Lipoproteins in the postprandial state

- studies on the metabolism of triglyceride-rich lipoproteins using multicompartmental models

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Abstract

Lipoproteins are lipid-containing spherical particles that circulate in the human blood stream. The reason for their intense study over the decades is their ability to cause atherosclerosis [1]. Atherosclerotic cardiovascular disease (ASCVD) remains as the single largest cause of death worldwide; and here in Sweden, around a third of all deaths can be attributed to the category: *diseases of the circulatory system* [2]. The strength of evidence that low-density lipoproteins (LDL) cause ASCVD is very high. In recent years evidence has emerged indicating that so-called triglyceride-rich lipoproteins (TRLs) also contribute causally to atherosclerosis [3]. The two sources of TRLs in the human circulation are the liver – which produces lipoproteins continuously, and the intestine – which produces lipoproteins in response to a meal. The liver-derived particles contain a structural surface protein called apolipoprotein B100 (apoB100) whereas particles from the intestine contain apolipoprotein B48 (apoB48). TRLs can also be further classified based on density into the following fractions: intermediate-density lipoprotein (IDL), very low-density lipoprotein 2 (VLDL2), very low-density lipoprotein 1 (VLDL1) and lastly the chylomicron (CM) fraction. Lipoproteins are studied in many ways, both in humans and in model organisms. In the current thesis I, study human lipoproteins *in vivo* with the help of multicompartmental modelling in conjunction with specifically designed metabolic studies involving stable isotopes. This enables quantification of the *fluxes* of lipoproteins as opposed to quantification of concentrations only. I developed a model able to study the postprandial, non-steady state, metabolism of both apoB48- and apoB100 containing lipoproteins. The model was primarily designed to study TRLs but was later adapted to also encompass LDL. After developing the model, it was first implemented for the purpose of studying whether the metabolism of TRLs differed across the range of subjects with low-to-high TRL levels. ApoB48 was found to track the metabolism of apoB100 which revealed a more complex picture of the state of hypertriglyceridemia (high levels of TRLs) with postprandial excursions of particles overlayered on a baseline steady-state level. Second, the model was implemented to study the effects of the antidiabetic drug liraglutide. We found that chylomicron secretion was specifically affected by liraglutide, an effect that was likely independent from the improvements in insulin sensitivity induced by the drug. Lastly, we implemented the expanded model to study the effects of the LDL-cholesterol lowering drug evolocumab. We found that evolocumab mostly affected apoB100-containing lipoproteins. Particles in the VLDL1 fraction remained unaffected but VLDL2, IDL and LDL concentrations were decreased with the largest effect size seen for LDL (around 80 % reduction). We further found evidence for the existence of two distinct LDL species with differing kinetic properties, that were differentially affected by evolocumab. In conclusion, here I present a new comprehensive multicompartmental model of lipoprotein metabolism. The model is designed to study apoB100- and apoB48 containing lipoproteins in the postprandial state. The model is used in the current thesis to study TRLs in hypertriglyceridemia and to study the effect of drugs on both TRLs and LDLs. The higher purpose for these studies is to add to the body of evidence for the role of lipoproteins in heart disease.

Sammanfattning

Lipoproteiner är lipid-innehållande sfäriska partiklar som cirkulerar i människans blodomlopp. Anledningen till att de har varit föremål för intensiva studier under många år är deras förmåga att orsaka hjärt-kärlsjukdom[1]. Hjärt-kärlsjukdom är den enskilt största orsaken till död runtom i världen, och här i Sverige kan en tredjedel av alla dödsfall härledas till kategorin *cirkulationsorganens sjukdomar* [2]. Evidensgraden för att LDL (low-density lipoprotein) orsakar hjärt-kärlsjukdom är mycket hög. På senare år har också så kallade triglyceridrika lipoproteiner (TRL) visat sig kunna orsaka hjärt-kärlsjukdom[3]. Det finns två källor till TRL; levern - som producerar lipoproteinpartiklar kontinuerligt, och tarmen - som producerar partiklar främst i respons till en måltid. Partiklar som härstammar från levern innehåller ett strukturellt ytprotein som heter apolipoprotein B100 (apoB100). Partiklar från tarmen däremot innehåller apolipoprotein B48 (apoB48). TRL kan vidare separeras baserat på densitet i följande fraktioner: IDL (intermediate-density lipoprotein), VLDL2 (very low-density lipoprotein 2), VLDL1 (very low-density lipoprotein 1) och slutligen CM (chylomicron). Lipoproteiner kan studeras på många sätt, både i människa och i olika modellorganismer. I denna avhandling studerar jag mänskliga lipoproteiner *in vivo* med hjälp av multikompartmentell modellering och därtill hörande speciellt designade metabola studier. Detta möjliggör kvantifiering av *flöden* av lipoproteiner i kontrast till statiska mätningar av koncentration. Jag har här utvecklat en modell som kan användas för att studera postprandiell metabolism (metabolism efter en måltid) av både apoB48- och apoB100 innehållande lipoproteiner. Modellen var främst utvecklad för att studera TRL-metabolism men var senare expanderad till att också inkludera LDL. Efter att ha utvecklat modellen var den först implementerad för att studera hur metabolismen av TRL-partiklar skiljer sig åt mellan personer som har låga, normala eller höga nivåer av triglycerider i blodet (så kallad hypertriglyceridemi). Vi fann att kinetiken av apoB48 speglade den för apoB100 och vi kunde avslöja en mer komplex bild av hypertriglyceridemi med postprandiell våg-liknande ackumulering av partiklar över en förhöjd basnivå. Modellen var sedan implementerad för att studera effekten av läkemedlet liraglutid som används för behandling mot typ2-diabetes. Vi fann en specifik reduktion av apoB48-sekretion från tarmen, en effekt som sannolikt var oberoende från den förbättrade insulinkänslighet som orsakas av läkemedlet. Till sist studerade vi effekten av det LDL-kolesterol sänkande läkemedlet evolocumab. Vi fann att evolocumab primärt påverkade apoB100-innehållande lipoproteiner. Partiklar i VLDL1-fraktionen påverkades inte av läkemedlet men nivån av partiklar i VLDL2, IDL och LDL reducerades; där störst påverkan sågs på LDL med en ca 80-procentig sänkning. Vi fann också evidens för existensen av två distinkta typer av LDL-partiklar med olika nedbrytningshastighet, som påverkades på olika sätt av läkemedlet. Sammanfattningsvis presenterar jag här en ny multikompartmentell modell som kan användas för att studera lipoproteinmetabolism i det postprandiella tillståndet. Modellen används här i syfte att studera TRL-partiklars beteende i personer med hypertriglyceridemi och för att studera effekten av läkemedel på både TRL- och LDL partikar. Det högre syftet med dessa studier är att addera förståelse för lipoproteiners roll i hjärt-kärlsjukdom.

List of publications

Publications included in the current thesis:

Björnson, E., Packard, C. J., Adiels, M., Andersson, L., Matikainen, N., Söderlund, S., ... & Borén, J. (2019). Investigation of human apoB48 metabolism using a new, integrated non-steady-state model of apoB48 and apoB100 kinetics. *Journal of internal medicine*, *285*(5), 562-577.

Björnson, E., Packard, C. J., Adiels, M., Andersson, L., Matikainen, N., Söderlund, S., ... & Borén, J. (2020). Apolipoprotein B48 metabolism in chylomicrons and very low-density lipoproteins and its role in triglyceride transport in normo-and hypertriglyceridemic human subjects. *Journal of internal medicine*, *288*(4), 422-438.

Taskinen, M. R., Björnson, E., Matikainen, N., Söderlund, S., Pietiläinen, K. H., Ainola, M., ... & Borén, J. (2021). Effects of liraglutide on the metabolism of triglyceride-rich lipoproteins in type 2 diabetes. *Diabetes, Obesity and Metabolism*.

Taskinen, M. R., Björnson, E., Kahri, J., Söderlund, S., Matikainen, N., Porthan, K., ... & Borén, J. (2020). Effects of Evolocumab on the Postprandial Kinetics of Apo (Apolipoprotein) B100-and B48-Containing Lipoproteins in Subjects With Type 2 Diabetes. *Arteriosclerosis, Thrombosis, and Vascular Biology*, ATVBAHA-120.

Additional publications not included in this thesis:

Gummesson, A., Björnson, E., Fagerberg, L., Zhong, W., Tebani, A., Edfors, F., ... & Bergström, G. (2021). Longitudinal plasma protein profiling of newly diagnosed type 2 diabetes. *EBioMedicine*, *63*, 103147.

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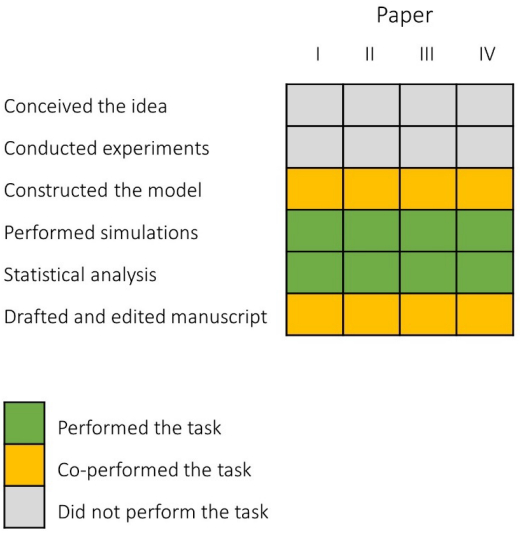
Contribution summary

My contributions to the papers are summarised below.



Preface

This dissertation serves as a partial fulfilment of the requirements to obtain a PhD degree of Medicine at Sahlgrenska Academy, Institute of Medicine, Department of Molecular and Clinical Medicine. The studies were supervised by professor Jan Borén and co-supervised by Martin Adiels and Jens Nielsen. It was mainly funded by the Swedish Research Council; the Swedish Heart and Lung Foundation and the Sahlgrenska University Hospital ALF research grants.

Elias Björnson, April 2021

Abbreviations

Apo	Apoprotein/apolipoprotein
ASCVD	atherosclerotic cardiovascular disease
CAD	coronary artery disease
CHD	coronary heart disease
CVD	cardiovascular disease
CM	Chylomicron
CETP	cholesteryl-ester transfer protein
CD36	cluster of differentiation 36
DGAT	diglyceride acyltransferase
FATP	fatty acid transport protein
FCR	fractional catabolic rate
GLP-1	glucagon-like peptide-1
GPIHBP1	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
GC/MS	gas chromatography mass spectrometry
HL	hepatic lipase
HSPG	heparan sulfate proteoglycan
IDL	Intermediate-density lipoprotein
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPL	lipoprotein lipase
LRP1	low-density lipoprotein receptor-related protein 1
MGAT	monoglyceride acyltransferase
MR	mendelian randomization
NPC1L1	Niemann-Pick C1-Like 1
ODE	ordinary differential equation
PCSK9	Proprotein convertase subtilisin/kexin type 9
Sf	Svedberg flotation rate
TRL	triglyceride-rich lipoprotein
VLDL1	very-low density lipoprotein 1
VLDL2	very-low density lipoprotein 2
VLDLR	very-low density lipoprotein receptor

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Table of Contents

Background

Every year, in the order of 10 million people world-wide die as a result of heart disease. Ischaemic heart disease is the single largest cause of death and was responsible for 16 % of all deaths in 2019 [4]. Lipoproteins are a major cause of heart disease and the study of lipoproteins is thus important for human life and health. There are many ways to study lipoprotein metabolism, and one such way is by means of multicompartmental mathematical modelling. These models, in conjunction with specifically-designed experiments involving administration of stable isotopes, can be used to quantify the fluxes of lipoproteins and further improve our mechanistic understanding of this metabolic system. Here, I developed a new comprehensive multicompartmental model of apoB48- and apoB100 lipoprotein metabolism. The model is designed to describe the kinetics of these lipoproteins in the postprandial state. In the current thesis, I used the model to study the metabolism of mainly triglyceride-rich lipoproteins and their remnants, but also the metabolism of low-density lipoproteins.

Preluding comments

Below, I will provide a brief layout of the relevant players in lipoprotein metabolism. I will focus on the triglyceride transport system since the model mainly is developed to describe TRLs. However, since we expand the model in paper IV to encompass LDL kinetics I provide some relevant background for LDL metabolism. We also study apoC-III kinetics and apoE kinetics (relevant for TRL- and TRL-remnant clearance) but since we do not study high-density lipoprotein (HDL) metabolism I will focus very little on this metabolic sub-system in the current report. I will also leave lipoprotein(a) metabolism to the side. Regarding the lipid particle content; the lipoprotein core contains mainly triglycerides and cholesteryl esters and is enclosed by a phospholipid layer. Several other exotic lipid species are also in and on the lipoprotein but the details of these will not be examined here. When discussing lipoproteins there can be some confusion regarding terminology. In the current thesis I would therefore like to highlight the following. The term apoprotein refer to the protein itself, the apoprotein in combination with the lipid particle is referred to as a lipoprotein and when merged the apoprotein can technically be referred to as an apolipoprotein. Regarding the density fraction terminology (see below for further details and definitions), I choose to use the terms loosely in the sense that a VLDL particle refers to an apoB100-containing particle in the VLDL density fraction if not explicitly stated otherwise. Technically, apoB48-containing lipoproteins can exist in the CM, VLDL1 and VLDL2 fractions. ApoB100-containing lipoproteins exist in VLDL1, VLDL2, IDL and LDL. Although no current clear definition exists on what a TRL *remnant* particle is, here I loosely define a remnant as any TRL particle that has been partly lipolyzed as opposed to newly secreted into the circulation. Lastly, the term *kinetic study* refers to the type of study described here; where the purpose is to evaluate the kinetics (i.e. fluxes - secretion rates and clearance rates) of lipoproteins. The terms *kinetic model* and *multicompartmental model* both refer to the type of mathematical model used in kinetic studies.

Overview of apoproteins

There are many apoproteins to consider which are relevant to lipoprotein metabolism. Broadly, apoproteins can be divided into the categories: A, B, C and E.

ApoAs: ApoA-I is the main apoprotein on the HDL particle. It has been shown to activate lecithin–cholesterol acyltransferase (LCAT) and interact with ATB-binding cassette protein

A1 (ABCA1), ABCG1 and class B, type I scavenger receptor (SR-BI) – proteins that are involved in reverse cholesterol transport. ApoA-II is the second most abundant apoprotein on HDL particles but its exact role is unknown. ApoA-IV is synthesized in the intestine during fat ingestion and chylomicron production. It is attached to newly synthesized chylomicrons but is also attached to HDL particles in the circulation [5]. ApoA-V is synthesized in the liver and is an activator of LPL [6].

ApoBs: ApoBs are the main focus of the current thesis and is divided into two types; apoB100 and apoB48. ApoB100 is the main structural protein of the endogenous lipoprotein pathway (particles produced in the liver) and is attached to lipoproteins in the VLDL/IDL/LDL density fractions. ApoB48 is the main structural protein of the exogenous lipoprotein pathway (intestinally-derived particles) and is attached to chylomicrons in the CM fraction as well as chylomicrons and chylomicron remnants in the VLDL1- and VLDL2 fraction. Both apoB100 and apoB48 are expressed from the same gene (APOB) but in the intestine, the mRNA editing complex apobec-1 modifies a cytosine to an uracil – introducing a stop codon into the apoB mRNA. This results in apoB48 (48 % of the length of apoB100) being expressed in the intestine whereas the liver expresses the full-length protein. As opposed to apoB100, apoB48 alone is not recognized by the LDL-receptor, and thus the catabolism of the two lipoprotein species differ. Both apoB100- and apoB48 containing lipoproteins only contain one apoB per particle, making them reflective of circulating particle number - a useful property when conducting kinetic studies.

ApoCs: ApoC-I is attached to TRLs and HDLs and can exchange freely between these particles. Fairly little is known about apoC-I, but it has been shown to be an inhibitor of LPL [7]. In addition, it has been shown to inhibit apoE-mediated receptor binding as well as hepatic lipase activity [8] thus tentatively regulating the plasma level of TRLs in VLDL and IDL. In contrast, apoC-II is a co-factor for LPL and thus promotes hydrolysis of TGs in TRLs [6]. Like apoC-I, apoC-III is an inhibitor of LPL and possibly also LDL-receptor and/or low density lipoprotein receptor-related protein 1 (LRP1) [9]. Plasma apoC-III correlates with plasma TG and we have previously shown that apoC-III secretion rate into circulation correlates with plasma apoC-III as well as plasma TG [10].

ApoE: ApoE is mainly synthesized in the liver and the intestine and can exchange between TRLs and some HDLs in plasma. ApoE functions as a ligand for the LDL-receptor family (including LRP1) and thus promotes clearance of both apoB100- and apoB48 containing lipoproteins [11]. There are three variants of apoE; E2, E3, E4, where the E2 variant is associated with lower clearance of TRL remnants due to its lower receptor binding affinity.

ApoB100- and apoB48 containing lipoprotein sizes and densities

ApoB48-containing lipoproteins can be found in the CM, VLDL1 and VLDL2 density fractions defined in the current thesis as a Svedberg flotation rate of: Sf > 400, Sf 60-400 and Sf 20-60 respectively. ApoB100-containing lipoproteins are found in the VLDL1, VLDL2, IDL and LDL fractions. Here we define IDL as a density of centrifugation at 1.035 g/ml (after removal of VLDL2) and LDL as a density of centrifugation at 1.063 g/ml. The corresponding rough diameters are: LDL 22-30 nm, IDL 30-45 nm, VLDL 45-75 nm and CM > 75 nm and up to around 600 nm. For a summary of the sizes and densities of these lipoproteins see Figure 1.

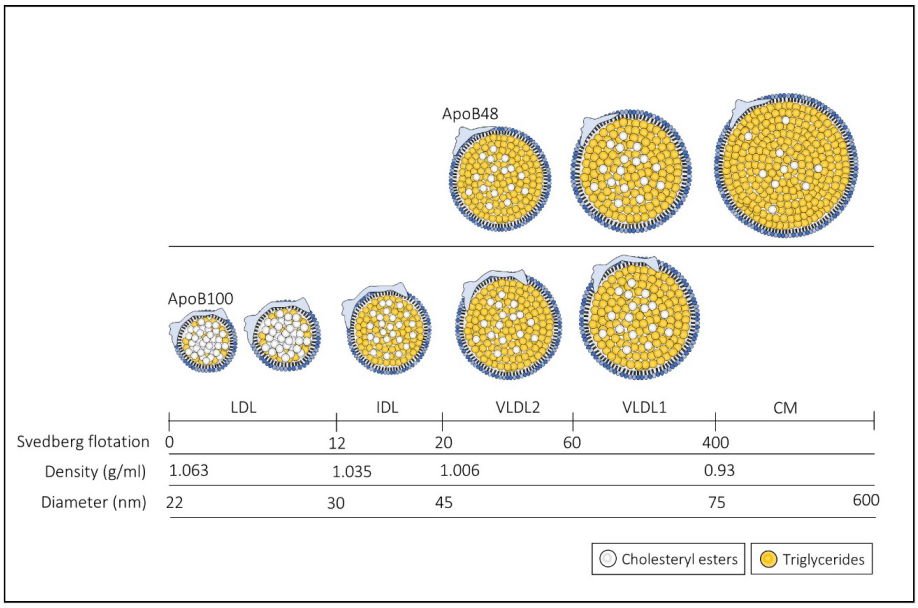

Figure 1: Size and density of lipoproteins are highly correlated. Lipoproteins are typically isolated by sequential ultracentrifugation whereby each fraction is harvested stepwise and the density of the solvent is controlled via addition of salt. The density fractions VLDL2, VLDL1 and CM are defined by Svedberg flotation rate (Sf) 20-60, 60-400 and >400 respectively. IDL is here defined by the material that is left when VLDL2 is removed and centrifugation at a density of 1.035 g/ml is performed. After removal of IDL, centrifugation at 1.063 g/ml is performed and LDL is subsequently isolated. As particles are delipidated they shrink in size, their density increases and they are relatively enriched in cholesterol.

Overview of lipoprotein receptors and enzymes

There are several receptors that can clear apoB-containing lipoproteins form the circulation and there are several enzymes that modify the lipoproteins. The most important enzyme that is responsible for the clearance of triglycerides from TRLs is lipoprotein lipase (LPL). LPL hydrolyses TGs from TRLs releasing free fatty acids which can be taken up by tissues such as adipose- and muscle. LPL is expressed in several tissues and is transported from the subendothelial space across the endothelium into the capillary lumen by a protein known as glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1). This protein also anchors LPL to the endothelium and aids in hydrolysis of TRLs [12].

Hepatic lipase (HL) resides at hepatocytes and hydrolyses IDL-and LDL sized lipoproteins. For this reason, it is important in the clearance of IDL (which results in LDL). It can also hydrolyse TGs and phospholipids in HDL (of which the latter task endothelial lipase also plays a role in).

Cholesteryl ester transfer protein (CETP) is an important protein involved in the reciprocal transfer of TGs and cholesteryl esters (CEs) between lipoproteins. More specifically, it transfers TGs from TRLs and LDLs to HDL; and CEs from HDL to TRLs and LDLs which results in a more CE rich TRL/LDL particle [13]. Inhibition of CETP leads to higher HDL-C and lower LDL-C [6].

The main receptor involved in clearance of apoB-containing lipoproteins from the circulation is the LDL receptor (LDLR). LDLR is expressed in the liver as well as many other tissues. ApoB100 is recognized by the LDLR and thus it mediates clearance of LDL particles but also of apoB100-containing IDL particles. LDLR also recognizes apoE and since chylomicron remnants are enriched in apoE, LDLR may mediate clearance of apoB48-containing lipoproteins as well. Upon internalization of the receptor-lipoprotein complex into the cell, the lipoprotein particle is degraded in the lysosome. The receptor itself may also be degraded or recycled back to the cell surface – a process influenced by proprotein convertase subtilisin/kexin type 9 (PCSK9). The number of LDL receptors on the cell surface is also influenced by the expression level of the protein; which in turn partly is a function of the cholesterol-status inside the cell. The transcription factor sterol regulatory element-binding protein (SREBP) is partly responsible for this feedback regulation. There are other receptors involved in the clearance of lipoproteins, namely the VLDL receptor and the LDL-receptor related proteins (LRPs) (mainly 1, 2, 6 and 8). All of these receptors are structurally related to the LDL receptor. The VLDL receptor is widely expressed in several tissues but not in the liver and may thus be responsible for extra-hepatic clearance of TRLs. LRP1 is expressed in most tissues, including the liver, and is involved in recognition of many ligands not related to lipoprotein metabolism. It does however recognize apoE and can therefore clear intestinally derived chylomicron remnants into hepatic tissue. The exact roles of LRP2 (also known as megalin), LRP5, LRP6 and LRP8 (also known as ApoER2) with regards to lipoprotein metabolism are less clear. The receptors have wider functions, including Wnt-signaling, renal re-absorption of various molecules and bone mineralization, but due to their structural similarity to LDLR, VLDLR and LRP1 may play roles in lipoprotein clearance [14]. Another class of proteins which can clear TRL remnants are heparan sulfate proteoglycans (HSPGs). Especially the hepatic HSPG syndecan-1, which may be considered as a separate remnant clearance receptor, independent from LDLR/LRP1 [15].

ApoB48 production and secretion

Upon ingestion, dietary triglycerides are hydrolysed in the stomach by acid lipase, and by pancreatic lipase in the small intestine. To increase surface area and thus enzyme reaction rate, bile salts help emulsify the TGs. The resulting monoacylglycerols (MGs) and free fatty acids (FFAs) then self-assemble in the intestinal lumen to micelles. The FFAs and MGs are able to migrate across the enterocyte cell surface; a process possibly aided by the scavenger receptor CD36 and/or the fatty acid transport protein FATP, and enter into the enterocyte. In the endoplasmic reticulum (ER) inside the enterocyte, the MGs are converted to DGs by the enzyme monoacylglycerol acyltransferase (MGAT) and the DGs are further converted into TGs by the enzyme diacylglycerol acyltransferase (DGAT). At this point, synthesis of chylomicrons can ensue. This occurs with the help of the enzyme microsomal transfer protein (MTP). TGs and cholesteryl esters are packaged and together with the structural apoB48 protein, constitute the growing chylomicron particle. The APOB gene is translated into the full apoB100 mRNA but it is post-transcriptionally edited by the enzyme Apobec-1 to form the truncated version – apoB48. Ready-made chylomicrons are subsequently secreted into the lymph and via the thoracic duct the particles reach systemic circulation [16].

ApoB48 catabolism

Upon secretion into the circulation, large TG-rich chylomicrons are a preferred substrate for LPL. LPL is expressed highly in e.g. muscle- and adipose tissue where it resides at the capillary

endothelial cell surface [17]. LPL hydrolyses triglycerides and releases free fatty acids which can be taken up by the tissues. When the chylomicrons are partially lipolyzed they shrink in size, become relatively enriched in cholesterol, and may at this point be referred to as chylomicron remnants. The remnants acquire apoE which makes the particles recognized by LDLR and LRP1.

ApoB100 production and secretion

The endogenous lipoprotein pathway occurs in the liver and the assembly of the particle is similar to the process in the intestine. In the endoplasmic reticulum, triglycerides and cholesteryl esters are merged with the growing apoB100 polypeptide by means of MTP. The so-called pre-VLDL is lipidated enough to be classified as a VLDL2-particle and by this point it is transferred to the Golgi apparatus. From here, the particle may be secreted directly or lipidated further and secreted as a more TG-rich VLDL1 particle [18]. Along this synthesis route there are two degradation options for the growing VLDL particle; one in the ER as a pre-VLDL (proteasomal degradation) and one in the Golgi apparatus as a larger particle (non-proteasomal degradation). These degradation pathways reveal the primary limiting factor to be TG-availability rather than apoB100 synthesis ability [19].

ApoB100 catabolism

The metabolic fate of apoB100-containing lipoprotein particles is partly overlapping as to the apoB48-containing particles (for a schematic summary of both apoB48- and apoB100 metabolism, see Figure 2). Like chylomicrons, apoB100-containing VLDLs are also hydrolysed by LPL, resulting in release of free fatty acids and a smaller particle. When partly hydrolysed, the resulting VLDL remnants may be directly cleared from circulationpossibly via VLDLR and/or accumulation of apoE and subsequent LDLR/LRP-mediated clearance. The particle may also be further lipolysed into an IDL particle. The IDL particle may be directly cleared via receptors or lipolysed further by hepatic lipase (HL) and form an LDL particle. The apoB100 on the LDL particle is itself a recognized ligand for the LDL receptor and can thus be cleared from circulation via this pathway. In addition, a large pool (up to two thirds) of LDL particles does not seem to be cleared by the LDL-receptor but rather by non-receptor mediated means [20].

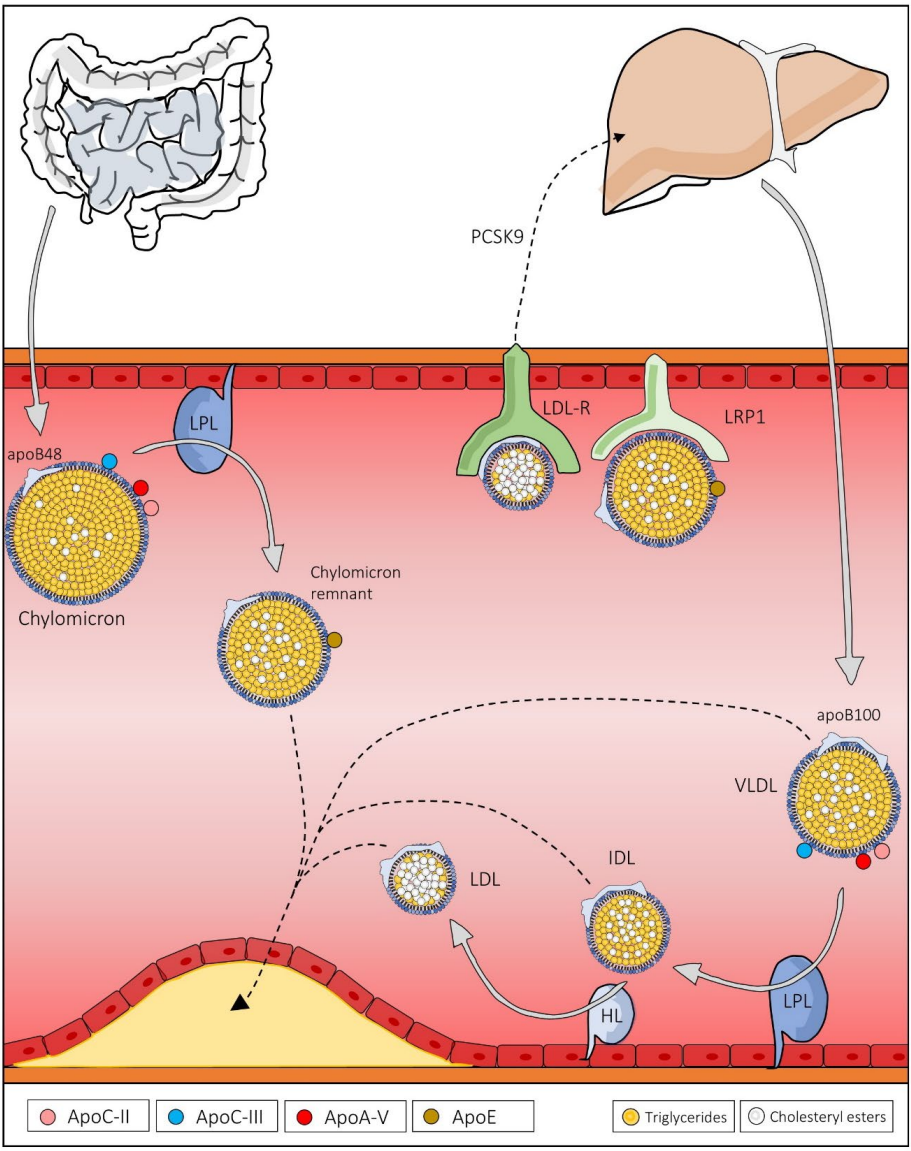

Figure 2: Overview of apoB48- and apoB100 lipoprotein metabolism. ApoB100-containing lipoproteins are produced by the liver and subsequently hydrolyzed by lipoporotein lipase (LPL) and hepatic lipase (HL). The LDL receptor (LDL-R) and the LDL-receptor related protein 1 (LRP1) are two main receptors responsible for removing apoB100 from the circulation. Other receptors may contribute (not shown here), and there are also other potentially contributing players such as heparan sulfate proteoglycans. ApoB48-containing lipoproteins are secreted from the intestine and are also hydrolyzed by LPL. The subsequently formed chylomicron remnants can be removed by LRP1 and may also be removed by LDLR if apoE is attached to the particle. As the lipoproteins become more and more delipidated they are relatively enriched in cholesteryl esters, although TRLs are cholesterol-rich in an absolute sense. There are several relevant additional apoproteins that modify the activity of LPL; where apoC-II and apoA-V stimulate lipolysis and apoC-III inhibit lipolysis. ApoE, as mentioned, functions as a ligand for receptor-mediated removal.

Multicompartmental modelling

Mathematical models in general are a broad category, but a common feature is their ability to represent and describe a system in the real world. One can classify models along two main axes: i) deterministic-stochastic and ii) empirical-mechanistic [21]. A deterministic model produces one and the same output given the same input. A stochastic model incorporates variance and may thus predict different answeres; or a set of answers, given the same input. An empircial model has no fundamental representations built into itself but merely seeks associations between variables for the purpose of predicting outcomes. A mechanistic model on the other hand, contains representations of actual physical occurances and can thus be used to gain information of a system if the parts of the system (on some level) are understood. Examples of an empirical, deterministic model is simple regression modelling – which is merely a statistical model that contains no fundamental mechanistic representations of the underlying system. An example of a mechanistic stochastic model is weather forecasting since it contains fundamental representations of reality but it also considers chance events. An example of a deterministic and mechanistic model is a structural engineering model for buildings.

In the current thesis, I have used mathematical representations of metabolic occurances inside the human body, which is an example of a mechanistic model. The model is constructed of so-called ordinary differential equations (ODEs) which make the model deterministic. A differential equation can be used to describe how a physical system changes over time. In this thesis, fluxes of e.g. apoB will move from one so-called "compartment" to another. Each compartment represent some physical entity; for example, one compartment may represent the amount of apoB100 in the VLDL1 fraction. If the liver produces apoB100 then there will be an influx into the compartment. And due to clearance of the particles there will be outflux from the compartment. The clearance can be mathematically described by means of a differential equation. In this example, the rate of clearance may be described by: $\frac{dQ}{dt} = -kQ$, where $\boldsymbol{Q}$ (for example) represent the amount of apoB100 in VLDL1, $\boldsymbol{t}$ represent time, and $\boldsymbol{k}$ represent the proportion of apoB100 in VLDL1 that is cleared in each unit of time. By letting an influx of material enter the pool and by a clerance-mechanism letting material exit the pool a corresponding pool size will emerge. This pool size may be measured experimentally and a model fit can be achieved (in this simple example by varying the parameter $\boldsymbol{k}$ and/or varying the influx parameter). The influx (also called production rate or secretion rate) is directly quantified by the model, and the so-called fractional catabolic rate (FCR) is directly proportinal to $\boldsymbol{k}$ or can be defined as flux per pool size. The above example is a simplified, but accurate, description of the model used in the current thesis. The main difference is that of complexity. In addition, the fluxes are non-steady state which adds another layer of complexity to the modelling process.

When a model is fitted to experimental data, the model parameters are adjusted in order to achive a satisfactoy fit. This is an iterative process whereby the model predictions are getting closer and closer to the experimental data. A satisfactory fit is necessary but, importantly, does not guarantee that the model has made correct predictions in the sense that the predicted secretion rates and clearance rates reflect the true values. Whether or not true values have been found depend also on the model structure. This issue is similar to the situtation in science in general; i.e. several competing hypotheses may all be consistent with the same experimental data.

A schematic overview of the relation between modelling and reality is found in Figure 3. The model structure is informed by previous fundamental knowledge in the area. The purpose of the experimental protocol for a particular so-called *kinetic study* is to generate subject-specific data, which the model will use to deduce the fluxes of the lipoproteins. The model fit will generate a prediction which may or may not be close to the true values. This depends in large part by the assumptions made when developing the model (the model structure) but also on the constraints of the parameters in the particular model. The better the model structure and the better the model fit to the data, the higher the probability that the made predictions are close to the true values.

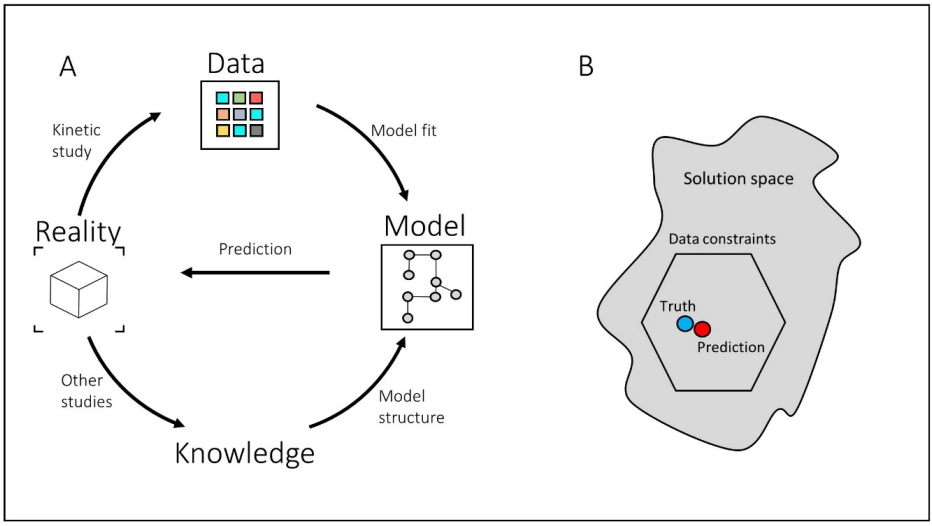

Figure 3: Schematic overview of the interplay of modelling and reality. A) Basic research informs our common understanding of the lipoprotein metabolic system. This knowledge is used to form the model structure. For a specific kinetic study, the experimental procedure; with administration of stable isotopes and subsequent measurement of experimental data; provides the basis for a particular model fit to the data. B) The solution space of a model is usually large, but shrinks firstly due to the choice of the model structure and secondly due to the experimental data. The model prediction may differ from reality since there can be several fits to the same data. But the final prediction should be close to ground truth given sound experimental- and model setup.

Do LDL- and TRL particles cause atherosclerotic cardiovascular disease?

The main motivation for studying lipoproteins are their role in human disease, more specifically atherosclerotic cardiovascular disease (ASCVD). In 2017 the European Atherosclerosis Society published a consensus statement on the evidence for the causality of LDL in CVD [22], and later a follow up article was published by Borén et al extending the first paper [23]. Several lines of evidence were presented, including: evidence from inherited disorders of lipid metabolism, evidence from prospective epidemiological studies, mendelian randomization studies and randomized controlled trials. The last two sources of evidence are particularly compelling. Briefly, there are several treatment approaches to reduce LDL particle count; i) HMG-CoA reductase inhibitors (statins), ii) NPC1L1 inhibitors (ezetimibe), iii) PCSK9 inhibitors and iv) bile acid sequestrants, all of which have been evaluated in meta-analyses of randomized controlled trials (RCTs). The combined results show a consistent

pattern of reductions in cardiovascular events in proportion to the reduction in LDL-cholesterol. The relative risk reduction was found to be around 22 % for every 1 mmol/l reduction in LDL-C, with a median of 5 years follow-up. Results from mendelian randomization studies are consistent with the results from the RCTs, in that there is a linear relationship between the magnitude of the LDL-C lowering effect of various genetic variants and the relative risk reduction in cardiovascular events. The slope of the line however is higher, indicating that duration of the reduction in LDL-C also constitute an independent effect. To achieve a 22 % relative risk reduction (as above, with a median follow-up time of 5 years) one only requires a 0.35 mmol/l reduction in LDL-C if it is life-long.

Historically, there have been uncertainty whether TRLs also cause ASCVD. In recent years, evidence have emerged that have reduced this uncertainty. In a 2013 study, 185 common genetic variants associated with plasma lipids (both TG and LDL-C) were examined [24]. The authors investigated whether there was an independent association of TG with coronary artery disease (CAD) and they concluded that the results support such an independent causal effect. In 2014, Thomsen et al. investigated associations of genetic variants in lipoprotein lipase and all-cause mortality in the Copenhagen City Heart Study [25]. Genetically lower non-fasting triglycerides of 1 mmol/l was associated with an odds ratio of all-cause mortality of 0.5. In another 2014 study, Crosby et al investigated the relative risk of CHD in carriers of genetic variants in APOC3 [26]. The authors found that a decrease in TG of 39 % was associated with a 40 % risk reduction of CHD. Similarly, another study in the Copenhagen City Heart cohort found that heterozygous carriers of mutations in APOC3 was associated with a 44 % reduction in plasma TG and a concomitant 41 % risk reduction in ischemic vascular disease [27]. In addition, in another 2015 mendelian randomization study, evidence for a causal effect of triglycerides on CHD was found [28].

In summary, it is well established that LDL causes ASCVD. Recent evidence also supports a causal role for TRLs. TRLs and their remnants contain large amounts of cholesterol and plausibly could explain considerable variation in the observed residual cardiovascular risk [29].

Methods

Here, I will briefly explain the model, the experimental setup (which is the same for all papers) and how the experimental data that serves as input to the model, is measured. For additional details, see corresponding papers.

Experimental setup

The basic experimental setup is the same for all studies included in the current thesis. First, subjects are fasted overnight and are given a bolus injection of the tracers (see also Figure 4). Then, a standardized meal is served containing both carbohydrates, protein and fat (the meal is composed in such a way to mimic a realistic meal that many people may consume on a daily basis). Blood samples are drawn at specific time-point intervals, from a few minutes up to several days later, to capture the behaviour of the various lipoprotein particles. The experimental data is constituted by pool sizes and enrichments for the different lipoproteins in the different density fractions, and this data is the basic input into the multicompartmental model. The model can utilize the experimental data for each study subject in order to fit the model parameters and calculate the kinetic parameters (i.e. secretion rates and clearance rates). For further details, see corresponding method section of each study.

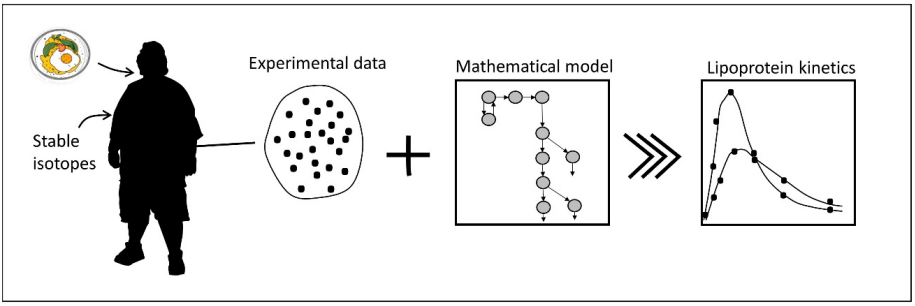

Figure 4: Basic experimental setup. Subjects are administered a test meal together with stable isotopes. After pre-determined time-intervals experimental data (tracers and tracees) are collected and quantified. The resulting data is used as input to the multicompartmental model. The output from the model (when a satisfactory fit is achieved) are predicted lipoprotein kinetic parameters, such as secretion rates (aka production rates) and clearance rates (aka catabolic rates, usually defined as a fraction relative to the pool size).

Tracers

In a steady-state system, the pool size of a given compartment can in theory be a result of an infinite number of combinations of secretion-and clearance rates. It is therefore of no use to experimentally measure only pool sizes and provide the model with this information. To reduce the solution space, so-called tracers are used. A tracer is normally an amino acid or other molecule where a stable isotope is incorporated. In our case, we use deuterium – which is a stable isotope of hydrogen. The most common form of hydrogen contains one proton and one electron but deuterium also contains one neutron in the nucleus. The two tracers in the current work are deuterated leucine ((5,5,5-D3), 7 mg/kg body weight) and glycerol (1,1,2,3,3-D5, 500 mg bolus dose). The D3-leucine is mixed with the leucine pool of the whole body, and is further incorporated into all newly synthesized proteins - including apoB48 and apoB100. Here, we experimentally quantify the incorporation of the deuterated leucine (the so-called enrichment, or tracer-tracee-ratio) in the plasma pool and in apoB100/apoB48 in the various density

fractions. The labelled glycerol is mixed with the glycerol pool of the body and incorporated into any newly synthesized triglyceride – including triglyceride secreted in hepatically derived lipoproteins. Glycerol is not incorporated into intestinally-derived lipoproteins since glycerol kinase is lowly expressed in enterocytes [30] [31].We quantify the enrichment of glycerol in TG in the VLDL1- and VLDL2 fractions.

Model

The model was constructed in the software SAAM II (SAAM Institute, Seattle, WA). Much of the model structure for the parts describing apoB100 and apoB100-TG is adapted from Adiels et al [32]. The key difference, of course, being the non-steady state nature of the current model and the incorporation of the apoB48- and apoB48-TG parts of the structure. The model structure in paper I-III does not include the IDL-and LDL fractions as shown in Figure 5, but for completeness the structure in paper IV is shown here.

SAAM II is not specifically designed for non-steady state models, so for this reason the model contains three separate "layers" describing three separate, but continuous occurances, namely: i) the flux of the tracer (labelled leucine and glycerol), ii) the steady-state flux of the tracee (apoB and TG) and iii) the non-steady state flux of TG and apoB induced by the test meal.

The model structure can be roughly divided into six different sub-structures describing the following: i) plasma leucine, ii) plasma glycerol, iii) apoB100, iv) apoB100-TG, v) apoB48 and vi) apoB48-TG.

When injecting the stable isotope, it will mix with the plasma leucine pool (compartment 31, see Figure 5) rapidly. Thereafter it will exchange with protein pools (compartment 32-33) that can re-release leucine and provide a slower appearance in plasma. Leucine is then (with a certain delay, represented by compartment 34/36) incorporated into intracellular pools in the liver (37) and intestine (35) respectively. The amino acids in the intracellular pools will be used for synthesis of the growing apoB protein thus the tracer will be incorporated into the newly synthesised apoprotein. Compartment 1 describes the influx of apoB100 in VLDL1 from the liver. The lipoprotein is lipolyzed and can follow down the delipidation chain through compartments 2, 4 and 5. ApoB100 in VLDL1 and VLDL2 can also be directly cleared from circulation (from compartments 2/3/5/6). Compartment 3 and 6 represent a slower turning over sub-pool of apoB100 in VLDL1 and VLDL2 respectively. The shrinking apoB100-containing lipoprotein in VLDL2 can be further lipolyzed and become an IDL-sized particle (compartment 7/8). IDLs may be cleared from circulation by receptor-mediated means or further delipidated to form an LDL particle (compartment 9/10) where it is lastly removed from circulation via receptor- or non-receptor mediated mechanisms. ApoB100 may be directly secreted from the liver as either VLDL1, VLDL2, IDL or LDL-sized particles, indicated by arrows from compartment 37 in Figure 5.

For the apoB48-system, the intracellular leucine in the intestinal cells will be used for newly synthesized apoB48. The black arrows from compartment 35 to 46/47 represent steady-state (basal) secretion of apoB48 into the VLDL1 and VLDL2 fractions. The blue arrows indicate non-steady state secretion in response to the meal; this type of secretion may enter both the CM, VLDL1 and VLDL2 fraction. Compartments 43-47 represent synthesis time and delay due to lymphatic transport before the chylomicrons arrive in systemic circulation. ApoB48 in the CM fraction is subdivided much like apoB100 in VLDL1/2 into three compartments with differing TG/apoB48 ratios and residence times. Here, we have assumed an existence of a

direct clearance route of apoB48 in the CM fraction from compartments 18 and 19. Compartments 20 and 21 represent apoB48 in VLDL1; and 21 is a slower turning over pool than 20. Correspondingly, 22 and 23 represent apoB48 in VLDL2. A two-compartment sub-structure were chosen for apoB48 in VLDL1/2 since this was enough to describe the experimental data. The apoB48-TG sub-structure mimics exactly the above described apoB48 sub-structure with the exception that it describes the triglyceride content in the particles and not the apoB48 itself.

A tracer for apoB48-TG is not included here. For apoB100-TG however, deuterated glycerol was used as tracer. For this reason, plasma glycerol was modelled similarly to our previous publication [32] whereby compartment 38 represents plasma glycerol and 39 represents extrahepatic glycerol pools. Through a delay due to apoB100 synthesis time, the enrichment may reach apoB100-TG in VLDL1 and VLDL2 in plasma. Compartments 11-16 are the TG-equivalents of compartment 1-6 described above; with the exception that triglycerides may also be cleared from 11 and 14 due to lipolysis.

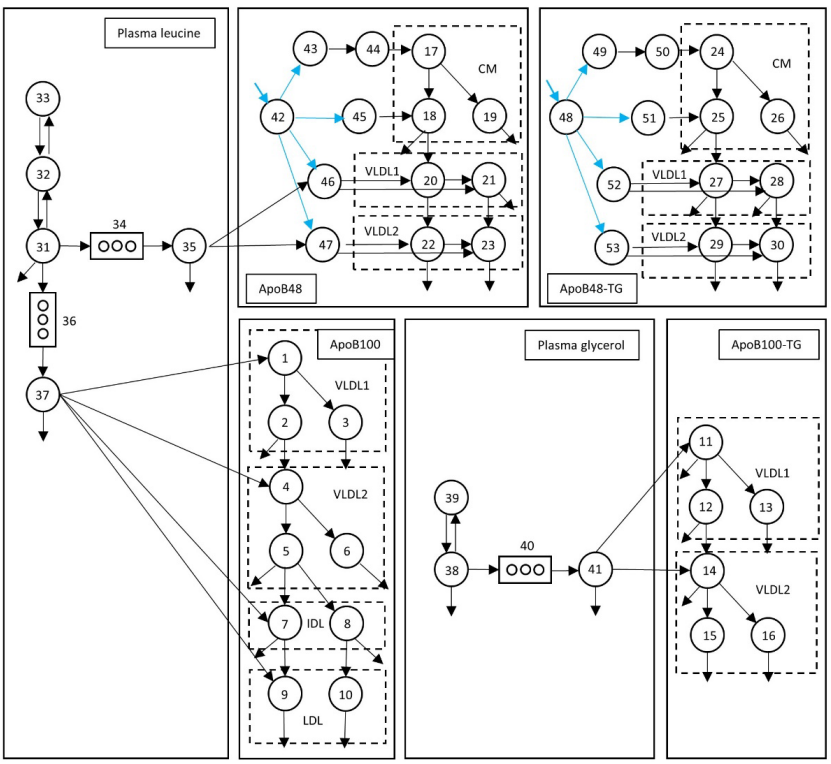

Figure 5: Overview of the kinetic model used in the current thesis. The IDL-and LDL compartments were only used in paper IV and not in paper I-III, but for completeness is shown here. The model structure contains compartments (circles) representing different physiological entities; for example, compartment 1-3 represent apoB100 in the VLDL1 density fraction. Arrows indicate fluxes between compartments; where black colour indicate steady-state conditions and blue arrows indicate flux specifically in response to the test meal. So-called delay compartments are represented by three circles in a rectangle and are used to introduce a time-shift in the model. For a fuller description of all the compartments and fluxes, see the method section of the current thesis.

Quantification of apoB100/TG pools and enrichments

The lipoprotein fractions were separated by means of centrifugation (same method also applies to isolation of apoB48 in the different fractions). Briefly, plasma samples were overlayered with salt solutions of varying density and centrifugation occurred in three steps. ApoB was isolated by adding isopropanol to remove other apolipoproteins and separated from the lipid-containing fraction by addition of ethanol/ether. The isopropanol/ethanol/ether supernatant contains the glycerol which is hydrolysed, derivatized and analysed by gas chromatography mass spectrometry (GC/MS). From the glycerol, a smaller amount is also removed which is used for subsequent analysis of *de novo* lipogenesis. The pellet contains apoB100 which was derivatized and analysed by GC/MS. For quantification of plasma leucine enrichment, the plasma is deproteinized, eluted through an ion exchange column, derivatized and finally analysed by means of GC/MS. Triglyceride and cholesterol were quantified by an automated enzymatic method.

Quantification of apoB48 pools and enrichments

For quantification of apoB48, a newly developed proteomics-based pipeline was used. Briefly, the samples were trypsin-digested and captured with an antipeptide antibody (specifically targeting a trypsin fragment unique for apoB48) to enrich the amount of apoB48 in the sample. Stable-isotope labelled apoB48 peptides were used as internal standards (not to be confused with the stable-isotope leucine in the endogenous apoB48 protein) in order to quantify apoB48 in an absolute manner. The samples were analysed by means of mass spectrometry which enabled quantification of both masses/concentrations of apoB48 in the CM, VLDL1 and VLDL2 as well as the enrichment (tracer-to-tracee ratio) in said fractions.

Results

Results from the four papers will be described separately below.

Model development (Paper I)

Before this work, there were no existing kinetic models which integrated both apoB48 and apoB100 metabolism in a non-steady state fashion. Previous models had either modelled only apoB100 or apoB48 separately [33 34 35 36 37 38 39 40]. Some previous apoB48 models had used a primed constant infusion design. This design introduces a need for pseudo steady-state conditions which can be enabled by feeding the test subjects many small meals. The disadvantage of this design is that there is no way to disentangle basal secretion (continuous secretion in the fasting state) from true postprandial secretion, and the physiological relevance of many small meals spread throughout the day is questionable. In another previous apoB48 model; where a more physiologic meal was provided as the test meal, the authors assumed apoB48 could be represented by one compartment [35]. Due to a pre-existing pool of apoB48 (i.e. during fasting conditions, before the test meal was provided), the basal secretion rate in this setup was found to be high in relation to the postprandial secretion. In addition, since the apoB48/TG ratio was assumed constant in fasting and during postprandial conditions; due to the single apoB48 compartment; the results pointed towards high fasting secretion of triglycerides and high between-subject variability in total TG-secretion - despite identical TG-content in the test meal.

We sought to make our model as reflective of the underlying physiology as possible and thus our model allowed apoB48 to exist not only in the CM fraction, but also in VLDL1 and VLDL2. More specifically, we enabled the model to secrete apoB48-containing lipoproteins directly into the CM and/or the VLDL1/2 fractions as this setup was found to best describe the experimental data (Supplementary Figure 2, paper I). This design also enabled the model to fit different clearance rates for apoB48 in CM, VLDL1 and VLDL2.

As can be seen in Figure 6, it is evident; particularly in the subject with the highest plasma TG, that a pre-existing pool of apoB48 is present, before any test-meal is given. As a control, we invited the subjects twice; once when a test-meal was given and once when they remained in a fasting state throughout the day. When analysing the fasting plasma apoB48 we saw a decrease (particularly in high-TG subjects) in apoB48 across the day. This indicates that apoB48 particles from the previous day's meals still lingered and that total elimination of these "excess" apoB48 particles would require fasting longer than overnight. There is indeed evidence that intestinal cytoplasmic lipid droplets can function as a lipid storage pool [41].

What is also evident in Figure 6 e-h is the non-zero enrichment curves. The fact that the lines are not flat show the presence of continuous basal secretion from the intestine, even during prolonged fasting. This basal secretion however, is found to be lower here than previously reported in terms of apoB48. But, more importantly, we find much lower basal intestinal triglyceride secretion due to the ability of the model to differentiate between VLDL-sized and CM-sized chylomicrons.

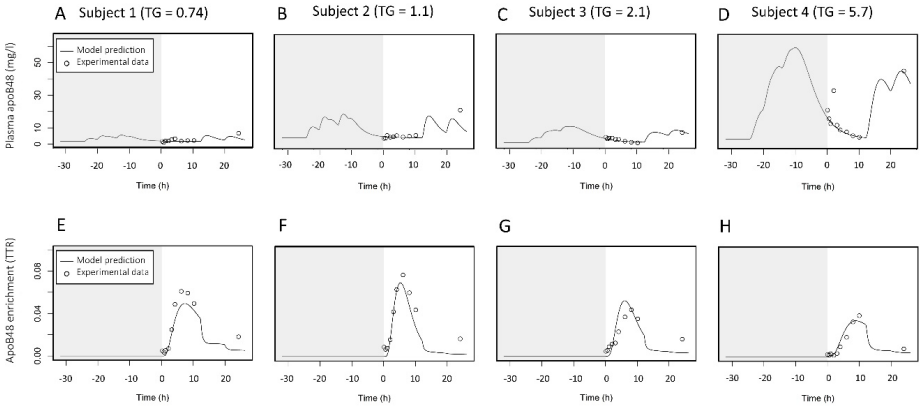

Figure 6: Plasma apoB48 and apoB48 enrichment (tracer-tracee-ratio, TTR) in four subjects with differing level of plasma TG, during the fasting test day, i.e. a day where no test meal was provided. A pre-existing pool of apoB48 is evident, particularly in the higher-TG subjects. Enrichment curves are non-zero indicating the presence of a continuous level of so-called "basal" apoB48 secretion.

A summary of the (group mean) fluxes in relation to the concentration for the different lipoproteins are found in Figure 7. Here we see that the flux of apoB100 in VLDL1 of around 40 mg/h is the largest flux of all lipoproteins; although at peak postprandial conditions the sum of the apoB48 fluxes are in the same order of magnitude. One should keep in mind that, since the molecular weight of apoB48 is around half of that for apoB100, the molecular fluxes should be multiplied by roughly two to display the per-particle fluxes. Nevertheless, one can also see that the flux of apoB48 varies considerably from fasting to peak postprandial conditions; the previous day probably reflects more accurately how a typical day may appear for most people. In Figure 7b we see that apoB100 in VLDL2 dominates the concentration in plasma, despite having a lower flux than apoB100. This is due to the lower clearance rate of apoB100 in VLDL2 compared to VLDL1. For apoB100 in VLDL1 we see a considerable variation in concentration despite a steady-state flux; which is a result of the competition from the chylomicrons for LPL postprandially. As for apoB48, the concentration depends on the feeding state but is a potentially clinically relevant part of the total pool of apoB in VLDL across the day. As for the flux of triglycerides (Figure 7c) the chylomicrons in the CM fraction dominates the total TG-flux into plasma on a daily basis. Despite this, the concentration of TG in VLDL1-particles from the liver is continuously higher. Clearly, CM-particles are catabolized more rapidly than VLDL particles.

In summary, here we present a new non-steady state kinetic model able to describe both apoB100- and apoB48 metabolism in the postprandial state. The model structure is informed by what we believe to be the best current knowledge of lipoprotein metabolism and is able to describe a range of subjects with low-to-high lipoprotein levels.

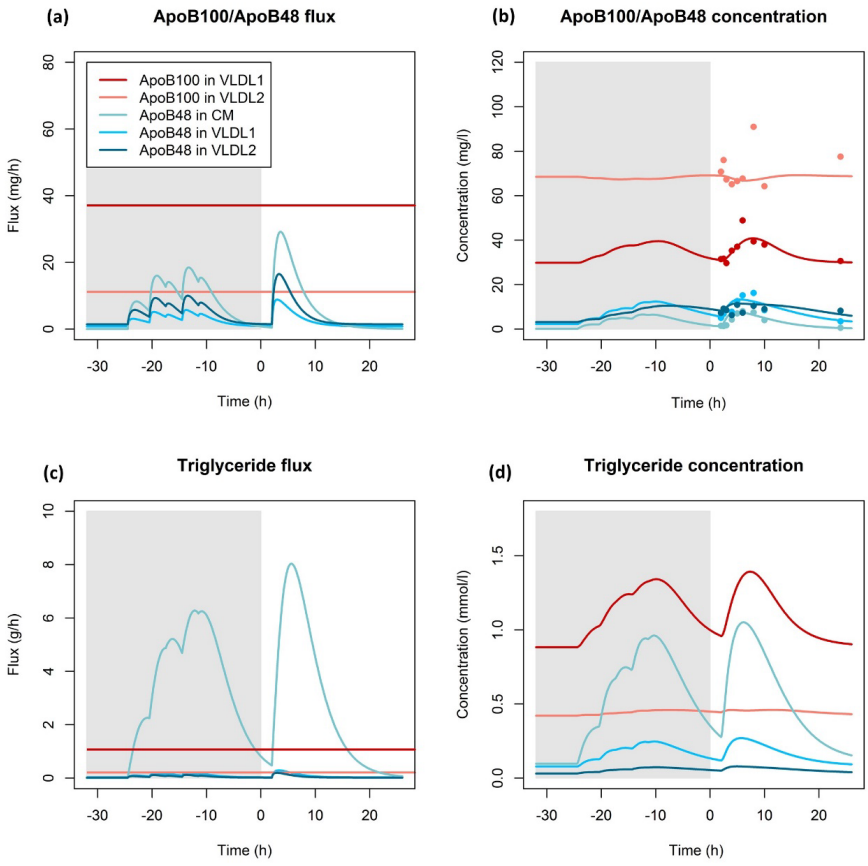

Figure 7: Overview of the lipoprotein fluxes and concentrations in the various density fractions. (a) Fluxes of apoB100 and apoB48 in CM, VLDL1 and VLDL2 in terms of mg/h. (b) Concentrations (mg/l) of apoB100 and apoB48 in CM, VLDL1 and VLDL2. Lines represent model predictions and closed circles represent experimental data. (c) Triglyceride fluxes and (d) Triglyceride concentrations in the CM, VLDL1- and VLDL2 density fractions. It is evident that most of the daily triglyceride flux stems from apoB48 in the CM fraction whereas the area under the curve for apoB48 in CM is relatively low. ApoB100 in VLDL2 is removed slowly and thus constitutes a large part of all lipoproteins despite its relatively low flux. ApoB48 in VLDL (where remnant apoB48 is found) varies depending on the feeding state. A representative previous day is shown with grey background and the test-day is shown with white background.

Lipoprotein kinetics in hypertriglyceridemia (Paper II)

Here we implemented the model we developed in paper I. Our research question can be stated as follows: how does the non-steady state apoB100- and apoB48 metabolism compare in subjects across the range of low-to-high levels of plasma triglycerides. To answer this question, we recruited 15 subjects with a large range of plasma triglycerides and divided them into three groups of five subjects each; one group with a mean triglyceride level of 0.9 mmol/l (the low-TG group), one group with a mean of 1.4 mmol/l (average-TG group) and one group with a mean triglyceride level of 3.6 mmol/l (high-TG group). As in paper I the subjects received a

standardized meal, and in addition we re-recruited nine of the subjects to be examined in fasting conditions as well.

Figure 8 presents an overview of the concentrations of the various lipoproteins in the three groups. Several things are noticeable. The apoB48 in the CM fraction does not vary greatly between the three groups – although there is a slight tendency towards being higher in the High-TG group. As expected, the apoB100 in VLDL1 and VLDL2 display a clear tendency towards increasing across the three groups (the total AUC is around three-fold higher in the High-TG group compared to the low-TG group). When examining the apoB48 in both CM, VLDL1 and VLDL2 it is noticeable that the total apoB48 AUC is constituted mainly by apoB48 in the VLDL fractions rather than in the CM fraction. One can also see that there is a similarly clear trend towards apoB48 in VLDL being increased across the range of the low-to the high-TG group. It appears that a high content of apoB100 remnants correlates with a high content of apoB48 remnants.

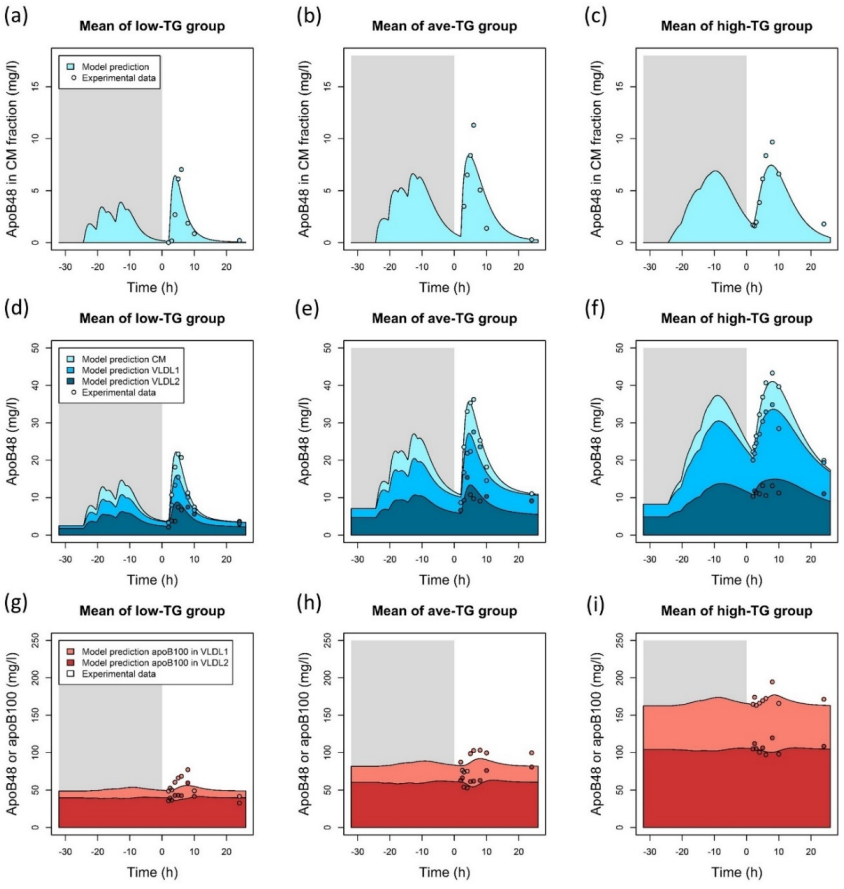

Figure 8: Overview of the lipoprotein concentrations in the CM, VLDL1- and VLDL2 density fractions for the three study groups (low-TG group, ave-TG group and high-TG group). It is evident that apoB100 in VLDL1 and VLDL2 increases in

relation to plasma TG. This pattern is apparent also for apoB48 - particularly for apoB48 in VLDL1 and VLDL2. ApoB48 in VLDL varies depending on the nutritional state but a baseline is present and is increased with increasing plasma TG.

When investigating the predicted kinetic parameters for the different lipoprotein classes, a pattern emerged. The production rates of both fasting and postprandial apoB48 in CM, VLDL1 and VLDL2 did not differ between the groups. Likewise, the production rates of apoB100 (total apoB100, apoB100 in VLDL1, apoB100 in VLDL2 as well as the triglyceride content of these particles) did not differ between the groups. In contrast, most of the clearance-rate related parameters, such as total apoB48 FCR, apoB48-VLDL1 FCR, apoB48-VLDL2 FCR, apoB100-VLDL1 FCR and apoB100-VLDL2 FCR showed an inverse association with plasma TG and was thus reduced with increasing degree of hypertriglyceridemia. These results indicate that the parallel increases in both apoB100-containing lipoproteins and apoB48-containing lipoproteins in the circulation are associated with a reduced clearance of these lipoproteins.

When investigating the shape of the enrichment curves (i.e. analysing only the raw data separate from the modelling results) we could see a typical pattern across the three groups of a less pronounced enrichment peak in the VLDL fractions as plasma TG rose (Figure 2 in the manuscript). This pattern is consistent with the notion that there is an underlying pool of particles, the pool size of which are increasing as plasma TG increases. This pool is not a result of increased production but of decreased clearance, and particles in this pool would likely fit the description of a remnant particle.

We know from previous research that apoC-III affects the clearance of apoB100-containing lipoproteins[42]. ApoC-III was found here to correlate with the fasting concentrations of triglycerides, apoB100 in VLDL and apoB48. In addition, apoC-III correlated negatively with total apoB48 FCR and total apoB100-VLDL FCR. It is therefore plausible that apoC-III also affects apoB48 clearance in the VLDL fraction and that increased levels of plasma apoC-III leads to the metabolic alterations across the groups observed in the current publication.

ApoB48 has been previously regarded as a negligible part of the lipoprotein pool in the VLDL fraction. When measuring apoB48 on a mg/l scale one ignores that the molar mass of apoB48 is roughly half that of apoB100. When taking this into account and further measuring apoB48 during the full range of fasting to postprandial states throughout the testing day, we found that apoB48 constituted around 25 % (23-30 %) of the total particle pool in the VLDL fraction. This may vary between subjects and it will be influenced of the overall dietary fat intake but this contribution may not be negligible in terms of CVD risk.

In summary, we found that as plasma triglycerides increase so does apoB100-and apoB48 containing lipoproteins in the VLDL fraction. The VLDL apoB48 originates both from hydrolysis of CM-sized particles but are also directly secreted from the intestine, both during postprandial- and fasting conditions. The type of particle that accumulates in the VLDL fraction as plasma triglycerides rise is a long-lived remnant-like particle which is accumulating due to reduced clearance, likely related to an increased concentration of apoC-III. Thus, hypertriglyceridemia is a symptom of an underlying phenotype characterized by the above features and the CVD-risk related consequences of this phenotype may be better understood in light of the current investigation.

Effect of liraglutide on postprandial lipoprotein kinetics (Paper III)

In this study we aimed to investigate whether the antidiabetic drug liraglutide affected postprandial lipoprotein metabolism in subjects with type 2 diabetes.

What is liraglutide?

Liraglutide is a glucagon like peptide-1 (GLP-1) receptor agonist, meaning it mimics the effect of GLP-1 by binding to the GLP-1 receptor. Unlike GLP-1 however, which has a half-life of minutes in circulation, liraglutide stays in the circulation for many hours [43]. The GLP-1 receptor is expressed in the pancreas, the heart, the brain and in the gastrointestinal tract; and GLP-1 has shown to enhance insulin secretion, increase heart rate, decrease appetite and delay gastric emptying [44 45 46]. GLP-1 receptor agonists were originally used to lower glycemia but have been found to cause weight loss. Liraglutide is today approved by the Food and Drug Administration (FDA) as a therapy for weight loss in non-diabetic individuals [43].

Study setup

The current study was a continuation of a previously published study [47] where we examined the lipoprotein profile and postprandial responses without performing kinetics analyses. Here, 18 type 2 diabetic subjects (14 in the intervention arm and 4 placebo subjects) were included. Briefly, the subjects in the intervention arm received liraglutide (titrating up to 1.8 mg/day) for 16 weeks whereas the placebo subjects were encouraged to lose weight by means of a life-style intervention, to achieve a similar weight loss as the subjects receiving liraglutide.

Impact on apoB48 and apoB48-TG

As seen in Figure 9 there was a reduction in chylomicron-TG on liraglutide. There was an even more pronounced reduction in apoB48 in the CM-fraction. Total plasma apoB48 AUC fell by 17% which was explained by a 54 % reduction in CM-apoB48. This was reflected in the kinetic results which showed a 60 % reduction in CM-apoB48 production. No change in apoB48 production in the VLDL1 or the VLDL2 fraction was noted. As seen in Figure 9 the enrichment curves were unaffected indicating no change in clearance rate of either apoB48 in CM, VLDL1 or VLDL2. Indeed, no change in CM-apoB48 clearance rate was found. However, the chylomicron-triglyceride clearance rate was increased by around 40 %. Direct clearance of chylomicrons, a pathway present before treatment, was reduced greatly on liraglutide. We further found an increased TG/apoB48 ratio in the chylomicron fraction in response to treatment.

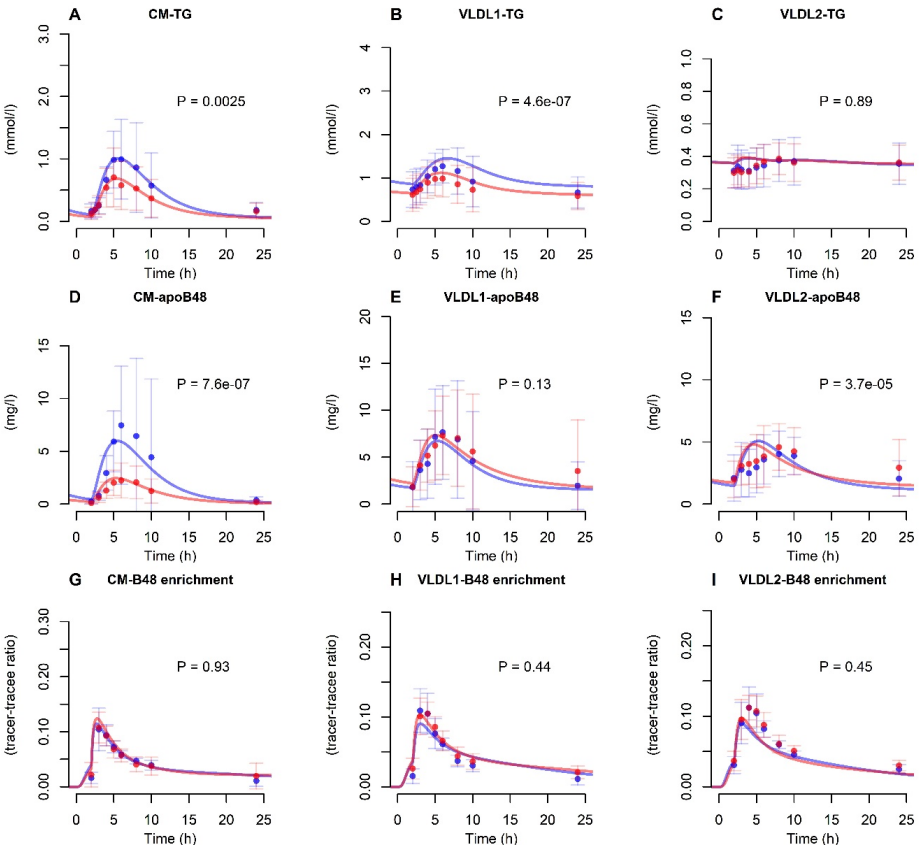

Figure 9: Model predictions (solid line) and experimental data (closed circles) before (blue) and after (red) 16 weeks of liraglutide intervention. CM-TG and CM-apoB48 are different between the visits whereas no discernible differences are noted in terms of the enrichment curves or the VLDL1/2 apoB48 concentrations. A difference of lower magnitude was noted in VLDL1-TG but not in VLDL2-TG. P-values have been calculated using repeated measures ANOVA.

Impact on apoB100

As seen in Figure 9, the VLDL1-TG was lower on liraglutide. The kinetic parameters indicated a trend towards decreased apoB100 secretion in VLDL1 and a significant decrease of VLDL1-TG secretion. These results may be an effect of the lower liver fat in response to the treatment, as we have seen from previous research that liver fat content and VLDL1-TG secretion correlates positively [48].

Looking at the summarized results depicted in Figure 10, the overall results suggest that the intestine, in response to liraglutide, secreted fewer CM-sized chylomicrons. However, since the triglyceride absorption was intact these particles were larger. This resulted in a faster hydrolysis but on a per apoB48 basis an unaltered clearance rate.

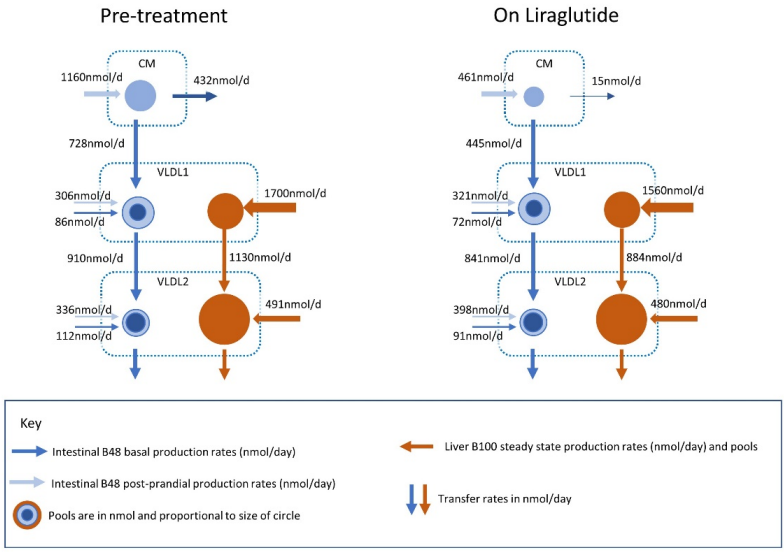

Figure 10: Schematic overview of the lipoprotein fluxes and concentrations before and after 16 weeks of liraglutide. The CM-flux is lower and the CM-pool size is lower after the intervention whereas other pool sizes and fluxes are largely unaffected. Fluxes are shown in nmol/day and pools are shown as nmol in order to adjust for the differences in molecular weight between apoB48 and apoB100.

Effects of evolocumab on lipoprotein metabolism (Paper IV)

In this study, we investigated to which extent the PCSK9 inhibiting, LDL-cholesterol lowering drug evolocumab, affected TRL metabolism.

What is evolocumab and PCSK9?

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein that plays a large role in regulating LDL cholesterol levels. The main function of the protein is to bind to the LDL receptor and (intracellularly) target it for degradation in the lysosome [49]. PCSK9 thus reduces the number of LDL receptors on the cell surface and increases the plasma concentration of cholesterol. Inhibition of PCSK9 therefore increases the number of available LDL receptors and lowers plasma cholesterol.

In this paper we wanted to examine the effect of 12 weeks of PCSK9 inhibition, using the monoclonal antibody drug called evolocumab, on the postprandial kinetics of apoB48 and of the kinetics of apoB100 in VLDL, IDL and LDL. We therefore expanded the model to also include compartments describing IDL and LDL metabolism. The subjects in the study were 13 type 2 diabetic males. All subjects were on current statin treatment (a treatment not halted during the study period due to ethical reasons).

Effects on apoB100 metabolism

As shown in Figure 11, the overall effects on the metabolism of apoB100-containing lipoproteins can be summed up as follows. There is no discernible effect on the metabolism of apoB100 in VLDL1. In VLDL2, the blue enrichment curve (which shows the data from the subjects on evolocumab treatment) we observe an apparent faster clearance. In IDL we were constrained by the number of subjects with available data (N=6) but the trend seems to go in the same direction. In the LDL fraction there is a very clear difference in the kinetics (i.e. apparent higher clearance of apoB100 in LDL on evolocumab). Further on, the pool sizes of apoB100 in VLDL1 appears unaffected, whereas in VLDL2 the pool is lower on treatment. The same is true for IDL, and for LDL the pool size is greatly diminished on evolocumab.

When investigating the kinetic parameters, the results echo the emerging picture as shown in Figure 11. We observe no change in either the production of VLDL1 particles, or the clearance of apoB100 in VLDL1 or the triglyceride content in VLDL1. As for VLDL2, we did observe an impact on the clearance of apoB100 and TG in this fraction, which both increased by around 45 %. The IDL clearance increased by 54 % albeit non-significantly (P=0.15); the lack of some data points and thus statistical power may explain this lack of a clear identified effect on clearance of IDL. LDL clearance on the other hand was markedly increased (2.4-fold) explaining the large drop in LDL cholesterol.

Effects on apoB48 metabolism

For apoB48 the effects were less clear. We observed a tendency for the total apoB48, CM-apoB48, VLDL1-apoB48 and CM-TG to be reduced at 6 to 8 hours after the test meal (Supplementary Figure 2, manuscript). The kinetic modelling results however did not show a significant effect on the clearance rates of apoB48-containing lipoproteins. Production rates were also unaffected by evolocumab.

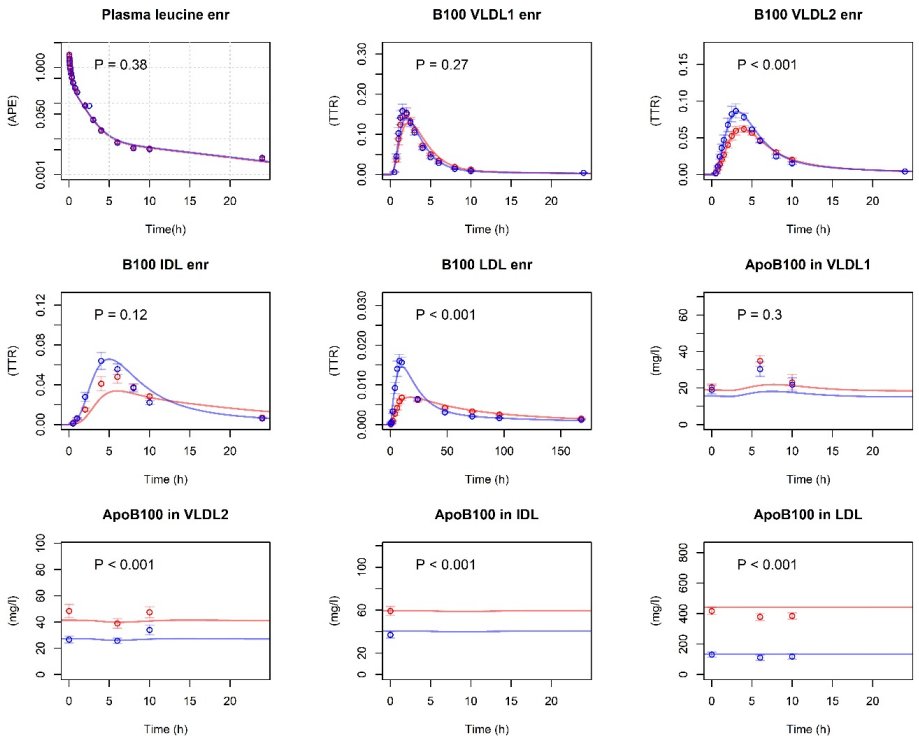

Figure 11: Model predictions (solid line) and experimental data (circles) of apoB100 enrichments and pool sizes before (red) and after (blue) 12 weeks of evolocumab treatment. VLDL1 kinetics appear to be unaffected by the intervention whereas VLDL2 clearance rate appear to have increased; resulting in a reduced VLDL2 pool size. The IDL pool is reduced and the enrichment curve appears to be affected (but this is hard to definitively determine due to non-complete data). LDL metabolism seems heavily affected where the pool size is greatly reduced and the enrichment curve indicate a change in the catabolism of the particles. P-values are calculated using a repeated measures ANOVA.

Metabolic heterogeneity in LDL

During the model development process, we noticed that a model with only a single LDL compartment was able to fit the experimental data before evolocumab treatment but was unable to fit the experimental data on treatment. The reason for this can be seen in Figure 12. When examining the LDL enrichment curve on a log-scale, it is evident that the curve on evolocumab display a bi-exponential appearance. In order to fit a bi-exponential curve the model needs two compartments with differing kinetics – one with a higher and one with a lower clearance rate. We named these two pools LDL-A and LDL-B respectively. Looking at Figure 12, two things are evident. First, the relative abundance of LDL-A to LDL-B has changed on treatment because the bi-exponential curve has emerged. The logical interpretation is that the pool of LDL-B has been reduced, in relation to LDL-A. Second, the clearance rate of LDL-B has not changed since the red line and the blue line are parallel at the later time-points. Indeed, when examining the results as predicted by the model we see a 4-fold drop in LDL-B pool size but only a 2-fold drop in LDL-A pool size (Figure 13). We see no change in LDL-B FCR but an increase in LDL-A FCR. We also see that the flux from IDL to LDL-B has decreased 4-fold.

These results indicate that the reduction in LDL cholesterol is mostly explained by a reduced IDL-to-LDL-B conversion but also by an increased clearance rate of LDL-A.

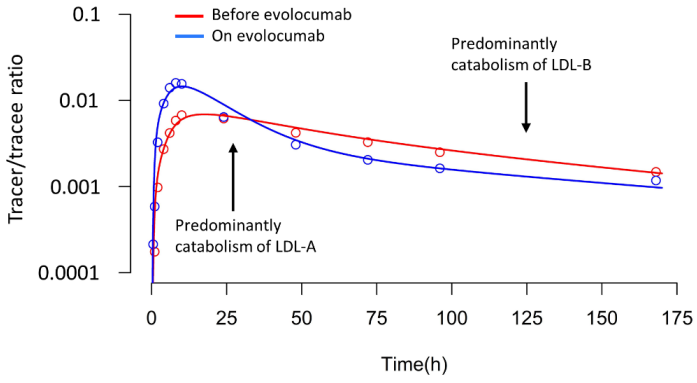

Figure 12: Metabolic heterogeneity in LDL. The LDL-enrichment curves before (red) and after (blue) 12 weeks of evolocumab treatment indicate the presence of two pools of LDL (two different slopes on the blue line) with differing kinetic properties. One pool (LDL-A) has a faster clearance and one pool (LDL-B) has a slower clearance. The pool size of LDL-B seems to be reduced on evolocumab without the kinetics of LDL-B itself being affected (one can see that the slopes on the latter part of the curve before and on treatment are similar).

ApoC-III and apoE kinetics

The kinetics of apoC-III and apoE were also examined in the current report. The plasma concentration of apoC-III was slightly reduced after treatment, but the pool size, production rate and clearance rate were not found to be significantly altered. ApoE on the other hand showed a 33 % reduction in concentration which was mostly explained by an increased clearance rate of apoE. This increased clearance rate was further shown to associate with the reduction in VLDL2 and IDL. This parallel clearance of these particles is in line with the notion that VLDL2/IDL-sized particles accumulate apoE and are removed by the LDL recptor; and that this pathway is upregulated on evolocumab.

Remnant cholesterol: TRL-C vs RLP-C

We used two different assays to measure “remnant” cholesterol: TRL-C and RLP-C. TRL-C is thought to measure apoB100+apoB48 remnants and RLP-C is thought to measure only apoB48 remnants. It was noted that TRL-C decreased on treatment but RLP-C did not. In addition, apoB48 in VLDL2 was not altered. These results may indicate a differential effect of PCSK9 inhibition on apoB48-containing remnant particles in comparison to apoB100-containing remnants.

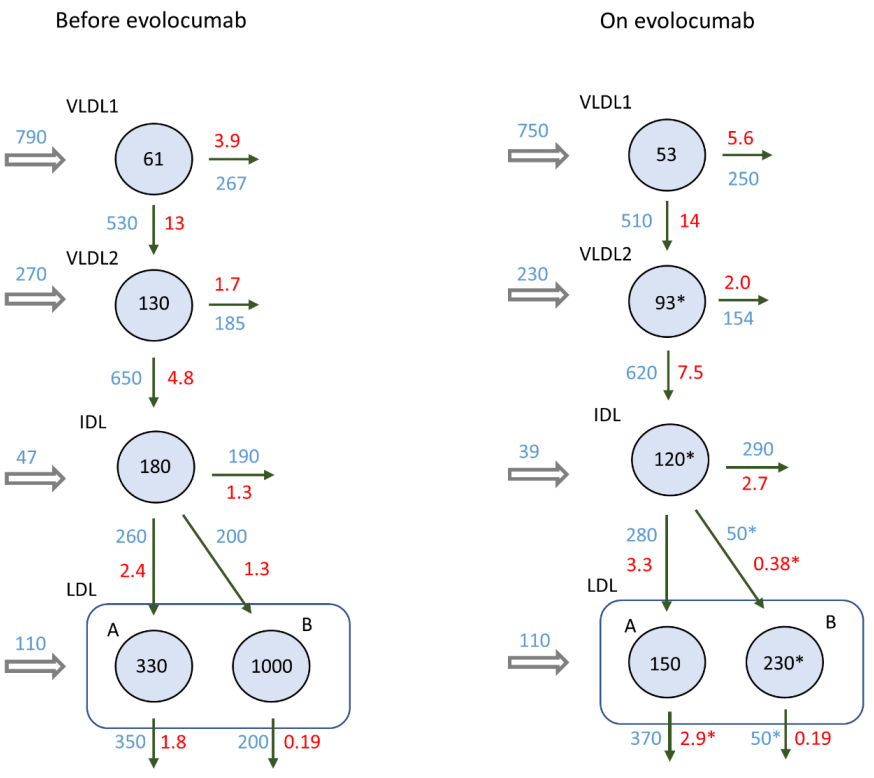

Figure 13: Overview of the fluxes and pool sizes of the different apoB100-containing lipoproteins before and on evolocumab. The kinetics of VLDL1 is unaffected whereas the clearance of VLDL2 has increased. IDL pool size is reduced likely as a result of increased direct catabolism of these particles. LDL-A fractional clearance is increased resulting in a reduced pool size. LDL-B is most heavily affected by the treatment as the pool size is reduced by a factor 4. The FCR is however unchanged; instead the production rate of LDL-B (conversion from IDL) is reduced, also by a factor 4.

Discussion

In this thesis, I developed a non-steady state kinetic model of the human triglyceride- and cholesterol transport system. The narrow purpose for developing the model was to describe human lipoprotein metabolism in the postprandial state. The broader purpose was to better understand the consequences of this system for the risk of cardiovascular disease. In the ensuing text, I first discuss the particular results in the current papers. After that, I broaden my perspectives and discuss things relating to the properties of the lipid transport system itself, and the atherogenic potential of the different lipoproteins.

Model development – paper I

Humans are in a postprandial state most of the day [50] and our triglyceride-rich lipoproteins are thus in a non-steady state much of our lives. In order to fully understand human lipoprotein metabolism, we believe we need to understand the postprandial state.

In paper I, we saw a need to improve previously developed kinetic models describing apoB48 [33 34 35 36 37 38 39 40]. There were two main criticism against these previous models: i) they utilised a primed constant infusion design and thus were forced to employ a micro-meal feeding pattern to simulate steady-state like conditions, ii) they represented apoB48 particles mathematically as one compartment in the model. I believe these limitations narrows their interpretability. If we instead want a more accurate understanding of the postprandial state, we realized that we needed to overcome these limitations by developing a new model. I built upon the previous work done in our group [32]. I adapted the steady-state apoB100 model to a non-steady state structure and expanded the model to include mathematical representations of apoB48. We studied our subjects in both fasting and postprandial conditions, and we observed that apoB48 during the fasting day behaved as if it had a slowly turning over pool; since it decreased throughout the day. In addition, thanks to the fasting day we could more easily disentangle basal apoB48 secretion from true postprandial secretion.

We wanted to describe apoB48 not only in the CM-fraction but also in VLDL1 and VLDL2. Here we had the opportunity to do so – thanks to our new proteomics-based analysis pipeline. During the model development process there are choices that needs to be made. One such choice is whether to constrain the model to only secrete CM-sized apoB48 particles or whether to also let the model opt to secrete VLDL-sized particles directly. We first tried the constrained approach but noticed that the model fitted the experimental data poorly. In particular, the rise of the enrichment curve in CM, VLDL1 and VLDL2 appeared to be simultaneous in the experimental data but was time-shifted due to the lipidation cascade in the constrained model. We therefore let the model secrete apoB48 directly into VLDL1 and VLDL2, which alleviated this discrepancy.

Previously, there have been various observations of apoB48 in plasma even after an overnight fast. Typically, the amount is in the order of 5 mg/l but with a large range [51]. If the turnover rate of chylomicrons truly can be measured in minutes one would expect the plasma concentration to be zero after an overnight fast. Here, the low clearance rate of apoB48 in VLDL1 and VLDL2 and the low-level basal secretion into these fractions explain these findings without invoking high levels of continuous fasting secretion of large chylomicrons.

The overall results are consistent with the idea of a “conveyer belt” chylomicron production; i.e. the intestine never shutting off apoB48 production completely but instead loading the

particles with triglycerides if present. If low levels of triglyceride are available the particle may be broken down intracellularly or secreted as a VLDL-sized particle.

These relatively long-lived apoB48 particles in the VLDL fractions may have atherogenic properties since they are in the right size-range to enter the arterial wall. Most of the particles would be classified as chylomicrons remnants and we observe a wave-like remnant area under the curve throughout the day depending on the postprandial state.

Model limitations

There are several limitations mostly relating to experimental setup and analytical approaches, but the most interesting criticism of the model relates to the structure. When developing the model, we used existing biological knowledge in conjunction with observations relating to how the potential model candidates fitted the experimental data. In science, there are often competing hypotheses that are consistent with a set of observations. Here too, we could have assumed several different model structures which may have also fitted the experimental data. To be specific, we could have assumed only one VLDL1/2 compartment for apoB48, or we could have assumed basal secretion into only the CM-fraction, or no postprandial secretion into VLDL1/2 etc. We realize these limitations but are fairly confident that our structure is more physiologically relevant than previously published models and enough so to to infer clinically relevant conclusions.

Paper II

In paper II, we further implemented the model on subjects with a high range of triglyceride levels. We found a clear pattern of accumulation of apoB48 in the VLDL1 and VLDL2 fractions as triglyceride levels increased. This accumulation was a result of slower clearance of the particles and this slower clearance correlated with increased apoC-III levels. Thus, we believe that apoB48 remnants behave much like apoB100 remnants in that they both accumulate when clearance is slowed due to higher apoC-III. The difference between apoB48 remnants and apoB100 remnants is that the latter is more affected by the postprandial state as we see a large difference between the peak postprandial level and the over-night fasting level. Hypertriglyceridemia is defined by a high fasting plasma triglyceride level but here we show that hypertriglyceridemia could be viewed as a representation of a specific metabolic phenotype. A phenotype characterized by wave-like alterations in intestinally derived remnants overlayered on top of an underlying liver-derived remnant pool.

It is tempting to speculate that this phenotype could be partly alleviated by reducing fat intake (and thus reduce production of intestinally derived particles) which could constitute a sizeable reduction in total remnant-particle burden. However, increased carbohydrate may increase apoC-III [52] and thus substituting fat for carbohydrates may not lead to improvements in this regard. A higher protein intake may be a viable option or fasting entirely for certain periods. But the best solution would be to increase the clearance rate of both apoB100 and apoB48 remnant particles. In 2015, the first antisense oligonucleotide study in humans was published (Gaudet et al., 2015). The primary outcome was of course plasma triglycerides but the authors also measured VLDL-cholesterol and apoB48, both of which decreased substantially. This indicates that reduction in apoC-III indeed causes a reduction in both liver-derived and intestinally-derived remnant lipoproteins.

Paper III

In paper III, we implemented the model on subjects receiving the drug liraglutide. We found increased chylomicron-triglyceride clearance rate but not increased apoB48 clearance rate in CM, together with an increase in the TG/apoB48 ratio. These results suggest that liraglutide may reduce the CM-apoB48 production rate but pack more triglyceride onto every particle that is secreted. In rat models where apoB48 protein translation is inhibited the formed chylomicrons are larger [54]. This could explain the increased CM-TG clearance rate since large triglyceride rich chylomicrons are likely a preferred substrate for LPL. Speculatively, this finding may help explain our previous finding of reduced remnant cholesterol during liraglutide treatment [47], although this may also be a result of lower apoC-III in that study. Our results are broadly in agreement with previous studies of GLP-1 receptor agonists which also found an apoB48 production-lowering effect [55] [56]. We could also show that the effect seems to be independent of an overall glycaemic-lowering effect due to improvements in insulin sensitivity.

The mechanism of this potential apoB48 production-specific effect of liraglutide is currently unknown. It is also unknown whether this translates into specific improvements in cardiovascular risk, independent from the general weight reduction induced by GLP-1 receptor agonists.

Paper IV

In paper IV we aimed to detail the metabolic effects of evolocumab, beyond the known LDL-C lowering effect. To our surprise we could not fit the simpler option of a one-compartment LDL model to the experimental data when the subjects were on treatment. It seemed that evolocumab treatment itself perturbed the metabolic system enough to reveal information about the system - information we wouldn't have been able to retrieve otherwise. We were forced to include one compartment with a higher clearance rate (LDL-A) and one with a lower clearance rate (LDL-B). This finding is itself interesting, and it raises several questions. Has this been discovered before? What are the properties of the particles in these two pools? Do they differ in atherogenicity? What does this say about normal LDL particle clearance?

In 1979, Shepherd et al used radiolabelled LDL to investigate the clearance rate of LDL [20]. The authors isolated LDL and treated it with two distinct radiolabels. One of the labelled particles were also treated with 1,2-cyclohexanedione which blocks LDL-receptor activity. By means of this setup they could quantify the relative contribution of receptor-mediated clearance versus non-receptor mediated clearance of LDL particles. They found a majority of LDL particles to be cleared by non-receptor mediated means. It has also been shown that urinary excretion of radioiodinated LDL is non-uniform [57]. The urinary excretion fell from around 0.5 pools/day at day 2 to 0.25 pools/day at day 12; indicating that LDL consists of (at least) two distinct pools with differing clearance rates.

In an upcoming study we extend paper IV and perform lipidomics analysis on the LDL fraction before and on treatment. We find that the triglyceride-to-cholesteryl ester ratio in the LDL fraction is increased on evolocumab. This result indicates that LDL-A is a relatively triglyceride-rich particle and that LDL-B is a relatively CE-rich particle. Importantly, the LDL diameter did not change in response to evolocumab, indicating that LDL-A and LDL-B does not map directly onto the definition of large vs small (dense) LDL. We will also investigate aggregability of LDL before and on evolocumab, which will enable inference on possible differential aggregability between LDL-A and LDL-B.

Regarding the classical definition of large LDL vs small dense LDL it has been previously shown that small dense LDL correlates with the phenotype also characterized by high triglycerides and low HDL; and that small dense LDL associates with CHD [58]. In a recent meta-analysis this association was also confirmed [59]. Whether there is any difference between our LDL-A and LDL-B particle in their relative atherogenicity can only be speculated upon at the present moment.

An additional finding in paper IV was that TRL-cholesterol (which should be a measure of the cholesterol content of all remnant particles) but not RLP-cholesterol (which should measure remnant cholesterol in apoB48 remnant particles) was reduced with evolocumab treatment. ApoE is a ligand for the LDL-receptor and apoB48-containing remnants contain apoE. Thus, in the presence of increased levels of available LDL-receptors these particles should in theory be cleared more rapidly - however this was not the case. If apoB100 remnants are cleared mainly by the LDL-receptor while apoB48 remnants are cleared mainly by another receptor, such as LRP1, this would fit the results found in the current report. If this is the case however, one would not expect to find higher apoB48 levels in people with mutations in the LDL receptor (familial hypercholesterolemia), but these subjects do indeed display higher apoB48 levels [60].

System properties of triglyceride- and cholesterol transport

In many biological systems, feedback is used to achieve homeostasis. Sometimes, but not always, negative feedback stabilizes a system, and sometimes positive feedback can do the same [61]. Indeed, there are several activators and inhibitors of the metabolic cascade involved in the lipid transport system. Most notably, apoC-III, ANGPTL3 and ANGPTL4 inhibit lipolysis whereas apoA-V and apoC-II enhance lipolysis. Likewise, PCSK9 inhibit LDL-receptor surface presence and thereby LDL particle uptake. The LDL-receptor gene expression is also under some regulation. It may seem strange that the body produces LPL in order to lipolyze particles and then, on the other hand, produces several inhibitors of LPL. This is likely best understood in the light of feedback-based homeostatic control.

The human triglyceride transport system has a fairly high dynamic range. A typical dietary fat intake is in the order of 100 grams per day, with a peak postprandial flux of roughly 5-25 grams of fat per hour. Comparing this with the more or less steady-state endogenous TG flux of 1-3 g/h we see that intestinal TG flux can be an order of magnitude higher than the hepatic TG flux. The pool of plasma TG in fasting conditions is typically 3-6 grams in the whole circulation. The peak postprandial pool is normally around a factor 2-3 higher than during fasting. The pool of plasma *glucose* in fasting conditions is typically 2-5 grams in the whole circulation. The fasting endogenous hepatic secretion of glucose is typically 5-8 g/h [62], whereas the peak postprandial flux can be in the order of 50 g/h. Again, the peak flux is an order of magnitude higher than the basal flux. And the peak postprandial pool is (similarly to TG) typically only a factor 2-3 higher than during fasting.

There are thus clear parallels of plasma glucose- and triglyceride pools and fluxes. And whenever there is i) a large range of possible fluxes, ii) high peak turnover and iii) narrow range of normal physiological concentrations; these properties indicate regulation of the system. In the case of glucose, we know this is a highly regulated system whereby insulin is released in response to a glucose load which activates the GLUT4 transporter and keeps the glucose concentration within a narrow range. In the case of regulation of plasma triglycerides, we haven't traditionally thought of this system as regulated in the same way. However, I

propose we should perhpa view it similarly to the glucose system and that apoC-III (and/or other inhibitors of LPL) could be the main regulator of plasma TG much like insulin is the main regulator of plasma glucose. Since apoC-III inhibits LPL activity in a manner non-linearly proportional to the apoC-III/TG ratio per particle [7], the presence of apoC-III in the circulation creates a lower level under which the concentration will not fall regardless of the influx. Above this lower level, the clearance capacity rapidly increases as concentration increases, creating a "pseudo negative feedback" loop which keeps plasma TG within a narrow range. My own modelling confirms these system properties (unpublished) and further predicts that hepatic TG production should explain roughly ¼ of the variation in plasma TG whereas clearance capacity should explain roughly ¾ , a prediction which is supported empirically by previous results from our group [63]. The modelling further predicts that external manipulation of plasma apoC-III (or any LPL inhibitor) should result in a linearly proportional change in plasma TG, which appears to be the case [53].

PCSK9 may fill a similar function in creating homeostatic regulation of LDL-cholesterol levels. The exact system properties of PCSK9 is however, to my knowledge, not completely understood. It may rather serve a cell/tissue specific regulatory role in that it may protect cells from internalizing too much cholesterol. The LDL-receptor clears a large portion of all circulating LDL particles but far from all. The non-receptor driven clearance of LDL particles; which could be constituted by non-specific pinocytosis distributed throughout the body, is a substantial part of the total clearance. Since this clearance is likely not under feedback control (i.e. it doesn't respond to plasma levels) it may be the case that LDL-cholesterol is more sensitive to changes in production than VLDL is. In this view, the VLDL secretion rate will result in a surprisingly low effect on the number of VLDL particles but may instead drive the accumulation of LDL.

Surely, there is much yet to discover about the regulatory properties of the lipid transport system. Future models of an even more detailed mechanistic nature could perhaps elucidate these underlying characteristics.

Are all apoB-containing lipoproteins equally atherogenic?

The main motivation behind the current thesis and the general research in the area of triglyceride-rich lipoproteins are their atherogenicity. But how atherogenic are they? In 2019, Ference et al addressed this question by performing a mendelian randomization study. The authors constructed two genetic scores, one for LPL and one for the LDL receptor. The LPL score was associated with lower plasma triglycerides and the LDL receptor score with lower LDL-C. When investigated per 10 mg/dl change in apoB the two scores showed an equally large association with reduced risk of CHD. In addition, when performing a multivariable mendelian randomization analysis, the TG and LDL-C associations with CHD became null after adjusting for apoB [64]. Similarly, in a recent multivariable mendelian randomization study, Richardson et al investigated the effect of TG, LDL-C and apoB on CHD. In a univariate analysis, all three measures were associated with the outcome, but in the multivariable analysis apoB retained the strongest effect. Although plasma TG was found to still be associated with CHD when adjusting for apoB, the effect size shrunk from an OR of 1.34 to 1.12 [65]. In another mendelian randomization study by Zuber et al, the authors had access to high-throughput NMR data from the Nightingale platform (118 variables) and used so called Baysean model averaging to select biomarkers most likely independently related to coronary artery disease (CAD). The authors found apoB as the primary determinant of CAD, however in a subset/sensitivity

analysis in UK Biobank, triglycerides in extra small VLDL was ranked as the most significant causal factor and apoB as the second most significant factor [66]. More recently, Si et al performed a two-sample mendelian randomization study investigating the causal role of remnant lipid-measures on CHD. They found apoB to be the strongest independent risk factor, and interestingly they found medium- and small sized VLDLs to have a causal effect on CHD but not large- and very large VLDLs [67]. Even more recently, Johansen et al investigated risk of myocardial infarction in relation to apoB particle number in 10 subfractions (from XXL VLDL down to to S LDL) in a subset of the Copenhagen General Population Study [68]. The authors found that the multivariable-adjusted hazard ratio on a particle basis was considerably higher for the VLDL fractions compared to that in IDL/LDL.

The above results combined reinforces the notion that remnants are causally related to CAD/CHD. The results from Ference and colleagues as well as the main analysis from Zuber et al suggest that apoB-containing lipoproteins in the VLDL fraction and in the LDL fraction are equally atherogenic. On the other hand; the results from Johansen et al and the sensitivity analysis from Zuber et al suggest that particles in the VLDL fraction may be more atherogenic per apoB. In earlier studies of APOC3 mutations and risk of CHD it was found that a roughly 40 % genetically lower plasma TG was associated with around 40 % lower risk of CHD. In these studies however, the APOC3 mutations were also associated with a 16 % lower level of apoB [27] and LDL-C [69], which would overestimate the effect size of the triglyceride-lowering. In fact, the LDL-C lowering in the paper by Crosby et al [26] was around 0.6 mmol/l. Given that a life-long 0.35 mmol/l LDL-C reduction should result in a 22 % relative risk reduction [1] the 0.6 mmol/l would translate to a roughly 40 % risk reduction.

Whether these earlier studies were confounded by LDL-C lowering the totality of the evidence points towards TRLs having a causal effect on CAD/CHD. But the question whether all apoB-containing lipoproteins are equally atherogenic remains to be definitively answered. In addition, it is to date not known whether apoB48-containing remnants are more or less atherogenic than apoB100-containing remnants. It has been shown that chylomicron remnants contain more cholesterol per particle [70] and since apoB48-containing remnants could constitute around a quarter to a third of the number of particles in the VLDL fraction, as shown in the current thesis, the cholesterol content in apoB48-containing remnants could be up to half of the total cholesterol in the VLDL fraction. Indeed, we have shown that RLP-C area under the curve in a postprandial setting is around half that of TRL-C [47]. The relative atherogenicity of apoB48 remnants in relation to apoB100 remnants will be difficult to untangle, but warrants further studies.

Common causes of increased lipid levels

One obvious question a lipoprotein-naïve person may ask is: What causes increased levels of lipids in the blood? Below, I briefly summarize what is known on this topic.

Explanatory factors of LDL-C

Twin studies are used to investigate how much of the variation in a trait can be accounted for by genetic and environmental factors. In the case of LDL cholesterol this question has been investigated in several studies [71] [72]. In general, around 50-65 % of the variance in LDL-C can be accounted for by genetic factors. Currently around a few percent of the variation in LDL-C can be explained by a polygenic risk score [73] [74]. This research shows that most of the genetically-derived factors that affect LDL-cholesterol levels are of unknown genetic origin.

Environmental effects that increase LDL-cholesterol levels mostly are diet-related. In a 1997 meta-analysis of 395 metabolic ward studies, Clarke et al investigated the relationship between (isocaloric) macronutrient intake, dietary cholesterol intake and LDL-C levels [75]. They found that isocaloric substitution of 10% of total energy intake of saturated fatty acids for complex carbohydrates results in a 0.36 mmol/l lower LDL-C level. They further found that replacing 5 % of energy intake from complex carbohydrates with poly unsaturated fatty acids results in a 0.11 mmol/l lower LDL-C. Lastly, a 200 mg/day lower intake of dietary cholesterol results in a 0.1 mmol/l lower LDL-C. Currently in Europe the dietary intake of saturated fat ranges between 9 % and 16 % of daily calories [76]. This means that, on a population level, reducing saturated fat intake of 5 percentage points (and replacing with complex carbohydrate, which is realistic in several countries currently) would reduce the population level of LDL-C by around 0.2 mmol/l. On a population level this would have a noticeable effect on CAD/CHD risk of around 10 % or so assuming life-long exposure.

Weight loss have also been shown to decrease LDL-cholesterol. Dattilo and colleagues showed in a meta-analysis that a 1kg decreased body weight associated with a 0.02 mmol/l lower LDL-cholesterol [77]. In the context of normal weight reduction this effect size can be considered modest. Even bariatric surgery, which leads to much greater weight reductions have shown to reduce LDL-C by around 0.5-0.6 mmol/l in average [78].

In summary, most of the variation between people in the general population cannot be attributed to identifiable environmental effects, such as life-style and diet. Instead, a majority of the variation can be explained by unknown genetic factors. Given that the population level of non-HDL cholesterol is commonly around 3-4 mmol/l in many countries to date [79] any public health strategy aiming to offer help to members of the general population wishing to optimize their LDL-C/apoB levels will, realistically, have to involve pharmaceutical interventions.

Explanatory factors of plasma triglycerides

The heritability for plasma triglycerides has been estimated to vary with age but hover around 50 % [80]. Recently, a polygenic hyperlipidemic score explained around 5 % of the variation in plasma TG in the population [81]. Thus, like for LDL-C much of the variation in plasma TG can be explained by unknown genetic influences. As for environmental contributions to triglycerides, diet is one such factor. In the same meta-analysis referenced above a 1 kg weight loss was associated with a 0.015 mmol/l lower plasma TG [77], which can be considered a modest effect size. Mechanistically, apoC-III has been shown to causally affect plasma TG [42]. In our cohorts we have noticed that apoC-III typically explains a majority of the variation in plasma TG, which indicates that it has the possibility of being a major contributor to explaining the variation in triglycerides in the general population. We have shown that a low carbohydrate diet is associated with a lower plasma apoC-III and a correspondingly lower plasma TG [82]. This finding is in line with a meta-analysis investigating the effect of a longer-term low-carbohydrate diet on plasma lipids; showing a lower plasma TG, compared to a low-fat diet [83]. Indeed, glycemia has been linked to the expression of apoC-III, in that glucose may stimulate apoC-III gene expression through upregulating hepatic nuclear receptor 4 (HNF4) and carbohydrate-responsive element binding protein (ChREBP) [84]. We have also shown that the (hepatic) secretion rate of apoC-III correlates with plasma apoC-III levels [10]. Taken together, this research indicates that the environmental factors that affect hepatic apoC-III expression may explain a non-trivial part of the variation in plasma TG in the general population. To my

knowledge however, there have been no investigations to confirm or reject this association in a large random selection of the population.

Population prevention strategies

The current treatment paradigm for prevention of ASCVD is based partly on various risk scores, such as SCORE [85] or Framingham risk score [86]. In this view, treatment with lipid-lowering therapy is offered when the predicted risk is above a certain threshold. Is this the optimal approach to reduce the burden of ASCVD in the general population? The risk scores are designed to predict (10-year) risk of ASCVD but this prediction does not necessarily correlate with benfit of treatment. If one examines the variable importance of the risk scores, age and sex dominates the prediction. In practice this means the basis of treatment will, on a population level, be initiated at a relatively old age when the disease has progressed to a later stage.

When examining the development of atherosclerotic vascular disease in an individual, the initiation phase begins decades before any development of symptoms. In patients with familial hypercholesterolemia the process is accelerated, and in subjects which are heterozygote for a PCSK9 loss-of-function mutation the process is markedly slowed [87]. Regression of this process may be possible, and is likely a function of the stage of the atherosclerotic disease and the degree of lipoprotein lowering. It thus follows that early intervention; particularly in individuals who otherwise would have experienced an accelerated atherosclerotic process, will yield more benefit than later intervention. In 2018, Robinson and colleagues suggested this as a new paradigm for ASCVD prevention [88]. They further suggested this hypothesis to be tested in a rigorous trial (CURE ATHERO), as specified in the paper. In this paradigm, treatment may begin when people are in their 30's or 40's. The initial treatment would consist of aggressive lipid lowering to induce regression, followed by long-term maintenance therapy to halt progression.

Usually, when a disease afflicts a large portion of the population and is preventable, a default approach is to prevent the disease as early as possible. Public health autorities currently deploy this strategy to many infectious diseases, by means of vaccines. This approach has been very effective in reducing several serious diseases during the last century [89]. By the same logic, there are currently a similar approach under development using CRISPR. The first long-term demonstration of liver-specific PCSK9 knock-out in primates was recently published [90]. The gene editing lipid nanoparticle delivery system was administered using an intravenous infusion, and the treatment resulted in a large and immediate LDL-C reduction which was sustained for a three-year period. This stratgey comes closer to the analogy to a vaccine against ASCVD since the treatment is a one-time occurrence but the effect is life-long. An alternative, and perhaps more realistic, strategy has emerged with the drug inclisiran. Inclisiran is a silencing-RNA molecule that also targets PCSK9. In contrast to PCSK9 monoclonal antibodies it can be administered twice per year and in contrast to the CRISPR-based strategy, it is reversible. The effect size is large and it has a favourable safety profile [91]. Whether any of these approaches becomes implemented on large portions of the populations remains to be seen.

A new tool in assessig risk is to incorporate a polygenic risk score (PRS). In a recent pre-print, the authors argue that risk of CAD is a function of the interaction of LDL-C and a newly developed PRS [92]. They show, for example, that some individuals with borderline LDL-C (3.4-4.1 mmol/l) but high PRS still have a high risk of CAD. Conversely, people with high PRS but

low LDL-C was not at increased risk. Whether or not PRSs will be incorporated into traditional risk scores remains to be seen but since a PRS can be measured at any time in a person's life it enables early identification of high-risk individuals.

Perspectives

The grand objective with the totality of the research on atherosclerotic cardiovascular disease must surely be the eradication of the disease itself. Complete understanding of the lipoprotein transport system would enable more audcious modelling schemes. One such strategy would be a deeply mechanistic model, whereby all steps involved in the process; from the emulsification of the dietary fats in the stomach and the translation of the mRNA coding for apoB100 in the liver to all the enzymatic/receptor and non-receptor mediated fluxes and reactions occuring in the circulatory system incorporating all enhancers and inhibitors and feedback-regulatory mechanisms. Even the entrapment in the arterior wall would be modeled and the downstream consequences from that. Such a deeply mechanistic model would enable hypothesis-testing schemes such as knocking out a protein to see how the system responds or manipulating enzymatic reactions via hypothetical drugs. In this world we would of course also have perfect information on the relative atherogenic potential of each individual lipoprotein. In addition, by knowing the mapping of the genome to the lipoprotein metabolic system we could; by having acces to the full genomic profile of a particular person; predict the behaviour of that person's lipoprotein system. This persepctive is far away but steps toward this vision may be closer at hand.

If I were to speculate, the most common causes of death of ancestral humans (infections and violence [93]) would not put enough evolutionary pressure on reducing risk of atherosclerosis in order for the current population to enjoy low risk; especially given our current environment. The naturalistic fallacy dictates that what is natural is not always healthy - indeed many unnatural things has greatly improved human health. To fully prevent ASCVD today, we likely cannot rely on nature to run its course but we will instead require interventions. In a not-so-distant future, we may have several options to prevent ASCVD, such as using CRISPR to knock out for example PCSK9, APOC3 or ANGPTL3, or consuming lipid-lowering agents from a younger age with or without periods of more aggressive treatment with the use of e.g. PCSK9 inhibitors or silencing-RNA type drugs. The former strategy is life-long whereas the latter requires continuous intervention but can also be titrated or stopped depending on other factors. Technologies such as RNA-based theraputics were considered unrealistic not long ago, but occasionally brekathroughs occur. It remains to be seen if and how our lives are affected by these and other upcoming opportunities in the field of cardiovascular disease research.

Conclusions

It is well established that LDL causes ASCVD [1]. In recent years, TRLs and their remnants have emerged as additional causative agents which may play a central role in residual cardiovascular risk. The study of these lipoproteins is of importance for providing fundamental knowledge in this area. Here, I present studies of the metabolism of TRLs and LDLs with the help of a newly developed multicompartmental, kinetic model. In the current thesis, I show how the model can be applied to gain insight into the postprandial metabolism of these lipoproteins in hypertriglyceridemia and during administration of the two drugs liraglutide and evolocumab. This thesis demonstrates the value of a systematic modelling-approach for the study of lipoprotein metabolism.

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