DOCTORAL THESIS

Calorimetry as a Tool for Mechanistic Studies of Lipolytic Enzymes under in vivo like Conditions

Robert Risti

TALLINNA TEHNIKAÜLIKOOL
TALLINN UNIVERSITY OF TECHNOLOGY
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Calorimetry as a Tool for Mechanistic Studies of Lipolytic Enzymes under in vivo like Conditions

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Robert Risti

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Kalorimeetria rakendamine lipolüütiliste ensüümide mehhanismide uurimiseks

ROBERT RISTI

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Villo L*, **Risti R***, Reimund M, Kukk K, Samel N, Lookene A. Calorimetric approach for comparison of Angiopoietin-like protein 4 with other pancreatic lipase inhibitors. Biochim Biophys Acta Mol Cell Biol Lipids. 2020 Feb; 1865(2):158553.

II. Reimund M, Wolska A, **Risti R**, Wilson S, Sviridov D, Remaley AT, et al. Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach. Biochem Biophys Res Commun. 2019;519(1).

III. **Risti R**, Gunn KH, Hiis-Hommuk K, Seeba NN, Karimi H, Villo L, Vendelin M, Neher SB, Lõokene A. Combined action of albumin and heparin regulates lipoprotein lipase oligomerization, stability, and ligand interactions. PLoS One. 2023 Apr 12;18(4)

*Equal contribution

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I. The author participated in planning the experiments and in analyzing and interpreting the results. The author performed measurements with isothermal titration calorimetry, fluorimetry, and spectroscopy, and contributed to writing of the manuscript.

II. The author participated in planning the experiments and performed chromatography, density gradient ultracentrifugation, spectroscopy, fluorimetry, and part of isothermal titration calorimetry experiments. The author contributed to analyzing and interpreting the results, as well as writing of the manuscript.

III. The author was responsible for carrying out isothermal titration calorimetry, fluorimetry, and part of surface plasmon resonance measurements, and for analyzing and interpreting the results. The author wrote the manuscript and visualized the data, with contributions from the co-authors.

OTHER PUBLICATIONS BY THE AUTHOR

The following publications are not discussed in this thesis, but the calorimetric method was used extensively in publication 2 and serves as an example of routine clinical analysis of lipolysis in blood plasma.

1. Rump A, **Risti R**, Kristal ML, Reut J, Syritski V, Lookene A, Ruutel Boudinot S. Dual ELISA using SARS-CoV-2 nucleocapsid protein produced in E. coli and CHO cells reveals epitope masking by N-glycosylation. Biochem Biophys Res Commun. 2021 Jan 1;534:457-460. doi: 10.1016/j.bbrc.2020.11.060. Epub 2020 Nov 20. PMID: 33276951; PMCID: PMC7678427.
2. M. Metz, M. Beghini, P. Wolf, L. Pfleger, M. Hackl, M. Bastian, A. Freudenthaler, J. Harreiter, M. Zeyda, S. Baumgartner-Parzer, R. Marculescu, N. Marella, J.T. Hannich, G. Györi, G. Berlakovich, M. Roden, M. Krebs, **R. Risti**, A. Lõokene, M. Trauner, et al., Leptin increases hepatic triglyceride export via a vagal mechanism in humans, Cell Metab, (2022).

INTRODUCTION

Pancreatic lipase (PL) and lipoprotein lipase (LPL) are central elements of triglyceride metabolism. PL is produced by pancreatic acinar cells and secreted to the duodenum where it governs the influx of lipid nutrients by breaking down dietary triglycerides. The released components are absorbed, reassembled, and packed into triglyceride rich lipoproteins (TRLs) to be sent into blood circulation. Regardless of the nutritional state of the body, endogenous TRLs are also constantly produced and secreted by the liver. Triglycerides in TRLs are hydrolyzed into free fatty acids (FFAs) by LPL for utilization in skeletal muscle and heart, or for energy storage in adipose tissue. LPL is produced in parenchymal cells of the same tissues, transported through the subendothelial space, and finally attached to the luminal side of capillary endothelial cells.

Numerous epidemiological and genetic studies have identified both pre- and post-prandial hypertriglyceridemia (HTG) as an independent and causal risk factor for the development of cardiovascular diseases (CVDs). Furthermore, HTG can cause hepatic steatosis and life-threatening acute pancreatitis. Statistics show that one in four Europeans or half of the US population suffers from moderate HTG. The causes behind HTG seldom originate from a single reason. Rather, elevated triglycerides are a combination of polygenic factors, poor life-style choices, and other conditions such as type 2 diabetes. Multiple therapeutical strategies have been developed for combating HTG. PL is targeted with inhibitors to reduce dietary triglyceride absorption and caloric intake in obese patients with HTG. At the same time, LPL activity is increased by activators or by suppression of endogenous LPL inhibitors to reduce the residence time of atherogenic lipoproteins in circulation.

Therefore, mechanistic studies of PL or LPL and their regulators are of great interest to pharmacological companies for developing new therapies that address HTG. Additionally, accurate and rapid measurement of PL or LPL activity in blood is needed in clinical situations to respectively determine pancreatitis or causes behind HTG. Both PL and LPL function in highly crowded complex environments where a multitude of factors can influence their activity. Stemming from this fact, and considering the broad substrate specificity of both lipases, many assays have been developed for determining lipase activity. However, most approaches utilize water-soluble synthetic substrates for continuous measurements, otherwise sacrifices in sensitivity and throughput must be made to accommodate more physiological-like conditions.

The current thesis explores the use of a calorimetric method to investigate PL and LPL regulators in physiologically relevant environments. We utilized calorimetry to study PL in a model substrate environment that contained components of intestinal emulsions. This approach was subsequently applied to elucidate how intestinal ANGPTL4 affects PL activity through interactions with substrate emulsions. Calorimetry was also used to evaluate a novel LPL activator in undiluted human plasma. Our approach highlighted multiple differences with previous results obtained in non-physiological conditions. Finally, calorimetric studies were combined with surface plasmon resonance, raster image correlation spectroscopy, and negative stain transmission electron microscopy to describe a novel mechanism where albumin and heparin regulate LPL stability, oligomerization, and ligand interactions.

ABBREVIATIONS

ANGPTL	angiopoietin-like protein
ASCVD	atherosclerotic cardiovascular disease
ASO	antisense oligonucleotide
CCD	coiled-coil domain
CTD	C-terminal domain
CVD	cardiovascular disease
EGCG	(−)-epigallocatechin-3-gallate
EPL	ε-polylysine
FCS	familial chylomicronemia syndrome
FLD	fibrinogen-like domain
GPIHBP1	glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1
HDL	high-density lipoprotein
HDX-MS	hydrogen-deuterium exchange mass spectrometry
HSPG	heparan sulfate proteoglycan
HTG	hypertriglyceridemia
ITC	isothermal titration calorimetry
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LMF1	lipase maturation factor 1
LRP	LDL-receptor related protein
nsTEM	negative stain transmission electron microscopy
NTD	N-terminal domain
PNPB	p-nitrophenyl butyrate
RICS	raster image correlation spectroscopy
SAXS	small-angle X-ray scattering
SDC1	Syndecan-1
SEC	size-exclusion chromatography
SEL1L	Sel-1 suppressor of Lin-12-like 1
THL	tetrahydrolipstatin
T2D	type 2 diabetes
TGN	trans-Golgi network
TRL	triglyceride-rich lipoprotein
VLDL	very low-density lipoprotein
WAT	white adipose tissue

1 LITERATURE OVERVIEW

1.1 Lipoproteins

Proper lipid homeostasis is essential for normal bodily function, as lipids are used for energy, cell signaling or membrane components. Many tissues require lipids such as triglycerides, cholesterol, and phospholipids for their operation but how to transport water-insoluble lipids in the aqueous environment of the bloodstream at mg/dl concentrations? This problem is solved by joining the lipids with amphipathic proteins (apolipoproteins) into lipid-protein emulsions in the liver and intestine. These soluble globular complexes are known as lipoproteins and their assembly is highly sensitive to nutritional, hormonal, and genetic factors.

All lipoproteins are related by a common structural characteristic — amphipathic phospholipids and unesterified cholesterol form an outer spherical phospholipid shell with apoliproteins, which encloses a core of triglycerides, cholesteryl esters and fat-soluble vitamins [1]. The differences arise from the size, density, function, and protein/lipid content of the lipoproteins. The most common classification of lipoproteins is based on lipoprotein density, which is dictated by their relative contents of protein and lipids. Lipoproteins are divided into four classes, from least dense to most: chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (**Table 1**). Additionally, intermediate-density lipoproteins (IDL) are placed as a separate class between VLDL and LDL densities in many classifications [2–4]. It is important to consider that this discrete classification is quite crude in the sense that lipoproteins are continuously acted on by plasma enzymes and undergo constant shuttling of their lipids and proteins. Each lipoprotein class can therefore be further divided into numerous subclasses (e.g., HDL_2, HDL_3) or even be considered a continuous spectrum of lipoprotein particles [1,2], all of which can have vastly different metabolic and atherogenic effects on the body.

The distinct function of each lipoprotein class is determined by their apolipoprotein and lipid content. Chylomicrons, with a protein content of 1–2% and sizes reaching 1200 nm, are synthesized in the intestine from dietary triglycerides. VLDLs on the other hand, are created from endogenous triglycerides in the liver, while IDL and LDL are the products of enzymatic VLDL degradation in blood circulation. HDL is also assembled in the liver. HDL contains an almost equal percentage of proteins and lipids. HDL particles can be tiny and dense, with diameters as small as 5 nm. Chylomicrons and VLDLs are called triglyceride-rich lipoproteins (TRLs) and their respective function is to deliver dietary or endogenic triglycerides to adipose tissue, skeletal muscle and heart for energy storage or consumption. Contrarily, the main role of LDL is the transport of cholesteryl esters to extrahepatic tissues and liver. LDL is known for being the carrier of so-called “bad cholesterol” (LDL-C), which concentration in plasma has been shown to be correlated with the risk of atherosclerotic cardiovascular disease (ASCVD) by numerous epidemiological and genetic studies [4].

HDL also contains primarily cholesteryl esters/cholesterol and phospholipids, but its function is to instead acquire excess cholesterol from extrahepatic tissues in a process coined “reverse-cholesterol transport”, which transports cholesterol back to the liver for excretion [1,2]. Another function of HDL is to act as an apolipoprotein reservoir. Apolipoproteins found in plasma can be broadly classified as either non-exchangeable or

Table 1. *Composition and physical parameters of major lipoprotein classes* [1,2].

	Chylomicron	**VLDL**	**LDL**	**HDL**
Density (g/ml)	<0.94	0.94–1.006	1.006–1.063	1.063–1.210
Diameter (nm)	80–1200	30–80	18–25	5–12
Major apoliproteins	B-48, A, C, E	B-100, C, E	B-100	A, C, E
% of total lipoprotein weight				
Protein	1–2	8–10	20–25	52–60
Lipids	98–99	90–92	75–80	40–48
% of total lipid weight				
Triglycerides	81–89	50–58	7–11	6–7
Unesterified cholesterol	1–3	4–9	10–12	6–8
Cholesteryl esters	2–4	15–23	47–51	24–45
Phospholipids	7–9	19–21	28–30	42–51

exchangeable. HDL shuttles exchangeable apolipoproteins (e.g., apoA, apoC, apoE) to or from circulating chylomicrons, VLDLs and LDLs, contributing to their maturation or catabolism. There has also been a resurgence in the importance of another class of lipoproteins called lipoprotein(a). Lp(a) is a <70 nm diameter LDL particle where an apo(a) moiety is covalently bound to apoB-100. Its size enables it to readily penetrate the endothelial barrier, where it can be retained similarly to LDL and thus pose as a risk for ASCVD [4].

As mentioned, lipoproteins contain non-exchangeable apolipoproteins (apoB-100, apoB-48), these are tightly bound to lipoproteins throughout their metabolic cycle and are only finally cleared from plasma through various receptors. The non-exchangeable 512 kDa apoB-100 and 242 kDa apoB-48 (48% of apoB-100 sequence, hence the name) are the main protein component of VLDLs/LDLs (apoB-100) or chylomicrons (apoB-48). The rest are exchangeable apolipoproteins, including apoA, apoC and apoE, and they are considerably smaller, ranging from 7 kDa to 44 kDa. These can transfer between lipoproteins in plasma and go through conformational changes depending on the properties of associated lipoproteins [3]. The amphipathic nature of exchangeable apoliproteins can be traced to the existence of an amphipathic α-helix in their structure. The hydrophobic side of the helix interacts with lipids while the hydrophilic face is in contact with the aqueous environment and its ligands. The amphipathicity and structural flexibility of apolipoproteins is the basis for their ability to gather lipids and stabilize lipoproteins, bind to various receptors for guiding lipoprotein endocytosis or inhibit and activate enzymes in lipoprotein metabolism, all depending on the lipid composition of the underlying lipoprotein [1,3,5].

1.2 Metabolism of intestinal-derived lipoproteins

TRLs in blood circulation can originate from either the intestine or the liver. There is an important balance between triglycerides and its constituent free fatty acids (FFA). Bulky triglycerides cannot penetrate cell membranes and must be degraded into FFAs for uptake. At the same time, FFAs are formed into triglycerides for transportation and storage in lipid droplets because of their tendency to form micelles that can disrupt cell membranes. As such, the journey of dietary triglycerides starts with the hydrolysis of dietary fat by gastric lipase and lingual lipase in the stomach, accounting for 10–30% of all absorbed fats [6]. Following this, the majority of triglycerides are hydrolyzed in the

small intestine by pancreatic lipase (PL) (**Figure 1**). Prior to the action of PL, the gall bladder or liver release bile acids (BAs), which act as detergents that emulsify fats and therefore increase the available substrate surface for PL. The emulsified fat particles are joined by another crucial component, a cofactor of PL called colipase, which facilitates binding of PL to the lipid surface and stabilizes the active conformation of PL [7]. Dietary fat also contains phospholipids, sterols, and fat-soluble vitamins. The hydrolysis of phospholipids is catalyzed by phospholipases that are secreted by the pancreas after meal consumption. The released FFAs and lysophosphatidylcholine are then incorporated into micelles upon interacting with bile salts. The dominant dietary sterol is cholesterol, which mostly exists in free form and can be packed into bile acid micelles. About 10–15% of cholesterol is present as cholesteryl esters; these need to be hydrolyzed by cholesterol esterase to release free cholesterol for packing [8]. The micelles containing lipolysis products are taken up by endothelial cells that line the small intestine (enterocytes) via various membrane proteins [6,8]. The absorbed fatty acids are reassembled as triglycerides and joined with apoB-48 in the ER to produce nascent chylomicrons whose size is influenced by the composition of dietary lipids [6]. The size of a chylomicron can play an important role because LPL has a higher affinity towards larger chylomicrons [9]. Cholesteryl esters, phospholipids, fat-soluble vitamins, and apoA5 are also added during assembly in the ER [8]. Additionally, apoA-I is attached as chylomicrons traverse the Golgi [10]. This process of chylomicron production and secretion is dependent on an ER luminal microsomal triglyceride transport protein (MTP) which transfers lipids to apoB and assists its folding [11]. Nascent chylomicrons loaded with lipids are secreted from the cells, transported through the thoracic duct, and distributed into the bloodstream via the liver [2]. Once in circulation, maturation of chylomicrons occurs via accusation of various apolipoproteins (e.g., apoC-I, apoC-II, apoC-III, apoE, apoA5) from HDL particles [8]. Interestingly, not all chylomicrons have to be assembled from freshly digested material. Studies in humans have demonstrated intestinal lipid retention after a fat-rich meal, where triglycerides from a previous meal can appear in new chylomicrons produced after a second meal [12]. Chylomicron production and secretion is also triggered in the cephalic phase. Research with human subjects has shown that oral fat tasting after a previous fat-containing meal leads to elevated triglycerides already in as early as 30 minutes. Oral glucose has been shown to play a similar role in mobilizing intestinal lipid reserves [12].

The triglyceride content of mature chylomicrons is rapidly hydrolyzed by LPL that is anchored to glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) on the luminal surface of capillary endothelial cells of heart, skeletal muscle, and adipose tissue. Although there are competing VLDLs in concurrent circulation, it has been demonstrated that LPL prefers chylomicrons over VLDLs [9]. LPL activity is tightly regulated post-translationally in a tissue specific manner through activation [13] (apoC-II) or inhibition [14] (angiopoietin-like proteins 3,4,8), which ensures that fatty acids reach the location where they are currently needed the most. The fatty acids released during lipolysis can be bound by albumin, which contains at least seven binding sites that differ in affinity [15]. The bound or unbound FFAs must then cross the endothelial barrier to be taken up by underlying parenchymal cells. Two possibilities have been proposed for the cellular uptake of fatty acids: FFAs could enter cells using membrane receptors or through a non-specific pathway (e.g., passive diffusion referred to as “flip-flop”) [16]. Recent evidence suggests that both modes of transport can occur, depending on the local concentration of FFAs at the endothelial

surface. During chylomicron lipolysis, when the FFA concentration is high, paracellular movement between endothelial cells is thought to happen. At lower FFA concentrations without chylomicrons, a scavenger receptor named cluster of differentiation 36 (CD36) has been shown to play an important role in parenchymal cell FFA uptake in CD36-knockout mice and CD36 deficient individuals [16]. Exchangeable apolipoproteins (apoCs, apoA) from chylomicrons are transferred back to circulating HDL particles as the triglyceride core is depleted and the formation of cholesterol-rich remnant chylomicrons occurs. Some triglycerides are also redistributed from chylomicrons to HDL by cholesteryl ester transfer protein (CETP), which circulates in plasma, mainly on HDL. CETP exchanges cholesteryl esters, triglycerides, and a small number of phospholipids between HDL and other lipoprotein classes [17]. Chylomicron remnants can also be subjected to further lipolysis by hepatic lipase [18]. Finally, chylomicron remnants are cleared from circulation in the liver through endocytosis via the LDL-receptor (LDLR) and LDL receptor-related protein (LRP), or by binding to heparan sulfate proteoglycans (HSPGs) [19,20]. Lipoproteins are shuttled to lysosomes for degradation, releasing FFAs, cholesterol, glycerol and amino acids, and the liberated receptors are recycled to the cell surface [21]. An LPL-independent pathway for chylomicron clearance has also been proposed where TG-rich particles are internalized by endothelial cells through scavenger receptor class B type 1 (SR-B1) and degraded within lysosomes [16]. The majority of chylomicrons are cleared from plasma within three to four hours after eating [22].

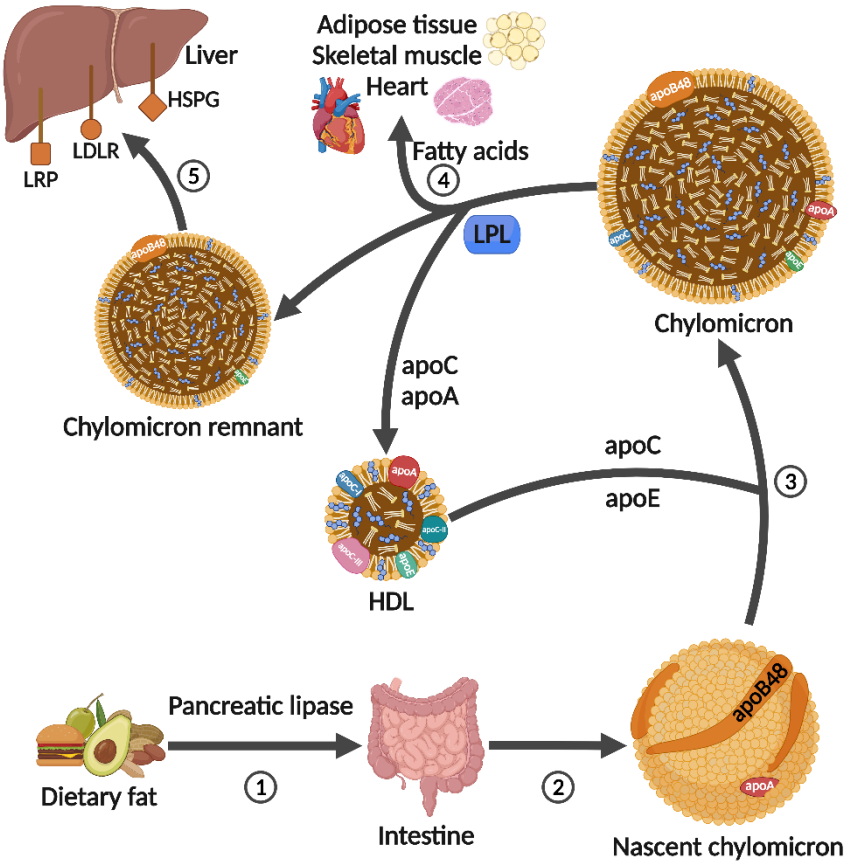

Figure 1. *Metabolism of dietary triglycerides in chylomicrons. (1) The majority of dietary triglycerides are digested by PL in the intestine. (2) Nascent chylomicrons are produced in enterocytes, loaded with dietary fat, and secreted into blood circulation through the thoracic duct. (3) Chylomicrons mature by acquiring apoCs and apoE from circulating HDLs. (4) The triglyceride content of mature chylomicrons can be hydrolyzed by endothelial-bound LPL in adipose tissue, skeletal muscle, and heart. The released FFAs and monoglycerides are taken up by the same underlying cells. Exchangeable apolipoproteins (apoCs, apoA) transfer back to HDLs during lipolysis. (5) Chylomicron remnants are cleared by the liver through apoE interactions with various receptors and degraded in lysosomes of hepatocytes.*

1.3 Endogenous lipoprotein metabolism

Lipid influx must be constant to ensure proper bodily function, which means TRL production has to occur independently from fasting and feeding cycles throughout the day. Specifically, VLDL is produced in the liver from endogenous triglycerides assembled from FFA sources such as lipids stored in adipose tissue, hepatic *de novo* lipogenesis or hepatic lipolysis of absorbed circulating lipoproteins [11]. Nevertheless, VLDL production and secretion can be increased in the fed state as the triglyceride content in the liver increases. Dietary triglycerides are already incorporated into VLDL during the early stages

in the postprandial period as shown with lipoproteins isolated from human subjects given a standardized meal [23]. Furthermore, the type of dietary fat content in chylomicron remnants delivered to the liver was shown to influence VLDL secretion [24]. Resulting elevated triglyceride secretion can be attributed to an increase in either VLDL particle size or particle number [11,25]. At the same time, insulin inhibits the production of VLDL, likely by decreasing FFA influx to the liver through reducing adipose tissue lipolysis [26].

Figure 2 outlines the VLDL metabolism pathway from creation to absorption. Nascent VLDL assembly starts with the co-translational translocation of a single apoB-100, the full-length variant of apoB. Similar to chylomicron production with apoB-48, MTP facilitates the transfer of lipids and folding of apoB-100 in the ER. This process creates a primordial VLDL particle that then fuses with cytosolic triglyceride-rich particles [27]. Insufficiently lipidated apoB-100 is directed to intracellular degradation, which ensures low VLDL secretion when FFAs are scarce [11,26,27]. Nascent VLDLs acquire additional apolipoproteins (apoCs, apoE, and apoA5) from circulating HDLs upon secretion from hepatocytes. The triglyceride core of mature VLDLs undergoes similar LPL-mediated lipolysis to chylomicrons at the vascular endothelium of adipose tissue, skeletal muscle, and heart. Likewise, there is also a lipid exchange mediated by CETP between VLDLs and HDLs, where cholesteryl esters are transferred to VLDLs, and triglycerides are received by HDLs [17]. The formed triglyceride-poor VLDL remnant, named IDL, can be taken up by the liver or be further hydrolyzed into cholesterol and cholesterol-ester rich LDL by hepatic lipase [18]. The binding affinity towards exchangeable apolipoproteins is decreased as VLDL is converted to IDL and LDL, with the latter containing only apoB-100 [19], which considerably reduces the efficiency of LPL-mediated lipolysis. IDL is cleared from circulation through its apoE interaction with the LDL receptor family [11]. Meanwhile, removal of LDL is mediated by apoB-100 interaction with LDLR, which happens mainly in the liver but can occur in all extrahepatic tissue [11,21]. This takes place at a considerably slower pace compared to IDL (hours vs minutes), which contributes to the atherogenic effect of LDL [19]. Finally, internalized lipoproteins are degraded into their basic constituents in lysosomes [21].

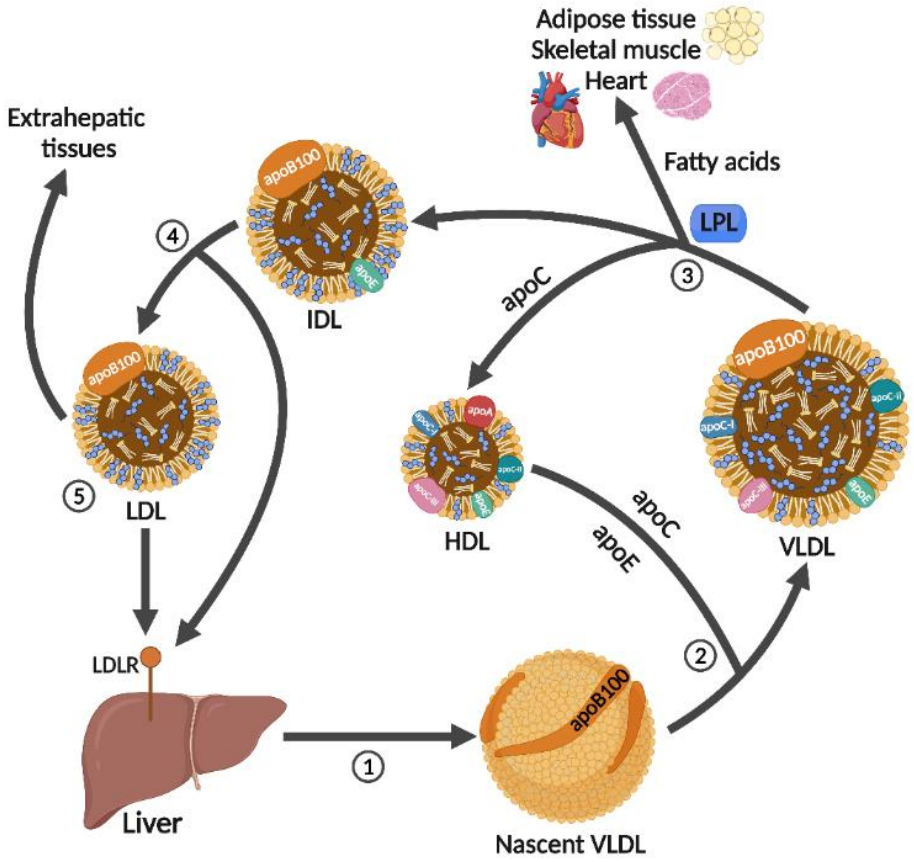

Figure 2. *Metabolism of endogenous triglycerides in VLDL. (1) Nascent VLDLs are synthesized in hepatocytes and secreted into the bloodstream. (2) Exchangeable apolipoproteins (apoCs, apoE) from circulating HDLs are transferred to secreted VLDLs. (3) The triglyceride core of mature VLDL is acted upon by endothelial-bound LPL in adipose tissue, skeletal muscle, and heart. Fatty acids released during lipolysis are taken up by underlying cells and apoCs move back to HDL. (4) VLDLs are transformed into smaller IDLs during lipolysis, which can be taken up by the liver via interactions with LDLR or they can be further degraded into cholesterol-rich LDLs by hepatic lipase. (5) LDL is finally internalized through LDLR in the liver or extrahepatic tissues.*

1.4 Lipoprotein lipase

LPL is a crucial enzyme in lipid metabolism which mediates the clearance and partitioning of plasma triglycerides [13]. LPL is anchored to the vascular endothelium of adipose tissue, heart or skeletal muscle and acts on circulating triglyceride-rich chylomicrons and VLDLs by catalyzing the hydrolysis of their core triglycerides. Lack of LPL activity leads to accumulation of these lipoproteins and results in chylomicronemia, a condition described by severe fasting hypertriglyceridemia (HTG) and an increased risk of life-threatening pancreatitis [22,28]. Reduced LPL activity, commonly originating from a polygenic background with heterozygous mutations, is associated with a significantly elevated risk of ASCVD [29,30]. Multiple regulators and factors in the rate-limiting step of TRL clearance

by LPL have been identified as potential targets for emerging pharmaceutical therapies, making the LPL system a lucrative approach for combating HTG [31].

The maturation and action of LPL on the endothelial surface is outlined in **Figure 3**. LPL is expressed in parenchymal cells[1] of lipolytic tissues where the nascent protein is N-glycosylated and reaches maturation in the ER, a process critically supported by the transmembrane proteins lipase maturation factor 1 (LMF1) [32] and sel-1 suppressor of Lin-12-like 1 (SEL1L) [33]. LPL is misfolded and degraded without LMF1 or SEL1L and loss-of-function mutations in the LMF1 gene manifest as severe HTG with associated phenotypes [32,34]. In addition to the aforementioned chaperones, multiple protein disulfide isomerases are needed for proper disulfide bond formation. Also, Ca^{2+} ions have been proposed to be necessary for the formation of catalytically active LPL in the ER[35]. LPL is further modified in the Golgi apparatus and undergoes glycan trimming by mannosidases, which is crucial for the secretion of LPL, albeit not for its enzymatic activity [36]. After movement to the trans-Golgi network (TGN), mature LPL may form oligomers (**Figure 4**, top-left corner) [37] that can bind to the HSPG Syndecan-1 (SDC1) [38]. SDC1 stabilizes the LPL oligomer and directs it to co-secretion in sphingomyelin-rich secretory vesicles [37,38]. Secretion of LPL has recently been shown to be stimulated by treating cultured adipocytes with insulin or the Ca^{2+} ionophore, ionomycin [39].

Upon secretion to the interstitial space**,** LPL oligomers dissociate into monomeric or dimeric LPL, which then attaches to HSPGs at the cell surface or extracellular matrix [37]. The monomers or dimers are then transferred to the membrane protein GPIHBP1 associated with capillary endothelial cells. GPIHBP1 transports LPL from the abluminal side to the vascular surface of the endothelium where LPL can act on circulating VLDLs and chylomicrons [40]. LPL experiences directional movement from HSPGs surrounding parenchymal cells to those near endothelial cells even in the absence of GPIHBP1, likely through transient interactions with HSPGs that have varying degrees of sulfation [41] as the affinity between LPL and HSPG is known to depend on the latter's negative charge [42–44]. However, LPL cannot escape the interstitial space without the acidic domain of GPIHBP1, which was recently shown to prevent accumulation of interstitial LPL by blocking electrostatic interactions between LPL and abluminal HSPGs [45].

After LPL is translocated to the capillary lumen, multiple LPL-GPIHBP1 complexes [46] begin to act on circulating TRLs simultaneously to produce fatty acids and monoglycerides [13]. Transport of LPL and subsequent TRL binding has been proposed to occur in the empty spaces between the glycocalyx that covers vascular endothelial cells [47]. LPL catalyzes the hydrolysis of triglyceride ester bonds at sn-1 and sn-3 fatty acid positions [48,49]. The reaction produces fatty acids and monoglycerides, which are then taken up by the underlying parenchymal cells. Lipolysis is likely stopped through product inhibition — fatty acids accumulate at the LPL-lipoprotein interface, which signals that the tissue has reached saturation, and results in the dissociation of the complex [50,51]. While TRLs and their triglycerides are the preferred target of LPL action, it can also target phospholipids in other lipoproteins. LPL catalyzes the hydrolysis of phospholipid ester bonds in the sn-1 position, which forms lysophospholipids as products, albeit at only 5% of the rate of triglyceride hydrolysis [52]. The size and lipid content of lipoproteins is animportant factor for LPL activity [53], but LPL can also be influenced by numerous apolipoproteins that are carried by circulating TRLs, and which can inhibit (apoC-I, apoC-III) or activate (apoC-II, apoA5) LPL.

[1] Some LPL can be expressed in endothelial cells of cold-activated BAT, but its activity is not involved in lipoprotein clearance[318]

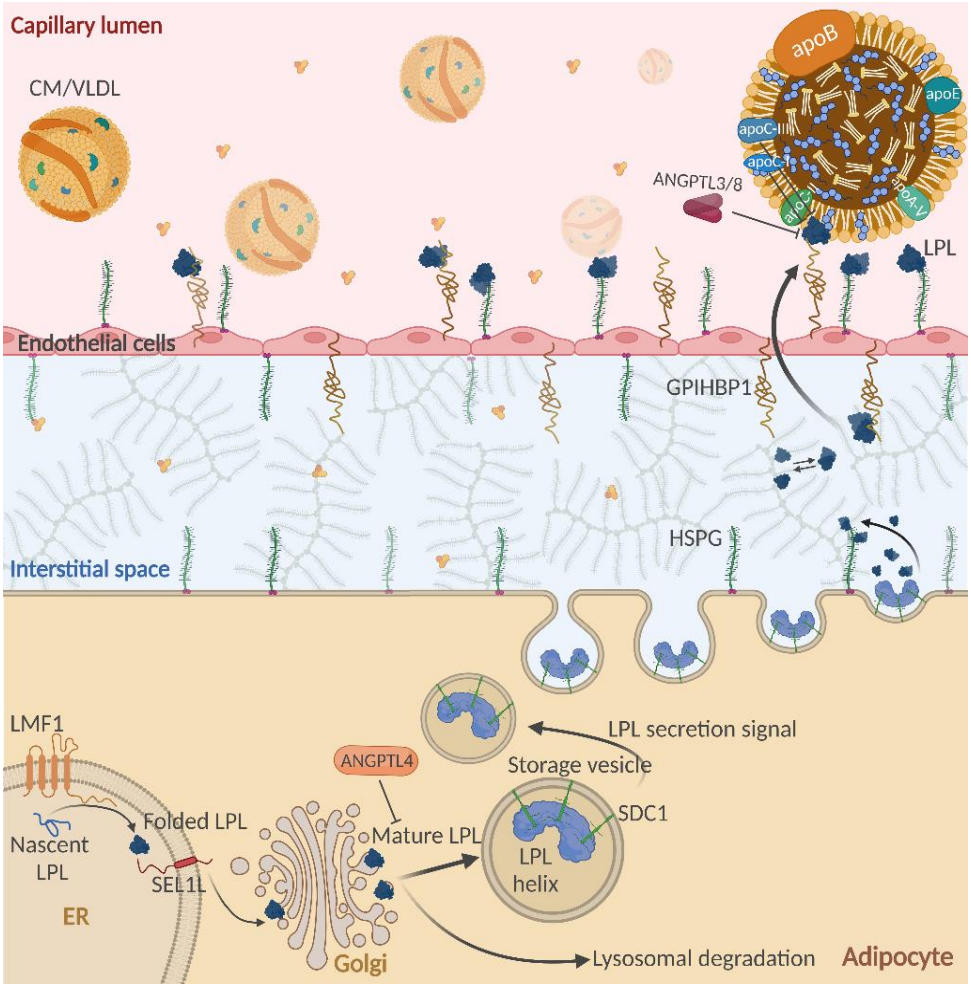

Figure 3. *LPL-mediated endothelial lipolysis. LPL is synthesized in the ER of parenchymal cells with the help of LMF1 and SEL1L. Matured LPL can form SDC1-stabilized helical oligomers after movement through the Golgi. These oligomers are packed into secretory vesicles that are released to the interstitial space upon signaling. Secreted LPL dissociates into monomeric or dimeric LPL, which attaches to HSPGs at the cell surface or extracellular matrix. LPL moves towards abluminal GPIHBP1, which transports the enzyme to the vascular surface of the endothelium. After transfer to the capillary lumen, multiple LPL-GPIHBP1 complexes start to act simultaneously on circulating TRLs. Lipoproteins contain multiple apolipoproteins, which activate (apoC-II and apoA5) or inhibit (apoC-I and apoC-III) LPL. There are also circulating ANGPTL3/8 complexes and ANGPTL4 that can inhibit LPL, although it has been suggested that binding of LPL to GPIHBP1 shields LPL from their effect. Additionally, ANGPTL4 can inactivate LPL in the subendothelial space and intracellularly. Finally, LPL-mediated hydrolysis of triglycerides produces fatty acids and monoglycerides, which are then taken up by the underlying parenchymal cells.*

Additionally, circulating ANGPTL3/8 complexes orANGPTL4 can also inhibit LPL in the capillary lumen. The expression and action of these regulators are finely tuned in response to the metabolic demand of the organism to ensure proper lipid homeostasis. LPL ligands and their effects are discussed more thoroughly in chapter 4.3 “Physiological regulators of lipoprotein lipase activity.” Lastly, some LPL remains associated with circulating lipoproteins after depletion of their triglyceride content. Bound LPL has been demonstrated to play two different roles in this case. LPL can participate in the hepatic uptake of remnant lipoproteins through interactions with various receptors [20]. It was also proposed that VLDL-bound LPL could be reactivated by arterial HSPGs, which triggers remodeling of the VLDL particle and its maturation into LDL [54].

The 55 kDa human LPL monomer spans 448 amino acid residues and belongs to the same serine hydrolase protein family as PL, endothelial lipase, and hepatic lipase [42]. The crystal structure of human LPL in complex with GPIHBP1 (**Figure 4**) was identically solved in 2019 by two independent groups [55,56]. LPL was shown to consist of two domains connected by a hinge region. The N-terminal domain (NTD) has an α/β-hydrolase fold with one antiparallel and seven parallel β-strands between five α-helices. The C-terminal domain (CTD) has eight antiparallel β-strands that form a β-sandwich region. The LPL NTD includes four disulfide bonds, while the LPL CTD has one. The serine protease-like catalytic triad (Ser^{159}, Asp^{183}, His^{268}) is found in the LPL NTD, together with an oxyanion hole (Trp^{82}, Leu^{160}), a bound Ca^{2+} ion and a lid region (residues 245-265) which moves to cover the active site or opens to reveal hydrophobic residues that facilitate binding to lipoproteins. Additionally, a recent work revealed a hydrophobic active site pore for the unidirectional movement of FFAs through LPL upon triglyceride hydrolysis [57]. The N-terminal domain has been implicated as the cause for the inherent instability of LPL by being unstable at body temperature [58,59]. The LPL CTD houses the binding site for the GPIHBP1 LU domain, and the Trp-rich loop which has been shown to be important for interacting with lipoproteins [60] or for the oligomerization of LPL [37]. Furthermore, a continuous basic batch with many lysine and arginine residues spans across the C-terminal LPL domain, hinge region and the neighboring region of the N-terminal domain. This surface-exposed charged region is responsible for the electrostatic interactions with negatively charged LPL ligands such as the intracellular SDC1 and subendothelial or vascular HSPGs and N-terminal GPIHBP1. LPL also has two glycosylation sites, Asn^{70} and Asn^{386} with the latter being essential for LPL activity [61]. Finally, while LPL was previously shown to form a heterodimer with GPIHBP1 [55,56], a recent work demonstrated a homodimer with a C-terminal to C-terminal interaction. The homodimer was shown to be catalytically active and hypothesized to remain bound to lipoproteins after dissociation from GPIHBP1, facilitating uptake of remnant lipoproteins via LRP [57].

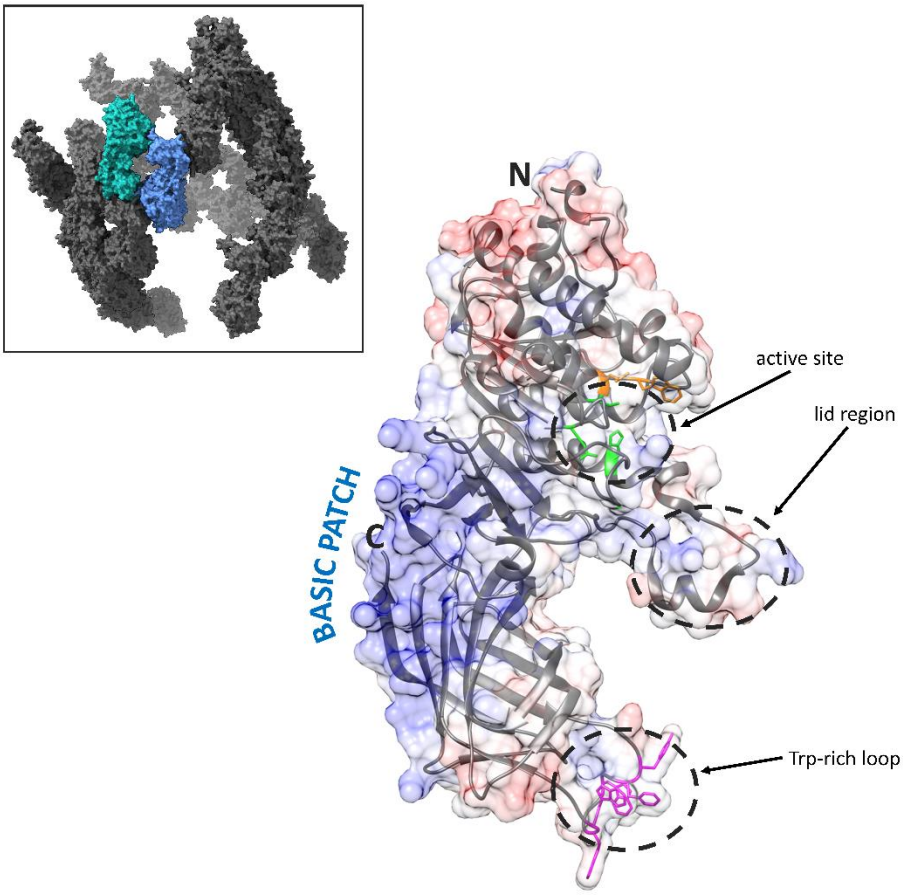

Figure 4. *Crystal structure of monomeric LPL* [55]. *The LPL NTD contains the catalytic triad (green), oxyanion hole (orange), Ca^{2+} ion (not depicted) and a lid region. The tryptophan rich lipid binding region (magenta) is located on the LPL CTD. The blue region indicates the positively charged basic patch. The lid is in an open conformation. A helical LPL oligomer is displayed in the top-left corner. Colored LPL units represent the dihedral LPL subunit of which the helix is comprised* [37].

1.5 Physiological regulators of lipoprotein lipase activity

LPL transcription is unaffected by fasting or feeding cycles which results in a necessity to control tissue specific LPL activity in a post-translational manner in response to physiological stimuli [42,62]. Additionally, LPL requires various cofactors and ligands for its transport, localization, and activity [37,63,64]. The most prominent regulators of LPL activity are the lipoprotein-bound apolipoproteins [65] and intracellular or circulating angiopoietin-like proteins [14]. The following chapters give a brief overview of major LPL influencers and their role in lipoprotein metabolism, as well as physiological consequences of various genetic disorders.

1.5.1 GPIHBP1

GPIHBP1 is a 25 kDa glycoprotein and an indispensable partner of LPL, responsible for shuttling LPL from the subendothelial space to the capillary lumen. The metabolic role of GPIHBP1 became clear when the first GPIHBP1-deficient mice were shown to exhibit severe chylomicronemia with serum triglyceride levels comparable to a lack of LPL [66]. The cause is now known — LPL remains trapped in the interstitial space without GPIHBP1 and is unable to fulfill its role on the luminal side [45,67]. The movement of GPIHBP1 has been shown to be bidirectional, GPIHBP1 transports LPL from the abluminal side to luminal surface and can return to shuttle more LPL from the subendothelial space [68]. GPIHBP1 is also required for the margination of TRLs along capillaries [47], and for increasing the thermal stability of LPL, as well as suppressing ANGPTL4-mediated LPL inactivation [59].

GPIHBP1 is found in the capillaries of all peripheral tissues, but more importantly, it is expressed in capillary endothelial cells of the same tissues (heart, skeletal, adipose) as LPL [40]. Interestingly, GPIHBP1 is present only in mammals although LPL can be found in all animals [69]. The structure of GPIHBP1 includes an intrinsically disordered N-terminal acidic domain with a long stretch of aspartate or glutamate residues, a cysteine-rich three-fingered fold (LU domain) with 3-5 disulfide bonds, which is finally followed by a C-terminal GPI membrane anchor [55,56]. While the LU domain does not differ much between species, the N-terminal domain can be quite varied in length. For example, the human N-terminal domain of GPIHBP1 contains 21 negatively charged residues while the mouse or opossum respectively have 17 or 32 acidic residues [40]. The N-terminal domain also contains a conserved O-sulfated tyrosine, which is important for increasing the affinity of the GPIHBP1-LPL complex [41]. Additionally, there are two glycosylation sites, Asn^{78} and Asn^{82}; the former is required for intracellular trafficking, as GPIHBP1 is otherwise retained in the ER [70], and the latter is only glycosylated when Asn^{78} has been mutated [55]. The crystal structures of the GPIHBP1-LPL complexes [55,56] revealed that the interaction between GPIHBP1 and LPL is largely hydrophobic with a large surface area, as the LU domain was bound to the LPL CTD in the solved structure. Although they were not able to elucidate the binding location of the N-terminal acidic domain, it is likely interacting with the basic patch of LPL via electrostatic forces as this LPL region is responsible for interacting with polyanionic HSPGs. This is supported by the notion that heparin competes with GPIHBP1 for binding to LPL [41,71]. LPL is also known to be quickly released from the endothelial surface into the bloodstream by intravenous admission of heparin [72]. SPR studies have also demonstrated that only LPL attached to N-terminal GPIHBP1 can bind to lipoproteins [71].

The importance of GPIHBP1 is supported by genetic studies in patients with severe HTG and familial chylomicronemia syndrome (FCS), where the LPL levels in pre- and post-heparin plasma are extremely low[69]. Many different mutations in the GPIHBP1 gene have been found, most of which disrupt the LPL-GPIHBP1 LU domain interaction [73–75]. Although, severe HTG manifests in the case of homozygous mutations but not heterozygous [76]. Most identified mutations involve one of the conserved cysteines in the LU domain [69] and to date no mutations in the N-terminal GPIHBP1 domain have been identified that cause FCS [40]. There are also known cases of GPIHBP1 autoantibodies, which bind to the LU domain and block interactions with LPL [69].

1.5.2 Heparan-sulfate proteoglycans

Heparan-sulfate proteoglycans are polyanionic glycosaminoglycans that are created in the Golgi from heparan sulfate chains covalently attached to a core protein. The chains are linear polysaccharides composed of repeating disaccharide units: N-acetylated or N-sulfated D-glucosamine joined with uronic acid [77]. HSPGs are highly heterogenous in structure and charge, capable of binding a host of different proteins. HSPGs are created in almost all animal cells, where they can be membrane-spanning, bound to cell surfaces, or secreted into the extracellular matrix [77].

HSPGs play an important role in triglyceride metabolism by binding lipases and remnant lipoproteins [42]. The HSPG SDC1 is expressed by hepatocytes and has been shown to be the major proteoglycan receptor for the clearance of lipoprotein remnants [78]. LPL interacts with HSPGs via its basic patch at multiple locations: inside parenchymal cells (SDC1-coated vesicles) [39], in the subendothelial space (transport of LPL to GPIHBP1) [63], and on the luminal surface of endothelial cells (TRL lipolysis) [43,54]. LPL has a very high affinity for HSPGs and heparin (K_D in the low nanomolar range), and the electrostatic interaction is characterized by fast association and dissociation values [43]. The interstitial space and endothelial surface are densely populated with HSPGs and such it was suggested that LPL is at a dynamic equilibrium, where a rapid exchange of LPL molecules between HSPGs occurs [43]. LPL activity is closely correlated to its ability to bind HSPGs and heparin. Inactive, conformationally altered LPL has lower affinity towards heparin, which is the basis of LPL purification by heparin-Sepharose chromatography [79].

Heparin is a smaller highly sulfated form of heparan sulfate synthesized in connective tissue-type mast cells [80]. Fractionated heparin, enriched in fully sulfated chains, is widely used by pharmaceutical companies for its anticoagulant abilities. Its high negative charge and availability are also extremely useful for studying HSPG-binding proteins such as LPL [80]. Similar to GPIHBP1-bound LPL [81], binding of LPL to heparin increases the thermostability of the enzyme [43]. Additionally, intravenous injection of heparin releases LPL from the vascular endothelium to blood circulation as the high negative charge of heparin competes with cell surface GPIHBP1 or HSPGs [42]. This approach is used to draw so-called “post-heparin” blood from patients to identify possible LPL-deficiencies [82].

1.5.3 Apolipoproteins C-I and C-III

ApoC-I and apoC-III are expressed primarily in the liver as secretory proteins with respective molecular weights of 6.6 kDa and 8.8 kDa after signal peptide cleavage [83]. The proteins rapidly associate with circulating lipoproteins after secretion and equilibrate between lipoprotein subclasses, preferentially being associated with TRLs in the fed state and HDLs in the fasted state [83]. Surprisingly, radiolabeling experiments

have demonstrated that there is a 30–60% fraction of total apoC-III that does not exchange between VLDLs and HDLs [84]. The concentrations of apoC-I and apoC-III in plasma of normolipidemic individuals are respectively 6–12 μM and 6–20 μM [85].

Loss-of-function mutations in apoC-III are well documented to be correlated with lower levels of plasma triglycerides and reduced risk of cardiovascular disease (CVD) [86,87]. Inversely, heightened concentration of circulating apoC-III is a widely studied cause of HTG and a potential therapeutic target for reducing triglycerides [88,89]. Results with apoC-I mutations are less conclusive, studies with transgenic mice have demonstrated HTG both in apoC-I overexpressing and deficient models [90]. Furthermore, no known mutations for apoC-I have been discovered in humans which would result in increased or reduced plasma triglyceride levels [62].

ApoC-I and apoC-III do not inhibit LPL directly but rather block binding of LPL to the lipoprotein surface [91] or increase the residence time of TRLs and remnants in circulation. ApoC-I has been shown to interrupt apoE and apoB-mediated receptor interactions, which blocks lipoprotein binding to LDLR or LRP and disrupts remnant lipoprotein endocytosis [92–94]. While apoC-III does not influence interactions with LRP [94], it has a major inhibitory effect on apoB-mediated binding of lipoproteins to LDLR [95]. The LPL-independent pathway for lowering plasma triglyceride by apoC-III inhibition has been clinically demonstrated in LPL-deficient patients who were administered an apoC-III antisense oligonucleotide (ASO) [96]. Regarding lipolysis, a more recent study has demonstrated that GPIHBP1-bound LPL is capable of binding apoC-III enriched TRLs but is blocked from accessing core triglycerides for hydrolysis [97]. Additionally, apoC-III may also promote VLDL production and secretion [98]. ApoC-I and apoC-III might also influence other proteins involved in lipoprotein metabolism [62]. For example, apoC-I has been shown to inhibit CETP [90] and apoC-III to inhibit hepatic lipase [99].

1.5.4 Apolipoprotein C-II

ApoC-II is a lipoprotein-bound cofactor of LPL that is necessary for lipolysis to occur [64]. Genetic studies have demonstrated that individuals lacking apoC-II experience severe HTG and accompanying risks, similarly to LPL deficiency [83,100]. Patients have highly elevated levels of TRLs and small, dense atherogenic LDLs, also reduced HDLs [100]. As with most causes of monogenic HTG, bi-allelic apoC-II deficiency is scarce, with an estimate of 1.5 individuals per 100 000 or even less [101]. Interestingly, overexpression of apoC-II has been also shown to be detrimental in transgenic mice as it causes HTG, likely because of reduced LPL activity or impeded access of LPL to lipoproteins [102]. Excess apoC-II has been also shown to impair apoE and apoB-mediated receptor interactions and could therefore reduce TRL catabolism [100]. HDL maturation is also hindered because of disruptions in reverse cholesterol transport [100].

ApoC-II is mainly expressed in and secreted from the liver, but it is also synthesized in other tissues such as the intestine, where it likely influences LPL on a local scale [101]. Additionally, apoC-II is produced in macrophages, where it may promote triglyceride uptake [101]. ApoC-II circulates on lipoproteins and is exchanged between TRLs and HDLs. ApoC-II desorbs from TRLs in response to an increase in surface pressure when the triglyceride core of TRLs is depleted and moves to HDLs which act as a reservoir for apoC-II [5]. The half-life of apoC-II in normolipidemic plasma is about 26 h, which means that apoC-II can be recycled multiple times to be used when new TRLs enter the bloodstream [103]. The concentration of apoC-II in normolipidemic plasma ranges from 2 to 7 μM (1.8–6.2 mg/dl) [85] but it can be significantly elevated in the case of HTG [100].

The mature full-length apoC-II is 79 amino acids long after cleavage of its 22 amino acid signal peptide, with a molecular weight of 8916 Da and does not undergo any significant glycosylation [104]. As with other exchangeable apolipoproteins, apoC-II reversibly binds circulating lipoproteins at the phospholipid interface. This triggers a change in apoC-II's secondary structure where the apolipoprotein becomes more ordered and develops α-helical structures [105,106]. The major lipid binding region of apoC-II is an amphipathic α-helix located on the N-terminal half of apoC-II (residues 13–39). The C-terminal end of apoC-II (residues 63–76) is responsible for activating LPL and guiding substrates to the LPL active site [107]. Notably, residues Tyr^{63}, Ile^{66}, Asp^{69}, Gln^{70}, which are conserved among various animal species, and all reside on the same face of the C-terminal helix, have been shown to be crucial for this function [108]. Additionally, Leu^{72} has been shown to be important for activating LPL. Mutations at this position, which break the α-helical structure of the C-terminus, result in HTG [109,110]. These two helices are connected by a central region (residues 40–65), which is more disordered but is predicted to form an amphipathic α-helix (residues 45-58) flanked by two connecting regions (residues 39–44 and 59–66) when interacting with LPL [105,107]. This region has been shown to bind LPL independent of an available lipid surface [111].

LPL can be active without apoC-II against short-chain triglycerides (e. g tributyrin [112]) but is only slightly active towards long-chain triglycerides [113] (e. g Intralipid) or inactive regarding physiological substrates such as chylomicrons [114]. While LPL activity against emulsions of long-chain triglycerides can be restored by the C-terminal helix of apoC-II alone, the full-length cofactor is required for lipoprotein lipolysis to occur [103]. This is not caused by a lack of binding as LPL binds lipoproteins even in the absence of apoC-II but rather LPL is unable to access the substrate and catalyze the hydrolysis of the triglyceride core [113,114]. The exact mechanism for LPL activation by apoC-II is unclear but a pressure-dependent model was proposed where the apoC-II C-terminal helix desorbs from the lipoprotein surface upon increase in surface pressure when lipolysis occurs [5]. This was thought to be important for promoting LPL-mediated triglyceride hydrolysis as apoC-II keeps LPL tethered at higher surface pressures [5]. Additionally, it has been proposed that apoC-II facilities entry of long-chain triglycerides into the LPL active site by orienting LPL on the lipid surface [107] and providing better access to the triglyceride core by triggering changes in the phospholipid bilayer [115].

1.5.5 Apolipoprotein E

ApoE has a molecular weight of 34.2 kDa after cleavage of its signal peptide and additionally to the liver, is also expressed in numerous extra-hepatic tissues [116]. ApoE circulates bound to plasma TRLs and HDLs with a concentration of around 1–2 μM [65]. ApoE plays a crucial role in remnant lipoprotein clearance as it mediates interactions between lipoproteins and hepatic receptors [116]. ApoE can also mediate cholesterol efflux and has anti-inflammatory or anti-oxidative properties [65].

ApoE is comprised of multiple bundled helices — N-terminal region (residues 1–164) with four α-helices connected to a C-terminal region (residues 206–299) with three amphipathic α-helices via a hinge domain (residues 168–205) that has two helices [117]. The N-terminal domain interacts with receptors while the C-terminal domain is responsible for lipid binding [65]. Like other apolipoproteins, lipid binding triggers changes in the tertiary structure. ApoE adopts an active conformation upon binding to lipoproteins or HSPGs, which unmasks its N-terminal domain and enables interactions with LDLR and LRP [65,117].

ApoE exists in three isoforms with varying degrees of prevalence. The isoforms represent mutations at amino acid positions 112 and 158, and are titled apoE2, apoE3 and apoE4. The most common variation is apoE3 (specifically the E3/E3 genotype), with a cysteine at position 112 and arginine at 158 [65,116]. High concentration of serum apoE correlates with increased triglycerides, regardless of the isoform distribution [118]. Although, an important factor to consider is how apoE is distributed among various lipoprotein subclasses, as paradoxically, low levels of apoE on HDL result in increased severity of CVD [119] while high levels of apoE on VLDL limit lipolysis [120]. Additionally, exogenously added apoE3 and apoE4 have been shown to inhibit LPL in a dose-dependent manner in an assay with VLDL [120]. Beyond this, the apoE4 isoform is a risk factor for Alzheimer's disease due to increased neurodegenerative effects compared to other isoforms [121]. ApoE4 is also associated with increased LDL-C, Lp(a) and apoB concentrations [116]. ApoE2 has an opposite effect, where LDL and apoB concentrations in plasma are lowered, which is thought to stem from the lack of ability for apoE2 to interact with LDLR [122]. Finally, studies with apoE-knockout mice have shown that lack of hepatic apoE expression severely reduces VLDL secretion [11]. Overall, apoE seems to have an interesting duality to its nature when it comes to regulating plasma triglyceride levels — it is a needed ligand for TRL clearance but at the same time acts as a lipolysis inhibitor.

1.5.6 Apolipoprotein A5

ApoA5 is an exchangeable apolipoprotein, which circulates blood at low nanomolar concentrations as a 39 kDa monomer after cleavage of its signal peptide [123]. This is highly different from other apolipoproteins, which exist in the micromolar range. ApoA5 is expressed in hepatocytes and upregulated during fasting or downregulated in response to insulin [123]. Secreted apoA5 binds to circulating TRLs and HDLs (but not LDL) [124] or alternatively, interacts with the ANGPTL3/8 complex [123]. Additionally, apoA5 contains a positively charged domain that can interact with GPIHBP1 [125] and HSPGs or heparin [126,127]. A contrast to other lipoproteins is the relatively short half-life of apoA5, about 2 hours against 26 hours for apoC-II as an example [128].

Multiple studies with apoA5 overexpression or knock-out mice [129–132] and genome-wide association studies of apoA5 mutations in humans [133–136] have demonstrated that higher apoA5 concentrations correlate with lower circulating triglyceride levels and vice versa. It is quite surprising that apoA5 has such a strong effect on triglycerides despite its low concentration compared to other apolipoproteins. There is about one apoA5 per 24 VLDL particles [128] so it seems unlikely that apoA5 is only limited to a direct stimulation of LPL activity. A definite explanation for the anti-atherogenic effect of apoA5 has not been declared yet but multiple mechanisms have been proposed. One explanation is the association of lipoprotein-bound apoA5 with heparin and HSPGs, which might promote lipolysis by LPL or facilitate lipoprotein remnant uptake [126,137]. ApoA5 also interacts with members of the LDLR family, which could assist in receptor-mediated lipoprotein clearance [127]. Recently, a new function for apoA5 was found where apoA5 binds a known LPL inhibitory complex of ANGPTL3/8 and suppresses its influence [123]. A strong argument for this is the production of all three proteins in hepatocytes at similar concentrations, and the concurrent insulin-sensitive production of apoA5 and ANGPTL8. Finally, apoA5 might also play an intracellular role in hepatocytes by interacting with hepatic lipid droplets and thereby withholding triglyceride secretion [128].

1.5.7 Angiopoietin-like proteins 3, 4 and 8

Angiopoietin-like proteins 3, 4 and 8 (ANGPTL3,4,8) are a group of physiological LPL antagonists involved in fatty-acid partitioning during fasting or feeding. ANGPTLs enable tissue-specific LPL inhibition in response to the metabolic demand of the organism. LPL activity is downregulated by ANGPTL4 in adipose tissue during fasting so triglycerides can be used for energy in skeletal muscle. The situation is flipped in the postprandial state where LPL activity is reduced in skeletal muscle by ANGPTL3/8 to enable lipid storage in white adipose tissue (WAT). [138]

ANGPTLs consist of a coiled-coil N-terminal domain (CCD), which contains the LPL binding site, and a fibrinogen-like C-terminal domain (FLD) separated by a furin-cleavage site [139]. ANGPTL8 is an exception and lacks the C-terminal FLD [140]. ANGPTLs circulate the bloodstream at ng/ml concentrations as either full-length or truncated variants [14,141]. Loss-of-function mutations in the N-terminal domain, such as the ANGPTL4 E40K variant, have been shown to be associated with lower levels of circulating triglycerides and a reduced risk of coronary artery disease [142–145]. The effect of ANGPTLs is further demonstrated by animal studies that show reduced levels of circulating plasma triglycerides after treatment with ANGPTL3 [146] and ANGPTL4 [142] monoclonal antibodies or deletion of the ANGPTL3, 4 or 8 gene [147–149]. In addition to impeding LPL, there is also evidence of ANGPTL3 and 4 lowering triglyceride levels through inhibition of hepatic lipase [150–152] and of ANGPTL3 regulating HDL cholesterol by suppressing endothelial lipase [153]. The C-terminal FLD is not required for LPL inhibition but the FLD of ANGPTL4 has been shown to stimulate lipolysis in adipose tissue [154] and the FLD of ANGPTL3 is possibly involved in inflammation and atherosclerotic lesion formation [155].

ANGPTL4 is a fasting- [138] and fatty acid-induced [156–158] 50 kDa secretory protein discovered simultaneously in 2000 by three independent groups [159–161]. ANGPTL4 is expressed in many tissues and cells, including white and brown adipose tissue, liver, kidney, heart, skeletal muscle, macrophages, and cancer cells [162]. Adipocytes primarily secrete full-length ANGPTL4 while the N- and C-terminal domains cleaved by proprotein convertases are released from hepatocytes [163]. FLD-ANGPTL4 makes up for the majority of circulating ANGPTL4 with a minute proportion of full-length or CCD-ANGPTL4 being available, although most of the latter is in complex with ANGPTL8 [164]. Hydrogen-deuterium exchange mass spectrometry (HDX-MS) experiments have pinpointed that ANGPTL4 binds to LPL near the active site at the lid domain and a nearby α-helix [165]. The higher order structure of CCD-ANGPTL4 was recently revealed to be an elongated, flexible trimer [166], previous studies have also reported the formation of dimers, tetramers, and hexamers [167,168]. The function of this oligomerization is not entirely clear, as mutations that disrupt oligomerization show severely reduced capacity for LPL inhibition [169], but at the same time, monomeric ANGPTL4 with reduced and alkylated disulfide-bonds is still capable of inhibiting LPL [170]. Production of ANGPTL4 is upregulated in adipocytes during fasting to direct energy-rich fatty acids from circulating TRLs to skeletal muscle and the heart. Inversely, ANGPTL4 expression is reduced in the fed state to increase LPL activity in the adipose tissue and enable storage of triglyceride-derived fatty acids [140]. The role of ANGPTL4 in skeletal muscle is likely attributed to partitioning fatty acids between active and non-active muscles [171]. Additionally, ANGPTL4 is downregulated in brown adipose tissue (BAT) and upregulated in WAT during sustained cold exposure to increase LPL activity and re-route fatty acids to BAT [172]. Genetic studies have shown that whole

body overexpression of ANGPTL4 in the fasted state leads to increased levels of circulating triglycerides, reduced TLR clearance and triglyceride uptake into adipose tissue [173,174]. Accompanying ANGPTL4 inactivation studies have demonstrated an increase in adipose tissue LPL activity, enhanced TRL clearance and triglyceride uptake [175]. Although ANGPTL4 is thought to regulate LPL activity in adipocytes, liver-specific overexpression of ANGPTL4 has been shown to be accompanied by reduced post-heparin LPL activity and increased levels of circulating triglycerides. Furthermore, intravenous injections and in vitro studies with recombinant ANGPTL4 show reduced mouse and human LPL activity. It is therefore possible that the effect of ANGPTL4 extends beyond its autocrine function [176,177].

ANGPTL3 is a 52 kDa secreted glycoprotein mainly produced in the liver [178] and unlike ANGPTL4, its expression is largely independent of the nutritional state [179]. CCD-ANGPTL3 forms trimers similarly to CCD-ANGPTL4 but can also appear as a hexamer that does not interconvert [166]. Also contrary to ANGPTL4, truncated ANGPTL3 is less effective at inhibiting LPL than the full-length version [180]. ANGPTL8, a 22 kDa key partner of ANGPTL3, is also expressed in the liver, where it forms a complex inside the hepatocyte with ANGPTL3, possibly through the CCD region of ANGPTL3 [180]. Recent HDXMS investigations revealed that this complex formation unmasks a unique ANGPTL3/8 LPL binding epitope, which consists of N-terminal regions from both ANGPTLs [181]. Complex formation between the two proteins requires co-expression or co-refolding after denaturing, indicating that the interaction likely occurs within the cell and then ANGPTL3 and 8 are secreted together. Furthermore, the majority of ANGPTL3 that is released without ANGPTL8 from hepatocytes to the bloodstream is in an uncomplexed form [164]. ANGPTL3 has some inhibitory activity towards LPL alone at supraphysiological concentrations but inhibition is greatly enhanced in the presence of ANGPTL8 [164,180,182]. Meanwhile, ANGPTL8 does not inhibit LPL by itself and requires ANGPTL3 for successful expression and secretion [180,183]. Interestingly, ANGPTL3 can be inactive towards LPL but still form a functioning ANGPTL3/8 complex, given that the inhibitory motif of ANGPTL8 is unhindered [183]. It was recently shown that the lower inhibitory effect of ANGPTL3 alone might be attributed to a DNA contaminant which co-purifies with recombinant CCD-ANGPTL3. LPL inhibition by CCD-ANGPTL3 was increased after removal of the DNA contaminant, making the protein a potent inhibitor even without ANGPTL8. It was speculated that ANGPTL8 acts as an activator which clears potential obstructions from ANGPTL3 through structural rearrangements [166]. Expression of ANGPTL8 is upregulated by nearly 60-fold [140] in the postprandial state to increase LPL inhibition in skeletal muscle and heart by circulating ANGPTL3/8 [184]. LPL activity is simultaneously increased in adipose tissue, where locally produced ANGPTL8 forms a complex with ANGPTL4, acting as an ANGPTL4 antagonist and reducing its inhibitory effect on LPL [182,185]. Furthermore, the ANGPTL4/8 complex might offer protection from circulating ANGPTL3/8 complexes [164] or promote intracellular degradation of ANGPTL4 in adipocytes [185]. It has also been recently proposed that the hepatocyte secreted apoA5 suppresses ANGPTL3/8 mediated inhibition of LPL [123]. ApoA5 binds to the same ANGPTL3/8 region as LPL, suggesting a competitive protective mechanism [181]. ApoA5 secretion in hepatocytes is suppressed by insulin which could help direct fatty acids to adipose tissue in the postprandial state [123]. At the same time, insulin has also been shown to decrease ANGPTL4 and increase ANGPTL8 mRNA levels [138].

Multiple mechanisms of action have been proposed for ANGPTL-mediated inhibition or inactivation of LPL. In the interstitial space [186] or capillary lumen, ANGPTL3 and 4

may promote the conversion of catalytically active dimeric LPL to an inactive monomeric form which cannot convert back to dimers [187,188]. Intracellularly, unfolding of the LPL hydrolase domain by ANGPTL4 [170,189,190] in adipocytes triggers a cascade where LPL is cleaved by PCSK-3 and subsequently degraded [191]. Alternatively, reversible inhibition has been proposed instead where ANGPTL4 acts as a noncompetitive inhibitor that binds close to the catalytic site of LPL, reducing its catalytic activity until dissociation of ANGPTL4 [166,192]. Furthermore, inactivation of LPL by ANGPTL3 or 4 abolishes binding to GPIHBP1 but meanwhile, LPL that is already bound to GPIHBP1 is shielded from ANGPTL inhibition [180,193,194]. Overall, these mechanisms are not necessarily mutually exclusive, the function of ANGPTLs in the presence of lipoproteins might differ from interstitial or cellular inhibition.

1.6 Pancreatic lipase

Pancreatic lipase (PL) belongs to the same family of fat-digesting enzymes as LPL, but targets emulsified dietary triglycerides for hydrolysis instead of triglycerides in lipoproteins [195]. As with other digestive enzymes, PL is produced by pancreatic acinar cells to be included in pancreatic juice [195] and its secretion to the duodenum is triggered by the peptide hormone cholecystokinin [6]. The release of cholecystokinin is itself stimulated by fat/fatty acids or proteins/amino acids reaching the duodenum and its concentration remains elevated for up to five hours [196]. Secretion of cholecystokinin is also accompanied by an induction of satiety [196]. The average production of pancreatic lipase in humans following a meal has been observed to be between 200–250 mg, depending on the meal [197]. Studies with rats and pigs have shown that PL expression might be affected by the saturation and amount of dietary fat [6]. As discussed earlier, dietary triglycerides cannot penetrate intestinal enterocytes and must be degraded into FFAs and monoglycerides for absorption. PL acts on these triglycerides with the support of its cofactor, colipase, which is required for the enzymatic activity of PL [7]. Mutations in the PL gene (e.g., [198,199]) characterized by PL deficiency result in increased fat content in stool (steatorrhea), causing fluid losses and nutrient malabsorption, which leads to weight loss, poor growth, and deficiencies of fat-soluble vitamins [200]. About 50–60% of dietary fats are not absorbed in the absence of PL [200]. At the same time, one of the hallmarks of obesity is increased body weight caused by excess fat stored in white adipose tissue because of net positive caloric intake. Inhibition of PL can tackle this cause by reducing the amount of absorbed dietary fat and therefore lowering energy consumption and subsequent storage [201]. Furthermore, reducing PL activity can be beneficial in cases of postprandial HTG and improve insulin sensitivity in obese insulin-resistant patients [202]. Normally, there is also a small amount of PL in blood serum (100–400 pM [203]). However, its level there is elevated in the case of acute pancreatitis where pancreatic secretion is blocked and the acinar cells leak PL into circulation [204]. PL concentration that exceeds three times the normal value is considered a clinical marker for the diagnosis of acute pancreatitis [204].

PL has been extensively studied and characterized as its crystal structure alone [205] or in complex with colipase [206] was already solved three decades ago. Glycosylated and secreted PL has a molecular weight of 46 kDa (449 amino acids) after cleavage of its 16 amino acid signal peptide [201]. The overall tertiary structure of PL is similar to LPL and other lipolytic enzymes, defined by two distinct structural domains. A globular NTD (residues 1–335) with an α/β-hydrolase fold which contains a Ca^{2+}-binding site and the typical catalytic triad of serine hydrolases (Ser^{153}, Asp^{177}, His^{264}) [7,200]. The active site of

PL targets fatty acids in the sn-1 and sn-3 position, with the hydrolysis of triglycerides resulting in the release of sn-2 monoacylglycerol (MAG) and FFAs [6]. While acyl chain length does not play a role in substrate specificity, PL prefers saturated or monounsaturated substrate over polyunsaturated [207]. The CTD (residues 336–449) forms a β-barrel structure and is necessary for interacting with lipid particles and colipase [7,205]. The domains are stabilized by seven disulfide bonds and separated by an unstructured stretch of amino acids [200]. The catalytic site is covered by a lid region of 23 amino acids (loop between the disulfide of Cys^{238} and Cys^{262}), which moves when PL binds to a lipid-water interface and creates a secondary interaction site for colipase [7]. This creates the so-called "open conformation" of PL, while "closed conformation" refers to PL in an aqueous environment, where access to the catalytic site is blocked by the lid. Targeted mutations in the lid, which affect the open conformation of PL, have been demonstrated to affect substrate specificity and PL activity [208]. The open or closed lid is stabilized by various electrostatic interactions and is sensitive to changes in pH [209]. Human PL is exposed to a pH of 5.5–6.5 in the small intestine of healthy individuals to as low as pH 2.0–3.0 in some pathological cases [209]. This means that PL must be able to retain its conformation and activity at various pH levels. Studies of human PL at low pH showed that PL can bind to lipid-water interfaces between pH values of 4.0–6.5 and its activity can later be restored when determined at pH 7.5 [209].

PL acts on the lipid-water interface of bile-salt stabilized emulsion particles formed from dietary lipids. Bile salts block adsorption of PL to these micelles but this is overcome by the help of colipase and adoption of an open conformation by PL [210]. PL in an aqueous environment, where the closed conformation is preferred, is thought to be activated by a formation of a ternary complex with colipase and a micellar surface [211–213]. This complex maintains the open conformation of PL and enables adsorption to emulsified dietary triglycerides. Furthermore, the composition and size of the micelles might also be crucial. Micelles smaller than 13 Å fail to activate PL and mixed micelles have been observed to be more efficient activators of lipolysis [211].

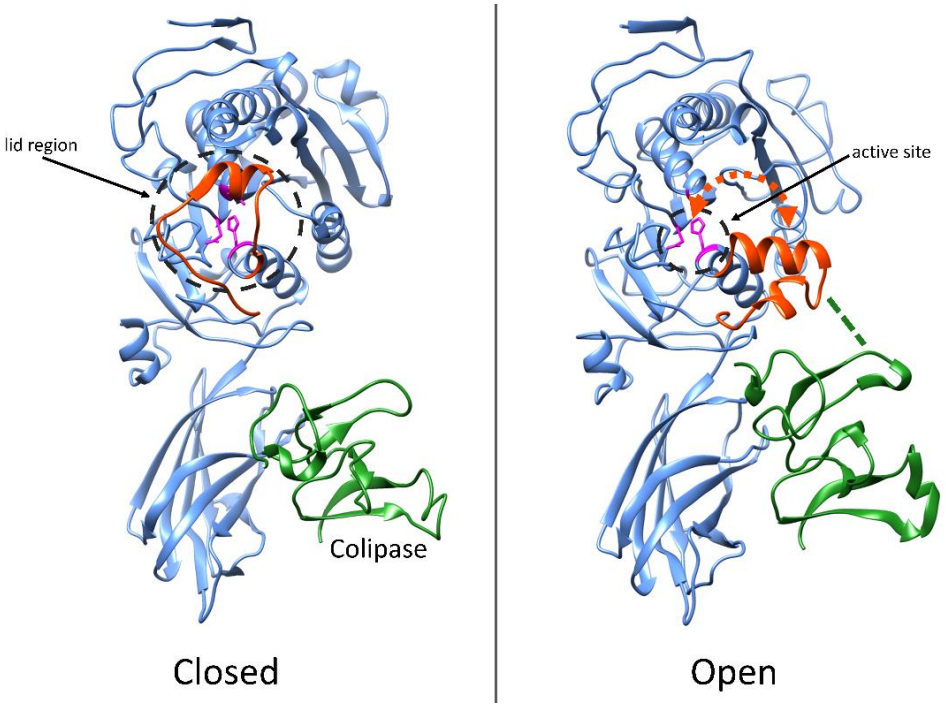

Figure 5. *Crystal structures of human PL in closed* [206] or *open conformation* [214] *with porcine colipase. The PL NTD contains the catalytic triad (magenta), Ca^{2+} ion (not depicted) and a lid region(orange). Colipase (green) is bound to the CTD of PL. Left panel — PL is in its closed conformation with the lid occluding the active site and colipase weakly bound to the CTD. Right panel — Binding to a lipid-water interface triggers conformational changes where the lid moves to uncover the substrate channel and catalytic triad. The new position of the lid enables colipase to interact with it, stabilizing the open conformation of PL.*

1.6.1 Colipase

Lipolysis of emulsified dietary triglycerides by PL is dependent on its cofactor colipase. The triglyceride core of lipid particles in the intestine is surrounded by bile salts, dietary proteins, and phospholipids which suppress PL adsorption [210]. Colipase helps PL overcome the inhibition conferred by these components while additionally anchoring the lipase and stabilizing its open conformation [195]. Genetic colipase deficiency leads to nutrient malabsorption and steatorrhea [215]. Colipase is not needed under non-physiological conditions when the substrate is comprised of only triglycerides or other non-polar substrates [210] (e.g., p-nitrophenyl butyrate).

Colipase is secreted by the pancreas as procolipase and is subsequently cleaved in the intestinal lumen by trypsin [216] although the pro-protein is equally capable of activating PL [217]. Cleavage of procolipase results in the release of colipase and a pentapeptide called enterostatin, which has been shown to reduce fat intake through various metabolic effects [218]. Alternatively, procolipase can also be found in the stomach [219]. The crystal structure of porcine colipase has been obtained in complex with inhibited human PL [217]. Mature colipase has a molecular weight of 10 kDa and its key structural elements are conserved among various species [217]. Namely, there are five conserved disulfide bridges, several conserved residues for interacting with PL, and a hydrophobic region with three conserved tyrosines which have been shown to be

involved in lipid binding [217]. The overall flat structure of colipase resembles a packed core with four distinct protruding loops ("fingers"). The lipid binding three tyrosines are located on a single overhanging region (residues 47–64 on so-called fingertip) and PL-binding sites are found on hairpin loops connecting the regions (residues 44–47 and 64–67) [217]. Colipase is also considerably heat and acid resistant [210].

Colipase is exclusively bound to the CTD of PL in the absence of substrate, where the PL active site lid is in its closed position [206,214](**Figure 5, left panel**). The interaction between PL and colipase is mediated by electrostatic forces and hydrogen bonds [217]. Binding to substrate triggers conformational changes in the NTD of PL and a movement of the lid to the open position, bringing it in contact with the N-terminal part of colipase [7](**Figure 5, right panel**). This creates a second site of interaction between PL and colipase where three hydrogen bonds are formed with the lid [217] and which stabilize the active open conformation of PL [7]. Opposite from the PL CTD binding site are conserved hydrophobic residues necessary for the interaction of colipase with lipid-water interfaces [217]. These hydrophobic regions penetrate the surface layer of substrate particles, causing conformation changes in colipase [213] and a redistribution of surface lipids which subsequently facilitates PL adsorption [210]. The preference of colipase for binding to a surface layer has been demonstrated by multiple studies where colipase does not significantly bind monomers of bile acids but rather interacts with micelles [211].

1.6.2 Bile acids

Bile acids (BAs) are amphipathic molecules which act as detergents and emulsify dietary triglycerides to enable their lipolysis by PL. BAs are produced in the liver from cholesterol and subsequently transferred to the gallbladder for storage. From there, they are secreted into the lumen of the intestine. About 95% of BAs can be later reabsorbed by enterocytes after facilitating lipolysis [220]. Small amounts of BAs (~1% of total intestinal BA) can also find their way to blood serum and urine [221]. BAs are conjugated with taurine or glycine prior to their secretion to create bile salts, improving their solubility and tendency for micelle formation [222]. However, unlike BAs, bile salts cannot penetrate membranes and must be moved by transmembrane transporters [222]. The millimolar concentration of BAs (2–5 mM in the fasted state, 8–15 mM in the fed state [223]) in the intestine is well above their critical micellar concentration, enabling efficient formation of mixed micelles within the intestine [220]. Absorbed intestinal BAs reach the liver on chylomicron remnants for re-use and in addition suppress VLDL production [11]. Disruption of normal BA metabolism is associated with a multitude of conditions such as malabsorption of lipids and fat-soluble vitamins, inflammation, gallstones, and cardiovascular or neurological diseases [222].

The primary BA components of human bile are cholic acid, chenodeoxycholic acid and deoxycholic acid (DOC) [220]. From these, the salt of deoxycholic acid (sodium deoxycholate) and additionally sodium taurodeoxycholate (NaTDC) are the most used bile salts for studying lipases. For PL, NaTDC is used in activity assays for mimicking conditions of duodenal lipolysis [224]. Meanwhile, DOC is used for increasing the stability and solubility of LPL [225,226].

1.6.3 Intestinal ANGPTL4

As reviewed earlier, ANGPTL4 is a fasting-induced potent inhibitor of LPL in multiple tissues which directs fatty acids based on metabolic demands [175]. However, ANGPTL4 is also expressed by intestinal cells [227] where it has been shown to be upregulated in mice by high fat feeding [175] or short-chain fatty acids [228]. The role of intestinal ANGPTL4 has not been fully elucidated but multiple mechanisms for its action in the intestine have been proposed.

Firstly, ANGPTL4 has been thought to serve as a PL inhibitor to limit lipid uptake following fat-rich meals [229]. The study demonstrated that ANGPTL4 knock-out mice have significantly higher bodyweight regardless of diet. ANGPTL4 did not influence triglyceride secretion from the intestine to circulation but rather regulated lipid absorption to enterocytes by inhibiting PL. Exogenous addition of ANGPTL4 to a substrate emulsion with PL resulted in a dose-dependent loss of PL activity.

Secondly, in addition to promoting PL-mediated lipolysis, bile acids and their composition have been shown to affect gut microbiota. Oppositely, at the same time gut microbiota might also influence the bile acid content of the host body. Bile acid supplementation has been shown to have a triglyceride lowering effect and an impact on intestinal ANGPTL4 secretion in vitro [227]. It has been postulated that bile acids and gut microbiota might play a role in ANGPTL4-mediated regulation of triglyceride levels [230]. The role of gut microbiota on ANGPTL4 mRNA levels has been also observed in mice receiving fecal microbiota from pigs [231]. Janssen et al [230] found that taurocholic acid supplementation in wild-type or ANGPTL4 knock-out mice has a similar effect on triglycerides, indicating that ANGPTL4 does not affect the triglyceride lowering effect of bile acids. However, they discovered that bile acid absorption is lower in ANGPTL4 knock-out mice compared to wild-type, but this difference is abolished after treatment with antibiotics. They concluded that ANGPTL4 might promote bile acid absorption via a mechanism that is dependent on the gut microbiota.

Lastly, CCD-ANGPTL4 is predicted to contain two amphipathic α-helices and positively charged stretch capable of binding heparin [166]. These structural characteristics might provide a platform for an interaction between ANGPTL4 and lipid surfaces. CCD-ANGPTL4 has been shown to bind fatty acids [232] and additionally, other basic [233] or hydrophobic proteins [234,235] have been shown to inhibit PL by interacting with emulsion particles or blocking adsorption of PL to lipid surfaces. Inhibition of PL by these proteins was seen to be dependent on the composition of the substrate emulsion. The lipid binding properties of CCD-ANGPTL4 were also demonstrated in our published study and are discussed in detail under the results section of this thesis (Publication I). CCD-ANGPTL4 was shown to interact with various emulsion components and indirectly inhibit PL by destabilizing the substrate emulsions.

1.7 Measuring lipoprotein lipase activity

A large variety of methods and substrates exist for determining exogenous or pre/post-heparin LPL activity. The latter is assessed routinely in clinical situations for lipolysis defects, while exogenous LPL is used for studying the effects and mechanisms of LPL activators and inhibitors. Measurements of LPL activity are also of great interest in the dairy industry where active LPL can be found in milk. Lipolysis releases free fatty acids that may result in unwanted flavors [236].

LPL activity can be determined in vitro using water-soluble synthetic fluorogenic or chromogenic substrates. However, while these assays are cost-effective and convenient to use, there are some considerations to be made. As mentioned under chapters 1.3 and 1.4, in vivo LPL functions on the phospholipid interface of circulating lipoproteins and requires apoC-II to do so. Additionally, there are several other circulating or lipoprotein-bound LPL regulators that influence LPL activity (ANGPTLs, apolipoproteins). Human plasma also contains a high concentration of proteins (80 g/l [237]) which creates a strong macromolecular crowding effect that could influence LPL activity as has been shown for some other enzymes [238]. While some more physiological assays include emulsified (radiolabeled) substrates or isolated TRLs, these approaches must yield in either sensitivity, throughput or they are not suitable for continuous measurement of LPL activity. Radiolabeling also poses possible health and environmental risks. Combining sensitivity and physiological conditions of the assay is especially challenging for determining LPL deficiency from pre- or post-heparin plasma where the concentrations of LPL are around 1 nM and 5 nM, respectively [239]. The gap in LPL activity is even larger as most of circulating pre-heparin LPL is inactive — pre-heparin LPL activity makes up only 1% of post-heparin LPL activity [240]. Additionally, post-heparin LPL activity alone might not be reflective of LPL activity or TRL clearance in vivo [241]. A more accurate description of LPL action can be obtained by measuring LPL activity directly in the plasma or serum obtained from donors [53,242]. In this case, the environment contains concentrations and ratios of endogenous LPL regulators and lipoproteins characteristic to the specific patient.

Broadly speaking, LPL assays can be divided into two groups based on whether the cofactor apoC-II and albumin as an FFA-acceptor are required for LPL activity. The assays without these requirements measure so-called esterase activity of LPL, using water-soluble synthetic substrates (e.g., DGGR, EnzChek, p-nitrophenyl butyrate (PNPB)), or emulsions of short-chain triglycerides (e.g., tributyrin). **Table 2** provides examples of known esterase assays and some associated parameters estimated from an example article. Microplate readers are the go-to strategy for rapid quantification of LPL activity with water-soluble substrates. These methods often boast fast turnaround times (a few minutes) and relatively high sensitivity when using fluorescence. Radiolabeling or a pH-stat is used with emulsion substrates. The former requires separation of released FFAs, making it quite time consuming but its upside is extremely high sensitivity. For continuous measurement of LPL activity, a pH-stat can be used but the assay volumes are considerably larger compared to other methods and the sensitivity is lower.

Table 2. *Examples of assays for measuring esterase activity of LPL.*

Substrate	Detection	LPL concentration	Real-time?	Total volume	Turnaround time
DGGR [166]	Fluorescence	2.5 nM	✔	100 µl (96-well plate)	Minutes
EnzChek [243]	Fluorescence	100 pM	✔	100 µl (96-well plate)	Minutes
PNPB [244]	Colorimetry	10 nM	✔	100 µl (96-well plate)	Minutes
Tributyrin	Radioactivity [245]	pM (LPL from culture medium)	✘	300 µl	Hours
	pH-stat [112]	22 nM	✔	5 ml	Minutes

DGGR – 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6' methylresorufin) ester

Emulsions of long-chain triglycerides (Intralipid [112], triolein [112]) or isolated TRLs are used to measure lipolytic activity of LPL. While LPL has some minor activity against long-chain triglycerides without apoC-II, its addition is needed for reaching maximal activity [113]. In the case of TRLs, apoC-II is crucial [114]. **Table 3** summarizes widely used or novel methods for determining lipolytic activity of LPL. The accompanying parameters were based on the associated example articles. The most applied method for determining lipolytic LPL activity with isolated TLRs is the NEFA kit which quantifies released FFAs by measuring enzyme-coupled absorption after lipolysis has occurred. [246] The assay can be quite sensitive, depending on the timepoints and substrate concentration chosen but dilution of samples is often needed to avoid interference from existing FFAs, signal saturation, and to obtain reliable results. Recently, a modified version for using a NEFA kit has been developed which enables continuous measurements of LPL activity against VLDLs using a fluorescent reporter (Amplex Red) [166,247]. NEFA kit can also be used with diluted human plasma as substrate [248] but it should be remembered that this also leads to the dilution of LPL regulators in the donor plasma. An additional method has been proposed which would account for endogenous LPL activators and inhibitors from a patient's serum [242]. In this assay, serum is added as a source of apoC-II and other LPL regulators to radiolabeled triolein substrate. The authors saw that serum from patients with HTG is worse at activating LPL. While the result is promising, the assay suffers from similar drawbacks to radiolabeled triolein or NEFA assays — low throughput, laborious, and requires dilution of donor serum. Currently, an isothermal titration calorimetry (ITC) based approach is the only method which enables continuous measurement of LPL activity in undiluted blood plasma. Minute LPL concentrations (50 pM [240]) can be used with this approach, but this sensitivity is highly dependent on the composition of donor plasma [53]. The method can be automated for higher throughput [53] and it is possible to follow the kinetics of complete lipolysis of available substrate [240].

Table 3. *Examples of assays for measuring lipolytic activity of LPL.*

Substrate	Detection	LPL concentration	Real-time?	Total volume	Turnaround time
Intralipid	pH-stat [112]	36 nM	✓	5 ml	Minutes
	NEFA kit [248]	36 nM	✗	50 µl (96-well plate)	7.5 min to hours
Triolein	pH-stat [112]	22 nM	✓	5 ml	Minutes
	Radioactivity [242]	~50 pM post-heparin LPL	✗	200 µl	Hours
Isolated TRLs	NEFA kit [246]	~70 pM post-heparin LPL	✗	730 µl	3 hours
VLDL-Amplex Red	Fluorescence [166]	10 nM	✓	100 µl (96-well plate)	Minutes
Diluted blood plasma	NEFA kit [248]	36 nM	✗	50 µl (96-well plate)	7.5 min to hours
Triolein with diluted serum	Radioactivity [242]	9 nM	✗	200 µl	Hours
Undiluted blood plasma	Nano ITC [240]	50 pM	✓	1,035 µl	1 hour
	PEAQ-ITC [53]	1 nM	✓	300 µl	15 min

Several observations highlight the major differences between using non-physiological substrates (i.e., measuring esterase activity) or long-chain triglycerides/lipoproteins to determine LPL activity. Firstly, choice of substrate system could be important when following thermostability of LPL. Inactivation of LPL at 20 °C was found to vary depending on whether tributyrin or Intralipid was used as a substrate to measure remaining LPL activity [249]. Secondly, LPL cleaved by chymotrypsin has been observed to have normal catalytic activity against tributyrin and p-nitrophenyl butyrate but at the same time, reduced activity against Intralipid or even non-existent activity when chylomicrons are used [112]. Thirdly, the effect of some LPL regulators depends on the substrate system used. Full-length or C-terminal apoC-II has a different effect on LPL activity when comparing VLDL and Intralipid as substrates [114]. Additionally, ANGPTL3 and 4 have been measured to be more than twice as potent in reducing LPL activity towards VLDL than DGGR [166]. Lastly, LPL rapidly loses its activity in ordinary buffers but at the same time, exogenously added LPL has been observed to be stable for hours in undiluted human plasma [240]. This indicates that a biological environment might be more suitable for studying LPL as the combined effect of components of blood plasma could affect LPL stability, activity, and potentially ligand interactions. Taken together, it is possible that measuring esterase activity could sometimes lead to confounding results and it should be carefully considered whether made observations hold true under in vivo conditions.

1.8 Measuring pancreatic lipase activity

Methods for measuring PL activity largely overlap with LPL assays as both enzymes bind to lipid-water interfaces and catalyze the same hydrolysis reaction of triglyceride ester bonds. The most widely used methods for the determination of PL activity include titrimetry, spectroscopy, turbidimetry, and radioactivity [250]. Some examples of related assays are outlined in **Table 4.** In addition to research assays for studying PL and its regulators, PL activity is also determined in a clinical setting from blood serum for the diagnosis of pancreatitis. The latter has proven challenging as high sensitivity is needed and serum can inhibit PL in many assays [251].

A widespread assay for the rapid determination of PL activity and the effect of its regulators employs PNPB as substrate. This method is simple, cost-effective and allows continuous monitoring of lipase activity. Coupled with automation and high throughput, it can be seen why this could be a lucrative approach, especially in clinical laboratories where high reproducibility is required. Unfortunately, this assay neglects the fact that in vivo lipolysis by PL occurs on the lipid-water interface in the presence of bile salts and colipase, as these components are not needed in the case of PNPB. Hydrolysis of triglycerides by PL releases sn-2 monoglycerides in addition to FFAs. Accumulation of sn-2 monoglycerides has been demonstrated to inhibit PL activity by expelling the enzyme from the lipid-water interface, an effect that would be ignored with PNPB [252]. Furthermore, in addition to low sensitivity, PNPB also lacks in specificity as multiple esterases, proteases, and even albumin or the non-catalytic domain of PL can hydrolyze nitrophenyl ester bonds [250]. This makes the assay heavily reliant on using highly purified PL for obtaining reliable results. Despite this, often crude (porcine) pancreatic lipase preparations are used which also contain amylase and protease activity (e.g., Sigma-Aldrich #L3126). Better sensitivity can be obtained with the water-soluble fluorescent DGGR which has even been shown to be able to determine PL from serum [253]. The authors added bile salts and colipase to the DGGR substrate mixture, presumably to counteract the interference of serum because these components are not needed in this assay when using purified PL.

Multiple substrates and environments have been proposed which would better resemble conditions of intestinal lipid digestion. These assays contain millimolar concentrations of bile salts (preferably a mixture), colipase, a source of phospholipids, short-chain (tributyrin) or long-chain triglycerides as substrate (e.g., soybean or olive oil) and a buffer with sodium and calcium [223]. None of the assays reviewed here contained the prevailing dietary sterol, cholesterol, in their substrate mixture. Use of p-nitrophenyl palmitate (PNPP) instead of PNPB has been proposed for differentiating between the esterase and lipolytic activity of PL [254]. The use of a long-chain fatty acid and bile salts would bring this assay at a middle ground between the more physiological-like conditions and water-soluble substrates. A pH-stat is commonly used for the investigation of PL kinetics, and a large degree of freedom is given when choosing assay conditions which makes this method especially suitable for research laboratories [255]. The drawbacks include large volumes and low sensitivity. Meanwhile, very high sensitivity can be achieved using radiolabeled substrates [255], but this approach does not allow for real-time measurements and requires separation of products. Another discontinuous method for using physiological substrates is the NEFA kit approach for determining the concentration of released FFAs [256].

Overall, these approaches rectify the shortcomings of synthetic water-soluble substrates by providing a physiological-like lipid-water interface for PL. This is a crucial step in the right direction as binding of lipases and their regulators is known to be affected by the composition lipid surfaces [252]. Inhibitors can accumulate at the lipid-water interface as illustrated by ε-polylysine which has no inhibitory effect on PL against water-soluble substrates but is potent when lipid emulsions are used [257]. Another inhibitor, epigallocatechin-3-gallate (EGCG), also interacts with components of substrate emulsions [258]. Similar differences between assays and substrates were observed in our comparison of NTD of ANGPTL4 (CCD-ANGPTL4) with other known PL inhibitors (Publication I of this thesis). These observations suggest an important role of studying PL inhibitors in more physiological-like conditions.

Table 4. *Examples of assays for measuring PL activity.*

Substrate	Detection	Bile salts	Gum arabic or phospholipids	Colipase	PL conc.	Real-time?
PNPB [259]	Colorimetry	✗	✗	✗	2 µM (pPL)	✓
DGGR [253]	Fluorescence	1.7 mM DOC 7 mM TDC	✗	✓	pM (serum)	✓
PNPP [254]	Colorimetry	5 mM DOC	✗	✗	21.7 µM*	✓
4-MUO [260]	Fluorescence	✗	✗	✗	217 nM*	✓
Tributyrin [261]	pH-stat	20 mM DOC	Gum arabic	✓	3.6 µM*	✓
Triolein	Radioactivity [255]	4 mM TDC	✗	✓	30 nM	✗
	Turbidimetry [251]	20 mM DOC	✗	✓	0.8 nM (hPL)	✓
	NEFA-kit [256]	1 mM TDC	Phosphatidyl-choline (0.55 mg/ml)	Not specified	1 µM*	✗
Tripalmitate [261]	pH-stat	20 mM DOC	Gum arabic	✓	3.6 µM*	✓

PNPP – p-nitrophenyl palmitate; 4-MUO – 4-Methylumbelliferyl oleate; * – Porcine pancreatic lipase type II from Sigma-Aldrich (#L-3126); pPL – porcine pancreatic lipase; hPL – human pancreatic lipase

1.9 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is a quantitative method for measuring thermodynamic parameters of interactions. A single ITC experiment provides information about the affinity (K_D), stoichiometry (n), free energy (ΔG), enthalpy(ΔH), entropy(ΔS) of an interaction [262]. These values are calculated from the heat released or absorbed upon binding of two molecules based on the expression of Gibbs free energy $\Delta G = RT\ln K_D$. Gibbs free energy is described by two components, enthalpy, and entropy, written as $\Delta G = \Delta H - T\Delta S$. An ITC experiment directly measures a change in heat (ΔH) at a constant temperature (T). K_D and stoichiometry are calculated from the binding curve, after which ΔG and ΔS can be deduced.

ITC can also be used to study the rate and kinetics of enzymatic reactions [240,263,264]. The enzymatic conversion of substrate is associated with a change in heat rate which can be detected by ITC. Heat is continuously released or absorbed in proportion to the reaction rate. Importantly, measurements are done in real-time without the need for labels or purified and diluted conditions. Macromolecularly crowded [263,264] and physiological environments such as undiluted human plasma [240] can be used without interference on the enzymatic reaction. This can be crucial for studying lipases in a more in vivo-like setting — both LPL and PL act in highly crowded environments. Macromolecular crowding can affect both k_{cat} and K_M as demonstrated with PEG, Ficoll, and albumin [238,263]. There are two fundamentally different experimental setups for measuring enzyme activity — the substrate can be injected into an enzyme solution [263,264] or vice versa [240].

In the first case, one or more injections of substrate are made. For a single injection experiment, the heat rate of the reaction is followed until no more changes are detected after which the total amount of heat released or absorbed can be found by integration. The quantity of heat involved in the conversion of n moles of substrate to product can be written as

$$\mathrm{Q} = \mathrm{n}\Delta\mathrm{H}_{\mathrm{app}} = [\mathrm{P}]_{\mathrm{total}}\mathrm{V}\Delta\mathrm{H}_{\mathrm{app}}$$

where $\Delta\mathrm{H}_{\mathrm{app}}$ is the total molar enthalpy for the reaction as measured by ITC, P is the concentration of product and V is the volume of the cell (reaction volume) [265]. The reaction rate can be derived from measuring changes in power since

$$\mathrm{power} = \frac{dQ}{dt} = \frac{\mathrm{d}[\mathrm{P}]}{dt}\mathrm{V}\Delta\mathrm{H}_{\mathrm{app}}$$

where d[P]/dt is equal to the reaction rate [265]. Rearrangement of this equation gives

$$\mathrm{rate} = \frac{1}{\mathrm{V}\Delta\mathrm{H}_{\mathrm{app}}}\frac{\mathrm{dQ}}{dt}$$

For experiments with multiple injections, increasing concentrations of substrate are injected until saturation (V_{max}) is reached. In this case, changes in steady-state heat rate rather than total heat released are followed. The concentration of enzyme in the cell is generally lower and the amount of product formed is considered negligible [264]. Afterwards, the substrate concentration is plotted against the reaction rate, giving k_{cat} and K_M from the fitted Michaelis-Menten equation [263]. Injecting substrate into an enzyme solution enables the use of very low concentrations of enzyme because the enzyme is not being noticeably diluted. Porcine PL has been studied in a crowded environment using this approach [264]. The authors found that macromolecular crowding

induces conformation changes which affect the active site of PL. The polysaccharide Ficoll400 was observed to increase PL activity while Dextran20 lowered catalytic efficiency. It should be noted that colipase, phospholipids, and cholesterol were not used in the assay. Also, only sodium cholate was present as an emulsifier which means that these conditions might not entirely reflect physiological substrates and conditions.

In the opposite setup, injecting the enzyme into substrate requires a concentrated stock solution to ensure that enough enzyme is used for detection. However, this approach enables to observe lipases in a crowded environment where substrate is in excess. This is a better reflection of in vivo conditions as lipases are secreted into mediums where substrate is already present. Our group demonstrated in 2017 that the activity of LPL can be directly measured in undiluted human plasma [240]. This assay allows the direct measurement of LPL activity in its physiological environment that is macromolecularly crowded, containing natural substrate and LPL regulators at physiologically relevant concentrations. A linear relationship between reaction rate and heat rate was observed despite the complexity of the system. Both initial rates under zero-order kinetics, and kinetics of complete substrate lipolysis can be studied using this method. Similar to the other approach, the reaction rate can be obtained from calculation of apparent enthalpy (ΔH) using the equation for heat (Q) released [240].

Figure 6 provides a schematic model of an ITC system for studying enzyme activity. Experimental setup for measurement of LPL activity in undiluted human plasma [53,240] is used as an example as this is more relevant in the context of this thesis. An ITC instrument is composed of a reference and a sample cell located inside an adiabatic jacket. Both cells are kept at a set temperature by heaters. The cells are separated by a feedback sensor which detects temperature difference between the sample and reference cell. Heat is either absorbed or released when an interaction or enzymatic reaction occurs. The temperature in the sample cell changes and then the feedback sensor relays this information to the sample cell heater which adjusts its power to match the temperature in the sample cell with the reference cell. This change in power is the signal that is tracked by the ITC, expressed as μJ/s. In a typical experiment with LPL, the sample cell is filled with undiluted human plasma and the reference cell contains water. LPL is injected via a syringe-stirrer that is inserted into the sample cell. The relative heat rate between two cells remains zero while no lipase is present in the sample cell. Once LPL is injected then a continuous heat will be released because of the hydrolysis of triglyceride ester bonds. A steady state baseline is achieved where heat is constantly produced because the concentration of substrate greatly exceeds that of LPL. These injections can be repeated to achieve new steady states. There is a proportional relationship between the concentration of LPL and the reaction rate as each injection increases the heat rate nearly equally. Addition of inhibitors reduces the change of heat rate while addition of activators increases it, allowing the quantification of their effect on LPL activity.

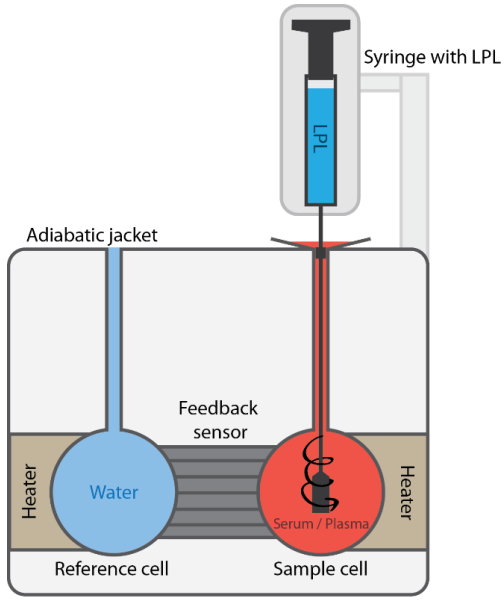

Figure 6. *Schematic representation of ITC for measuring LPL activity in serum or plasma. A reference cell and sample cell are located inside an adiabatic jacket. The cells are kept at an equal temperature by two heaters. A feedback sensor detects fluctuations in the sample cell, directing the sample cell heater to increase or reduce power to maintain equal temperature between two cells. In a standard experiment with LPL and undiluted blood plasma, LPL is injected from the syringe-stirrer into the sample cell containing plasma or serum. Heat will be released as LPL begins to hydrolyze the triglyceride core of TRLs in plasma. Less power is needed to heat the sample cell when lipolysis occurs. This drop in power is recorded by the ITC and can be used to quantify LPL activity and the effect of its regulators.*

1.10 Clinical importance of determining lipase activity: hypertriglyceridemia

LDL-C has been in the focus of attention for decades as a recognized risk factor for CVD. Reduction of LDL-C is associated with reduced morbidity and mortality from CVD [266]. However, a spotlight has been lately shined on triglycerides as epidemiological and genetic studies have now demonstrated that elevated plasma triglycerides play an independent and casual role in the development of CVD [266,267]. HTG can lead to conditions such as acute pancreatitis, atherosclerosis, and hepatic steatosis. These illnesses can be extremely dangerous, as for example acute pancreatitis has a mortality rate of 5–6% per occurrence [65]. All apoB-containing lipoproteins less than 70 nm in diameter are capable of penetrating the endothelial barrier, where they can become trapped [4]. Elevated triglycerides cause continued exposure to these particles, which leads to accumulation of lipoproteins in the artery wall and formation of atherosclerotic plaques. Some patients who receive LDL-C lowering therapy still possess residual risk for the development of ASCVD [268]. This is now known to be caused by the fact that remnant TRLs contain 5–20 times more cholesterol per particle than LDL [269] and convey a similar risk for ASCVD per particle as LDL [30].

There are different classifications of HTG, but the broadest categories can be defined as follows: circulating fasting triglyceride concentrations under 150 mg/dl (1.7 mM) are considered normal, anything between 150 mg/dl to 880 mg/dl (1.7–9.9 mM) is defined

as HTG, and everything exceeding that (>880 mg/dl; >10 mM) is considered as severe HTG [270]. However, it is important to note that optimal fasting triglycerides for lowest risk of cardiovascular diseases may be below 100 mg/dl [270].

Severe HTG is quite uncommon with a prevalence of 0.10 to 0.20%, and triglyceride levels exceeding 1800 mg/dl (20 mM) are even rarer, with a frequency of 0.01% [31]. Monogenic causes behind severe HTG result in FCS with an estimated occurrence of 1 in 10 million [31]. This condition is caused by biallelic mutations in five major genes associated with lipoprotein metabolism: LPL, APOC2, APOA5, LMF1, and GPIHBP1 [31]. A much more likely cause of primary (genetic) HTG arises from a polygenic background with various environmental influences [270,271]. Secondary HTG, where a specific genetic cause can't be distinguished, may caused by drug abuse, an unhealthy lifestyle (e.g. low physical activity, smoking) or by bad nutritional choices (over-eating, excessive alcohol consumption) [272]. Elevated plasma triglycerides can also be a consequence of medical disorders such as (uncontrolled) diabetes, obesity, or metabolic syndrome [270]. These are associated with an increase in VLDL production and secretion as well as a reduction in VLDL clearance [270,272]. VLDL synthesis and secretion have been shown to be enhanced due to insulin resistance [27].

Statistics from 2010 and 2016 show that 47% of adults in the USA and 27% in Europe suffer from hypertriglyceridemia [266]. Obesity runs rampant in the modern world, with over 650 million people affected in 2016 according to the World Health Organization by what has been dubbed as the global obesity pandemic. The total medical cost in the USA due to obesity among adults was $260.6 billion in 2016 [273], putting a massive economic burden on the healthcare system. This has created a giant need for prevention and treatment of CVD, including lowering of LDL-C and circulating triglycerides through various novel therapies. It is a multi-billion industry for pharmaceutical companies to take advantage of. As such, accurate and reliable determination of post-heparin LPL activity is crucial to ascertain causes behind elevated triglycerides. Is the condition triggered by LPL-deficiency, reduced LPL activity mediated by LPL regulators, or instead related to lipoprotein processing and catabolism? Measurement of purified LPL activity in physiological-like conditions is equally important to properly elucidate the mechanisms behind LPL-mediated HTG and develop potential lipid lowering therapies.

1.11 Strategies for managing hypertriglyceridemia

The first line of defense against HTG are lifestyle interventions and establishing control of secondary factors [274]. This means reducing caloric intake and alcohol consumption while increasing exercise to induce weight loss and improving glycemic control when needed [266,274]. Unfortunately, the results are often unreliable as strong self-control is required for adherence to lifestyle changes [31]. In the case of primary hypertriglyceridemia, lifestyle interventions are similarly applied but pharmacotherapy is simultaneously needed to achieve necessary goals [270]. A summary of known triglyceride-lowering therapies can be found in **Table 5**.

Statin treatment is the recommended approach for 40–75 years old patients with HTG who don't respond to changes in lifestyle or improvements in secondary factors [267]. Statins significantly lower LDL-C and have a mild effect on circulating triglyceride concentrations by increasing chylomicron catabolism [267]. However, it is worth pointing out that studies and clinical trials indicate increased risk of developing type 2 diabetes (T2D) following long-term statin treatment [275]. Patients with severe HTG, especially those exceeding triglyceride levels of 1000 mg/dl (>11.3 mM) are first recommended to be

put on a very-low-fat diet (10–15% of daily caloric intake) [267]. Depending on the risk of acute pancreatitis, fibrates and omega-3 fatty acids are recommended in addition to statin therapy. Both reduce VLDL production and fatty acids also increase TRL clearance [267].

Table 5. *Available triglyceride lowering therapies.*

Therapy	Mode of action	Triglyceride reduction (%)
Healthy lifestyle [103]	Weight loss, physical activity, alcohol abstinence	~20% [270]
Statins	Increased chylomicron remnant catabolism	10–20% [276]
Fibrates	PPARα agonist; decreases VLDL production	30–50% [276]
Omega-3 fatty acids	Reduced VLDL production and increased TRL clearance	30–40% [276]
Volanesorsen (Waylivra)	ASO against apoC-III; Increases TRL catabolism and reduces VLDL production,	30–70% [276]

Many patients with increased circulating lipids do not reach their goals, despite these existing treatments [277]. Multiple novel strategies (**Table 6**) have been developed for these patients to increase the efficacy of lipolysis and regulate lipoprotein production or clearance. A number of these therapies are designed to promote LPL activity as this has been well-documented to reduce the risk of CVD [276]. This can be achieved by reducing and blocking LPL inhibitors (apoC-III, ANGPTLs) or by increasing LPL activity directly with activators. Currently, the only approved novel (in EU) therapy for treatment of FCS is the ASO targeting apoC-III mRNA (**Table 5**, known as Volanesorsen, marketed under Waylivra). It was shown to lower plasma apoC-III and triglyceride levels by 84% and 77% respectively [267]. A liver-specific APOC3-ASO has been also developed (IONIS-APOCIII-LRx), which grants the same benefits as Volanesorsen but considerably lower doses can be used [278]. Monoclonal antibodies (STT505/STT5058) and siRNA targeting apoC-III are additional promising leads in reducing circulating triglycerides and improving TRL uptake [267]. For ANGPTL3, clinical trials with HTG subjects have confirmed the efficacy of an antibody treatment as a promising lead for reducing triglycerides and cardiovascular risk [279]. Currently, an ANGPTL3 antibody referred to as Evinacumab (sold under the brand name Evkeeza) has already been approved by the FDA and EMA for treatment of patients with homozygous familial hypercholesterolaemia [277]. Another option to reduce plasma ANGPTL3 levels utilizes an ANGPTL3 ASO (Vupanorsen) with a high specificity for the liver [31]. Studies in humans showed marked reductions in circulating triglyceride, LDL or remnant cholesterol, apoB, and apoC-III levels. Similar results have been achieved with ANGPTL4 inhibition. Homozygous carriers of the ANGPTL4 E40K mutation demonstrate higher LPL activity and reduced triglyceride levels, indicating that lowering ANGPTL4 might be a viable strategy for combating HTG [142]. However, ANGPTL4 inhibition must be liver specific as whole body ANGPTL4 knock-out leads to inflammation in gut lymph nodes [164,175]. As such, a ANGPTL4 ASO has been developed which is conjugated with N-acetylgalactosamine (GalNAc) for specific delivery to the liver [280]. The ASO-ANGPTL4 successfully lowered hepatic ANGPTL4 mRNA and protein, circulating triglycerides levels, and body weight in fasted or fed mice. While inhibition of ANGPTLs has a major benefit on reducing HTG, a question arises whether there are long-term effects of suppressing ANGPTLs. Are there concerns for ectopic fat deposition since these LPL inhibitors are thought to regulate tissue-specific LPL activity? A dose-dependent increase in liver fat has been noted as an adverse effect

of ANGPTL3 inhibition [278]. Additionally, a study found that carries of the ANGPTL4 E40K mutation have an increased risk of CVD despite favorable lipid profiles [278].

Use of mimetic peptides has been proposed for stimulating LPL activity. A dual anti-apoC-III/apoC-II mimetic peptide (D6PV), that displaces apoC-III from TRLs and activates LPL, was shown to reduce plasma triglycerides by more than 80% in mice lacking apoC-II [115]. The peptide was also shown to reduce triglyceride levels by 50% in LPL-knockout mice. Another potential peptide is the apoC-II mimetic peptide which was demonstrated to lower triglycerides in various animal models with HTG [248,281,282]. The peptide is comprised of a designed N-terminal lipid binding helix and the C-terminal helix of apoC-II for activating LPL.

Table 6. *Novel triglyceride lowering therapies in development.*

Therapy	Mode of action	Triglyceride reduction (%)
Antibodies		
Evinacumab (Evkeeza)	Monoclonal antibody against ANGPTL3; reduces VLDL secretion, reduces plasma triglycerides and LDL-C	47–80% [31]
STT505/STT5058	Monoclonal antibody against apoC-III; lowers plasma apoC-III and triglycerides, promotes TRL clearance	~40% in mice expressing human apoC-III [88]
siRNA		
ARO-APOC3	siRNA targeting apoC-III mRNA; reduces plasma apoC-III and triglycerides	Up to 95% [267]
ARO-ANG3	siRNA targeting ANGPTL3 mRNA; reduces plasma ANGPTL3, LDL-C and triglycerides	Up to 67% [277]
ASO		
IONIS-APOCIII-L_{Rx}	Liver-specific ASO against apoC-III; same effect as Volanesorsen but lower doses can be used	23–60% [278]
IONIS ANGPTL3-L_{Rx} (Vupanorsen)	ASO against ANGPTL3; Increases TRL clearance, reduces LDL-C and hepatic triglyceride content	33.2–63.1% [31]
ASO-ANGPTL4	ASO against ANGPTL4; reduces hepatic ANGPTL4 expression, triglyceride levels, and body weight	46–63% in fasted or fed mice [280]
Mimetic peptides		
D6PV	apoC-II mimetic peptide and apoC-III antagonist; activates LPL and displaces apoC-III from TRLs, increases hepatic TRL uptake	80% in apoC-II deficient mice, 50% in LPL-knockout mice [115]
C-II-a peptide	apoC-II mimetic peptide; activates LPL, lowers plasma triglycerides	78–91% in apoE-knockout mice [248]

1.11.1 Reducing postprandial hypertriglyceridemia

While multiple guidelines reflect fasting triglycerides, it is important to consider that postprandial HTG is also an independent risk factor for the development of CVDs [274]. Under conditions of normal metabolism, triglycerides in blood reach their peak 3–4 hours after meal consumption and stay elevated for up to 6 or 8 hours [274,283]. However, triglyceride concentrations and the residence time of atherogenic particles can be greatly increased for up to 12–14 hours when lipoprotein metabolism is compromised [272].

It should also be taken into account that modern humans consume multiple meals throughout the day. Additionally, a spike in triglyceride levels occurs already 10–30 min after consuming a fat-containing meal, indicating that there is a pool of triglycerides ready to be secreted from enterocytes accounting to a previous meal [283]. Taken together, it can be seen that humans are in a constant postprandial state throughout the day [284] which can be problematic with impaired metabolism.

More than 95% of dietary triglycerides are absorbed under normal digestive function [6] with an estimate of 50–70% hydrolyzed by PL [197]. Currently the only FDA approved purchasable PL inhibitor is tetrahydrolipstatin (THL, approved under the name Orlistat) which binds covalently to the active site serine of PL, thus blocking any further hydrolysis from occurring [197]. Gastric lipase and PL inhibition by commercially available THL (marketed as Xenical®) results in a 30% reduction of triglyceride absorption [285]. THL also inhibits other lipases (LPL [286], endothelial lipase [287]), cholesterol esterase, and serine hydrolases [197]. Although, its absorption from the intestine to circulation is negligible [197]. PL supplements can also be important. They are used for therapeutic purposes in patients with conditions that result in nutrient malabsorption and who require exogenous digestive enzymes [195].

Unfortunately, the use of THL is accompanied by multiple gastrointestinal adverse effects and fat-soluble vitamin deficiencies, which can negatively affect patient compliance in the long-term [202]. This has led to the search for new and improved drug candidates without major side effects for inhibiting PL. A vast number of possible compounds have been screened to date and more are being rapidly discovered. These inhibitors are often aromatic and of natural origin, i.e., plants or their extracts and phytochemicals such as flavonoids, terpenoids, saponins, phenols, and alkaloids [288]. Plant-based materials are preferred over synthetic compounds because of their reduced side-effects and lower toxicity [289]. PL inhibitors can also be found in bacteria, fungi, or some marine species [289].

Flavonoids are polyphenolic compounds found in leaves, fruits, and vegetables. Many results indicate that plants rich in flavonoids can inhibit PL [288]. A promising example is the green tea derived EGCG with an IC_{50} of 1.8 μM [260]. Administration of EGCG to mice significantly reduces body weight and body fat [288]. The widely studied alkaloids for PL inhibition are caffeine, theophylline, and theobromine which have been demonstrated to have a dose-dependent inhibitory effect on the hydrolysis of tributyrin and tripalmitate [261]. Use of theobromine and caffeine leads to a loss of body weight and body fat, as well as a reduction in triglycerides and cholesterol in mice fed a high fat diet [288]. Saponins from oolong tea have shown a similar potential for dose-dependent PL inhibition [202]. Another subset of PL inhibitors includes positively charged proteins and peptides [233,257]. A thoroughly investigated charged peptide is the antibacterial ε-polylysine (EPL) which also has a strong inhibitory effect on PL. IC_{50} for PL inhibition by EPL (0.12 μM) is eight times lower than for THL [290]. This value was highly dependent on the substrate mixture used with up to 230-fold variations. At the same time, EPL did not interact strongly with PL, indicating that the inhibitory effect on PL is likely mediated by EPL interacting with emulsion particles [290]. Use of EPL resulted in lower body fat, triglyceride, and cholesterol levels in mice [290]. Many synthetic PL inhibitors have been also created, usually with a common motif of being highly lipophilic and based on the triglyceride structure [202,291]. Many of these results should be interpreted with caution as the go-to assay for measuring PL uses PNPB which is accompanied by several limitations as discussed previously.

2 AIMS OF THE STUDY

- To investigate the effect of CCD-ANGPTL4 on PL by using an ITC-based method that enables determination of PL activity in complex substrate mixtures

- To apply the calorimetric approach for measuring LPL activity in undiluted human plasma for studying an apoC-II mimetic peptide activator of LPL

- To elucidate the effect of albumin on LPL oligomerization, stability, activity

3 MATERIALS AND METHODS

More detailed information about materials and methods are provided in the corresponding publications.

Publication I

- Recombinant human PL was expressed in the yeast *P. pastoris* and purified using Ni-affinity chromatography.
- Bovine LPL (bLPL) was purified from fresh milk using heparin chromatography.
- Recombinant His-tagged CCD-ANGPTL4 (residues 26-184) was expressed in *E. coli* and purified using Ni-affinity chromatography, anion-exchange, and size-exclusion chromatography (SEC).
- Surface plasmon resonance (SPR), fluorescence and UV-Vis spectroscopy, and isothermal titration calorimetry (ITC) were used to compare CCD-ANGPTL4 to other known PL inhibitors.

Publication II

- Human plasma was purchased from Tallinn Blood Centre.
- Recombinant His-tagged apoC-II was expressed in *E. coli* and purified using Ni-affinity chromatography, anion-exchange chromatography, and SEC
- ApoC-II mimetic peptides were provided by Dr. Alan T. Remaley from National Institutes of Health, USA.
- SEC was used to fractionate human plasma. Additionally, density gradient ultracentrifugation was used to isolate chylomicron, VLDL, LDL and HDL fractions from human plasma.
- ITC was used to measure bLPL activity in undiluted or fractionated human plasma.
- NEFA kit (FUJIFILM Wako Diagnostics, USA) was used as another method for determining bLPL activity in lipoprotein fractions by measuring the generation of fatty acids.

Publication III

- Recombinant ANGPTL4 was purchased from BioVendor. Recombinant GPIHBPI was purchased from Sino Biological. Recombinant human LPL (hLPL) was purchased from Bio-Techne.
- Lipoprotein free human plasma (LFHP) was obtained by flotation ultracentrifugation.
- ITC and fluorescence spectroscopy were used to measure bLPL activity under various oligomeric conditions.
- Fluorescence spectroscopy was used to compare thermostability of bLPL and hLPL in the presence of BSA.
- SPR was used to probe the interaction between bLPL and BSA.
- Negative-stain transmission electron microscopy measurements were performed by Dr. Saskia Neher and Dr. Kathryn Gunn from The University of North Carolina, USA to assess oligomerization of bLPL.
- RICS measurements were done by Dr. Marko Vendelin and Kristofer Hiis-Hommuk from Tallinn University of Technology, Estonia to investigate oligomerization of bLPL.

4 RESULTS AND DISCUSSION

4.1 Using ITC to study pancreatic lipase under in vivo-like conditions (Publication I)

In this study, we demonstrate that ITC can be used to measure PL activity in complex substrate environments which resemble conditions of in vivo PL-mediated lipolysis more so than previous assays. We apply the developed method to compare the effect of CCD-ANGPTL4 on PL activity in various substrate systems with other well-known PL inhibitors. Namely, the antiseptic EPL, green tea flavonoid EGCG, and the over-the-counter drug THL. ANGPTL4 has been widely investigated as an endogenous LPL inhibitor in adipocytes or blood circulation, but ANGPTL4 is also present in the intestine where its effects on PL have not been elucidated. It is not entirely clear whether ANGPTL4 influences triglyceride absorption directly, by inhibiting PL, or indirectly, by other mechanisms associated with dietary fat uptake. A seemingly direct effect has been previously observed where ANGPTL4 inhibited PL in a dose-dependent manner [229]. An indirect approach could be caused by a synergistic effect between ANGPTL4, gut microbiota, and bile acids [230].

ITC can be used to measure PL activity in complex substrate environments

We opted for a calorimetric approach to better clarify the uncertainty surrounding the effect of intestinal ANGPTL4. ITC allows us to use complex environments in real-time measurements and has been previously utilized for studying lipases [240,264]. In vivo PL adsorbs to an oil-water interface consisting of colipase, polar lipids, phospholipids, bile salts, fatty acids, triglycerides, and cholesterol surrounding a core of dietary triglycerides. Dietary oligosaccharides and denatured dietary proteins can also be found on the surface of these particles [292]. As such, intestinal fluid and the oil-water interface of bile salt stabilized emulsion particles are both highly crowded environments with many components which could affect PL-mediated lipolysis or the effect of PL inhibitors.

In our experimental setup, purified hPL expressed in yeast is placed in the syringe-stirrer, and the substrate mixture is loaded into the calorimetric cell. Our substrate emulsions included either 2% tributyrin as short-chain or 5% olive oil (55–83% oleic acid, 3–21% linoleic acid [293]) as long-chain triglycerides. These substrates were mixed in NaCl/$CaCl_2$ containing buffer with NaTDC (0.4 or 4 mM), a mixture of phospholipids (0.8 mg/ml) (soybean lecithin), and cholesterol (10 µM). A standard measurement saw five sequential 360 pM hPL injections into substrate with 84 nM colipase — a 233 to 47-fold excess of colipase which should be enough to overcome any inhibition caused by the composition of the surface [251]. These conditions were chosen in accordance with other published methods which have sought to mimic intestinal lipolysis, apart from cholesterol which was added because of its presence in the intestine.

Our results demonstrated that the highest sensitivity (1.5 pM injection of hPL) can be obtained by measuring hPL activity in tributyrin with 0.4 mM NaTDC (**Figure 7**). This is considerably more sensitive than other assays that allow continuous measurement of PL activity (chapter 1.8). PL is known to have higher activity against short-chain triglycerides [294], and to be inhibited by high concentrations of bile salts [295]. Despite this, high catalytic activity could be obtained even with 10 mM NaTDC, indicating that great sensitivity can also be achieved at physiological bile salt concentrations. Activity of hPL could be detected in all tested substrate systems when 0.4 mM NaTDC was used. In the

case of olive oil, addition of phospholipids and cholesterol to the substrate mixture increased hPL activity, likely because a ternary complex with mixed micelles have been observed to be better at activating PL [211].

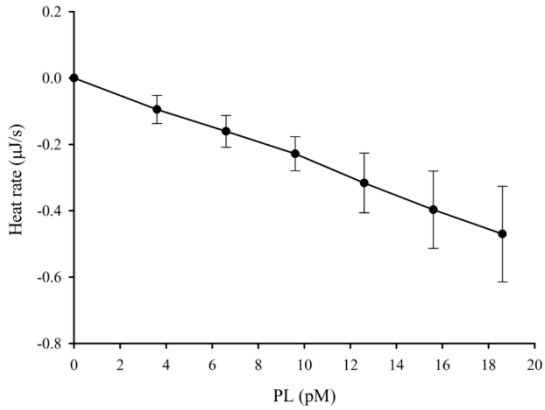

Figure 7. *hPL activity expressed as heat rate (µJ/s) and determined with ITC by sequential injections of hPL into an emulsion of 2% tributyrin and 0.4 mM NaTDC.*

Unfortunately, we were unable to obtain desired results with 4 mM NaTDC, olive oil, lecithin, and cholesterol. It is possible that reorganization of the substrate lipid surface upon PL-mediated lipolysis produces an endothermic effect which overshadows the exothermic reaction of hydrolysis. Formation of NaTDC (mixed) micelles is exothermic at room temperature [296,297] but at the same time demicellization and desorption of surface components or products is an endothermic process [298]. Olive oil or triolein with various concentrations of bile salts and phospholipids has been previously successfully used to determine PL activity using many different methods [251,255,256]. Therefore, it is unlikely that PL is inactive in our case, but rather the activity cannot be followed by monitoring heat effects. Since ITC experiments could be carried out at 37 °C instead of 25 °C, it is possible that an increase in temperature could provide better sensitivity [298]. A temperature-dependent increase in catalytic activity of pPL has been observed when colipase and NaTDC are used [299]. Additionally, thermodynamic parameters of NaTDC (de)micellization are also dependent on temperature. However, we used 25 °C as this is what has been validated previously with LPL [240].

Comparison of ITC with other novel methods for investigating PL inhibitors

Multiple novel methods for determination of PL activity and rapid screening of PL inhibitors have been proposed. A voltammetric PL assay using a modified carbon paste electrode has been demonstrated to provide good LOD using dilinolein [300]. The method demonstrated good correlation with titrimetry when the extent of pPL inhibition by THL was measured. However, they neglected the use of multiple intestinal emulsion components (e.g., bile salts) in their substrate mixture which could affect results when this assay is applied for screening of PL inhibitors. An interesting new approach involves the use of carboxyl-activated magnetic nanoparticles that are coated with pPL [301]. The beads are mixed with PNPP and are separated by a magnet after 10 minutes of hydrolysis. The liberated product is determined using HPLC and a p-nitrophenol standard. The advantages of this assay are the reusability of the nanoparticles and thermal stability

of the immobilized pPL. They also noticed a considerable increase in substrate affinity as K_m had increased 14-fold compared to free pPL. However, similarly to the voltammetric assay, this approach does not utilize other components of intestinal substrate emulsions, and continuous measurement of PL activity is not possible. Another example of an immobilized PL assay is the use of hollow polypropylene fibers [302]. PPL is bound to the fiber matrix and incubated in natural extracts to detect potential inhibitors. Bound and unbound compounds are separated by washing and detected with LC-MS. While convenient for screening inhibitors from complicated mixtures, the binding assays lacks substrate emulsion particles and can therefore detect only inhibitors that interact directly with PL. However, as apparent from chapter 1.11.1, many PL inhibitors influence PL indirectly by interacting with emulsions components and not PL. HPLC–MS ultrafiltration is a method that has been successfully used to rapidly screen PL inhibitors and has led to the identification of sixteen new natural inhibitors [303]. They incubated pPL with inhibitors in a 96-well plate. Following a 60-minute incubation period, the solutions were filtrated (10 kDa MWCO) and analyzed by HPLC-MS. The analysis then reveals reductions in the peak area of inhibitors when binding to pPL occurs. Activity of potential inhibitors was later assayed with the fluorescent 4-methylumbelliferyl oleate as a substrate. This method avoids isolation and purification of bioactive compounds but remains qualitative in nature as the authors demonstrated that the degree of binding observed by HPLC-MS does not correlate to actual IC_{50} values. Similar to the previous method with PL immobilized to fibers, this assay only detects direct inhibitor binding to PL in the absence of substrate. Thin-layer chromatography (TLC) is an alternative method proposed to detect PL inhibitors with high sensitivity [304]. Inhibitors are separated by TLC, and pPL with PNPB are added. Formation of p-nitrophenol on the plate is then observed using a pH-indicator which visualizes blue spots on a green background at locations where lipase is inactive. While it is sensitive and crude extracts can be used, the method itself is time-consuming as multiple steps of incubations and drying are required, and additionally suffers from any weaknesses associated with the use of PNPB.

Overall, many new possibilities have arisen for the detection of PL and its inhibitors from complex heterogenous mixtures. These novel methods are often sensitive and have identified numerous natural inhibitors of PL [291]. Unfortunately, the assays seem to suffer from similar drawbacks to older approaches. Measurements are not carried out in an environment that would reflect the conditions of intestinal lipolysis, and for the most part, PL activity cannot be followed in real-time. These shortcomings can hide the effect of natural emulsion components and macromolecular crowding on PL inhibitors and their influence on enzyme kinetics. ITC alleviates these issues by providing a label-free platform for measuring real-time lipolysis in physiological-like conditions with a high sensitivity. However, ITC cannot identify specific inhibitors from crude extracts but rather characterizes the effects of purified compounds on PL activity and kinetics.

CCD-ANGPTL4 is a lipid-binding protein that inhibits PL by destabilizing the substrate emulsion

We tested inhibition of hPL by CCD-ANGPTL4 in three different substrate systems, using fluorimetry for DGGR, and ITC for mixtures of tributyrin/NaTDC or olive oil/NaTDC/lecithin/cholesterol. We observed inhibition of PL in all substrate emulsions by submicromolar concentrations of CCD-ANGPTL4. Throughout the experiments we noticed that addition of CCD-ANGPTL4 to substrate emulsions induced the formation of precipitates (tributyrin, ρ = 1.03 g/cm^3) or a less dense layer on top of the emulsion

(olive oil, ρ = 0.917 g/cm^3). This visual inspection was afterwards confirmed by turbidity measurements where addition of CCD-ANGPTL4 resulted in a dose-dependent decrease in optical density. Additionally, ITC, SPR, and fluorimetry experiments demonstrated that CCD-ANGPTL4 interacts with components of substrate emulsions. We observed heat effects when CCD-ANGPTL4 is injected into olive oil emulsions or saw interactions between CCD-ANGPTL4 and NaTDC, phospholipids, and DGGR. Interestingly, changes in turbidity and the biggest shifts in heat effects appeared at the same concentration range where inhibition of PL was previously detected. Furthermore, CCD-ANGPTL4 failed to inhibit PL when preincubated together prior to dilution into substrate mixture. This is unlike LPL, where a 10-fold lower concentration of CCD-ANGPTL4 resulted in a 90% loss of LPL activity. Taken together, we have demonstrated that CCD-ANGPTL4 inhibits PL by destabilizing substrate emulsions through direct interactions with its components.

No data has surfaced before or after our publication on the interaction between ANGPTL4 and intestinal lipid emulsions. However, basic [233] or hydrophobic proteins [234,235] have been previously demonstrated to accumulate on lipid surfaces and block adsorption of PL. CCD-ANGPTL4 contains a positively charged stretch of amino acids, has two amphipathic α-helices, and has been shown to bind heparin [166] and fatty acids [232]. It is therefore likely that CCD-ANGPTL4 interacts with emulsion components through these structural characteristics. This is further demonstrated by the fact that we saw how the positively charged EPL or hydrophobic EGCG interacts similarly with emulsions, resulting in their destabilization.

The apparent direct inhibition of PL by ANGPTL4 observed by Matijssen et al. is likely also caused by changes in the substrate emulsion [229]. Their assay for measuring PL activity uses a fluorescent emulsified substrate (Roar LPL Activity Assay Kit) which would be similarly exposed to the destabilizing effect of ANGPTL4. They saw a 60% reduction in PL activity using 226 nM full-length ANGPTL4. This submicromolar value does not differ much from our measurements with CCD-ANGPTL4 and DGGR. Any deviations could be caused by using full-length mouse ANGPTL4 instead of human CCD-ANGPTL4 or by using a different substrate system.

The ITC assay can reveal differences between using water-soluble substrates or physiological-like emulsions

Comparison of PL inhibitors in three different substrate systems revealed that their effect is dependent on the environment of the measurement assay (**Table 7**). THL is a competitive inhibitor of PL which could explain its weaker effect in substrate systems where binding of PL to substrate is not hindered by bile salts or phospholipids, as our DGGR assay did not contain any other components besides substrate. An opposite effect was observed for CCD-ANGPTL4, EPL, and EGCG, which had to be at a higher concentration in tributyrin or olive oil than in DGGR to achieve the same inhibitory impact on hPL. Only 2-fold differences between DGGR and other substrates were detected for CCD-ANGPTL4. As demonstrated by our binding experiments, CCD-ANGPTL4 indiscriminately interacts with most lipid compounds. This could explain why inhibition of PL happens at similar concentrations regardless of the substrate system used. However, there were massive variations in IC_{50} for EGCG and EPL, depending on the substrate system used.

EGCG has been shown to reduce the solubility of micelles consisting of phosphatidylcholine, bile salt, and cholesterol [258]. The authors proposed a hydrophobic interaction between bile salts and EGCG. This could explain why higher concentrations of EGCG were needed in our experiment with tributyrin/4 mM NaTDC compared to olive

oil and 0.4 mM NaTDC, as less EGCG is needed when the bile salt concentration is reduced. This correlation can also be seen in an assay where bile salts were not used and EGCG was more potent [301]. The authors recorded an IC_{50} of 55.00 ± 0.50 μM for EGCG-mediated PL inhibition which is considerably lower than our results with environments including bile salts. Interestingly, unlike in the case of CCD-ANGPTL4, we also observed a direct inhibition of PL by EGCG when the two were preincubated prior to injection into substrate. A formation of an inactive complex between PL and EGCG has also been previously observed [305]. This demonstrates that even the same compound may have multiple inhibitory mechanisms, solidifying the role of investigating PL inhibitors in a more physiological-like environment.

Table 7. *IC_{50} values of PLPNLIP inhibitors measured in three different substrate systems.*

Inhibitor	**IC_{50}**		
	DGGR	Tributyrin/4 mM NaTDC	Olive oil/0.4 mM NaTDC/lecithin/cholesterol
CCD-ANGPTL4	0.60 ± 0.27 μM	1–2 μM	1–2 μM
EPL	No effect detected	332–375 nM	16.6 ± 3.8 μM
EGCG	1.03 ± 0.20 μM	1.5–2 mM	220.8 ± 50.9 μM
THL	3.08 ± 1.16 μM	217.0 ± 37.6 nM	12.0 ± 1.9 nM

Surprisingly, EPL had no inhibitory effects when DGGR was used as a substrate. Instead, EPL was a potent inhibitor in the tributyrin/NaTDC system and about 50-fold weaker when olive oil was used. This could indicate that the effect of CCD-ANGPTL4 in the DGGR system is not mediated by a positive charge but rather through hydrophobic interactions as seen with EGCG. The inhibitory effect of EPL has previously been reported to be independent of PL concentration, indicating an indirect mode of action [290]. The authors also saw that the effect of EPL was strongly dependent on the substrate system — use of anionic surface components hindered the effect of EPL. They proposed that EPL forms a surface-active complex with anionic taurocholate which can then bind to a zwitterionic phosphatidylcholine interface. This effect was also observed with a synthetic non-ionic surfactant (Tween 20), where inhibition of PL by EPL was greater compared to emulsions with an anionic detergent [306]. Similarly, our results with EPL demonstrate increased potency in a neutral tributyrin/anionic NaTDC system compared to emulsions with olive oil and lecithin that contain a degree of anionic phospholipids.

In addition to the differences in IC_{50} of PL inhibitors between substrate systems, the kinetic curves for inhibitors that interact with substrate components were also highly different (**Figure 8**). The composition and stability of substrate environments can play a major role in how PL inhibitors destabilize the emulsions. Inhibition of PL by EGCG or EPL happened over a wider range of concentrations and appeared as a more typical inhibition curve when the olive oil/0.4 mM NaTDC/lecithin/cholesterol mixture was used (**Figure 8**, left column). In the case of tributyrin, acute changes in PL activity and emulsion stability appeared in a narrow concentration range (**Figure 8**, right column).

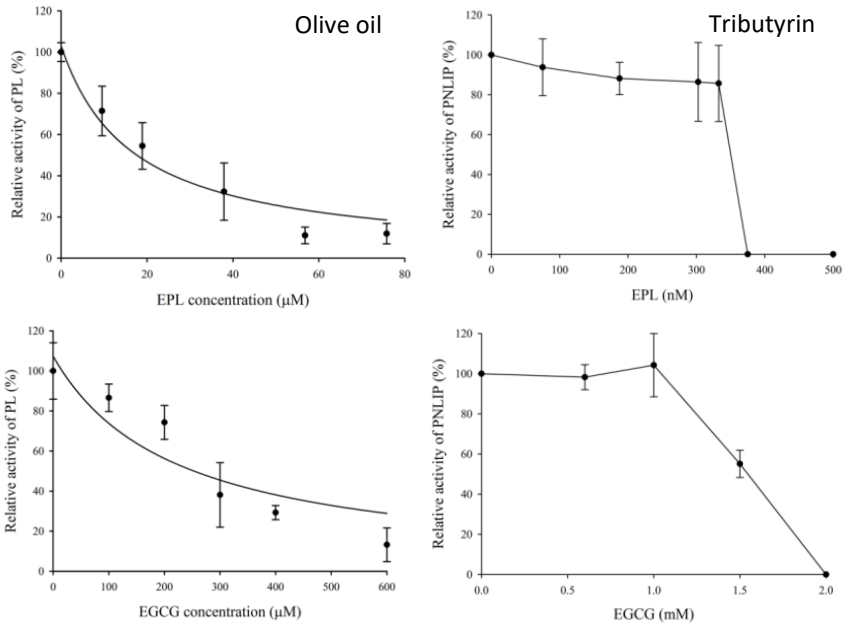

Figure 8. *Comparison of EGCG and EPL inhibitory curves in olive oil/0.4 mM NaTDC/lecithin/cholesterol (left column) or tributyrin/4 mM NaTDC (right column). Inhibitors were mixed with emulsions and loaded into the ITC cell, after which five sequential injections of 0.36 nM HPL were conducted. The choice of substrate system greatly affected the efficacy and kinetics of PL inhibitors.*

Perspectives of using ITC to determine PL activity from human serum for the detection of pancreatitis

Measurement of serum PL for diagnosis of pancreatitis is preferred over amylase tests and could help reduce costs in healthcare systems [204]. Current colorimetric assays (DGGR, triolein) for PL suffer from problems with specificity as they have been demonstrated to provide false positive results [307]. In our case, the use of high concentrations of NaTDC should suppress the activity of pre-heparin LPL [308], providing specificity for PL. Even the low 100 to 400 pM concentrations of PL in healthy human plasma should be sufficient for determination of PL activity in our assay thanks to the extremely high sensitivity provided by ITC. However, our initial results revealed that PL incubated in human plasma was inactive towards the substrate emulsions. Injection of plasma with THL and without PL into 2% tributyrin/0.4 mM NaTDC resulted in a continuous heat effect and the appearance of precipitates. This indicates that there was a constant heat flow that did not originate from lipase activity. A possible cause would be the presence of fibrinogen in plasma which has been shown to associate with olive oil droplets and cause aggregation with thrombin [309]. Additionally, our injections of plasma also introduce 0.75 mg/ml albumin to the substrate mixture which has been shown to inhibit PL by accumulating at the oil-water interface [235]. It has been shown that using very high concentrations of colipase (250-fold molar excess) helps overcome the inhibition of PL by human serum [251]. In the same study, they also used 20 mM DOC and observed that a 1110-fold excess of colipase is even more potent at activating lipase. The 84 nM colipase in our emulsions is in a 237-fold molar excess of PL which should be

sufficient for eliminating the effects of human plasma. It is therefore possible that our limitations could be overcome by switching from plasma to serum which does not contain fibrinogen, and by using high concentrations of bile salts to prevent adsorption of albumin to the surface of substrate.

4.2 Evaluating the efficacy of potential triglyceride-lowering drug candidates in human blood plasma using ITC (Publication II)

Our work here explores the capabilities of using a calorimetric approach in a physiological-like setting to study a potential triglyceride-lowering peptide that activates LPL. An apoC-II mimetic peptide, dubbed 18A-CII-a, has been previously shown to lower plasma triglycerides in various animal models with HTG [248,281,282]. The authors demonstrated that 18A-CII-a activates LPL similarly to wild type apoC-II when Intralipid or highly diluted mouse/human plasma (1:50 to 1:250 final dilution) is used as a substrate. In addition to dilution of plasma samples, LPL activity in these cases was monitored using a NEFA kit which does not enable continuous measurement of lipolysis or initial reaction rates. As ITC addresses these aspects, we sought to characterize the 18A-CII-a peptide using real-time measurements in nearly undiluted human plasma.

18A-CII-a activates LPL in normolipidemic and hypertriglyceridemic plasma

We first investigated the effects of 18A-CII-a or apoC-II on exogenously added LPL activity in plasma obtained from healthy volunteers or patients with HTG. Our experimental setup was based on the description given in **Figure 6** under chapter 1.9. This allowed us to quantify the effects of LPL activators in an environment that is macromolecularly crowded and contains relevant concentrations and ratios of physiological LPL regulators. Measurements of initial reaction rates under zero-order conditions demonstrated that both 18A-CII-a and apoC-II could activate LPL in normolipidemic plasma, starting from 5 µM and 10 µM concentrations, respectively. However, the effect of apoC-II saturated when added concentrations exceeded 20 µM in normolipidemic plasma. Going even higher and using additions of 40 to 50 µM apoC-II started to have a negative effect on LPL activity compared to baseline levels of apoC-II. At the same time, addition of 50 µM 18A-CII-a increased LPL activity even further, by roughly 77%. Addition of apoC-II failed to increase LPL activity in plasma from HTG patients while 18-CII-a had a moderate statistically significant effect. This difference with normolipidemic plasma could be explained by the increased concentration of apoC-II in hypertriglyceridemic plasma [100]. An inactive analog of the mimetic peptide (18A-CII-I) that contains four Ala substitutions in the LPL-activating region was also tested. The peptide decreased LPL activity up to 32% at 50 µM concentration in plasma from healthy volunteers. Lastly, concerns for immunogenicity have been raised because of the synthetic nature of the 18A helix [115]. As such, we have also tested an approach where the 18A peptide was replaced with a FFA as a possible alternative to 18A-CII-a (unpublished data). However, our initial results did not provide satisfactory results at concentrations comparable to the mimetic peptide or full-length apoC-II.

ITC reveals that human plasma is not always saturated with apoC-II

It was a surprising result that the addition of apoC-II up to 20 µM is beneficial to LPL activity. The concentration of plasma apoC-II in healthy people is in the range of 2 to 7 µM [85] and this has been thought to be sufficient for full activation of LPL [53,100].

Additionally, previous measurements with ITC and Intralipid showed that already 100 nM apoC-II yields maximal LPL activity [240]. This is in accordance with the observation that heterozygotes for apoC-II deficiency have normal circulating triglyceride levels [100]. Instead, it has been observed that high concentrations of apoC-II have a positive correlation with plasma triglyceride levels [100,102]. Experiments with Intralipid [310] and triolein [102] saw inhibition of LPL by excess apoC-II already at concentrations as low as 2.2 µM or 17 µM, respectively. This is considerably lower than our results obtained with undiluted human plasma, indicating that the substrate and measurement environment can noticeably affect how regulators influence LPL activity. Furthermore, since addition of apoC-II up to 30 µM could increase LPL activity, it seems that LPL is not always saturated by apoC-II in some individuals. A similar effect was seen in mice who were injected recombinant apoC-II which led to moderately decreased plasma triglyceride levels [311]. Moreover, intravenous injection of apoC-II mimetic peptide to wild type mice helps overcome post-prandial hypertriglyceridemia when mice are administered Intralipid [282]. The beneficial effect of increased apoC-II concentrations has also been demonstrated by Li et al who noticed lowered plasma triglyceride levels in mice after enhancing apoC-II expression [312]. Our findings also suggest that previously recorded cases of high plasma triglyceride levels arising from increased apoC-II concentrations [100] are not the result of reduced LPL activity on TRLs. Rather, it is possible that superfluous apoC-II instead hampers TRL clearance by blocking apoE and apoB-mediated binding to hepatic receptors [100] or by interfering with the margination of circulating TRLs to endothelial-bound LPL [102].

Following kinetics of complete lipolysis using ITC can reveal additional information about the efficacy of LPL regulators

We used the calorimetric approach to investigate complete lipolysis of all available substrate in plasma for LPL after addition of apoC-II or mimetic peptides (**Figure 9**). Both kinetics of the lipolysis rate and total heat released could be followed. The former demonstrates how efficiently LPL hydrolyzes lipoproteins while the latter shows how much substrate is available to LPL. Additionally, lipoproteins in human plasma or lipid emulsions in the intestine are under constant change in their composition and surface properties because of ongoing lipolysis. Lipid components and apolipoproteins are released, and the size of the particles is continually decreasing while the surface pressure is increasing. Changes in the physical properties of lipoproteins greatly affects the subsequent efficacy of lipolysis [5,53]. Following complete lipolysis can help clarify any changes in the transformation of lipoproteins in response to lipid-binding lipase regulators. These continuous alterations in lipoproteins cannot be observed under zero-order conditions where the concentration of LPL is too low to induce a noticeable reduction in available substrate [240].

Our analysis revealed that the amount of total heat released is increased after addition of 18-CII-a or apoC-II as illustrated by the increased area under the curves in **Figure 9A**. Total heat production has been previously shown to be in a proportional relationship with the triglyceride concentration of the sample [240], indicating that the addition of activators increased the amount of available substrate for LPL. Furthermore, plotting the amount of heat released (available substrate) against the heat rate (reaction rate) allows to evaluate the efficiency of lipolysis (**Figure 9B**). It can be seen that the substrate available after addition of 18A-CII-a or apoC-II is hydrolyzed at a slower pace compared to reference plasma. This analysis also illustrates the additional information

that can be gained compared to measurements of initial reaction rates. Previous experiments under zero-order conditions demonstrated inhibition of LPL by 18A-CII-i, but here we can see that addition of 18A-CII-i does not affect the amount of substrate hydrolyzed. Interestingly, neither was the rate of lipolysis affected, despite the inhibition we previously observed at lower LPL concentrations.

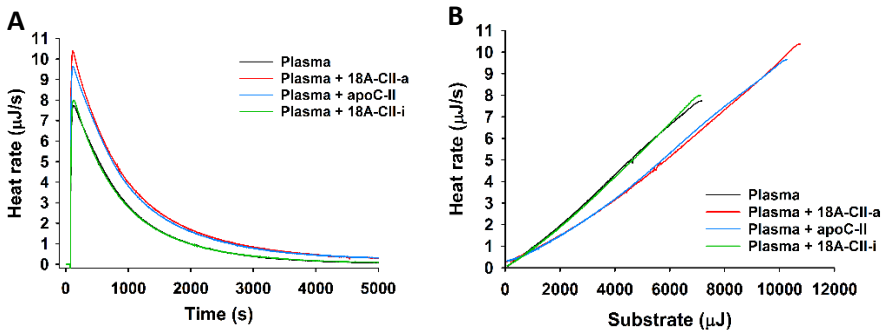

Figure 9. *Lipolysis of all available substrate for LPL as measured by calorimetry.* ***A*** *— A single injection of 10 nM LPL into human plasma with no added ligands (**black**) or 10 µM 18A-CII-a (red), 10 µM apoC-II (blue), 10 µM 18A-CII-I (green). Addition of 18A-CII-a or apoC-II increased the total amount of heat produced, indicating that more substrate became available to LPL.* ***B*** *— Values from panel A plotted as total heat released (area) against heat rate. This transformation of data demonstrates that the additional substrate gained with 18A-CII-a or apoC-II is less effectively hydrolyzed.*

Another advantage of ITC is that the reaction of complete lipolysis can be stopped at any time point and the hydrolyzed plasma can be removed from the ITC cell for further analysis. Because of the non-destructive nature of the assay, the lipoprotein content, FFA concentration, or distribution of drug candidates on lipoprotein classes can be detected. This could provide additional information about how the lipid or protein composition of lipoproteins has changed after certain time points, or how the therapeutic agent is distributed among lipoprotein classes at a given step in complete lipolysis. For example, by separating plasma after complete lipolysis, our unpublished data has shown that the degree of complete lipolysis can be highly variable between individuals. A few samples display a complete lack of TRLs after hydrolysis of all available substrate while others demonstrate only moderately reduced levels. The calorimetric assay can then provide information about whether only the amount of available substrate is reduced in some individuals or has the efficiency of lipolysis also decreased.

18A-CII-a stimulates LPL activity in isolated smaller size lipoproteins

The superior affinity of 18-CII-a towards some lipoprotein classes was observed in calorimetric measurements with lipoproteins isolated by SEC or ultracentrifugation. 18-CII-a was significantly more potent in activating LPL in remnant lipoproteins, LDL, and HDL than apoC-II. ApoC-II has been previously shown to dissociate from lipoproteins that have undergone lipolysis as this leads to the release of surface components and an increase in surface pressure [5]. The 18-CII-a peptide is designed to have very high affinity towards lipoproteins because of its synthetic 18A lipid binding region. This stronger interaction could allow the peptide to associate with lipoproteins that are unavailable to apoC-II because of increased surface pressure, even under saturation concentrations.

As a result, 18-CII-a could bind to many different lipoprotein subclasses while excess apoC-II preferentially accumulates on TRLs or HDLs.

Interestingly, 18-CII-a nor apoC-II stimulated the lipolysis of large TRLs, and apoC-II even mildly lowered LPL activity in TRLs and remnant particles. Kovrov et al have shown that LPL is most active against large VLDLs but suffers from reduced activity against small VLDLs [53]. It is possible that large TRLs are already saturated with apoC-II since apoC-II prefers surfaces with low crowding and surface pressure [53]. At the same time, less apoC-II is present on smaller TRLs, explaining why addition of 18A-CII-a could increase LPL activity in those fractions. On the other hand, addition of apoC-II slightly reduced LPL activity in smaller TRLs. The reduced activity of LPL in smaller TRLs might instead be explained by the non-productive manner of how apoC-II and LPL are bound to the surface. The C-terminal helix of apoC-II has been proposed to desorb first in response to increased surface pressure, rendering apoC-II unable to activate LPL [5]. Increasing the concentration of apoC-II might cause further rises in surface pressure by additional binding of apoC-II to lipoproteins, causing even more apoC-II to release their third helix. This effect is not seen with 18A-CII-a as it likely has a stronger affinity for lipoproteins and can activate LPL at higher surface pressures.

The results with 18A-CII-a contradict previous observations where biggest changes were noticed in VLDL after injection of 18A-CII-a to apoE-knockout mice [248] or apoC-II mutant mice [281]. However, it should be considered that the lack of apoE and apoC-II in these mice models can affect the potency of mimetic peptides. There is less competition for available lipid surface under these conditions, giving 18A-CII-a the possibility to interact with lipoproteins more readily. Still, our measurements were performed with isolated lipoprotein fractions where the exchange of apolipoproteins, cholesteryl esters, and triglycerides does not happen between lipoprotein classes. Moreover, use of isolated lipoproteins could skew how well LPL hydrolyzes certain fractions. For example, while LPL has been shown to have some activity against HDL in an solitary setting, the activity is minimal under more physiological-like conditions [313]. Measurements were also based on lipoproteins obtained from a single donor. Exogenous LPL activity and the distribution of lipoprotein classes is known to vary by a large margin between individuals [53]. It would be interesting to repeat our experiments with multiple patients or pooled samples, and additionally, reintroduce isolated lipoproteins to lipoprotein free plasma, providing a natural matrix without interference from other lipoproteins.

The notion that 18A-CII-a activates LPL in remnant lipoproteins and LDL supports the use of the peptide as anti-atherogenic agent. Remnant lipoproteins and LDL both contain cholesterol and provide a similar increase in the risk of developing ASCVD [30]. 18A-CII-a makes these atherogenic lipoproteins more readily hydrolysable, reducing their residence time in circulation and decreasing inflammatory responses. Intriguingly, 18A-CII-a also increased LPL activity in HDL fractions. Larger HDLs with increase triglyceride content are associated with metabolic syndrome and risk of CVD [313]. Furthermore, LPL activity is lower against these detrimental larger HDLs [53]. Stimulation of LPL activity by 18A-CII-a might reduce the triglyceride content of HDLs and prove beneficial in combating CVD.

Application of the calorimetric method in personal medicine and drug development

As stated previously, 18A-CII-a was a significantly more potent activator of LPL compared to apoC-II, which differs from what has been previously observed where both had a comparable effect on in vitro lipolysis [248]. The key difference of our assay is the use of nearly undiluted human plasma compared to the 50 to 250-fold dilution used in previous assays with 18A-CII-a. It is clear that the measurement environment, as well as the substrate concentration and composition can greatly affect how regulators influence lipases. This was also seen in Publication I where the effect of PL inhibitors was considerably varied between different substrate systems. Therefore, an approach is required that could enable the measurement of lipolytic activity in more complex and crowded situations.

Multiple clinical uses can be envisioned for our ITC assay as demonstrated here and in a published work where exogenous LPL activity in plasma samples from over thirty patients were analyzed by ITC [314]. The calorimetric assay enables us to rapidly evaluate how the overall composition of patient plasma affects LPL-mediated lipolysis. Each person has their own ratio of LPL activators and inhibitors, as well as a distinct distribution of lipoprotein subclasses and plasma FFAs. Kovrov et al used ITC with undiluted human plasma and demonstrated that added LPL activity was mostly dependent on the size of VLDL and HDL — greater LPL activity was seen in samples with increased concentrations of larger VLDL and smaller HDL particles [53]. Saturated FFAs were also positively correlated with LPL activity. This matches our unpublished results where we compared exogenous LPL activity in serum taken from people following vegan or omnivorous diets. However, Kovrov et al did not observe strong correlations between plasma LPL regulators and LPL activity. It is possible that the circulating concentrations of ANGPTLs in fasting normolipidemic plasma are too low to affect exogenous LPL activity. ANGPTLs are thought to regulate LPL activity locally — ANGPTL4/ANGPTL8 in adipose tissue, and ANGPTL3/ANGPTL8 in skeletal muscle [14,140]. Circulating ANGPTL4 is mostly in its inactive FLD-ANGPTL4 form, and ANGPTL3 is likewise greatly reduced in potency when released without ANGPTL8 under fasting conditions. It is more likely that the calorimetric approach could help identify individuals with abnormal concentrations of regulators rather than detect minute changes arising from fluctuations in normal concentration ranges. Still, moving forward, it is worth considering whether the assays should be carried out at 37°C as LPL is known to be more affected by its regulators at body temperature compared to room temperature [81,187].

A surprising phenomenon that has shined through from every cohort that we have studied illuminates how individuals, who possess similar plasma triglyceride concentrations, can have multiple fold differences in exogenous LPL activity. We observed this in a collaboration with Metz et al [314], as well as in healthy volunteers or patients with T2D [315], and in our unpublished data with healthy people following a vegan diet. It is clear that triglyceride concentration is not the deciding factor for LPL activity. ITC could help ascertain whether heightened triglyceride levels are the result of an unfavorable composition of plasma or caused by other factors, such as defects in the patient's LPL system, reduced lipoprotein clearance or increased TRL production.

Finally, as demonstrated in the current work with CII-18-a, ITC can be successfully used to gauge the efficacy of synthetic LPL activators in undiluted human plasma. Potential drug candidates can be screened in a physiological environment where other LPL regulators are present and multiple lipoprotein (sub)classes are affected simultaneously. Furthermore, the usefulness of available drugs could be evaluated in personal medicine

— pre-heparin plasma is obtained from a patient, and the effect of a compound on exogenous LPL activity can be directly studied in that specific plasma. Additionally, changes in plasma composition in response to medication that affect LPL activity could be detected. Ideally, post-heparin plasma would be also drawn from the same patient, resulting in a laboratory assay where all major components of endothelial lipolysis are derived from the patient under question. This could be used to evaluate how patients respond to certain treatments and could help fine-tune strategies for combating hypertriglyceridemia by increasing LPL activity.

4.3 Combined action of albumin and heparin regulates lipoprotein lipase oligomerization (Publication III)

The aim of this study was to investigate the synergistic effect of albumin and heparin on LPL oligomerization. LPL has been shown to form oligomeric structures in the presence of HSPGs inside vesicles prior to secretion to the interstitial space [37]. However, it was not established how LPL dissociates into its active form after secretion or how the composition of the subendothelial space influences LPL oligomerization. We demonstrate that albumin, which is abundant in the interstitial space together with HSPGs, directly interacts with LPL and is capable of dissolving oligomeric LPL helices induced by heparin.

ITC revealed changes in LPL activity that were not observed with synthetic substrates

As previously discussed, LPL inside the cell, in the interstitial space, or on the endothelial surface can be influenced by a horde of regulators or by the macromolecular crowding environment.

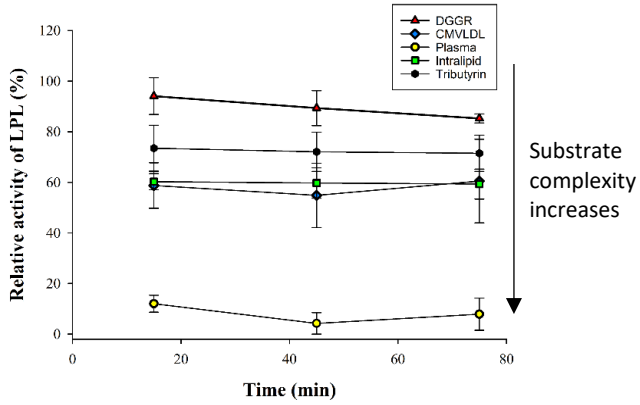

Figure 8. *LPL activity measured in various substrate systems after incubation with albumin. The activity of LPL is provided relative to incubation with both heparin and albumin, as LPL activity was observed to be the highest and most stable under those conditions. The apparent change in LPL activity was larger in more complex substrate systems, with human plasma being highly different from other substrates.*

Additionally, LPL can exhibit both esterase and lipolytic activity, depending on the substrate used. Our initial goal was to examine LPL activity in different substrate systems to assess their use in studying LPL and its regulators. We used albumin (BSA) as an LPL stabilizer prior to diluting the enzyme into substrate, as our measurements with DGGR had demonstrated that albumin can keep LPL active for at least 75 minutes (**Figure 8**, red

triangles). ITC enabled us to determine LPL activity in four different substrate systems with increasing complexity, tributyrin<Intralipid<mixture of TRLs<undiluted human plasma. Surprisingly, results in other substrate systems were discordant with those observed with DGGR, as major differences in residual LPL activity were detected. The biggest drop in LPL activity was observed with undiluted human plasma (**Figure 8**, yellow circles), indicating that this medium is the most sensitive to fluctuations in LPL activity. It was clear that there are changes in the conformation of LPL that are triggered by the presence of albumin, and that these changes are perceived differently based on which substrate system is used to determine LPL activity.

Albumin directly interacts with LPL and influences the enzyme's stability

The variable activity of LPL in the presence of albumin raised a question of what happens to LPL that would warrant these differences between substrate systems. Additionally, is the interaction between albumin and LPL specific or are the changes merely a result of macromolecular crowding as seen with multiple other enzymes [238]?

We compared LPL stability with albumin to two widely used macromolecular crowders, PEG or dextran, and to various proteins (casein, β-lactoglobulin, lysozyme). Macromolecular crowding did not stabilize LPL to a considerable extent and neither did it have any effect on LPL activity when measured with a mixture of CM/VLDL. On the other hand, casein and β-lactoglobulin were comparable to albumin in their ability to stabilize LPL while lysozyme was not. LPL has a large basic patch spanning both of its domains that interacts with many molecules which are negatively charged [55,56]. LPL also contains many exposed hydrophobic residues in the lid and CTD. A common motif of the proteins we assayed is their hydrophobicity, but this does not explain why lysozyme could not stabilize LPL. Instead, the negative charge of albumin, casein, and β-lactoglobulin at pH 7.4 could be the deciding factor as lysozyme is instead strongly positively charged at this pH.

A direct interaction between albumin and LPL was confirmed by ITC, SPR and thermostability measurements. The latter indicated that detectable stabilization of LPL begins already at albumin concentrations as low as 0.4 mg/ml and all three methods demonstrated binding at physiologically relevant albumin concentrations. SPR also showed that the interaction between albumin and LPL is short lived, with a complex lifetime of less than a few seconds. We had confirmed that the stabilization of LPL by albumin observed with the DGGR assay is a result of a direct interaction. However, this did not explain why LPL remains active in the DGGR system but not in other substrate environments. Heparin is another well-known stabilizer of LPL [43], so we compared the activity of LPL with heparin to LPL with albumin using ITC and undiluted human plasma as a substrate. Surprisingly, even with heparin, LPL activity was still lowered instantly when measured in a physiological setting, but not when DGGR was used.

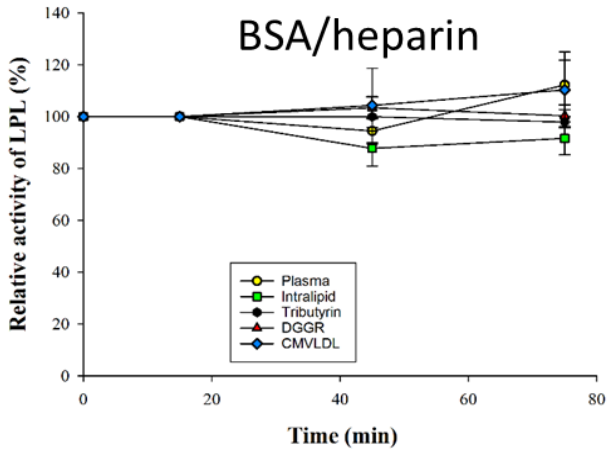

Figure 9. *LPL activity measured in various substrate systems after simultaneous incubation with both albumin and heparin. The differences observed previously in incubations with only albumin are gone when heparin is added.*

However, when we combined both albumin and heparin, we obtained the best stability and activity in every tested substrate system (**Figure 9**). Based on previous published data that LPL conforms to an inactive oligomeric state in the presence of heparin [37], we hypothesized that the same might be true for LPL with albumin as both incubations resulted in a similar loss of activity when human plasma was used. Additionally, we saw that heparin-induced loss of LPL activity is reversible by diluting the LPL oligomers into buffer containing albumin, LFHP, or Triton X-100. Likewise, when heparin was added to an incubation with LPL and albumin, activity was once again returned to its maximal value. This would explain why LPL activity is highest when heparin and albumin are combined, and why differences were not observed with the DGGR assay as it contains Triton X-100.

Combination of albumin and heparin regulates LPL oligomerization

We used the fluorescent diffusion-based method of raster image correlation spectroscopy (RICS) to examine the size of dye-conjugated LPL particles in solution, and negative stain transmission electron microscopy (nsTEM) to visualize unlabeled LPL on grids. Results with RICS showed large aggregates of LPL in buffer with albumin under conditions used for measurements of LPL activity using ITC. Addition of heparin to this solution homogenized the LPL particle size distribution observed with RICS. This effect coincided with the notion that LPL activity is restored after addition heparin to an incubation with albumin. The same conditions were also probed with nsTEM and revealed similar changes in LPL structure — large aggregates of LPL were present with albumin, but they disappeared after addition of heparin.

Gunn et al previously demonstrated that LPL forms large reversible helical structures at concentrations above 1 μM, and that these inactive oligomers are stabilized by heparin when LPL is diluted down to 31 nM [37]. Our results with ITC indicated that 200 nM LPL loses over 60% of its activity in 15 minutes after incubation with heparin. Because this loss of activity could be reversed, we postulated that 200 nM LPL could also form reversible inactive helical structures in the presence of heparin. However, RICS did not reveal particles of LPL with heparin whose size would be akin to those observed with LPL

and albumin. The diffusion coefficients were rather similar to those seen with active LPL (heparin + albumin) or with LPL at 10 nM concentration. It is likely that the 200 nM concentration of LPL is too low for the initial formation of proper helical structures. Rather, LPL must be already oligomeric and stabilized by heparin at higher concentrations, only then can it be diluted to a nanomolar range while maintaining its helical form. While there were some helices detected by nsTEM at this concentration, most of the LPL seems to be in a smaller oligomeric or inactive dimeric form as revealed by RICS. Our unpublished data has previously also shown dimeric or oligomeric LPL under high salt concentrations. It was obtained via crosslinking by using disuccinimidyl tartrate at 4 °C and in buffer containing 1 M NaCl. Mass-spectrometry analysis of the crosslinked molecules revealed that LPL can form dimers in the head-to-tail or tail-to-tail orientation. Lastly, Gunn et al also observed stabilization of LPL helices by NaCl as low as 575 mM [37].

It has been previously demonstrated that LPL filaments can be broken down in vitro by substrate (Intralipid). However, interstitial space does not contain substrate for LPL, so it was proposed that dilution is the in vivo driving force behind oligomer dissociation. Our results with ITC contradict this effect because we are performing a 40-fold dilution of reversibly inactive 200 nM LPL into substrate, but LPL activity is not restored. From our work and previously published data, it is apparent that the formation of LPL oligomers is highly dependent on the concentration of LPL. However, it is difficult to predict the local concentration of LPL on the parenchymal cell surface or interstitial space. It is possible that dilution helps dissociate LPL oligomers, but this effect is highly dependent on the initial LPL concentration and dilution factor. At the same time, we observed that dilution is not necessary when heparin is added to LPL with albumin or that albumin dissolves heparin-induces LPL helices with minimal dilution.

Oligomerization of LPL protects it from ANGPTL4

ANGPTL4 and LPL colocalize inside adipose cells [191] and in the subendothelial space [186], providing ample chance for ANGPTL4 to inactive LPL [170,189,190] even before LPL reaches the luminal side of the endothelium. HDX-MS experiments have demonstrated that CCD-ANGPTL4 binds LPL near the active site lid [165]. However, considering that this region is sterically obstructed in the interface of inactive dimers that make up the helical LPL oligomer [37], we hypothesized that triggering LPL oligomerization by heparin or albumin would protect the enzyme from ANGPTL4-induced inactivation. Measurements of LPL activity with human plasma confirmed our suspicions. ANGPTL4 reduced LPL activity more effectively when LPL was fully active, i.e., incubated with both heparin and albumin. Additionally, ANGPTL4 inhibited LPL similarly under all conditions when LPL concentration was lowered to the point where oligomerization does not happen. LPL activity was not fully protected under oligomeric conditions, but it is possible that some portion of LPL remains active during incubation. The LPL concentration we used was considerably lower than previously demonstrated to be needed for initial oligomer formation [37]. Considering these results, we propose that the oligomeric form of LPL protects vesicle-stored LPL from intracellular inactivation by ANGPTL4. This could be a fail-safe to protect LPL from unnecessary degradation under feeding conditions since low levels of free ANGPTL4 are still present in addition to ANGPTL4/8 complexes [140]. Lastly, LPL transcription is not significantly altered by fasting-feeding cycles [42,62], therefore it is possible that this mechanism also ensures that a pool of vesicle-stored LPL remains quickly available in the presence of ANGPTL4 when transitioning from fasting to feeding.

How does albumin affect LPL oligomerization and interactions with heparin?

Several observations in three different experimental setups for SPR indicated that the interaction between albumin and LPL is not limited to the basic patch of LPL. Firstly, the presence of negatively charged heparin did not interfere with binding of albumin to GPIHBP1-bound LPL. Additionally, there is 35-50 mg/ml [237] of albumin in human plasma but this does not dissociate LPL bound to endothelial cell surface HSPGs or GPIHBP1. Secondly, heparin is known to compete with the NTD of GPIHBP1 for binding to LPL [41,71] but we did not observe any dissociation of LPL from the sensor chip when heparin was introduced to the running buffer. This hints that LPL was bound to the LU domain of GPIHBP1 via its hydrophobic region in the CTD as observed in published crystal structures of LPL-GPIHBP1 [55,56]. As albumin was still able to interact with LPL and did not dissociate LPL from GPIHBP1, it is possible that this hydrophobic region does not play a major role in the interaction between albumin and LPL. Thirdly, albumin could not bind to LPL immobilized via the 5D2 antibody. This antibody has been pinpointed to bind to the Trp-rich loop located on the other side of the CTD than the hydrophobic region where the LU domain of GPIHBP1 is bound [316]. Lastly, we also noticed that the binding of 5D2 to GPIHBP1-bound LPL was severely diminished in the presence of albumin (data not shown in publication). Taken together, stability and SPR experiments seem to indicate that albumin binds to the Trp-rich loop of LPL while also simultaneously interacting with the positively charged region. Furthermore, the binding between LPL and albumin did not reach saturation in our experiments, indicating that multiple albumin molecules can bind to LPL simultaneously.

The Trp-rich loop has been shown to be a crucial part of the interface that forms between inactive LPL dimers in the helical oligomeric structure of LPL with heparin [37]. As we have observed albumin to compete for this region with 5D2, it is possible that albumin dissolves LPL filaments by disrupting the interaction between the inactive dimers. On the other hand, electrostatic interactions between albumin, casein or β-lactoglobulin are important for the interaction with LPL as observed with stability measurements and SPR. This suggests that this interaction is important for the formation of reversible unstructured LPL aggregates that were observed with nsTEM and RICS. Heparin has high affinity for this region [43] which could interfere with the interaction between albumin and LPL, resulting in the dissociation of inactive LPL aggregates into active LPL as demonstrated by ITC and confirmed by nsTEM and RICS. Considering these observations, we propose an in vivo model for the oligomerization of LPL in the interstitial space (**Figure 10**). As previously demonstrated, LPL is tightly packed in SDC1 vesicles as helical oligomers consisting of inactive LPL dimers [37,38]. Upon signaling, oligomeric LPL is secreted into the interstitial space of parenchymal cells where albumin and HSPG are both plentiful [77,317]. Albumin dissolves the HSPG-stabilized LPL helices, and this process is accelerated by the dilution of LPL. Subsequently, albumin also stops the reformation of oligomers when LPL is traversing the HSPG-filled space. At the same time, interstitial HSPG blocks the formation of albumin-induced LPL aggregates, ensuring the transportation of active LPL monomers or dimers to the surface of endothelial cells via GPIHBP1. Additionally, the oligomeric form of LPL inside secretory vesicles protects it from inactivation by intracellular ANGPTL4. During fasting, ANGPTL4 can still act on LPL in the ER and Golgi of adipocytes [191] or inactivate interstitial LPL that has been unnecessarily secreted and converted into monomers/dimers by albumin and HSPGs.

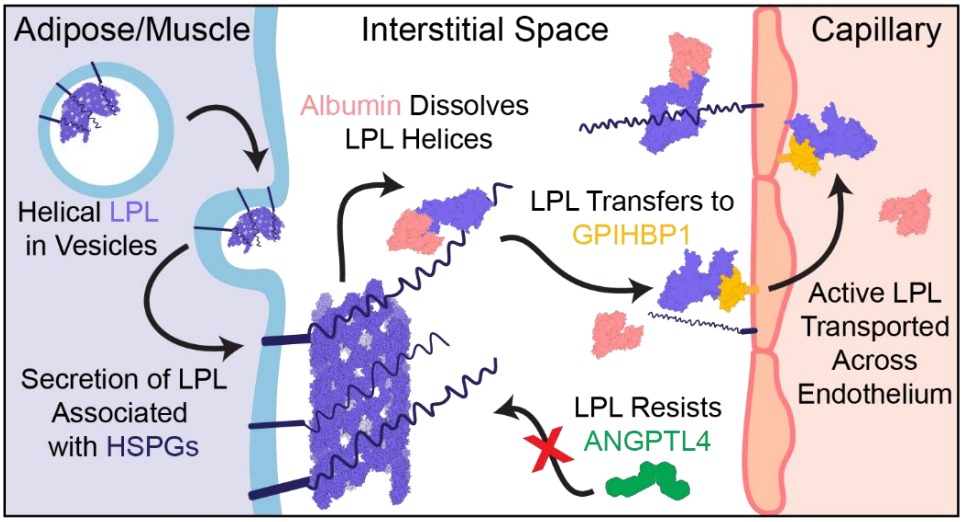

Figure 10. *Proposed model for the oligomerization of LPL. LPL forms oligomeric helical structures inside vesicles. These oligomers are broken apart by albumin after secretion to the interstitial space. The combined action of albumin and subendothelial HSPGs keeps LPL in its active form and stops LPL from aggregating. The oligomeric form of LPL is resistant to ANGPTL4-mediated inactivation.*

CONCLUSIONS

- Calorimetry can be used to measure PL activity in real-time with picomolar sensitivity using physiologically relevant emulsions. Furthermore, the composition of substrate emulsions can affect the efficacy of PL inhibitors. Contradicting results could be obtained between synthetic and physiological substrates.

- A novel mechanism for inhibition of PL by ANGPTL4 was proposed. CCD-ANGPTL4 inhibits PL indirectly by destabilizing emulsions through interactions with components of substrate emulsions. This effect is similar to two other known PL inhibitors — EGCG and EPL.

- Calorimetry is an effective tool for studying potential anti-hypertriglyceridemic drug candidates in undiluted human plasma using continuous measurements of LPL activity. This approach provides a natural matrix of lipoproteins and LPL regulators at physiological concentrations and ratios.

- The calorimetric approach demonstrated that apoC-II mimetic peptides could be used as potential triglyceride-lowering therapies with anti-atherogenic properties. The mimetic peptide was shown to increase LPL activity in normolipidemic and hypertriglyceridemic by increasing the efficacy of lipolysis in all lipoprotein classes.

- A mechanism was proposed where interstitial oligomerization of LPL is regulated by the combined action of HSPGs and albumin. LPL is secreted to the interstitial space in an oligomeric form and albumin induces the dissociation of these oligomers into active LPL monomers or dimers. Albumin and subendothelial HSPGs keep LPL in its active form as LPL traverses the interstitial space towards endothelial GPIHBP1.

- The oligomeric form of LPL protects it from intracellular degradation induced by ANGPTL4. This could assist in ensuring secretion of active LPL under feeding conditions or storage of LPL during fasting.

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ABSTRACT

Calorimetry as a Tool for Mechanistic Studies of Lipolytic Enzymes under in vivo like Conditions

The leading cause of death in the world are cardiovascular diseases (CVD). Hypertriglyceridemia (HTG), characterized by elevated plasma triglycerides, has been identified as an independent and causal risk factor for CVD by numerous epidemiological and genetic studies. HTG can also cause hepatic steatosis and life-threatening acute pancreatitis. Moderate HTG is a consequence of multiple polygenic reasons, life-style factors, and medical conditions, such as obesity and type 2 diabetes. Obesity and diabetes are extremely widespread in the modern world, with hundreds of millions of people affected. As a result, statistics show that one in four Europeans or half of the US population suffers from moderate HTG.

Absorption of dietary triglycerides is regulated by pancreatic lipase (PL) which catalyzes their hydrolytic breakdown into free fatty acids (FFA) in the intestine. PL is produced by pancreatic acinar cells and secreted to the duodenum in response to a meal. FFAs are resynthesized into triglycerides and packaged into triglyceride-rich lipoproteins (TRLs) in intestinal cells. These TRLs are sent into blood circulation where their triglyceride content can be readily hydrolyzed by endothelial-bound lipoprotein lipase (LPL) in adipose tissue, skeletal muscle, or heart. LPL is produced in parenchymal cells of the same tissues and transported to be attached to the luminal side of capillary endothelial cells. The body requires a constant influx of FFAs, therefore endogenous TRLs are produced and secreted by the liver even under fasting conditions.

Mechanistic studies of PL or LPL and their regulators are of great interest to pharmacological companies for developing new therapies that combat HTG. PL is targeted with inhibitors to reduce dietary triglyceride absorption and caloric intake in obese patients with HTG. At the same time, LPL activity is increased by activators or by suppression of endogenous LPL inhibitors to reduce the residence time of atherogenic lipoproteins in circulation. Additionally, accurate and rapid measurement of PL or LPL activity in blood is needed in clinical situations to respectively determine pancreatitis or causes behind HTG. Both PL and LPL function in highly crowded complex environments where a multitude of factors can influence their activity. Stemming from this fact, and considering the broad substrate specificity of both lipases, many assays have been developed for determining lipase activity. However, most approaches utilize water-soluble synthetic substrates for continuous measurements, otherwise sacrifices in sensitivity and throughput must be made to accommodate more physiological-like conditions.

The current thesis explores how a calorimetric approach can be utilized to study PL and LPL regulators in physiological-like or complex substrate environments. The first publication demonstrates that PL activity can be measured with picomolar sensitivity in conditions that contain crucial components of intestinal substrate emulsions. We also describe a novel indirect mechanism of action for angiopoietin-like protein 4 (ANGPTL4) mediated inhibition of PL activity. In addition to participating in LPL regulation, ANGPTL4 has been also identified in the intestine. However, its effect on lipid absorption has not been elucidated. We revealed that ANGPTL4 destabilizes substrate emulsions through interactions with lipid components. Similar mechanisms were observed for other known PL inhibitors, namely, epigallocatechin-3-gallate and ε-polylysine (EPL). Our comparison

of ANGPTL4 with three previously described PL inhibitors revealed that the choice of substrate system can have an enormous effect on the efficacy of PL inhibitors. For example, EPL did not exhibit any inhibitory activity against PL when using a synthetic substrate but was already effective at nanomolar concentrations when triglycerides and bile salts were used.

The second publication focuses on utilizing calorimetry to evaluate the efficacy of a potential therapeutic LPL activator (apolipoprotein C-II mimetic peptide) in nearly undiluted human plasma. This approach ensures a physiological environment for LPL with concentrations and ratios of lipoproteins and LPL regulators native to the capillary medium. Consequently, some of the results were strikingly different to previous observations made in 50 to 250-fold diluted plasma or simplified substrate systems. Addition of apoC-II or the apoC-II mimetic peptide (18A-CII-a) to human plasma increased LPL activity and the amount of substrate available to LPL. However, 18A-CII-a was considerably more effective than full-length apoC-II at increasing LPL activity in normolipidemic and hypertriglyceridemic plasma. The effect of 18A-CII-a was largest in smaller size lipoproteins, some of which have atherogenic properties. It was additionally revealed that contrary to previous observations, human plasma is not always saturated with apoC-II. In some cases, increasing the concentration of apoC-II can be a viable strategy to promote LPL activity. Taken together, these results support the use of calorimetry for studying potential therapeutic LPL agents in undiluted human plasma, and that apoC-II mimetic peptides could be used to combat HTG and reduce risk of CVD.

In the third and final publication of this thesis, calorimetric studies were combined with surface plasmon resonance, raster image correlation spectroscopy, and negative stain transmission electron microscopy to describe a novel mechanism where albumin and heparin regulate LPL oligomerization. Previous studies have shown that LPL is secreted to the interstitial space in an oligomeric form induced by heparan-sulphate proteoglycans (HSPGs). However, it was not known how these oligomers convert into active LPL. We demonstrated that albumin causes the dissociation of heparin-induced LPL oligomers into active LPL monomers or dimers. Meanwhile, heparin was capable of dissolving albumin-induced aggregates. This mutual mechanism could keep LPL in its active form as LPL traverses the interstitial space towards endothelial cells. Lastly, the oligomeric form also protected LPL from inactivation by ANGPTL4. This could assist in ensuring secretion of active LPL under feeding conditions or storage of LPL during fasting.

KOKKUVÕTE

Kalorimeetria rakendamine lipolüütiliste ensüümide mehhanismide uurimiseks

Südame- ja veresoonkonnahaigused (SVH) on peamiseks surma põhjuseks arenenud riikides. Mitmed epidemioloogilised ja geneetilised uuringud on näidanud, et kõrgenenud triglütseriidide tase veres ehk hüpertriglütserideemia (HTG) on sõltumatu ja põhjuslik ohutegur SVH kujunemisel. HTG võib lisaks veel põhjustada rasvmaksa ning eluohtlikku ägedat pankreatiiti. Piiripealne kuni mõõdukas HTG võib tekkida mitmete tegurite koosmõjul — polügeenne taust, ebatervislikud eluviisid või haiguslikud seisundid nagu diabeet ning rasvumus. Suurimaks HTG põhjustajaks ongi viimased kaks, sest sajad miljonid inimesed kannatavad rasvumuse või diabeedi all. Seda kinnitab ka statistika, mille järgi esineb HTG igal neljandal eurooplasel või ligikaudu poolel USA elanikkonnast.

Pankrease lipaas (PL) reguleerib toiduga saadud triglütseriidide imendumist soolestikus, lõhkudes neid vabadeks rasvhapeteks. PL-i toodetakse pankrease atsinaarrakkude poolt ja sekreteeritakse peensoolde toitumisest tuleneva signaali tõttu. Imendunud rasvhapped konverteeritakse enterotsüütide poolt tagasi triglütseriidideks ning pakitakse triglütseriidirikastesse lipoproteiinidesse (TRL). Valminud TRL-d saadetakse vereringesse, kus lipoproteiinlipaas (LPL) hakkab lagundama nendes sisalduvaid triglütseriide. Reaktsiooni tulemusena tekivad vabad rasvhapped, mida kasutatakse rasvkoes energia talletamiseks või lihaskoes ning südames energia tootmiseks. LPL toodetakse samade kudede parenhüümsetes rakkudes, misjärel ensüüm transporditakse vaskulaarse endoteeli pinnale. TRL-e toodetakse ka maksas, et kindlustada pidev vabade rasvhapete olemasolu vajalikes kudedes isegi paastunus olekus.

Farmaatsiaettevõtted on HTG raviks suunanud oma tähelepanu PL-i ja LPL-i uurimisele ensüümide keskse rolli tõttu triglütseriidide metabolismis. PL-i inhibeerimine ja seeläbi triglütseriidide imendumise takistamine on üks mooduseid kuidas vähendada toitumisjärgset hüpertriglütserideemiat. LPL-i puhul on eesmärgiks ensüümi aktiivsuse tõstmine või endogeensete inhibiitorite mõju vähendamine, et kiirendada TRL-de koristamist vereringest. Lisaks on olulisel kohal ka PL-i või LPL-i aktiivsuse määramine verest. PL-i puhul jälgitakse selle kaudu pankreatiidi ohtu ning LPL-ga seotud mõõtmised võimaldavad tuvastada HTG põhjuseid. Mõlemad ensüümid töötavad makromolekulaarselt tihedalt asustatud keskkondades, kus nende aktiivsust mõjutavad väga palju erinevad tegurid ja ligandid. Lisaks sellele on PL ja LPL ka laia substraadi spetsiifilisusega ning see on viinud suure hulga aktiivsuse määramise meetodite välja töötamiseni. Suurem osa lähenemistest, mis võimaldaks pidevat aktiivsuse mõõtmist, kasutavad vesilahustuvaid sünteetilisi substraate. Saadaval on ka meetodid füsioloogilisemate tingimuste ja substraatidega, kuid sellisel juhul kannatavad tihti tundlikkus ja läbilaskevõime.

Antud doktoritöö raames uuriti kalorimeetria kasutamise võimalusi PL-i ja LPL-i ning nende regulaatorite uurimiseks füsioloogilisele sarnastes või keerukates keskkondades. Töö esimeses osas näidati, et PL-i aktiivsust on võimalik pikomolaarse tundlikkusega mõõta segus, mis sisaldab soolestiku rasvemulsiooni komponente. Lisaks näidati, et angiopoietiini-sarnane valk 4 (ANGPTL4) inhibeerib kaudselt PL-i. ANGPTL4 on põhjalikult uuritud LPL-i endogeenne inhibiitor. Samas leidub ANGPTL4 ka soolestikus, kuid seal pole tema mõju toidurasvade lagundamisele täielikult teada. Töös pakuti uus mehhanism,

mille kohaselt ANGPTL4 interakteerub lipiididega ning destabiliseerib seeläbi rasvemulsioone. Sarnane mõju emulsioonidele tuvastati ka kahe varasemalt kirjeldatud loodusliku PL-i inhibiitori puhul. Lisaks näitas ANGPTL4 võrdlemine tuntud PL-i inhibiitoritega, et substraadi ja keskkonna valik võib mängida olulist rolli regulaatorite mõju avaldumisel. Näiteks ei omanud ε-polülüsiin PL-i inaktiveerivat toimet vesilahuses sünteetilise substraadiga, kuid mõjus juba nanomolaarses kontsentratsioonis emulsioonide kasutamisel.

Doktoritöö teises osas rakendati kalorimeetriat LPL-i aktivaatori ja potentsiaalse ravimikandidaadi (apolipoproteiin C-II analoogpeptiid) uurimiseks inimese lahjendamata vereplasmas. Antud lähenemine võimaldab LPL-i aktiivsust mõõta keskkonnas, kus LPL-i regulaatorite ja substraadi hulk on lähedane füsioloogilistele tingimustele. Sellest tulenevalt leiti, et mitmed tulemused erinevad võrreldes varasemate katsetega, mis on läbi viidud 50 kuni 250 korda lahjendatud vereplasmas või lihtsustatud süsteemides. Apolipoproteiin C-II (apoC-II) ja apoC-II analoogpeptiidi (18A-CII-a) lisamine inimese plasmasse tõstis LPL-i aktiivsust ja ensüümile kättesaadava substraadi hulka. 18A-CII-a peptiid osutuks efektiivsemaks LPL-i aktivaatoriks kui apoC-II, seda nii tervete doonorite kui ka kõrgenenud triglütseriidide tasemega doonorite plasmades. Peptiidi mõju oli suurim väiksemate lipoproteiinide klassides, kus paljud osakesed kujutavad endast kõrgenenud ateroskleroosi riski. Lisaks avastati, et vastupidiselt varasemale arvamusele ei ole inimese vereplasmas alati piisavalt apoC-II LPL-i täielikuks aktiveerimiseks. Osadel juhtudel viib apoC-II kontsentratsiooni tõstmine vereplasmas LPL-i aktiivsuse suurenemiseni. Kokkuvõttes demonstreeriti kalorimeetria tugevusi ja eeliseid potentsiaalsete ravimikandiaatide uurimises inimese lahjendamata vereplasmas. Lisaks ilmnes, et apoC-II analoogpetiid võib olla hea lähenemine HTG vähendamiseks ning SVH riski leevendamiseks.

Doktoritöö kolmandas ja viimases osas näidati mitmete biofüüsikaliste meetoditega, et albumiini ja hepariini koosmõju reguleerib LPL-i oligomerisatsiooni. Eelnevate uuringute põhjal on teada, et LPL moodustab rakusisestes vesiikulites helikaalseid oligomeere heparaansulfaat proteoglükaanide juuresolekul. Signaali saabumisel sekreteeritakse oligomeerne LPL rakkudevahelisse ruumi, kuid siiani polnud teada kuidas inaktiivne helikaalne LPL agregaat laguneb aktiivseteks LPL-i ühikuteks. Käesolevas töös näidati, et albumiin kutsub esile hepariini poolt tekitatud LPL-i oligomeeride lagunemise aktiivseteks LPL-i monomeerideks või dimeerideks. Lisaks oli vastupidiselt ka hepariin võimeline lõhkuma albumiini tekitatud LPL-i agregaate. Tulemuste põhjal pakuti välja mudel, mille kohaselt albumiini ja hepariini vastastikune koosmõju hoiab LPL-i aktiivsena rakkudevahelises ruumis. Viimaks näidati, et ANGPTL4 ei ole võimeline inaktiveerima LPL-i oligomeere. Antud nähtus võib soodustada LPL-i ladustamist rasvkoes paastumisel või kaitsta LPL-i lagundamise eest toitumisel.

PUBLICATION I

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Calorimetric approach for comparison of Angiopoietin-like protein 4 with other pancreatic lipase inhibitors

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ABSTRACT

Pancreatic lipase (PNLIP) is a digestive enzyme that is a potential drug target for the treatment of obesity. A better understanding of its regulation mechanisms would facilitate the development of new therapeutics. Recent studies indicate that intestinal lipolysis by PNLIP is reduced by Angiopoietin-like protein 4 (ANGPTL4), whose N-terminal domain (nANGPTL4) is a known inactivator of lipoprotein lipase (LPL) in blood circulation and adipocytes. To elucidate the mechanism of PNLIP inhibition by ANGPTL4, we developed a novel approach, using isothermal titration calorimetry (ITC). The obtained results were compared with those of well-described inhibitors of PNLIP – ε-polylysine (EPL), (−)-epigallocatechin-3-gallate (EGCG) and tetrahydrolipstatin. We demonstrate that ITC allows to investigate PNLIP inhibition mechanisms in complex substrate emulsions and that the ITC-based assay is highly sensitive - the lowest concentration for quantification of PNLIP is 1.5 pM. Combining ITC with surface plasmon resonance and fluorescence measurements, we present evidence that ANGPTL4 is a lipid-binding protein that influences PNLIP activity through interactions with components of substrate emulsions (bile salts, phospholipids and triglycerides), and this promotes the aggregation of triglyceride emulsions similarly to the PNLIP inhibitors EPL and EGCG. In the absence of substrate emulsion, unlike in the case of LPL, ANGPTL4 did not induce the inactivation of PNLIP. Our data also prove that due to various interactions with components of substrate systems, the effect of a PNLIP inhibitor depends on whether its effect is measured in a complex substrate emulsion or in a simple substrate system.

1. Introduction

Pancreatic lipase (PNLIP) plays a major role in the hydrolysis of dietary triglycerides (fats) into fatty acids and monoglycerides in the intestine. It has been estimated that 50–70% of dietary triglycerides are degraded by PNLIP [1,2]. This process is essential for acquiring fat-stored energy - intestine enterocytes absorb lipolysis products, fatty acids and monoglycerides, but not triglycerides. Reducing PNLIP activity by inhibitors has been considered a promising treatment for lowering body weight – less intestinal lipolysis leads to less uptake of energy-rich fatty acids and monoglycerides. In addition to the anti-obesity effect, inhibitors of PNLIP may reduce postprandial hypertriglyceridemia and improve conditions of glycemic control in obese type 2 diabetic patients [3]. In vivo PNLIP acts in a complex environment at the surface of insoluble triglycerides, and its activity is influenced primarily by bile salts, colipase and fatty acids [4]. Several dietary components may also influence PNLIP activity in the intestine [4]. In addition to these regulators, Mattijssen et al. [5] reported recently that the intestinal lipolysis by PNLIP is also reduced by ANGPTL4. Using ANGPTL4 knockout mice, this study presents evidence that ANGPTL4 acts as an endogenous inhibitor of PNLIP. Moreover, ANGPTL4 may also influence the interaction of bile salts with microbiota [6]. PNLIP belongs to the lipase family and shares structural similarity with lipoprotein lipase (LPL), endothelial lipase and hepatic lipase [7]. Numerous studies suggest that LPL activity in the bloodstream and adipocytes is negatively regulated by the N-terminal domain of ANGPTL4 (nANGPTL4) [8–13]. This inhibitory effect is likely caused by the binding of ANGPTL4 to LPL, followed by the conversion of active

Abbreviations: PNLIP, pancreatic lipase; ANGPTL4, Angiopoietin-like protein 4; nANGPTL4, N-terminal domain of Angiopoietin-like protein 4; ITC, isothermal titration calorimetry; EPL, ε-polylysine; EGCG, (−)-epigallocatechin-3-gallate; LPL, lipoprotein lipase; THL, tetrahydrolipstatin; DGGR, 1,2-Di-O-lauryl-rac-glycero-3-glutaric acid 6-methylresorufin ester; NaTDC, Na-taurodeoxycholate; BSA, bovine serum albumin; Lcth, lecithin; Chol, cholesterol; SPR, surface plasmon resonance

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LPL dimers into inactive monomers [9,10]. Alternatively, it has been proposed that at the substrate surface nANGPTL4 and LPL form an inactive reversible complex [14]. It is tempting to assume that the ANGPTL4 effect on PNLIP is also due to the formation of a complex. However, to date there is no experimental evidence to support this mechanism. Due to the complexity of the PNLIP system, the suppression of PNLIP activity can occur through various mechanisms. In addition to the formation of a complex with PNLIP, inhibitors can accumulate at bile salt-stabilized triglyceride emulsion particles, and thereby affect the binding and activity of the enzyme [15]. It is well documented that both the activity and binding of any lipase are strongly influenced by the surface composition of emulsified substrates [16]. Changes in the surface composition may also promote the aggregation of the emulsion particles, and this can lead to reduced PNLIP activity [17]. Furthermore, associations of inhibitors with PNLIP regulators may mask their interaction with PNLIP. Thus, the inhibitory effect of any compound on PNLIP can be either increased or decreased under physiological conditions.

Numerous structurally diverse natural and synthetic compounds have been demonstrated to reduce PNLIP activity in artificial substrate systems, usually using simplified substrate systems in the absence of the main PNLIP regulators, such as colipase and bile salts. It has been shown that PNLIP can be inhibited by some alkaloids, carotenoids, glycosides, polyphenols, polysaccharides, saponins and terpenoids [3,18]. Positively charged proteins and peptides form a special group of PNLIP inhibitors [19,20]. However, despite extensive investigations, there is only one approved PNLIP-inhibiting drug on the market – Orlistat - whose active component, tetrahydrolipstatin (THL), reacts with the active site serine of PNLIP [21,22]. In addition to THL, ε-polylysine (EPL) and (−)-epigallocatechin-3-gallate (EGCG) are two other potent PNLIP inhibitors which have been thoroughly investigated [19,23–26]. The comparison of ANGPTL4 with well-described PNLIP inhibitors would help to explain its mechanism and provide information about its efficacy. In addition, to elucidate mechanisms of PNLIP inhibitors, experiments should not be limited in testing their binding to PNLIP and measuring their inhibitory effect in simplified substrate systems. It would also be important to find out whether the inhibitors interact with components of triglyceride emulsions.

So far, titrimetry, turbidimetry and radiometric assays are the most common techniques used for the measurement of PNLIP activity in emulsified substrate systems [27]. These techniques have been used to unravel several important aspects of the action of PNLIP. However, it has been demonstrated that titrimetry and turbidimetry are not sufficiently sensitive and their use in complex substrate systems can be problematic, while assays based on using radio-labeled triglycerides are laborious and not suitable for continuous monitoring of lipolytic reactions [27].

Here we introduce a calorimetric approach for measuring PNLIP activity and for the investigation of its inhibitors in complex substrate systems. The ITC assay is based on the registration of heat rate changes derived from the lipolysis of triglycerides. In general, there is a proportional relationship between heat rate and reaction rate. Previous studies have demonstrated that ITC is applicable in studies of lipolytic enzymes [13,28,29]. For example, we have recently shown that ITC enables to study LPL in undiluted human plasma [13,29]. In the current study, the results obtained by ITC and other techniques suggest that ANGPTL4 is a lipid-binding protein that inhibits human PNLIP activity via interactions with components of substrate emulsions, such as bile salts, phospholipids and triglycerides.

2. Materials and methods

2.1. Reagents

Tetrahydrolipstatin (THL), colipase from porcine pancreas, cholesterol, tributyrin, 1,2-Di-O-lauryl-rac-glycero-3-glutaric acid 6-methylresorufin ester (DGGR), Na-taurodeoxycholate (NaTDC), (+)-biotin-N-hydroxysuccinimide and neutravidin were purchased from Sigma Aldrich, (−)-epigallocatechin gallate (EGCG) from Calbiochem, bovine serum albumin (BSA) from Amresco, ε-polylysine (average MW 3500–4500 Da) from Carbosynth, lecithin (egg) from Fischer Chemical, heparin from LEO Pharma, and commercial extra virgin olive oil was used as the lipase substrate. The amino coupling kit (containing N-hydroxysuccinimide, N-ethyl-N9-[(diethylamino)propyl] carbodiimide, 1 M ethanolamine) and BIAcore sensor chips were purchased from GE Healthcare. nANGPTL4, residues 26-184 of ANGPTL4, was expressed in *E. coli* and purified as described [30]. Lipoprotein lipase was purified from bovine milk [31].

2.2. Plasmid construction and expression of human pancreatic lipase

The sequence encoding human pancreatic lipase (hPNLIP) without signal peptide and with a C-terminal hexahistidine tag was amplified from cDNA of the pancreas. Human and porcine PNLIP have been previously expressed in the yeast *Pichia pastoris* [32,33]. Here, the PCR product was ligated into the yeast expression vector pPIC9 (Thermo Fisher Scientific) and transformed into *P. pastoris* GS115 (Thermo Fisher Scientific). The hPNLIP expression strains were grown in BMGY/BMMY media (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 1% glycerol or 0.5% methanol), whereas the expression was carried out at 20 °C and 260 rpm for 72 h. The expression was continuously induced by adding methanol to a final concentration of 0.5% every 24 h. After 72 h, the medium was collected, frozen in liquid nitrogen and stored at −80 °C. The protein was purified using a HisTrap Ni-affinity column. Protein analysis on the SDS-PAGE showed a single homogeneous band in an expected MW region. The fractions were dialyzed to 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer and aliquots were stored at −80 °C. The concentration of PNLIP was determined by absorption at 280 nm using a calculated extinction coefficient of 61,725 $M^{-1}\,cm^{-1}$. Detailed information is provided in the supplementary material.

2.3. Preparation of substrate emulsions and liposomes

Substrate emulsions of olive oil or tributyrin were prepared according to Tavridou et al. [34] and Wulff-Perez et al. [35]. Briefly, solutions of tributyrin or olive oil were mixed with a solution of 20 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 8.4 and NaTDC at various concentrations. Some emulsions contained lecithin (Lcth) as a source of phospholipids and cholesterol (Chol). The composition of the substrate emulsions was as follows: 1) tributyrin/NaTDC: 2% tributyrin, NaTDC at concentration 0.4 mM, 4 mM or 10 mM; 2) olive oil/NaTDC: 5% olive oil, 4 mM NaTDC; 3) tributyrin/NaTDC/Lcth/Chol: 2% tributyrin, 0.4 mM NaTDC, 0.8 mg/ml lecithin, 0.01 mM cholesterol; and 4) olive oil/NaTDC/Lcth/Chol: 5% olive oil, 0.4 mM NaTDC, 0.8 mg/ml lecithin, 0.01 mM cholesterol. The emulsions were sonicated by ultrasound for 3 × 5 s. Colipase (84 nM final concentration) was added after sonication. All samples were degassed under vacuum for 15 min before the ITC experiments with Nano ITC.

Liposomes were prepared from lecithin (egg) which was dissolved in chloroform. N_2 was used to evaporate the chloroform and the remaining lecithin layer was dried for 1 h under vacuum. A buffer containing 50 mM Tris, 100 mM NaCl, pH 7.4 was added to create a 25 mg/ml solution. The mixture was incubated for 1 h at room temperature, after which it was shaken for 2 min and sonicated by ultrasound for 15 × 5 s, with 1 min pauses between sets. Finally, the solution was centrifuged at 16,000*g*, 4 °C for 15 min.

2.4. Determination of PNLIP and LPL activity with fluorogenic substrate DGGR

PNLIP and LPL activity on DGGR was measured on a

spectrofluorophotometer Shimadzu RF-5301 PC (Shimadzu Corporation, Japan). The reaction was monitored over a period of 3 min using an excitation wavelength of 572 nm and an emission wavelength of 605 nm. Measurements with PNLIP were performed in a buffer of 20 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 8.4 at 25 °C. For the determination of IC_{50} values, inhibitors at various concentrations were mixed with a buffer and DGGR. Next, PNLIP was added and its remaining activity was determined immediately by measuring fluorescence. The concentration ranges of inhibitors were as follows: 0 to 1.6 μM for nANGPTL4, 0 to 10 μM for EGCG or THL and 0 to 250 μM for EPL. The final concentrations of PNLIP and DGGR were 1.6 nM and 24 μM, respectively. For measurement of time dependent stability of PNLIP with or without nANGPTL4, 63 nM PNLIP was incubated in the buffer described above with 4 mM NaTDC and the remaining PNLIP activity was determined using 24 μM DGGR at the indicated time points. In the case of LPL, 10 nM LPL was incubated in the absence or presence of 100 nM nANGPTL4 in a solution of 20 mM HEPES, 150 mM NaCl, 20 IU/ml heparin, 50 mg/ml BSA, pH 7.4 at 25 °C. At the indicated time points, LPL activity was determined using 24 μM DGGR and 0.5% Triton X-100 (final concentrations) [36]. A stock solution of 4 mM DGGR was made in ethanol.

2.5. Interaction measurements using fluorescence spectroscopy

nANGPTL4 interactions with DGGR, NaTDC or lecithin liposomes were determined using fluorescence spectroscopy. Measurements were performed in a solution of 50 mM HEPES, pH 7.4, 0.15 M NaCl (DGGR, NaTDC) or 20 mM HEPES, pH 7.4, 0.15 M NaCl (liposomes). The final concentration of nANGPTL4 was 2 μM. nANGPTL4 has one tryptophan residue, which was excited at 280 nm. The changes in the emission spectra were observed in the range 310 to 410 nm.

2.6. ITC experiments

Experiments were performed on a Nano ITC model 5300 (TA Instruments) and MicroCal PEAQ-ITC (Malvern) at 25 °C, as previously described by Reimund et al. [13].

In an experiment performed by Nano ITC, the substrate mixture was placed in the calorimetric cell (1035 μl) and the syringe (250 μl) was filled with PNLIP in buffer with NaTDC. The reference cell contained MilliQ water (1032 μl). The stirring speed in the sample cell was 400 rpm. The baseline was left to stabilize for about 1 h before PNLIP was injected. The first injection was 2 μl and after that sequential injections of 10 μl were made. The interval between the injections was 300 s. After each experiment, the Nano ITC sample cell and syringe were washed with the following solutions: MilliQ water, 2% SDS, 40 mM NaOH and finally 95% ethanol, rinsing with water in-between.

Experiments on MicroCal PEAQ-ITC (Malvern Instruments Plc) were performed as follows: the substrate mixture was placed in the calorimetric cell (200 μl) and the syringe (40 μl) was filled with PNLIP in buffer with NaTDC. The reference cell contained MilliQ water (200 μl). The stirring speed in the sample cell was 1000 rpm. The automatic baseline stabilization took 5–10 min before PNLIP was injected. The first injection was 0.4 μl and after that sequential injections of 1 μl were made. The interval between the injections was 100 s. After each experiment, the sample cell and syringe were washed with the following solutions: 10% Decon, MilliQ water and methanol (syringe only).

2.7. Turbidity measurements

The turbidity of the substrate emulsions in the absence or presence of inhibitors at different concentrations was measured using a UV–visible spectrophotometer (UV5Nano, Mettler Toledo). The substrate emulsions were mixed and incubated with the inhibitors as described in Fig. 3. After incubation, the top layer was removed, and the optical density was measured at 600 nm in the bottom layer after 50× or 25× dilution.

2.8. Surface plasmon resonance measurements

SPR measurements were performed on a Biacore 3000 instrument. nANGPTL4 was covalently immobilized on a sensor chip CM5 using the experimental conditions described by Robal et al. [30]. Next, increasing concentrations of PNLIP, NaTDC or colipase were injected over the surface. Biotinylated PNLIP attached to a CM5 sensor chip via neutravidin that was pre-immobilized to the chip using the amine coupling kit (GE Healthcare). To evaluate associations, increasing concentrations of ANGPTL4 or EGCG were injected into flow-cells with immobilized PNLIP. The binding of EPL to PNLIP and NaTDC was investigated using a hydrophobic sensor chip HPA. The surface of the HPA sensor chip was covered by PNLIP or NaTDC by injecting 1 μM PNLIP (60 μl, 2 μl/min) or 0.4 mM NaTDC (60 μl, 5 μl/min), respectively, into a flow-cell of the HPA sensor chip. Next, increasing concentrations of EPL (25 μM–400 μM) were injected over the surfaces. All experiments were carried out at 25 °C in 20 mM HEPES, pH 7.4, 0.15 M NaCl (running buffer).

PNLIP was biotinylated with (+)-Biotin-N-hydroxysuccinimide in bicarbonate buffer at pH 8.5. (+)-Biotin-N-hydroxysuccinimide was diluted in DMSO and added to 2.5 μM PNLIP in final concentration of 50 μM. The reaction was carried out at 4 °C in 5 h. Biotinylated PNLIP was dialyzed to a buffer of 20 mM HEPES, 0.15 M NaCl, pH 7.4 and the protein concentration was measured at 280 nm.

2.9. Dynamic light scattering

Particle size analysis for DGGR was performed with Nano-S ZEN1600 (Malvern Instruments Plc) on a 24 μM DGGR solution in 20 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 8.4, which was prepared from a 4 mM DGGR stock solution in ethanol. Buffer without DGGR was used as a reference. All buffers were filtered through a 0.22 μm pore size filter. The sample was equilibrated for 120 s and measured at 20 °C.

2.10. Data analysis

The SPR data were analyzed by BIAEvaluation 3.1 software. The inhibition data obtained by ITC or fluorescence measurements were analyzed using Sigma Plot 13 software. IC_{50} values were determined using the hyperbolic decay equation:

$$a = b + (a_o \cdot IC_{50})/(IC_{50} + C),$$

where (IC_{50}) – the concentration of an inhibitor at which 50% of PNLIP activity was inhibited, C – the concentration of the inhibitor, a – activity of PNLIP, b – the activity of PNLIP in the complex with an inhibitor, and a_o – the PNLIP activity in the absence of inhibitors.

3. Results

3.1. ITC can be used for determination of PNLIP activity in a complex substrate system

To assess the applicability of ITC in investigations of PNLIP, the enzymatic activity of PNLIP was measured in four different substrate emulsions: 1) tributyrin/NaTDC, 2) olive oil/NaTDC, 3) tributyrin/NaTDC/Lcth/Chol and 4) olive oil/NaTDC/Lcth/Chol. The first two emulsions have been widely used for the determination of PNLIP activity using other techniques, such as titrimetry [27]. The two other emulsions were chosen because they contained Lcth and Chol which are components of substrate emulsions in the intestine [37]. This provided an opportunity to test the limits of the method using a more complex system. To avoid possible artifacts and to estimate the sensitivity of the assays, experiments were performed with highly purified recombinant human PNLIP. The reaction media contained other necessary

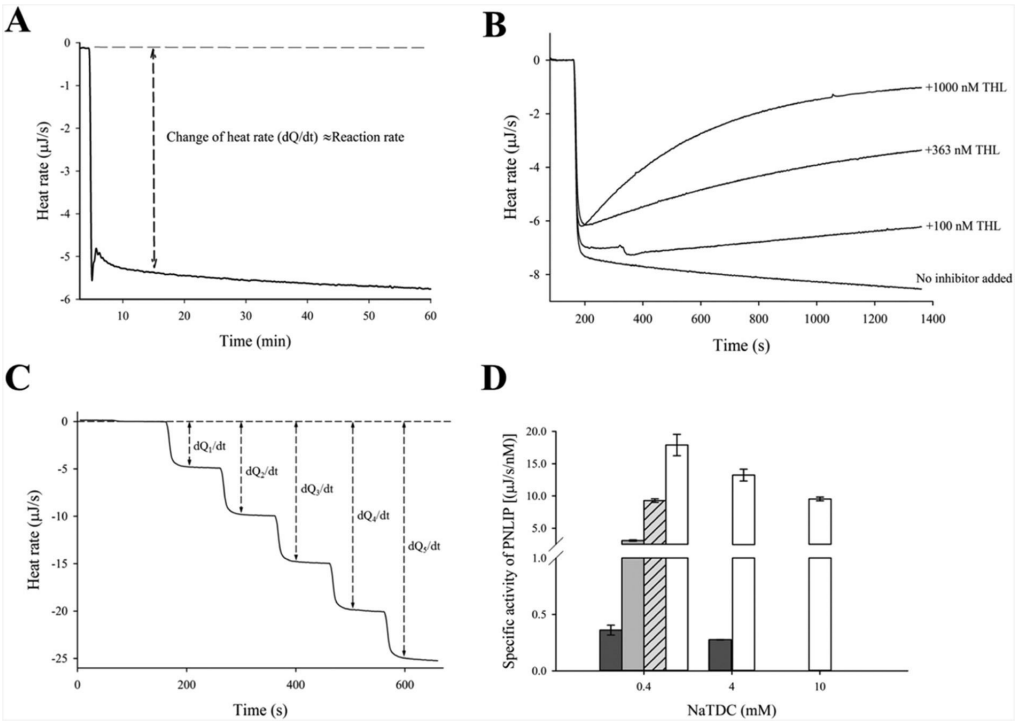

Fig. 1. Catalytic activity of PNLIP as measured by ITC under various conditions. A: Raw thermogram of heat rate change as a result of a single injection of 0.36 nM PNLIP into ITC cell containing emulsion tributyrin/4 mM NaTDC; B: Inhibition kinetics of PNLIP by THL under zero-order reaction conditions as measured by ITC. THL was mixed with the emulsion tributyrin/4 mM NaTDC at the concentrations indicated. Heat rate changes were monitored after a single injection of 0.72 nM PNLIP; C: Sequential injections of PNLIP into ITC cell containing emulsion tributyrin/4 mM NaTDC. Each injection increased PNLIP concentration by 0.36 nM; D: Specific activity of PNLIP in different emulsions. Dark gray – olive oil/NaTDC, light gray – olive oil/NaTDC/Lcth/Chol, striped – tributyrin/NaTDC/Lcth/Chol, white – tributyrin/NaTDC. The values are mean ± SD of three independent measurements.

components for PNLIP activity – colipase along with Ca^{2+}. Fig. 1A shows an experiment that was performed with the substrate emulsion tributyrin/4 mM NaTDC. As can be seen, an injection of PNLIP into this emulsion resulted in a decrease in the heat rate to a new level that remained almost constant at least for 60 min. This indicated that the reaction was exothermic and that the PNLIP activity did not change during this period. To confirm the correspondence of the observed heat effects to PNLIP activity, THL, a selective and potent inhibitor of PNLIP, was added to the substrate emulsion. The inhibition time courses for the four THL concentrations presented in Fig. 1B show concentration dependent inhibition of PNLIP by THL.

Sequential injections of equal amounts of PNLIP into the ITC cell containing substrate emulsion resulted in a step-wise decrease in the heat rate (Fig. 1C). The data obtained by these titrations for each emulsion were converted to a plot heat rate versus PNLIP concentration. A nearly proportional correlation was observed for all tested emulsions (data not shown). The slope of this relationship indicates sensitivity of the substrate system and represents apparent specific activity (SA) expressed in the unit $\mu J \cdot s^{-1} \cdot nM^{-1}$. The SA values of the assays are presented in Fig. 1D. As can be seen, the substrate emulsion tributyrin/0.4 mM NaTDC was the most sensitive substrate system. Somewhat lower sensitivity was detected for emulsions tributyrin/4 mM NaTDC and tributyrin/10 mM NaTDC, which is in agreement with previous works that demonstrate how higher concentrations of NaTDC reduce PNLIP activity [38]. In comparison with the emulsion tributyrin/0.4 mM NaTDC, 60 times lower sensitivity was determined for the emulsion olive oil/NaTDC. The data obtained for olive oil or tributyrin substrate emulsions with 4 mM NaTDC/Ltch/Chol are not presented. This was due to very low heat effects that did not provide satisfactory results - to reliably measure PNLIP activity, impractically high concentrations of PNLIP and colipase would have to be used.

To determine the quantification limit, it was based on the general assumption that the signal-to-noise ratio of the heat rate change must be over 10. The noise level in the substrate emulsion tributyrin/0.4 mM NaTDC, calculated as a standard deviation of the heat rate level, was found to be 4 nJ/s. Hence, for reliable determinations of PNLIP activity, the change in heat rate must be over 40 nJ/s, which corresponds to the PNLIP concentration 1.5 pM (Fig. S1). The ITC assay is approximately 500–1000 times more sensitive than the standard pH stat assay [27]. The high sensitivity of the ITC assay prompted us to test whether the ITC assay could be used for determination of PNLIP activity in human plasma. This could have been applicable in the diagnosis of pancreatitis, however, PNLIP was completely inactive in human plasma.

3.2. Comparison of PNLIP inhibitors in substrate emulsions

In the next set of experiments, ITC was used to investigate the effects of PNLIP inhibitors. All inhibitors – nANGPTL4, THL, EGCG and EPL, were tested in the substrate emulsions olive oil/0.4 mM NaTDC/Lcth/Chol and tributyrin/4 mM NaTDC (Fig. S2 and Fig. 2, respectively). In addition, ANGPTL4 was tested in the substrate system tributyrin/10 mM NaTDC. The aim was to compare nANGPTL4 with other PNLIP inhibitors. nANGPTL4 appeared to be an efficient PNLIP inhibitor – in all substrate emulsions, its effect on PNLIP activity was

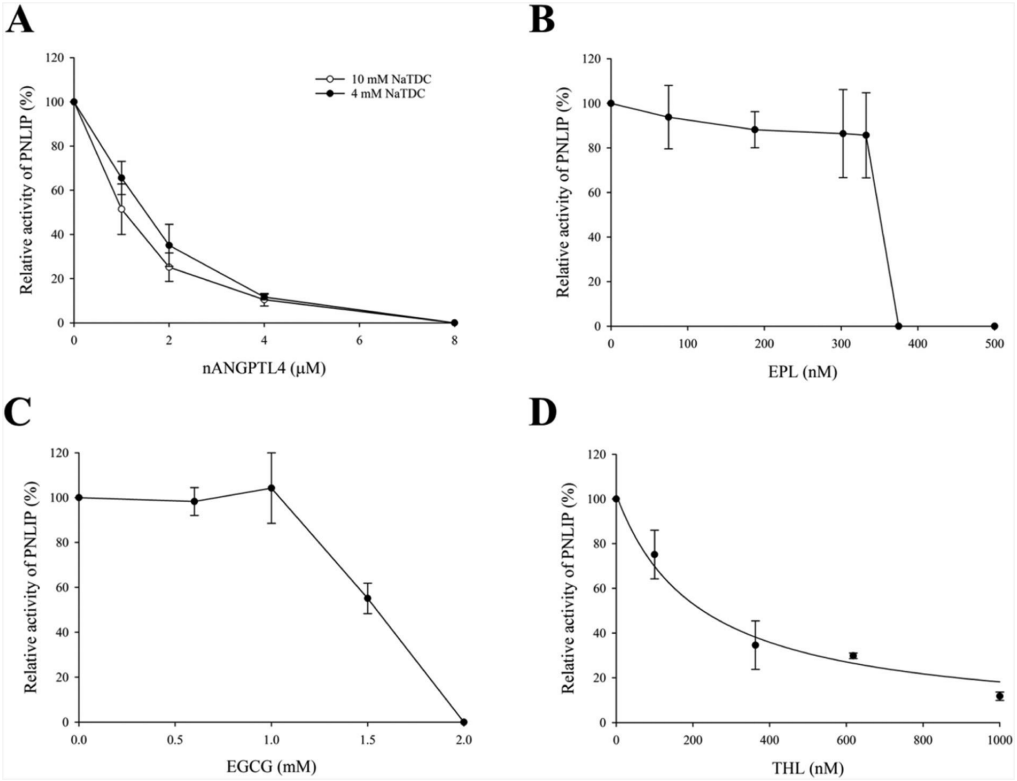

Fig. 2. Effect of inhibitors on PNLIP activity as determined by ITC in substrate emulsion of tributyrin/NaTDC. For all inhibitors, measurements were performed in the presence of 4 mM NaTDC (●). For nANGPTL4, additional measurements in the presence of 10 mM NaTDC were carried out (○). The inhibitors were mixed with the substrate emulsion in the ITC cell before injections of PNLIP. In the case of nANGPTL4, the mixture was further allowed to equilibrate in the cell for 5 min. Measurements with nANGPTL4, EGCG and EPL were carried out as demonstrated in Fig. 1B (sequential injection of PNLIP). In the case of THL, a single 0.72 nM PNLIP injection was made, and PNLIP activity was calculated from the heat rate at 30 min after injection. The measurements were performed under zero order reaction conditions. The values are mean ± SD of three independent measurements.

Table 1
IC_{50} values of PNLIP inhibitors measured in substrate systems DGGR, tributyrin/4 mM NaTDC and olive oil/0.4 mM NaTDC/Lcth/Chol and DGGR.

Inhibitor	IC_{50}		
	DGGR	Tributyrin/4 mM NaTDC	Olive oil/0.4 mM NaTDC/Lcth/Chol
nANGPTL4	0.60 ± 0.27 μM	1–2 μM	1–2 μM
EPL	No effect detected	332–375 nM	16.6 ± 3.8 μM
EGCG	1.03 ± 0.20 μM	1.5–2 mM	220.8 ± 50.9 μM
THL	3.08 ± 1.16 μM	217.0 ± 37.6 nM	12.0 ± 1.9 nM

detectable already at submicromolar concentrations (Figs. 2A and S2A). In the substrate system tributyrin/4 mM NaTDC, EPL was the most efficient inhibitor and EGCG was the poorest inhibitor. Surprisingly, instead of a gradual process, a very small change in the EPL concentration range between 350 and 390 nM resulted in a sudden and complete loss of PNLIP activity and formation of precipitates in the substrate emulsion. Interestingly, in the substrate emulsion tributyrin/4 mM NaTDC, THL was a less potent inhibitor than nANGPTL4 or EPL. In the substrate emulsion olive oil/0.4 mM NaTDC/Lcth/Chol, THL was the most potent PNLIP inhibitor and EGCG was the poorest inhibitor. The reducing effect of THL appeared already in the nanomolar concentration range while the EGCG inhibitory effect was detectable at concentrations higher than 100 μM (Fig. S2). To quantitatively evaluate inhibition efficacy, an IC_{50} value was estimated for each inhibitor in different substrate systems (Table 1). While the inhibition data of THL in both substrate system and ECGC and EPL in the substrate system olive oil/0.4 mM NaTDC/Lcth/Chol could be fitted to a hyperbolic decay model, the inhibition by nANGPTL4 followed a more complex mechanism in both emulsions. For comparison, the inhibitory effects were examined using DGGR, a fluorogenic triglyceride analog (Table 1). These measurements were performed in the absence of PNLIP regulators colipase, bile salts and phospholipids. The results obtained by this system were different from that of the substrate emulsion olive oil/0.4 mM NaTDC/Lcth/Chol and tributyrin/4 mM NaTDC. Firstly, the inhibitory effects of nANGPTL4, THL and EGCG were comparable – for all these three substances, inhibition was observed in the range of micromolar concentrations (Table 1). Secondly, even millimolar concentrations of EPL did not lower the PNLIP activity on DGGR, suggesting that the inhibitory effect of EPL in the substrate emulsion olive oil/0.4 mM NaTDC/Lcth/Chol was not due to its interactions with PNLIP. Thirdly, in this simple substrate system even the inhibition by nANGPTL4 followed a simple hyperbolic decay model. Considering the results obtained with these two different substrate systems, it is obvious that the inhibitory effect strongly depends on the substrate system. The variability of the shapes of the inhibition curves suggests that the components of the substrate emulsions interact differently with the inhibitors.

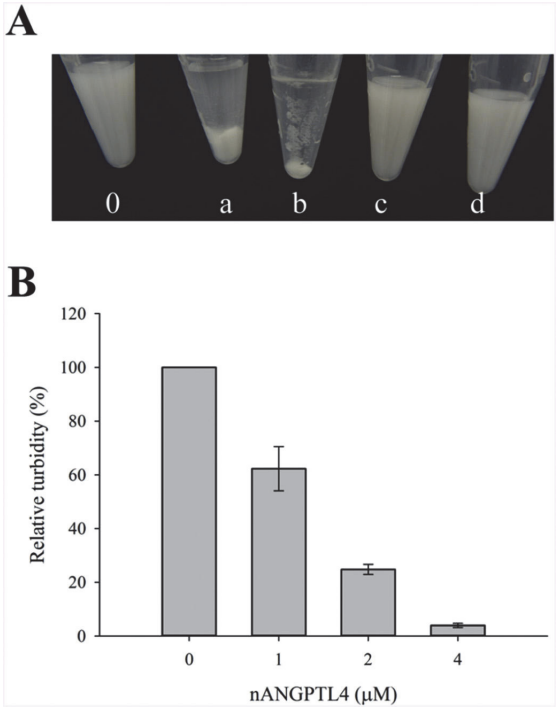

Fig. 3. Effect of PNLIP inhibitors on turbidity of substrate emulsion tributyrin/4 mM NaTDC. A: 0 – d Visible appearance of emulsions after addition of PNLIP inhibitors (0 – no inhibitor, a – 8 μM nANGPTL4, b – 500 nM EPL, c – 2 mM EGCG, d – 1000 nM THL). B: Measurement of turbidity in the emulsion tributyrin/4 mM NaTDC in the absence or presence of nANGPTL4. Substrate with nANGPTL4 was incubated for 5 min after mixing. Turbidity was then estimated using optical density values at 600 nm after 50× or 25× dilution. The values are mean ± SD of three independent measurements.

3.3. Interaction of PNLIP inhibitors with substrate emulsion olive oil/NaTDC/Lcth/Chol

Visual inspection revealed that nANGPTL4, EPL and EGCG induced changes in turbidity, promoting the aggregation of substrate emulsions. In the substrate emulsion tributyrin/4 mM NaTDC, the addition of nANGPTL4 resulted in the appearance of precipitates (Fig. 3A) while in the emulsion olive oil/0.4 mM NaTDC/Lcth/Chol, the addition of nANGPTL4 resulted in a creaming effect, so that the middle part became less cloudy and a less dense layer appeared on top (Fig. S3A). Similar effect in the emulsion olive oil/0.4 mM NaTDC/Lcth/Chol was observed after the addition of EPL or EGCG. The different appearances of these two emulsions were due to the different densities of the insoluble substrates – triolein is lighter and tributyrin heavier than water. In contrast to the other inhibitors, no visually detectable changes for either substrate were observed after the addition of THL. The addition of EGCG to tributyrin/4 mM NaTDC also did not lead to any visible changes. It was noteworthy that the changes in the turbidity appeared at the concentrations of nANGPTL4, EGCG and EPL that corresponded to their concentrations at which PNLIP inhibition was observed. To quantitatively evaluate the changes in turbidity, the absorbance at 600 nm was measured after the removal of either the precipitates or the top layer. Fig. 3B shows a clear decrease in the turbidity of the substrate emulsion tributyrin/4 mM NaTDC when nANGPTL4 is added. In Fig. S3B, the turbidity of the emulsion olive oil/0.4 mM NaTDC/Lcth/Chol mixed with nANGPTL4, EPL or EGCG was also remarkably decreased, supporting the visual observations in Fig. S3A. In control experiments, the addition of THL to emulsion olive oil/0.4 mM NaTDC/Lcth/Chol had no effect on the turbidity. These data suggest that nANGPTL4, EPL and EGCG interact with the substrate components. ITC experiments supported the results of the turbidity measurements – nANGPTL4, EGCG and EPL interacted with the components of the complex emulsions (Fig. 4). For all three inhibitors, measurable heat effects were detected in their inhibition concentration range. Dividing the total heat released from sequential injections with the amount of inhibitor added showed that the interaction of nANGPTL4 and EPL with the components of the substrate emulsion were endothermic, with average heat effects of 182.9 μJ/μmol and 5.3 μJ/μmol, respectively. At the same time, the EGCG effect was exothermic, with an average heat effect equal to −2.0 μJ/μmol. Excluding NaTDC from the emulsion only slightly changed the heat effects of the titrations (Fig. 5), suggesting that for all three inhibitors the heat effect was not due to their interactions with NaTDC. However, it should be noted that the lack of heat effect does not mean that the inhibitors do not form a complex with NaTDC. SPR measurements showed that NaTDC binds to EPL (Fig. S4) and to nANGPTL4, as discussed in the next section.

3.4. nANGPTL4 interacts with components of substrate emulsions

Detailed ITC analysis revealed that nANGPTL4 interacted with most of the components of the emulsion olive oil/0.4 mM NaTDC/Lcth/Chol (Fig. 6). SPR and fluorescence measurements supported these observations – nANGPTL4 interacted with NaTDC (Fig. 7A and B), phospholipids (Fig. 7C) and even the triglyceride analog DGGR (Fig. 7D). According to the SPR data, the nANGPTL4/NaTDC interaction was weak – the estimated Kd value was 3.0 ± 0.3 mM (Fig. 7A). However, since the bile salt concentration in the intestine is in a similar, millimolar range [39], it is reasonable to assume that this complex forms in vivo. In the concentration range up to 1 mM, the binding of NaTDC to nANGPTL4 resulted in an increase in the intrinsic fluorescence of nANGPTL4, but at higher NaTDC concentrations the fluorescence started to decrease (Fig. 7B). Lecithin liposomes (Fig. 7C) and DGGR (Fig. 7D) also decreased the intrinsic fluorescence of nANGPTL4.The estimated Kd values of the interactions between lecithin liposomes and DGGR with nANGPTL4 were 1.0 ± 0.2 mg/ml and 23.4 ± 1.3 μM, respectively.

To clarify if DGGR forms any aggregates in solution, particle size analysis on a standard measurement DGGR solution (24 μM) was performed using dynamic light scattering (DLS). It revealed that DGGR is highly aggregated at that concentration, with an average particle size of 341.2 ± 5.3 nm. This could explain how nANGPTL4 can affect PNLIP activity through interactions with DGGR, despite the large excess of DGGR in solution compared to nANGPTL4. Instead of forming a complex with the substrate molecule, it is likely that nANGPTL4 interacts with the surface of the large aggregated particles.

3.5. Direct interaction between PNLIP and inhibitors

To investigate whether nANGPTL4 or the other inhibitors form a complex with PNLIP, binding experiments were performed using SPR. Biotinylated PNLIP was attached to pre-immobilized neutravidin, and the binding of nANGPTL4 and EGCG to this surface was examined. In the case of EPL, PNLIP was attached to the hydrophobic surface of the HPA sensor chip and EPL was injected over the surface. Of these three inhibitors, only experiments with EGCG were successful (Fig. S5). The corresponding sensograms were best fitted to a binding model according to which EGCG binds to several binding sites on PNLIP. In addition, mixing EGCG and PNLIP prior to injection into an ITC cell containing substrate emulsion completely abolished PNLIP activity at concentrations comparable to measurements where EGCG was added to substrate before the injection of PNLIP (data not shown). No detectable binding of EPL to PNLIP was observed even at a concentration of

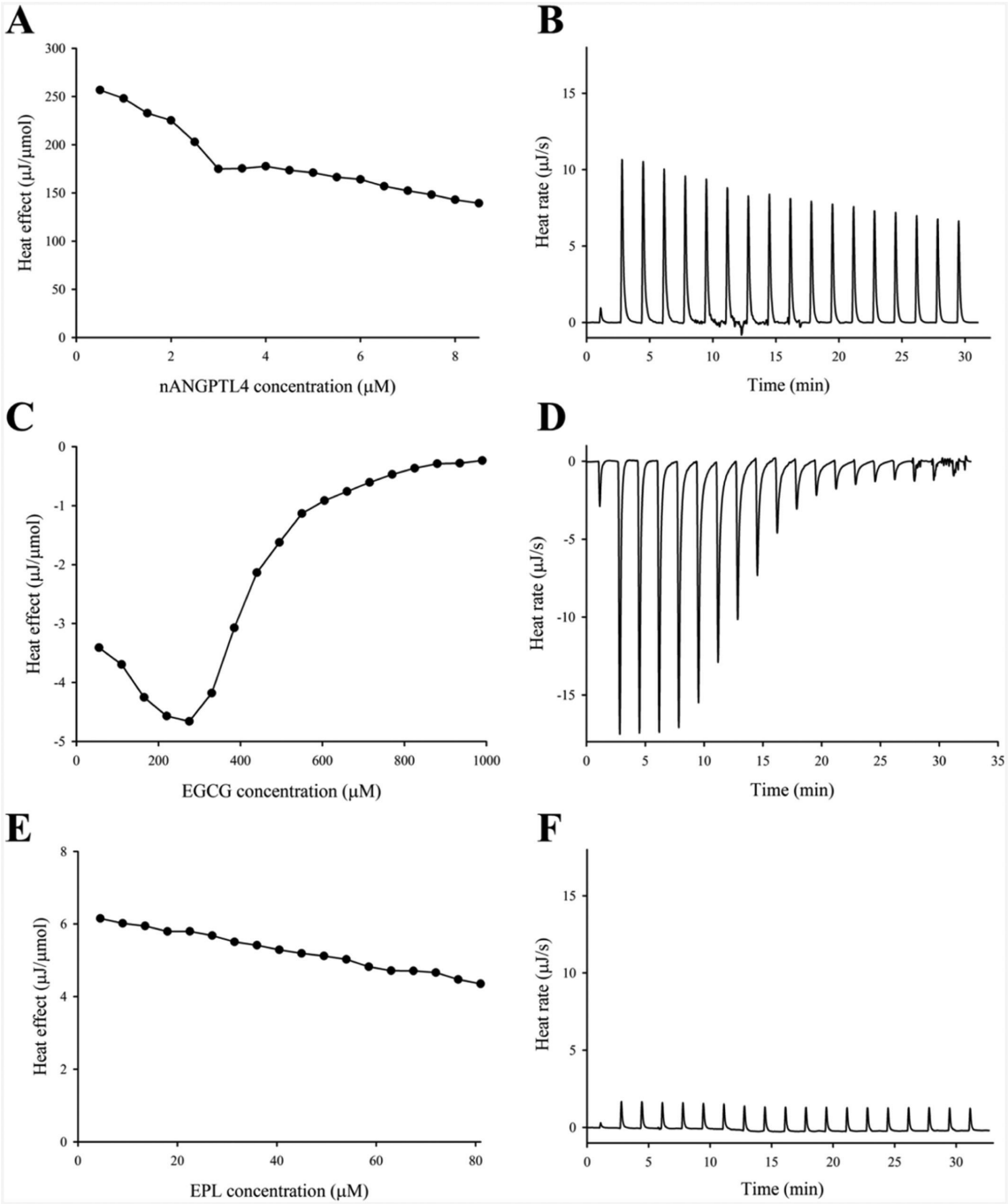

Fig. 4. Examples of raw ITC data obtained by titration of olive oil/0.4 mM NaTDC/Lcth/Chol with inhibitors nANGPTL4, EGCG or EPL. The ITC cell was filled with the substrate emulsion olive oil/0.4 mM NaTDC/Lcth/Chol and sequential injections of each inhibitor were made. Concentrations of inhibitors in the cell were in the same range of their inhibitory concentrations (see Fig. 3). A, C, E: heat effects calculated per amount of added inhibitor and B, D, F: Corresponding raw thermograms. A: Each injection increased the cell concentration of nANGPTL4 by 0.5 µM. C: Each injection increased the cell concentration of EGCG by 55 µM. E: Each injection increased the cell concentration of EPL by 4.5 µM.

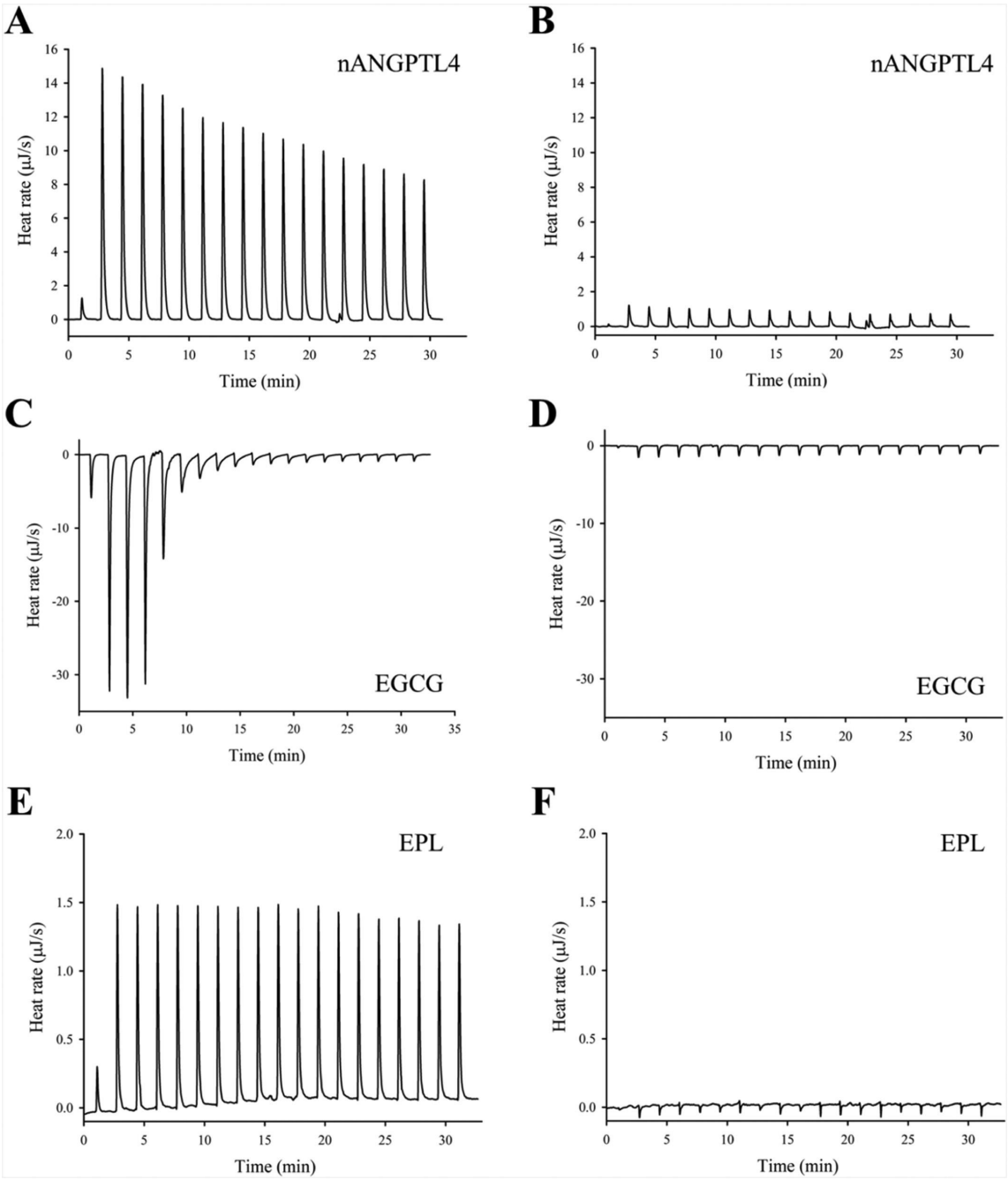

Fig. 5. Examples of raw ITC data for titration of PNLIP inhibitors to emulsion of olive oil/Lcth/Chol or to solution of 0.4 mM NaTDC. A, B: Titration of nANGPTL4 to olive oil/Lcth/Chol (A) or 0.4 mM NaTDC (B). Each injection increased the cell concentration of nANGPTL4 by 0.5 μM. C, D: Titration of EGCG to olive oil/Lcth/Chol (C) or to 0.4 mM NaTDC (D). Each injection increased the cell concentration of EGCG by 55 μM. E, F: Titration of EPL to olive oil/Lcth/Chol (E) or 0.4 mM NaTDC (F). Each injection increased the cell concentration of EPL by 4.5 μM.

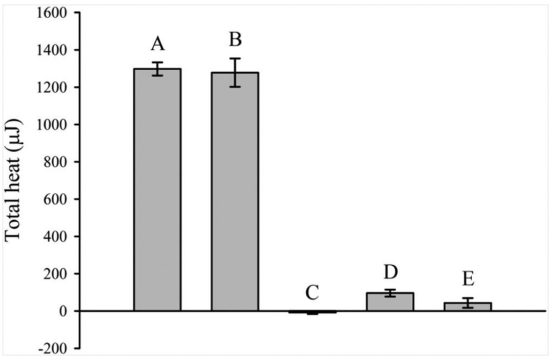

Fig. 6. Heat effects of interactions of nANGPTL4 with components of substrate emulsion olive oil/Lcth/0.4 mM NaTDC/Chol. The presented total heat effects are obtained by summarizing heat effects of 16 sequential 2 μl injections of 50 μM nANGPTL4 into the 200 μl ITC cell that was filled with 20 mM Tris, 0.15 M NaCl, 2.5 mM $CaCl_2$ pH 8.4 and in addition contained the following components: A – olive oil/Lcth/0.4 mM NaTDC/Chol; B – olive oil/Lcth/Chol; C – 0.4 mM NaTDC; D – Olive oil/0.4 mM NaTDC; E – 0.8 mg/ml Lcth liposomes. The values are mean ± SD of three independent measurements.

400 μM. Due to the very high non-specific binding of nANGPTL4 to the matrix of the CM5 sensor chip, it was not possible to ascertain whether PNLIP and nANGPTL4 formed a complex. The possibility of a complex formation between nANGPTL4 and PNLIP or between nANGPTL4 and colipase was also tested using covalently immobilized nANGPTL4. Even in these cases the association of colipase or PNLIP with nANGPTL4 was not observed.

In the next experiment, it was tested whether nANGPTL4 induces irreversible structural changes in PNLIP in the absence of substrate emulsion. It is well documented that according to this mechanism, ANGPTL4 destabilizes LPL, the enzyme that is structurally similar to PNLIP [9,10]. In contrast to LPL, preincubation of PNLIP with nANGPTL4 prior mixing with substrate did not change the PNLIP activity (Fig. 8A). In the control experiment, nANGPTL4 from the same batch efficiently inactivated LPL (Fig. 8B).

4. Discussion

Several observations of this study suggest that ITC is a promising technique for the investigation of PNLIP in complex substrate emulsions. ITC allows determination of PNLIP activity, as well as studying its interactions in emulsified substrate systems. The ITC assay has several advantages over other techniques. Firstly, the broad linear relationship between PNLIP concentration and heat rate enables to measure PNLIP activity in various samples. Secondly, the ITC assay is simple, label-free

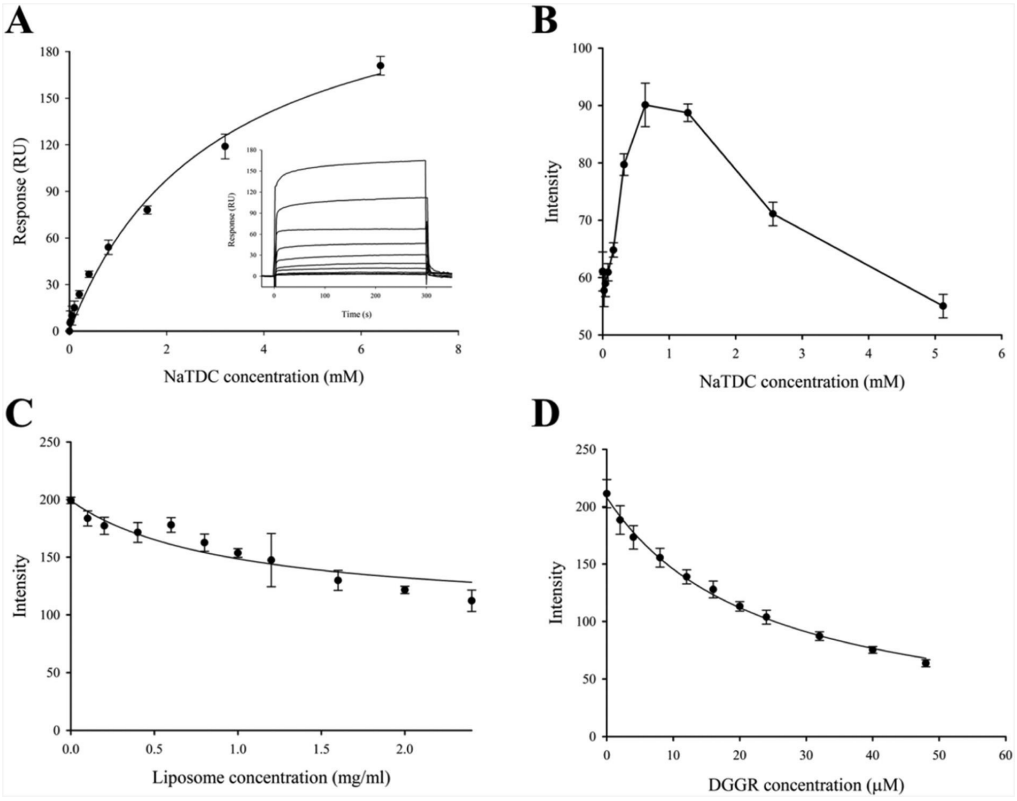

Fig. 7. Interaction of nANGPTL4 with the components of substrate systems as studied by SPR and fluorescence measurements. A: Steady-state SPR analysis of binding of NaTDC to nANGPTL4 which was immobilized on a CM5 sensor chip. Concentrations of NaTDC were as follows: 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 mM. Corresponding SPR sensorgrams are shown in the inset. B: Binding of NaTDC to nAngpt4 as measured using changes in tryptophan fluorescence of nANGPTL4; C: Binding of lecithin liposomes to nANGPTL4 as measured using changes in tryptophan fluorescence of nANGPTL4; D: Binding of DGGR to nANGPTL4 as measured using changes in tryptophon fluorescence. The values are mean ± SD of three independent measurements.

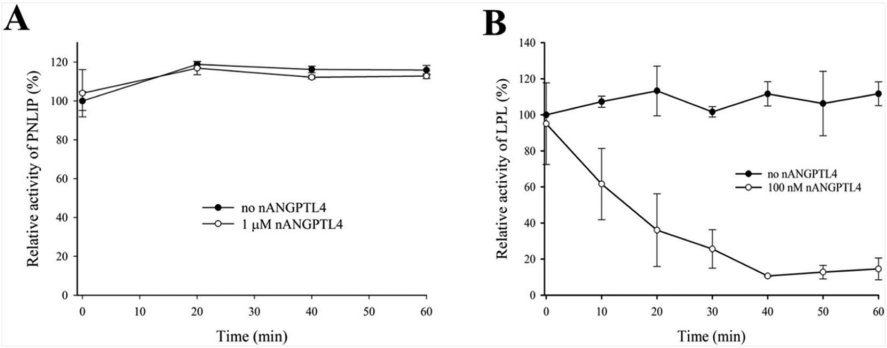

Fig. 8. Effect of nANGPTL4 on stability of PNLIP and LPL in the absence of components of PNLIP substrate emulsions. A: 63 nM PNLIP was incubated in the absence or presence of nANGPTL4 in 20 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$, 4 mM NaTDC, pH 8.4 at 25 °C. At the indicated time points, aliquots of PNLIP reaction mixtures were withdrawn and diluted to 2 nM PNLIP concentration. Remaining activity of PNLIP was determined using DGGR as substrate; B: 10 nM LPL was incubated in the absence or presence of 100 nM nANGPTL4 in a solution of 20 mM HEPES, 150 mM NaCl, 20 IU/ml heparin, 50 mg/ml BSA, pH 7.4 at 25 °C. At the indicated time points, aliquots of LPL reaction mixtures were withdrawn for activity measurements using DGGR as substrate. Both PNLIP and LPL activities are expressed as percent remaining of the initial activity at time 0. The values are mean ± SD of three independent measurements.

and direct; there is no need to separate products from substrates. Thirdly, the ITC assay is highly sensitive – when tributyrin was used as a substrate, the detection limit was as low as 1.5 pM. The sensitivity of the ITC assay is comparable to that of PNLIP assays which use radiolabeled substrates, and is about 500–1000 times more sensitive than the pH-stat assay [27]. Fourthly, the reaction rate can be followed continuously, which can give additional information about the mechanisms behind PNLIP inhibition. We demonstrate that the ITC measurements of PNLIP activity can be done using both submicellar and micellar concentrations of NaTDC. In general, the complexity of the substrate system should not be a limitation to perform ITC measurements. However, in some cases ITC measurements can be problematic in complex substrate emulsions composed of many components. We were unable to obtain satisfactory results using substrate emulsion composed of olive oil, lecithin, cholesterol and 4 mM NaTDC. We propose that lipolysis products in this emulsion can trigger essential reorganisation processes. While PNLIP catalysed hydrolysis of triglycerides is exothermic, the reorganisation in the substrate emulsion may lead to compensatory endothermic effects. This may also result in low sensitivity, complex kinetics or high noise level.

ITC in combination with other techniques enabled us to investigate the mechanism that underlies the inhibition of PNLIP by nANGPTL4. A comparison of nANGPTL4 with the well-described PNLIP inhibitors THL, EPL and EGCG also provided information about its efficacy. Since obtaining intestinal fluid, which is the physiological substrate emulsion of PNLIP, is an invasive procedure and it already contains PNLIP, mechanistic investigations were performed using artificial substrate emulsions. We performed measurements in the substrate emulsion tributyrin/4 mM NaTDC that is a generally accepted model system for testing PNLIP inhibitors and for investigations of structure/function relationships of PNLIP or colipase [40–42]. In addition, we used the substrate emulsions that contained the main components of physiological intestinal lipids - triglycerides, NaTDC, phospholipids and cholesterol [43]. Previous studies suggest that the inhibition of PNLIP by THL, EPL and EGCG in triglyceride emulsions is likely to occur through different mechanisms. It is well established that THL reacts with the active site serine of PNLIP [21,22]. On the other hand, like a previous study [23], our results suggest that EPL does not interact with PNLIP, but rather influences lipolysis by destabilizing substrate emulsion. One might think that the direct binding to PNLIP is the most effective mechanism for inhibition. However, it has been shown that in some substrate systems EPL is an even more efficient PNLIP inhibitor than THL [23]. EGCG, a flavonoid component of green tea, has been demonstrated to form a non-covalent inactive complex with PNLIP [24]. The binding of EGCG to PNLIP was also observed in the current study using SPR. On the other hand, EGCG also interacts with bile salts and cholesterol, which are components of intestinal triglyceride emulsions [44]. It is likely that the aggregation of olive oil substrate emulsion by EGCG observed in this study is due to these interactions. Thus, there is some ambiguity regarding the effect of EGCG on PNLIP activity. The experiments with EGCG indicated that the inhibitory effect on PNLIP may depend on the order of mixing of inhibitor, substrate emulsion and PNLIP. When an inhibitor is incubated with PNLIP before the addition of substrate emulsion, it is possible that due to slow kinetics the inhibitor/PNLIP complex does not dissociate during the time period of activity measurements. Alternatively, in the absence of substrates, an inhibitor may induce irreversible structural changes in PNLIP, similarly to the nANGPTL4 effect on LPL [9]. Our results also demonstrated that EGCG had a much larger inhibitory effect when mixed with PNLIP prior to addition to the substrate when compared to experiments where EGCG was preincubated with substrate prior to the addition of PNLIP, indicating a direct interaction between EGCG and PNLIP, which results in the formation of an inactive form of PNLIP. In contrast to EGCG, the preincubation of PNLIP with nANGPTL4 at submicromolar concentrations prior to mixing with substrate emulsion did not lead to the inhibition of PNLIP. Thus, we could not demonstrate association of nANGPTL4 with PNLIP. This suggests that nANGPTL4 influences PNLIP differently from LPL, which forms an unstable complex with nANGPTL4. Furthermore, the presence of substrates protects LPL from the inhibitory effect of nANGPTL4 [45]. A comparison of the nANGPTL4 effect on these two lipases can be important in terms of the development of antihypertriglyceridemic drugs that would block the binding of nANGPTL4 to LPL [46].

Our data suggest that the inhibitory effect of nANGPTL4 is due to its interaction with the components of substrate emulsion. In particular, this conclusion is based on the observation that the concentration range of the inhibition of PNLIP coincided with the concentrations at which nANGPTL4 caused the destabilization of substrate emulsions. This destabilization is the result of the interaction of nANGPTL4 with the components of the substrate emulsions: triolein, phospholipids and bile salts. Considering that nANGPTL4 also binds fatty acids [30], it is reasonable to conclude that nANGPTL4 is a lipid binding protein which is able to interact with both lipid aggregates and lipid monomers. To some extent, the nANGPTL4 inhibition mechanism is similar to that of EPL – both destabilize substrate emulsions. Like EPL, nANGPTL4 is positively charged – it contains a cluster of lysine and arginine residues

in region 136–139. Since other basic proteins (protamine, histones) have also been shown to inhibit PNLIP activity on emulsified substrates, it is likely that this effect is a general property of highly positively charged proteins [20]. It is also possible that the three predicted helices of nANGPTL4, which have an amphipathic nature, can participate in the interaction with the hydrophobic part of the lipids. Hydrophobic proteins, such as BSA and β-lactoglobulin, inhibit PNLIP activity toward emulsified substrates via their accumulation at substrate particles [47]. It is possible that the effect of nANGPTL4 on substrate emulsions is also a nonspecific protein effect. However, the inhibitory effect of nANGPTL4 was detectable at even 1000 times lower concentrations than that of BSA or β-lactoglobulin. An important conclusion of this study is that the effect of an inhibitor on PNLIP depends strongly on a substrate system. Compounds that efficiently inhibit PNLIP activity in simplified substrate systems can be remarkably less efficient in emulsified substrate mixtures which contain bile salts, phospholipids and colipase. This difference was observed for EGCG and EPL. EGCG effectively reduced PNLIP activity on DGGR but was much less effective in reducing PNLIP activity in emulsified substrate mixtures. The inhibitory behavior of EPL was completely opposite that of EGCG – it was efficient in emulsified substrate systems but did not influence the PNLIP activity on DGGR. The erratic, and in some cases atypical (Fig. 2B, C), appearance of the inhibition curves in different substrate systems can be explained by variability of the interactions between the inhibitors and the components of substrate mixtures. The sharp reduction of PNLIP activity in the narrow range of EGCG or EPL in the substrate emulsion tributyrin/4 mM NaTDC (Fig. 2B, C) is likely to be due to inhibitor induced coalescence of this emulsion. In the case of more stable emulsions such like olive oil/0.4 mM NaTDC/Lcth/Chol (Fig. S2), the EGCG or EPL induced changes would occur more smoothly and appear within their wider concentration range.

In summary, in this report we have demonstrated that ITC is a powerful tool for characterizing PNLIP interactions with its inhibitors. In comparison with other techniques, ITC has several advantages – simple assay, high sensitivity and an opportunity to continuously monitor the lipolytic reaction in complex substrate systems. Using ITC in combination with other techniques, we provide evidence that nANGPTL4 is a lipid-binding protein that influences PNLIP activity through interactions with components of substrate emulsions. We conclude that the inhibition mechanism of nANGPTL4 on PNLIP is different from its effect on LPL.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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PUBLICATION II

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Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach

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ABSTRACT

Elevated plasma triglyceride (TG) levels are associated with higher risk of atherosclerotic cardiovascular disease. One way to reduce plasma TG is to increase the activity of lipoprotein lipase (LPL), the rate limiting enzyme in plasma TG metabolism. An apolipoprotein (apo) C-II mimetic peptide (18A—CII—a) has been recently developed that stimulated LPL activity *in vitro* and decreased plasma TG concentration in animal models for hypertriglyceridemia. Since this peptide can serve as a new therapeutic approach for treatment of hypertriglyceridemia, we investigated how 18A—CII—a peptide influences LPL activity in human plasma. We used recently described isothermal titration calorimetry based approach to assess the peptide, which enables the analysis in nearly undiluted human plasma. The 18A—CII—a peptide was 3.5-fold more efficient in stimulating LPL activity than full-length apoC-II in plasma sample from normolipidemic individual. Furthermore, 18A—CII—a also increased LPL activity in hypertriglyceridemic plasma samples. Unlike apoC-II, high concentrations of the 18A—CII—a peptide did not inhibit LPL activity. The increase in LPL activity after addition of 18A—CII—a or apoC-II to plasma was due to the increase of the amount of available substrate for LPL. Measurements with isolated lipoproteins revealed that the relative activation effects of 18A—CII—a and apoC-II on LPL activity were greater in smaller size lipoprotein fractions, such as remnant lipoproteins, low-density lipoproteins and high-density lipoproteins. In summary, this report describes a novel mechanism of action for stimulation of LPL activity by apoC-II mimetic peptides.

1. Introduction

Epidemiological and genetic studies have clearly established that elevated triglycerides (TGs) in plasma are an independent risk factor of atherosclerotic cardiovascular disease (ASCVD) [1,2]. Increasing the activity of lipoprotein lipase (LPL), the main enzyme responsible for the hydrolysis of plasma TGs, is one possible approach to reduce the risk of ASCVD [3]. LPL requires apolipoprotein C-II (apoC-II) for its activity in plasma; homozygotes for apoC-II deficiency have severe hypertriglyceridemia, similarly to genetic LPL deficiency [4]. ApoC-II is a small protein that exchanges between triglyceride-rich lipoproteins (TRLs), such as chylomicrons (CMs) and very low-density lipoproteins (VLDLs), and high-density lipoproteins (HDLs). The mean apoC-II concentration in normolipidemic human plasma is approximately 4.5 μM [5]. Several studies

Abbreviations: apo, apolipoprotein; ANGPTL, angiopoietin-like protein; ASCVD, atherosclerotic cardiovascular disease; CETP, cholesteryl ester transfer protein; CM, chylomicron; HDL, high-density lipoprotein; ITC, isothermal titration calorimetry; LDL, low-density lipoprotein; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; SEC, size-exclusion chromatography; TG, triglyceride; TRL, triglyceride-rich lipoprotein; VLDL, very low-density lipoprotein.

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performed with isolated lipoproteins and artificial TG emulsions suggest that normal apoC-II levels in plasma are sufficient for full activation of LPL [5–7]. A recent study has revealed that an apoC-II mimetic peptide, named 18A–CII–a, efficiently decreases plasma TG concentration when given to apoE-knockout mice [8]. This was unexpected as apoC-II concentration has been shown to be positively correlated to TG levels in human plasma [9]. Furthermore, transgenic mice overexpressing human apoC-II have hypertriglyceridemia [10].

18A–CII–a is a bi-helical peptide that contains an 18-residue long amphipathic α-helix called 18A in its N-terminus, which binds to lipoproteins with a high affinity. 18A is linked via proline residue to another amphipathic α-helix containing residues 59–79 of human apoC-II that are needed for LPL activation. 18A–CII–a activated LPL similarly to full-length apoC-II when synthetic emulsion of TG was used as a substrate [8]. It also increased LPL activity in *ex vivo* plasma assays from patients with hypertriglyceridemia [8,11]; however, for practical reasons, plasma samples were at least 50-fold diluted in these assays. Dilution of the plasma may influence the effect of different ligands on LPL stability and activity. In addition to apoC-II, plasma contains numerous components that together with apoC-II influence LPL activity and/or stability. ApoC-I and apoC-III on TRLs and circulating angiopoietin-like proteins (ANGPTLs) 3, 4, 8 all inhibit LPL activity, whereas apoA-V increases LPL activity [12]. LPL activity is also stabilized by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 and heparan sulfate proteoglycans [12–14].

We have recently developed an isothermal titration calorimetry (ITC) assay that allows the analysis of LPL activity, stability and ligand interactions in nearly undiluted human plasma [7]. This assay is based on the detection of the heat rate accompanying LPL hydrolysis of lipids. The ITC-based assay was successfully used to study the effects of apoC-III, apoA-V, ANGPTL3 and ANGPTL4 on LPL activity in undiluted human plasma [7]. In the present study, we applied our ITC-based approach to investigate the mechanism for how the 18A–CII–a peptide and apoC-II influence the activity of LPL in nearly undiluted human plasma.

2. Materials and methods

2.1. Reagents

LPL was purified from bovine milk and dialyzed to buffer containing 10 mM TRIS (pH 8.5, 4 °C) and 4 mM sodium deoxycholate [7]. Recombinant human apoC-II with a C-terminal His_6 tag was expressed in *E. coli* BL21 containing a pET29a plasmid, using an auto-induction protocol [15]. Following expression, cells were lysed in buffer containing 6 M GuHCl and purified with Ni-affinity (HisTrap HP), anion-exchange (HiTrap Q FF) and size-exclusion chromatography (SEC) (Superdex 200 10/300 GL) following standard protocols (GE Healthcare Life Sciences) using the indicated buffers containing 6 M urea. The purified protein was dialyzed against 10 mM ammonium bicarbonate and lyophilized. The 18A–CII–a peptide and an inactive analog called 18A–CII–i were synthesized as previously described [8]. Four residues critical for LPL activation were changed in the 18A–CII–i peptide to alanine [8]. Peptides and apoC-II were dissolved in 35 mM $NaHCO_3$, pH 8.5 buffer, kept at 4 °C and used within one week. Samples of non-fasting human EDTA-plasma were purchased from the Tallinn Blood Centrum. Normolipidemic plasma samples were aliquoted, stored at −80 °C and were used immediately after thawing. Hypertriglyceridemic plasma samples were only used fresh.

2.2. Human plasma separation by SEC

Human plasma was separated using Superose 6 10/300 GL (GE Healthcare Life Sciences) column. 500 μl of nonfasting human plasma (TG = 111 mg/dl) was injected to the column. Elution was performed in a 20 mM TRIS, 0.15 M NaCl, 1 mM EDTA, pH 7.4 buffer. Fractions of 500 μl were collected. TGs and cholesterol were measured in each fraction, using Triglyceride Colorimetric assay kit (Cayman, USA) and Cholesterol Fluorometric Assay Kit (Cayman, USA).

2.3. ITC measurements

Experiments were performed on a Nano ITC model 5300 (TA Instruments, USA) or MicroCal PEAQ-ITC (Malvern Panalytical) at 25 °C [7]. Undiluted human plasma samples were mixed with 20 mM TRIS pH 7.4 buffer and with 18A-CII peptides, apoC-II or 35 mM $NaHCO_3$, pH 8.5 buffer. This resulted in only a 1.2-fold dilution of the plasma samples. Next, plasma samples were loaded to the ITC sample cell. Syringe was filled with purified LPL. To determine initial reaction rates (Fig. 1 and Fig. 3B), five sequential injections of 0.2 nM LPL were made into the ITC cell. The resulting slope of the relationship between heat rate and LPL concentration represented specific activity of LPL. Relative activity of LPL (%) in Fig. 1 was calculated as a ratio of the slope of the experiment where 18A-CII peptides or apoC-II were added to the slope of the experiment where no ligands were added. Sample cells were washed after each experiment [7].

2.4. Non-esterified fatty acid (NEFA) assay for measurement of LPL activity

LPL activity was determined by measuring the generation of NEFAs after lipolysis of human lipoproteins [8] (Fig. 3C) which were normalized to contain 1.7 μg of TGs in incubation mixtures, except HDL, which contained 1.3 μg of TGs. In the case of HDL this was the highest achievable amount. 10 μM 18A–CII–a or apoC-II, 7 nM LPL, 1% BSA, and PBS were added to yield a final reaction volume of 50 μl. Reaction mixtures were incubated for 30 min on ice and then for 1 h at 37 °C. NEFAs were determined using NEFA-HR (2) (FUJIFILM Wako Diagnostics, USA) kit.

3. Results

3.1. Effects of 18A-CII peptides and apoC-II on LPL activity in human plasma

The effects of 18A–CII–a, its inactive analog 18A–CII–i, and full-length apoC-II on LPL activity were first studied by ITC in undiluted human plasma obtained from a subject with normal TG level. The experiments were performed under conditions in which the concentration of LPL was sufficiently low (0.2–1 nM) that consumption of substrate did not influence the reaction rate, meaning that initial rates were determined under zero-order conditions. As can be seen in Fig. 1A, the peptides and apoC-II had different effects on LPL activity. Addition of 18A–CII–a to human plasma increased LPL activity in a concentration-dependent manner, reaching a 77% increase at 50 μM, the highest tested concentration (Fig. 1A). In contrast, apoC-II increased LPL activity up until 20 μM of apoC-II was added, reaching only a maximum increase level of 20%. Higher concentrations led to an inhibition of LPL activity; 14% lower activity compared to baseline was detected when 50 μM of apoC-II was added (Fig. 1A). 18A–CII–i decreased LPL activity in a concentration-dependent manner. The maximum inhibition of 32% occurred when 50 μM

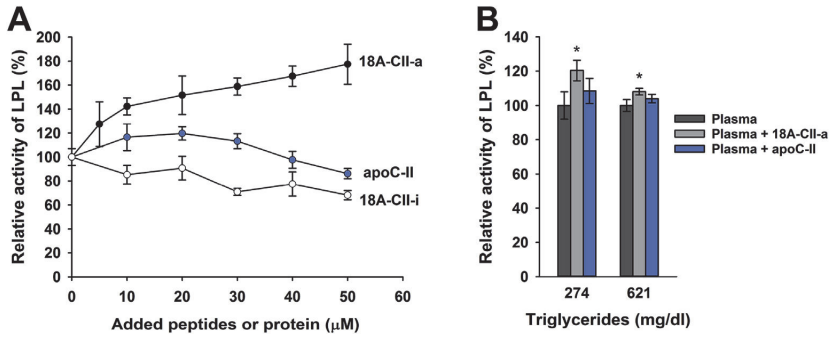

Fig. 1. Effects of 18A-CII peptides and apoC-II on LPL activity in normolipidemic (A) and hypertriglyceridemic (B) human plasma samples as measured by ITC. TG concentration of plasma sample in panel A was 111 mg/dl. In panel B, 10 μM 18A–CII–a, 10 μM apoC-II or buffer was added to pooled hypertriglyceridemic plasma samples with an average of 274 or 621 mg/dl TG concentration. The values are mean ± SD of three different measurements. In panel B, data were compared by two-tailed *t*-test; *P < 0.05 compared with experiments where no ligands were added.

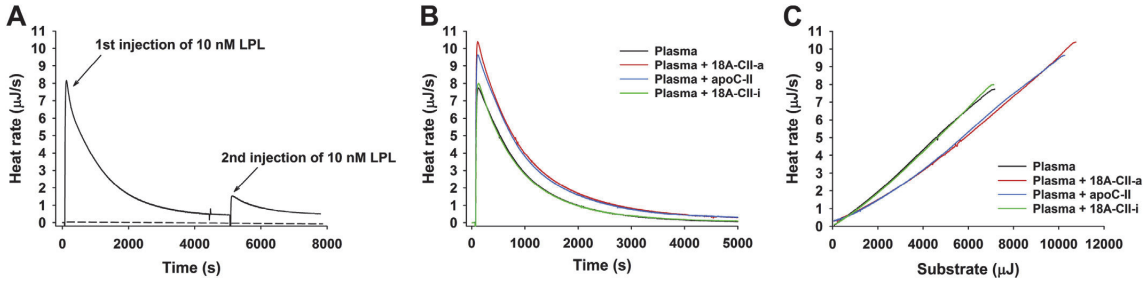

Fig. 2. Complete hydrolysis of available substrate in human plasma by LPL as measured by ITC. (**A**) Two injections of LPL were made into ITC cell containing human plasma. (**B**) One injection of 10 nM LPL was made into ITC cell containing human plasma with no added ligands or with added 10 μM 18A–CII–a, 10 μM apoC-II or 10 μM 18A–CII–i. (**C**) Data presented in panel B was transformed into the relationship between heat rate and remaining substrate concentration (area). TG concentration of the plasma samples were 62 mg/dl. One representative curve from three different measurements is presented for each condition.

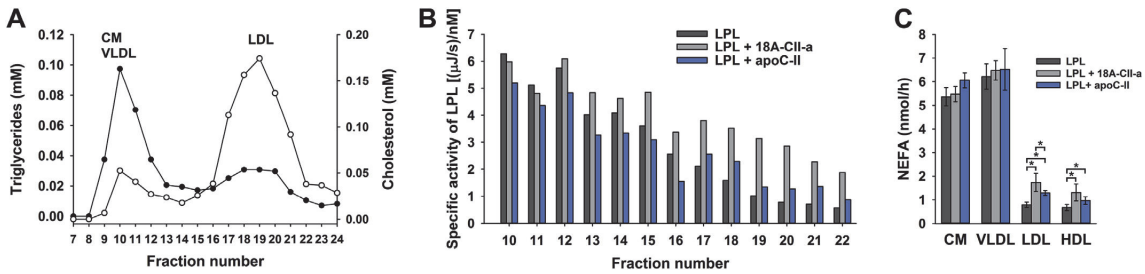

Fig. 3. Effects of 18A-CII-a and apoC-II on LPL activity in human plasma fractions. (A) Human plasma separation using SEC. TGs (●) and cholesterol (○) were measured in each fraction. **(B)** LPL activity determination by ITC in SEC fractions. The fractions were concentrated 5-fold and mixed with 10 μM 18A–CII–a, 10 μM apoC-II or buffer and 10 mg/ml BSA and 10 IU/ml heparin. **(C)** Effects of 18A–CII–a and apoC-II on LPL activity in lipoproteins isolated by density gradient ultracentrifugation as measured by NEFA assay. The values are mean ± SD of four measurements. Data were analyzed by Mann-Whitney test, *P < 0.05.

of 18A–CII–i was added (Fig. 1A). The effects of 18A–CII–a and apoC-II were also tested in pooled hypertriglyceridemic plasma samples (Fig. 1B). Addition of 10 μM 18A–CII–a peptide to pooled plasma samples with a mean TG level of 274 mg/dl (n = 6) or 621 mg/dl (n = 3) resulted in a 20% or 8% increase in LPL activity, respectively. In contrast, increasing apoC-II concentration by 10 μM in these plasma samples did not cause a statistically significant increase in LPL activity.

3.2. Effects of 18A-CII peptides and apoC-II on complete hydrolysis of available substrate in human plasma by LPL

Next, using ITC, we investigated how the addition of 18A-CII peptides or apoC-II influenced the kinetics of complete hydrolysis of available substrate lipids in plasma by LPL. To carry out these measurements within a traceable time period, LPL concentration was increased up to 10 nM, which was 50-fold higher than in the

initial rate experiments presented in Fig. 1. In the first set of experiments, LPL was injected into plasma that did not contain the test peptides or additional apoC-II. After 5000 s, the heat rate level was close to the initial level, suggesting that the reaction catalyzed by LPL was almost complete (Fig. 2A). To be confident that this was not influenced by the inactivation of LPL, a second injection of LPL was made. This resulted only in a relatively small increase of the heat rate, confirming that complete hydrolysis of the available substrate was nearly achieved during the first injection.

The complete hydrolysis experiments were then performed after addition of 10 μM 18A–CII–a, 10 μM 18A–CII–i or 10 μM apoC-II (Fig. 2B). Areas under the complete hydrolysis curves of plasma samples with added 18A–CII–a (area = 10408 ± 953 μJ, mean of 3 measurements ± SD) or apoC-II (area = 9892 ± 1125 μJ) were ~1.5-fold larger than for plasma samples without added ligands (area = 7109 ± 292 μJ). When 18A–CII–i was added there was almost no effect on the area of the complete hydrolysis curve (area = 6913 ± 851 μJ) compared to the reference curve (Fig. 2B). The area under a corresponding thermogram is equal to the total heat production, which is proportional to the hydrolyzed TG concentration of the plasma sample [7]. Thus, these experiments indicated that the addition of 18A–CII–a or apoC-II to human plasma increased the amount of available substrate for LPL.

To further analyze the complete hydrolysis results, the data presented in Fig. 2B were transformed into a relationship between heat rate (reaction rate) and remaining substrate concentration (area) (Fig. 2C). This was obtained by subtracting the hydrolyzed substrate concentration at a chosen time point (area up to this point) from the total substrate concentration (the total area). These relationships revealed how the addition of the peptides or apoC-II influenced reaction rate dependency on substrate concentration. Although there was more available substrate after the addition of 18A–CII–a or apoC-II, the "new available substrate" was not hydrolyzed as efficiently by LPL as the substrate that was first available without the addition of 18A–CII–a or apoC-II (Fig. 2C). The kinetics of lipid hydrolysis by LPL was not changed after addition of 18A–CII–i compared to the experiment where no ligands were added.

3.3. Effects of 18A–CII–a and apoC-II on LPL activity in human plasma fractions

To investigate which lipoprotein fractions LPL activity was increased by the 18A–CII–a peptide, human plasma was separated by SEC (Fig. 3A). The effects of 18A–CII–a and apoC-II were investigated on each plasma fraction separately using ITC (Fig. 3B). As expected, the specific activity of LPL was the highest in the fractions 10–13 that contained TRLs. Interestingly, the addition of 18A–CII–a to fraction 10 or 11 did not increase LPL activity. However, 18A–CII–a modestly increased LPL activity in fractions 12 and 13, containing smaller size TRLs. Addition of extra apoC-II to these fractions even reduced the activity of LPL. Specific activity of LPL decreased as the following fractions containing smaller size lipoproteins were used as a substrate. At the same time, the relative effect of 18A–CII–a on LPL activity started to increase with the following fractions containing remnant lipoproteins. In Table 1, LPL activities from Fig. 3B are presented as a ratio of the specific activity of LPL with added 18A–CII–a or apoC-II to the specific activity of LPL without the added peptide or protein. The relative effect of 18A–CII–a on LPL activity was the highest with fraction number 20, which contains low-density lipoproteins (LDLs) and likely smaller size remnant lipoprotein particles (Table 1 and Fig. 3B). Addition of the peptide to this fraction caused a 3.7-fold increase in LPL activity. Effect of apoC-II in this fraction calculated as a fold change in LPL activity was more than 2 times lower (Table 1).

Table 1
Fold change in LPL activity after addition of 18A-CII-a or apoC-II to human plasma fractions. Fold change was calculated from data presented in Fig. 3B as a ratio of the specific activity of LPL with added 18A–CII–a or apoC-II to the specific activity of LPL without the added ligands.

Fraction number	Fold change in LPL activity	
	18A–CII–a	apoC-II
10	0.95	0.83
11	0.94	0.85
12	1.06	0.84
13	1.2	0.82
14	1.13	0.82
15	1.35	0.86
16	1.32	0.6
17	1.79	1.21
18	2.22	1.45
19	3.1	1.34
20	3.67	1.64
21	3.23	1.93
22	3.26	1.51

Next, the effects of 18A–CII–a and apoC-II on LPL activity were examined in CM, VLDL, LDL and HDL fractions isolated from human plasma by density gradient ultracentrifugation, using a NEFA assay (Fig. 3C). Although the addition of 18A–CII–a or apoC-II to CM or VLDL samples tended to somewhat increase LPL activity, these effects were not statistically significant. Both 18A–CII–a and apoC-II had their largest relative effects on LPL activity in LDL and HDL fractions.

4. Discussion

The novel ITC assay of LPL that we developed enabled us to more carefully investigate drug candidate properties of the 18A–CII–a peptide, whose TG-lowering effect has been shown in various mouse models for hypertriglyceridemia [8,16,17] and in apoC–II–knockout zebrafish [18]. The main advantage of the ITC assay is that it makes it possible to perform experiments in human plasma samples with only minimal dilution, thus insuring that the concentration and proportions of lipoproteins and LPL regulators (activators and inhibitors), as well as other plasma components that can influence the LPL activity remained physiological. Furthermore, the results of measurements in undiluted plasma also take into account the possible macromolecular "crowding effect" of plasma on LPL activity and interactions [19].

Although addition of both apoC-II and 18A–CII–a peptide to plasma increased LPL activity, 18A–CII–a peptide was a more potent activator of LPL than apoC-II. The maximum increase in LPL activity after addition of 18A–CII–a to normolipidemic plasma sample was 3.5-fold greater than in the case of extra apoC-II. Unlike apoC-II, high concentrations of 18A–CII–a did not inhibit LPL activity. The inhibitory properties of apoC-II can possibly be attributed to its N-terminal domain, since this region is different in 18A–CII–a and apoC-II. Although 18A–CII–a increased LPL activity in all tested concentrations in both normolipidemic and hypertriglyceridemic plasma samples, the effect was larger in the samples obtained from normolipidemic individuals.

The observation that the addition of apoC-II or 18A–CII–a peptide to normolipidemic human plasma increased LPL activity was unexpected based on clinical reports of apoC-II deficiency. In general, heterozygotes for apoC-II deficiency have normal TG levels in plasma, suggesting that their plasma contains sufficient amounts of apoC-II for full LPL activation [5]. Our results demonstrate, however, that at least in some cases, LPL is not saturated by apoC-II. This is in line with a recent study that showed that the

downregulation of a long non-coding RNA, termed liver-specific TG regulator (lncLSTR), increased plasma apoC-II levels and LPL activity in mice, which resulted in decreased plasma TG levels [20]. Also, it was recently demonstrated that endogenous apoC-II levels are insufficient in mice to rapidly clear TGs after intravenous injection of TG-rich emulsion. Injection of apoC-II mimetic peptide to these mice helped to overcome the insufficiency of apoC-II and TG-rich emulsion was rapidly cleared [17].

The increase in LPL activity after addition of 18A–CII–a or apoC-II to plasma was due to the increase of the amount of available substrate for LPL. This indicates that part of the substrate in plasma was not available for LPL without addition of 18A–CII–a or extra apoC-II. This also suggests that LPL was not saturated with the substrate, meaning that part of LPL was not active in plasma. It is likely that "inactive" LPL was bound to lipoproteins but was not hydrolyzing TGs. After addition of 18A–CII–a or apoC-II, this part of LPL became activated. It has been shown that while LPL prefers TRLs as a substrate, it can distribute between all classes of lipoproteins in plasma [21,22]. It is possible that some of the particles which became available for LPL after addition of 18A–CII–a simply did not contain enough apoC-II. While the relative effects of 18A–CII–a and apoC-II on LPL activity were greater in smaller size lipoproteins, such as remnant lipoproteins, LDLs and HDLs, 18A–CII–a also slightly enhanced LPL activity in smaller size TRL fractions. Thus, it is likely that fraction of TGs in almost all lipoprotein classes was not available for LPL and became available only after addition of the peptide. Overall, our data suggest that 18A–CII–a helps to hydrolyze smaller TRLs and makes otherwise poorly hydrolysable remnant lipoproteins, LDLs and HDLs more available for LPL.

The relatively small stimulating effect of 18A–CII–a on the hydrolysis of isolated TRLs was also unexpected as it was previously shown that 18A–CII–a lowers VLDL-TG concentration in apoE-knockout mice [8] and also in mice with a defective truncated form of apoC-II [16]. This suggests that, *in vivo*, there may be additional mechanisms responsible for the TG lowering effect of the peptide. At least in humans, TG rapidly equilibrates *in vivo* between lipoproteins by cholesteryl ester transfer protein (CETP) and thus a significant fraction of TG hydrolysis can possibly occur on lipoprotein particles besides TRLs. The main focus on CETP has been its role in equilibrating cholesteryl esters between lipoproteins, but in terms of energy metabolism it may be that one of its main roles is the transfer of TG from TRL to other lipoproteins to enhance overall lipolysis. From an energy delivery standpoint, the re-delivery of any unhydrolyzed TG to the liver by remnant uptake defeats the main purpose for VLDL secretion. It is important to note, however, while the relative effects of 18A–CII–a and apoC-II were larger on smaller size lipoproteins, much more TGs are present in TRLs than in LDLs and HDLs. Thus, the small relative effect of 18A–CII–a on TRL degradation may have larger impact, than the effect on smaller particles, on total reduction of TGs *in vivo*. Finally, high levels of TGs in remnant lipoproteins and in LDLs are associated with increased cardiovascular mortality [2,23]. In fact, it was recently shown in ARIC, a large epidemiologic study of heart disease, that the TG content of LDL was a better predictor of ASCVD risk than LDL-Cholesterol [24]; thus, the lowering of TG on remnants and LDL could be beneficial for reducing ASCVD risk. At the same time, further investigations are needed to ascertain whether the increased lipolysis of HDL by LPL has any impact on ASCVD risk.

In conclusion, our results demonstrate that apoC-II mimetic 18A–CII–a has different properties in promoting lipolysis than the human endogenous apoC-II protein. The 18A–CII–a peptide is significantly more efficient in stimulating LPL activity in human plasma and it does not inhibit LPL activity at higher concentrations unlike apoC-II. Thus, while increasing the concentration of apoC-II in individuals with common forms of hypertriglyceridemia would likely not be beneficial, based on our findings treating such patients with 18A–CII–a could be an effective way to lower TG and thus supports future development of this peptide.

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PUBLICATION III

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Combined action of albumin and heparin regulates lipoprotein lipase oligomerization, stability, and ligand interactions.

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RESEARCH ARTICLE

Combined action of albumin and heparin regulates lipoprotein lipase oligomerization, stability, and ligand interactions

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Abstract

Lipoprotein lipase (LPL), a crucial enzyme in the intravascular hydrolysis of triglyceride-rich lipoproteins, is a potential drug target for the treatment of hypertriglyceridemia. The activity and stability of LPL are influenced by a complex ligand network. Previous studies performed in dilute solutions suggest that LPL can appear in various oligomeric states. However, it was not known how the physiological environment, that is blood plasma, affects the action of LPL. In the current study, we demonstrate that albumin, the major protein component in blood plasma, has a significant impact on LPL stability, oligomerization, and ligand interactions. The effects induced by albumin could not solely be reproduced by the macromolecular crowding effect. Stabilization, isothermal titration calorimetry, and surface plasmon resonance studies revealed that albumin binds to LPL with affinity sufficient to form a complex in both the interstitial space and the capillaries. Negative stain transmission electron microscopy and raster image correlation spectroscopy showed that albumin, like heparin, induced reversible oligomerization of LPL. However, the albumin induced oligomers were structurally different from heparin-induced filament-like LPL oligomers. An intriguing observation was that no oligomers of either type were formed in the simultaneous presence of albumin and heparin. Our data also suggested that the oligomer formation protected LPL from the inactivation by its physiological regulator angiopoietin-like protein 4. The concentration of LPL and its environment could influence whether LPL follows irreversible inactivation and aggregation or reversible LPL oligomer formation, which might affect interactions with various ligands and drugs. In conclusion, the interplay between albumin and heparin could provide a mechanism for ensuring the dissociation of heparan sulfate-bound LPL oligomers into active LPL upon secretion into the interstitial space.

Introduction

Lipoprotein lipase (LPL) is a crucial enzyme in the intravascular hydrolysis of triglyceride-rich lipoproteins (TLRs). LPL deficiency causes hypertriglyceridemia, which is causally linked to

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cardiovascular disease (CVD) and pancreatitis[1–5]. LPL and its regulators are potential drug targets for the treatment of hypertriglyceridemia[6–8]. LPL is mainly produced by adipose and muscle parenchymal cells and secreted into the interstitial space where a capillary endothelial protein called glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) transports LPL to the endothelium of the vascular lumen[9, 10]. At this site, LPL catalyzes the hydrolytic degradation of triglycerides in chylomicrons (CM) and very low-density lipoproteins (VLDL). In addition to GPIHBP1 and lipoproteins, secreted LPL is known to interact with apolipoprotein CII[11], fatty acids[12, 13], heparan sulfates [14], and angiopoietin-like proteins (ANGPTLs) 3, 4, and 8 [15]. LPL activity is also indirectly affected by apolipoproteins CIII, CI [16], and AV [17]. Combined action of these ligands may play a key role in the extracellular regulation of LPL activity [17, 18]. Regulators and ligands can affect LPL in a variety of extracellular regions, including the interstitial space and vascular endothelium. In the postprandial state, LPL is also associated with circulating TRLs and is involved in the uptake of remnant lipoproteins by the liver [19].

Although significant progress has been made in understanding the complex regulation system of LPL, there are still several aspects that require further investigation. This is because the basic mechanistic knowledge about LPL and its regulators is primarily based on experiments performed with purified proteins and synthetic lipid substrates in conventional buffer solutions. However, it should be considered that in the native capillary plasma environment, which contains thousands of proteins and other molecules, extracellular effects on LPL can occur. The high plasma protein content (80 g/liter) is sufficient to produce a macromolecular crowding effect [20] that may affect LPL and its ligands. Many studies indicate that the stability, structure, and aggregation of proteins in general can be significantly affected by the macromolecular crowding effect [21–23]. In addition to the crowding effect, LPL may be affected by plasma components which have not been identified yet.

Numerous studies indicate that LPL can exist in different conformational and oligomeric states. It has been shown by several studies that purified and active LPL appears as a dimer. These dimers rapidly exchange subunits and can irreversibly dissociate into inactive monomers [24, 25]. According to this mechanism, a part of the active LPL pool is always in monomeric form. Recent studies have indicated that LPL can be in an active monomeric form under certain conditions [26]. In addition, based on recent x-ray crystal studies, monomeric LPL forms a stable 1:1 complex with its transporter GPIHBP1 [27, 28]. This state has been proposed to be the active form of LPL at the vascular endothelium [29]. Under physiological salt concentration, temperature and pH, isolated LPL is very unstable and loses most of its activity within minutes. This inactivation occurs due to conformational changes in the N-terminal domain of LPL and leads to the irreversible formation of inactive monomers that can form amorphous aggregates [25]. The inactive monomeric LPL has lower affinity for heparin than the active form, this difference is used for their separation using heparin-chromatography [30]. ANGPTL3, 4 and 8 actively regulate the ratio between inactive and active LPL as a means of post-translational regulation of LPL in tissues [31]. Similar to the thermal inactivation, ANGPTL4 induces conformational changes in the N-terminal domain of LPL [32]. However, the states of LPL are not limited to dimer and monomer, as recent data revealed that natively folded LPL can form helical oligomers at high concentrations, which are stabilized by the presence of heparin [33]. These oligomers are composed of dihedral LPL dimers, and unlike the amorphous irreversible aggregates of LPL, their dissociation restores the catalytic activity of LPL. The existence of inactive, helical form of LPL raises the question of under which conditions is LPL activity irreversibly vs. reversibly lost. In addition, there is the question of where different oligomeric forms of LPL are present in the body, and how LPL transitions between these forms.

LPL oligomerization varies as it travels through the cell, interstitial space, and vascular endothelium. Prior to secretion, it has been shown that LPL forms helical oligomers in the presence of heparan sulfate proteoglycans (HSPG) attached to syndecan-1 (SDC1) inside of adipose cell vesicles [33]. Following secretion, LPL has been found to linger at the cell surface in HeLa cells, tethered there by HSPGs [34]. This raises the question of what precipitates LPL dissociation from the HSPG, permitting LPL to transfer across the interstitial space and reach GPIHBP1. During this time in the interstitial space, it is crucial that LPL does not degrade prior to binding GPIHBP1, which facilitates LPL's transfer into the capillaries. LPL is known to be stabilized by heparin, which is comparable to HSPGs [14, 33], which are present in the interstitial space. However, it begs the question whether there are additional components that influence LPL in the interstitial space, given the propensity of LPL to oligomerize in the presence of HSPGs.

In the present study we show that albumin, the major protein in blood plasma, forms a complex with LPL and has a significant effect on LPL stability and oligomerization. Surface plasmon resonance (SPR) measurements revealed that the LPL-albumin complex is dynamic, with a lifetime of less than few seconds. The equilibrium dissociation constant of the complex is 30–70 μM, which is comparable to the albumin concentration in the interstitial space [35], suggesting the physiological relevance of the LPL-albumin interaction. Negative stain transmission electron microscopy (nsTEM) and raster image correlation spectroscopy (RICS) measurements indicate that albumin, like heparin, induces reversible concentration dependent oligomerization of LPL. However, the oligomers decomposed when both heparin and albumin were present, indicating that albumin may play a role in liberating LPL from HSPGs allowing LPL to transit across the interstitial space. The reversible oligomerization also protected LPL from inactivation by its physiological regulator ANGPTL4. Based on these observations, we conclude that the role of albumin in the LPL system is more diverse than the previously known role of binding lipolysis-derived fatty acids in the capillaries.

2. Materials and methods

2.1 Reagents

Normolipidemic non-fasting plasma samples were purchased from Tallinn Blood Centrum, aliquoted and stored at -80˚C. Triglyceride-rich lipoprotein fractions (CM/VLDL) were isolated from human plasma by density gradient ultracentrifugation. Goat serum as a source of apoC-II was obtained from Invitrogen (#10000C). Bovine LPL was purified from bovine milk [36] and dialyzed against 1 M NaCl and 20 mM NaH_2PO_4, pH 7.4 or 20 mM Bis-Tris, pH 6.5. Recombinant human LPL was purchased from Bio-Techne (#9888-LL) with a specific activity of >2,500 pmol/min/μg as determined with 4-nitrophenyl butyrate. Recombinant N-terminal domain of ANGPTL4 (nANGPTL4$^{26\text{-}184}$) was expressed in *E. coli* and purified as previously described [37]. Full-length ANGPTL4 was purchased from BioVendor (#RD172073100-HEK). Human GPIHBP1 was purchased from Sino Biological (#15388-H02H). Both proteins were assessed by reducing and non-reducing SDS-PAGE prior to use. 1,2-Di-O-lauryl-rac-glycero-3-glutaric acid 6-methylresorufin ester (DGGR) (#30058) and bovine serum albumin (#A7906) were purchased from Sigma Aldrich. Na-deoxycholate (#218590250) and heparin (#411212500) were purchased from Acros Organics. Polyethylene glycol 6000 (PEG 6) was purchased from Alfa Aesar (#A17541) and dextran 40000 (dextran 40) from Sigma Aldrich (#31389). The amino coupling kit (containing N-hydroxysuccinimide, N-ethyl-N9 [(diethylamino)propyl]carbodiimide, 1M ethanolamine) and BIAcore sensor chips were purchased from GE Healthcare. Fluorescence reagent ATTO610-NHS ester was purchased from Merck (#93259). Lipoprotein free human plasma (LFHP) from a single donor was obtained by

flotation ultracentrifugation at d = 1.215 g/ml [38] and dialyzed against 150 mM NaCl. Residual triglyceride and cholesterol concentrations in LFPH were respectively 40 μM and 10.34 μM.

2.2 Determination of LPL activity using isothermal titration calorimetry (ITC)

Catalytic activity of LPL was measured using an ITC assay that allows to determine LPL activity in complex substrate systems including undiluted human plasma [39]. The assay is based on the detection of changes in heat rate as a result of LPL catalyzed hydrolysis of lipids. The heat rate detected by ITC corresponds to the release of fatty acids and is related to the catalytic activity of LPL. There is a linear correlation between heat production and the number of fatty acids released. LPL activity is expressed as μJ/s (microjoule per second). The experiments in this study were performed on a MicroCal PEAQ-ITC (Malvern) as previously described [40]. In a standard measurement, the calorimetric cell (200 μl) was filled with a substrate mixture and the syringe (40 μl) contained 200 nM LPL in 150 mM NaCl, 20 mM HEPES, pH 7.4 buffer with 50 mg/ml BSA and 10 IU/ml heparin. The reference cell was filled with MilliQ water (200 μl). The stirring speed in the sample cell was set to 1000 rpm in all experiments. Automatic baseline stabilization took 5–12 min, after which a 0.4 μl LPL injection was made. Following that, a 5 μl LPL injection was made, which increased LPL concentration in the cell by 5 nM and the new baseline was measured for 3 min. All incubations and measurements were carried out at room temperature. The sample cell and syringe were washed with 10% Decon 90 and rinsed with MilliQ water after each experiment. The syringe was additionally rinsed with methanol.

2.3 Determination of LPL activity with DGGR

LPL activity was determined with fluorogenic substrate DGGR, using a spectrofluorophotometer (Shimadzu RF-5301 PC, Shimadzu Corporation, Japan). The reaction was followed for 3 minutes at 25˚C, using an excitation wavelength of 572 nm and an emission wavelength of 605 nm. Samples were prepared by incubating 200 nM LPL at room temperature in a 150 mM NaCl, 20 mM HEPES, pH 7.4 buffer with various supplements or in LFHP. The incubation mixture was diluted to 10 nM LPL in a measurement buffer containing 150 mM NaCl, 20 mM HEPES, 10 IU/ml heparin, 24 μM DGGR and 0.5% Triton X-100. A 4 mM stock solution of DGGR was prepared in ethanol.

2.4 Determination of the affinity for the interaction of LPL-BSA using ITC

The binding affinity between bLPL and BSA was determined using MicroCal PEAQ-ITC. The calorimetric cell was filled with 1.695 μM bLPL and 10 IU/ml heparin in 20 mM HEPES, 150 mM NaCl, pH 7.4 buffer. The syringe contained 752 μM BSA in the same conditions and the reference cell was filled with degassed MilliQ water. Titration was carried out at 25˚C with a reference power set to 41.9 μJ/s and at a stirring speed of 750 rpm. Binding affinity was estimated based on 38 sequential injections of BSA where each injection increased BSA concentration in the cell by 3.76 μM. A control experiment (dilution of BSA) was carried out in the same conditions but without bLPL in the measurement cell. Data analysis and K_D estimation was performed with MicroCal PEAQ-ITC Analysis Software (Malvern).

2.5 Stabilization measurements for estimation of affinity for the interaction of LPL-BSA

Human or bovine LPL (200 nM) was preincubated alone or with various concentrations of BSA at room temperature in a solution of 20 mM HEPES, 150 mM NaCl, pH 7.4. After 60

minutes, LPL activity was determined at 25˚C using DGGR as a substrate. Stabilization effect of BSA was calculated by subtracting the LPL activity at 60 minutes from the initial LPL activity. The affinity (expressed as K_D) of BSA for bLPL or hLPL was calculated by the equation:

$$v = \frac{V_m \cdot [BSA]}{K_D + [BSA]} + v_0 \quad (1)$$

where v is the catalytic activity of hLPL or bLPL at any BSA concentration, [BSA]—BSA concentration, v_0 –catalytic activity of bLPL or hLPL in the absence of BSA, V_m–maximal bLPL or hLPL activity achievable by increasing the BSA concentration. To obtain estimation for K_D, SigmaPlot software (SPSS, Chicago, IL, USA) and its hyperbolic curve fit function was used for fitting the experimental data according to Eq 1.

2.6 Surface plasmon resonance measurements

SPR experiments were performed on a Biacore 3000 instrument using CM5 sensorchips (GE Healthcare). The binding of BSA to bLPL was analyzed using two experimental set ups. In one experiment, biotinylated bLPL was attached to the surface of a CM5 sensor chip in 20 mM HEPES, 150 mM NaCl, pH 7.4 buffer via neutravidin that was covalently pre-immobilized using the amino coupling kit (GE Healthcare). It has been previously demonstrated that bLPL immobilized in this manner retains its ability to bind lipoproteins and ANGPTL4 [41]. 1074 RU of biotinylated bLPL was bound to the surface of the sensor which corresponded to a surface density of 19.5 fmol/mm2. In the other experiment, bLPL in 20 mM HEPES, 150 mM NaCl, 10 IU/ml heparin, 2 mg/ml BSA, pH 7.4 buffer was attached to GPIHBP1 that was covalently pre-immobilized in 10 mM acetic acid (pH 3.5) using the amino coupling kit. In this case, 215 RU of bLPL was bound to the immobilized GPIHBP1 corresponding to surface density of 3.9 fmol/mm2. Anti-LPL monoclonal antibody 5D2 was immobilized to a sensor chip according to Mysling et al. [42]. In this case, bLPL was bound to the immobilized 5D2 in 20 mM HEPES, 150 mM NaCl, 10 IU/ml heparin, pH 7.4. The surface density of bound LPL was 1.7 fmol/mm2. Binding of BSA to the sensor chips with bLPL were carried out at 25˚C in a buffer that contained 20 mM HEPES, 150 mM NaCl, pH 7.4. For data analysis, nonspecific binding and bulk effect was subtracted from the total binding.

For calculation the number of BSA molecules bound to immobilized LPL (n), we used the following equation:

$$n = \frac{RU_A / Mw_A}{RU_L / Mw_L} \quad (2)$$

where RU_A is the response for binding of BSA to the immobilized bLPL at the steady state; RU_L is the amount of immobilized LPL expressed in response units; Mw_A and Mw_L are molecular weights of BSA (66 kDa) and bLPL (55 kDa), respectively.

2.7 Determination of diffusion coefficients using raster image correlation spectroscopy

LPL was labelled with ATTO610-NHS ester for these experiments. This LPL conjugate was prepared as follows: LPL was dialyzed against 200 mM $NaHCO_3$, 1 M NaCl, pH 8.4 buffer, after which LPL was incubated with ATTO610 at a 1:5 molar ratio for 2 h at 4˚C. The active fluorescent LPL was purified using heparin chromatography and the concentration and degree of labeling was determined by measuring optical densities at λ_{280} and λ_{616}. Samples for RICS were prepared by incubating 10 nM LPL-ATTO610 with 190 nM unlabeled LPL at room

temperature for up to 2 hours in buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl and either 2 or 50 mg/ml BSA or 10 IU/ml heparin. Heparin was added after 15 minutes into LPL incubation with BSA in measurements with both BSA and heparin. LPL was incubated for at least 2 hours in incubations with 1 M GuHCl to allow for monomerization of LPL to happen [43].

Raster image correlation spectroscopy (RICS) was used to determine diffusion coefficient assuming that the sample consisted of a single component. Previously developed RICS extensions were used [44, 45]. Namely, sample was scanned at three different line scanning frequencies and imaging a region of 20 x 20μm. The signal was acquired during half of the scan time with the other half used for flyback with each image containing 1000 lines and pixel time set to 1μs. Images were acquired in sets of 90 (line scanning frequency 500 Hz), 60 (289 Hz), and 30 (167 Hz) frames with the order of the used frequencies randomized. Measurements were performed on custom built confocal microscope as previously described [45]. Analysis of the samples involved in this work required additional filtering step. Namely, samples contained a small fraction of large particles that were visible on some of the images. Such large particles disturbed the correlation analysis and were removed from overall diffusion coefficient determination, as described in Results. To filter the images with the large particles, each acquired image was split into four equal sections and correlation functions (CF) for each section was found. CF from each section was fitted by single-component diffusion model and the diffusion coefficient together with the number of particles in the confocal volume. For image sections with the large particles, estimated diffusion coefficient and number of particles in confocal volume were much smaller than for the image sections without such particles (see Results for examples). The sections without large particles were later analyzed by finding CF on the basis of all data and fitting CF obtained for each line scanning frequency with the same model parameters. The used models assumed Gaussian form of the point spread function and triplet state. Measurements and analysis of RICS data was done blindly with the samples prepared and enumerated by personnel not involved in the measurements and analysis of RICS data to avoid any bias.

2.8 Negative stain transmission electron microscopy (nsTEM)

Samples were prepared as described for RICS. Briefly, LPL was diluted to 200 nM in buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, and 2 mg/ml BSA (A7906, Sigma) or 10 IU/ml Heparin (H19, Fisher scientific) and incubated for 30 min. For samples with both BSA and heparin, heparin was added 15 min into incubation. For samples with both heparin and triton X-100, triton X-100 was added 15 min into incubation. Following incubation, 5 μl of each sample was applied to a glow discharged ultrathin carbon nsTEM grid (CF300_CU_UL, EMS). Grids were glow discharged with a PELCO easiGlow for 25 seconds to ensure hydrophilization of the grid carbon. Sample was incubated on the grid for 1 min followed by a wash with 100 μl of 20 mM HEPES pH 7.4 and 150 mM NaCl. The grid was then stained for 1 min with a filtered 2% uranyl acetate solution in 50% ethanol, blotted, and allowed to dry. Grids were imaged using a TECNAI T12 microscope equipped with a Gatan Rio camera. Images were collected using DigitalMicrograph and analyzed with ImageJ.

3. Results

3.1 Effect of BSA on LPL stability

LPL irreversibly loses most of its catalytic activity in ordinary buffers within minutes but is stable for several hours in undiluted human plasma as shown by the measurements of ITC [39]. This stabilization could be due to the binding of LPL to lipoprotein surfaces, as this effect has

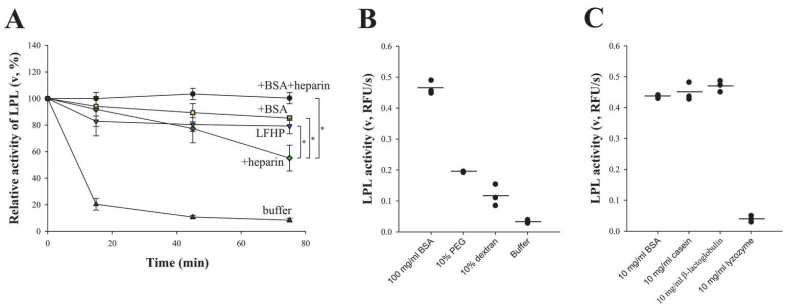

Fig 1. Catalytic activity of LPL measured with DGGR after incubation in various conditions. (A) 200 nM LPL was incubated in buffer A (▲), which contained 10 IU/ml heparin (◆), 50 mg/ml BSA (■) or both (●). Alternatively, LPL was incubated in LFHP (▼). LPL activity is expressed relative to the initial activity of the experiment that contained both heparin and BSA. Results at 75 minutes were compared by two-tailed Student's *t*-test. *P<0.05. LPL loses its activity quickly in plain buffer but is stabilized by lipoprotein free human plasma (LFHP) or its main constituent albumin (BSA). The most significant stabilization was observed when both BSA and heparin were used together. **(B)** 200 nM LPL was incubated in macromolecularly crowded buffer A. LPL activity is expressed as relative fluorescence units per second (RFU/s). PEG 6 nor dextran 40 could stabilize LPL like BSA, indicating that macromolecular crowding alone is not sufficient for LPL stabilization. **(C)** 200 nM LPL was incubated in buffer A with various proteins. Casein and β-lactoglobulin were as efficient as BSA in stabilizing LPL, however lysozyme failed to exert any effect. This suggests that LPL stabilization by proteins depends on their specific characteristics such as charge or hydrophobicity.

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also been observed in some artificial substrate emulsions [46]. To examine whether other plasma components affect LPL stability, incubations were performed in lipoprotein free human plasma (LFHP), and in various other solutions for comparison: a) 150 mM NaCl, 20 mM HEPES, pH 7.4 buffer (buffer A), b) buffer A supplemented with BSA, the main plasma protein, c) buffer A supplemented with heparin (a well-known stabilizer of LPL), d) buffer A supplemented with both BSA and heparin. LPL activity was measured at multiple time-points using the fluorogenic substrate DGGR. As seen in **Fig 1A**, LPL lost almost 80% of its initial activity within 15 min in buffer A. Significant stabilization was recorded in all other solutions used. LFHP and BSA were somewhat more effective stabilizers than heparin. The most significant LPL stabilization was observed when both BSA and heparin were present, as LPL was completely stable for 75 minutes in this case. The similar stabilization effect of BSA and LFHP suggests that albumin is a major stabilizer of LPL in plasma.

We next wanted to address the mechanism by which BSA might stabilize LPL. We first investigated whether BSA was acting as a macromolecular crowder, effectively stabilizing LPL by altering the properties of the surrounding buffer environment. For this purpose, BSA was replaced with 10% PEG or 10% dextran in the LPL incubation mixture with buffer A. The concentration of 10% for PEG or dextran was chosen to produce a crowding effect that is comparable or higher than that of BSA at the concentration 100 mg/ml [47]. The results of these experiments are presented as residual LPL activity determined after 60 minutes of incubation (**Fig 1B**). The loss of LPL activity was greater in buffer conditions without crowders, but neither PEG nor dextran stabilized LPL to the extent BSA did. LPL activity was at least two-fold higher when incubated with BSA instead of PEG or dextran. This observation suggests that macromolecular crowding alone is not sufficient to cause the stabilization of LPL.

We did find that BSA was not the only protein that stabilized LPL as the milk proteins casein and β-lactoglobulin had a similar ability (**Fig 1C**). However, lysozyme, a small antibacterial protein, lacked this property. Thus, the stabilization of LPL seems not to be a general property of proteins, but rather depends on their specific characteristics. The effect of casein agrees with previous studies (casein stabilizes LPL in milk [48]) and β-lactoglobulin may also

play a similar role. These proteins were chosen for comparison randomly based on their stability and solubility at room temperature.

The above experiments suggest that albumin plays a role in stabilizing LPL in the absence of substrate, the situation that occurs in vivo in the interstitial space after secretion of LPL from parenchymal cells. However, albumin is also required as a fatty acid acceptor to keep LPL active on triglycerides of long chain fatty acids. In vivo this occurs at the vascular endothelium when LPL binds lipoproteins and hydrolyzes their triglycerides. In the absence of albumin, long chain fatty acids inhibit LPL activity [12]. Here, we investigated whether the effect of albumin on LPL is limited to the fatty acid binding during lipolysis of lipoproteins by replacing BSA with another fatty acid acceptor, β-cyclodextrin, in the calorimetric assay (**Fig 2**). LPL activity was determined with ITC using a mixture of isolated triglyceride-rich lipoproteins CM/VLDL (adjusted to 0.5 mM triglycerides) accompanied by BSA, PEG or dextran. The concentrations of BSA (0.75 mM) and β-cyclodextrin (8.81 mM) were chosen to be well above the required limit to bind all FFAs released during lipolysis. The lack of difference in LPL activity between the use of two different FFA acceptors, BSA and β-cyclodextrin, allows to conclude that the role of albumin during endothelial lipolysis is confined to binding released FFAs. Introducing a crowded environment did not seem to significantly affect LPL activity towards TRLs. Only a 20% increase in LPL activity was noticed when dextran was used as a macromolecular crowder, and no change was detected when BSA or PEG was involved. This implies that the role of albumin in stabilizing LPL is likely to occur prior to its movement onto the endothelial surface.

3.2 LPL forms a complex with BSA

Next, we investigated whether LPL was able to form a direct complex with BSA. The formation of the bLPL-BSA complex was first demonstrated by ITC by titrating BSA into a solution with

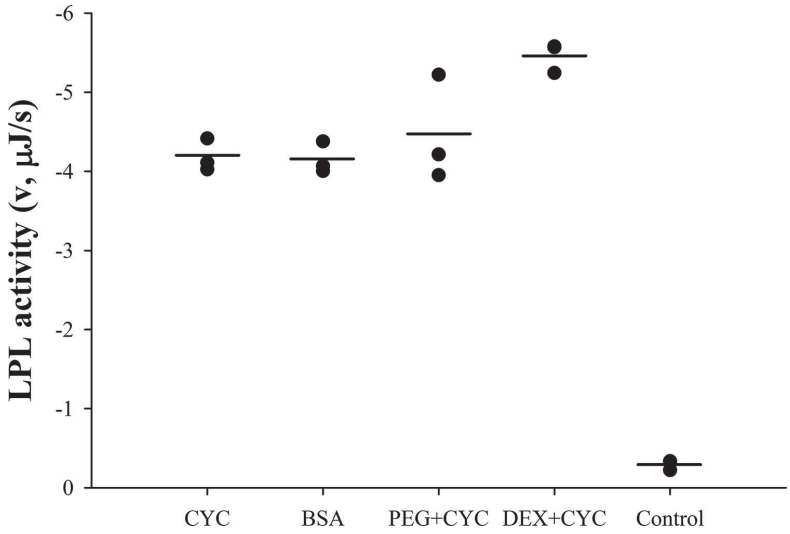

Fig 2. Catalytic activity of LPL on triglyceride-rich lipoproteins in the presence of fatty acid acceptors and macromolecular crowders. Measurements were performed using ITC. LPL activity is expressed as heat rate (μJ/s). The substrate mixture contained CM/VLDL (adjusted to 0.5 mM triglycerides), 50 mg/ml BSA (0.75 mM) or 10 mg/ml β-cyclodextrin (8.81 mM) (CYC) and either 10% PEG 6 or 10% dextran 40 (DEX).

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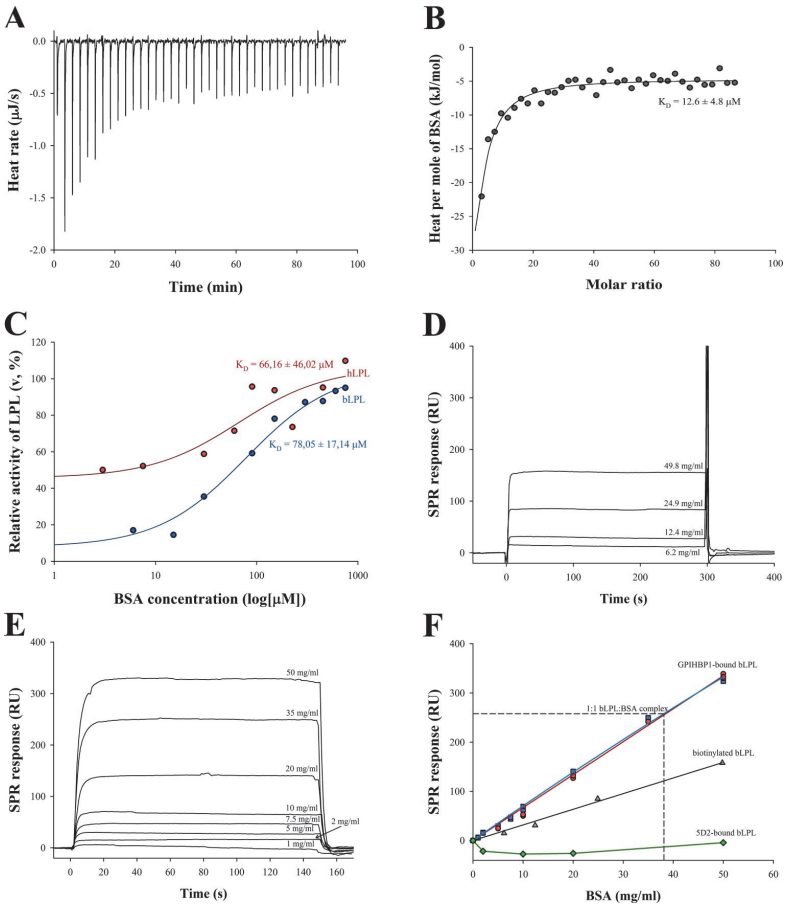

Fig 3. Interaction of LPL with BSA as studied using ITC, stabilization of LPL, and SPR. (A) An example ITC thermogram for the titration of BSA into a solution with bLPL, obtained after subtraction of the BSA dilution effect. The concentration of bLPL in the cell was 1.695 μM. **(B)** Fitted isotherm for the binding between BSA and bLPL from the ITC experiment on panel A. **(C)** Enzymatic stability of LPL in the presence of BSA as determined with DGGR. 200 nM bLPL or hLPL was incubated for 60 minutes in buffer A at various BSA concentrations. The values are calculated relative to the initial activity of LPL in the same conditions. BSA stabilized both bLPL and hLPL in a concentration-dependent manner. **(D)** SPR sensograms showing binding of BSA to biotinylated bLPL that was attached to pre-immobilized neutravidin. **(E)** SPR sensorgrams showing BSA binding to bLPL that was attached to pre-immobilized GPIHBP1. In D and E, BSA concentrations are shown on sensograms. Non-specific binding sensorgrams of BSA to streptavidin and GPIHBP1, respectively, have been subtracted. **(F)** Plateau values of sensorgrams plotted against BSA concentration. ●—Binding of BSA with 0.1 IU/ml heparin to GPIHBP1-bound bLPL. ▲—Binding of BSA to biotinylated bLPL. ◆—Binding of BSA to 5D2-bound bLPL. Dashed line—1:1 ratio of immobilized bLPL to bound BSA in the experiment with GPIHBP1-bound bLPL. The results indicate that BSA can bind to neutravidin-bound LPL or GPIHBP1-bound LPL but not 5D2-bound LPL.

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bLPL (Fig 3A and 3B). This experiment was performed in the presence of heparin to stabilize bLPL. The bLPL-BSA interaction was exothermic and BSA concentrations above 3 μM were required to record a measurable heat effect. The low solubility of bLPL, the weakness of the interaction, and the solubility limit of BSA did not allow us to perform experiments under

optimal conditions where all the parameters characterizing the interaction could be determined. Therefore, the ITC titration data could only be used to estimate the apparent affinity of the BSA-bLPL interaction. These data suggested a complex type of interaction: after an initial saturable binding phase, additional and almost constant heat production was recorded at higher BSA concentrations. The second weaker binding event was not well-defined to allow for the estimation of K_D. When the second weaker binding phase was not taken into account, the calculated apparent K_D for the initial binding was 12.6 μM. However, our various simulations involving both phases confirmed that any additional binding has a negligible effect on the K_D of initial binding.

We also investigated the interaction between LPL and BSA by measuring the enzymatic stability of bovine LPL (bLPL) and human LPL (hLPL) at different BSA concentrations (Fig 3C). BSA stabilized both LPL homologs in a concentration-dependent manner and the stabilization effect was already detectable when BSA concentration was as low as 6 μM (0.4 mg/ml). A hyperbolic relationship was observed between the relative increase of bLPL or hLPL activity and BSA concentration. The calculated apparent K_D for the interaction between bLPL-BSA and hLPL-BSA were 78 μM (5.2 mg/ml) and 66 μM (4.4 mg/ml), respectively. The reason for the difference in the K_D values between the ITC and the stabilization study is likely due to the presence of heparin in the ITC study.

In addition to ITC and enzymatic stability measurements, the interaction between bLPL and BSA was investigated by SPR. Three separate BSA binding experiments were performed using a Biacore 3000 (GE Healthcare): 1) with biotinylated bLPL non-covalently attached to neutravidin that was directly pre-immobilized on the sensor chip 2) with bLPL non-covalently attached to the chip surface via pre-immobilized GPIHBP1 3) with bLPL non-covalently attached to pre-immobilized 5D2 antibody. In all systems, dissociation of bLPL from the sensor chip was negligible. In the experiments with the neutravidin-bound biotinylated bLPL, a detectable association of BSA was observed when its concentration was above 6 mg/ml (91 μM) (Fig 3D) while in the case of GPIHBP1-bound bLPL, a detectable binding of BSA was observed at just 1 mg/ml (Fig 3E). In the case of 5D2-bound bLPL, at lower BSA concentrations of 5–20 mg/ml, the non-specific binding to 5D2 was higher than the binding to bLPL-5D2, however, minor association was observed when BSA concentration was as high as 50 mg/ml. We conclude that 5D2 strongly hinders association of BSA with LPL. The rectangular shape of the sensorgrams of the systems with GPIHBP1 and neutravidin indicated very fast off-kinetics, BSA was completely dissociated within few seconds. Such rapid dissociation made the correct calculation of rate constants impossible. The rapid dissociation kinetics also indicated that the lifetime of the bLPL-BSA complex is very short, less than a few seconds. For estimation of the equilibrium dissociation constant K_D, the plateau values were plotted against the BSA concentrations in the injected solutions (Fig 3F). In both systems, this relationship remained linear over the range of BSA concentrations used (0–910 μM), meaning that no saturation was achieved. Higher BSA concentrations were not possible with the BIAcore system. Additionally, the presence of 0.1 IU/ml heparin did not affect the binding of BSA to GPIHBP1-bound bLPL (Fig 3F, red circles). Although the apparent binding kinetics of BSA to neutravidin-bound LPL and to GPIHBP1-bound LPL were similar, much less of the GPIHBP1-bound LPL than the neutravidin-bound LPL was required for detectable BSA binding. The surface density 3.9 fmol/mm^2 of the GPIHBP1 bound LPL was sufficient to observe the interaction while a measurable association was detected when the surface density of neutravidin-bound biotin-bLPL was 19.5 fmol/mm^2. When measuring 50 mg/ml BSA binding, the steady-state bound BSA/LPL molar ratio was 0.12:1 for neutravidin-bound LPL and 1.2:1 for GPIHBP1-bound LPL. This indicates that the main part of the neutravidin-bound LPL was not able to interact with BSA. At the same time, a significant fraction, if not all, of the LPL

bound to GPIHBP1 interacted with BSA. Non-saturable linear binding, as well as a greater than one molar ratio of bound BSA/LPL, suggest that each molecule of LPL attached to GPIHBP1 can simultaneously interact with multiple BSA molecules. In summary, all three different binding studies confirm that LPL forms a complex with BSA at physiologically relevant concentrations, as circulating albumin concentrations range from 35 to 50 mg/ml and 4.4–15.7 mg/ml in the interstitial space, the latter depending on the tissue [35].

3.3 Interplay between albumin and heparin differs from their individual effects

Building on the data that LPL forms a complex with BSA and previous works showing that LPL interacts with heparin, we wanted to investigate the independent and combined effects of BSA and heparin on LPL when LPL activity is determined with a natural substrate. When 200 nM LPL was incubated for 15 minutes with BSA or heparin alone, its activity on TRLs in human plasma, as measured by ITC, was much lower than when incubated in the presence of both heparin and BSA (Fig 4A). Our data therefore shows that when acting on a natural substrate, both heparin and BSA are needed during incubation for maximum LPL activity. This difference was not observed in the DGGR assay (Fig 1A) under the same incubation conditions, nor when LPL activity was measured using the tributyrin/gum arabic substrate system (S1 Fig). To elucidate the reason for this discrepancy between the assays, effects of various incubation conditions were tested. We found that LPL, which has been incubated with heparin alone for 15 minutes regains its maximal activity after dilution to a solution containing BSA (Fig 4B). A similar increase in LPL activity was also observed when the dilution was carried out into LFHP or Triton X-100 (Fig 4B). These data suggest that the low activity of LPL measured in human plasma when preincubated with heparin (Fig 4A) was due to reversible changes in the state of LPL. It has recently been shown that heparin induces formation of reversible inactive helical LPL oligomers that dissociate into active LPL in the presence of the

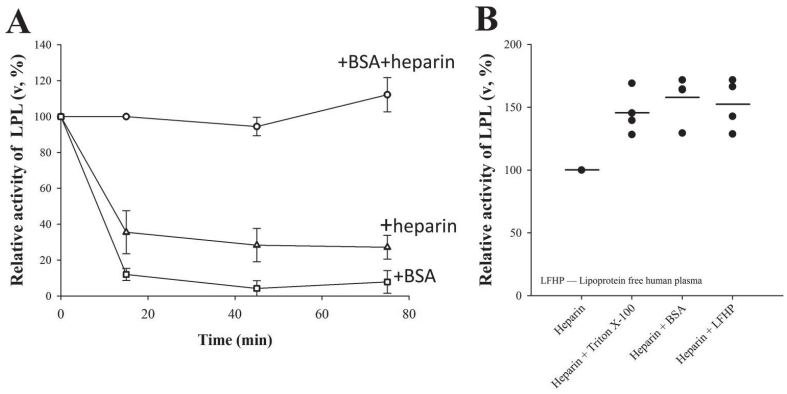

Fig 4. Effect of BSA, Triton X-100 or LFHP on LPL activity in the presence of heparin. (A) 200 nM LPL was incubated for the indicated timepoints with 50 mg/ml BSA, 10 IU/ml heparin or both in buffer A. The remaining activity (expressed as heat rate, μJ/s) was determined with ITC after a single 5 nM LPL injection into human plasma that contained 1.31 mM triglycerides. LPL activity is expressed relative to the initial activity of the experiment that contained both heparin and BSA and the data is presented as mean ± SD of three independent measurements. LPL activity was significantly lower when only BSA or heparin was used. **(B)** 1 μM LPL was incubated with 10 IU/ml heparin in buffer A and diluted 5-fold to buffer A with heparin and 0.5% Triton X-100, 50 mg/ml BSA or LFHP. The remaining LPL activity was determined in the same manner as panel A. LPL activity was restored equally with every additive.

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surfactant deoxycholate [33]. Our data here indicate that the LPL helical oligomers do not dissociate into the active form in undiluted human plasma but do so in the substrate systems containing surfactants such as Triton X-100 in the DGGR assay or gum arabic in the tributyrin assay. The LPL helical oligomers also dissociated in the substrate-free preincubation solutions with Triton X-100, BSA, or LFHP. The observation that the oligomers did not dissolve in undiluted human plasma but did when lipoproteins were removed from plasma—that is, in LFHP, —suggests that the presence of lipoproteins hindered this process.

When LPL at various concentrations was preincubated with BSA alone prior to activity determination in human plasma by ITC, the observed catalytic activity of LPL was higher at its lower preincubation concentrations. A significant increase in the activity of LPL was observed when preincubation concentration was reduced from 200 nM to 20 nM (Fig 5). This sharp change in LPL activity suggests that BSA, like heparin, causes reversible oligomerization of LPL. Like heparin induced LPL oligomers, BSA induced LPL oligomers did not dissociate into active form in human plasma. The LPL concentration effect on its activity disappeared when heparin was combined with BSA during preincubation. In this case, LPL activity was the same for every concentration (Fig 5).

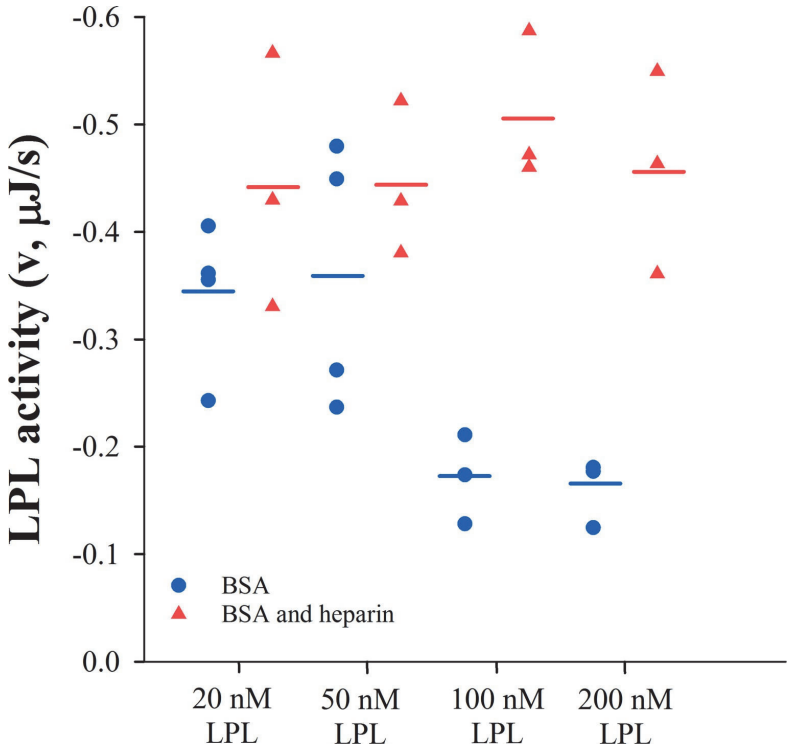

Fig 5. Effect of LPL incubation concentration on its activity as measured with ITC. LPL activity, expressed as heat rate (μJ/s), after incubation of LPL at various concentrations with 50 mg/ml BSA with (▲) or without (●) 10 IU/ml heparin in buffer A. The changes in residual LPL activity suggest that LPL oligomerization triggered by BSA is dependent on LPL concentration. This dependence disappears with the combined use of heparin and BSA which blocks the formation of LPL oligomers.

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3.4 Oligomerization of LPL as studied by RICS and Transmission Electron Microscopy (TEM)

To further characterize the LPL oligomerization occurring in the presence of heparin and BSA, we used RICS technology to determine diffusion coefficients (D) and estimate the relative content of LPL species with different sizes. Larger particles will have a lower D value while smaller particles are characterized by high D values. We chose RICS technology instead of the previously used standard sedimentation method [14, 26] because the high sucrose concentration used in the latter would cause additional crowding effect [49, 50]. In the RICS experiments, LPL was labelled with the fluorescent marker ATTO610, and measurements were performed in the presence of either BSA, heparin, or both. No fluorescence correlation was observed in measurements performed with BSA alone in solution. Straightforward fitting of autocorrelation functions, as a part of RICS analysis, produced poor fits (results not shown). This was caused by the presence of a population of large bright particles which disturbed autocorrelation significantly and made it impossible to fit with the diffusion models. To resolve this issue, the recorded images were split into subsections and the diffusion coefficient with the number of particles (N) in the confocal volume was estimated for each of these subsections. As it is shown in **S2A Fig**, the estimated D varied from subsection to subsection throughout the experiment, including some with relatively small values. When plotting D against N, one can observe that there is a fraction of subsections with small D that have small N (bottom left corner of **Fig 6A, 6C and 6E**). This is consistent with large bright particles moving through the experimental area–leading to low D and small N values. The high variability of D and N estimates is expected as we used small datasets to make those estimations. Subsections with the small estimated D and N were removed and the average correlation functions for the remaining subsections were found and fitted with the diffusion model (**S2B Fig**). Analysis of the LPL diffusion coefficients for different samples is summarized in **Table 1**, the average diffusion coefficient takes into account all observed larger and smaller species. RICS experiments were conducted using both 200 nM and 10 nM LPL to observe differences in the average diffusion coefficients resulting from LPL concentration.

LPL at 200 nM formed larger particles in the presence of 50 mg/ml BSA, as indicated by a low average D (7.4 ± 1.2 $\mu m^2/s$). In addition, as shown in **Fig 6A**, the sample was heterogeneous, leading to formation of tail-like structure on N-D scatter plot (red circle). We predict that the large particles represent the reversible oligomerized state of LPL identified with BSA. Oligomerization of LPL was also seen with 10 IU/ml heparin (**Fig 6C**), albeit in a smaller proportion as indicated by larger average D of ~20 $\mu m^2/s$ (**Table 1**). Similar average D (~22 $\mu m^2/s$) was recorded after addition of 10 IU/ml heparin to the LPL+BSA mixture homogenized the LPL distribution (**Fig 6E**). This agrees with ITC measurements that show LPL activity of ATTO610 labeled LPL is restored after addition of heparin to an LPL-BSA incubation (**S3 Fig**), similarly to how BSA was previously able to restore LPL activity in a heparin containing solution (**Fig 4B**). The same pattern was observed at 2 mg/ml BSA concentration (**Table 1**). However, on the basis of D measurements, it is impossible to explain the differences in activity of LPL with heparin alone and BSA with heparin. In solution, LPL is likely in a similar size, but inactive conformation, in the presence of heparin at this LPL concentration, as indicated by activity measurements.

TEM experiments were used to examine the appearance of LPL aggregates under the conditions used in the RICS measurements, but without ATTO610 labeled LPL. LPL aggregates were observed with BSA (**Fig 6B**), which were not seen with BSA alone (**S4A Fig**). LPL helical oligomers were observed when incubated with heparin (**Fig 6D**). LPL aggregates were not observed when combined with both BSA and heparin (**Fig 6F**), although combining heparin

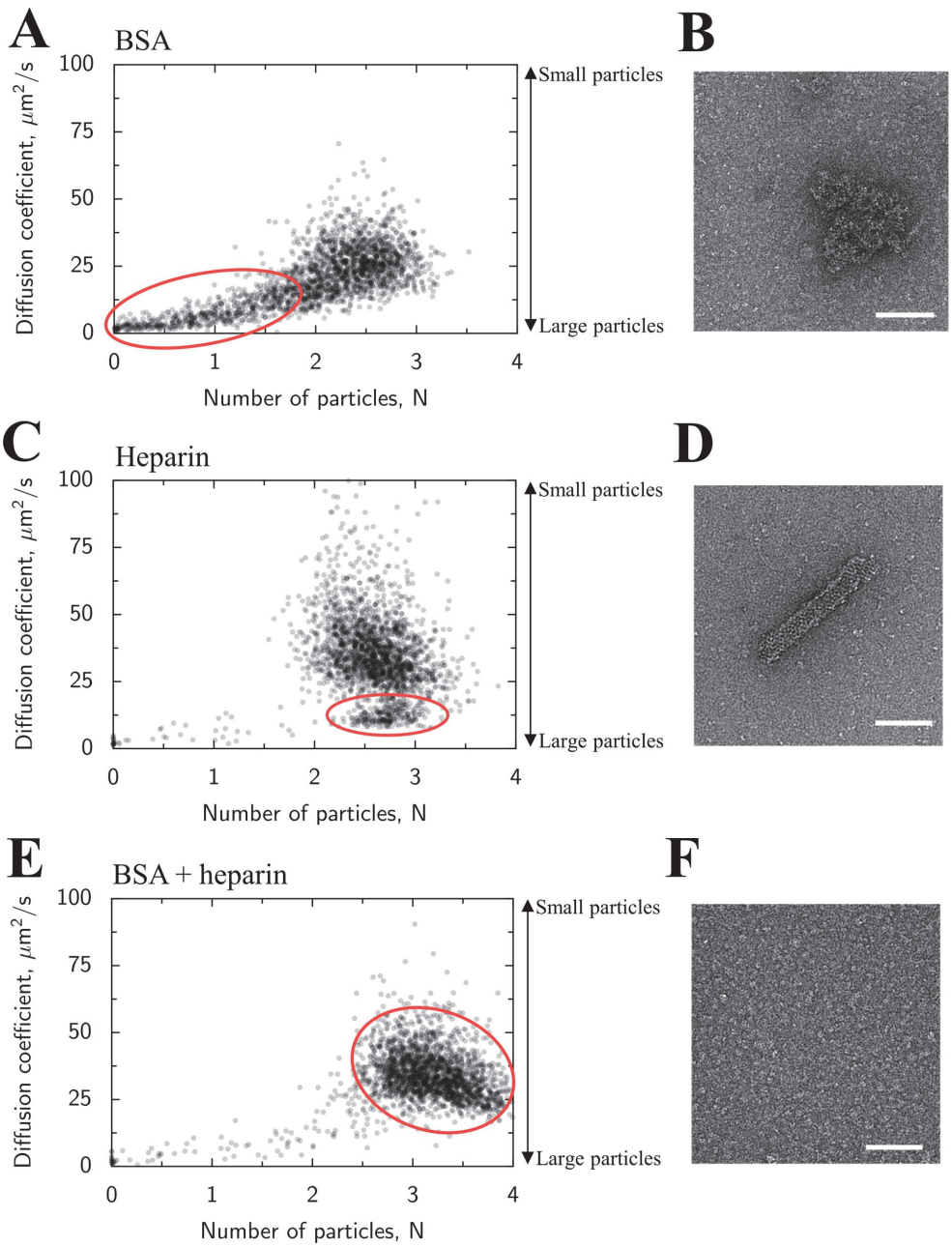

Fig 6. Oligomerization of LPL as studied by RICS and TEM. 10 nM LPL-ATTO610 with 190 nM unlabeled LPL was incubated in buffer A with the indicated supplements for up to two hours at room temperature. **(A)** Relationship between diffusion coefficient (D) and number of particles (N) in confocal volume (proportional to concentration) for image sectors recorded in an experiment with 200 nM LPL and 50 mg/ml BSA. Larger particles have lower D values. Notice that a large fraction of measurements leads to estimates with low D and N values (red circle)

(B), (D), (F) Representative nsTEM micrographs of unlabeled LPL in similar conditions as RICS experiments. Scale bars are 100 nm. **(C)** Interaction of LPL and heparin leads to formation of a fraction of estimates with lower D values at N values between 2–3. This is visible in the plot as a smaller set of points grouped around D = 10 $\mu m^2/s$ (red circle) with the larger set of estimates above it. **(E)** Relationship between D and N for an experiment with LPL, BSA and heparin. Notice that most of the image sectors lead to D and N estimates in a certain region of the plot (red circle).

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and BSA did lead to some aggregation of the BSA (S4B Fig). The data from RICS supports that these BSA aggregates in the presence of heparin do not contain LPL. LPL aggregates were also observed with 200 nM LPL by itself (S4D Fig). Finally, LPL oligomers in the presence of heparin were dissolved by treatment with Triton X-100 (S5 Fig), which is in agreement with LPL activity measurements that show an increase in LPL activity after introduction of Triton X-100 to oligomeric LPL conditions (Fig 4B).

To test the effect of LPL concentration on formation of LPL oligomers, measurements were performed with 10 nM LPL and 50 mg/ml BSA, in the absence or presence of 10 IU/ml heparin. Measurements with 10 nM LPL and 50 mg/ml BSA exhibited only slight oligomerization (Table 1, average D ~20 $\mu m^2/s$) and the results were comparable to 10 nM LPL with BSA and heparin together. This agrees with the concentration dependence demonstrated in Fig 5A. Finally, 10 nM LPL was denatured with 1 M GuHCl to produce partially unfolded monomeric LPL [43] as a comparison (S2C Fig). In this case, the LPL solution is inhabited by smaller particles whose average Ds were approximately twice as high as that observed when LPL was incubated with heparin and BSA.

3.5 Oligomeric form of LPL is more resistant to inactivation by ANGPTL4

We next asked if ANGPTL4 inactivation of LPL could be influenced by the formation of the LPL-BSA and LPL-heparin oligomers. At a relatively low LPL concentration of 20 nM where we do not expect to see significant oligomerization, we found the rate of inactivation by ANGPTL4 was similar in all conditions (Fig 7A). When the LPL concentration was raised to 200 nM (Fig 7B), the difference between the incubation conditions was noticeable—after 130 minutes of incubation, the residual LPL activity was 55% of the initial activity in the presence of heparin or BSA alone and 35% in the presence of both. These results indicate that conditions that promote oligomer formation, such as higher LPL concentrations and the presence of either BSA or heparin alone, reduce the rate of ANGPTL4 inactivation. Thus, it is reasonable to assume that LPL oligomers are resistant or less affected by the action of ANGPTL4. LPL was not fully resistant to inactivation by ANGPTL4, likely due to our previous observation that LPL is not entirely oligomeric in these conditions. Some fraction of total LPL remains catalytically active in these cases as seen in **Figs** 4 and 5A.

Table 1. Calculated diffusion coefficients of main LPL component from RICS measurements.

LPL	BSA	10 IU/ml heparin	Diffusion coefficient (D, $\mu m^2/s$)
200 nM	50 mg/ml		7.0 ± 1.2
	2 mg/ml		13.0 ± 2.9
		+	19.9 ± 3.4
	50 mg/ml	+	21.7 ± 0.8
	2 mg/ml	+	25.7 ± 2.6
10 nM	50 mg/ml		20.5 ± 1.4
	2 mg/ml		28.7 ± 2.2
	50 mg/ml	+	24.0 ± 1.0
10 nM LPL + 1 M GuHCl			42.5 ± 7.2

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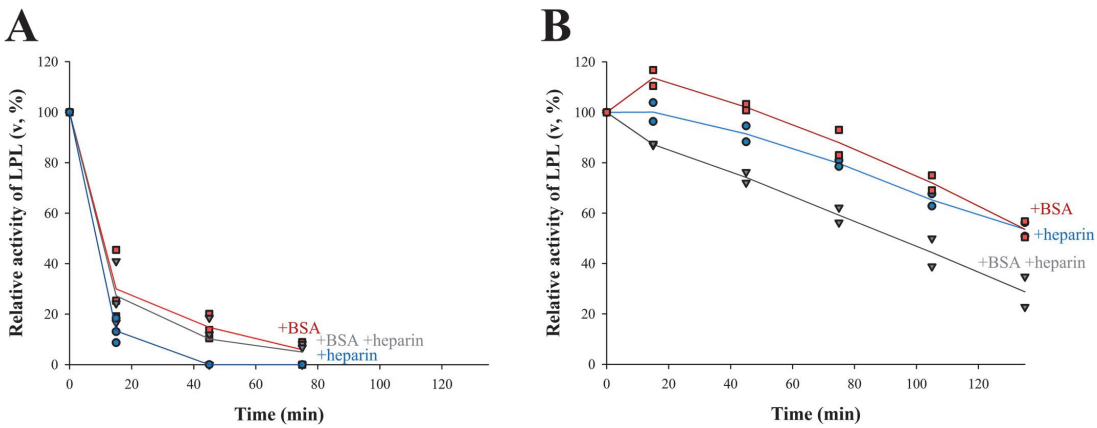

Fig 7. Inactivation of oligomeric LPL by ANGPTL4. 20 nM LPL **(A)** or 200 nM LPL **(B)** was incubated in the presence of 1 μM ANGPTL4 and 10 IU/ml heparin (●) or 50 mg/ml BSA (■) or both (▼) in buffer A. Remaining LPL activity was determined by ITC using undiluted pooled human plasma (0.89 mM triglycerides, n = 5). LPL activity is expressed relative to initial activity in the same conditions without ANGPTL4. Symbols represent individual measurements and lines correspond to their average values. The results suggest that the rate of LPL inactivation by ANGPTL4 depends on the degree of LPL oligomerization.

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3.6 Albumin amplifies the inactivation of LPL by nANGPTL4 in the presence of substrate

In addition to the interstitial space, ANGPTL4 can affect LPL at the surface of lipoproteins [51].To investigate whether albumin affects the ANGPTL4/LPL interaction in this case, inactivation kinetics of LPL by ANGPTL4 were measured at various BSA concentrations. These measurements were performed by ITC using a mixture of triglyceride-rich lipoproteins as substrate. We found that the rate of inactivation of LPL by nANGPTL4 was increased at higher BSA concentrations (**Fig 8A**). The inactivation curves followed the first-order kinetics and the inactivation rate constant (k_i) in the presence of 10 mg/ml or 50 mg/ml BSA was about 1.6 time higher than for 2 mg/ml BSA. Since albumin does not affect LPL activity and stability at substrate surfaces, we wondered if there might be a direct interaction between albumin and nANGPTL4. Indeed, SPR measurements showed association between human serum albumin (HSA) and immobilized nANGPTL4 (**Fig 8B**). This interaction was rather weak (K_D = 500 ±51 μM), but still sufficient to have physiological significance in the capillary.

Discussion

In the present study, we show that albumin, the major protein component of plasma, increases the stability of LPL via reversible oligomerization, dissolves heparin-induced LPL oligomers, and can affect the LPL-ANGPTL4 interaction. Thus, our results suggest that the role of albumin in the LPL system is more diverse than the previously known role of binding lipolysis-derived fatty acids. Albumin, usually as BSA, has been also previously included in LPL assays [52–54] but its specific role has never been studied before this study. Although most of the experiments in this study were performed with bovine LPL and BSA, it is likely that the results of the proteins from other sources will not differ because our experiments with human LPL and BSA did not differ significantly from those with bovine LPL. Also, LFHP, which contains HSA, affected LPL stability similarly to BSA. LPL is exposed to albumin and other plasma components in the vascular endothelium, interstitial space, the surface of parenchymal cells,

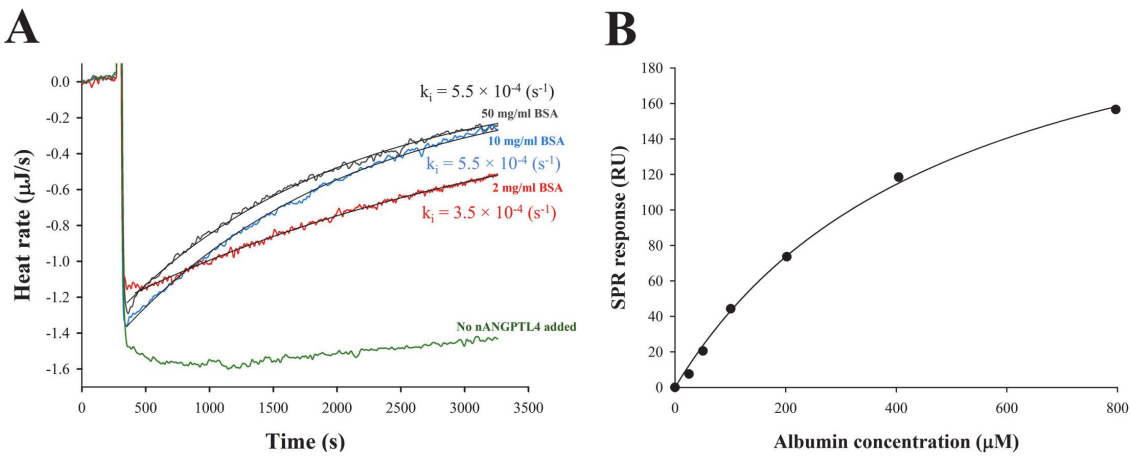

Fig 8. Interaction of albumin with nANGPTL4. (A) Effect of BSA at various concentrations on nANGPTL4-induced inactivation of LPL during lipolysis of triglyceride-rich lipoproteins. Raw ITC thermograms of lipolysis of CM/VLDL (adjusted to 0.75 mM triglycerides) by LPL in the presence of 100 nM nANGPTL4 and various concentrations of BSA (2 mg/l, 10 mg/ml, 50 mg/ml). The lipolysis rate is expressed as heat rate, μJ/s. The control experiment was performed in the absence of nANGPTL4 and presence of 50 mg/ml BSA. k_i represents inhibition rate constant of LPL by nANGPTL4 calculated from the data (n = 2). The results demonstrate that the rate of LPL inactivation by nANGPTL4 is increased at higher BSA concentrations. (**B**) SPR analysis of binding of various concentrations of HSA to immobilized nANGPTL4. Plateau values of SPR sensorgrams were plotted against HSA concentrations after subtracting non-specific binding. The results indicate that HSA interacts with nANGPTL4.

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and when attached to lipoprotein remnants [55]. The plasma concentration of albumin is 35–50 mg/ml and its concentration in the interstitial space of adipose tissue and skeletal muscle is 4.4–10 mg/ml and 9.7–15.7 mg/ml, respectively [35]. Since the K_D values of the LPL-albumin complexes were lower than or comparable to albumin concentrations in all extracellular locations of LPL, it is reasonable to assume that albumin affects LPL *in vivo*. Our data also indicate that albumin affects LPL stability via direct interaction, and macromolecular crowding may have only a limited role in the effect. The short lifetime of the LPL-albumin complex, as seen in the SPR experiment, may be important for the rapid transition of LPL to other complexes in the interstitial space to enable efficient shuttling of LPL to the vascular endothelium.

The observation that casein and β-lactoglobulin also stabilize LPL, but lysozyme does not, raises the question of what properties are required for a protein to interact with LPL. Our measurements were performed at pH 7.4, where albumin, casein and β-lactoglobulin are negatively charged due to their low pI, while lysozyme with its very high pI (11.1) is positively charged. The overall positive charge of LPL may explain its interaction with negatively charged albumin, casein and β-lactoglobulin, and also the avoidance of positively charged lysozyme. Exposed hydrophobicity, which plays a role in the thermal stabilization of several proteins, is likely not the only reason for stabilization of LPL by these proteins. This is mainly because lysozyme has a significantly large exposed hydrophobic area. However, the structural properties of proteins that determine their ability to stabilize LPL require further investigation.

The most intriguing observation in this study was the interplay of BSA and heparin in the reversible aggregation / oligomerization of LPL. As shown by ITC, TEM, and RICS measurements, both heparin and albumin alone induced reversible oligomerization of LPL. The LPL aggregates disappeared when both heparin and BSA were present in the incubation solution. This observation suggests that albumin binds to the LPL region involved in heparin-induced oligomerization and that heparin binds to the LPL region that plays a role in albumin-induced

aggregation. TEM analysis revealed that albumin-induced LPL aggregates differed from that of heparin-induced LPL helices (**Fig 6**). Unlike heparin-induced higher-order structure helices, albumin-induced aggregates did not have a well-defined regular structure. Despite structural differences, both oligomers were soluble in detergents such as Triton X-100, gum arabic or deoxycholate and dissociated at low LPL concentrations. The solubilization effect of the detergents suggests that the hydrophobic effect may play a role in the formation of both types of oligomers. LFHP was as effective as albumin in dissolving the LPL oligomers induced by heparin, indicating that albumin is the major component of plasma responsible for this effect.

LPL is mainly synthesized by parenchymal cells of adipose tissue and skeletal muscles, secreted into the interstitial space, and transported by GPIHBP1 to the vascular endothelium. Prior to secretion from cells, LPL has been shown to be transported in intracellular vesicles in which it is complexed with HSPG SDC1 [34] and has acquired filamentous distribution [33]. Upon secretion, the LPL filaments arrive in the interstitial space where vast amounts of albumin and glucose-aminoglycans, including HSPGs, are present. To become enzymatically active, the filaments must dissociate into an active form of LPL. This dissociation can be ensured by the coaction of interstitial albumin and HSPG. Thus, albumin can have a role in creating a suitable environment that turns oligomerized LPL into its active form (**Fig 9**).

It is possible that ANGPTL4 can act on LPL either in the cells that produce them [56], in the subendothelial space right after their secretion [57] or at the luminal side of vascular endothelium [41, 51, 58]. Our experiments show that the ANGPTL4 induced inactivation of LPL occurs faster when both albumin and heparin are present, i.e. in an environment in which LPL is not oligomerized. Both heparin-induced and albumin-induced oligomerization protected LPL from the action of ANGPTL4. It has been shown that nANGPTL4 binds to the lid-domain and the lid-proximal helix of LPL [59]. According to the cryo-EM structure of helical oligomers of LPL, the lid region is involved in the formation LPL helices. Thus, the ANGPTL4 binding region could be occluded in oligomeric LPL, and this might prove to be a fail-safe to protect vesicle-stored LPL in adipose tissue. Inversely, albumin and HSPG are present in the subendothelial space, which might provide a suitable environment for the action of ANGPTL4 on LPL.

Albumin acts as a FFA acceptor *in vivo* during lipolysis and has to be used *in vitro* with substrates that contain long-chain triglycerides to bind released FFAs which would otherwise inhibit LPL. We did not observe any changes to LPL activity when another fatty acid acceptor, cyclodextrin, was used in place of albumin. This indicates that when LPL is in the presence of substrate, the role of albumin is limited to its ability to bind FFAs–and does not rely on a direct physical interaction between LPL and albumin. Further suggesting that the realm of LPL-albumin interactions might be prior to transfer to the vascular endothelium. LPL stabilization has been previously observed when LPL is bound to surfaces of artificial substrate emulsions [46]. It is likely that LPL is also stable on the surface of lipoproteins and is not influenced by albumin or macromolecular crowding of plasma.

A model for describing our observations on the oligomerization of LPL is proposed in **Fig 9**. Depending on the conditions, LPL can appear as an irreversible aggregate, a heparin-induced reversible helical oligomer [33], a reversible albumin-induced aggregate, or in its active form—which could be a properly folded monomer or dimer. The presence of both heparin and albumin leads to dissociation of both reversible type aggregates [42].

In summary, this study suggests that albumin can strongly affect the action and properties of LPL. Albumin may play a role in the decomposition of helical LPL oligomers induced by heparin or HSPGs. The dissociation of LPL oligomers or the avoidance of their formation can be crucial for LPL's interaction with its ligands as it transits the interstitial space. *In vitro*, both albumin alone and heparin alone promote the concentration-dependent formation of LPL

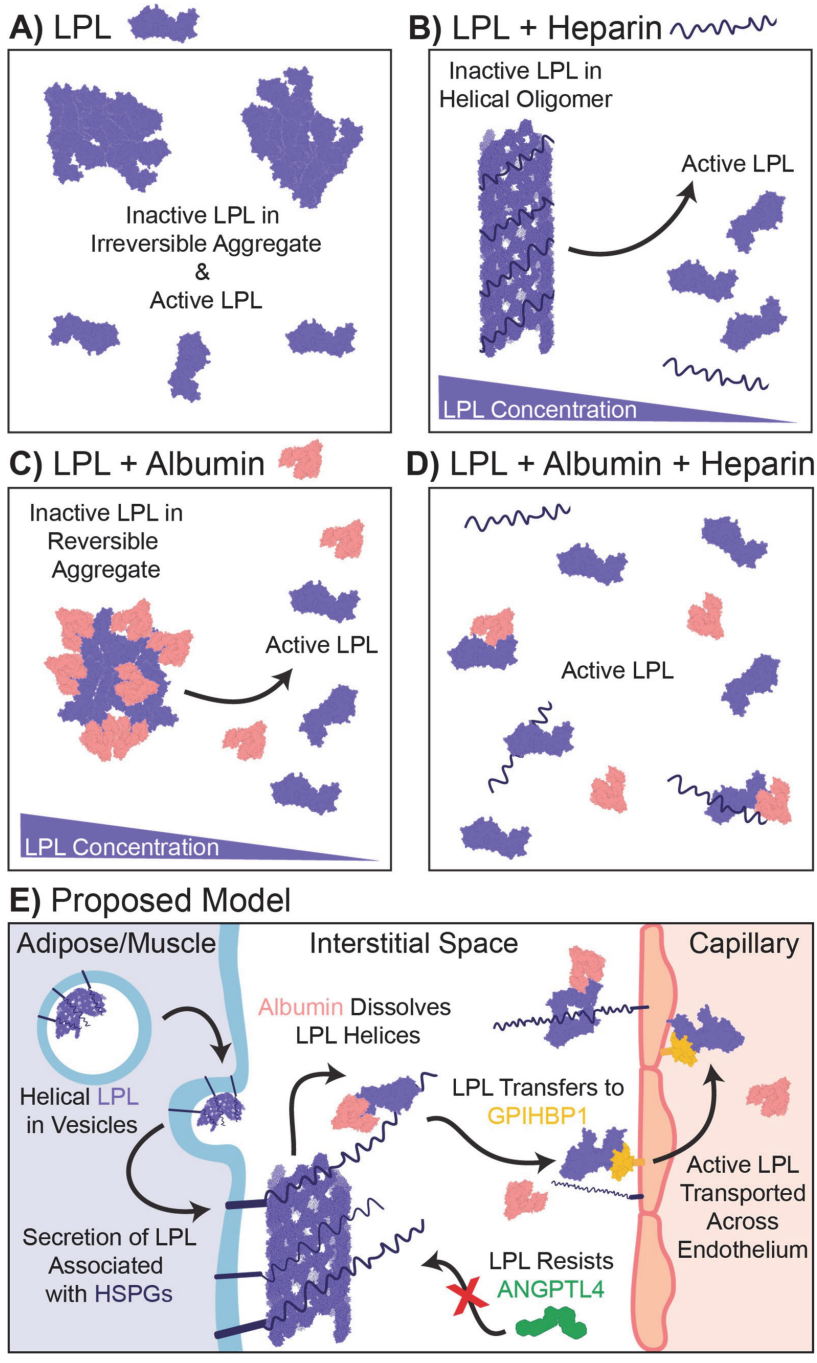
A) LPL
Inactive LPL in
Irreversible Aggregate
&
Active LPL
B) LPL + Heparin
Inactive LPL in
Helical Oligomer
Active LPL
LPL Concentration
C) LPL + Albumin
Inactive LPL in
Reversible
Aggregate
Active LPL
LPL Concentration
D) LPL + Albumin + Heparin
Active LPL
E) Proposed Model
Adipose/Muscle
Interstitial Space
Capillary
Albumin Dissolves
LPL Helices
Helical LPL
in Vesicles
LPL Transfers to
GPIHBP1
Active LPL
Transported
Across
Endothelium
Secretion of LPL
Associated
with HSPGs
LPL Resists
ANGPTL4

Fig 9. Summarized results and proposed model for oligomerization of LPL. The model describes possible states of LPL according to in vitro investigations.

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oligomers. These oligomers are reversible and dissociate into catalytically active LPL upon dilution and/or treatment with Triton X-100. Great consideration into LPL concentration and buffer environment should be taken in studies to distinguish between irreversible inactivation or aggregation and reversible LPL oligomer formation, which might affect interactions with various ligands and drugs.

Supporting information

S1 Fig. LPL activity measured in various substrate systems. LPL activity was measured in substrate systems with increasing complexity: soluble fluorescent substrate DGGR < Tributyrin < Intralipid < Isolated triglyceride-rich lipoproteins (CM/VLDL) < Undiluted human plasma. 200 nM LPL was incubated for 15, 45 or 75 min in 20 mM HEPES, 150 mM NaCl, pH 7.4 buffer with 50 mg/ml BSA. Remaining LPL activity was determined with ITC for all substrates except DGGR, where fluorimetry was used instead. A single 5 nM LPL injection was made in the ITC experiments and the final concentration of LPL with DGGR was 10 nM. Results are presented as mean ± SD of three independent measurements and calculated relative to measurements where heparin was added in addition to albumin.
(TIF)

S2 Fig. Determining diffusion coefficients of LPL using RICS. (A) Estimation of diffusion coefficient (D) for all image sectors acquired with 200 nM LPL, 50 mg/ml BSA and 10 IU/ml heparin. Notice the spread of Ds and that some of the estimates had rather low value, corresponding to images with the larger particles. **(B)** Fit of fluorescence autocorrelation obtained in RICS experiment at 289 Hz scanning speed after analyzing average correlation and filtering the data (see main text for details). **C**—10 nM LPL-ATTO610 incubated in 1 M GuHCl for 2 hours at room temperature. There are only a few bright particles in these conditions (red circle).
(TIF)

S3 Fig. Addition of heparin to LPL incubation with albumin. 190 nM LPL with 10 nM LPL-ATTO610 was incubated at room temperature in 20 mM HEPES, 150 mM NaCl, pH 7.4 buffer with 50 mg/ml BSA to mimic conditions used for FCS measurements. After 15 minutes, final concentration of 10 IU/ml heparin or water was added to the mixture. LPL activity expressed as heat rate was measured with ITC at 25˚C in 1.31 mM triglycerides human plasma after a 5 nM LPL injection. Data is presented as mean of two independent measurements.
(TIF)

S4 Fig. BSA and heparin occasionally form aggregates. (A) Aggregates of BSA were not observed when 2 mg/ml BSA was applied to a TEM grid. **(B)** However, aggregates were sometimes observed when 2 mg/ml BSA and 10 IU/ml heparin were mixed together. **(C)** These BSA/heparin aggregates were also seen in a minority of 200 nM LPL with 2 mg/mL BSA and 10 IU/mL heparin micrographs. Scale bars 100 nm. **D**—200 nM LPL in 20 mM HEPES, 150 mM NaCl, pH 7.4 buffer.
(TIF)

S5 Fig. Incubation of heparin treated LPL with triton X-100 abolishes formation of LPL helices. When 200 nM of LPL is mixed with 10 IU/ml heparin, the formation of LPL helices was observed (Fig 6D). When 200 nM LPL with 10 IU/ml heparin was treated with increasing

concentrations of the detergent triton X-100, the LPL helices were progressively dissolved. **(A)**—At 0.005% triton X-100 concentration the beginning of LPL helix dissolution can be observed, with the helices pulling apart (upper panel) or dissolving from one end of the helix (lower panel). **(B)**—At 0.05% triton X-100 LPL helices are no longer observed, although some clumps of LPL are visible. **(C)**—at 0.5% triton X-100 the LPL particles are disperse and do not appear aggregated. Scale bars are 100 nm.
(TIF)

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Methodology: Robert Risti.

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Writing – original draft: Robert Risti, Aivar Lõokene.

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CURRICULUM VITAE

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Education

2017–…	Tallinn University of Technology, Chemistry and Gene Technology, PhD
2015–2017	Tallinn University of Technology, Applied Chemistry and Biotechnology, MSc (cum laude)
2011–2014	Tallinn University of Technology, Applied Chemistry and Biotechnology, BSc
1999–2008	Gustav Adolf Grammar School

Languages

Estonian (Native), English (Fluent), French (Basic)

Professional employment

2017–...	Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology, Early Stage Researcher

Honors and awards

2020	Teadus Vikipeediasse 2020 — Prize for Estonian Wikipedia article about hypertriglyceridemia
2017	National Contest for University Students — letter of appreciation, Master's students, biosciences and environment

Supervised dissertations

Natjan-Naatan Seeba, Master's Degree, 2022, (sup) Aivar Lõokene; Robert Risti, Effect of vegan diet on exogenic lipoprotein lipase in healthy individuals, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology

Merilin Grossthal, Master's Degree, 2020, (sup) Kaia Kukk; Robert Risti, Expression of human angiopoietin-like protein 4 in the yeast Pichia pastoris and effect of the N-terminal domain on lipoprotein lipase, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology

Robert Uibu, Master's Degree, 2019, (sup) Robert Risti; Aivar Lõokene, Functional studies of lipoprotein lipase and endothelial lipase in physiological conditions using isothermal titration calorimetry, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Riin Lepik, Bachelor's Degree, 2018, Effect of apolipoprotein C-II on lipoprotein lipase studied in different lipoprotein subclasses, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Andreas Remmel, Bachelor's Degree, 2019, Expression and purification of human apolipoprotein C-II, angiopoietin-like protein 3 and 8, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Laura Kaar, Bachelor's Degree, 2020, Environments and substrates affecting the activity and stability of lipoprotein lipase, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Teaching

2018–2022	Interactomics (Master's and PhD students), supervision of practical works
2018	Biochemistry (Bachelor's students), supervision of practical works

Publications

Risti, Robert; Gunn, Kathryn; Hiis-Hommuk, Kristofer; Seeba,Natjan-Naatan; Karim, Hamed; Villo, Ly; Vendelin, Marko; Neher,Saskia B.; Lõokene, Aivar. Combined action of albumin and heparin regulates lipoprotein lipase oligomerization, stability, and ligand interactions. PLoS One. 2023 Apr 12;18(4).

Metz, Matthäus; Beghini, Marianna; Wolf, Peter; Pfleger, Lorenz; Hackl, Martina; Bastian, Magdalena; Freudenthaler, Angelika; Harreiter, Jürgen; Zeyda, Maximilian; Baumgartner-Parzer, Sabina; Marculescu, Rodrig; Marella, Nara; Hannich, J. Thomas; Györi, Georg; Berlakovich, Gabriela; Roden, Michael; Krebs, Michael; Risti, Robert; Lõokene, Aivar; Trauner, Michael ... Scherer, Thomas (2022). Leptin increases hepatic triglyceride export via a vagal mechanism in humans. Cell Metabolism. DOI: 10.1016/j.cmet.2022.09.020.

Rump, A.; Risti, R.; Kristal M.-L.; Reut, J.; Syritski, V.; Lõokene, A., Rüütel Boudinot, S. (2021). Dual ELISA using SARS-CoV-2 N protein produced in E. coli and CHO cells reveals epitope masking by N-glycosylation. Biochemical and Biophysical Research Communications, 534, 457–460. DOI: 10.1016/j.bbrc.2020.11.060.

Villo, Ly; Risti, Robert; Reimund, Mart; Kukk, Kaia; Samel, Nigulas; Lõokene, Aivar (2020). Calorimetric approach for comparison of Angiopoietin-like protein 4 with other pancreatic lipase inhibitors. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1865 (2), UNSP 158553. DOI: 10.1016/j.bbalip.2019.158553.

Reimund, Mart; Wolska, Anna; Risti, Robert; Wilson, Sierra; Sviridov, Denis; Remaley, Alan T.; Lõokene, Aivar (2019). Apolipoprotein C-II mimetic peptide is an efficient activator of lipoprotein lipase in human plasma as studied by a calorimetric approach. Biochemical and Biophysical Research Communications, 519 (1), 67−72. DOI: 10.1016/j.bbrc.2019.08.130.

Conference presentations (poster)

2022 "Albumin affects the stability, oligomerization and ligand interactions of lipoprotein lipase", Gordon Research Conference "Lipoprotein Metabolism", Southbridge, MA, USA

2022 "Albumin affects the stability, oligomerization and ligand interactions of lipoprotein lipase", 90th EAS Congress, Milan, Italy

2021 "A calorimetric approach for investigating anti-hypertriglyceridemic drugs in a native-like environment", Buchheim 200, Tartu, Estonia

2021 "Calorimetric assay reveals the importance of substrate choice for determination of lipoprotein lipase activity", 89th EAS Virtual Congress, Helsinki, Finland

2018 "Calorimetry for activity and inhibition studies of pancreatic lipase under physiological-like conditions", 3rd European MicroCal User Meeting, London, UK

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Hariduskäik

2017–…	Tallinna Tehnikaülikool, keemia ja geenitehnoloogia, PhD
2015–2017	Tallinna Tehnikaülikool, rakekenduskeemia ja biotehnoloogia, MSc (cum laude)
2011–2014	Tallinna Tehnikaülikool, rakekenduskeemia ja biotehnoloogia, BSc
1999–2008	Gustav Adolfi Gümnaasium

Keelteoskus

Eesti (emakeel), inglise (kõrgtase), prantsuse (algtase)

Teenistuskäik

2017–...	Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut, doktorant-nooremteadur

Teaduspreemiad ja tunnustused

2020	Teadus Vikipeediasse 2020 — ergutuspreemia hüpertriglütserideemia artikli eest Vikipeedias
2017	Üliõpilaste teadustööde riiklik konkurss — tänukiri, magistrandid, bio- ja keskkonnateaduste valdkond

Juhendatud väitekirjad

Natjan-naatan Seeba, magistrikraad, 2022, (juh) Aivar Lõokene; Robert Risti, Vegandieedi mõju eksogeense lipoproteiinlipaasi aktiivsusele, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

Merilin Grossthal, magistrikraad, 2020, (juh) Kaia Kukk; Robert Risti, Inimese angiopoietiini-sarnase valgu 4 ekspresseerimine pärmis Pichia pastoris ja valgu N-terminaalse domeeni mõju uurimine lipoproteiinlipaasile, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

Laura Kaar, bakalaureusekraad, 2020, (juh) Ly Villo, Robert Risti, Lipoproteiinlipaasi aktiivsust ja stabiilsust mõjutavad keskkonnad ja substraadid, Tallinna Tehnikaülikool, Loodusteaduskond, keemia ja biotehnoloogia instituut.

Robert Uibu, magistrikraad, 2019, (juh) Robert Risti; Aivar Lõokene, Lipoproteiinlipaasi ja endoteeli lipaasi toimemehhanismide uurimine füsioloogilistes tingimustes isotermilise tiitrimis-kalorimeetria meetodil, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut.

Andreas Remmel, bakalaureusekraad, 2019, (juh) Robert Risti, Inimese angiopoietiini-sarnaste valkude 3 ja 8 ning apolipoproteiin C-II ekspressioon ja puhastamine, Tallinna Tehnikaülikool, Loodusteaduskond, keemia ja biotehnoloogia instituut.

Riin Lepik, bakalaureusekraad, 2018, (juh) Robert Risti, Apolipoproteiin C-II mõju uurimine lipoproteiinlipaasi aktiivsusele erinevates lipoproteiini klassides, Tallinna Tehnikaülikool, Loodusteaduskond, keemia ja biotehnoloogia instituut.

Õppetöö

2018–2022	Interaktoomika (magistritudengid ja doktorandid), praktiliste tööde juhendamine
2018	Biokeemia (bakalaureusetudengid), praktiliste tööde juhendamine

Publikatsioonid

Risti, Robert; Gunn, Kathryn; Hiis-Hommuk, Kristofer; Seeba,Natjan-Naatan; Karim, Hamed; Villo, Ly; Vendelin, Marko; Neher,Saskia B.; Lõokene, Aivar. Combined action of albumin and heparin regulates lipoprotein lipase oligomerization, stability, and ligand interactions. PLoS One. 2023 Apr 12;18(4).

Metz, Matthäus; Beghini, Marianna; Wolf, Peter; Pfleger, Lorenz; Hackl, Martina; Bastian, Magdalena; Freudenthaler, Angelika; Harreiter, Jürgen; Zeyda, Maximilian; Baumgartner-Parzer, Sabina; Marculescu, Rodrig; Marella, Nara; Hannich, J. Thomas; Györi, Georg; Berlakovich, Gabriela; Roden, Michael; Krebs, Michael; Risti, Robert; Lõokene, Aivar; Trauner, Michael ... Scherer, Thomas (2022). Leptin increases hepatic triglyceride export via a vagal mechanism in humans. Cell Metabolism. DOI: 10.1016/j.cmet.2022.09.020.

Rump, A.; Risti, R.; Kristal M.-L.; Reut, J.; Syritski, V.; Lõokene, A., Rüütel Boudinot, S. (2021). Dual ELISA using SARS-CoV-2 N protein produced in E. coli and CHO cells reveals epitope masking by N-glycosylation. Biochemical and Biophysical Research Communications, 534, 457−460. DOI: 10.1016/j.bbrc.2020.11.060.

Villo, Ly; Risti, Robert; Reimund, Mart; Kukk, Kaia; Samel, Nigulas; Lõokene, Aivar (2020). Calorimetric approach for comparison of Angiopoietin-like protein 4 with other pancreatic lipase inhibitors. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1865 (2), UNSP 158553. DOI: 10.1016/j.bbalip.2019.158553.

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2022 “Albumin affects the stability, oligomerization and ligand interactions of lipoprotein lipase”, Gordon Research Conference “Lipoprotein Metabolism”, Southbridge, MA, Ameerika Ühendriigid

2022 “Albumin affects the stability, oligomerization and ligand interactions of lipoprotein lipase”, 90th EAS Congress, Milano, Itaalia

2021 “A calorimetric approach for investigating anti-hypertriglyceridemic drugs in a native-like environment”, Buchheim 200, Tartu, Eesti

2021 “Calorimetric assay reveals the importance of substrate choice for determination of lipoprotein lipase activity”, 89th EAS Virtual Congress, Helsingi, Soome

2018 “Calorimetry for activity and inhibition studies of pancreatic lipase under physiological-like conditions”, 3rd European MicroCal User Meeting, London, Inglismaa

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