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Apolipoprotein B48 metabolism in chylomicrons and very low-density lipoproteins and its role in triglyceride transport in normo- and hypertriglyceridemic human subjects

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Abstract. Björnson E, Packard CJ, Adiels M, Andersson L, Matikainen N, Söderlund S, Kahri J, Hakkarainen A, Lundbom N, Lundbom J, Sihlbom C, Thorsell A, Zhou H, Taskinen M-R, Borén J (University of Gothenburg, Gothenburg, Sweden; University of Glasgow, Glasgow, UK; University of Helsinki; Helsinki University Hospital; University of Helsinki, Helsinki, Finland; Aalto University School of Science, Espoo, Finland; University of Gothenburg, Gothenburg, Sweden; Merck & Co. Inc., Kenilworth, NJ, USA; Sahlgrenska University Hospital, Gothenburg, Sweden). Apolipoprotein B48 metabolism in chylomicrons and very low-density lipoproteins and its role in triglyceride transport in normo- and hypertriglyceridemic human subjects. *J Intern Med* 2020; **288**: 422–438.

Background. Renewed interest in triglyceride-rich lipoproteins as causative agents in cardiovascular disease mandates further exploration of the integrated metabolism of chylomicrons and very low-density lipoproteins (VLDL).

Methods. Novel tracer techniques and an integrated multi-compartmental model were used to determine the kinetics of apoB48- and apoB100-containing particles in the chylomicron and VLDL density intervals in 15 subjects with a wide range of plasma triglyceride levels.

Results. Following a fat-rich meal, apoB48 appeared in the chylomicron, $VLDL_1$ and $VLDL_2$ fractions in all subjects. Chylomicrons cleared rapidly from the circulation but apoB48-containing VLDL accumulated, and over the day were 3-fold higher in those with high versus low plasma triglyceride. ApoB48-containing particles were secreted directly into both the chylomicron and VLDL fractions at rates that were similar across the plasma triglyceride range studied. During fat absorption, whilst most triglyceride entered the circulation in chylomicrons, the majority of apoB48 particles were secreted into the VLDL density range.

Conclusion. The intestine secretes apoB48-containing particles not only as chylomicrons but also directly into the $VLDL_1$ and $VLDL_2$ density ranges both in the basal state and during dietary lipid absorption. Over the day, apoB48-containing particles appear to comprise about 20–25% of circulating VLDL and, especially in those with elevated triglycerides, form part of a slowly cleared 'remnant' particle population, thereby potentially increasing CHD risk. These findings provide a metabolic understanding of the potential consequences for increased CHD risk when slowed lipolysis leads to the accumulation of remnants, especially in individuals with hypertriglyceridemia.

Keywords: apoB48, apoB100, hypertriglyceridemia, kinetics, postpranidal lipid metabolism, stable isotopes.

Introduction

Dysregulation of chylomicron-mediated lipid absorption has been purported to lead to excess formation of remnant lipoproteins that are believed

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to contribute significantly to the process of atherogenesis [1–4]. Investigating this pathway, however, has proved problematic due to its dynamic nature—chylomicrons appear as a wave following fat meals—and the need to distinguish it from very low-density lipoprotein (VLDL) mediated triglyceride transport that originates in the liver. Retinyl palmitate and apoB48 (a protein found solely in particles of intestinal origin: apoB100 in VLDL is exclusively liver-derived) have been used as tools to follow chylomicron metabolism [5–7]. However, the low abundance of apoB48 and its transient behaviour have presented challenges in the conduct and interpretation of kinetic studies. Some investigations have addressed these issues by employing quasi-steady-state designs with participants fed micro-meals across the day [8–10], others by the use of simplified kinetic analysis [11]; neither of these approaches replicates the physiological situation of the overlay of a complex, nonsteady-state process—postprandial lipid absorption—on the relatively constant VLDL pathway.

Since humans are postprandial most of the day, the continuous generation of remnants after each meal may be an important causal risk factor for the development of atherosclerosis. Recent attention has therefore focused on modulation of postprandial lipemia by lifestyle changes and pharmacological interventions. Statins increase clearance of both LDL particles and of CM and VLDL remnants [12–14]. Monoclonal antibodies to proprotein convertase subtilisin/kexin type 9 (PCSK9) significantly lower LDL and fasting triglyceride-rich lipoprotein (TRL) levels [15–18], but their effect on postprandial lipid metabolism is not fully clarified. Furthermore, angiopoietin-like protein 3 (ANGPTL3) and apoC-III have emerged as important metabolic regulators of the metabolism of triglyceride-rich lipoproteins (TRLs) and novel candidate targets for intervention to correct the dyslipidaemia and ameliorate CVD risk [4]. In addition, the activity of cholesteryl ester (CE) transfer protein (CETP) is also important in the generation of remnants since long-lived particles enable more transfer of CE to take place [19].

Advances in mass spectrometric peptide determination have permitted the development of more sensitive methods for kinetic studies [10, 20, 21] and use of these techniques in a pilot investigation led to the development of a new, integrated model describing the nonsteady-state kinetics of chylomicrons, $VLDL_1$ and $VLDL_2$ [collectively referred to as triglyceride-rich lipoproteins (TRL)] during fat absorption [21]. The aim of the present study, which follows from this work, is to reveal the quantitative contribution that intestinal-derived lipoproteins make to 'VLDL' metabolism, their impact on the behaviour of liver-derived VLDL, and the potential for remnant formation from both tissue sources. Relationships are explored between the kinetics of apoB48 and apoB100 within TRL, and the association of apoB48 and apoB100 metabolism with potential regulatory factors such as apoC-III that appears to influence the clearance of chylomicrons and VLDL [22–25], and is being actively explored as a possible therapeutic target [24, 25].

These investigations are pertinent and timely since, with the publication of the positive results of REDUCE-IT [26], and the ongoing PROMINENT trial [27]—both of which recruited hypertriglyceridemic subjects—there is renewed interest in deciphering the basis of CVD risk in such individuals, and how it might be mitigated. An important component of this risk predictably will derive from aberrant metabolism of apoB48-containing lipoproteins.

Methods

Subjects

Fifteen healthy men with a wide range of plasma triglycerides were studied. The group was divided into thirds, that is five with the lowest plasma triglyceride (termed 'Low-TG'; 0.69–1.07 mmol L^{-1}), five with average triglyceride ('Average-TG'; 1.21–1.71 mmol L^{-1}) and five with the highest triglyceride levels ('High-TG'; 2.09–5.66 mmol L^{-1}). All subjects underwent an investigation of apoB48 and apoB100 kinetics in which a fat-rich meal was given 2 h into the turnover. Nine of the subjects (as noted in Table S2) were studied also in a prolonged fasting state in which the same metabolic protocol was followed but no meal was given at the 2-h time-point. This second turnover allowed in the same men a comparison of VLDL apoB100 and VLDL triglyceride kinetics in fed versus fasted conditions. Protocols were approved by the Helsinki University Ethics Committee, and the volunteers gave written informed consent. No subjects with type 2 diabetes or the apoE phenotype apoE2/2 were included in the study.

Metabolic protocol

The metabolic protocol is described in detail in a previous publication [21]. Subjects were admitted

to the metabolic ward of the Helsinki University Central Hospital after a 12 h overnight fast and at 8.00 am (0-h time-point), deuterated leucine (5,5,5-D3 Euriso-Top, d3-leucine) at a dose of 7 mg kg^{-1} body weight, and 500 mg of deuterated glycerol (1,1,2,3,3-D5 Euriso-Top, d5-glycerol) were given as a bolus. At the 2-h time-point subjects received a fat-rich mixed meal consisting of a standard breakfast (bread, cheese, ham, boiled eggs, fresh red pepper, low-fat (1%) milk, orange juice and tea or coffee) served with a cocoa/fat emulsion containing 40 g olive oil (Amway, Firenze, Italy). This meal provided 63 g carbohydrate, 69 g fat and 40 g protein and was consumed within 10 min. Blood sampling continued at frequent intervals until 10 h after tracer administration [21], at which time-point subjects were served a standard dinner. Subjects returned the following morning when the last blood sample was drawn at 24 h posttracer administration.

Lipoprotein isolation

Chylomicrons (Sf > 400), $VLDL_1$ (Sf 60-400) and $VLDL_2$ (Sf20-60) were isolated by preparative centrifugation as previously described [28]. Plasma samples were overlayered with a gradient of salt solutions of decreasing density and centrifugation performed in three steps. Lipoprotein fractions were harvested from the top of the centrifuge tube and stored at −80 °C prior to processing.

Quantification of apoB48 and tracer enrichment

As described in detail earlier [21], commercially prepared stable-isotope labelled apoB48 peptides were used as internal standards for quantification of apoB48. Tryptic digestion of plasma, $VLDL_1$, $VLDL_2$ and chylomicron fractions was performed as described [21] and apoB48 was then enriched by immunoadsorption prior to measurement of its concentration and the determination of tracer abundance of d3-leucine.

Determination of d3-leucine enrichment in apoB100 and d5-glycerol enrichment in triglycerides

ApoB100 isolated from $VLDL_1$ and $VLDL_2$ was hydrolyzed, derivatized and subjected to gas chromatography–mass spectrometry to measure tracer leucine enrichment [28]. Triglycerides were isolated from the two lipoprotein fractions and tracer glycerol enrichment determined as previously described [28].

Multi-compartmental modelling and parameter estimation

The multi-compartmental model (Figure S1) was developed using SAAM2 [28, 29]. The comprehensive data set used to derive rate constants is provided in the annotation to Figure S1 (see also Ref [21]). When subjects were studied in a prolonged fasting state, the same model was used but the meal-associated inputs were set to zero. Production rates for apoB48 in chylomicrons, $VLDL_1$ and $VLDL_2$ were derived for the basal situation and the increment following the fat meal; the latter was time-averaged over 24 h. Production rates were derived also for apoB100 in $VLDL_1$ and $VLDL_2$ in line with the steady-state approach used in previous models [28]. Individual fractional catabolic rates (FCRs) were determined for apoB48 in chylomicrons, $VLDL_1$ and $VLDL_2$ and for apoB100 in $VLDL_1$ and $VLDL_2$. A total FCR was calculated to describe overall apoB48 clearance from TRLs (i.e. chylomicrons plus $VLDL_1$ plus $VLDL_2$) and for apoB100 clearance from $VLDL_1$ plus $VLDL_2$.

When chylomicrons appear in the bloodstream there is competition with VLDL, especially the larger more triglyceride-rich $VLDL_1$ for available lipoprotein lipase [6, 30, 31]. Chylomicrons are the preferred substrate for the enzyme [30, 31] and so there is a transitory inhibition of VLDL lipolysis. This was modelled in Figure S1 by allowing the $VLDL_1$ fractional transfer rate (comprising the rate constant between compartments 1 and 2, and the rate constants exiting compartments 2 and 3 that are linked to the analogous constants in the VLDL-TG sub-model) to decrease as a dynamic function of the rate of chylomicron triglyceride hydrolysis (see Figure S2).

Determination of intra-abdominal fat depots

Proton magnetic resonance spectroscopy was performed using a 1.5-T whole-body device to determine liver fat content [32, 33], as well as subcutaneous abdominal and intra-abdominal fat [34]. All analyses of the imaging results were performed by one person (AH). Subjects were advised to fast for 4 h before imaging.

Biochemical analyses

Fasting plasma glucose, triglycerides, HDL-cholesterol, LDL-cholesterol and plasma liver enzymes were determined by automated enzymatic methods using the Konelab 60i analyser (Thermo Fisher Scientific,

Waltham, MA, USA). Plasma levels of apoC-III were measured immunoturbidometrically (Kamiya Biomedical Company, Seattle, WA, USA). Fasting and postprandial apolipoprotein (apo) B48 and apoB100 levels in total plasma were measured by ELISA (Shibayagi, Shibukawa, Japan). Plasma non-esterified fatty acids (NEFA) were analysed with an automated enzymatic colorimetric method (Wako Chemicals, Neuss, Germany). Postheparin LPL and HL activities were measured as described [35].

Statistical analysis

Correlation coefficients were assessed using R version 3.5.2 and GRAPHPAD PRISM 7 (GraphPad Software, San Diego, CA, USA). These were calculated using the Pearson method; nonnormally distributed variables were log-transformed before analysis. If transformation did not result in a normal distribution then a Spearman Rank test was used to evaluate associations. A paired *t*-test, with subsequent adjustment for multiple comparisons, was used to assess differences in kinetic parameters between the fed versus fasted state. Interpolations of observed data in chylomicrons, $VLDL_1$ and $VLDL_2$ fractions were performed using the 'pchip' (Piecewise Cubic Hermitean Interpolation Polynomials) function in R (package *pracma*) to allow for small variations in sampling times between subjects.

Results

Metabolic characteristics and lipid and lipoprotein levels of the 15 subjects are given in Table 1; for ease of presentation the group was divided into thirds according to plasma triglyceride level. Mean plasma triglyceride was 0.89 mmol L^{-1} in the third with the lowest values (termed 'Low-TG'), 1.43 mmol L^{-1} in the middle third ('Average-TG') and 3.61 mmol L^{-1} (with a range 2.09–5.66 mmol L^{-1}) in the 'High-TG' third. Total plasma apoB48, apoB100 in VLDL and plasma apoC-III all increased significantly as plasma triglyceride concentration rose (all $P < 0.001$). There was no apparent association of plasma triglyceride with lipoprotein or hepatic lipase activity. Plasma insulin ($P = 0.0011$) but not insulin resistance (Matsuda index, HOMA-IR2) was related positively to plasma triglyceride whilst beta-hydroxybutyrate showed an inverse association ($P = 0.023$).

Changes in apoB48 and apoB100 concentration after a fat-rich meal

Figure 1 panels a–f present the mean change in apoB48 concentration in the chylomicron, $VLDL_1$ and $VLDL_2$ fractions in the Low-TG, Average-TG and High-TG subjects. Baseline (fasting) plasma apoB48 ranged from 1.1 to 40.3 mg L^{-1} (median 8.5, interquartile range 3.7–14.6 mg L^{-1}) (data not shown) and was a substantial proportion of the total apoB present in the VLDL density range in High-TG subjects (Table 1). ApoB48 rose in response to the fat-rich meal, virtually simultaneously in the three TRL fractions and, after reaching a peak 2–6 h postingestion, levels fell, most rapidly in the chylomicron fraction and less quickly in $VLDL_1$ and $VLDL_2$ (Fig. 1 panels d–f). ApoB48 in chylomicrons returned to near zero at the 24-h time-point in all subjects (Fig. 1 panels a–c). In contrast, substantial levels of apoB48 in $VLDL_1$ and $VLDL_2$ were still present at 24 h in the High-TG subjects, and to a lesser extent in the Average-TG subjects (Fig. 1 panels d–f). Plasma apoB48 levels at 24 h were similar to those at time zero (panels d–f). These data indicate that whilst chylomicrons in the circulation were mostly cleared within a 12–14-h timeframe—the normal period of fasting prior to lipid analysis—the same is not true of intestinally derived lipoproteins present in the VLDL density range. Throughout the experimental day, these apoB48-containing lipoproteins were present in both $VLDL_1$ and $VLDL_2$ at increasingly higher concentration as plasma triglyceride rose and were added to during absorption of the fat meal. The area-under-curve (AUC) for total apoB48 in High-TG subjects was increased 1.7-fold compared to the Average-TG subjects, and 4.2-fold compared to Low-TG subjects, and most of this increment was in the VLDL fractions (Fig. 1 panels d–f).

During model development, it was necessary to account for the presence of apoB48 in the circulation in the baseline fasting state, and two constructs were used to accommodate this feature [21]. First, basal production of apoB48 from the intestine was permitted (i.e. continuous assembly and secretion of apoB48-containing particles; values are given in Table 2). Second, a 'previous day' (depicted in the shaded regions in Fig. 1) was simulated using the apoB48 kinetic rates in chylomicrons, $VLDL_1$ and $VLDL_2$ derived from the experimental day (shown in Table 2) with notional meal intakes at breakfast, lunch, dinner and evening snack (see reference [21] for more details). This simulation generated the appropriate amount of slowly metabolized apoB48-containing particles required to contribute to the fasting level of apoB48 and was especially important in explaining the

Table 1. *Subject group characteristics and lipid levels*

	Low-TG[a] (n = 5) Mean ± SD	Average-TG (n = 5) Mean ± SD	High-TG (n = 5) Mean ± SD	P[b] for association with TG across all subjects
Age (years)	56.0 ± 6.5	57.8 ± 6.5	52.8 ± 8.1	0.68
Weight (kg)	85.3 ± 9.0	93.4 ± 5.7	98.0 ± 9.3	**0.0093**
BMI (kg m^{-2})	27.0 ± 3.5	28.4 ± 2.2	31.6 ± 2.4	**0.022**
Waist (cm)	97.4 ± 5.7	107 ± 8.5	108 ± 10	0.14
Syst. BP (mmHg)	131 ± 18	139 ± 17	137 ± 19	0.13
Diast. BP (mmHg)	82 ± 6	85 ± 4	94 ± 11	**<0.001**
Lipids and lipoproteins				
Triglycerides (mmol L^{-1})	0.89 ± 0.16	1.43 ± 0.18	3.61 ± 1.5	NA
Plasma apoC-III (mg dL^{-1})	9.1 ± 2.8	11.3 ± 2.9	18.9 ± 4	**<0.001**
Plasma apoB (mg L^{-1})	763 ± 110	1150 ± NA	1290 ± 220	0.088
Plasma apoB48 (mg L^{-1})	3.1 ± 1.4	9.6 ± 3.8	21.3 ± 13	**<0.001**
ApoB48/apoB ratio	0.04 ± 0.02	0.04 ± NA	0.17 ± 0.11	0.16
ApoB100 in VLDL (mg L^{-1})	48 ± 12	87 ± 16	165 ± 58	**<0.001**
LDL-C (mmol L^{-1})	2.8 ± 0.5	4.0 ± 0.3	3.9 ± 0.8	0.18
HDL-C (mmol L^{-1})	1.8 ± 0.5	1.2 ± 0.3	0.9 ± 0.2	**0.012**
ApoA-I (mg dL^{-1})	161 ± 25	142 ± 19	139 ± 14	0.090
NEFA (μmol L^{-1})	556 ± 120	523 ± 40	384 ± 83	**0.030**
Metabolic indices				
Plasma glucose (mmol L^{-1})	5.6 ± 0.1	5.4 ± 0.3	5.6 ± 0.6	0.13
Insulin (μU mL^{-1})	7.5 ± 6.0	10.3 ± 4.9	18.0 ± 13	**0.0011**
Matsuda index	14.6 ± 11	3.8 ± 1.9	5.8 ± 4.2	0.31
HOMA2-IR	1.0 ± 0.8	1.4 ± 0.6	2.4 ± 1.7	**0.0011**
CRP (mg L^{-1})	1.5 ± 1.8	2.2 ± 1.1	1.1 ± 0.6	0.37
β-OHB (mg dL^{-1})	1.1 ± 0.2	1.2 ± 0.7	0.3 ± 0.1	**0.023**
ALT (U L^{-1})	24 ± 9	31 ± 8	27 ± 12	0.26
LPL activity (mU mL^{-1})	170 ± 28	155 ± 43	165 ± 40	0.48
HL activity (mU mL^{-1})	252 ± 120	259 ± 110	278 ± 87	0.66
Liver fat (%)	6.6 ± 6.4	4.7 ± 2.7	9.1 ± 7	0.21
VAT (cm^3)	2220 ± 1100	2630 ± 420	3060 ± 1400	0.70
SAT (cm^3)	3390 ± 500	3670 ± 1100	4240 ± 1200	0.055

ALT, Alanine transaminase; BMI, body mass index; CRP, C-reactive protein; Diast. BP, diastolic blood pressure; HL, hepatic lipase; LPL, lipoprotein lipase; NEFA, nonesterified fatty acids; SAT, subcutaneous adipose tissue; Syst. BP, systolic blood pressure; VAT, visceral adipose tissue; β-OHB, β-hydroxybutyrate.
Bold indicates $P < 0.05$.
[a]The group of 15 subjects was divided into thirds on the basis of plasma triglyceride
[b]P-value for Pearson correlation with fasting TG across subjects in all groups (n = 15).

findings in the High-TG subjects. (The alternative of attributing all fasting apoB48 to basal production was discounted for reasons explained in reference [21]).

Change in apoB100 in $VLDL_1$ and $VLDL_2$ during the experimental day is shown in Fig. 1 panels g–i. In all subjects, there was a rise in $VLDL_1$ apoB100 that coincided with the appearance of apoB48 particles in the bloodstream, but little change in $VLDL_2$ apoB100. The increment in VLDL apoB100 (AUC) was around 3-fold greater in the High-TG compared to Low-TG subjects (panels g–i).

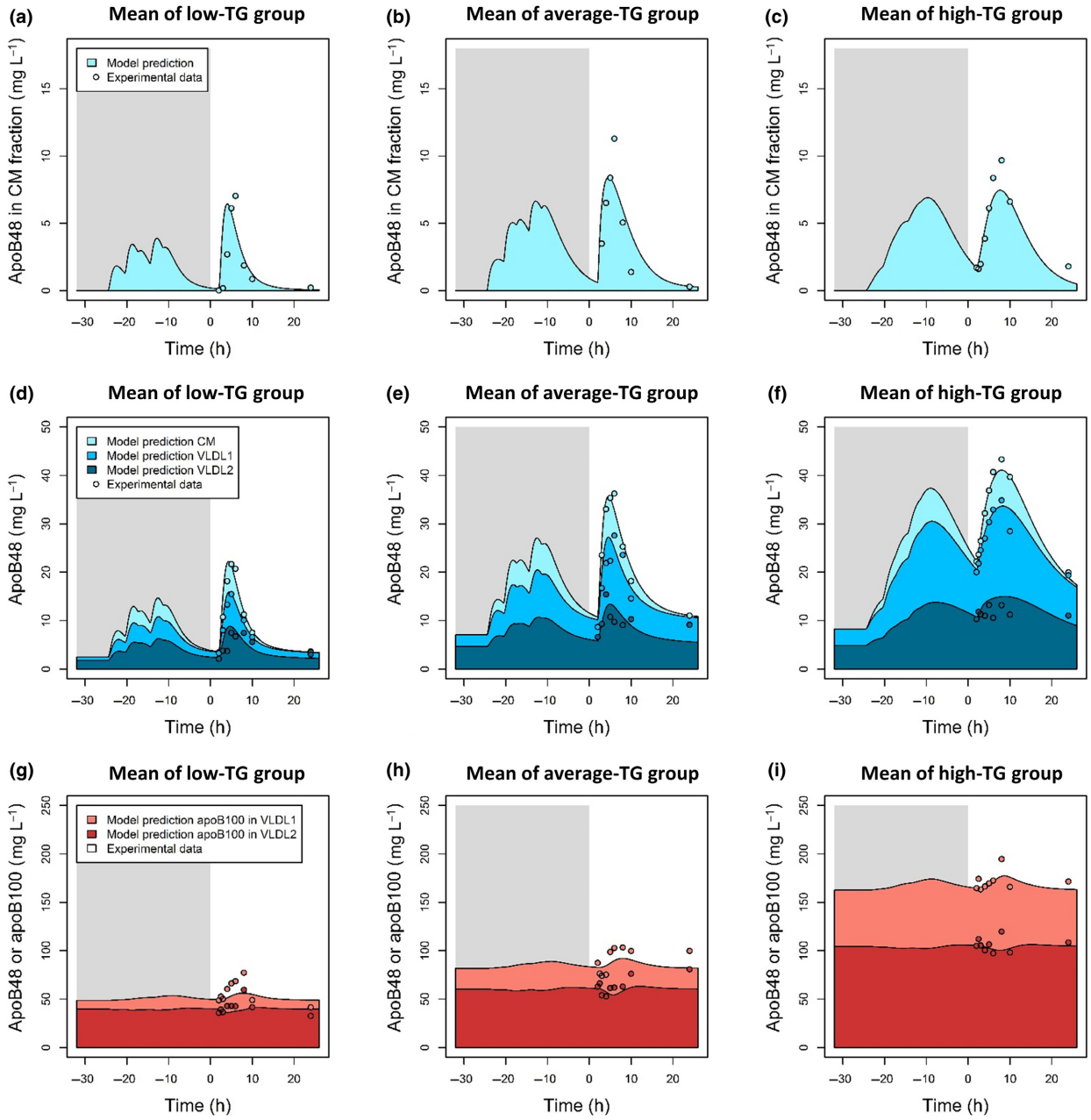

Fig. 1 *Change in apoB48 and apoB100 levels during fat meal test day. Mean concentrations for the three subject groups (Low-TG, Average-TG and High-TG) are presented for apoB48 in the chylomicron (panels a–c) and VLDL$_1$ and VLDL$_2$ (d–f) fractions, and for apoB100 in VLDL$_1$ and VLDL$_2$ (g–i). Observed values measured during the experimental day are shown as individual data points on the right-hand side of each panel (0–24 h). A 'previous day' (−24 to 0 h) was simulated using the apoB48 and apoB100 kinetics derived from the observed data using the multi-compartmental model in Figure S1 (see also Ref [21]) and this is shown as the continuous line on the left-hand side of each panel (grey background). Using this simulation, which incorporated meals at notional times, the existence of VLDL-apoB48 at the start of the experimental day could be accounted for satisfactorily. The line during the experimental day represents the fit to the observed data. The plots in d–i are stacked (meaning all concentrations are added on top of each other in each time-point) to illustrate the contribution of the different parts to the total.*

Table 2. *Metabolism of apoB48, apoB100 and triglycerides in chylomicrons and VLDL*

	Low-TG Mean ± SD	Average-TG Mean ± SD	High-TG Mean ± SD	P^a for association with TG across all subjects
ApoB48 production rates				
ApoB48 basal VLDL1 production (mg day^{-1})	9.4 ± 2.1	21.6 ± 3.9	22 ± 14	0.08
ApoB48 basal VLDL2 production (mg day^{-1})	26.1 ± 14	35.8 ± 16	31.1 ± 23	0.46
ApoB48 total basal production (mg day^{-1})	35.5 ± 13	57.4 ± 13	53.1 ± 28	0.51
ApoB48 CM postprandial production (mg day^{-1})	141 ± 59	186 ± 75	90.1 ± 38	0.47
ApoB48 VLDL1 postprandial production (mg day^{-1})	10.6 ± 11	60 ± 53	51 ± 29	0.47
ApoB48 VLDL2 postprandial production (mg day^{-1})	60.4 ± 61	45.4 ± 36	77.4 ± 95	0.33
Total apoB48 postprandial production (mg day^{-1})	212 ± 100	291 ± 70	219 ± 130	0.64
Total apoB48 production (mg day^{-1})	247 ± 95	348 ± 81	272 ± 120	0.75
ApoB100 production rates				
Total apoB100 VLDL production (mg day^{-1})	969 ± 300	973 ± 98	1260 ± 93	0.15
ApoB100 VLDL1 production (mg day^{-1})	709 ± 270	708 ± 170	982 ± 40	0.11
ApoB100 VLDL2 total production (mg day^{-1})	816 ± 260	730 ± 140	704 ± 360	0.35
ApoB100 VLDL2 direct production (mg day^{-1})	261 ± 91	265 ± 110	279 ± 57	0.98
VLDL-TG production rates				
VLDL-TG total production (g day^{-1})	28.8 ± 13	23 ± 3	36.3 ± 6.5	0.085
VLDL1-TG production (g day^{-1})	24 ± 12	19 ± 4.3	32.9 ± 7.2	0.06
VLDL2-TG total production (g day^{-1})	12.3 ± 6.3	11.1 ± 2.2	8.4 ± 4.5	0.81
VLDL2-TG direct production (g day^{-1})	4.8 ± 3.1	4.0 ± 1.7	3.4 ± 0.77	0.75
ApoB48 clearance rates				
Overall apoB48 FCR (pools per day)	15.6 ± 8.4	6.1 ± 2.2	3.2 ± 1.6	**0.0022**
ApoB48 CM FCR (pools per day)	47.7 ± 42	20.5 ± 8.7	11.9 ± 6.3	**0.035**
ApoB48 VLDL1 FCR (pools per day)	17 ± 9.2	13.9 ± 8.1	4.3 ± 2.2	**0.0054**
ApoB48 VLDL2 FCR (pools per day)	17.9 ± 10	11.5 ± 2.5	6.36 ± 2.2	**0.0026**
ApoB100 clearance rates				
ApoB100 VLDL1 FCR (pools per day)	24.1 ± 6.5	12.9 ± 9.1	6.3 ± 3.5	**<0.001**
ApoB100 VLDL1 FDC (pools per day)	3.2 ± 5.2	4.9 ± 3.8	4.8 ± 4.0	0.43
ApoB100 VLDL1 FTR (pools per day)	20.9 ± 10	8.1 ± 5.9	1.5 ± 0.7	**0.018**
ApoB100 VLDL2 FCR (pools per day)	6.4 ± 2.6	3.2 ± 0.4	1.8 ± 0.7	**0.021**
VLDL-TG clearance rates				
VLDL1-TG FCR (pools per day)	36.0 ± 12	18.7 ± 13	9.4 ± 4.8	**<0.001**
VLDL1-TG FDC (pools per day)	22.6 ± 12	12.2 ± 8.9	8.6 ± 5.1	**0.0067**
VLDL1-TG FTR (pools per day)	13.3 ± 9	6.5 ± 4.8	0.8 ± 0.4	**0.013**
VLDL2-TG FCR (pools per day)	33.9 ± 15	10.8 ± 2.1	4.2 ± 1.4	**0.0075**

Bold indicates $P < 0.05$.
[a]*P*-value for Pearson correlation of log-transformed values with fasting TG, across subjects in all groups ($n = 15$).

Kinetics of apoB48 and apoB100 in chylomicrons, VLDL$_1$ and VLDL$_2$

Mean enrichment curves for d3-leucine in apoB48 and apoB100 in VLDL$_1$ and VLDL$_2$ are shown in Fig. 2 for the Low-TG (panel a, b), Average-TG (panel c, d) and High-TG (panel e, f) subjects. To aid in comparisons, the VLDL$_1$ data for the three sets of subjects are superimposed in panels g, h. Following administration at 0 h, tracer appeared in apoB100 in VLDL$_1$ and reached peak enrichment at 2–4 h postinjection (panels a, c, e). Thereafter, there was a biphasic decline with an initially

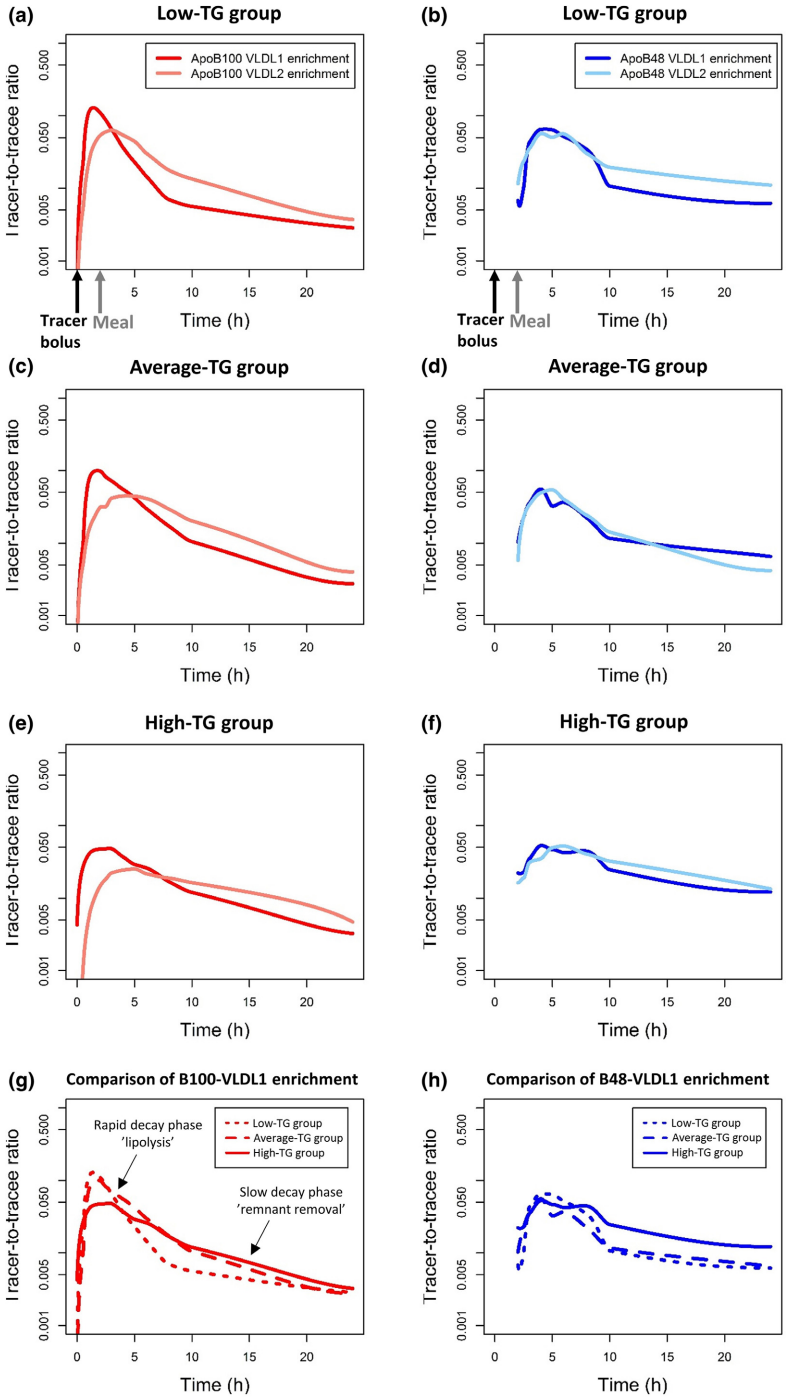

Fig. 2 *ApoB48 and apoB100 enrichment curves of d3-leucine VLDL$_1$ and VLDL$_2$. The log-linear plots show mean observed tracer enrichment data for each group. The lines represent interpolated values to allow for the slight differences in time of blood sampling between subjects. ApoB100 enrichment data in VLDL$_1$ and VLDL$_2$ are indicated in red (left panel; a, c, e, g), and apoB48 enrichment data are indicated in blue (right panel; b, d, f, h). Times of tracer administration (at 0 h, black arrow) and meal ingestion (at 2 h, grey arrow) are shown.*

rapid, followed by a much slower, decay (as depicted in panel g; the presence of clearly discernible exponential decays on the log-linear plot is consistent with two metabolically distinct clearance mechanisms). The enrichment curve for $VLDL_2$ apoB100 peaked later than for $VLDL_1$ and decayed more slowly in all subjects consistent with the usually observed precursor-product relationship between these VLDL fractions [28, 36]. The initial phase of relatively rapid decline in $VLDL_1$ apoB100 (Fig. 2 panels a, c, e) is likely due to lipolysis of the particles and (in part) conversion to $VLDL_2$. It can be seen that the gradient of this decline reduced as plasma triglyceride increased across the subjects (panel g), consistent with less efficient delipidation. The rate of decay in the second phase (attributed possibly to 'remnant removal' as depicted in panel g) appeared to be relatively slow in all subjects, and as plasma triglyceride rose there was a greater proportion of the slow versus fast decaying apoB100.

When the fat-rich meal was given at the 2-h timepoint, d3-leucine tracer appeared in chylomicron apoB48 (Figure S3), and $VLDL_1$ and $VLDL_2$ apoB48 (Figure 2 panels b, d, f). Enrichment in apoB48 rose to a peak 2–4 h after ingestion of the meal and then decayed. For apoB48 in $VLDL_2$ the rise and fall of the enrichment curves mirrored closely that seen for $VLDL_1$ indicating that apoB48 in both VLDL subfractions had a similar metabolic fate (Fig. 2 panels b, d, f), in contrast to the observations for apoB100. Again, two phases appeared to be present with the first decay component having a reduced gradient, and the second, slower decay being more prominent in the High-TG subjects compared to others (Fig. 2 panel h). An interpretation of these findings is that slowly metabolized apoB100- and apoB48-containing (remnant) particles were present to a degree in all subjects, and their relative abundance increased as plasma triglyceride levels rose.

Mean rate constants, calculated for each of the thirds of the plasma triglyceride distribution, are presented in Table 2 (individual data are given in Table S1). Basal apoB48 production at an overall mean of 49 mg day^{-1} was similar across the range of plasma triglyceride, as was the postprandial production rate of around 240 mg day^{-1} (data not shown) (note these latter rates are not steady-state inputs but the aggregate value over the 24-h period of observation). To fit the data in all TRL fractions, the model allowed direct release from the intestine of apoB48 particles into the $VLDL_1$ and $VLDL_2$ as well as chylomicron density ranges [21] (Figure S1). The total apoB48 FCR (over all TRL fractions) decreased markedly as plasma triglyceride increased ($P = 0.0022$, Table 2) falling from 15.6 pools per day in Low-TG subjects to 3.2 pools per day in High-TG subjects. FCRs for apoB48 in the chylomicron fraction were, as expected from Fig. 1, much higher than those seen for apoB48 in the VLDL subfractions. It should be noted that the metabolic characteristics of apoB48-containing particles in $VLDL_1$ and $VLDL_2$ reflect a combination of the kinetics of lipoproteins that were directly secreted into these density ranges (at about 150 mg apoB48 per day) and those derived from chylomicron lipolysis (at about 140 mg apoB48 per day) (data not shown). The majority of apoB48 secreted as chylomicrons appeared, postlipolysis, in VLDL (as depicted in Fig. 3).

Whilst the production rate of apoB100-containing $VLDL_1$ particles showed a modest correlation with plasma triglyceride ($r = 0.43$, $P = 0.11$, Table 2), the main driver of raised $VLDL_1$ apoB100 levels was the approximate 4-fold decrease in FCR as plasma triglyceride increased (Table 2, Table S1). Likewise, examination of the kinetics of triglyceride in VLDL revealed that secretion of $VLDL_1$-TG tended to rise, and clearance and transfer rates fell markedly as plasma triglyceride increased ($P < 0.001$ for the correlation of plasma triglyceride with $VLDL_1$-TG FCR, Table 2). For both apoB100 and TG, the fractional transfer rates (FTR) from $VLDL_1$ to $VLDL_2$ increased stepwise about 10-fold comparing High-TG, Average-TG and Low-TG subjects ($P = 0.018$ and $P = 0.013$, respectively, for association of $VLDL_1$-B100 FTR and $VLDL_1$-TG FTR with plasma triglyceride, Table 2).

To investigate further the contribution of apoB48 to VLDL particle metabolism, production rates were expressed in molar terms as depicted in Fig. 3. This allowed for the difference in molecular weight between apoB48 and apoB100, and since there is one apoB moiety per particle the values represent particle secretion rates (in nmol day^{-1}) and residence times (in hours) in the circulation (residence time is the reciprocal of the overall fractional clearance rate in hours, i.e. 24/FCR). The amount of apoB48 secreted in chylomicron particles did not differ significantly across plasma triglyceride range but the mean residence time was 0.5 h in Low-TG compared to 2 h in High-TG subjects. Total (basal plus postprandial) secretion of apoB48 particles

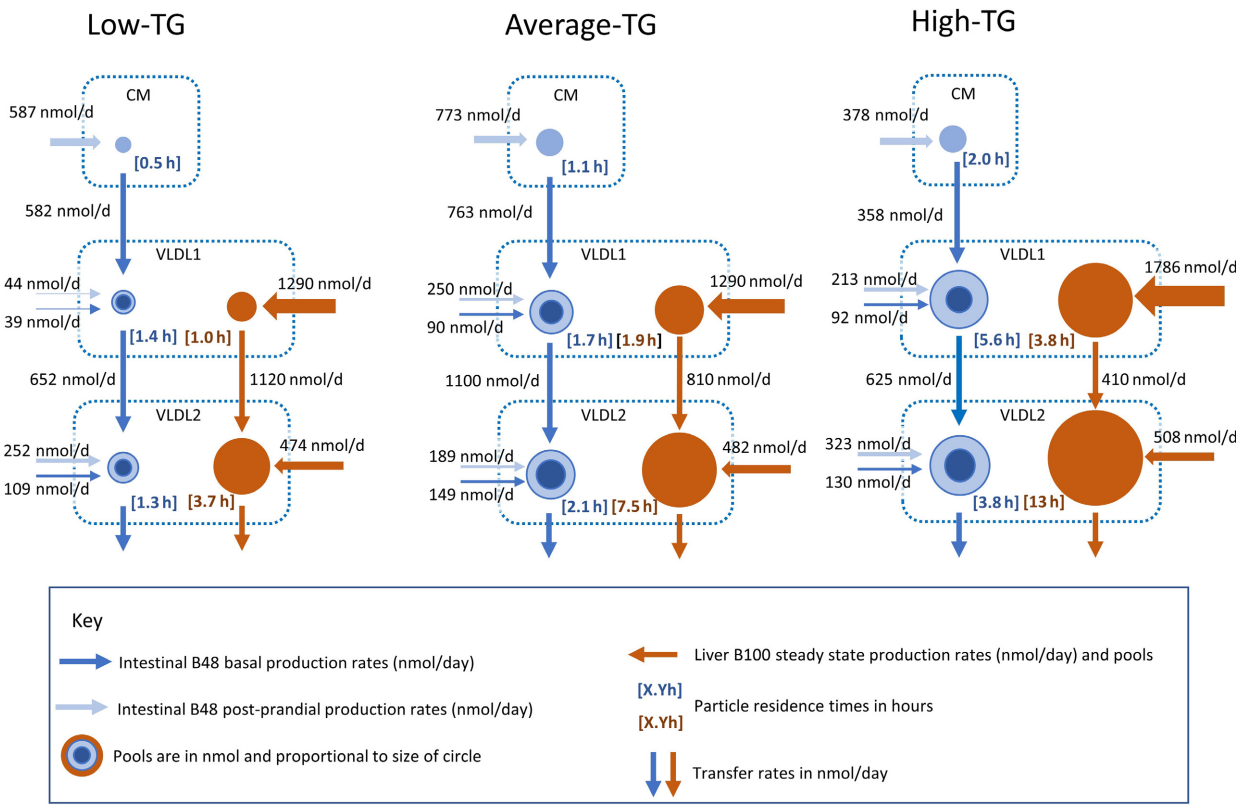

Fig. 3 *Metabolic flow of apoB48 and apoB100 in triglyceride-rich lipoproteins. Mean apoB100 pools and fluxes (in nmol day^{-1}) are indicated in red and apoB48 pools and fluxes (in nmol day^{-1}) are indicated in blue for each of the thirds of the plasma triglyceride distribution. The areas of the circles are directly proportional to pool sizes compared on a particle-count basis. Light blue circles indicate the peak pool sizes of apoB48 and dark blue circles indicate steady-state (fasting/basal) pool sizes of apoB48. Residence times are shown in brackets [ ] in hours. Secretion rates of apoB48 and apoB100 are indicated with horizontal arrows, the size/width of which is proportional to the measured flux. Transfer rates (from one fraction to another) are indicated with vertical arrows. Both fasting fluxes and postprandial fluxes are indicated for apoB48.*

into $VLDL_1$ did not vary significantly by plasma triglyceride (Table 2), but, especially in the Average-TG and High-TG subjects was a notable proportion of the apoB100 secretion rate (comparing the means of 340 and 305 nmol day^{-1} for apoB48 versus 1290 and 1786 nmol day^{-1} for apoB100, respectively, in the Average-TG and High-TG subjects) (Fig. 3). The amount of direct secretion of apoB48 particles into $VLDL_2$ was high in all subjects at 300–450 nmol day^{-1} and was of the same order of magnitude as the direct secretion of apoB100 particles into $VLDL_2$ at 470–510 nmol day^{-1}.

Residence times of both apoB100 and apoB48-containing particles increased as plasma triglyceride rose, from about 1 to 4 h in Low-TG to 4 to 13 h in High-TG subjects (Fig. 3). As might be predicted from Figs 1 and 2, apoB48 particles in $VLDL_1$ and $VLDL_2$ fractions had mean residence times that were up to 2-fold longer than that of apoB48-containing chylomicrons. Further, the predicted residence times for apoB48 particles during the slow decay phase shown in Fig. 2 were, on average, 6–8 h for all subjects (4–5 h for Low-TG, 7–11 h for Average-TG, and 11–13 h for High-TG subjects). Consideration of particle abundance (represented by the size of the coloured circles in Fig. 3) revealed that across the day apoB48 particles (from both basal secretion and postprandial production) accounted for about 23%-30% of the total number of $VLDL_1$ and $VLDL_2$ particles present in the circulation. These observations (Table 2, Figs 1–3) taken together revealed that High-TG subjects

in particular accumulated long-lived apoB48- and apoB100-containing lipoproteins and that in many subjects the intestine made a significant quantitative contribution to triglyceride transport within the VLDL density range.

Impact of postprandial lipid absorption on VLDL apoB100 metabolism

In the multi-compartmental model shown in Figure S1, the impact on VLDL metabolism of the wave of chylomicrons appearing in the bloodstream was modelled using a time-based parameter that decreased the $VLDL_1$ apoB100 and $VLDL_1$-TG fractional transfer rates (FTR; i.e. $VLDL_1$ lipolysis) as a function of the amount of chylomicron triglyceride lipolysis occurring at any given point (see Figure S2). Using this approach, it was observed that after the meal was ingested (at 2 h) chylomicron triglyceride concentration reached a peak at about the 6-h time-point and at this time the $VLDL_1$ apoB100 FTR was reduced on average by 60%. This change accounted adequately for the transient rise in $VLDL_1$ apoB100 (Figure S2). The ability of the model to generate acceptable VLDL apoB100 production and clearance rates once the dynamic phenomenon of competition for lipolysis had been taken into account was tested by comparing the results in the nine subjects studied twice; once using the regular protocol where the fat-rich meal was given and another occasion where it was not. As shown in Table S2, the rate constants did not differ significantly between the two investigations.

Metabolic determinants of triglyceride transport

To explore the links between TRL apoB48 and apoB100 metabolism, we examined relationships between kinetic parameters, and the associations of production and clearance rates with the potential regulatory factors shown in Table 1. Correlation analysis revealed significant associations between $VLDL_1$-apoB100 FCR and total and $VLDL_1$-apoB48 FCR (Fig. 4 panels a, b see Figure S4 for a more complete correlation matrix). There was no significant relationship between $VLDL_2$-apoB100 FCR and total apoB48 FCR ($r = 0.39$, $P = 0.15$), between $VLDL_2$-apoB48 FCR and $VLDL_2$-apoB100 FCR ($r = 0.37$, $P = 0.18$) nor between the FCR of apoB48 and apoB100 in $VLDL_2$ ($r = 0.37$, $P = 0.18$) (Figure S4).

Plasma apoC-III correlated positively and significantly with the fasting concentrations of plasma

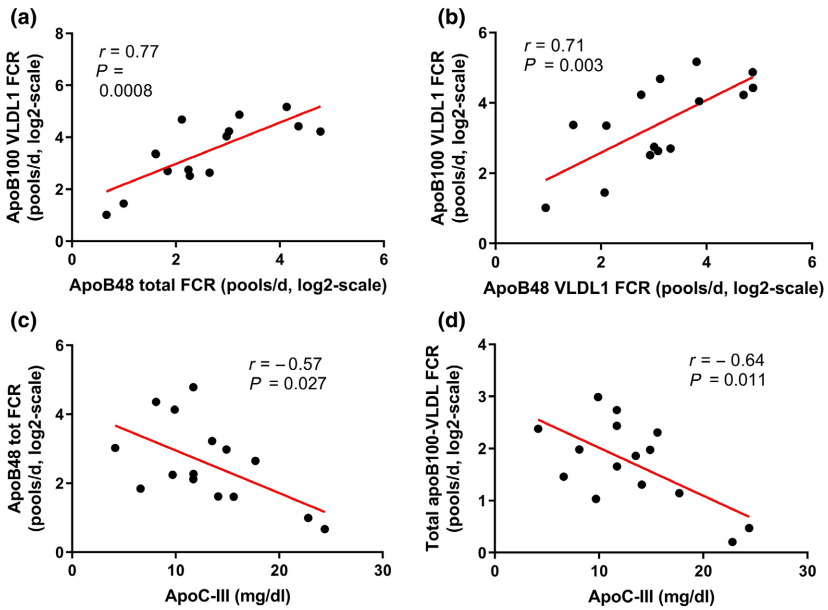

Fig. 4 *Correlations of apoB48 and apoB100 catabolism and apoC-III. Relationships between apoB48 and apoB100 kinetics (panels a, b), and between plasma apoC-III concentration in mg dL^{-1} and overall apoB48 FCR and $VLDL_1$ apoB100 FCR (panels c, d).*

triglyceride, total apoB100 in VLDL and plasma apoB48 (Figure S4). As shown in Fig. 4, panels c, d, plasma apoC-III concentration exhibited negative associations with the total FCR for apoB48 in TRL ($r = -0.57$, $P = 0.027$) and the FCR for apoB100 in total VLDL ($r = -0.64$, $P = 0.011$). Additionally, we observed correlations between apoC-III concentration and the FCRs for $VLDL_1$ apoB100 ($r = -0.63$, $P = 0.011$, data not shown) and for $VLDL_2$ apoB100 ($r = -0.55$, $P = 0.032$, data not shown). Plasma apoC-III was also negatively correlated with $VLDL_1$-TG FCR ($r = -0.63$, $P = 0.012$, data not shown) and $VLDL_2$-TG FCR ($r = -0.66$, $P = 0.0071$, data not shown).

Discussion

With the emergence of new findings from genomics [37–39], epidemiology [1–4, 40, 41] and clinical trials [26, 27, 42, 43], regarding the relationship between triglyceride-rich lipoproteins and CHD risk, and the identification of associated potential novel targets for intervention [24, 25, 44], there is a pressing need to understand in greater detail the integrated metabolism of chylomicrons and VLDL. The present study addresses this topic and, in particular, provides a metabolic explanation for the fact (as observed by us here and others before [5–7]) that apoB48-containing triglyceride-rich lipoproteins appear in both the VLDL and chylomicron density range during fat ingestion and, in our investigation, across the day accounted for around 25% of 'VLDL' particles. Further key findings were not only that there was continual low-level basal secretion of apoB48-containing particles by the intestine (i.e. in the fasting state—see also reference [21]) but also that direct secretion of apoB48-containing $VLDL_1$ and $VLDL_2$ increased during lipid absorption. Our kinetic analysis revealed that over the postprandial period about half of the apoB48-containing particles in VLDL were derived from chylomicron lipolysis and half from direct secretion. Of particular significance also was the observation that apoB48-containing VLDL had prolonged residence times (unlike chylomicrons that were rapidly cleared from the bloodstream) and on this basis apoB48 carrying 'remnants' persisted in the circulation even in fasted individuals. Additionally, we observed that the metabolism of triglyceride and apoB100 in VLDL was affected by the appearance of chylomicrons, with inhibition of $VLDL_1$ lipolysis rates and an increment in the plasma concentration of $VLDL_1$ apoB100 in line with previously published observations [5–7, 30, 31, 36].

These features of apoB48 and apoB100 metabolism in VLDL, and in particular the magnitude of the fat-rich meal-induced perturbations were found to be related strongly to the plasma triglyceride level as depicted conceptually in Fig. 5. This metabolic pattern may underpin the increased CHD risk associated with elevated concentrations of nonfasting plasma triglyceride [1–4] and remnant lipoproteins [1–4, 45, 46] that appears independent of low-density lipoprotein (LDL) linked risk [46] and persists even in statin-treated patients [46–48]. It is noteworthy that the High-TG subjects had plasma triglyceride levels similar to those seen in recruits to REDUCE-IT and PROMINENT [26, 27] and, therefore, our findings may provide insight into the potential disturbances in triglyceride metabolism in the subjects of these important outcome trials. We propose that elevated fasting (or nonfasting) plasma triglyceride (with accompanying raised plasma apoB48) could be regarded as a 'reporter biomarker' signalling the presence of a history of significant metabolic dysregulation (as seen in Fig. 1). With this in mind, when judging the impact of a triglyceride-lowering therapy, it is worth noting that measurement of fasting plasma triglyceride is an assessment made at the nadir of triglyceride transport and that during a day of normal meal consumption, TRL and their long-lived remnants would accumulate in the circulation [3]. Further, a change in fasting plasma triglyceride concentration will be accompanied by a greater effect on plasma apoB48 than plasma apoB100 as reported for both high dose fish oil [49] and pemafibrate (the drug used in PROMINENT) [50]. This is an important distinction in the light of the recent report [51] that the cardiovascular benefits of triglyceride lowering are linked to change in apoB, that is, particle number. Our observations suggest additionally that the impact of such agents on apoB48 may be due not only to altered chylomicron metabolism but also to an effect on intestinally derived VLDL.

The concept that triglyceride-rich lipoproteins and their remnants contribute to atherogenesis and CHD risk arose from epidemiological surveys [1–4, 52] and pathological studies [4, 44, 53, 54] showing that smaller TRLs and remnants entered the artery wall and there could contribute to cholesterol deposition and inflammation. Indeed, the molar content of cholesterol (free cholesterol and cholesterol esters) in remnant particles is in the range of that of LDL [55–58]. Most early surveys were conducted with individuals fasted overnight

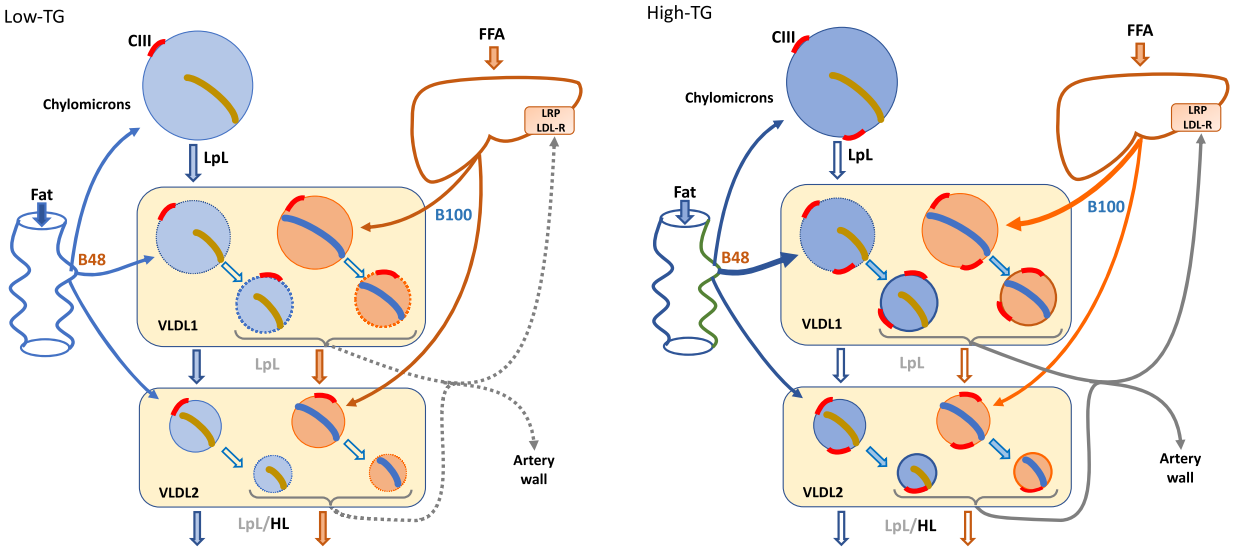

Fig. 5 *Integrated scheme for chylomicron and VLDL metabolism. Schematic representation of the integrated metabolism of apoB48 and apoB100 particles within triglyceride-rich lipoproteins in the basal and postprandial states. Separate schemes are given for subjects with Low- and High-TG. This conceptual depiction should be viewed alongside Fig. 3, which gives the flows into each of the particle types. The current study provides evidence that the intestine, following a fat load, releases TG-rich, apoB48-containing particles across the density range from chylomicrons to $VLDL_2$. Chylomicrons when present are superior substrates for lipoprotein lipase and interfere with the efficient lipolysis of $VLDL_1$ apoB100 particles (depicted as grey 'LpL'). Higher levels of apoC-III (red arcs) lead to inhibition of lipolysis of chylomicrons and $VLDL_1$/$VLDL_2$ (depicted as arrows with no infill) and the development of hypertriglyceridemia. During fat feeding in high-TG subjects, the inhibition of particularly VLDL lipolysis leads to the generation of long-lived remnant lipoprotein species within $VLDL_1$ and $VLDL_2$. These remnants are cleared by putative lipoprotein receptors and if present in excess can contribute to atherogenesis and lipid deposition in the artery wall.*

and hence the initial focus was on VLDL, neglecting the postprandial state. The recognition that the fat absorption pathway transported large quantities of triglyceride led investigators to recognize that the 'fed' state, in which we spend most of our daily life, should be considered in assessment of the consequences of dysregulation of triglyceride transport and the generation of remnants [59, 60]. As noted above, studies of apoB48 kinetics are challenging due to its low abundance and nonsteady-state dynamics [8–11, 21, 61, 62]. A quasi-steady state in which to measure apoB48 turnover can be obtained by feeding micro-meals across the day but this approach may not generate physiologically relevant results [8, 10, 61, 62]. However, values seen in previous studies for the overall apoB48 FCR of about 4 pools per day are similar to those found in the present investigation. As might be expected, rates of apoB48 production across the day when very small meals were given to normal subjects—about 70 mg day^{-1} [8]—were less than those seen here with our fat-rich mixed meal—about 240 mg day^{-1}, although much higher rates have been reported in subjects with type 2 diabetes [10]. Where earlier apoB48 turnover studies used a physiological test meal [11], FCRs were similar to our findings but the increment in secretion at 70 mg over 10 h was considerably below the values we observed.

Most earlier investigations due to the low abundance of apoB48 examined total TRL, a pragmatic approach that was unable to resolve either the important contribution that apoB48 particles make to overall VLDL metabolism during lipid absorption, or the distinct kinetics of apoB48 in VLDL versus chylomicrons. A study using a micro-meal protocol [61] in a group of five women with normal lipid levels found evidence for secretion of apoB48-containing lipoproteins into large and small VLDL in line with the present report. However, echoing what is stated above, the authors of this study acknowledged that the metabolic patterns they reported may not

represent the situation during ingestion of a physiologically relevant meal [61]. In the current investigation, we found in response to a fat-rich mixed meal that intestinally derived lipoproteins containing apoB48 are indeed released across a wide range of particle size (and density). Those appearing as VLDL had a metabolism that was different from those secreted as chylomicrons, and within the $VLDL_1$ and $VLDL_2$ density ranges apoB48 and apoB100 kinetics differed (in contrast to the earlier finding [61]). Why the intestine secretes continuously apoB48-containing VLDL particles at a low level in the fasting state and at a higher rate during fat absorption, in addition to assembling and releasing the much larger chylomicrons, is not yet clear. A clue may come from recent studies that indicate that triglycerides may be stored in the enterocytes as lipid droplets and then used for lipoprotein production pathways [60, 63].

We observed that the clearance rates of total apoB48 and $VLDL_1$-apoB100 were associated with each other and that within $VLDL_1$ apoB100- and apoB48-particle removal rates were related positively to each other. Of the potential determinants of TRL removal that were examined, plasma apoC-III levels exhibited the strongest and most consistent associations. ApoC-III correlated with plasma triglyceride, plasma apoB48 and VLDL apoB100 concentration, and this apolipoprotein has been proposed as a major regulator of the lipolysis, particle uptake and possibly secretion of TRL [25, 39, 64–66]. In our subjects who had a wide range of plasma triglyceride levels, significant inverse relationships were seen between plasma apoC-III concentration and the total FCR of apoB48, and the FCR of apoB100 and TG in $VLDL_1$. In contrast, no relationship was observed between chylomicron and apoB100-$VLDL_1$ clearance rates and lipoprotein lipase ($r = 0.49$, $P = 0.065$ and $r = 0.33$, $P = 0.22$, respectively), possibly because this enzyme is measured after release from tissue sites by heparin and not *in situ*. The specific association of apoC-III with regulation of VLDL lipolysis (FCR) is in accord with a recent report [65] showing that genetic deficiency of apoC-III led to an acceleration of VLDL conversion to intermediate- and low-density lipoprotein but no change in the rate of direct removal of VLDL. In that study, a near 50% reduction in plasma apoC-III was associated with a doubling of the FCR for VLDL [65], and the regression line between plasma apoC-III and $VLDL_1$ apoB100 FCR that we observed had a similar gradient (i.e. as plasma apoC-III rose from 10 to 20 mg dL^{-1} the FCR fell from 20 to 10 pools per day). Our findings suggest that a further impact of apoC-III may be, by inhibiting lipolysis, to promote the generation of remnants from chylomicron and VLDL and these particles accumulate in the $VLDL_1$ and $VLDL_2$ fractions (Fig. 5). These observations support the concept that agents such as anti-sense oligonucleotides designed to disrupt apoC-III synthesis could be useful lipid-regulating drugs.

The advantages of the present study are, first, the deployment of technical advanced methods to permit quantitation and tracer enrichment measurement in VLDL and chylomicrons separately; second, the development of a combined steady-state: nonsteady-state compartmental model to describe the dynamics of TRL metabolism; and third, the use of a physiologically relevant feeding protocol. Its main limitations are that we recruited only men, also there was a relatively small number of subjects (not unusual for such a detailed kinetic investigation) and there were constraints on the number of blood samples that could be drawn thereby limiting the data set on which to base the multi-compartmental model (see supplementary information to reference [21]).

In conclusion, the importance of the present study lies in the revelation that the metabolism of apoB48-containing lipoproteins is more complex than previously thought. ApoB48-containing particles are secreted not only as chylomicrons but also directly into the $VLDL_1$ and $VLDL_2$ density ranges both in the basal state and during dietary lipid absorption. Within VLDL long-lived (remnant) particles of both liver (containing apoB100) and intestinal (containing apoB48) origin accumulate, becoming quantitatively more important as plasma triglyceride rises. These findings provide a metabolic understanding of the potential consequences for increased CHD risk when slowed lipolysis leads to the accumulation of remnants, especially in individuals with hypertriglyceridemia who are increasingly the focus of clinical trials of novel interventions.

Conflict of interest statement

None of the authors reports any potential conflicts of interest for any aspect of this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Kinetics of apoB48, apoB100 and TG in all subjects.

Table S2. VLDL1 TG and B100 kinetics in fed vs fasted states.

Figure S1. Integrated model structure.

Figure S2. Impact of chylomicronemia on VLDL metabolism.

Figure S3. Interpolation of experimental enrichment data for apoB48 in the chylomicron (CM) fraction.

Figure S4. Correlation matrix of clinical variables and kinetic parameters.■

