	698.19
7	226.03	153.77	376.59	306.16	158.22	84.14	1218.02	979.51

Table R-2: Response to Oral Fat Tolerance Test in WT and EL-KO mice on chow and high-fat diet

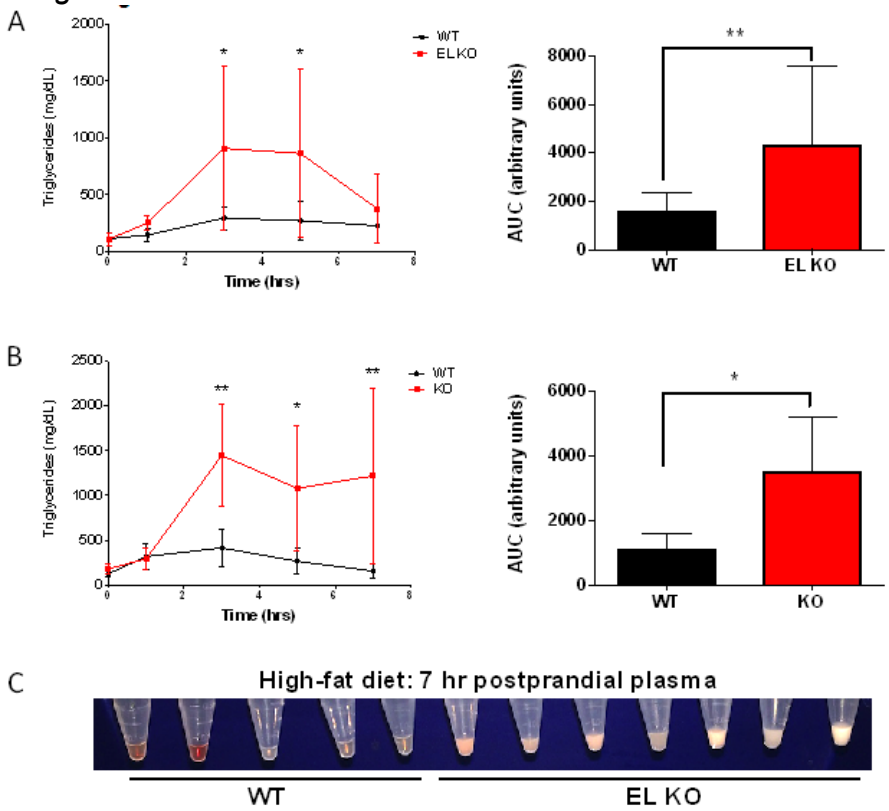

Figure R-2.6: Oral fat Tolerance Test (OFTT) in EL WT vs. KO mice fed a chow diet and high-fat diet for 10 weeks.

Postprandial plasma triglycerides in EL WT vs. KO mice fed a chow (Panel A-B) or high fat diet (Panel B), following oral gavage with olive oil. Mice (6 per group) were fasted overnight, and gavaged with 10 µl/g body weight of oil olive. Plasma was collected at the indicated time-points. Plasma TG were measured by colorimetric plate assays. Panel C: Tubes containing plasma from the 7-hour time point following olive oil gavage from mice on high-fat diet. Data are expressed as mean ± SD *P<0.05, **P<0.01, ***P<0.001, Student's unpaired t-test

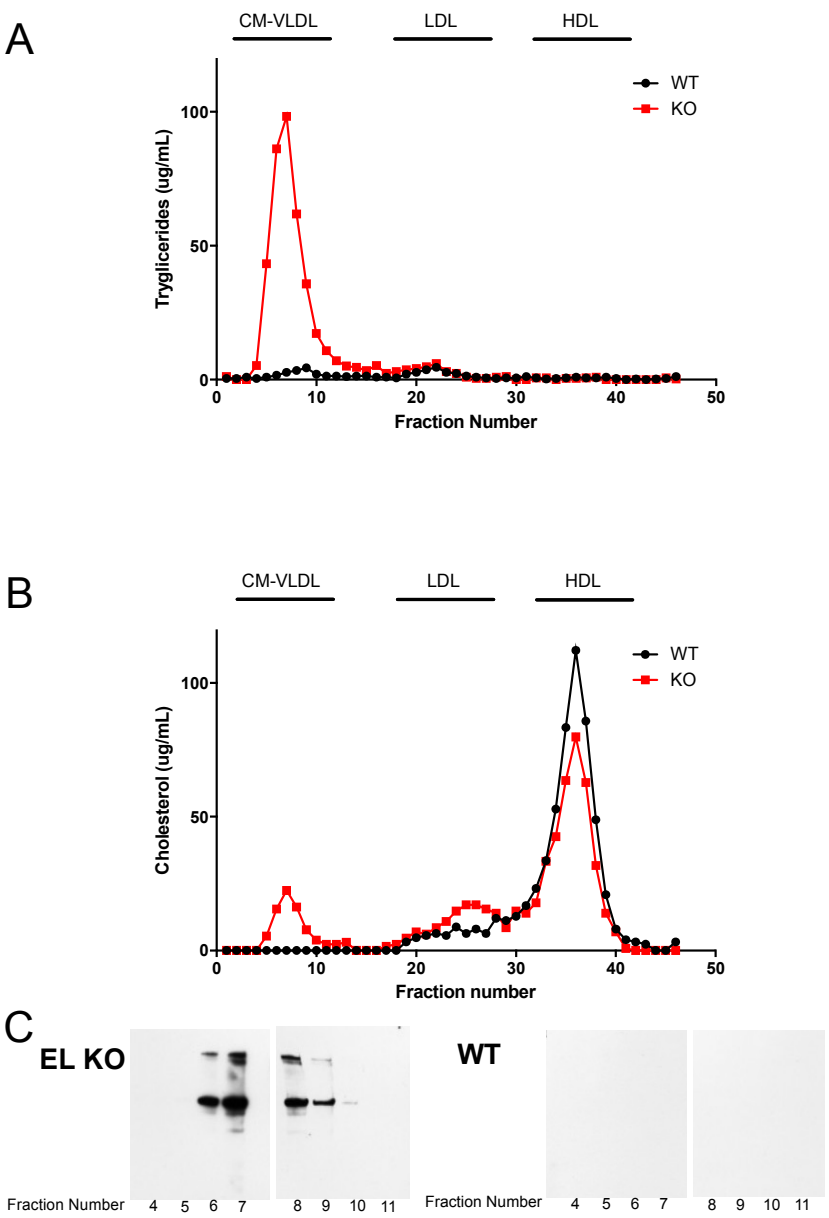

Figure R-2.7: FPLC profile of plasma lipoproteins from WT and EL-KO mice 7 hours post olive oil gavage

Plasma from WT and EL-KO mice was collected 7 hours post gavage of olive oil, pooled and fractionated into lipoproteins using FPLC. Triglycerides (Panel A) and cholesterol (Panel B) were measured by colorimetric plate assays.
Panel C: Western blot analysis of apoB content of fractions 4-11 (VLDL-CM peak).

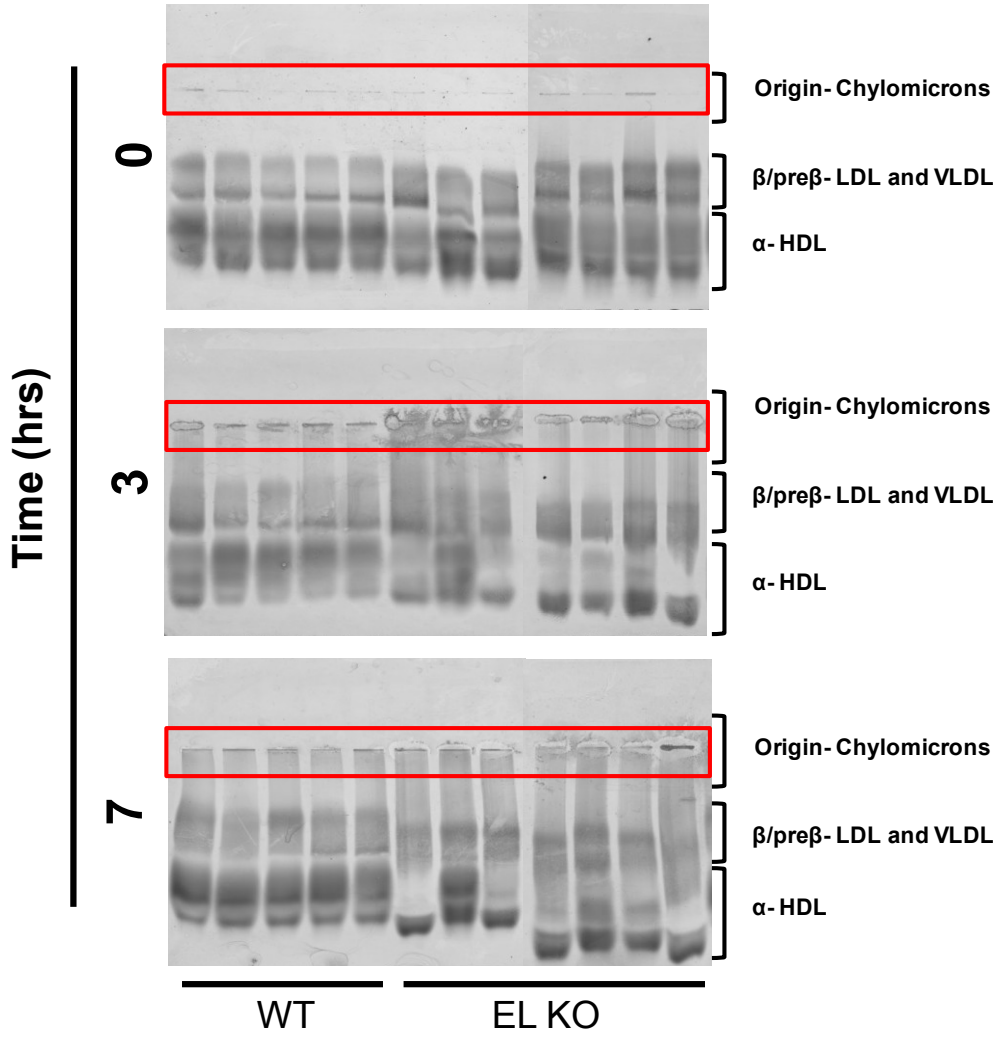

Figure R-2.8: Agarose-gel electrophoresis of plasma lipoproteins from WT and EL-KO mice at 0,3 and 7 hours after gavage

Plasma from WT and EL-KO mice was collected before and at 3 and 7 hours post gavage of olive oil, pooled and separated by charge/size using agarose gel electrophoresis. Lipoproteins were detected by Fat Red neutral lipid staining. Red boxes highlight the chylomicron region (origin of electrophoretic migration).

2.5 Evaluation of potential mechanisms responsible for increased TG-rich lipoprotein accumulation

Collectively, results from the previous experiments indicated that EL-deficiency was associated with impaired post-prandial TG clearance. The further investigation of potential mechanisms responsible for the observed phenotype was done by directly assessing TG-rich lipoprotein catabolism. When endogenous and dietary TG enter the circulation via VLDL and CM, they are readily hydrolyzed by plasma lipases and released FA are taken up by peripheral cells. This process is very rapid and it's believed to be mainly driven by LPL activity.

The impact of EL deficiency on TG-rich lipoprotein lipolysis was evaluated through *in vivo* kinetic experiments. TG-rich lipoproteins from non-fasted human plasma were isolated by density-gradient ultracentrifugation (d<1.006) and labeled with ^{3}H-triolein. WT and EL-KO mice on high fat diet for 12 weeks were then intravenously injected with the substrate and plasma was serially collected to determine the activity decay. In order to selectively investigate the lipolytic-driven lipoprotein catabolism and minimize the contribution of further lipoprotein remodeling, the experiment was terminated at 15 min after injection and animals were sacrificed. The analysis of plasma ^{3}H-triolein activity revealed that the removal of TG-derived FA from the circulation was slower in EL-deficient mice (figure R-2.9A). Residual ^{3}H activity was significantly higher in EL-KO mice at all the indicated time points (figure R-2.9A) and that translated in a significantly reduced Fractional Catabolic Rate (FCR) (figure R-2.9A).

To examine whether enhanced clearance of triglycerides was also due to enhanced particle uptake by peripheral tissues, the same experiment was repeated using ^{125}I-labeled lipoproteins (Figure R-2.9B). In this setting, ^{125}I turnover was considered a marker for whole particle clearance. No difference was observed in ^{125}I clearance between WT and EL-KO mice (Figure R-2.9B).

Finally, to assess whether EL-deficiency could possibly affect the hepatic VLDL-TG secretion, WT and EL deficient mice were injected with Pluronic to

inhibit endogenous lipolysis. TG accumulation at different time-points (0, 1, 2, and 4 hours after injection) was measured with colorimetric assay and the TG production rate was calculated. EL-KO mice showed a trend-to decrease in TG production that was more evident in the later time-points. However, this difference didn't translate into a significantly different production rate (Figure R-2.10).

Results from these three experiments collectively lead to the conclusion that in EL-deficient mice, TG accumulation was due to impaired TG clearance from the circulation and this was mainly explained by impaired lipolysis rather than to impaired particle uptake.

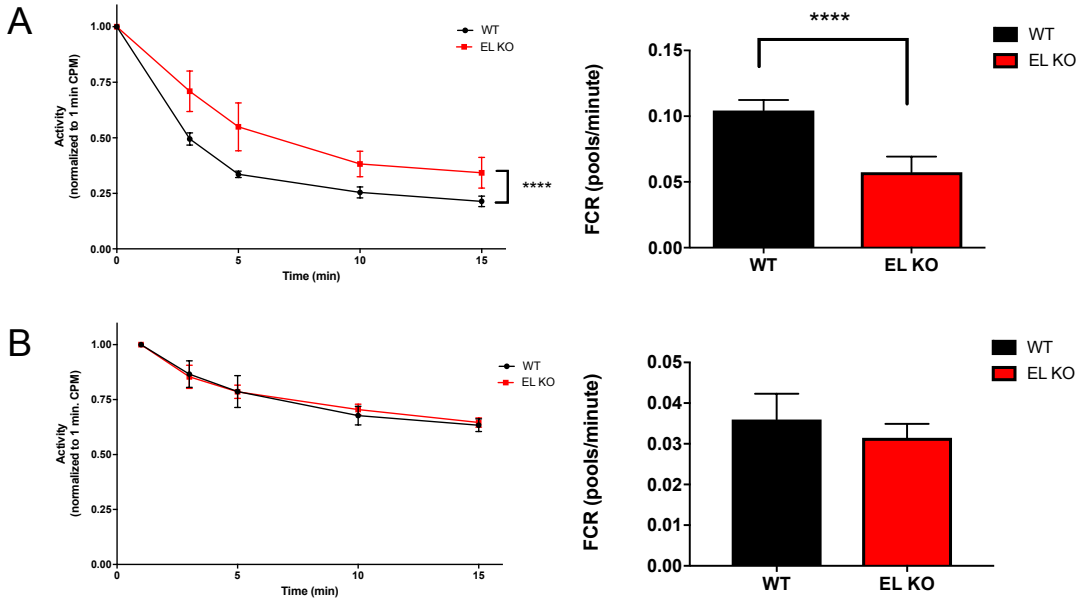

Figure R-2.9: Plasma clearance of ^{3}H-triolein and ^{125}I labeled human TG-rich lipoproteins in EL WT vs. KO mice fed a high-fat diet for 12 weeks.

Human lipoproteins were isolated by density gradient ultracentrifugation (d<1.006 g/mL) and labeled with ^{3}H-triolein or ^{125}I. They were then injected intravenously and mice were bled for plasma activity measurements at the indicated time-points. The experiment was terminated 15 min after injection. Panel A: ^{3}H oleate plasma clearance and Fractional Catabolic Rate (FCR) Panel B: ^{125}I-protein plasma clearance and Fractional Catabolic Rate (FCR). Data are expressed as mean ± SD *P<0.05, **P<0.01, ***P<0.001, ****P<0.000. Student's unpaired t-test.

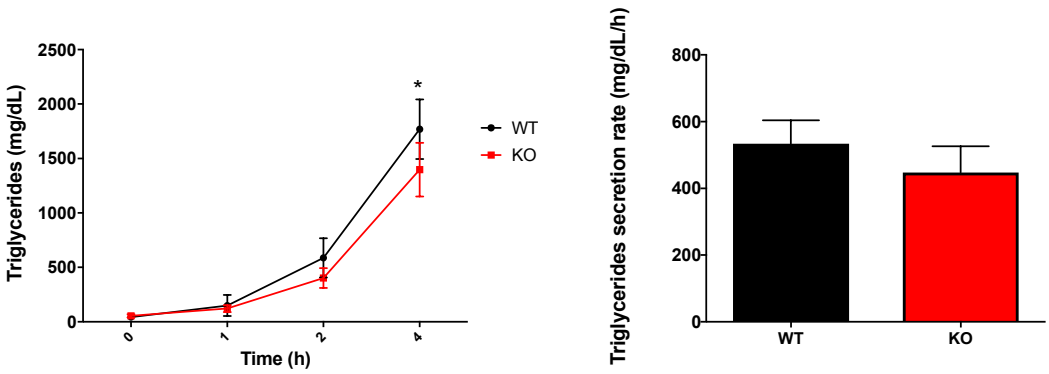

Figure R-2.10: TG secretion rate in WT vs. EL-KO mice fed a high-fat diet for 12 weeks.

WT and EL-KO mice (10 per group) were fasted for 4 hours and intraperitoneally injected with the detergent Pluronic to inhibit lipolysis. Plasma was collected serially 1, 2, and 4 hours after Pluronic injection and TG concentration was measured with a colorimetric assay. Panel A: plasma TG concentration at indicated time points. Panel B: Calculated VLDL-TG secretion rate. Data are expressed as mean ± SD *P<0.05. Student's unpaired t-test.

2.6 Impact of EL deficiency on expression and activity of other extracellular lipases

The observation that EL-deficient mice displayed severely impaired TG-lipolysis raised the question whether the observed effect was due to a direct, previously underappreciated, activity of EL towards TG or to an indirect effect of EL on other extracellular lipases. This question has been addressed through two different strategies.

The first set of experiments was designed to test the ability of EL and LPL to hydrolyze TG on a synthetic substrate containing ^{3}H-triolein. This assay allows one to determine TG activity independently of the endogenous substrate. To test the specific affinity of EL and LPL towards TG, media containing each enzyme were obtained by adenoviral-mediated gene transfer in COS-7 cells. Increasing volumes of EL and LPL-media were incubated with a ^{3}H-triolein labeled emulsion substrate and liberated fatty acids were quantified by scintillation counting. Consistent with previously published data, LPL exhibited a significantly higher TG activity compared to EL (Figure R-2.11).

The TG lipase potential of WT and EL-deficient plasma was assessed according to a similar experimental approach. Post heparin plasma from WT and EL-KO mice was incubated with ^{3}H-triolein labeled synthetic substrate and TG lipase activity was quantified as previously described. Results indicated that EL deficiency did not affect post-heparin plasma (PHP) TG lipase activity (Figure R-2.12A). Since LPL is believed to be the main responsible for TG lipolysis in plasma, the total expression of this protein has been assessed by WB. No difference was observed between the two experimental groups (Figure R-2.12B).

These results demonstrated that PHP from EL-KO mice retained the ability to hydrolyze TG and that was likely due to an intact LPL functionality. However, these data were in contrast with the *in vivo* observation that EL-deficient mice had a significantly impaired post-prandial lipolytic activity.

In order to test whether EL deficiency impaired TG clearance by altering LPL interaction with the substrate, a second set of experiments was designed. In these experiments, increasing volumes of EL and a constant volume of LPL conditioned media were incubated with human ^{3}H-triolein-labeled-TG-rich lipoproteins. After incubation, lipids were extracted, separated by Thin Layer Chromatography (TLC) and the percentage of released FA was quantified by scintillation counting. Results indicated that EL had very little activity on isolated TG-rich lipoproteins compared to LPL (figure R-2.13A). Surprisingly, when EL and LPL were co-incubated, the experimentally measured TG lipase activity was significantly higher than what expected by summing the contributions (figure R-2.13B).

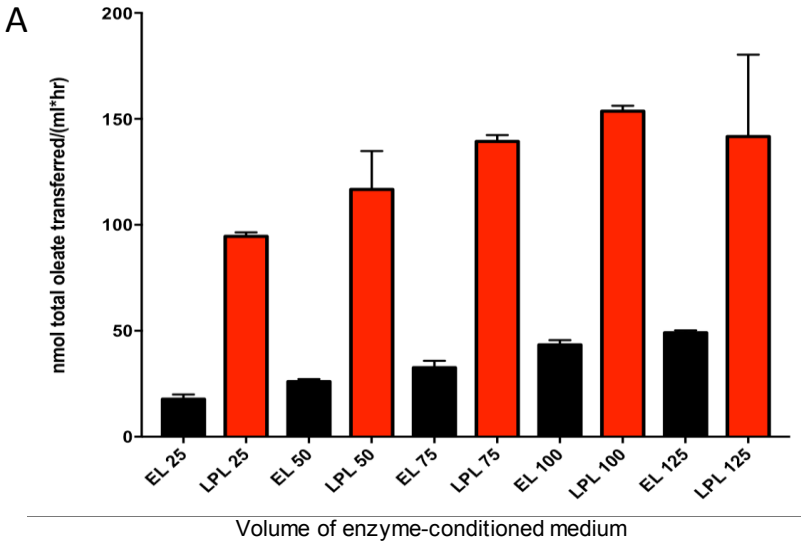

Figure R-2.11: TG-lipase activity of EL and LPL conditioned media towards synthetic substrate

Media containing EL or LPL were obtained by adenoviral-mediated transfection of COS-7 cells . The media were then used as source of enzyme for further activity assays. Increasing volumes of EL and LPL-media were incubated with a ^{3}H-triolein labeled emulsion substrate. Products of reaction were extracted and the liberated fatty acids (FFAs) were quantitated by scintillation counting. Panel A: Volume/TG-lipase activity plot

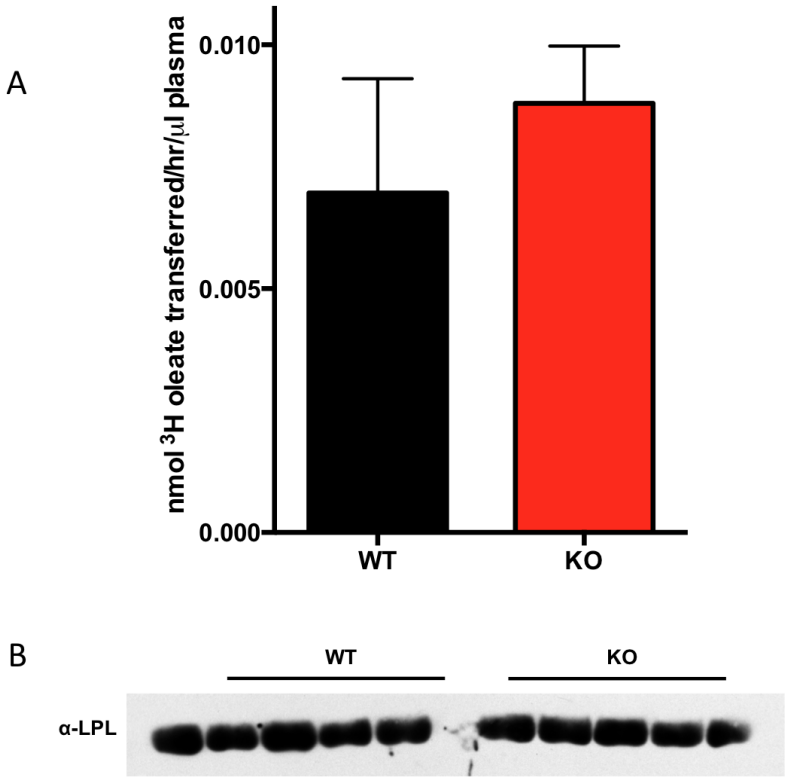

Figure R-2.12 TG-lipase activity of post-heparin plasma from WT and EL-KO mice

Panel A: Post heparin plasma TG-lipase activity in WT and EL-KO mice (8 per group) was measured by incubating the samples with a ^{3}H-triolein emulsion substrate. The products of reaction were extracted and the liberated fatty acids (FFAs) were quantitated by scintillation counting. Panel B: western blot analysis of post heparin plasma from WT and KO mice showing LPL protein levels.

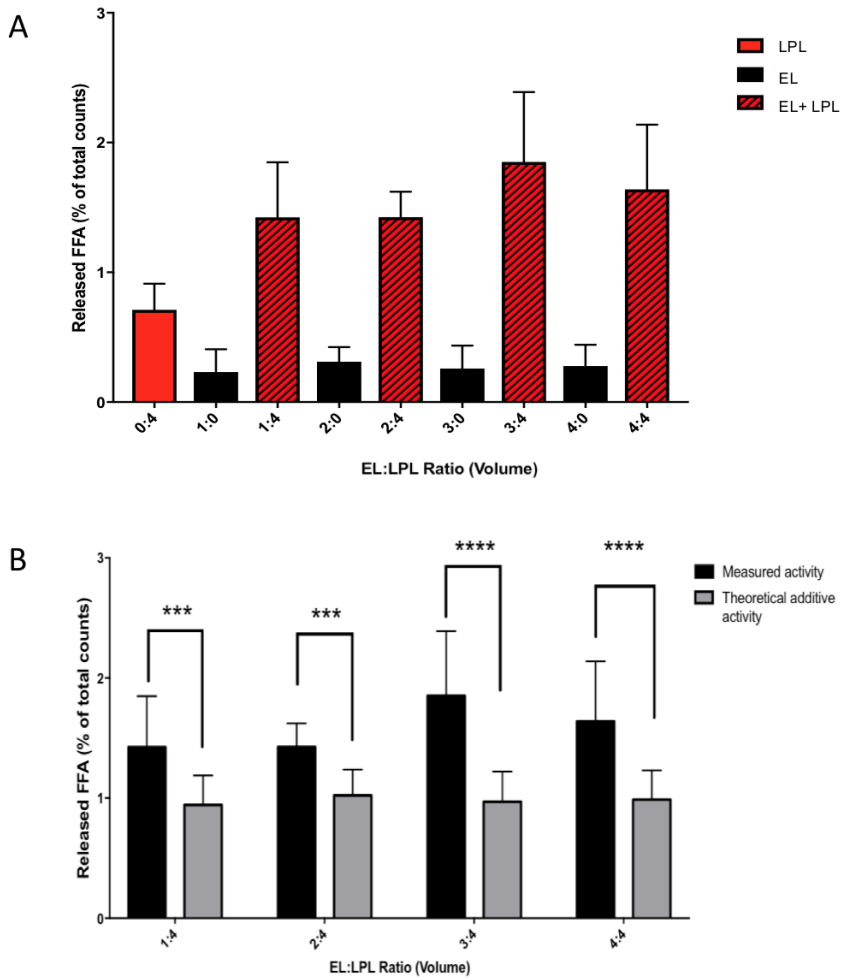

Figure R-2.13: Single ad combined TG-lipase activity of EL and LPL towards human TG-rich lipoproteins.

Media containing EL or LPL were obtained by adenoviral-mediated transfection of COS-7 cells. Human TG-rich lipoproteins were isolated from human plasma by density-gradient ultracentrifugation and labeled with ^{3}H-triolein. This substrate was used to determine the TG-lipase activity of LPL and EL alone or in combination. Products of reaction were extracted and lipids were separated with Thin Layer Chromatography (TLC) and counted. Results are expressed as % Free Fatty Acids activity vs. Total activity. Panel A: TG-lipase activity of a fixed volume of LPL, Increasing volumes of EL and co-incubation of the two enzymes. For the co-incubation assay a constant volume of LPL and increasing volumes of EL were added to the reaction mix. Panel B: Direct comparison between experimentally measured and calculated (sum of single contributions) TG-lipase activity of media containing both EL and LPL at different volume ratios. Data are expressed as mean ± SD *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Student's unpaired t-test with adjustment for multiple comparison.

Specific Aim 3: Defining the impact of EL deficiency on liver lipid metabolism.

3.1 EL deficiency is associated with increased hepatic steatosis

EL is physiologically expressed in adult liver and may contribute to hepatic lipid sensing and regulation of hepatic lipid storage. Absence of EL can thus potentially alter hepatic lipid homeostasis and lead to abnormal fat deposition. To test this hypothesis livers from WT and EL-KO mice on high fat diet for 12 weeks were collected, weighted and processed for H&E and Oil Red O staining. Liver weight was significantly higher in EL-KO mice compared to WT (1.6 ± 0.04 and 1.9 ± 0.16 g, in WT and KO mice, respectively n=6) and the overall appearance was consistent with the development of steatosis. The histological analysis confirmed that EL deficient mice were subjected to a significantly higher neutral lipid deposition (figure R-3.1A) and highlighted the presence of all the morphologic features of steatosis. To confirm these findings, liver tissues from WT and EL-KO mice were homogenized and lipids were solubilized using sodium deoxycholate. Tissue TG and cholesterol levels were then determined using a colorimetric assay. The analysis confirmed that the hepatic lipid content was significantly higher in EL-KO mice compared to WT (figure R-3.1B).

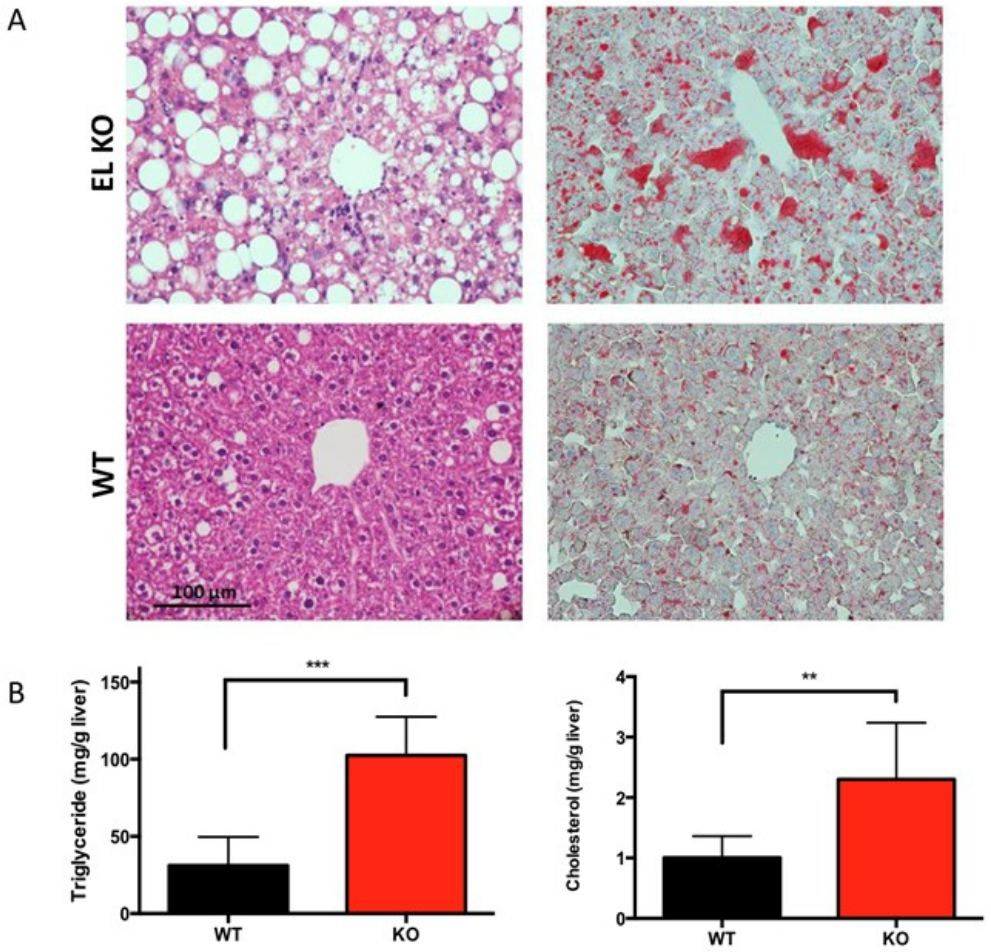

Figure R-3.1 Hepatic lipid accumulation in WT vs. EL-KO mice.

Panel A: Histological analysis of livers from WT and EL-KO mice after 12 weeks of high fat diet. In order to detect lipid depot, H&E staining (left) and Oil Red O staining of neutral lipids (right) were performed. Panel B: Hepatic triglycerides (left) and cholesterol (right) from the same mice. Liver homogenates from mice were solubilized using sodium deoxycholate and lipids were measured by colorimetric plate assay. Data are expressed as mean ± SD *P<0.05, **P<0.01, ***P<0.001, Student's unpaired t-test

3.2 Defining the mechanisms involved in increased hepatic lipid accumulation in EL-deficient mice

In animal models, excess in dietary-derived TG translates in obesity and this condition is often associated with steatosis. Prolonged high-fat feeding, in fact, leads to the release of high concentrations of FA into the portal circulation. FA can be taken up by the liver via hepatic fatty acid translocase [372]. This process is upregulated during obesity-induced inflammation. Cytokines upregulate the expression of FA transporters at the hepatocyte membrane and further enhance the flux of FA entering the liver.

EL-KO mice develop a significantly more severe obesity, hypertriglyceridemia and reactive inflammatory response. In order to test whether this phenotype could contribute to hepatic steatosis, human ^{3}H-TG-labeled lipoproteins were prepared as previously described and they were injected in WT and KO-mice. The experiment was terminated 15 minutes after injection and mice were sacrificed to permit tissue collection. The ^{3}H activity in each tissue, was considered as representative of the total TG tissue uptake. Surprisingly, despite the increased TG plasma levels and the markedly more severe obesity, hepatic FA uptake was significantly impaired in EL-deficient mice (figure R-3.2). Since EL and HL, but not LPL are physiologically expressed in the adult mouse liver, it has been hypothesized that EL-deficiency could impair the hydrolysis of TG at the hepatic endothelial surface thus negatively impacting FA uptake. To address this question, we measured the gene and protein expression levels of HL and LPL. Results indicated a significant increase in LPL gene and protein levels in EL-KO compared to WT whereas HL expression was comparable in the two groups (figure R-3.3).

Since EL is believed to have a higher specificity for HDL-derived PL compared to TG, and PL-derived FA can modulate liver function, a similar experiment has been conducted using a synthetic PL-labeled substrate. In this experiment, fluorescently labeled palmitoyl-PC (TopFluorPC) was used to synthetize reconstituted liposomes (diameter 12nm), that were injected in WT and EL-KO mice on chow diet. Since the HDL turnover requires a longer time

compared to TG-rich lipoprotein lipolysis, mice were sacrificed at 6 hours after injection and tissues were collected. Similar to what observed in the ^{3}H-TG-labeled lipoproteins, we observed a significantly reduced PC-derived FA uptake in the liver of EL-KO mice (figure R-3.4)

To address whether increased TG accumulation was a result of *de novo* lipogenesis, the expression levels of key genes involved in the regulation liver lipid metabolism was tested. Results indicated an overall upregulation of lipogenic genes in EL-KO mice compared to WT and significantly increased SCD1 protein levels (figure R-3.5).

Thanks to a collaboration with PV Subbaiah, at the University of Illinois, we were able to further explore this hypothesis. The analysis of liver FA in WT and EL-KO mice showed that the dietary intervention lead to significant changes in the total FA mass and distribution in both groups. On chow diet, WT and KO mice displayed a comparable liver FA content (41.16±9.21 and 43.26 ± 5.62 μg/mg of tissue, in WT and KO respectively). However, EL-KO mice had a 1.6 times higher response to high fat-feeding, that resulted in significantly higher FA mass at 12 weeks on diet (138.44±28.91 and 232.89 ± 55.13 μg/mg of tissue, in WT and KO respectively). The relative abundance of FA species was also deeply affected by the diet (Table R-3,R4). The percentages of most of the saturated fatty acids decreased (16:0, 18:0, 20:0, 22;0), as well as the percentage of some selected polyunsaturated FA (20:4 and 22:6) (Table R-3,R4 figure R-3.6) . This was compensated by a significant increase in 14:0, 16:1, 18:1, 20:1 and 22:5. These changes were more pronounced in EL-KO mice compared to WT (Table R-3,R4 figure R-3.6). The ratios of 18:1/18:0, 16:1/16:0, that are considered indicators of SCD activity, were disproportionately increased in KO livers compared to WT, thus supporting the idea that EL deficient mice may have increased hepatic *de novo* lipogenesis (18:1/18:0 WT=1.52 on chow diet and 9.14 on high fat diet; 18:1/18:0 KO= 1.47 on chow diet and 15.10 on high-fat diet; 16:1/16:0 WT= 0.07 on chow diet and 0.19 on high fat diet; 16:1/16:0 KO=0.08 on chow diet and 0.13 on high-fat diet).

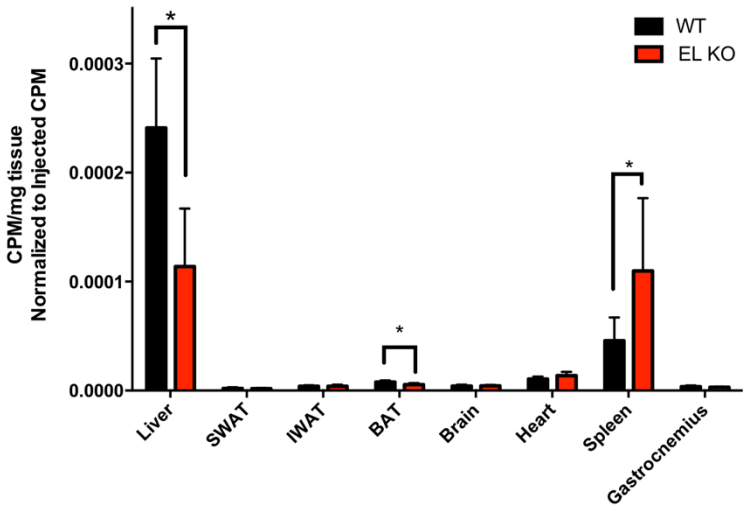

Figure R-3.2 ^{3}H oleate tissue uptake.

Human lipoproteins were isolated by density gradient ultracentrifugation (d<1.006 g/mL) and labeled with ^{3}H-triolein. They were then injected intravenously in WT and EL-KO mice. Animals were sacrificed 15 min after injection for tissues collection. Data are expressed as mean ± SD *P<0.05, Student's unpaired t-test.

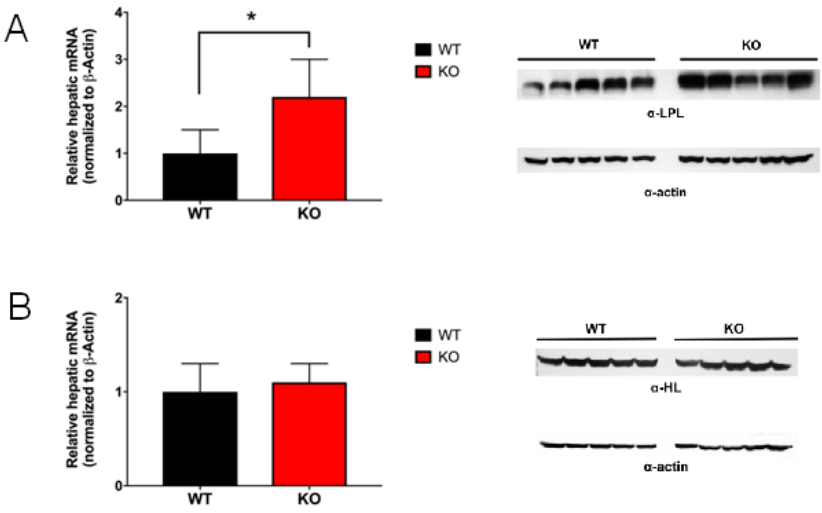

Figure R-3.3 Hepatic LPL and HL gene and protein expression in EL WT vs. KO mice fed a high-fat diet for 12 weeks.

Panel A: Hepatic LPL gene (left) and protein (right) expression in EL WT vs. KO mice fed a high-fat diet for 12 weeks. Panel B: Hepatic HL gene (left) and protein (right) expression in EL WT vs. KO mice fed a high-fat diet for 12 weeks. Gene and protein expression were normalized to expression of actin. Data are expressed as mean ± SD *P<0.05, Student's unpaired t-test

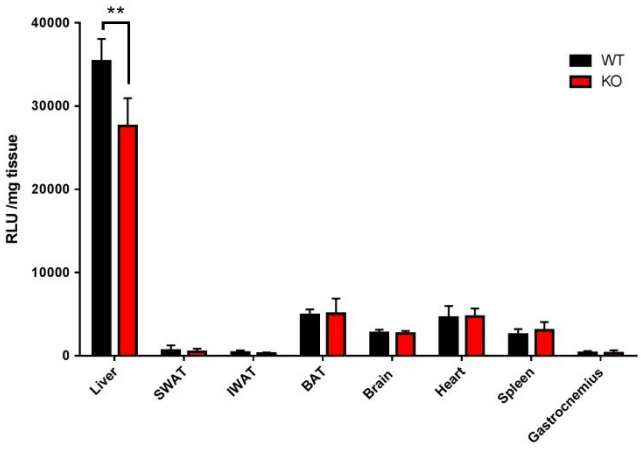

Figure R-3.4 TopFluor-PC tissue uptake.

WT and EL-KO mice (n=6) were intravenously injected with synthetic liposomes containing TopFluor-PC and they were sacrificed 6h after injection for tissue collection. The fluorescence intensity is expressed as RLU/mg tissue (mean ± SD). *P<0.05, **P<0.01, Student's unpaired t-test.

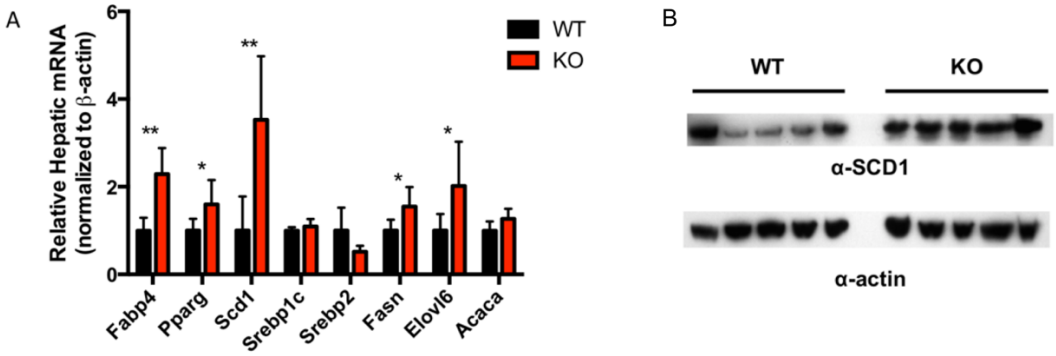

Figure R-3.5 Hepatic gene and protein expression in EL WT vs. KO mice fed a high-fat diet for 12 weeks.

Panel A: Hepatic gene expression in EL WT vs. KO mice fed a high-fat diet for 12 weeks. Gene expression was normalized to expression of actin. Panel B: Total hepatic immunoblots of LPL and SCD1 from the same mice. Data are expressed as mean ± SD *P<0.05, **P<0.01, Student's unpaired t-test.

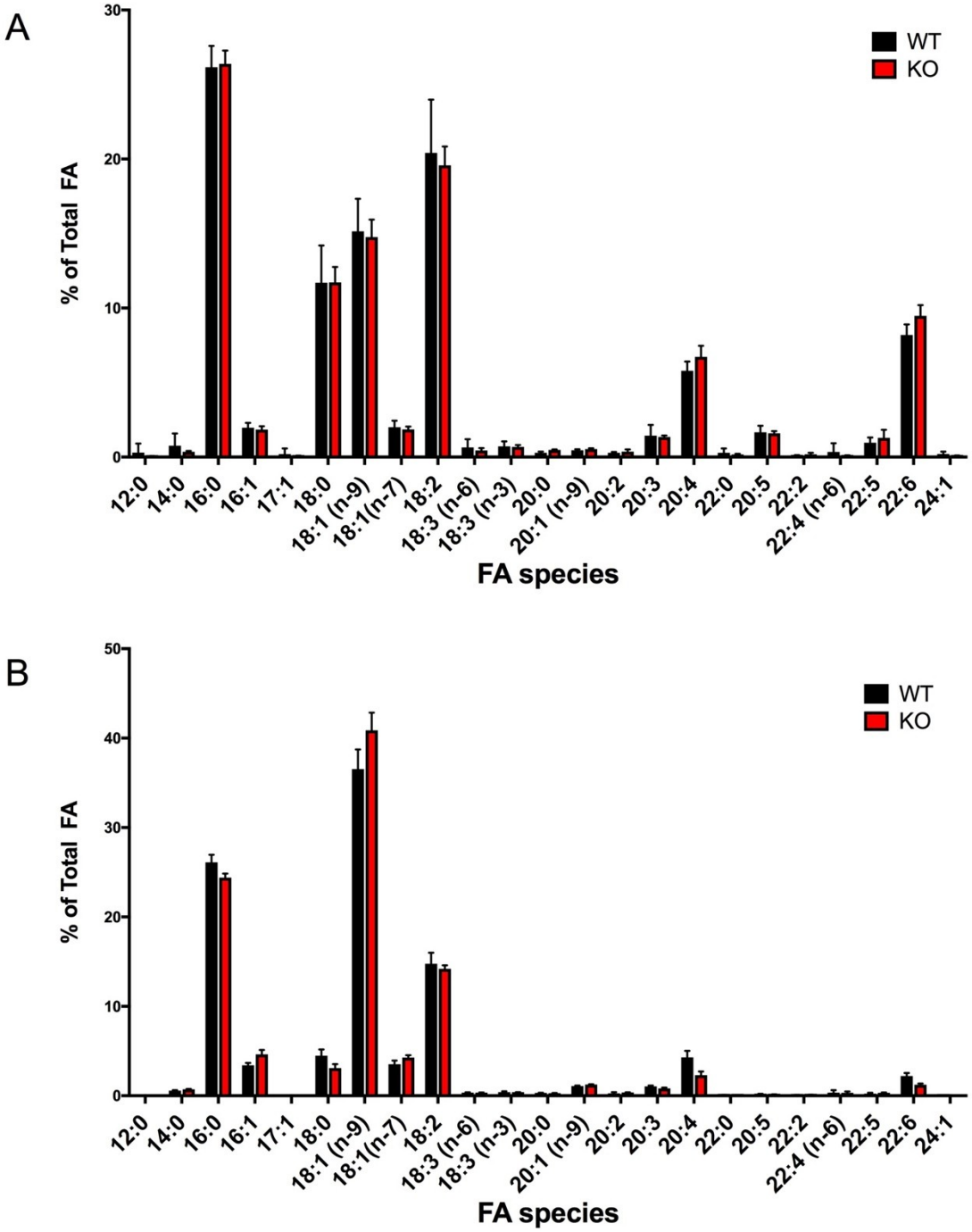

Figure R-3.6 Lipidomic analysis of Hepatic Fatty Acid content of mice on chow vs. high fat diet

Total fatty acid composition of WT and EL-KO livers was determined by GC/MS. The plots show the % of each FA species vs. total FA of WT and EL-KO mice on chow diet (Panel A) and after 12 weeks on high-fat diet (Panel B) Data are expressed as mean ± SD. For each dietary condition, the relative % of FA species has been compared for WT vs. EL-KO mice. For clarity, single data and significant results are reported in Table R-3 (chow diet) and R4 (high fat diet).

FA species	WT		KO		Difference	SE	Adjusted P Value
	% of Total FA	SD	% of total FA	SD			
12:0	0.30	0.61	0.05	0.02	-0.24	0.27	0.9978
14:0	0.76	0.82	0.36	0.06	-0.40	0.37	0.9959
16:0	26.17	1.43	26.39	0.90	0.22	0.75	0.9996
16:1	1.96	0.33	1.84	0.23	-0.12	0.18	0.9985
17:1	0.19	0.39	0.06	0.04	-0.13	0.18	0.9985
18:0	11.70	2.50	11.72	1.03	0.02	1.21	0.9996
18:1 (n-9)	15.15	2.19	14.76	1.18	-0.39	1.11	0.9996
18:1 (n-7)	2.00	0.44	1.86	0.19	-0.14	0.21	0.9985
18:2	20.41	3.57	19.57	1.27	-0.84	1.70	0.9991
18:3 (n-6)	0.65	0.55	0.44	0.17	-0.21	0.26	0.9985
18:3 (n-3)	0.71	0.35	0.69	0.12	-0.03	0.16	0.9996
20:0	0.29	0.07	0.48	0.04	0.19	0.04	0.0239
20:1 (n-9)	0.45	0.07	0.54	0.05	0.09	0.04	0.7076
20:2	0.28	0.07	0.37	0.14	0.09	0.07	0.9933
20:3	1.44	0.72	1.35	0.10	-0.09	0.33	0.9996
20:4	5.79	0.62	6.72	0.75	0.93	0.43	0.7378
22:0	0.28	0.30	0.14	0.09	-0.14	0.14	0.9959
20:5	1.66	0.45	1.59	0.15	-0.07	0.21	0.9996
22:2	0.08	0.05	0.16	0.13	0.08	0.06	0.9947
22:4 (n-6)	0.35	0.58	0.07	0.05	-0.28	0.26	0.9959
22:5	0.96	0.35	1.29	0.54	0.33	0.29	0.9959
22:6	8.20	0.70	9.47	0.72	1.28	0.45	0.3858
24:1	0.20	0.17	0.07	0.04	-0.12	0.08	0.9485

Table R-3: GC/MS analysis of Hepatic FA content of WT and KO mice on chow diet.

Relative % of each FA species/total FA content (mean and SD) in WT (n=8) and EL-KO mice (n=5) on chow diet. The three columns on the right show the results from the statistical comparison of the relative FA abundance in the two strains (Multiple t-test with significance assessed with Holm-Sidak method, with alpha = 0.05). Significant results ($p<0.05$) are highlighted in grey.

FA species	WT		KO		Difference	SE of difference	Adjusted P Value
	% of Total FA	SD	% of total FA	SD			
12:0	0.01	0.01	0.01	0.00	0.00	0.00	0.9484
14:0	0.58	0.05	0.72	0.05	0.14	0.03	0.0154
16:0	26.10	0.85	24.41	0.45	-1.70	0.42	0.0324
16:1	3.41	0.26	4.64	0.49	1.23	0.21	0.0021
17:1	0.01	0.01	0.00	0.01	-0.01	0.01	0.7377
18:0	4.49	0.69	3.08	0.45	-1.40	0.35	0.0354
18:1 (n-9)	36.54	2.20	40.87	1.98	4.33	1.21	0.0627
18:1 (n-7)	3.52	0.42	4.27	0.26	0.75	0.21	0.0627
18:2	14.76	1.25	14.20	0.39	-0.55	0.58	0.9484
18:3 (n-6)	0.34	0.04	0.34	0.02	0.00	0.02	0.9975
18:3 (n-3)	0.41	0.08	0.40	0.01	-0.01	0.04	0.9975
20:0	0.29	0.05	0.23	0.08	-0.06	0.04	0.7812
20:1 (n-9)	1.08	0.06	1.24	0.04	0.16	0.03	0.0037
20:2	0.20	0.21	0.37	0.02	0.16	0.09	0.7377
20:3	1.03	0.10	0.82	0.10	-0.21	0.06	0.0466
20:4	4.29	0.74	2.30	0.42	-1.99	0.37	0.0042
22:0	0.07	0.04	0.06	0.03	-0.01	0.02	0.9897
20:5	0.11	0.10	0.11	0.06	-0.01	0.05	0.9975
22:2	0.05	0.05	0.09	0.03	0.03	0.03	0.8684
22:4 (n-6)	0.35	0.28	0.31	0.17	-0.04	0.14	0.9975
22:5	0.13	0.20	0.30	0.07	0.18	0.10	0.6699
22:6	2.21	0.33	1.23	0.12	-0.98	0.16	0.0015
24:1	0.02	0.01	0.01	0.00	-0.01	0.01	0.2656

Table R-4: GC/MS analysis of Hepatic FA content of WT and EL-KO mice after 12 weeks on high-fat diet

Relative % of each FA species/total FA content (mean and SD) in WT and EL-KO mice on high-fat diet for 12 weeks (n=5 per group). The three columns on the right show the results from the statistical comparison of the relative FA abundance in the two strains (Multiple t-test with significance assessed with Holm-Sidak method, with alpha = 0.05). Significant results ($p<0.05$) are highlighted in grey.

Discussion

Endothelial lipase (EL) is a major determinant of HDL-C levels [89, 90, 218]. Given the established inverse correlation between HDL-C and cardiovascular disease, early human studies have focused on investigating the relationship between EL activity, pro-atherogenic lipid profile and CVD risk. Most of the studies that directly measured EL mass or activity were conducted in populations of individuals with traits of metabolic syndrome [228, 330, 373]. At the time of the studies several pieces of evidence had already demonstrated that EL activity was inversely correlated to HDL-C levels and its expression was positively regulated by inflammation [89, 224, 228, 334]. Metabolic syndrome was thus an ideal model to assess whether EL could affect the development of CVD by reducing plasma levels of atheroprotective HDL. These studies consistently demonstrated a strong correlation between EL mass and several traits of metabolic syndrome such as body mass index (BMI), visceral adiposity, inflammation, insulin resistance and atherogenic lipoprotein levels [228, 229, 330]. However, the directionality of the association is still not known and whether EL mass or activity could drive the progression of one or multiple of the indicated traits it's not clear. Furthermore, although *LIPG* genetic variants are associated with increased HDL-C levels in humans, their association with the risk of CVD has not been convincingly supported (table 4).

First objective of this thesis was to establish the effect of EL deficiency on the onset of clinical hallmarks of metabolic syndrome. In mice, most of the traits of metabolic syndrome can be induced by high-fat feeding. When EL-deficient mice were kept at low-fat (chow) diet, their body weight was comparable to that of WT. Surprisingly, on high-fat diet EL-deficiency was associated with significantly increased weight gain. The analysis of body composition highlighted the presence of increased fat mass and decreased lean mass. The fat accumulation was more pronounced in the epididymal region and the analysis of the fat morphology revealed that it was due to an increased number and size of adipocytes. The relatively higher weight gain was not explained by increased food intake or decreased energy expenditure, thus suggesting a dysfunctional utilization of FA. The mechanism underlying these abnormalities has not been fully elucidated but the expression analysis of

genes involved in intracellular FA trafficking and *de novo* lipogenesis revealed an upregulation of these pathways in adipose tissues with no major changes in heart and skeletal muscle. Notably adipocyte LPL mRNA levels were significantly increased in EL-KO mice compared to WT. It has been demonstrated that in murine lipoprotein-deficient adipose tissue, endothelial lipase represents an alternative pathway for FFA uptake [357]. Data from this study support this hypothesis and suggest that in absence of EL, LPL may be subjected to a compensatory upregulation.

Abnormalities in glucose metabolism and low-grade inflammation are features commonly associated with obesity and represent peculiar traits of metabolic syndrome [374]. These conditions are intimately related. High fat feeding induces constitutively high TG plasma levels. Adipose tissue is an insulin sensitive compartment and responds to calories in excess by storing FA in the form of TG. This process induces adipocyte hypertrophy and predispose to cell dysplasia and subsequent increased macrophage infiltration. At the same time, hypertrophic adipocytes are characterized by a hyperlipolytic state that is resistant to the antilipolytic effect of insulin. This condition further increases the FA flux to the liver and impairs liver glucose and fat metabolism [374]. The efficiency of glucose metabolism in WT and EL-KO mice was assessed by measuring the glucose tolerance (defined as clearance of an intraperitoneal glucose load) and insulin tolerance (glucose response to an intraperitoneal injection of insulin). On low-fat diet, EL-deficient mice displayed a normal response to glucose loading but progressively developed a pre-diabetic phenotype when fed hypercaloric diet. At 12 weeks, EL-KO mice showed a frank glucose intolerance whereas insulin sensitivity was preserved. Similarly, high fat-feeding seemed to induce a metainflammatory status in EL-KO mice. At the baseline both groups of mice had similar levels of TNFα, but when they were exposed to a pro-inflammatory stimulus, EL-KO mice displayed a significantly higher inflammatory response. In humans there is a strong correlation between plasma levels of EL and cytokines in obese and healthy individuals. It has been proposed that obesity, in particular visceral adiposity may be sufficient to induce and sustain EL upregulation in individuals with metabolic syndrome. Data from this study show that in a high-fat dietary

setting, absence of EL is associated with earlier and a more severe obesity. Furthermore, the onset of the different traits suggests that, in this model, obesity may be a primary event in the development of metabolic syndrome whereas glucose intolerance and low-grade inflammation seem to be secondary. Taken altogether these observations support the hypothesis that increased EL expression may be a physiological compensatory mechanism in response to diet-induced metabolic abnormalities rather than a causal factor.

One of the main characteristics of the metabolic syndrome is dyslipidemia, characterized by increased cholesterol and TG levels, and reduced HDL-C levels. Previously published studies widely demonstrated that EL mediates HDL catabolism by promoting PL hydrolysis. Furthermore, EL can promote the clearance of apoB-containing lipoproteins and this process is dependent on its hydrolytic activity [211, 216, 222]. Very little is known about the role of EL in TG-rich lipoprotein metabolism. LPL is thought to be the main responsible for the catabolism of TG-rich lipoproteins but isolated reports suggest that in certain conditions EL-mediated hydrolysis may represent an alternative pathway to release FA in peripheral tissues [2, 357, 375]. The second aim of this thesis was thus to determine the role of EL in plasma TG-rich lipoprotein metabolism. This aspect has been extensively elucidated in this study by characterizing the lipid profile of WT and EL-deficient mice in response to a high fat diet. Consistent with previous reports, in this study we found that on low-fat dietary setting, EL deficiency is associated with increased cholesterol and PL plasma levels and this effect is explained by a significant increase in both HDL and non-HDL-C levels [356]. As expected, the high-fat dietary intervention induced significant changes in plasma lipids in both WT and EL-deficient mice and all the measured lipid parameters were significantly increased after 12 weeks. However, although the levels of FA, PL, and non-HDL cholesterol increased at a similar rate in both strains, HDL-C increased at a relatively lower rate in EL-KO mice. Finally, TG plasma levels seemed to be the most severely affected by the dietary treatment, showing a two-fold higher change compared to WT. Since there was no difference between WT and KO mice at the baseline, it can be deduced that the latter change was triggered by the prolonged dietary intervention. Given

the enzymatic properties of EL, we hypothesized that EL-deficiency may directly or indirectly impair the TG-rich lipoprotein remodeling and that this condition may become more evident upon a chronic high-TG dietary supplementation. Further analyses were then specifically aimed to characterize the features of plasma lipoproteins in WT versus EL-deficient mice in response to the chronic high-fat feeding and to detect the remodeling efficiency in the transition between fasted and fed state. The FPLC fractionation revealed that EL-KO mice displayed a significantly higher cholesterol and TG-enrichment of apoB-containing lipoproteins and these changes became even more dramatic upon re-feeding. The apoB-48 and apoB-100 content was also significantly higher in EL-deficient mice, thus suggesting a parallel increase in the number of particles and a possible retention of post-prandial CM remnants. This possibility has been directly tested by performing an oral-fat tolerance test, followed by qualitative analyses of plasma lipoproteins (FPLC and agarose gel electrophoresis). Results demonstrated that EL-deficiency is associated with persistent post-prandial hyperlipemia and that is due to the accumulation of apoB-100 and apoB-48-containing lipoproteins, at least in part identifiable as chylomicrons. Notably, this trait was already detectable in mice on chow diet, thus suggesting that it may be a pre-existing feature directly associated with EL deficiency and not secondary to other causes. In this scenario, the chronical administration of high fat diet may exacerbate an already pre-existing phenotype. Different mechanisms can concur to the retention of post-prandial TG-rich lipoprotein remnants. Physiologically, a key event in CM and VLDL clearance, is lipase-mediated hydrolysis. The efficiency of this process in WT and EL-KO mice was specifically tested through an *in vivo* kinetic experiment where WT and EL-KO mice were injected with either protein and TG-labeled TG-rich lipoproteins and their clearance was evaluated over 15 minutes. Results indicated that EL deficiency was associated with significantly impaired ^{3}H-TG clearance whereas the ^{125}I-protein clearance was not affected. Together these findings indicate that, in this experimental setting, the impaired clearance is primarily attributable to a reduction of TG lipolysis and not to an impaired particle uptake by peripheral tissues. Another mechanism that can contribute to TG-rich lipoprotein accumulation is hepatic production of VLDL.

We then measured the hepatic production of VLDL-TG by pharmacologically inhibiting plasma lipases and then measuring the plasma TG accumulation over time. Results show that EL-KO mice displayed a moderate trend to decreased TG production, thus indicating that hepatic VLDL secretion is not a major contributor to plasma TG levels in EL-deficient mice. The potential biochemical causes underlying the observed phenotype were then explored by testing EL and LPL lipase activity *in vitro*. Consistent with previously published data, EL and LPL displayed a different activity towards a synthetic ^{3}H-TG-labeled VLDL-like emulsion [211]. Although EL was capable of an appreciable dose-dependent TG lipase activity, the quantitative relevance of this reaction was significantly lower compared to LPL. However, the activity of plasma lipases can be modulated by several inhibitors *in vivo* and different lipases can exert cooperative effects on the same substrate. The post-heparin plasma activity assay represents a useful tool to evaluate the potential influence of these different factors on the TG-lipase activity and has been widely used in several animal and human studies. In this study, we didn't observe any difference in the post-heparin plasma activity of EL-KO and WT mice. However, when we co-incubated different ratios of EL and LPL with ^{3}H-TG-labeled isolated VLDL, we observed that the resulting activity was higher than what expected by calculating the sum of the contributions. These results support the conclusion that EL may modulate plasma TG levels by facilitating LPL activity in a synergistic fashion. This effect seems to be dose-dependent and requires the presence of a lipoprotein substrate. Furthermore, since EL does not display a significant TG activity on isolated lipoproteins *per se,* the observed effect is likely a consequence of its phospholipase activity. The overall effect of this synergistic activity *in vivo* is difficult to predict. Lipases are synthetized by different cell types and they are then secreted in the vascular space where they bind to heparan sulfate proteoglycans. As a result, different lipases coexist on the endothelial wall and it is possible that this spatial proximity may also translate in a functionally complementary activity. Supporting this concept, in healthy subjects, simultaneous heterozygosity for SNPs in different lipase genes (EL 2,237 3 untranslated region, EL Thr111Ile, HL 514C/T, and LPL HindIII) had synergistic association with plasma TG, beyond individual gene effects [376].

Increased adiposity, glucose intolerance and hypertriglyceridemia are associated with an increased risk of developing fatty liver disease in humans [221]. The phenotype observed in EL-KO mice fed a high-fat diet recapitulates all these pro-steatotic conditions. Therefore, the third objective of this study was to define the impact of EL deficiency on liver FA metabolism. When compared to WT, EL-deficient mice showed a significantly more pronounced steatosis. The uptake of ^{3}H-TG-derived FA by the liver was 50% decreased, and this was accompanied by the upregulation of lipogenic genes. The lipidomic analysis of hepatic FA content confirmed these findings and showed not only a significantly increased fat content in the livers of EL-deficient mice but also a disproportionately higher increase in monounsaturated fatty acid. The 18:1/18:0 and 16:1/16:0 ratios were in fact 1.7 and 1.5 folds higher in EL-KO mice compared to WT. These indexes are established markers of SCD1 activity, the enzyme that catalyzes the limiting step of monounsaturated fatty acid synthesis and are positively correlated with triglyceride generation. Notably, LPL gene and protein were both significantly more expressed in EL-KO mice, whereas HL levels were comparable. This finding was unexpected because hepatic LPL expression levels are physiologically high during embryogenesis but they progressively drop after birth and they are negligible in adult animals. Once again, these findings are supportive of a model where different lipases cooperate in the metabolism of circulating lipoproteins and suggest a potentially previously underappreciated crosstalk in the expression of EL and LPL. Whether this mutual influence in the regulation of expression is representative of partially overlapping function is not known and further studies will be required to address this point. However, according to the present data hepatic LPL upregulation is not sufficient to normalize the hepatic FA uptake.

In conclusion, the current thesis work demonstrated that EL deficiency in the high-fat dietary setting is associated with the development of earlier and more severe traits of metabolic syndrome, namely obesity, visceral adiposity, glucose intolerance and metainflammatory status. Furthermore, deficiency of EL in mice results in significantly impaired lipolytic catabolism of TG-rich particles and this effect seems to be mediated by an indirect impairment of

LPL activity. Finally, these changes result in a subsequent decreased delivery of TG-derived FA to the liver and a parallel upregulation of lipogenic pathways.

Collectively these data support a model by which EL is required for efficient triglyceride-rich lipoprotein clearance and the absence of this function impairs hepatic fat intake and results in compensatory *de novo* lipogenesis, which may contribute to systemic obesity and insulin resistance (figure C-1).

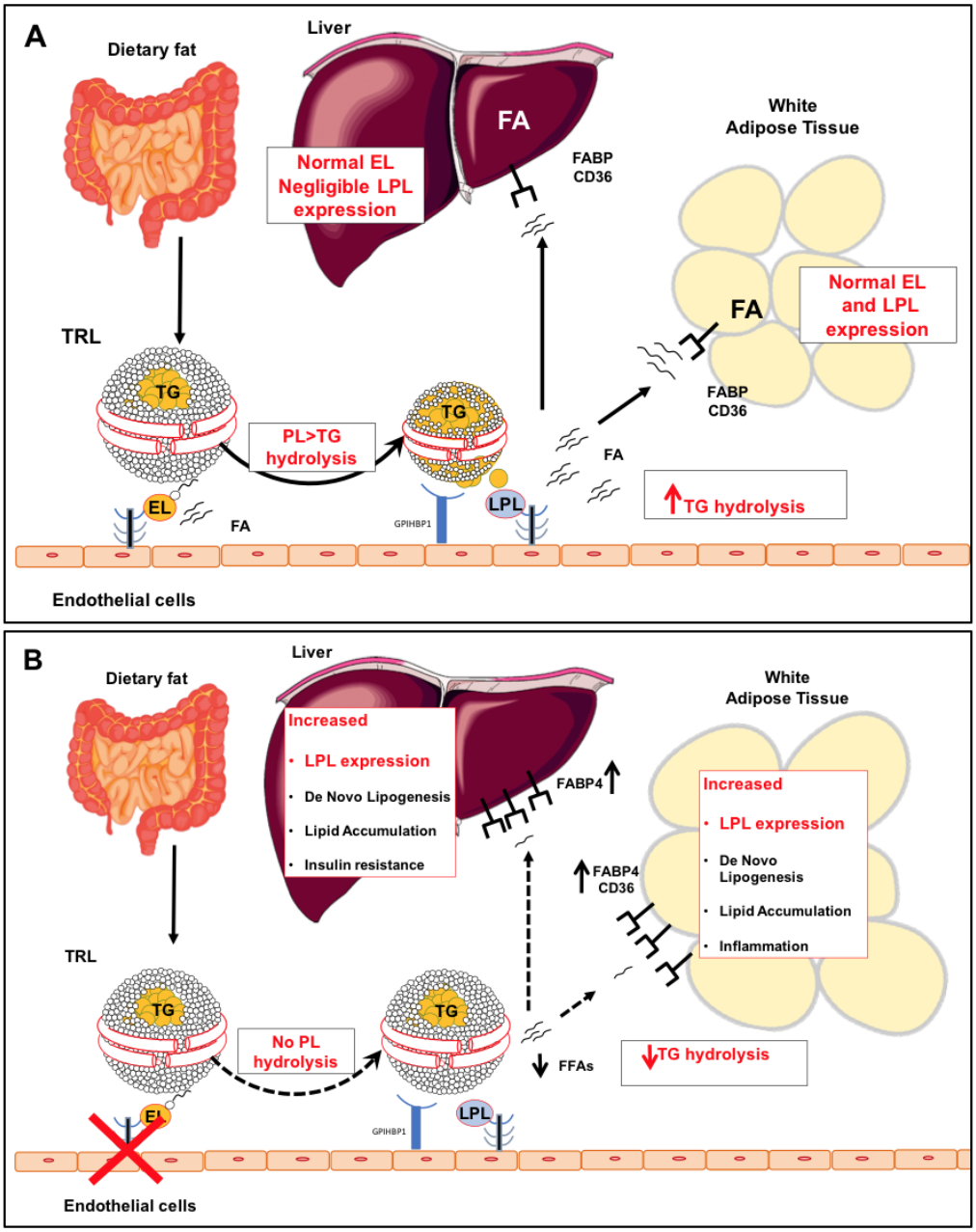

Figure C-1: Proposed model of EL deficiency-induced abnormalities in plasma, liver and adipose tissue metabolism.

Panel A: EL hydrolyzes TG and surface PL in TRL. This process facilitates the further hydrolysis of TG by LPL. Released FA can be taken up by liver and adipose tissue through specific transporters. Panel B: in absence of EL, LPL has access to a limited amount of TG. This impairs the availability of FA to adipose tissue and liver. This is associated with hepatic upregulation of LPL, lipid transporters and lipogenic genes and translates into increased *de novo* lipogenesis, steatosis and insulin resistance. In the adipose tissue, lack of EL induces increased expression of membrane lipid transporters and lipogenic genes. This leads to increased lipid storage and predispose to inflammation.

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