SUPPLEMENT MATERIAL TO:

Apolipoprotein C-III and the metabolic basis for hypertriglyceridemia and the dense LDL phenotype

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Supplemental Methods:

***Subjects*:** Nine patients with moderate hypertriglyceridemia and twelve normolipidemic participants were recruited into the study. Participants with secondary hyperlipidemia, diabetes, (APO)E2/E2, E4/E4, E2/E4 genotypes, and use of medications that affect lipid metabolism were excluded from the study. The study was approved by the Human Subjects Committees at Harvard School of Public Health and Brigham and Women's Hospital. All participants gave informed consent.

***Controlled dietary intake*:** All participants entered a 3-week controlled dietary period before starting the tracer studies. The diet consisted of 20% energy from fat (7% from saturated fat, 8% from mono-unsaturated fat, and 5% from polyunsaturated fat), 65% from carbohydrate (48% from complex carbohydrate, and 17% from simple carbohydrate) and 15% from protein. The entire diet was provided to the participants as outpatients, and they were asked not to consume alcoholic beverages or any other source of caloric intake. Dietary energy levels were adjusted to reports of hunger or satiety, and trends in body weight which were measured every other day. The diet was prepared by the metabolic kitchen of the General Clinical Research Center at Brigham and Women's Hospital (Boston, MA).

***Tracer infusion*:** Participants were admitted to the General Clinical Research Center of Brigham and Women's Hospital in the evening prior to study. After an overnight fast, they received a priming dose of 4.2 μmol/kg [D_3] L-Leucine (Tracer Technologies, Cambridge, MA), followed by a constant infusion of [D_3] L-Leucine leucine at 4.8 μmol/kg/hour for 15 hours. A bolus injection of [D_5] L-Phenylalanine, 7.3 μmol /kg, was also administered at the same time. We used two tracer techniques mainly

to reduce measurement errors. Both tracers, when modeled independently, gave similar parameter estimates in this study and previous ones [1, 2]. But by combining both tracer data in the same model, the coefficients of variation for parameter estimates were substantially lowered.

Blood samples were collected at baseline, every 20 minutes in the first 2 hours after the infusion started, and hourly thereafter. For the first 4 hours, participants were restricted to noncaloric drinks. After this, they were given a standardized lunch and supper that had no fat, leucine or phenylalanine, and that contained 60% of daily required calories to avoid discomfort and abnormal metabolic effects of prolonged fasting.

***Sequential immuno-affinity chromatography*:** Plasma was first incubated with an anti-apoE immunoaffinity column made from an affinity purified polyclonal antibody against human apoE (Academy-Biomedical, Houston TX). The unbound fraction (E-) and the bound fraction (E+) were then incubated with anti-apoC-III columns. This sequential immuno-affinity column procedure separated plasma into four immuno-fractions: those with both apoE and apoC-III (E+CIII+), those with apoE but not apoC-III (E+CIII-), those with apoC-III but not apoE (E-CIII+), and those without apoE or apoC-III (E-CIII-). Column efficiency was 98% for both apoE and apoC-III. Validation studies were performed to evaluate the effects of freezing and storage of plasma, and they were found not to affect the subsequent immuno-affinity columns [2, 3].

***Ultracentrifugation*:** The above four immuno-fractions were then centrifuged separately at 25000 rpm on a Ti 25 rotor in an L8-70M instrument (Beckman, Brea CA) to isolate light VLDL (Svedberg units of flotation: 60~400), dense VLDL (S_f: 20~60), IDL (1.006~1.025 g/mL), LDL-1 (1.025~1.032 g/mL), LDL-2 (1.032~1.038 g/mL), and

LDL-3 (1.038~1.050 g/mL) using a modification of the Lindgren methods [4]. A density of 1.050 g/mL was selected as the cutoff point for LDL to avoid contamination by Lp(a), which is concentrated at densities between 1.050~1.080 g/mL [5].

***Determination of lipids and apolipoproteins*:** Triglyceride and cholesterol concentrations were determined enzymatically by Infinity© kits (Thermo Electron, Melbourne Australia). Concentrations of apoE, apoC-III, and apoB were determined by sandwich ELISA using affinity-purified antibodies (Academy-Biomedical, Houston TX). Intra-assay coefficients of variances for lipid and apolipoprotein measurements were less than 5%, and inter-assay coefficients of variances were less than 10% [2, 3]. Plasma glucose was determined enzymatically by a Glucose Assay kit (Sigma, St. Louis, MO). Insulin was determined by an ELISA kit from Abcam (Cambridge, MA).

***Measurement of tracer enrichment and pool size*:** Apolipoprotein B was precipitated from the lipoprotein fractions, and tracer enrichment and tracee mass were measured by GC-MS as described previously [1, 2]. In all participants, plasma apoB concentrations stayed constant during the infusion protocol with less than 10% variation. Under the study dietary conditions, there is a negligible contribution of apoB48 to the precipitated apoB in the VLDL (<5%) and LDL (<1%) fractions. For participants with body mass index (BMI) between 18.5~29.9 $kg \cdot m^{-2}$, plasma volume (L) was assumed to be 4.4% of body weight (kg). For obese participants (BMI>30.0), plasma volume was calculated by an equation described by Nikkila and Kekki [6]: plasma volume (L) = ideal body weight (kg) X 0.044 + excess weight (kg) X 0.010.

***Model development and kinetic analysis*:** Tracer enrichment and apoB were measured in 24 apoB lipoprotein fractions (four immuno-fractions further separated into

six density fractions) for each sample. A multi-compartment model was employed to find the best fit to the observed data by using SAAM II software, Version 1.2.1 (SAAM Institute, Seattle WA) following established methods [7-10] as described previously [1, 2]. **Figure 2** shows the diagram of the model. Plasma apoB lipoproteins were represented by 21 compartments separated in density by ultracentrifugation and in apoE and apoC-III content by immuno-affinity chromatography. Three LDL types, LDL-1, -2 and -3 containing apoE and not apoC-III, had concentrations that were too low to measure and they were not studied.

The model was developed by designating every physically separated lipoprotein fraction to a single compartment. A plasma amino acid forcing function was followed by a hepatic intracellular delay compartment accounting for the time required for the synthesis and secretion of apoB100 into plasma. The model allowed apoB secretion from the liver into each plasma compartment, as well as direct clearance from the circulation. Metabolism of plasma apoB lipoproteins includes conversion of larger particles to smaller ones. Modeling results suggested that for apoB lipoproteins with the same apoE and apoC-III composition, stepwise delipidation as the primary metabolic pathway usually fitted the data best (e.g. E-CIII+ VLDL → E-CIII+ IDL → E-CIII+ LDL). In addition, we also tested conversion pathways among lipoproteins with different apoE and apoC-III compositions, and found some that were required to improve the fitting. For example, conversion from E-CIII+ light VLDL to E-CIII- dense VLDL represents such a pathway, indicating during lipolysis, some E-CIII+ light VLDL lose all of their apoC-III content and are converted to dense VLDL without apoC-III. When evaluating these potential conversion pathways between apoB lipoproteins with different apoE and apoC-

III composition, we first compared the enrichment curves of the originating and destination compartments to eliminate pathways that prohibit a precursor-product relationship. Next, qualified pathways were added to the existing model one by one, and were fitted to the data. Pathways for which rate constants were zero or negligible were eliminated. Average particle size and triglyceride content were also taken into consideration when evaluating such a pathway.

After the model structure was established by the mean tracer and tracee data of all participants, each participant's data were fitted individually to obtain the parameter values. For each individual, both [D_5] L-Phenylalanine and [D_3] L-Leucine tracer data were included in the same model, and the data were solved simultaneously by making the rate constants equal for leucine and phenylalanine experiments. Thus for each participant a single set of rate constants was produced. This model was able to generate excellent fits to tracer enrichments, and apoB masses. The coefficients of variation for most parameter estimates were less than 25%, and for the major pathways usually less than 15%.

Regression analysis: To determine the major kinetics parameters that predict plasma triglyceride levels, we performed simple and multiple linear regression analysis with SAS (Version 9.1, SAS Institute, Cary NC). We used plasma triglyceride concentration (mg/dL) as the dependent variable and apoB secretion rates or fractional catabolic rates as independent variables. In multiple regression analysis, in addition to the above kinetic parameters, independent variables also included body mass index and gender, or fasting glucose, fasting insulin, or HOMA index.

***Statistical analysis*:** Comparisons between hypertriglyceridemics and controls were performed using unpaired t-test with SAS software (Version 8). The results are

presented as mean ± standard error of the mean (SEM) unless specified otherwise. A *p*-value ≤ 0.05 (two-sided) is considered statistically significant.

Supplemental References:

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Supplemental Table 1: Comparison of kinetics parameters between hypertriglyceridemic patients and normotriglyceridemic controls.

	Flux Rates (mg•day^{-1}•kg^{-1})						Rate Constants (pools•day^{-1})					
	NTG		HTG				NTG		HTG			
	Mean	**SEM**	**Mean**	**SEM**	**Change**	p-value	**Mean**	**SEM**	**Mean**	**SEM**	**Change**	p-value
Direct Removal Pathways												
E-CIII- Va	**0.06**	0.04	**0.28**	0.18	386%	0.166	**0.42**	0.16	**0.42**	0.20	-2%	0.962
E-CIII- Vb	**0.35**	0.08	**0.53**	0.15	54%	0.327	**1.38**	0.21	**1.14**	0.25	-17%	0.469
E-CIII- IDL	**0.21**	0.10	**1.30**	0.20	534%	0.007†	**0.64**	0.11	**1.12**	0.47	76%	0.044*
E-CIII- LDL1	**0.61**	0.22	**1.54**	0.30	153%	0.042*	**0.40**	0.11	**0.99**	0.12	148%	0.009†
E-CIII- LDL2	**2.12**	0.50	**1.53**	0.34	-28%	0.446	**0.42**	0.13	**0.78**	0.22	86%	0.163
E-CIII- LDL3	**10.20**	0.90	**9.82**	1.35	-4%	0.823	**1.17**	0.13	**0.75**	0.13	-36%	0.042
E-CIII+ Va	**0.02**	0.00	**0.05**	0.09	105%	0.278	**0.25**	0.17	**0.21**	0.21	-16%	0.892
E-CIII+ Vb	**0.05**	0.02	**0.16**	0.10	221%	0.304	**0.40**	0.21	**0.48**	0.32	20%	0.830
E-CIII+ IDL	**0.10**	0.05	**0.33**	0.15	228%	0.179	**0.38**	0.20	**0.64**	0.32	68%	0.476
E-CIII+ LDL1	**0.20**	0.08	**0.35**	0.20	75%	0.523	**0.58**	0.19	**0.75**	0.33	29%	0.629
E-CIII+ LDL2	**0.13**	0.06	**0.12**	0.05	-5%	0.950	**0.33**	0.20	**0.39**	0.21	18%	0.835
E-CIII+ LDL3	**0.13**	0.06	**0.27**	0.11	103%	0.328	**0.32**	0.21	**0.60**	0.30	88%	0.422
E+CIII- Va	**0.40**	0.07	**0.25**	0.06	-37%	0.205	**9.36**	1.72	**2.77**	0.17	-70%	0.009†
E+CIII- Vb	**0.65**	0.14	**0.31**	0.08	-52%	0.109	**20.63**	2.05	**8.58**	0.43	-58%	0.007†
E+CIII- IDL	**0.39**	0.05	**0.25**	0.08	-36%	0.179	**7.76**	1.29	**5.23**	0.33	-33%	0.114
E+CIII+ Va	**3.26**	0.51	**1.58**	0.24	-51%	0.037*	**8.20**	0.75	**3.68**	0.21	-55%	0.0001†
E+CIII+ Vb	**3.19**	0.25	**2.47**	0.43	-22%	0.215	**9.55**	1.52	**5.38**	0.72	-44%	0.049
E+CIII+ IDL	**2.39**	0.23	**1.44**	0.27	-40%	0.032*	**5.48**	0.43	**5.00**	1.12	-9%	0.663
E+CIII+ LDL1	**1.22**	0.18	**0.48**	0.07	-60%	0.011*	**4.77**	0.99	**3.57**	0.75	-25%	0.372
E+CIII+ LDL2	**0.35**	0.06	**0.30**	0.06	-14%	0.643	**2.34**	0.50	**5.07**	0.78	117%	0.010†
E+CIII+ LDL3	**0.24**	0.03	**0.20**	0.05	-19%	0.477	**1.96**	0.56	**3.33**	1.39	70%	0.326
Conversion Pathways												
E-CIII- Va to E-CIII- Vb	**0.56**	0.12	**1.07**	0.26	92%	0.116	**2.13**	0.33	**1.47**	0.07	-31%	0.104
E-CIII+ Va to E-CIII- Vb	**0.83**	0.15	**1.96**	0.30	136%	0.007†	**5.40**	1.70	**3.08**	0.42	-43%	0.260
E-CIII- Vb to E-CIII- IDL	**1.98**	0.26	**3.60**	0.50	82%	0.043*	**9.06**	0.36	**7.91**	0.85	-13%	0.190
E-CIII+ Vb to E-CIII- IDL	**2.15**	0.28	**2.69**	0.37	25%	0.331	**9.83**	1.83	**5.86**	0.47	-40%	0.045*
E-CIII- IDL to E-CIII- LDL1	**3.86**	0.41	**4.46**	0.67	16%	0.504	**3.08**	0.34	**3.24**	0.38	5%	0.756
E-CIII+ IDL to E-CIII- LDL1	**1.81**	0.31	**2.30**	0.33	27%	0.370	**5.89**	1.77	**3.66**	0.32	-38%	0.296
E+CIII+ IDL to E-CIII- LDL1	**0.75**	0.20	**0.42**	0.18	-44%	0.339	**3.14**	1.66	**0.95**	0.23	-70%	0.273
E-CIII- IDL to E-CIII- LDL2	**1.63**	0.49	**1.26**	0.41	-22%	0.656	**1.56**	0.52	**1.72**	0.95	10%	0.874

E-CIII- LDL1 to E-CIII- LDL2	**3.33**	0.42	**3.00**	0.47	-10%	0.678	**1.09**	0.14	**1.63**	0.23	50%	0.049*
E-CIII+ LDL1 to E-CIII- LDL2	**1.68**	0.29	**1.12**	0.20	-33%	0.232	**3.58**	0.42	**3.29**	0.91	-8%	0.762
E-CIII- LDL1 to E-CIII- LDL3	**3.52**	0.29	**2.97**	0.68	-16%	0.504	**1.91**	0.81	**1.54**	0.24	-19%	0.704
E-CIII- LDL2 to E-CIII- LDL3	**5.27**	0.57	**4.26**	1.14	-19%	0.484	**0.90**	0.11	**1.33**	0.17	48%	0.043*
E-CIII+ LDL2 to E-CIII- LDL3	**0.32**	0.10	**0.57**	0.07	82%	0.100	**1.13**	0.37	**1.72**	0.23	52%	0.237
E-CIII+ LDL3 to E-CIII- LDL3	**0.44**	0.08	**0.92**	0.16	108%	0.026*	**1.18**	0.35	**1.71**	0.62	45%	0.439
E-CIII+ Va to E-CIII+ Vb	**1.09**	0.28	**3.37**	0.62	210%	0.006†	**6.27**	1.73	**8.85**	2.86	41%	0.426
E-CIII+ Vb to E-CIII+ IDL	**2.23**	0.35	**3.63**	0.31	63%	0.022*	**10.88**	2.03	**9.32**	1.94	-14%	0.596
E-CIII+ IDL to E-CIII+ LDL1	**1.40**	0.26	**1.85**	0.26	32%	0.322	**4.48**	1.13	**4.29**	1.55	-4%	0.920
E+CIII+ IDL to E-CIII+ LDL1	**0.52**	0.21	**0.35**	0.14	-33%	0.615	**0.91**	0.39	**1.24**	0.51	36%	0.611
E-CIII+ LDL1 to E-CIII+ LDL2	**0.58**	0.09	**1.09**	0.17	89%	0.025*	**1.46**	0.26	**2.77**	0.54	90%	0.026*
E-CIII+ LDL2 to E-CIII+ LDL3	**0.40**	0.06	**0.83**	0.09	107%	0.032*	**1.46**	0.26	**2.77**	0.54	90%	0.026*
E+CIII- Va to E+CIII- Vb	**0.19**	0.03	**0.11**	0.03	-41%	0.115	**5.09**	2.41	**1.17**	0.03	-77%	0.189
E+CIII+ Va to E+CIII- Vb	**0.11**	0.03	**0.10**	0.03	-6%	0.892	**0.37**	0.12	**0.21**	0.07	-43%	0.311
E+CIII- Vb to E+CIII- IDL	**0.10**	0.02	**0.05**	0.01	-55%	0.122	**2.63**	0.26	**1.44**	0.08	-45%	0.009†
E+CIII+ Vb to E+CIII- IDL	**0.05**	0.03	**0.12**	0.06	162%	0.313	**0.15**	0.10	**0.36**	0.20	135%	0.346
E+CIII+ Va to E+CIII+ Vb	**3.34**	0.70	**1.81**	0.34	-46%	0.151	**9.05**	1.70	**4.97**	1.69	-45%	0.121
E+CIII+ Vb to E+CIII+ IDL	**3.39**	0.46	**2.23**	0.40	-34%	0.144	**8.44**	1.88	**4.83**	0.54	-43%	0.122
E+CIII+ IDL to E+CIII+ LDL1	**0.98**	0.16	**0.48**	0.06	-51%	0.039*	**2.13**	0.25	**2.01**	0.48	-6%	0.805
E+CIII+ LDL1 to E+CIII+ LDL2	**0.21**	0.03	**0.25**	0.06	19%	0.611	**0.85**	0.10	**1.72**	0.18	102%	0.010†
E+CIII+ LDL2 to E+CIII+ LDL3	**0.14**	0.03	**0.12**	0.04	-16%	0.711	**0.85**	0.10	**1.72**	0.18	102%	0.010†

ApoB flux rates (mg•day^{-1}•kg^{-1}) and rate constants (pools•day^{-1}) in hypertriglyceridemic patients (HTG, N=9) and normotriglyceridemic controls (NTG, N=12). "Change" column indicates percentage changes of HTG values compared to corresponding controls, i.e. (HTG-NTG)/NTG. *P*-values indicate difference between the two groups by unpaired t-test. *: $p<0.05$, †: $p<0.01$. Flux rates data were used to prepare Figure 6A, and rate constants data were used to prepare Figure 6B. Va: Light VLDL; Vb: Dense VLDL.