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To Mr. Shankly !

Kinetics of Apolipoproteins in Plasma of Normolipidemic and Dyslipidemic Subjects: An Endogenous Labeling Study

A Thesis Submitted to the Faculty of Graduate Studies and Research, McGill University, in Partial Fulfillment of the Requirements of the Degree of Doctor of Philosophy

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List of Abbreviations

[D_3] leucine	Deuterium-labeled leucine
Apo	Apolipoprotein
CE	Cholesterol ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CLAS	Cholesterol Lowering Atherosclerosis Study
EGF	Epidermal Growth Factor
FCH	Familial combined hyperlipidemia
FCR	Fractional catabolic rate
FH	Familial hypercholesterolemia
FHD	Familial HDL deficiency
FHTG	Familial hypertriglyceridemia
FPR	Fractional production rate
FSR	Fractional synthetic rate
FTR	Fractional transport rate
GC-MS	Gas chromatography-mass spectroscopy
gp330	Glycoprotein 330
HDL-C	High density lipoprotein cholesterol
HL	Hepatic lipase
HMG-CoA red.	3-hydroxy 3 methylglutaryl coenzyme A reductase
HTG	Hypertriglyceridemic

IDL	Intermediate density lipoprotein
IEF	Preparative isoelectric focusing
ISL	Intermediate density lipoprotein
LCAT	Lecithin: cholesterol acyl transferase
LDL	Low density lipoprotein
LDL-C	LDL-cholesterol
LDL-R	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LRCFS	The Lipid Research Clinic's Follow-up Study
LRP	LDL-R related protein
LSR	Lipolysis stimulated receptor
MARS	Monitored Atherosclerosis Regression Study
MIDA	Mass Isotopomer Distribution Analysis
MTP	Microsomal TG transfer protein
NL	Normolipidemic
PC-PLC	PC-specific phospholipase C
PC-PLD	Phosphatidylcholine-specific phospholipase D
PKC	Protein kinase C
PPAR	Peroxisome proliferator activated receptor
PR	Production rate
PT-III	Pseudotype III dyslipidemia
RCT	Reverse cholesterol transport
RER	Rough endoplasmic reticulum

RFLP	Restriction fragment length polymorphism
RT	Residence time
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SER	Smooth endoplasmic reticulum
SIM	Selected Ion Monitoring
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
TD	Tangier disease
TG	Triglyceride
TR	Transport rate
TRL	Triglyceride rich lipoprotein
TypIII	Type III hyperlipoproteinemia
VLDL	Very low-density lipoprotein
VLDL-R	VLDL receptor
Z(t)	Tracer to tracee ratio

Abstract

Apolipoproteins play a central role in controlling the plasma metabolism of cholesterol- and triglyceride-rich lipoproteins, and in regulating their levels. Plasma concentrations of apolipoproteins, as well as their relative distribution on different lipoprotein fractions are determined by their rates of production and catabolism. In order to investigate the plasma kinetics of these proteins in normolipidemic and dyslipidemic individuals, an endogenous labeling procedure was set up, in which a primed constant (12 h) infusion of deuterium-labeled leucine is administered to fasted study subjects. Fractional rate of appearance of newly-synthesized, stable isotope-enriched, apolipoprotein in plasma pools is determined by gas chromatography-mass spectrometry (GC-MS). Residence times (RT) are derived from tracer to tracee ratios using SAAM II computer software, and transport rates (TR) are calculated from residence times and pool sizes (measured by ELISAs). Two kinetic studies were conducted using this procedure. In the first, plasma kinetics of apolipoprotein (apo) C-III and apoE were simultaneously investigated in normolipidemic (NL) subjects (n=5), hypertriglyceridemic (HTG) patients (pts.) (n=5) and in pts. (n=2) with type III (TypIII) hyperlipoproteinemia (both groups of patients having reduced catabolism of very low-density lipoprotein (VLDL) apoB-100). Elevated total plasma and VLDL apoC-III concentrations in HTG and TypIII pts., although associated with lower rates of apoC-III catabolism, were mainly due to significantly increased rates of apoC-III production (plasma apoC-III TR (mean ± SE): (NL) 2.05 ± 0.22, (HTG) 4.90 ± 0.81 ($P < 0.01$), and (TypIII) 8.78 $mg.kg^{-1}.d^{-1}$; VLDL apoC-III TR: (NL) 1.35 ± 0.23, (HTG) 5.35 ± 0.85 ($P < 0.01$), and (TypIII) 7.40 $mg.kg^{-1}.d^{-1}$). Elevated total plasma and VLDL apoE concentrations in HTG and particularly in TypIII pts. were associated with increased VLDL apoE RT (0.21 ± 0.02, 0.46 ± 0.05, and 1.21 days, NL vs. HTG vs. TypIII, respectively), as well as significantly increased apoE TR (plasma: (NL) 2.94 ± 0.78, (HTG) 5.80 ± 0.59 ($P < 0.01$) and (TypIII) 11.80 $mg.kg^{-1}.d^{-1}$; VLDL: (NL)1.59 ± 0.18, (HTG) 4.52 ± 0.61 ($P < 0.01$) and (TypIII) 11.95 $mg.kg^{-1}.d^{-1}$). These results demonstrate that

hypertriglyceridemic patients, having reduced rates of VLDL apoB-100 catabolism (including patients with type III hyperlipoproteinemia) are characterized by overproduction of plasma and VLDL apoC-III and apoE. In the second study the plasma kinetics of apoA-I and apoA-II were investigated in control subjects (n=4) and pts. (n=2) with Familial HDL Deficiency (FHD). These pts. had a marked reduction in the plasma concentration of high-density lipoprotein (HDL) cholesterol and apoA-I but lacked clinical manifestations of Tangier disease (TD). Mature plasma apoA-I TRs were similar in pts. and controls (7.9 and 9.1 vs. 10.5 ± 1.7 $mg.kg^{-1}.day^{-1}$). RTs of mature apoA-I were however significantly less in FHD patients (0.79 and 1.66 days) compared to controls (5.32 ± 1.05 days). Normal levels of plasma proapoA-I (the precursor protein of apoA-I) in FHD patients were associated with normal plasma proapoA-I TRs (7.8 and 10.4 vs. 10.9 ± 2.6 $mg.kg^{-1}.day^{-1}$), and proapoA-I RTs (0.18 and 0.15 vs. 0.16 ± 0.03 days). The RTs of apoA-II were however less in patients (3.17 and 2.92 days) vs. controls (7.24 ± 0.71 days), while TRs of apoA-II were similar (1.8 and 1.9 vs. 1.7 ± 0.2 $mg.kg^{-1}.day^{-1}$). Increased plasma catabolism of apoA-II in FHD patients was associated with the presence in plasma of abnormal apoA-II-HDL without apoA-I. These results demonstrate that FHD in these pts. is characterized, like TD, by hypercatabolism of mature apoA-I and apoA-II, but unlike TD, by essentially normal plasma catabolism and concentration of proapoA-I.

Résumé

Les apolipoprotéines jouent un rôle majeur dans la régulation du métabolisme et de la concentration plasmatique des lipoprotéines riches en triglycérides ou en cholestérol. La concentration plasmatique des apolipoprotéines ainsi que leur distribution relative parmi les fractions lipoprotéiques sont déterminées par leurs taux de production et de catabolisme. Dans le but d'étudier la cinétique plasmatique de ces protéines chez des sujets normolipidémiques et dyslipidémiques, un protocole de marquage endogène a été mis au point dans lequel une dose initiale suivie par une infusion constante (12h) de leucine deutériée sont administrées aux sujets à jeun. Le taux fractionnel de formation des apolipoprotéines nouvellement synthétisées et enrichies en isotopes stables est mesurée dans les pools plasmatiques par chromatographie en phase gazeuse couplé à la spectrométrie de masse. Le temps de résidence (TR) est déterminé par ordinateur (SAAM II software) à partir du rapport traceur/tracé, et le taux de transport (TT) est calculé à partir du temps de résidence et de la taille des pools (mesurés par dosage ELISA). Deux études cinétiques ont été menées d'après ce protocole. Dans la première, les cinétiques plasmatiques de l'apolipoprotéine (apo) CIII et de l'apoE ont été étudiées simultanément chez des sujets normolipidémiques (NL) (n=5), hypertriglycéridémiques (HTG) (n=5) et hyperlipoprotéinémiques de type III (TypIII) (n=2) –les 2 groupes de patients ayant un catabolisme réduit d'apoB100 contenue dans les lipoprotéines de très faible densité (VLDL)-. Il en résulte une concentration élevée en apoCIII totale plasmatique ou en apoCIII associée aux VLDL chez les patients HTG et TypIII. Cette augmentation est cependant corrélée à un faible taux catabolique de l'apoCIII et peut être essentiellement attribuée à une élévation significative du taux de production d'apoCIII (apoCIII/plasma TT (moy ± SE): NL 2.05 ± 0.22, HTG 4.90 ± 0.81 ($p<0.01$) et TypIII 8.78 $mg.kg^{-1}.d^{-1}$; apoCIII/VLDL TT:NL 1.35 ± 0.23, HTG 5.35 ± 0.85 ($p<0.01$) et TypIII 7.4 $mg.kg^{-1}.d^{-1}$). Il en résulte également une concentration élevée en apoE plasmatique totale et dans les VLDL chez les patients HTG, plus marquée chez les TypIII, qui peut être associée à une

augmentation du TR de l'apoE des VLDL (0.21 ± 0.02, 0.46 ± 0.05 et 1.21 jours, NL, HTG, TypIII respectivement) ainsi qu' à une augmentation du TT de l'apoE (plasma: NL 2.94 ± 0.78, HTG 5.8 ± 0.59 ($p<0.01$) et TypIII 11.80 $mg.kg^{-1}.d^{-1}$; VLDL: NL 1.59 ± 0.18, HTG 4.52 ± 0.61 ($p<0.01$) et TypIII 11.95 $mg.kg^{-1}.d^{-1}$). Ces résultats démontrent que les patients HTG, ayant un taux catabolique réduit d'apoB-100 associée aux VLDL (patients TypIII compris), sont caractérisés par une surproduction d'apoCIII et d'apoE dans le plasma et les VLDL. Dans la seconde étude, les cinétiques d'apoAI et d'apoAII ont été mesurées dans le plasma de sujets témoins (n=4) et de patients (n=2) ayant une déficience familiale en HDL (FHD). Ces patients présentent une nette réduction de la concentration en cholestérol et apoAI associés aux lipoprotéines de haute densité (HDL) sans manifester aucun des traits cliniques de la maladie de Tangier (TD). Les TTs de l'apoAI plasmatique mature sont similaires chez les patients et les contrôles (7.9 et 9.1 vs 10.5 ± 1.7 $mg.kg^{-1}.d^{-1}$.). Néanmoins, les TRs de l'apoAI mature sont significativement inférieurs chez les patients FHD (0.79 et 1.66 jours) à ceux des témoins (5.32 ± 1.05 jours). Les taux plasmatiques de pro-apoAI (la protéine précurseur de l'apoAI) normaux chez les FHD sont associés aux TTs normaux (7.8 et 10.4 vs 10.9 ± 2.6) et aux TRs normaux (0.18 et 0.15 vs 0.16 ± 0.03 jours) de pro-apoAI. A l'inverse, les TRs de l'apoAII sont inférieurs chez les patients (3.17 et 2.92 jours) que chez les contrôles (7.24 ± 0.71 jours) tandis que les TTs sont équivalents (1.8 et 1.9 vs 1.7 ± 0.2 $mg.kg^{-1}.d^{-1}$). Le catabolisme plasmatique accru de l'apoAII chez les patients FHD a été associé à la présence de HDL-apoAII dépourvues d'apoAI, anormales dans le plasma. Ces résultats démontrent que FHD est caractérisée chez ces patients, comme TD, par un hypercatabolisme de l'apoAI mature et de l'apoAII. En revanche, FHD chez ces patients est associée à un catabolisme et à une concentration plasmatique de la proapoAI normaux, à l'inverse de TD.

Chapter 1

Literature Review

1.1. Atherosclerosis

Atherosclerosis is the major cause of morbidity and mortality in much of the world, particularly in Western societies, where it accounts for close to 50 % of deaths (1). Atherosclerosis is not merely a disease in its own right, but a complicated, multi-stage, multi-factorial process which is central to the pathogenesis of myocardial infarction, stroke, and peripheral vascular disease (2).

The development of atherosclerotic lesions is associated with the occurrence of cellular responses that define an inflammatory-fibroproliferative response to injury (the "response to injury" hypothesis) (3). Manifestations of such a response include: 1) smooth muscle proliferation, 2) expression of adhesive glycoproteins on the surfaces of endothelial cells, 3) recruitment of circulating monocytes and T lymphocytes, and their subsequent migration between endothelial cells into the intima (under the influence of growth regulatory molecules and chemoattractants), 4) differentiation of monocytes to macrophages, 5) extensive entrapment of lipids, 6) formation of fatty streaks, and 7) development of these streaks (with continuous cell influx and proliferation) into fibrous plaques and ultimately complicated lesions (Fig 1.1) (4-7).

1.2. Risk Factors in Atherosclerosis

Several risk factors have been identified in coronary heart disease (CHD) and atherosclerosis, namely, high levels of serum cholesterol, low levels of high

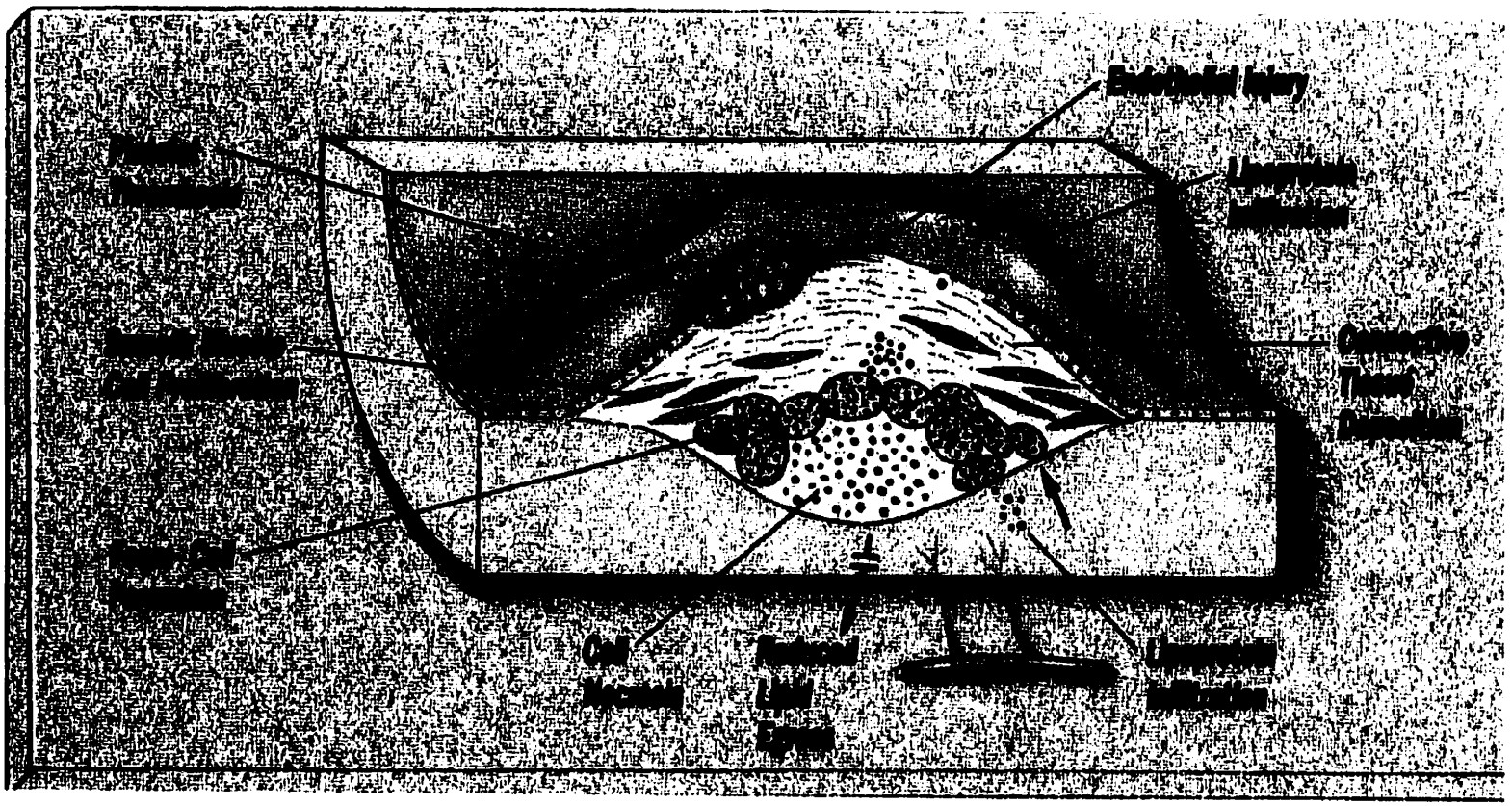


Figure 1.1. Pathological processes leading to the development of an atherosclerotic plaque (8).

density lipoprotein cholesterol (HDL-C), hypertension, smoking, diabetes, male sex, and family history. Additional less highly correlated risk factors include high dietary fat and cholesterol intake, high plasma triglyceride (TG) level, obesity, lack of physical activity, high plasma fibrinogen level, high leukocyte count, homocysteinemia, and baldness (9).

1.3. Plasma Lipoproteins and Atherosclerosis

Epidemiological and clinical evidence has demonstrated the existence of a strong association between elevated levels of serum cholesterol and the presence of CHD (Fig 1.2) (10). This association is due to an involvement of lipoproteins in the mechanism of atherosclerotic lesion formation, whereby certain lipoproteins (mainly cholesterol-rich low density lipoproteins (LDL), but also triglyceride rich lipoproteins (TRL) and their remnants), filter into the arterial wall and are trapped in the intima, due to their apoB attachment to proteoglycans. They undergo chemical modifications (particularly oxidation) and are taken up by macrophages, thus causing the formation of foam cells. The accumulation of foam cells leads to the development of fatty streaks which are the precursors of fibrous plaques-proliferative lesions whereby a fibrous cap covers a lipid core. Finally, a complicated lesion is formed. This lesion frequently underlies the acute clinical events of arterial occlusion, and is characterized by calcification, hemorrhage, ulceration and thrombosis (10). The theory that recognizes and explains the role of lipoproteins in the development of atherosclerosis and CHD is termed the lipid theory. The theory that addresses

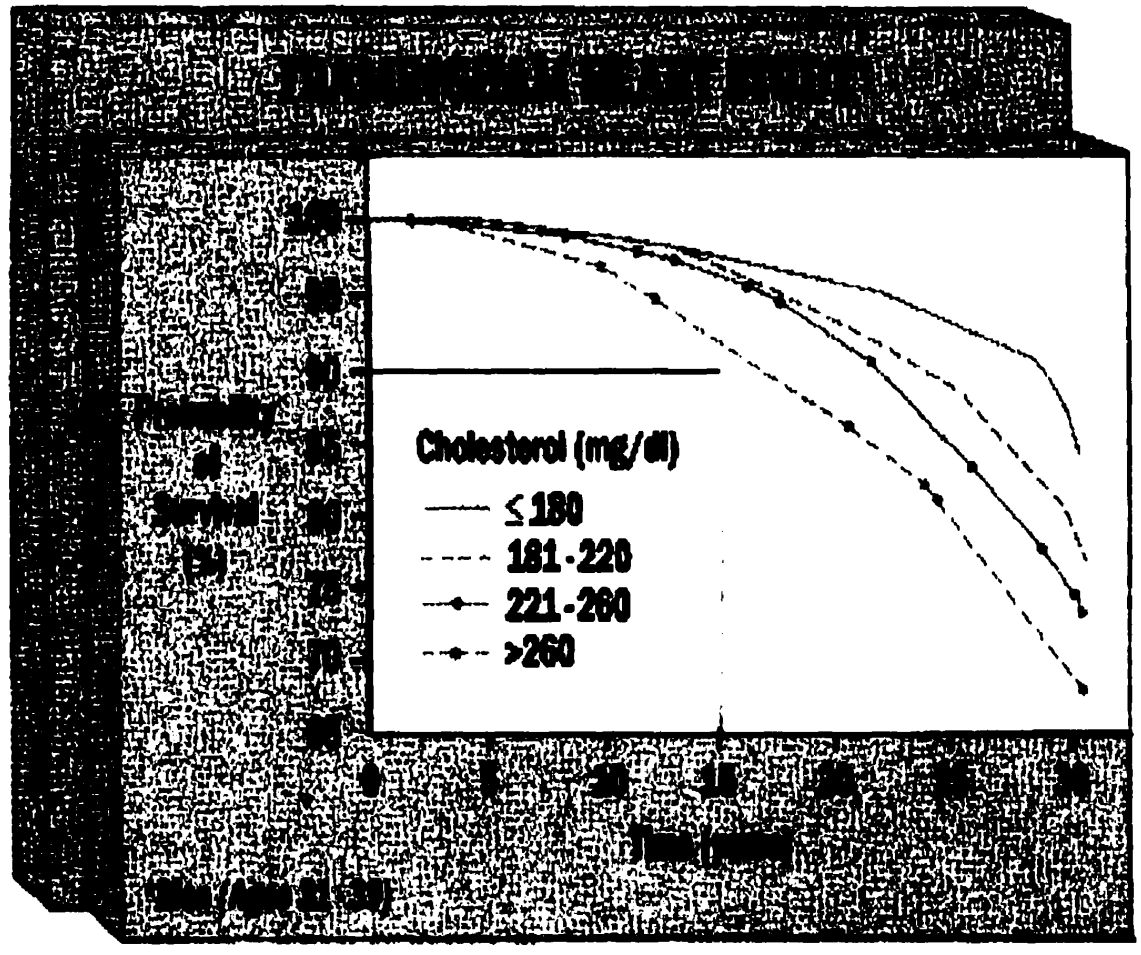


Figure 1.2. Thirty-year mortality by serum cholesterol level for men in the Framingham Heart Study (8).

the atherogenic role of oxidized lipoproteins (as opposed to native lipoproteins) is termed the oxidation theory. Ongoing research is nevertheless required to further define the physiology and pathophysiology of plasma lipoproteins.

1.4. Lipoproteins

Lipoproteins are lipid-protein spherical or discoidal complexes that are involved in lipid transport in the circulation. They make up a heterogeneous population of different-sized particles having a common general composition. They have a hydrophobic core consisting of neutral lipids (i.e. cholesterol esters (CE) and TG), and a more hydrophilic surface coat which contains polar lipids (i.e. unesterified cholesterol and phospholipids) as well as several protein molecules termed apolipoproteins or apoproteins (Fig 1.3) (8). Apolipoproteins have several functions: 1) they are structural proteins which serve to stabilize lipoprotein particles, 2) they are required for normal synthesis and secretion of lipoprotein particles, 3) they function as co-factors for plasma lipoprotein-processing enzymes, and 4) they are ligands for cell-specific receptors, which are involved in the uptake of lipoproteins. Lipoproteins can be separated according to density by ultracentrifugation, or to charge by electrophoresis. They have also been separated according to their size by gel permeation chromatography.

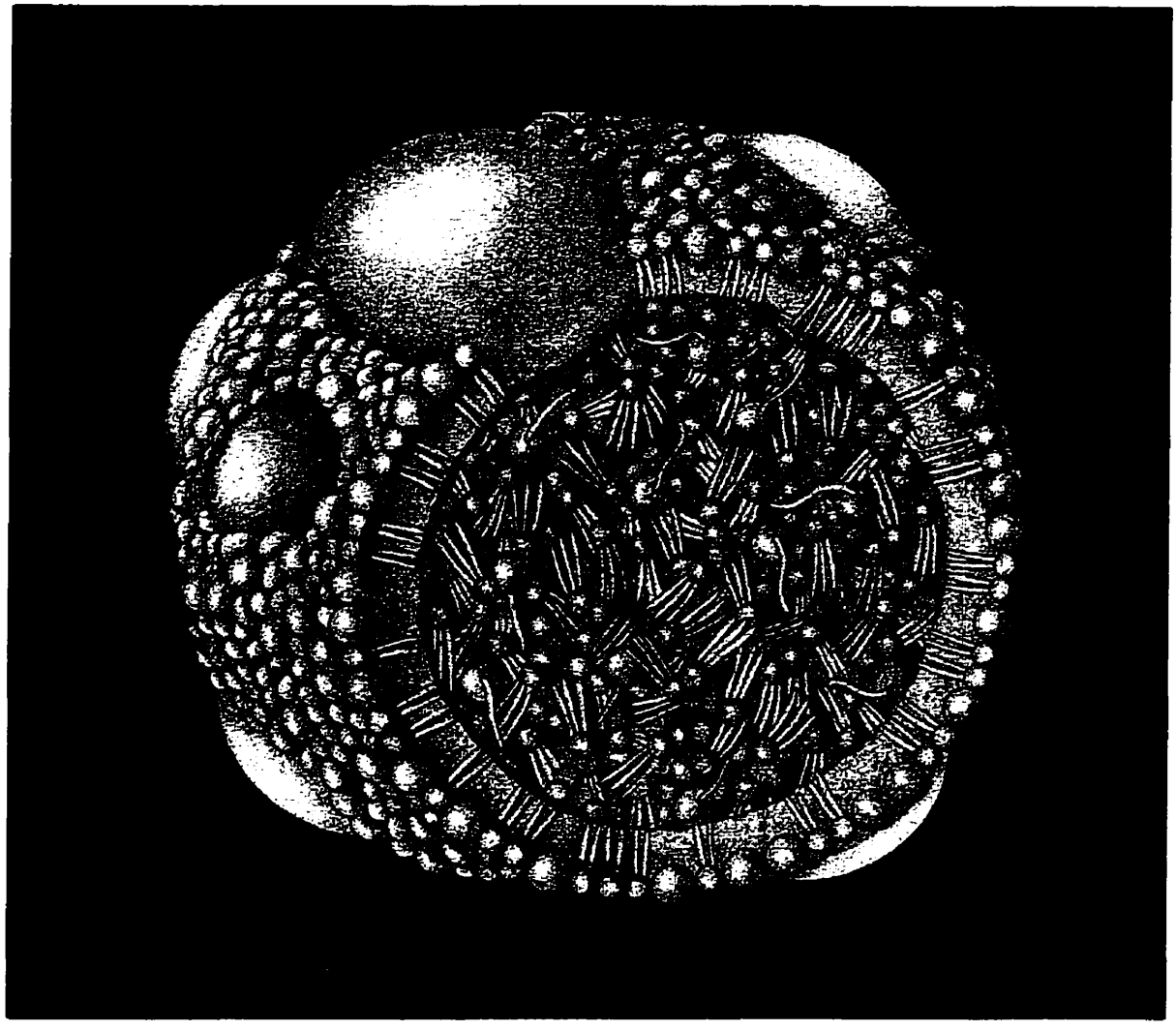


Figure 1.3. Schematic representation of the structure of one apolipoprotein, the very low density lipoprotein (VLDL) (8).

1.4.1. Chylomicrons

Chylomicrons are the largest and least dense of all lipoproteins, with a diameter of 75-450 nm and density of approximately 0.98 g/ml. They are of intestinal origin and are involved in the transport of dietary (exogenous) cholesterol and TG (11). The major constituents of the lipid core of chylomicrons are TG (85-92%) and cholesterol ester. In contrast, the surface coat is rich in phospholipids and also contains free cholesterol and apolipoproteins (apo)B-48, C-III, A-I, A-IV and E. The latter protein is not thought to be secreted on newly-synthesized particles but to be acquired in the mesentric lymph (11-13). ApoB-48, which is the product of the translation of an edited copy of apoB-100 mRNA, is synthesized only in the intestine and not in the liver in humans. Consequently, it is exclusively present on chylomicrons and their remnants. The nuclear multicomponent enzyme complex which mediates apoB-100 mRNA editing includes the catalytic subunit Apobec-1 (14). Once they reach the peripheral circulation, chylomicrons are lipolyzed by the enzymatic activity of lipoprotein lipase (LPL) which is attached to the surface of capillary endothelial cells through binding to proteoglycans. The enzyme hydrolyzes the TG-rich core, releasing fatty acids. Fatty acids either fuel muscular cells or are reesterified in adipocytes. Chylomicron remnants formed during lipolysis are relatively rich in CE and apoE, and are taken up by hepatic lipoprotein receptors (15,16) (the role of apoE in the metabolism of TRL and their remnants is discussed in section 1.8.2). Disruption of chylomicron catabolism due to LPL deficiency manifests in

chylomicronemia (type I hyperlipidemia) which is probably not associated with the presence of CHD but may cause acute pancreatitis (17).

1.4.2. VLDL

Very low-density lipoproteins (VLDL), as well as chylomicrons, are separated from plasma by ultracentrifugation at a density less than 1.006 g/ml, and by gel permeation chromatography. VLDL are however smaller than chylomicrons (400-700 Å), and unlike chylomicrons, they are mainly of hepatic origin and have apoB-100 as their structural protein. Furthermore, VLDL migrate as a distinct preβ band when separated from plasma of normolipidemic subjects by agarose gel electrophoresis, while chylomicrons remain at the origin. VLDL have a core which consists mainly of TG and some CE, and a surface coat that consists mainly of unesterified cholesterol, phospholipids, and apolipoproteins (apoB-100, apoE, apos C-I, C-II, and C-III). ApoB-100, the structural protein of VLDL, is synthesized in the rough endoplasmic reticulum (RER) in hepatic cells. A significant fraction of newly-synthesized apoB-100 molecules is degraded while being translocated across the RER membrane (18). Newly-synthesized apoB-100, and possibly apoE, pass to the smooth endoplasmic reticulum (SER) where they are assembled with phospholipids (and subsequently TG and CE) to form nascent VLDL (one apoB-100 molecule per particle) (19). Assembly of the VLDL particle is mediated by the microsomal TG transfer protein (MTP) (20). Nascent VLDL subsequently migrate through the Golgi, where their

apolipoproteins get glycosylated (21). Finally, VLDL are transported through vesicles and bud off the cellular membrane.

In plasma, VLDL make up a population of heterogeneous particles with densities ranging from Sf 400 to 20 (22). Mature VLDL particles are rich in apoE and apoC-III. They come second only to HDL in their content of these apoproteins in normolipidemic subjects. Nascent VLDL isolated from the Golgi have been found to contain newly-synthesized apoE and apoC-III (23). However, It is believed that during their maturation in plasma, VLDL acquire apoC-III, and apoC-II, and possibly more apoE from HDL (24,25). Once in plasma, VLDL are subjected to the lipolytic effect of LPL and hepatic lipase (HL) which hydrolyze the TG core of VLDL (Fig 1.4). The resulting fatty acids are taken up by muscle cells and adipocytes. VLDL TG can also be transferred to HDL in exchange for CE through the action of cholesteryl ester transfer protein (CETP) which is associated with HDL (27). The formation of VLDL remnants is thus characterized by a progressive absolute and relative enrichment with CE. These remnants can suffer one of two fates: 1) they can be cleared from plasma by binding lipoprotein receptors, such as the low density lipoprotein receptor (LDL-R), and apoE specific receptors, such as LDL-R related protein (LRP) and the VLDL receptor (VLDL-R) or 2) they can progress along a lipolytic cascade to eventually become more cholesterol-enriched and denser LDL particles. In normolipidemic subjects, 30% to 40 % of all VLDL remnants are converted to LDL (8). The relative size of this fraction, however, changes in certain dyslipidemias. The conversion to LDL is also characterized by conformational

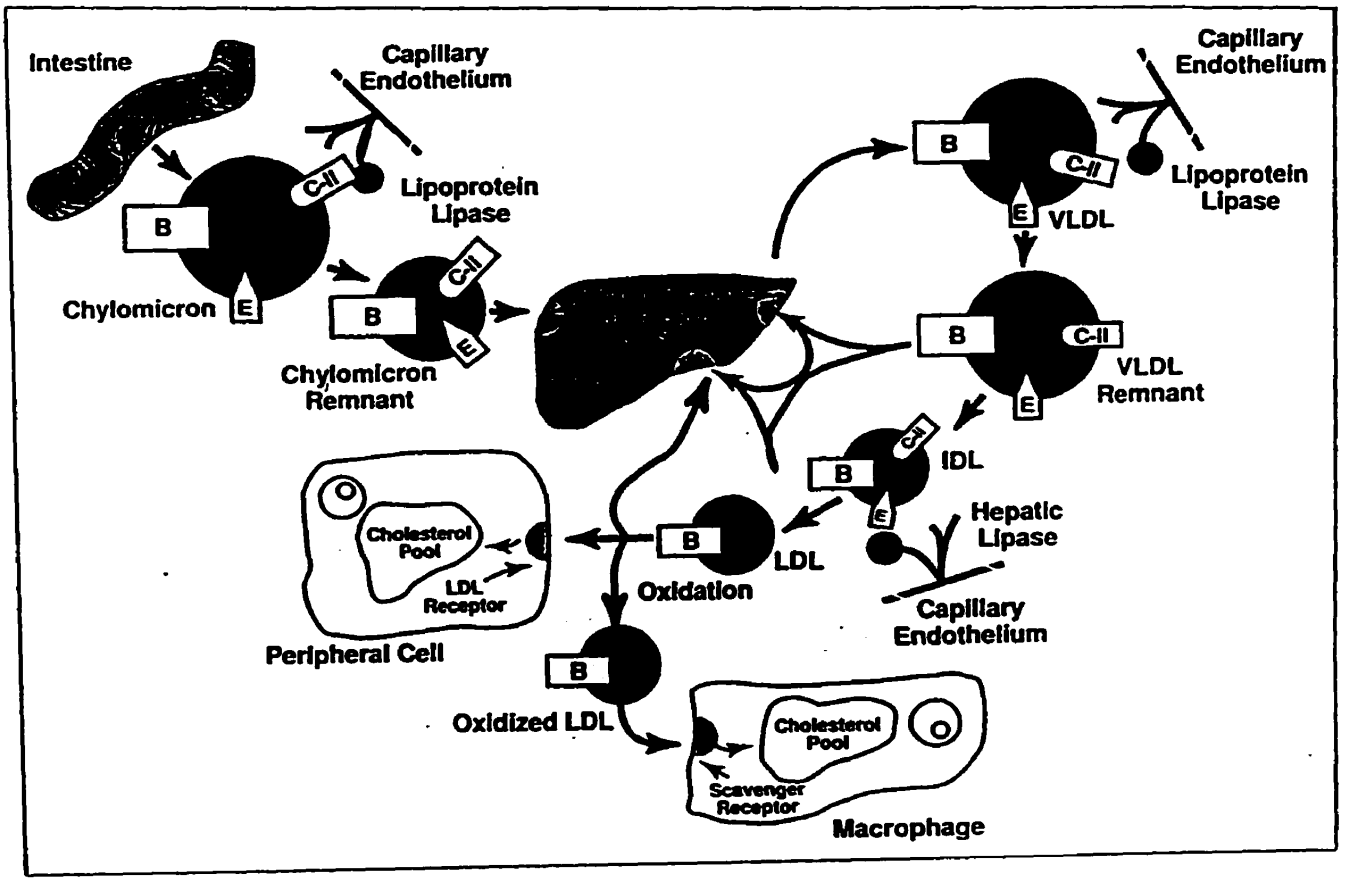


Figure 1.4. Schematic representation of the metabolic pathways of apoB-containing lipoprotein particles (26).

changes in apoB-100 which expose antigenic sites of this molecule, thus allowing its participation in binding to the LDL-R (28).

1.4.3. LDL

LDL are considered the most atherogenic of lipoproteins. Several epidemiological studies have confirmed a strong association between the presence of elevated plasma LDL-cholesterol (LDL-C) and CHD (11,29). LDL (diameter 225-275 ^{0}A) are smaller than TRL and are separated by ultracentrifugation at a density between 1.019 and 1.063 g/ml. They migrate on agarose gels to β position and are separated by gel permeation chromatography in the intermediate density lipoprotein (ISL) fraction. The core of LDL consists almost entirely of CE, while the surface consists of phospholipids and free cholesterol, with apoB-100 being the main apolipoprotein (30).

The LDL-R (or B/E receptor) is a specific receptor for LDL. It is responsible for the uptake of two thirds of plasma LDL (31). The binding of LDL to LDL-R, triggers an endocytic process, whereby the ligand-receptor complex is internalized into lysosomes. In lysosomes, apoB-100 and CE are hydrolyzed into amino acids, free cholesterol and fatty acids, through the action of proteases and acidic cholesterol esterase. The LDL-R is recycled back to the surface of the cell. Free cholesterol regulates the expression of several genes which are involved in intracellular cholesterol homeostasis, namely those expressing LDL-R (32), 3-hydroxy 3 methylglutaryl coenzyme A reductase (HMG-CoA reductase)

(33), and caveolin (34). It has been shown that free cholesterol exerts its regulatory effect by inhibiting release from the nuclear membrane and subsequent nuclear localization of the active segment of sterol regulatory element binding proteins (SREBP) (35). The active segment of SREBP acts as a transcriptional modulator of LDL-R, HMG-COA reductase and caveolin genes by binding to sterol regulatory elements (SRE) in their promoters (34-37).

A significant body of evidence supports a role for modified LDL, particularly oxidized LDL, in atherogenesis (38-43). Uptake and subsequent accumulation of LDL-C by monocyte-derived macrophages in the intima (responsible for the formation of foam cells) is probably due to non-regulated uptake of modified LDL through a scavenger receptor and not due to regulated uptake of native LDL through the LDL-R (44). Native LDL can be modified in several ways once trapped in the intima: 1) acetylation (evidence only *in vitro*) (45), 2) attachment of a malonaldehyde molecule to apoB-100 (46), and 3) oxidation in the presence of metallic cations (Fe^{+3} and Cu^{+2}) (47) through the enzymatic activity of lipoxygenases (48), cycloxygenases (49), and xanthine-oxidase (50) found in endothelial cells, macrophages, and smooth muscle cells. Platelets are also able to oxidize LDL *in vitro* (51). LDL oxidation modifies the secondary structure of apoB-100 (52). It also affects polyunsaturated fatty acids, and the whole structure of the particle, making it prone to engulfment by macrophages (48).

Several genetic defects in the LDL-R have been identified as the causes of familial hypercholesterolemia (FH), characterized by an elevation in plasma

LDL-C and increased risk of atherosclerosis and CHD (31). These genetic defects affect either the synthesis, maturation and transport to cell surface, ability to bind ligands, internalization or recycling of LDL-R. The clinical phenotype of FH has also been demonstrated in patients with a mutation in apoB ($Arg^{3500} \rightarrow Gln$) (53,54). The metabolism of LDL, the evidence supporting its atherogenicity, and the clinical manifestations and molecular bases of FH have been extensively reviewed (31).

One lipoprotein that is related to LDL is Lp(a) (reviewed in 55). This lipoprotein consists of an LDL particle to which a long polymorphic peptide chain, apo(a), is attached by a disulfide bridge. Lp(a) may have atherogenic as well as thrombogenic properties and its level is genetically determined.

1.4.4. HDL

HDL are the smallest of the lipoproteins (diameter 75-100 ^{0}A). They are separated by ultracentrifugation at a density 1.063-1.25 g/ml, and are the last particles to elute off the column in gel permeation chromatography. HDL is a heterogenous lipoprotein fraction, comprising several discrete subpopulations that differ in size, density, electrophoretic mobility and lipid and apolipoprotein composition. The majority of HDL migrate to an α position when separated from plasma by electrophoresis and the rest (2-10% in normolipidemic subjects) to a pre-β position and possibly γ (as revealed by two-dimensional gel electrophoresis) (56). HDL can be further subdivided by size and lipid content to at least 5 distinct subpopulations: Pre-β1, pre-β2, HDL_{2a}, HDL_{2b}, and HDL_3 with

HDL_{2b} being the largest and most lipid-enriched (57). Pre-β1 is a lipid-poor apoA-I particle with lecithin and sphingomyelin being its lipid constituents (58). Pre-β2 is a discoidal particle with apoA-I as its structural protein, and lecithin and cholesterol (and some sphingomyelin) as the lipid portion (58). The diameter of pre-β2 varies with lipid content and the number of apoA-I molecules per particle. In contrast, the thickness of the disc is constant, as the particle is made of a single lipid bilayer stabilized by a protein at the periphery (58). Larger HDL particles (HDL_2 and HDL_3) assume a spherical shape due to their lipid-rich core, composed mainly of CE. Spherical HDL also contain phospholipids, and their structural proteins are apoA-I (LpA-I particles) or both apoA-I and apoA-II (LpAI:AII particles) (58). Finally, apoE and apoC-III are present on different subpopulations of HDL and are exchangeable with those of VLDL (25, 59-65).

Epidemiological studies have consistently demonstrated that low plasma levels of HDL-C are associated with the presence of CHD (66). This association has been mostly attributed to a role of HDL in mediating reverse cholesterol transport (RCT) (67), whereby HDL mediate the transfer of cholesterol from peripheral cells to the liver, its site of elimination. The antiatherogenic role of HDL and their metabolism are discussed further in sections 1.9.1 and 1.9.2.

1.5. Apolipoproteins

1.5.1. ApoB

The human apoB gene contains 29 exons and 28 introns and spans approximately 43 Kb of the genome (68). ApoB is found in 2 major forms in

human plasma and thoracic duct lymph. One form, designated apoB-100, has a molecular weight of 549 KDa (4536 amino acid residues), and the other, apoB-48, has a molecular weight of 264 KDa (2152 amino acid residues). ApoB-48 is identical to the N-terminal 48% of apoB-100 (hence the name) (69), and is synthesized through the translation of an edited copy of apoB mRNA, as mentioned earlier (section 1.4.1). The multicomponent editing enzyme which includes apobec-1 replaces the nucleotide cytidine with uridine at position 6666 of apoB mRNA, changing the codon CAA (Glutamine 2153) to UAA (a stop codon) (69). In humans and non-rodent animals, apoB-48 is produced exclusively in the small intestine, while in rats and mice, the liver contributes significantly to apoB-48 synthesis (70-72). ApoB-100 is found in plasma as a sole copy on VLDL and their remnants, IDL, and LDL particles. It is also part of Lp(a) where it is found linked to apo(a) by disulfide bridging. ApoB-48 is found on chylomicrons and their remnants.

ApoB-100 is a glycosylated protein (16 N-linked glycosylations) (73,74). There are 25 cysteine residues in apoB-100 and they are asymmetric in distribution, with a concentration in the N-terminal region. Based on the Chou-Fasman algorithm, the predicted secondary structure of apoB-100 consists of 43% α-helices, 21% β-sheet structure, 20% random coil structure, and 16% β-turns. Immunoelectron microscopic analysis of apoB-100 on LDL suggests that the protein is extended and spans at least a hemisphere of the particle surface (75). By sequence alignment with LDL-R binding sequences of apoE, two regions in apoB-100 (amino acid residues 3147-3157 and 3359-3367) have been

identified as receptor binding sites (76). Being devoid of these regions, apoB-48 does not have any receptor binding activity. Two types of peptide sequences in apoB-100 are believed to be important in lipid binding: amphipathic α-helices, which are common among all apolipoproteins, and proline-rich hydrophobic sequences with β-sheet potential, which are unique to apoB (77,78).

In patients with familial hypobetalipoproteinemia, different lengths of N-terminal apoB sequences are synthesized. Studies on these patients (79,80), as well as expression of truncated forms of apoB in rat hepatoma cells (81), have shown that truncated copies of apoB are distributed differently between plasma lipoprotein fractions, depending on their length.

1.5.2. ApoA-I and apoA-II

1.5.2.1. ApoA-I

The apoA-I gene (1863 bp long) is found on the long arm of chromosome 11, and is clustered with 2 other apolipoproteins genes, apoC-III and apoA-IV (Fig 1.5) (82,83). ApoA-I (28 KDa) consists of 22-amino acid amphipathic α-helices separated by helix-breaking proline residues. Each 22 amino acid helix consists of two 11-amino acid subunits (85). The apoA-I gene is transcribed in the liver and the intestine (86). At least one cis-acting DNA element, required for intestinal expression of the apoA-I gene, is distinct from the liver elements (87). This element resides 3' to the gene between the apoC-III and apoA-IV genes. It is required for the intestinal expression of the apoA-I gene and may also control the intestinal expression of the entire apoA-I, apoC-III, and apoA-IV gene locus.

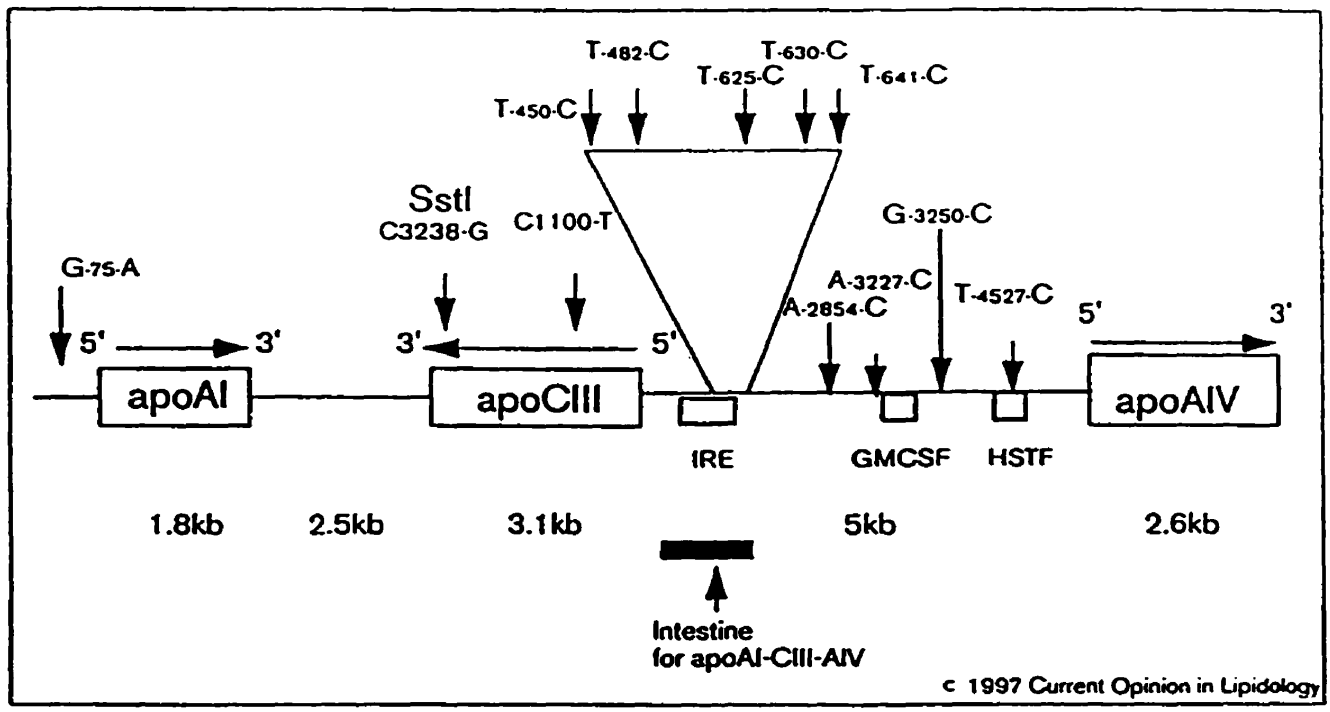


Figure 1.5. Schematic representation (not drawn to scale) of apoAI/C-III/A-IV gene cluster showing selected variability. GMCSF, concensus sequence of the granulocyte macrophage colony-stimulating factor; HSTF, consensus binding site for the heat -shock transcription factor; IRE, negative insulin-responsive elements (84).

Besides being the major structural protein in HDL, apoA-I has other functions: 1) it can activate lecithin: cholesterol acyl transferase (LCAT) (discussed in section 1.6.3), 2) it is an acceptor of free cholesterol derived from cells and thus an important participant in reverse cholesterol transport, and 3) apoA-I controls the selective uptake of HDL-C esters by the liver and by steroid hormone-producing (steroidogenic) tissues (88).

Overexpression of human apoA-I in transgenic mice, fed an atherogenic diet, increases plasma HDL levels and reduces atherosclerosis (89). Overexpression of human apoA-I in apoE-deficient transgenic mice increases HDL-C, and suppresses atherosclerosis and formation of fibroproliferative lesions (90). Overexpression of human apoA-I in transgenic rabbits, fed a cholesterol-rich diet, leads to an almost 50% inhibition of aortic lesions and cholesterol accumulation in the aortic wall (91).

At least one copy of apoA-I is present on each HDL particle in plasma of normolipidemic subjects. The preproapolipoprotein synthesized in hepatocytes and enterocytes is cleaved intracellulary to form a 249 amino acid proprotein, which is rapidly cleaved by a plasma protease to generate the mature 243 amino acid form (92). ApoA-I forms three types of stable structures with lipids: 1) small lipid-poor complexes (preβ-1 HDL), 2) flattened discoidal particles containing only polar lipids, namely cholesterol and phospholipids (preβ-2 HDL), and 3) spheroidal particles containing both polar and nonpolar lipids (α HDL) (Fig 1.6) (58). Preβ1-HDL are particles with a molecular weight of 60-70 KDa and a preβ-1 electrophoretic mobility on agarose gels. ApoA-I is the only protein on preβ-1

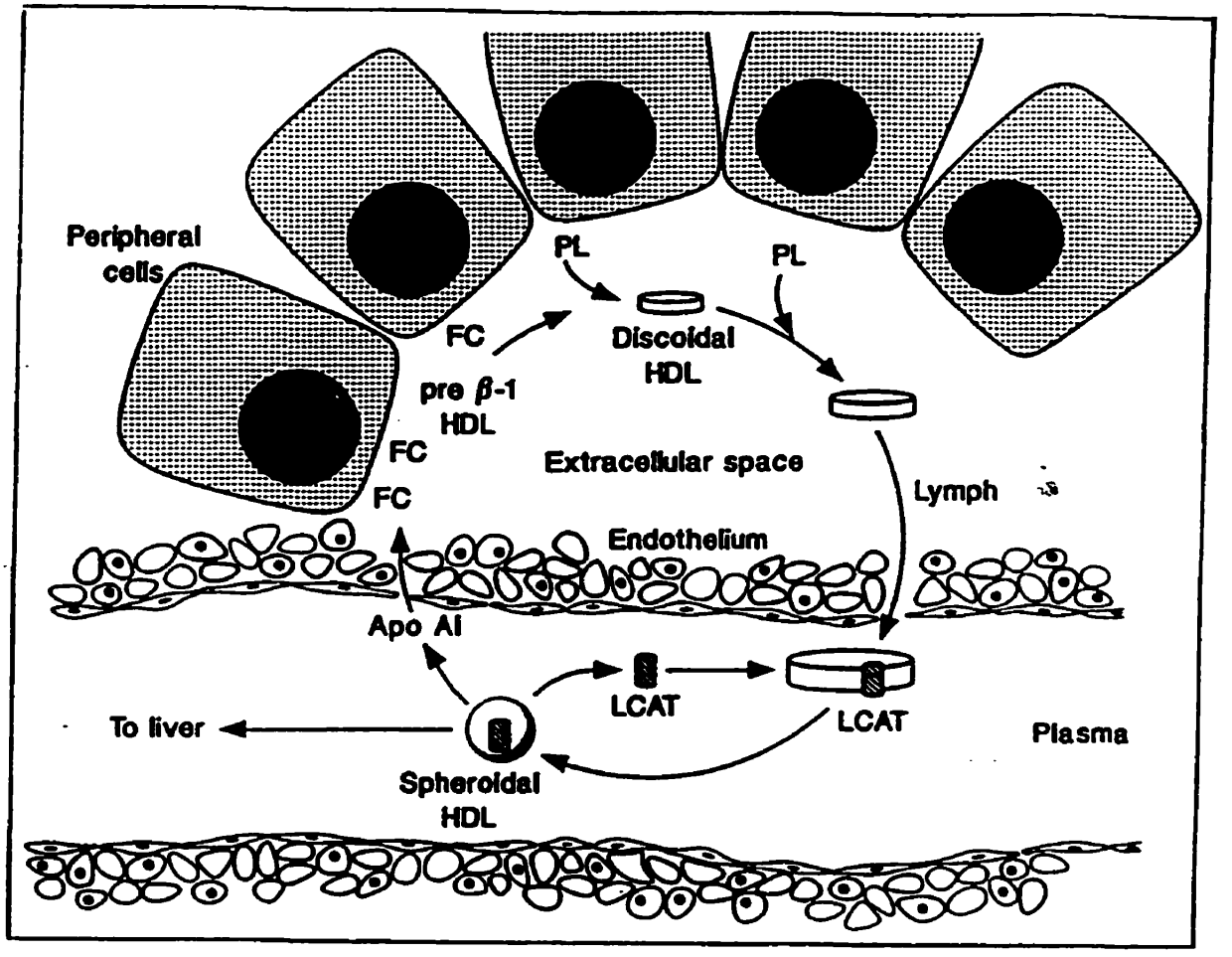


Figure 1.6. The HDL maturation cycle (58).

HDL. Their lipid content accounts for 10-40 % of their weight and they have equal molar amounts of lecithin and sphingomyelin (93). They are found in higher concentrations in the lymph (93). The conformation of apoA-I in preβ-1 HDL differs from other HDL particles by containing a lower α-helical content. A series of monoclonal antibodies recognizing different epitopes in apoA-I was raised in mice against delipidated apoA-I. An epitope consisting of residues 137-144 of the mature apoA-I polypeptide was recognized on preβ-1 HDL (94). Interestingly, in the presence of this antibody, the ability of preβ-1 HDL to promote the efflux of cholesterol from cell membranes was significantly inhibited. Other sequences in apoA-I of preβ-1 HDL accessible to monoclonal antibodies included the N-terminus (residues 1-10) and residues 167-174. ApoA-I of preβ-1 HDL did not react with a monoclonal antibody that recognized residues 93-99 in discoidal HDL (94), which suggests a unique organization of apoA-I in lipid-poor HDL.

Preβ-2 HDL also contains apoA-I as its only protein moiety. Preβ-2 HDL are rich in lecithin and contain a small proportion of sphingomyelin and cholesterol but not CE. The diameter of their discs vary depending on the lipid content and the number of apoA-I molecules per particle. The thickness, however, is constant. Sectional electron microscopy indicates that discoidal HDL are made of a single lipid bilayer (95) probably stabilized by protein at the periphery. Computer modeling of discoidal HDL suggests that the repeating 22 amino acid helices of apoA-I run from side to side of the disc (96). Like preβ-1 HDL, preβ-2 HDL are present in higher concentrations in the lymph (93). Preβ-2

HDL in plasma reacted strongly with a monoclonal antibody recognizing residues 93-99 but not with that recognizing residues 137-144 in preβ-1 HDL (94). The C-terminal part of apoA-I may be of significance in phospholipid binding in discoidal HDL; deletion of residues 212-243 by mutagenesis inhibited disc formation in one study (97), but shorter deletions within the same region did not (98).

Finally, most plasma HDL are in the form of spherical HDL (9-12 nm in diameter), with an α electrophoretic migration. A significant portion of spherical HDL contains a second structural protein, apoA-II, in addition to apoA-I (particles containing both proteins are termed LpA-I:A-II vs. LpA-I for those containing only apoA-I). Spherical HDL also contain apoE, which is possibly involved in the direct hepatic uptake of CE from HDL, as well as apoC-III and lipid processing enzymes such as LCAT and CETP. The lipid core is composed mainly of CE. Spherical HDL are a heterogeneous population. They can be divided, according to particle size into three subclasses: HDL_3, HDL_{2a}, and HDL_{2b}, in order of increasing size and lipid content. When monoclonal antibodies were raised against spherical HDL, most of them recognized discontinuous epitopes (99) suggesting that the organization of repeating sequences of apoA-I was disordered in spherical HDL compared to that in discs. β-turns from different apoA-I polypeptides may be in contact with each other on the surface of the sphere. In contrast, there seems to be few differences in the conformation of apoA-I in large and small spherical HDL (100).

1.5.2.2. ApoA-II

ApoA-II is the second most abundant apolipoprotein in HDL. It is present in plasma generally as a 17 KDa dimer of two 77-amino acid chains linked by a disulphide bridge (cysteine residue at amino acid 6) (101). ApoA-II consists of multiple repeats of 11 amino acids, and the two lipid-binding domains within it are located at opposite ends (one region includes amino acids 12-31, and the other amino acids 40-77) (77,102,103). ApoA-II is synthesized mainly in the liver as a preproapolipoprotein, which is cleaved cotranslationally to form proapoA-II. ProapoA-II is then cleaved both intracellularly and extracellulary to form the mature protein (101).

The human apoA-II gene is located on the long arm of human chromosome 1 and contains four exons and three introns (104). Studies of the 5' flanking sequence of the human apoA-II gene have identified five regulatory elements within the promoter region that control liver-specific expression of the gene (105,106). In addition, several distal and proximal regulatory elements were also shown to regulate its expression (107). Fibrates, activators of peroxisome proliferator activated receptor (PPAR), increase plasma apoA-II concentrations in humans by increasing its hepatic production via an upregulation of the transcription of A-II mRNA (108).

Unlike apoA-I, no exclusive function has yet been defined for apoA-II. In fact, members of a family with total apoA-II deficiency exhibited relatively normal levels of plasma lipoproteins (109). Several reports have suggested that LpA-I:A-II are less efficient at promoting cholesterol efflux from cells (110,111). This

however has been disputed (112,113). It has also been shown that the plasma clearance rate of LpA-I:A-II is less than LpA-I, suggesting that, as a structural protein, apoA-II serves to stabilize the HDL particle (114).

Overexpression of mouse apoA-II gene (2.5-fold increase in plasma apoA-II) in transgenic mice results in an increase in total plasma cholesterol and plasma HDL levels (115,116). Plasma TG levels increase several times as well, and this TG is found mainly in the VLDL fraction. Furthermore, transgenic mice which were maintained on a chow diet developed aortic fatty streak lesions, indicating that apoA-II overexpression promotes atherosclerotic development in the mouse (116). In contrast, overexpressing human apoA-II gene in transgenic mice had no effect on plasma lipids in one study (117) and, in another, led to 1) a marked reduction in mouse apoA-II, 2) a decrease in HDL-C and apoA-I, 3) an increase in TG and free cholesterol, and 4) a marked reduction in LCAT activity (118). Finally, coexpression of apoA-II with CETP in transgenic mice resulted in HDL particles that were more TG enriched and resistant to reductions in size and apoA-I content, reflecting inhibition of hepatic lipase by apoA-II (119). Further, apoA-II did not inhibit the lipid transfer activity of CETP *in vivo*.

Homozygous apoA-II knockout mice had marked reductions in HDL-C levels in the fasted and fed states, and HDL particle size was decreased (120). Reduction in HDL levels was due to both decreased transport rates and increased fractional catabolic rates (FCR) of cholesterol esters and apoA-I. Expression of the apoA-II deficiency trait on an apoE-deficient background resulted in a decrease in cholesterol levels and a reduction in the level of

remnant lipoprotein particles due to increased uptake (120). Also, apoA-II deficiency was associated with lower free fatty acids, glucose and insulin levels. In contrast to the evidence for inhibition of hepatic lipase by apoA-II in the transgenic mouse model, a study of substrate properties of different HDL particles for the enzyme showed that fatty acid release was two fold higher from apoA-II-containing HDL compared to HDL devoid of apoA-II. Further, apoA-II-containing HDL competed more effectively with small VLDL for binding to HL compared to HDL devoid of apoA-II (121).

1.5.3. ApoE

ApoE is a glycosylated protein with a molecular weight of almost 34 KDa. It is found in plasma almost equally distributed between VLDL and HDL (122). It is also found in association with chylomicron and VLDL remnants. ApoE has many functions related to the metabolism of lipoproteins and is a ligand for many lipoprotein receptors. Also, one variant of apoE (apoE4) has emerged recently as a major genetic risk factor for Alzheimer's disease (123), which is probably related to its role in transporting lipids in the central nervous system.

The apoE gene is found on chromosome 19 along with those of apoC-I, pseudo apoC-I, apoC-II, and the LDL-R (124,125). ApoE is produced in most organs including the liver, brain, spleen, lung, adrenal, ovary, kidney, and muscle (126-129). It is not produced by the epithelium of the intestine. Also, macrophages derived from the peritoneal cavity of mice or from human blood monocytes produce significant quantities of apoE (130). The major source of

plasma apoE production however is the liver which accounts for almost 90% of apoE in plasma (131). The second largest source, as determined by mRNA expression in tissues, is astrocytes in the brain (126). ApoE is synthesized as a prepeptide with an 18-amino acid NH_2-terminal signal which is cleaved intracellularly (132).

The predicted secondary structure of apoE, based on the Chou-Fasman algorithm predicts α-helices, β-sheets, β-turns, and random structure to make up 62%, 9%, 11%, and 18% of the protein, respectively (124). The conformation of the protein, however, is affected by the size of the lipoprotein particle to which it is attached (133). Like other apolipoproteins, apoE interacts with lipid by its amphipathic α-helical segments (133). Interestingly, apoE contains 2 separate domains: an amino- and a carboxyl-terminal domain, which are connected by a hinge region (approximately residues 165 to 215) (134). The crystal structure for the 22-KDa NH_2-terminal fragment (residues 1-191) of human lipid-free apoE shows that the protein forms an unusually elongated four-helix bundle (135). The basic amino acids which are important for LDL-R binding are clustered into a surface patch on helix-4 of the bundle that includes residues 140-150 (133, 134). Lysine residues are important for apoE binding to the LDL-R. $Lysine^{143}$, $lysine^{146}$, and $lysine^{157}$ are particularly important in this process (135).

ApoE is a polymorphic protein. Using isoelectric focusing and two dimensional electrophoresis, three major isoforms of apoE (apoE2, apoE3 and apoE4), were identified in the general population as gene products of three alleles (ε2, ε3, and ε4) at a single gene locus (136, 137). The three isoforms give

rise to 3 homozygous and 3 heterozygous phenotypes, with the phenotype E 3/3 being the most abundant and E 2/2 being the least abundant (136). The three apoE alleles differ in their amino acid sequence. ApoE4 differs from apoE3 by having an arginine residue instead of a cysteine residue at position 112. ApoE2 differs from apoE3 by having a cysteine residue instead of an arginine residue at position 158. These point changes in the amino acid sequence lead to differences between the three alleles in structure (138), lipoprotein distribution (139,140) and receptor binding properties (135,141).

The replacement of arg^{158} with cys in apoE2, which has weak affinity to the LDL-R, disrupts the naturally occurring salt bridge between Asp^{154} and Arg^{158} (141). A new bond between Asp^{154} and Arg^{150} is formed, shifting Arg^{150} out of the receptor binding region. Elimination of the Asp^{154} - Arg^{150} salt bridge by site-directed mutagenesis of Asp^{154} to Ala restores the receptor binding activity to near normal levels. The X-ray crystal structure of apoE2 Ala^{154} demonstrated that Arg^{150} was relocated within the receptor binding region (141). Reduced affinity of apoE2 to LDL-R shown *in vitro* (142,143) and subsequent delayed catabolism of apoE2 in plasma of patients with type III hyperlipidemia (144) is now believed to be the primary cause for accumulation of TRL and their remnants in these patients. The role of apoE in the metabolism of TRL and their remnants is further discussed in section 1.8.2.

ApoE4 has a preference for VLDL while apoE3 has a preference for HDL (139,145). The difference in lipoprotein preference between the two is attributed to an interaction between the amino- and carboxy- terminal domains in the case

of apoE4 and the absence of such interaction in apoE3. Arg^{112} was not directly involved in the interaction between the two domains (139). However, Glu^{109} formed a salt bridge with Arg^{112} and Arg^{61} side chain was displaced to a new position. The interaction between Arg^{61} in the amino terminus and Glu^{255} in the carboxy terminus was shown to be important in mediating the domain's interaction in apoE4 (140).

Finally, the apoE4 isoform is more common in patients with Alzheimer disease compared with the general population (123). Although the nature of the association between apoE4 and Alzheimer disease is not fully understood, it is suggested that apoE is involved in a final common pathway of neuronal repair and remodeling. ApoE3 is believed to support effective repair and remodeling after neuronal injury by noxious agents, while apoE4 is thought to be less effective in these processes (123).

A secondary form of apoE polymorphism is due to posttranslational glycosylation. The glycosylation (sialylation) occurs intracellularly through O-linkage to threonine residue 194 (132). Subsequent extracellular desialylation has been suggested as the mechanism responsible for the dominance of the asialylated form in plasma. However, Ghiselli et al. (146) have shown the lack of conversion of disialylated apoE to the asialylated form in 3 hypertriglyceridemic patients. Further, in hypertriglyceridemic patients, the ratio of monosialylated to nonsialylated apoE3 in VLDL was significantly higher compared to normal and hypercholesterolemic patients (147).

1.5.4. ApoC-I, C-II and C-III

The apoC group includes 3 apolipoproteins with different biochemical and physiological characteristics.

1.5.4.1. ApoC-I

ApoC-I is the smallest of three C apolipoproteins. It consists of 57 amino acids and has a molecular weight of 6.5 KDa (148). It is usually found on VLDL and to a lesser extent on HDL. ApoC-I is synthesized predominantly in the liver. However, the intestine also contributes to its synthesis (149). The apoC-I gene is found on chromosome 19 adjacent to that of apoE and apoC-II, as mentioned before. ApoC-I is possibly involved in the esterification of cholesterol through the activation of LCAT (150). However, this activation does not exceed 25% that of apoA-I (151). Furthermore, apoC-I inhibits the binding of apoE-enriched β-VLDL to LDL-R and LRP (152,153). The inhibitory effect could not be mapped to a certain region in the protein and is thought to be exerted by the whole molecule (154). It was hypothesized that apoC-I exerts its inhibitory effect by changing the conformation of apoE on β-VLDL, which in turn impairs binding to receptors (155).

1.5.4.2. ApoC-II

ApoC-II consists of 79 amino acids and has a molecular weight of 8.8 KDa (156). It is produced in the liver, and to a lesser extent the intestine, and is associated in plasma with chylomicrons, VLDL and HDL (157). Newly-

synthesized apoC-II gets glycosylated during its passage through the RE and the Golgi and is secreted either in a monosialylated or disialylated form (158). In plasma, the non-sialylated form is formed from the 2 sialylated forms (159). ApoC-II activates LPL (160). Residues 43-79 are apparently involved in this activation (161,162). Mutations in the carboxy terminal of apoC-II, such as apoC-$II_{Toronto}$ and apoC-$II_{St.Michel}$ disrupt this activity (163,164). Total or partial absence of plasma apoC-II is characterized by delayed catabolism and accumulation in plasma of TRL (165). Like apoC-I, apoC-II inhibits apoE-enriched β-VLDL binding to LDL-R and LRP (152,153).

1.5.4.3. ApoC-III

ApoC-III consists of 79 amino acids and has a molecular weight of 8.9 KDa (166). It is mainly found on TG-rich lipoproteins and their remnants and on HDL. It was also found *in vitro* and *in vivo* associated with particular lipoprotein subclasses, designated LpB:CIII, LpB:E:CIII and LpAI:AII:CIII (167-169). ApoC-III is predominantly synthesized in the liver and to a lesser extent in the intestine (12,170). The gene encoding apoC-III is found on chromosome 11 between that of apoA-I and apoA-IV and is oriented in the opposite direction to the two flanking genes (82,171). ApoC-III is a glycosylated protein. It is found in plasma in 3 isoforms: nonsialylated (apoC-III_0), monosialylated (apoC-III_1) and disialylated (apoC-III_2), with the monosialylated form being the prevailing one (172,173). The extent of sialylation of apoC-III on TRL may be critical for the interaction of TRL with LPL (174). Regulation of apoC-III gene expression

appears to occur primarily at the level of transcription. In hepatocytes, the transcriptional activity of the gene is regulated by a variety of agents, including inflammatory cytokines like IL-1 (175). Fibrates, a group of peroxisome proliferators, decrease human liver apoC-III gene expression, due to transcriptional suppression of hepatic nuclear factor (HNF-4), as well as displacement of HNF-4 from the apoC-III promoter (176,177). The hypotriglyceridemic action of these medications was suggested to be due to their effect on hepatic apoC-III gene expression (178). Also, the apoC-III gene is transcriptionally downregulated by insulin in animals and cultured hepatocytes (179). The role of apoC-III in the metabolism of TRL and their remnants is discussed in section 1.8.3.

1.5.5. Other Apolipoproteins

Human ApoA-IV is a 46 KDa protein which is synthesized predominantly in the small intestine (180,181), and is mostly associated with HDL in plasma. Several other apoliproteins, namely apolipoproteins C-IV (182), D (183), F (184), H (185) and J (186), are found in plasma in relatively small concentrations associated with lipoprotein particles. The presence of these proteins is not restricted to lipoproteins and they are thought to be less involved in plasma lipoprotein metabolism.

1.6. Lipoprotein Processing Enzymes

Several plasma enzymes play important roles in the metabolism of lipoproteins and apolipoproteins. A brief review of these enzymes and their roles is thus warranted.

1.6.1. Lipoprotein Lipase (LPL)

LPL is a heparin-releasable enzyme, bound to glycosaminoglycan components of the capillary endothelium and is particularly abundant in muscle, adipose tissue and macrophages (187). LPL hydrolyzes TG in chylomicrons and VLDL with apoC-II as an essential co-factor. LPL is a glycoprotein and is enzymatically active as a homodimer with each subunit of approximate molecular weight of 50 KDa. In addition to its role in catalyzing the lipolytic processing of VLDL and chylomicrons which leads to the formation of TRL remnants and LDL, LPL contributes to the clearance from plasma of TRL and their remnants through LPL-mediated lipoprotein binding to hepatic receptors. LPL binds with high affinity to LRP (188,189) and promotes the binding, uptake and degradation of TRL. LPL is also a ligand for VLDL-R (190). The involvement of the carboxy terminus of LPL, which is devoid of enzymatic activity, in this binding, as well as the ability of the monomeric form of the protein to bind LRP suggest that the lipase activity of LPL does not contribute to the binding process (191). In contrast, localization of LPL on cell surface and the presence of HSPG are important for binding (192-194). Several mutations in the LPL gene cause

familial LPL deficiency (type-I hyperlipidemia) (reviewed in 17), which is characterized by fasting chylomicronemia.

1.6.2. Hepatic Lipase (HL)

HL is a liver enzyme exhibiting phospholipaseA_1, as well as TG and monoglyceride hydrolase activities. HL deficiencies in humans are associated with a delayed clearance of chylomicron remnants (195), suggesting that HL contributes to chylomicron remnant clearance. HL-deficient mice however exhibit no apparent impairment in remnant clearance (196). Enrichment of rabbit β-VLDL or rat chylomicrons with HL leads to increased removal of these particles when injected intravenously into rabbits (197). Active and heat-inactivated HL stimulate the binding of chylomicron-remnant-like particles and their uptake by isolated rat hepatocytes (198). Further, when enriched with HL, the uptake of chylomicron remnants devoid of apoE is increased, and this effect is abolished when hepatocytes are preincubated with heparinase. Finally, rat hepatoma cells transfected with human HL cDNA have higher binding and uptake of remnants and this binding required HSPG and is independent of apoE (199).

In addition, HL plays an important role in HDL metabolism. Using sibling-pair linkage analysis, Cohen et al. (200) demonstrated that allelic variation in the gene encoding HL accounts for 25% of total interindividual variation in HDL-C levels. Also, HL mediates HDL-phospholipid hydrolysis *in vitro*, which in turn promotes the uptake of HDL-C by liver cells (200). The hydrolysis of HDL-TG mediated by HL, promotes shedding of apoA-I (201,202). Rat liver perfusion

with native HDL_2 in the presence of heparin leads to the formation of preβ-1 HDL and a 57% decrease in HDL_2 CE and TG (203). In contrast, when HDL_2 is delivered to HL-depleted livers, no preβ-1-HDL is formed. HDL_2 enriched in TG, by CETP-mediated transfer from VLDL, rapidly forms preβ-1 HDL during *in vitro* incubation with HL. Overexpression of HL in rabbits causes an 83% decrease in HDL-C, and this is accompanied by a decrease in the abundance of apoA-I, apoE, and apoC-III in the 1.06 - 1.2 density range (204,205). Finally, HL may also mediate the selective uptake of CE from HDL by the liver and steroidogenic tissues in the periphery (206).

1.6.3. Lecithin: Cholesterol Acyl Tranferase (LCAT)

LCAT plays an important role in the plasma metabolism and remodeling of HDL (207). LCAT is synthesized primarily by hepatocytes and mediates the reaction by which an acyl group is transferred from phosphatidylcholine (lecithin) to cholesterol forming CE and lysolecithin. LCAT is predominantly associated with HDL, especially Lp-AI, and is activated by apoA-I (208). It nevertheless acts on LDL (209). LCAT-mediated esterification of free cholesterol is a critical step in the maturation of HDL from discoidal to CE-enriched spherical particles (210), and is also important for reverse cholesterol transport. The formation of cholesteryl ester is thought to be necessary for the efflux of free cholesterol from peripheral cells to HDL down a concentration gradient (211). HDL maturation and reverse cholesterol transport are discussed in section 1.9.2.

The importance of LCAT in HDL metabolism is demonstrated in patients with mutations in the LCAT gene leading to total (classical or familial) or partial (Fish eye disease) LCAT deficiency (212). LDL particles in patients with total LCAT deficiency tend to be rich in unesterified cholesterol and lecithin, whereas HDL are predominantly discoidal in shape. Further, the few spherical HDL particles are small and are rapidly catabolized resulting in marked hypoalphalipoproteinemia. LCAT activity in LDL tends to be normal in patients with partial LCAT deficiency (212).

Overexpression of human LCAT in transgenic mice (213) and rabbits (214), leads to hyperalphalipoproteinemia. HDL in plasma of transgenic rabbits overexpressing LCAT are CE- and phosholipid-enriched in a gene dose-dependent manner. Furthermore, *in vivo* kinetic studies in these animals showed that the catabolism of apoA-I was slower compared to control animals, suggesting that reduced apoA-I FCR is the predominant mechanism by which human LCAT modulates HDL concentrations in this animal model (215). Studies of the kinetics of apoA-I and apoA-II in patients with LCAT deficiency is discussed in section 1.9.4.2.3.

1.6.4. Cholesterol Ester Transfer Protein (CETP)

Plasma CETP is predominantly bound to HDL and mediates the exchange of HDL CE with TG of TRL. Consequently, it plays an important role in reverse cholesterol transport and HDL remodeling (216). Cases of CETP deficiency in humans are common in the Japanese population. In fact, it has been estimated

that approximately 10% of the total phenotypic variation in HDL-C levels in Japanese men can be attributed to genetic variation in CETP (217). A point mutation (G→A substitution in intron 14 of the CETP gene), as well as a missense mutation leading to the substitution of Gly for Asp^{442} in CETP, were suggested to be responsible for CETP deficiency. At the plasma level, CETP deficiency is characterized by a marked increase in HDL-C and apoA-I concentrations. Furthermore, HDL particles in these cases are larger, and have decreased TG and increased apoE. The proportion of LpAI:AII relative to LpAI is also higher (217). The *in vivo* kinetics of apoA-I and apoA-II have been studied in subjects with CETP deficiency, using endogenous labeling (218). The fractional synthetic rates (FSR) of apoA-I and apoA-II in two homozygotes with CETP deficiency were lower than controls, indicating a reduced catabolism for apoA-I and apoA-II, while the production rate of both proteins were normal. In contrast, the kinetics of apoA-I and apoA-II in the CETP deficient heterozygote were not different from those in controls.

1.7. Lipoprotein Receptors

Lipoprotein receptors are membrane proteins which bind to lipoprotein particles and mediate their clearance from plasma. Characteristics of key lipoprotein receptors are briefly described in this section.

1.7.1. LDL Receptor (LDL-R)

The LDL-R (219) is a single transmembrane glycoprotein containing 839 amino acids. It is expressed in many tissues but mainly on the surface of hepatocytes and steroidogenic tissues. LDL-R is organized in five independent domains , each having an independent function and each contributing to the functional activity of the receptor (219). The first two domains are directly involved in binding lipoproteins. The first domain located at the cell surface is rich in cysteine. Cysteine residues form disulfide bonds within seven repeat sequences. The carboxy terminus of each repeat is rich in negatively-charged amino acids, which are complementary to positively-charged amino acids in the receptor's ligand. Cysteine-rich repeats are thus responsible for binding the ligands, apoB-100 and apoE. This binding however depends on the conformation of ligands on lipoprotein particles, and is modulated by the presence of other proteins such as apoC-III and LPL (220). The second domain consists of 3 sequences (A, B and C), and is homologous to the extracellular domain of the Epidermal Growth Factor (EGF) precursor. Repeat sequences in domains 2 - 7, as well as sequences A and B in the second domain are important for the optimal binding of apoB in LDL, whereas only repeat 5 is essential for the binding of apoE in β-VLDL (221).

1.7.2. LDL Receptor-Related Protein (LRP)

LRP is one of the largest plasma membrane proteins ever found, with a size 4 times that of LDL-R (4525 amino acids) (Fig 1.7) (222). LRP is found on

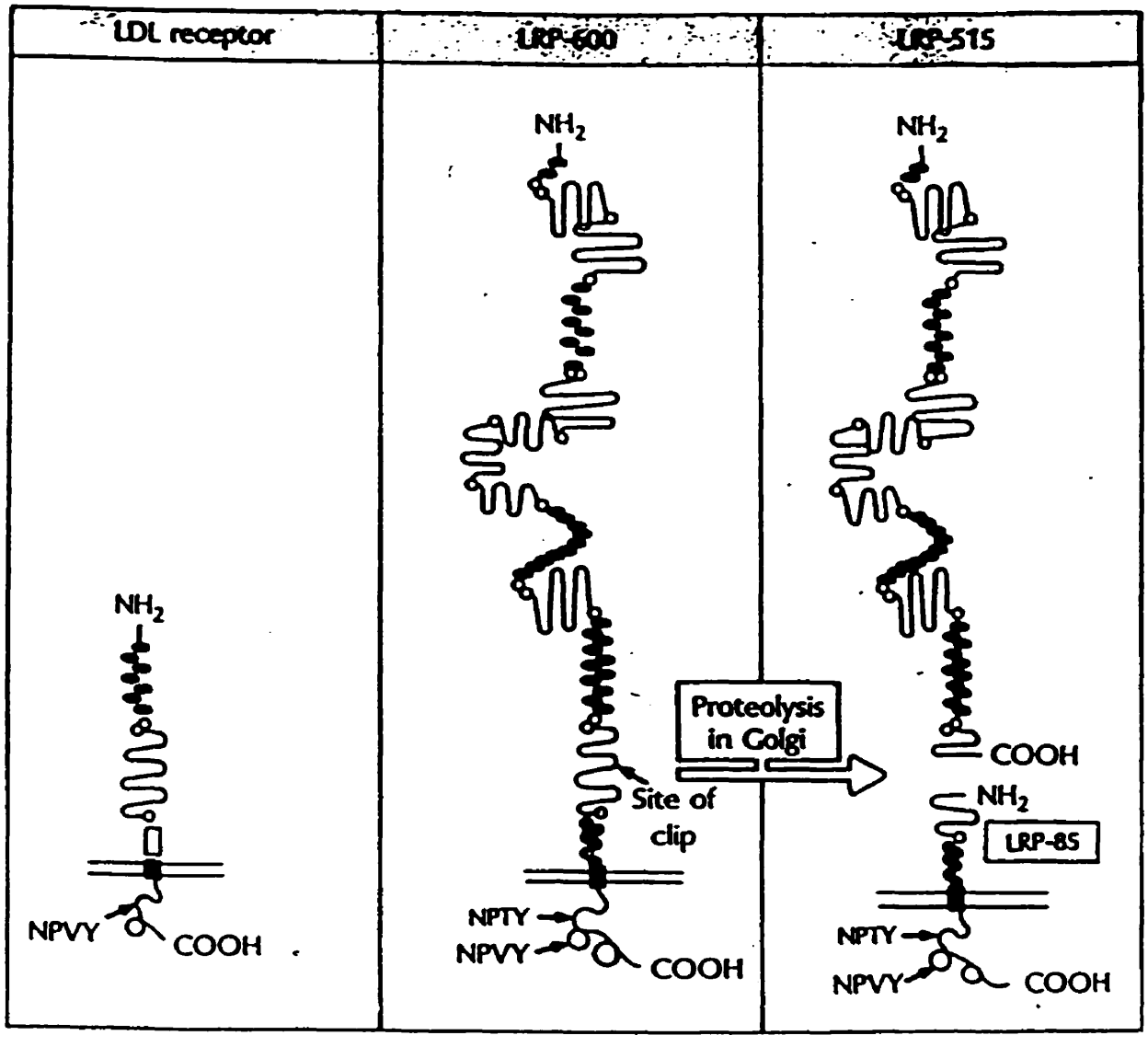


Figure 1.7. Schematic model of LDL-R and LRP. NPVY and NPTY: consensus sequences of internalization (220).

the cell surface as two non-covalently associated subunits: a large subunit of 515 KDa and a smaller one of 85 KDa (223). The larger subunit contains 31 cysteine-rich repeat sequences similar to the 7 cysteine repeats of LDL-R, and 22 sequences in the domain homologous to the EGF precursor. LRP is found mainly on the surface of hepatocytes and Kupffer cells (224) but is also found in the brain, lungs, intestine and muscles. LRP does not bind apoB-100 but binds apoE which makes it a candidate receptor for chylomicron remnants. In fibroblasts from patients with homozygous FH, LRP was shown to bind β-VLDL when enriched with apoE, and the binding was blocked by apoCs (152,153). Enrichment of chylomicron remnants with apoE may be a physiological process. Hepatocytes secrete apoE which in turn binds to their surface by interaction with proteoglycans or LRP itself, thus apoE molecules could be available to associate with remnants (225), and to target them to LRP for internalization (220,226). LRP also has a binding site for LPL (188). LRP also binds and internalizes other ligands such as the protease inhibitor α_2-macroglobulin, Vitellogenin, pseudomonas endotoxin A and lactoferrin (227-230). A small protein of 323 amino acids (39 KDa) called the Receptor Associated Protein (RAP) is associated with LRP (231). It inhibits the binding and internalization of α_2-macroglobulin and apoE/β-VLDL, and is thus a potential negative modulator for LRP (232-234). LPL is another potential modulator for LRP binding activity. It enhances the binding of VLDL and chylomicron remnants to LRP by binding to proteoglycans adjacent to LRP and functioning like a bridge (230).

1.7.3. Other Lipoprotein Receptors

Glycoprotein 330 (gp330), or megalin, is another member of the LDL-R family, and is the largest membrane protein identified so far in vertebrates (molecular weight of 330-600 KDa) (235). It is found in epithelial brush-border membranes, in particular in the kidney. gp 330 binds RAP and LPL (236,237), and its binding to apoE/VLDL is greatly increased in the presence of LPL. gp330 also has a role in receptor-mediated uptake and transport of apoJ in the central nervous system (238) and was found to be a major target antigen for Heyman nephritis, an autoimmune disease in rat (a model of human membranous glomerulonephritis).

The VLDL receptor (VLDL-R) is also a member of the LDL-R family and is the closest in structure to the LDL-R (239). VLDL-R is most abundant in the heart, skeletal muscle, and adipose tissue (240-244), where fatty acids are actively metabolized as an energy source. VLDL-R does not bind apoB-containing LDL but binds apoE-containing VLDL, IDL, and β-VLDL with high affinity (240,245), which makes it another candidate receptor for chylomicron remnants. VLDL-R also binds RAP with high affinity (233).

The Lipolysis Stimulated Receptor (LSR) is able to mediate LDL uptake and intracellular degradation once activated by the presence of free fatty acids (246,247). LSR binds different lipoproteins including those rich in CE such as LDL and β-VLDL. However, it has a higher affinity for TRLs (VLDL and chylomicrons) (248). Unlike LRP, LSR does not bind RAP.

Finally, several scavenger receptors are responsible for the binding and cellular uptake of modified lipoproteins including oxidized, acetylated, and maleylated LDL (249,250). Furthermore, they are involved in the selective uptake of HDL-CE by the liver and steroidogenic tissues (251-253).

Two original studies are presented in this thesis (chapters 3 and 4). The first addresses the plasma kinetics of apoE and apoC-III, in association with those of VLDL apoB-100, in normolipidemic subjects and hypertriglyceridemic patients. Section 1.8 reviews the current knowledge regarding triglyceride rich lipoproteins, the role of apoE and apoC-III in regulating their metabolism, and the plasma kinetics of apoE and apoC-III. The second study looks at the plasma kinetics of apoA-I, including proapoA-I, and apoA-II in normolipidemic subjects and patients with familial HDL deficiency. Section 1.9 discusses the metabolism of HDL, HDL deficiency (particularly familial HDL deficiency), and the plasma kinetics of apoA-I and apoA-II.

1.8. Triglyceride Rich Lipoproteins (TRL), ApoE and ApoC-III

1.8.1. TRL and Coronary Heart Disease (CHD)

Multivariate analysis of epidemiological data has often shown that elevated plasma TG concentration is not an independent risk factor for CHD (254). This interpretation, which is at variance with clinical evidence, does not take into consideration: a) the heterogeneity of diseases associated with hypertriglyceridemia, b) the wide range of environmental factors which can

influence plasma TG levels, and c) the type of lipoprotein particles which cause the accumulation of TG in a given individual (254). More recently, however, subgroup- and meta-analyses of epidemiological data, clinical trial results, and molecular evidence have supported the concept that elevated plasma TG level is a risk factor for CHD, especially when this parameter reflects an accumulation of atherogenic TG-rich TRL remnants.

1.8.1.1. Evidence from Epidemiological Studies and Clinical Trials

Epidemiological studies have shown that plasma TG concentration is predictive of CHD in women and diabetics (255-258). The Lipid Research Clinic's Follow-up Study (LRCFS) (259) has also shown that there is an independent association between TG level and coronary mortality in subgroups of men with low HDL cholesterol (< 0.9 mol/l), men with low LDL cholesterol (<4.1 mmol/l) and men and women of younger age (<70 years). This association, however, did not remain significant after controlling for plasma glucose. Also, in the Paris Prospective Study (260), when diabetes and other risk factors were considered among 3585 subjects with a serum cholesterol lower than 220 mg/dl, serum TG level remained an independent predictor of CHD.

A meta-analysis carried out by Hokanson and Austin (261) on 12 population-based prospective studies in men and 4 in women has shown that the multivariate summary for the association between TG levels and the incidence of cardiovascular disease remained significant for men and women even after the

adjustment for HDL-C, suggesting that this association is independent of HDL-C. Furthermore, postprandial triglyceridemia has been shown to be an independent predictor of CHD. Patsch et al. (262) showed that postprandial TG levels 6 and 8 h after a standardized fat load were highly discriminatory between 61 CHD cases and 40 control male subjects. Karpe et al (263) correlated the increase in postprandial remnants with changes in atherosclerosis progression score for 32 male patients who had survived a first myocardial infarction before the age of 45 years and who had participated in a 5-year angiography study of CHD progression. Patients with monogenic and polygenic hypercholesterolemia were excluded. The area under the curve of postprandial plasma levels of small chylomicron remnants (Sf 20-60, apoB-48 particles) was found to correlate with the rate of progression of coronary lesions, and the strength of this correlation was not affected when adjusted for HDL-C and dense LDL-apoB concentrations (263).

Several angiographic trials have also supported the atherogenic role of TRL and their remnants. In a trial to investigate the effect of a calcium channel blocker on the progression of coronary atherosclerosis as evaluated by angiography, Phillips et al (264) found that a measure of TRL remnants, which included IDL-C, was directly related to lesion progression and CHD clinical events, independent of plasma TG.

Quantitative angiographical data from the Monitored Atherosclerosis Regression Study (MARS) (265) have suggested that TG- and cholesterol-rich lipoproteins affect the progression of mild to moderate atherosclerotic lesions.

The results of MARS revealed that therapy with the HMG CoA reductase inhibitor (lovastatin) improved severe but not mild to moderate coronary lesions, and that the proportion of subjects with mild to moderate stenosis showing lesion progression was similar in placebo and in lovastatin-treated groups. In univariate analysis of the < 50% stenosis placebo group, the relative risk of progression increased with absolute TG increase or TC:HDL cholesterol increase from baseline. In multivariate analysis, however, TC:HDL-C was the only risk factor associated with progression, for mild to moderate lesions. In univariate analysis of the < 50% stenosis lovastatin-treated group, the relative risk of progression increased with on-trial changes in TG and apoC-III. In the multivariate model, the on-trial level of LDL-C:HDL-C was the single risk factor associated with progression. Interestingly, the greatest risk of progression was found in the fourth quartile of the distribution for apoC-III-heparin precipitate (apoC-III in TRL) for mild to moderate lesions (254,265).

Finally, Blankenhorn et al. (266) conducted a within-group analysis of the Cholesterol Lowering Atherosclerosis Study (CLAS) results and showed by multivariate analysis that HDL apoC-III was negatively related to disease progression in the patient group treated with colestipol and niacin. The results of MARS and CLAS show that the distribution of apoC-III between potentially atherogenic TRL and potentially protective HDL is of significance in defining the atherogenic role of TRL.

1.8.1.2. Evidence from Studies in Cell Cultures and Transgenic Mice

In vitro experiments with cultured cells have demonstrated that remnant lipoproteins can cause macrophage lipid accumulation, cytotoxicity and changes to endothelial cell function (267-270). Also, recent studies in transgenic and apo E gene-knock out mice have provided further evidence of the importance of TRL and their remnants in atherosclerotic events. This evidence, along with the role of apoE in TRL metabolism, are discussed in section 1.8.2.

1.8.1.3. Clinical Evidence

TRL and their remnants may be very damaging to the artery wall. TRL and their remnants have been isolated from human atherosclerotic plaques (271) and liposome-like particles isolated from these plaques have been shown to be structurally and compositionally similar to surface remnants of TRL (272).

Furthermore, the atherogenicity of TRL and their remnants has been demonstrated in certain familial forms of hypertriglyceridemia, namely type III hyperlipidemia, familial hypertriglyceridemia and familial combined hyperlipidemia (FCH).

1.8.1.3.1. Type III Hyperlipidemia

The lipoprotein abnormalities later reported as typical of Type III dyslipidemia or familial dysbetalipoproteinemia, namely the presence of VLDL with abnormal electrophoretic migration and an increase in plasma TG and cholesterol levels, was first described in 1954 by Gofman and his colleagues

(273). Fredrickson (274) identified the disorder as a distinct familial dyslipidemia. Havel and Kane (275) observed that the β electrophoretic migration of VLDL was associated with an elevated level of plasma apoE. Utermann and his colleagues, in turn, reported an association of type III dyslipidemia with the presence of one apoE isoform (276-278) and suggested, along with others, a role for this isoform in the etiology of the disorder (279,280). Subsequently, it has been shown that type III hyperlipidemia is associated with a low affinity of the variant apoE2 to the LDL-R of human fibroblasts, and membranes from liver and adrenal of rats, rabbits, and cows (281,142). The affinity of apoE2 to LDL-R was found to be only 2% that of apoE3 (143). Substitution of Cys for Arg 158 in apoE2 and the consequential changes in the structure of the protein are thought to be responsible for the impaired binding (142). Kinetic studies *in vivo* have shown that the clearance of apoE2 was slower than apoE3 in plasma of controls and patients with type III hyperlipidemia (Fig 1.8) (144). Kinetic studies also showed that treatment with bezafibrate, a peroxisome proliferator, reduced the plasma levels of VLDL apoB-100 by both decreasing its synthesis and increasing its clearance (282). Finally, there is evidence to suggest that delayed LPL-mediated lipolytic processing can contribute to remnant accumulation in type III patients. A subpopulation of VLDL rich in CE, apoB and apoE was resistant to conversion to LDL by LPL *in vitro* (283).

Type III hyperlipidemia rarely manifests itself before adulthood, and is more prevalent in men than in women. It is characterized clinically by the the occurrence of xanthomas, such as 1) planar xanthomas which are distinctive of

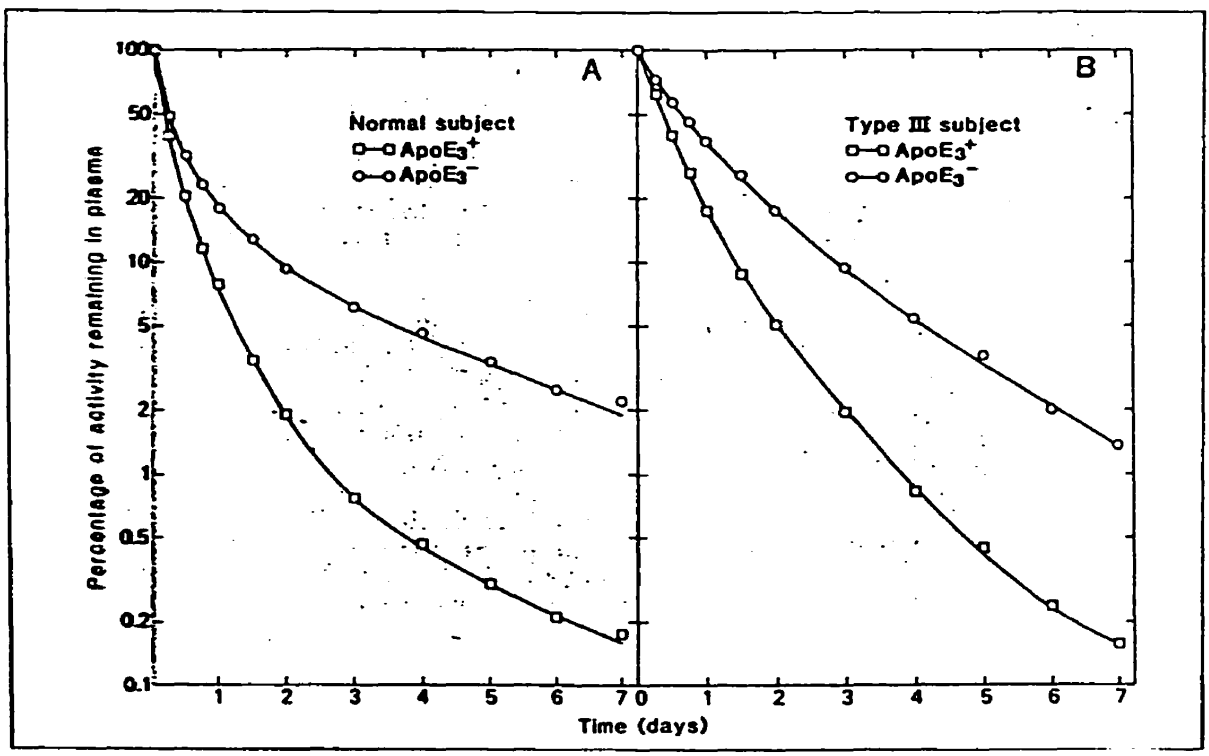


Figure 1.8. The clearance of simultaneously injected apoE3 (□) and apoE2 (O) from the plasma of a normolipidemic subject (A) and a type III hyperlipidemic patient (B). All activity values have been normalized to 100 perecent at day 0 (144).

disorder, 2) tuberous and tuberoeruptive xanthomas on the elbows, knees, buttocks, and knuckles, and 3) sometimes xanthelasmas and tendinous xanthomas (284). Premature or accelerated atherosclerosis occurs in one-third to one-half of individuals with this disorder. Peripheral vascular disease involving the lower extremities is almost as common as CHD (284). Importantly, homozygosity for apoE2 phenotype is not sufficient for the development of overt type III hyperlipidemia. In fact, the majority of apoE2 homozygotes do not display type III dyslipidemia. They have lower plasma cholesterol levels, lower LDL cholesterol and apoB levels, but higher plasma TG and apoE levels compared to apoE3 homozygotes (284). The hypocholesterolemic effect of apoE2 is suggested to be due to: 1) upregulation of hepatic LDL-R, although experimental evidence in animal models does not support this notion (285), 2) increased uptake of LDL by LDL-R due to impaired apoE2-mediated uptake of VLDL particles and their remnants (285), or 3) decreased lipolytic conversion of VLDL to LDL resulting from direct inhibition of LPL activity by apoE2 (286). Almost 1 % of the North American population is homozygous for apoE2, however, the overt manifestation of type III hyperlipidemia occurs in only 1 to 5 per 5000 in the general population (284). The discrepancy between these two occurrences suggests that this disorder is modulated by other genetic and environmental factors which affect the absolute levels of plasma lipids (284), and that a second hit must intervene for overt type III hyperlipidemia to occur. Metabolic diseases such as hypothyroidism (287,288) and diabetes (289) can contribute to the manifestation of type III hyperlipidemia. Other secondary

factors include obesity, high CETP, and low LDL-R activity (284). Finally, several mutations of apoE have been associated with a dominant form of type III (290).

Another familial dyslipidemia characterized by TRL accumulation, was discovered in five members of a French Canadian family of ten (291,292), and has been termed pseudotype III dyslipidemia (PT-III). This unique disorder shares some of the characteristics of type III dyslipidemia. Like in type III, plasma levels of cholesterol, TG, VLDL-C, VLDL-TG and apoE, as well as the VLDL-C/TG ratio are high and LDL-C and HDL-C tend to be low in PT-III. Further, the clearance of plasma TG after a fat load is impaired, and tubero-eruptive xanthomas, arcus cornea and manifestations of atherosclerosis are present in some patients. Unlike type III, however, PT-III dyslipidemia has been found in patients with different apoE phenotypes, such as E4/2, E4/3, and E3/2. None of the patients had an apoE mutant known to be associated with type III hyperlipidemia. Further, the VLDL-C/VLDL-B ratio was low, β-VLDL was absent, but a double pre-β band (normal pre-β-migrating and slow pre-β-migrating VLDL) was found on lipoprotein electrophoretic gels.

Initially, PT-III was hypothesized to be due to impaired interaction of TRL with their receptor(s). This hypothesis was tested by Giroux et al. (293), who measured the activity of 3 lipoprotein receptors implicated in the uptake of TRL, namely LDL-R, LRP and LSR, in PT-III and normal cultured fibroblasts. Specific cell association and degradation of ^{125}I-LDL by LDL-R-upregulated PT-III fibroblasts was normal, and so was the association and degradation of rabbit ^{125}I-β-VLDL. LRP activity was assessed by measuring the ability of PT-III cells to

bind α2-macroglobulin, lactoferrin and apoE-enriched rabbit β-VLDL and was found to be normal. LSR activity, as assessed by the cell association or degradation of ^{125}I-LDL by fibroblasts in the presence of 0.5 mM oleate and human leptin, was also normal in PT-III patients. Finally, no evidence was obtained for deficient cellular recognition of PT-III TRL by normal human fibroblasts or mouse macrophages (293). The etiology of PT-III dyslipidemia thus remains unknown.

1.8.1.3.2. Familial Hypertriglyceridemia

Familial hypertriglyceridemia (FHTG) (also termed isolated or endogenous hypertriglyceridemia, or type IV hyperlipidemia) is characterized by an increase in plasma VLDL and TG levels in affected family members at the fasting state (294). Some studies have supported the association of FHTG with CHD (295-297), while others have not been able to show such an association (298).

The etiology of FHTG is not well-defined, probably because of its heterogeneous nature. Elevated concentration of TRL in plasma of FHTG patients has been shown to result from overproduction and/or decreased catabolism of these TRL (299-302). The catabolic abnormality is thought not to be the result of a deficiency in LPL or HL as FHTG patients have been shown to possess normal levels of post-heparin plasma lipolytic activity (303). Nevertheless, there is evidence to suggest that delayed catabolism is a result of defective processing of TRL. Oschry et al. (304) have shown that large VLDL from hypertriglyceridemic patients are not converted into LDL-like particles during

incubations with LPL purified from bovine skim milk, and Evans and colleagues (301) have shown *in vitro* and *in vivo* that an apoE-poor VLDL subfraction in plasma of type IV patients is resistant to lipolysis by LPL relative to its apoE-rich counterpart. Chung et al. (305) have demonstrated defective *in vitro* lipolysis of type IV VLDL by bovine milk LPL, with the extent of defective lipolysis being proportional to the severity of the hypertriglyceridemia in these patients. Finally, Furukawa et al. (306) have reported that VLDL from hypertriglyceridemic nephrotic rats are relatively resistant to *in vitro* lipolysis by rat post-heparin plasma when compared with VLDL from normolipidemic nonnephrotic rats.

In addition to the metabolic abnormalities, there is evidence to suggest that there is also a compositional abnormality in accumulated TRL in type IV hyperlipidmia (307). The work of Evans et al. (301) as well as work in our laboratory (Marcoux C, unpublished data), have shown that type IV is characterized by an accumulation of apoE-poor but apoC-III enriched TRL particles.

1.8.1.3.3. Familial Combined Hyperlipidemia

Familial combined hyperlipidemia (FCH) is characterized by an expression of several lipoprotein phenotypes, such as IIa (increase in LDL) and IIb (increase in LDL and VLDL), as well as type IV, in different members of the same family (308). Unlike FHTG, FCH has been identified as a distinctive genetic disorder (308), and has been shown to be strongly associated with an increase in the risk for myocardial infarction (309). Genetic analyses indicated that the inheritance

pattern was complex and non-Mendelian with a major gene acting on TG levels (310). The locus with the greatest evidence for involvement in FCH is that of the apoA-I/C-III/A-IV gene complex (311-313). No associations were observed between markers near the apoB gene and the FCH phenotype (314,315), and LPL gene was excluded as a major cause of FCH by sib-pair analysis (316) despite decreased LPL activity found in 30% of patients (317-319).

FCH has generally been associated with an overproduction of hepatic apoB-100-containing lipoproteins resulting in increased plasma levels of VLDL and LDL (320-326). One study has examined VLDL kinetics in patients with FCH and FHTG and found an overproduction of VLDL apoB-100 in both groups compared to age and weight matched non-hyperlipidemic controls (324). However, production of VLDL apoB-100 was significantly higher in FCH than in familial hypertriglyceridemia while TG production in plasma was higher in familial hypertriglyceridemia. Another study has found that treatment with lovastatin reduced the production of apoB-containing lipoproteins into plasma in FCH subjects (327). Despite the evidence for apoB-100 overproduction in FCH, a recent study by Aguilar-Salinas and his colleagues (325) suggested that delayed catabolism of VLDL apoB-100 may also be an underlying factor in some cases of FCH. Five members of an FCH kindred (the M-kindred) had low VLDL apoB FCRs and low LDL apoB FCRs, with no evidence for an overproduction of VLDL apoB. In contrast, in two subjects drawn from two other FCH kindreds, VLDL apo B PRs were increased, and VLDL apoB FCRs were decreased. Treatment with pravastatin normalized LDL apoB FCR and did not change apoB PRs in

members of the M family. Small dense atherogenic LDL particles (LDL type B) are abundant in patients with FCH (328). The presence of these particles was attributed to an increased production and reduced direct clearance of large VLDL, which then underwent successive lipolysis to smaller remnant particles (329).

Scientific evidence is thus supportive of the notion that TRL remnants are atherogenic. It also reveals a central role for apoE and apoC-III in controlling the metabolism of TRL and their remnants, and determining their plasma levels. The next two sections review evidence for the involvement of apoE and apoC-III in the plasma metabolism of these lipoprotein particles.

1.8.2. ApoE and Metabolism of TRL

ApoE plays an important role in regulating the metabolism of TRL and their remnants. ApoE mediates hepatic recognition and uptake of TRL and their remnants through binding to LDL-R, LRP, and VLDL-R (330-332). As discussed previously, impaired binding of a structural variant of apoE (apoE2), and several apoE mutations, to these hepatic receptors is the primary metabolic defect behind type III dyslipidemia. The atherogenicity and dyslipidemic effects of these mutants have been demonstrated in transgenic mice models (333-335). Expression in mice of receptor-binding-defective apoE (Arg^{112}, Cys^{142}) is associated with increased susceptibility to diet-induced hyperlipoproteinemia and atherosclerosis (333,334). Expression of the apoE3-Leiden mutation causes

severe accumulation of VLDL remnants (335). This accumulation is strongly responsive to dietary changes and is linearly correlated with the development of atherosclerotic lesions. Also, apoE deficiency in humans (apoE0/0) is associated with hypercholesterolemia and elevated βVLDL (remnant accumulation) as well as premature atherosclerosis (336).

Plasma cholesterol and LDL concentrations are influenced by apoE polymorphism. It has been estimated that 60% of the variation in plasma cholesterol levels is genetically determined and that apoE polymorphism accounts for approximately 14% of this genetic variation (337). It has also been demonstrated in several populations that the ε4 allele is associated with the highest cholesterol and LDL levels, the ε2 allele with the lowest, and the ε3 allele with intermediate levels (338). Frequency of the ε2 allele was also shown to be higher in patients with familial hypertriglyceridemia (339) suggesting that the presence of a single copy of the ε2 allele could contribute to remnant accumulation. The phenotype of apoE affected also the conversion of VLDL2 (20-60 Sf) to LDL in normolipidemic subjects. Those with apoE3/3 phenotype converted 50% of VLDL2 into LDL as compared with 25% and 70% for E2/2 and E4/4, respectively (340). Furthermore, in normolipidemic subjects with an apoE2/2 phenotype, there was less production of VLDL1(60-400 Sf) but more of it was converted to VLDL2 (20-60 Sf). LDL levels were low due to increased direct catabolism of VLDL2 and IDL, and reduced efficiency of delipidation. In contrast, in subjects with an apoE4/4 phenotype, there was less direct catabolism of VLDL and FCR of LDL was also reduced. Total cholesterol and

LDL cholesterol were lower in apoE2/2 and higher in apoE4/4 compared to apoE3/3 homozygotes, while TG and VLDL cholesterol levels were elevated in apoE2/2 and apoE4/4 homozygotes (340). Another study (341) looked at the effects of apoE phenotype and polymorphic alleles of apoA-I, apoB, apoC-III, and LDL-R genes, separately and together, on regulation of serum LDL cholesterol level in 29 middle-aged men. Individuals with the apoE2 allele had the lowest LDL cholesterol and LDL apoB levels, and the highest clearance rate of LDL apoB (0.453 ± 0.03 vs 0.312 ± 0.01 pools/day, respectively). Individuals with an apoB gene variant, the EcoRI RFLP allele, had marked LDL elevation, especially in combination with the apoE4 and were non responders to dietary cholesterol.

Intravenous injection of apoE in Watanabe heritable hyperlipidemic rabbits, which are deficient in LDL-R, resulted in a rapid reduction in plasma cholesterol (342). VLDL- and IDL- cholesterol and apoB levels dropped rapidly during the first two hours after injection, followed by a reduction in LDL cholesterol and LDL apoB. In addition, the rate of removal of ^{125}I-labeled VLDL, preincubated with apoE, was 3-fold higher than that of unmodified VLDL. The changes were attributed to enhanced uptake of TRL and their remnants by the liver. In another study (343), chronic intravenous injections of apoE in Watanabe rabbits prevented the progression of atherosclerosis but did not affect the lipoprotein profile.

Overexpression of the rat apoE gene in transgenic mice resulted in decreased plasma cholesterol and TG levels. There was a reduction in plasma

levels of VLDL and LDL, due to an enhanced clearance of VLDL, LDL and chylomicron remnants (344,345). There was also a blunting of the hyperlipidemia induced by diets high in fat and cholesterol (344). ApoE deficient mice (apoE knock-out mice), created by gene-targeting of embryonic stem cells, developed severe hypercholesterolemia, atherosclerosis and arterial lesions on a normal chow diet (346,347). The hypercholesterolemia was due to an increase in plasma levels of VLDL and IDL (including an apoB48-enriched remnant). Accumulation of cholesterol-enriched remnant lipoproteins was associated with aortic foam-cell formation and the development of spontaneous atherosclerotic lesions, which are characteristic of those seen in humans and other species (348,349). Accumulation of cholesterol was due to a reduction in the FCR of total body cholesterol (350). Heterozygous apoE-deficient mice also developed hypercholesterolemia on an atherogenic diet and showed an increased susceptibility to atherosclerosis (351). Replacement of apoE, by bone marrow transplantation or by using adenovirus vectors, caused cholesterol and remnant lipoprotein levels to be normalized and atherosclerosis to be reduced (352-356). The plasma half-life of β-VLDL in transplanted mice, compared with control apoE-deficient mice, was shortened. Plasma lipoprotein distribution shifted also from primarily VLDL and LDL in the control mice to predominantly HDL in the adenovirus-infected group.

Transgenic rabbits expressing high levels of human apoE2 (30-70 mg/dL) have a lipid profile similar to that seen in type III hyperlipidemia (357). Male transgenic rabbits have more severe hyperlipidemia and more extensive

atherosclerosis than females. Transgenic mice expressing low and intermediate levels (<10 and 10-30 mg/dL, respectively) of human apoE2 are hypolipidemic, while those expressing high levels (>50 mg/dL) are hyperlipidemic and have an accumulation of cholesterol-enriched VLDL and IDL (358). Further, apoE2/apoB (intermediate expression of apoE2), apoE2/LDL-$R^{-/-}$, and apoE2/LDL-$R^{+/-}$ double transgenic mice have a lipid profile resembling human type III dyslipidemia, namely, an accumulation of VLDL and IDL and a reduction in LDL and HDL (358,359). The low levels of LDL-C in apoE2/LDL-$R^{-/-}$ mice suggests that apoE2 might inhibit LPL- mediated TRL lipolytic processing (360). Finally, expression in mice of receptor-binding-defective apoE (Arg 112, Cys 142) or apoE3-Leiden, which are associated in humans with dominantly-inherited forms of type III dyslipidemia, cause TRL metabolism to be perturbed, and increase susceptibility to diet-induced hyperlipoproteinemia and atherosclerosis (334,335)

1.8.3. ApoC-III and Metabolism of TRL

ApoC-III also plays an important role in TRL metabolism. Plasma apoC-III levels are positively correlated with the concentration of plasma TG (172,361). Hypertriglyceridemic patients with type III or type IV hyperlipoproteinemia (discussed earlier) have plasma apoC-III levels increased 3 to 4 times and TRL apoC-III levels increased 5 to 12 times compared to normolipidemic individuals (362). Two sisters with apoC-III/apoA-I deficiency had reduced levels of VLDL TG, and FCRs for VLDL apoB which were 6 to 7 times faster compared to normal subjects (363). The presence of a polymorphic Sst I site (S2 allele) in the

apoC-III 3' untranslated region of the apoC-III gene was associated with increased levels of plasma TG (364) and apoC-III (365,366). Further, subjects carrying a lysine 58 to glycine mutation in apoC-III were hyperalphalipoproteinemic with decreased apoC-III levels and low to normal plasma TG levels (367). Five sites of genetic variation have been identified in the C-III gene promoter and haplotyping utilizing these sites and the Sst I site has revealed three classes of apoC-III alleles, susceptible, neutral, and protective with regard to hypertriglyceridemia (368). Polymorphism at nucleotide -455 of the apoC-III gene promoter was associated with variation in plasma TGs in 509 adult aboriginal Canadians from an isolated community in Northern Ontario (369). Homozygosity for the presence of a C at nucleotide -455 and a T at nucleotide -482 was associated with significantly increased plasma TG in both men and women (370). Interestingly, sites -482 and -455 fall within an insulin response element (371) and their variation abolished the insulin-induced downregulation of apoC-III gene trancsription (371,372).

Transgenic mice expressing human apoC-III had increased levels of plasma TG, and hypertriglyceridemia was proportional to plasma apoC-III levels and to liver apoC-III gene expression (373). In contrast, a targeted disruption of the mouse apoC-III gene resulted in hypotriglyceridemia and protection from postprandial hypertriglyceridemia (374). In an apoC-III transgenic/apoE0 mouse model, there was severe hypertriglyceridemia due to decreased clearance and not overproduction of VLDL TG (375). Furthermore, LPL activity was shown to be inhibited and VLDL-glycosaminoglycan binding to be reduced independent of

apoE. It was concluded that the predominant mechanism of apoC-III-induced hypertriglyceridemia was decreased lipolysis at the cell surface (375).

High concentrations of apoC-III inhibits LPL and HL *in vitro* (376-378) and *in vivo* (379-381). Also, overexpression of apoC-III in transgenic mice showed that, while both LPL and apoE increase the ability of TRL to bind HSPG, apoC-III has the opposite effect (382). Sera from apoC-III/apoA-I deficient patients were not able to inhibit LPL activity in the same way as sera from normal individuals (381). ApoC-III has also been shown to inhibit the clearance of TRL by the liver (383-385), and there is evidence to suggest that it is the relative amount of apoC-III, compared to apoE, on TRL particles which regulates the uptake of these particles by lipoprotein receptors. In one study (386), the apoC group were found to inhibit predominantly apoE3-dependent interaction of TRL with LDL-R in cultured fibroblasts. The mechanism of inhibition reflected association of apoC proteins with lipoproteins and specific concentration-dependent effects on apoE3 at the lipoprotein surface. In another study (387), the LDL-R binding of immunoaffinity chromatography-isolated apoB-containing lipoprotein particles, artificially-enriched with TG, was almost completely abolished with the addition of apoC-III and to a lesser extent apoC-II but not apoC-I. Conversely, apoC-III had no effect on the binding activity of rabbit β-VLDL, preincubated with apoE, to LRP, while apoC-I did (388). Mice overexpressing apoC-III had VLDL enriched in apoC-III and deficient in apoE and they had a reduced VLDL FCR (389). Univariate analysis in subjects with a wide range of VLDL TG demonstrated that the FCR for VLDL TG was inversely related to the ratio of apoC-III/apoC-II in

VLDL, although this relationship was not significant in multivariate analysis (390). Accumulation of VLDL in apoC-III transgenic mice can be overcome by the expression of human apoE (382,391). Supplementation of ^{125}I-VLDL with apoC-III inhibited the LSR-mediated binding, internalization, and degradation of ^{125}I-VLDL in primary cultures of rat hepatocytes (392). This inhibitory effect was specific for apoC-III and not apoC-II, and was greater for apoC-III$_1$ than apoC-III$_2$. An apoC-III variant (Gln^{38} to Lys) identified in a large kindred of Mexican origin was associated with moderate hypertriglyceridemia.

Interestingly, several reports have suggested an association between polymorphism in the apoAI-CIII-AIV gene cluster and FCH. Indeed, a transgenic mouse model displaying some of the features of FCH was created by crossing mice expressing human apoC-III with mice deficient in LDL-R (393). A study of the frequency of restriction fragment length polymorphism (RFLP) in the apoAI-CIII-AIV gene cluster has suggested an association between an Xmn-I RFLP located 2.5 Kb upstream of the apoA-I gene and FCH (394). A subsequent study has confirmed this association (311). Three restriction enzyme polymorphisms, XmnI and MspI sites 5' of the apoA-I gene and the Sst-I site in the 3' untranslated region of exon 4 of the apoC-III gene, were examined in 18 families with FCH (18 probands, 179 hyperlipidemic relatives, 211 normolipidemic relatives and 177 spouses). The apoAI-CIII-AIV gene cluster was not found to be the primary cause of FCH, but to have a specific modifying effect on plasma TG and LDL cholesterol levels (312). The complexity of the contribution of the apoAI-CIII-AIV gene cluster to the FCH phenotype was

confirmed in a subsequent haplotype analysis (313). In another study (395) carried out in 16 FCH families (16 probands and 106 family members) together with 12 normolipidemic control families (63 members), a C1100-T polymorphism in exon 3 of the apoC-III gene was more common in the probands relative to control subjects and was associated with elevated concentrations of plasma TG and apoC-III, VLDL cholesterol, VLDL TG, IDL cholesterol, IDL TG, VLDL apoB and IDL apoB, indicating a relationship between this variation and increased TRL. It was concluded that the apoC-III gene might be acting as a modifier gene which is only expressed in the presence of other factors and is predisposing family members with that phenotype to FCH.

The roles of apoE and apoC-III in the metabolism of TRL and their remnants are governed by the plasma levels of both apolipoproteins, by the relative amount of each on TRL particles, and by their relative distribution on lipoprotein fractions. These parameters are in turn determined by the production and catabolism of plasma apoE and apoC-III, i.e. their plasma kinetics. The next two sections review the current knowledge regarding the plasma kinetics of apoE and apoC-III.

1.8.4. Plasma Kinetics of ApoE

Apo E is secreted either on lipoproteins, mainly VLDL, or in a free form (396). It is present in plasma on VLDL, intermediate density lipoprotein (IDL) and HDL (397) and is known to exchange between VLDL and HDL (398). During

alimentary lipemia in normal subjects, the mass of apoE redistributed towards TRL (44% increase in the portion associated with TRL) with no change in total plasma concentration (399). In contrast, postheparin lipolysis was associated with a reduction in plasma concentration of apoE and redistribution towards HDL. Further, some reports have suggested the presence of an exchange mechanism for apoE between VLDL and an extravascular compartment, where apoE is lost from VLDL and is subsequently reintroduced (400-402).

The production (i.e. synthesis and secretion) of apoE in humans has been studied in cultured hepatocytes and has been shown to be independent of the production of apoB-containing lipoproteins (403). Less information is available concerning the *in vivo* kinetics of apoE. In a study involving the use of ^{125}I- and ^{131}I- exogenously-labeled apoE, Gregg and his colleagues (404) measured the RT and PR of plasma apoE3 in nine normolipidemic subjects homozygous for the apoE3 phenotype. The RT was 0.73 ± 0.18 days (mean ± SD) and was longer in females compared to males (0.83 ± 0.16 vs 0.63 ± 0.15 days, respectively). The PR was 3.4 ± 1.5 mg/kg.day, and was less in females compared to males (2.60 ± 0.78 vs 4.20 ± 1.73 mg/kg.day, respectively). ApoE3 was also catabolized most rapidly from the VLDL and slowest from HDL, and the decay of radiolabeled apoE3 from the intermediate fractions, IDL and LDL, was slightly delayed suggesting at least a partial precursor-product relationship between VLDL apoE and IDL/LDL apoE (Fig 1.9) (404). No kinetic parameters for the individual lipoprotein fractions were, however, reported.

Homologous and heterologous dimerization of apoE affected its plasma residence time (RT); apoE-apoA-II heterodimer resided longer in plasma compared to the apoE-apoE dimer, while apoE monomer had the shortest RT (405). Sialylation of apoE was reported to modulate its plasma RT in hypertriglyceridemic subjects (406). Plasma RTs for autologous non-sialylated ^{131}I- and disialylated ^{125}I-apoE3 were 0.95 ± 0.16 and 0.74 ± 0.16 days, respectively, and no conversion from the sialylated to the non-silalylated form was observed.

Importantly, different isoforms of apoE were shown to differ in their plasma RTs. Heterologous radiolabeled apoE4 was catabolized twice as fast as apoE3 in apoE3 homozygous normal subjects, with a mean plasma RT of 0.37 ± 0.01 (days ± SEM) and 0.73 ± 0.05 ($p<0.001$), respectively (407). The largest fraction of radiolabeled apoE4 was found associated with VLDL, while that of apoE3 was associated with HDL. Further, the reduction in plasma concentration of apoE in apoE4 homozygotes compared to apoE3 homozygotes (3.11 vs. 4.83 ± 0.35 mg/dl, respectively) could be entirely explained by the shorter plasma RT of apoE4. Finally, both isoforms were shown to be catabolized faster on VLDL compared to HDL but no kinetic parameters were reported.

Another study by the same group (144) has shown that radiolabeled apoE2 resided longer in plasma of both normolipidemic subjects and patients with type III hyperlipidemia (0.77 ± 0.08 and 1.07 ± 0.21 days, respectively) compared to apoE3 (0.35 ± 0.04 and 0.59 ± 0.15 days, respectively), and this delayed clearance, as discussed above, is thought to be the primary defect

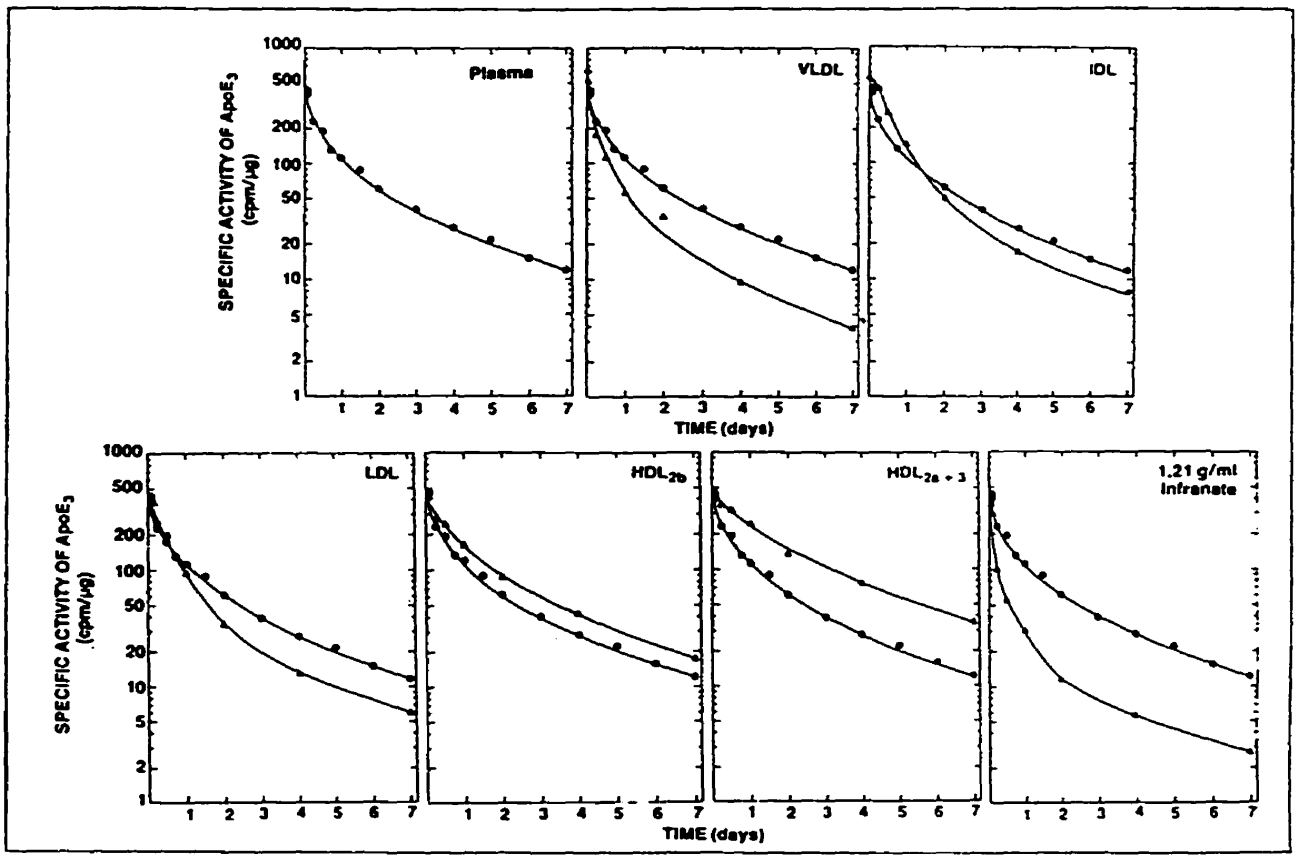


Figure 1.9. The specific activity (tracer to tracee ratio) decay curves of apoE from plasma and lipoprotein subfractions. Following the injection of radioiodinated apoE reassociated with lipoproteins, plasma was obtained at various time intervals and fractioned by ultracentrifugation. The specific activity decay curves of apoE were determined for each lipoprotein subfraction. The plasma specific activity decay curve (●---●) is plotted in each panel to facilitate the comparisons with the different lipoprotein subfractions (▲---▲) (404).

leading to TRL accumulation in these patients. Also, apoE1 ($Lys^{146} \rightarrow Glu$), an apoE mutant associated with the dominant expression of type III hyperlipoproteinemia in a kindred, resided longer in plasma of normal subjects compared to apoE3 (408), and the RT of apoE3 in the proband who has an apoE3/1 phenotype was longer compared to normal subjects. The PR of total apoE was two times higher. Binding assays *in vitro* revealed that apoE1 was defective in interacting with LDL-R, and its affinity to bind heparin was also reduced. In abetalipoproteinemia, a genetic disease in which apoB is absent from the plasma and HDL is the sole plasma lipoprotein, the RT of apoE was somewhat shorter than that of control subjects (0.50 vs 0.66 ± 0.15 days, respectively), while the PR was not "substantially" different (2.14 vs 1.55 ± 0.62 mg/kg.day, respectively) (405).

Finally, Millar et al. (409) have recently proposed a multicompartmental model which describes the *in vivo* kinetics of apoE, based on data obtained from normolipidemic subjects using endogenous labeling (primed constant infusion of $[D_3]$L-leucine). The selected model accounted for a direct production of apoE into HDL, TRL, and an extravascular compartment. It also accounted for mono-directional transfers from HDL and the extravascular compartment to TRL, and a direct loss only from TRL. Using this model, the mean RT for TRL apoE, measured in 6 subjects, was 0.11 days, and that of HDL was 2.96 days.

1.8.5. Plasma Kinetics of ApoC-III

The plasma kinetics of apoC-III in humans have been studied using exogenous labeling (410). Autologous ^{125}I-lableled VLDL (59), or ^{125}I-apoC-III incorporated into HDL (60) or whole plasma (411), were injected in human subjects, and the decay curves of radiolabelled apoC-III were followed in VLDL, IDL and HDL.

Interestingly, the kinetic data for plasma apoC-III, which were obtained using this method, lend themselves to opposing arguments. Regarding the site of entry of apoC-III to plasma, it was initially suggested that the first appearance in plasma of apoC is with HDL (25) and that the initial events associated with TRL entry into plasma are a concomitant transfer of both apoC-II and apoC-III from HDL (412). Conversely, kinetic data generated by Malmendier et al. (413) and the multicompartmental model which best fitted them, tended to support, at least in normal subjects, the largest entry of apoC first appearing in VLDL (up to 87%), and the remaining entry going into HDL. The same conclusion, that newly-synthesized apoC first appears in VLDL and from there is transferred to HDL, was reached by Noel and Rubenstein in experiments involving rat liver perfusion with radiolabeled amino acid (414).

It is well accepted that there is some degree of bidirectional transfer between VLDL apoC-III and HDL apoC-III. This exchange was first described by Bilheimer and colleagues (61). When ^{125}I-lableled VLDL was incubated with plasma *in vitro*, a rapid distribution of radioactivity took place, with a transfer of radiolabeled apoC primarily to HDL. The reverse of this exchange process

occurred when radiolabeled HDL was added to plasma. In other studies, heparin-induced lipolysis was shown to be followed by redistribution of apoC from VLDL to HDL (62), whereas during alimentary lipemia, apoC-III shifted towards VLDL (415). The notion of a bidirectional transfer of apoC-III between VLDL and HDL was further supported by subsequent work (59,60,63-65). The extent of this exchangeability has not however been defined, and it is not clear whether or not it involves the entire VLDL apoC-III and HDL apoC-III pools . Several investigators have concluded that apoC-III molecules readily exchange and fully equilibrate between different lipoprotein fractions (59,60,63). This was based on the finding that when apoC-III radiotracer was introduced into plasma, initial specific activities, which reflect the tracer to tracee mass ratios, were similar in different lipoprotein fractions. The notion of full exchangeability has not gone unchallenged, however. Using autologous ^{131}I-labeled VLDL and ^{125}I-labeled HDL, the presence of non-equilibrating pools of apoC-III in plasma lipoproteins accounting for 30-60% of the total apoC-III mass in each lipoprotein fraction has been demonstrated (64). When ^{125}I-labeled VLDL was injected, the specific activity of radiolabeled apoC-III in VLDL was found to be higher than that in HDL, with the difference being maintained throughout the sampling period (48-72 hours) (Fig 1.10). The ratios of respective specific activities ranged from 1.2 to 1.9 in the six studied subjects (two normolipidemics and four hypertriglyceridemics). When ^{125}I-labeled HDL was injected, however, the higher specific activity for apoC-III was associated with HDL fraction. The results obtained *in vivo* were further supported by findings from *in vitro* experiments.

^{125}I-labeled VLDL from a mildly hypertriglyceridemic subject was incubated with autologous unlabeled HDL and was found to maintain a specific activity that was 30% higher than HDL. When ^{125}I-labeled HDL from the same subject was incubated with unlabeled VLDL, final specific activity in VLDL was less than 10% that of HDL apoC-III. An important conclusion from this work is that any attempt to derive a detailed kinetic model for apoC-III metabolism would require 1) the introduction of multiple tracers simultaneously, 2) the follow-up of decay curves in VLDL, IDL+LDL, and HDL and 3) estimates of the relative distribution of exchangeable and non-exchangeable apoC-III within each lipoprotein density class. Another group of investigators (65) have used artificial TG emulsions to demonstrate the presence of non-equilibrating pools of apoC-II and apoC-III in human plasma lipoproteins. As the concentration of acceptor TG increased, a greater fraction of both apoC-II and apoC-III shifted away from the native plasma lipoproteins to the artificial lipid emulsions, but all of the apoC-II and apoC-III could not be removed from the native lipoproteins. Furthermore, when plasma samples from normotriglyceridemic subjects were used, HDL was the primary donor of apoCs, while VLDL was the primary donor when plasma from hypertriglyceridemic subjects was used.

Studies to date have shown that decay curves (specific activity vs. time curves) of VLDL- and HDL- radiolabeled apoC-III were superimposable. This was interpreted to mean that apoC-III had an identical FCR whether on VLDL or HDL and that plasma apoC-III is a homogenous kinetic pool (59). However, the tri-exponential shape of the plasma apoC-III decay curve and the biphasic nature

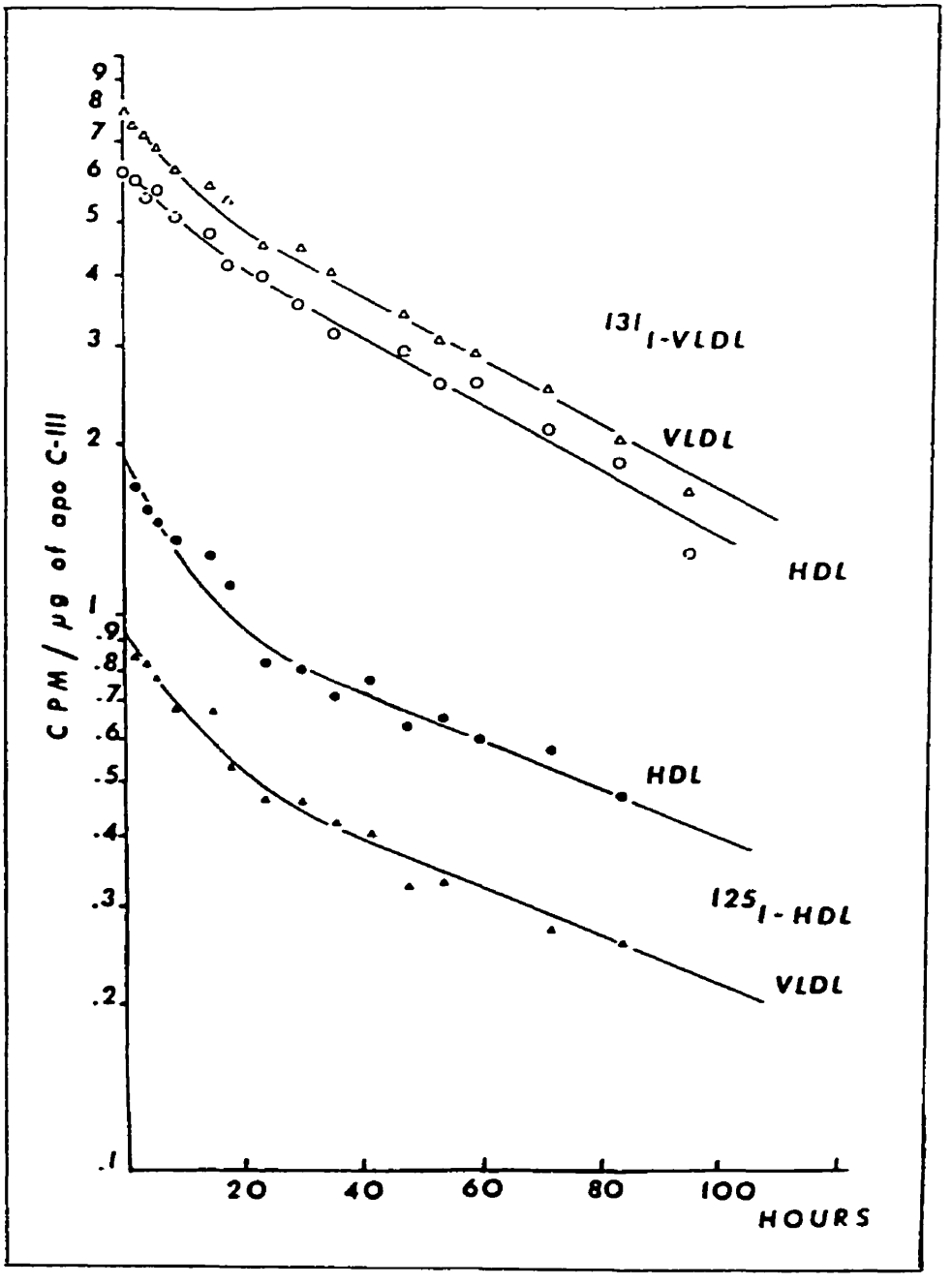


Figure 1.10. ApoC-III specific activity (tracer to tracee ratio) decay curves in a single subject following the injection of ^{131}I-labeled VLDL (open symbols) and ^{125}I-labeled HDL (closed symbols). After injection of the radio-labeled VLDL tracer, the specific activity curve for VLDL apoC-III (Δ) was consistently higher than that for HDL apoC-III (O). In contrast, when the tracer was radioiodinated HDL, the HDL apoC-III specific activity curve (●) was consistently higher than the curve for VLDL apoC-III (▲) (64).

of the urine/plasma radioactivity ratio curve (inconstant urine/plasma radioactivity ratio) observed in longer experiments (15 days follow up experiments) (60,411,416) did not support this notion (Fig 1.11). Malmendier and his colleagues (60) discussed the possibility that apoC-III metabolism could be heterogeneous due to the presence of 2 or more populations of apoC-III-containing lipoprotein particles with different clearance rates. They suggested that the first part of the urine/plasma ratio curve reflected the quickly metabolized particles, while the terminal slope reflected the slowly metabolized ones. In order to describe plasma apoC-III kinetics, Malmendier and colleagues used a multicompartmental model which accounted for "fast" and "slow" pathways for the removal of apoC-III, and suggested that the fast pathway represented large Lp-B:CII:CIII:E particles in the VLDL density range while the slow pathway represented LpA-I:A-II:C-II:C-III particles in the HDL range (416).

Finally, expansion in the pool size of apoC-III in hypertriglyceridemic subjects was attributed to both increased production and delayed catabolism in separate studies. Huff and colleagues (59) showed that the FCR of apoC-III and apoC-II were inversely related to apoC-III, apoC-II and TG pool size suggesting that increased plasma levels of apoC-III_1, apoC-III_2 and apoC-II were related to longer RT of TG-rich VLDL particles in plasma. When studying the effect of the consumption of high carbohydrate diets on apoC-III_1, apoC-III_2, apoC-II, and VLDL apoB, the same group showed that the increase in the mass of the apoCs associated with the consumption of high carbohydrate diet was due to overproduction and not lower fractional catabolism (412). The average half-lives

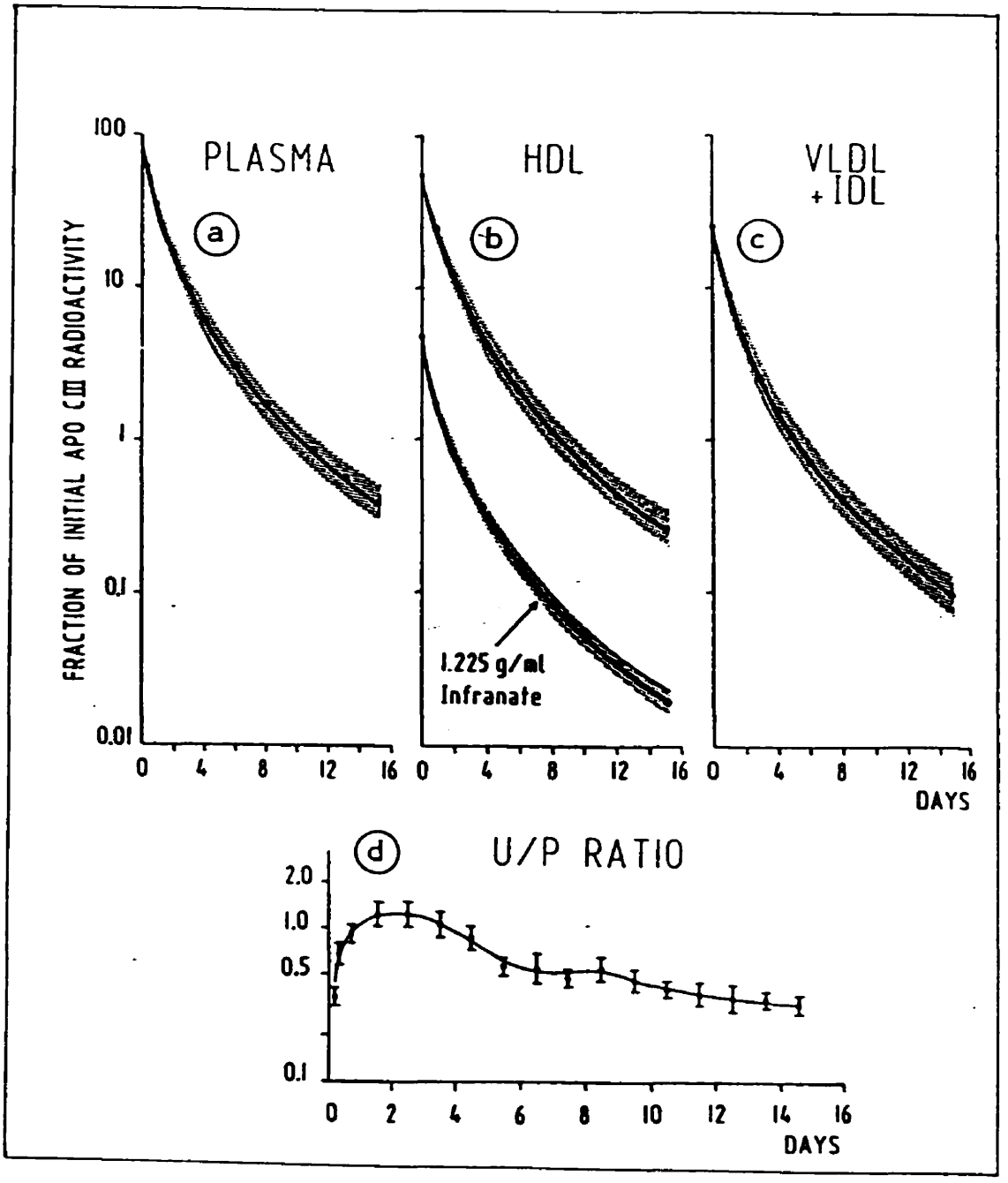


Figure 1.11. Mean radioactivity decay curves of plasma and lipoprotein subfractions as a fraction of initial apoC-III radioactivity. Shaded areas indicate 1 SD on either side of the mean. Mean urine/plasma radioactivity ratios are shown in d, vertical bars are SD (60).

of apoC-II, C-III_1, and C-III_2 were similar in both studied groups, and the increase in the mass of apoB was shown to be due to both overproduction and delayed catabolism in each study. Other reports by Malmendier and his co-workers have shown increased pool size of apoC-III in hypertriglyceridemic subjects to be a function of increased production (60,411,413,416). Table 1.1 summarizes the kinetic results reported for apoC-III in the most recent studies.

1.9. HDL Metabolism and Plasma Kinetics of ApoA-I and ApoA-II

1.9.1. HDL and Coronary Heart Disease

Epidemiological studies have consistently demonstrated that low plasma HDL levels are associated with the development of CHD (417). The prospective Framingham study (10) reported a highly significant inverse relationship between CHD and HDL-C on the basis of a 12-year follow-up. The relationship held after multivariate adjustment for total cholesterol systolic blood pressure, cigarette smoking, and body mass index. HDL-C levels showed a strong inverse relationship with CHD at low (<200 mg/dL), medium, and high (>260 mg/dL) total cholesterol levels. In another prospective study, the Lipid Research Clinics Primary Prevention Trial (29), HDL-C levels were a strong inverse predictor of coronary heart disease incidence in study subjects over the 7-10 year follow-up period. Subjects with HDL-C levels under 40 mg/dL had twice the incidence of CHD as those with levels over 50 mg/dL. Although the major finding was that CHD incidence was lowest among men with the greatest treatment-induced reductions in LDL-C levels, it was also found that disease incidence decreased

Table 1.1. Kinetic Results Reported for ApoC-III in Previous Studies*

Tracer	Subjects	ApoC-III RT (day)	ApoC-III TR ($mg.kg^{-1}.d^{-1}$)	Reference
^{125}I-VLDL	Normolipidemics (n=8)	1.54, 1.60**	2.6, 2.4	Huff et al. (59)
	Hypertriglyceridemics (n=7)	3.21, 2.98**	3.7, 2.5	
^{125}I-VLDL	Normolipidemics (n=6)	0.50, 0.48**	1.2, 1.1	Huff et al. (412)
	Normolipidemics on a high carbohydrate diet (n=6)	0.53, 0.52**	2.0, 1.9	
^{125}I-apoCIII-HDL	Normolipidemics (n=8)	1.24	2.3	Malmendier et al. (60)
^{125}I-apoCIII-plasma	Hypertriglyceridemics (n=4)	1.77	6.1	Malmendier et al (411)
	Hypertriglyceridemics on fenofibrate (n=4)	1.10	5.0	
^{125}I-VLDL	Normolipidemics (n=2)	0.83	NA	Bukberg et al. (64)
	Hypertriglyceridemics (n=4)	2.41	NA	

*Copied from a project proposal with the permission of Dr. Jeffrey Cohn, Hyperlipidemia and Atherosclerosis Research Group, Clinical Research Institute of Montreal.

** Results are reported for apoC-III_1 and apoC-III_2, respectively.

with treatment-induced HDL increments. In the Helsinki Heart Study (418), treatment of middle-aged men with LDL-C levels not higher than 200 mg/dL with gemfibrozil over 5 years decreased LDL-C levels by 8% and increased HDL-C levels by more than 10%. This caused a 34% reduction in the incidence of CHD which was much greater than expected from the degree of LDL-C lowering. The excess protection was attributed to increase in HDL-C. In a 21 year follow-up of more than 8500 middle-aged men (419), those with low plasma HDL-C (< 0.9 mmol/l), who represent more than one sixth of the healthy population studied, displayed a 36% higher CHD mortality than individuals with HDL-C greater than 0.9 mmol, independent of total plasma cholesterol concentration. In a study in which intravascular ultrasound imaging was used to evaluate the extent of atherosclerosis in 48 asymptomatic patients with FH or FCH (420), plasma HDL-C level and total/HDL-cholesterol ratio were more powerful predictors of coronary plaque burden than LDL-C levels, suggesting that HDL exert their protective effect by preventing early events that initiate the formation of atherosclerotic lesions.

The association between the presence of low HDL-C and CHD has been mostly attributed to the role that HDL play in reverse cholesterol transport (RCT) (67). HDL has other potentially protective functions, however, such as inhibiting LDL oxidation (421,422), reducing the expression of endothelial cell adhesion molecules (423), and inhibiting platelet aggregation (424,425). Finally, it is important to point out that decreased level of HDL-C associated with CHD is often accompanied by other changes in the lipid profile, namely elevated levels

of TG, VLDL-C, IDL-C and postprandial lipoproteins and by the presence of small dense LDL (426-428). The correlation between low levels of HDL-C and the occurrence of CHD could then be partially due to increased levels of other atherogenic lipoprotein particles.

1.9.2. Reverse Cholesterol Transport

Reverse cholesterol transport (RCT) is a pathway by which cholesterol is transported from extrahepatic tissues to the liver where it is eventually excreted into the intestine in bile. It has been suggested, although not proven, that impairment of this pathway may lead to atherosclerosis and CHD (57,58).

The RCT process can be artificially divided into three consecutive steps: 1) efflux of cholesterol from cells to HDL in the extracellular space, 2) esterification of HDL free cholesterol by LCAT, and 3) CETP-mediated transfer of CE from HDL to other lower density lipoproteins, or direct delivery of CE to the liver and steroidogenic tissues.

Firstly, the efflux of cholesterol from peripheral cells may take place by two possible mechanisms. In one, cholesterol diffuses from cell plasma membrane to the extracellular fluid where it associates with phospholipid-containing acceptor particles without the need for direct contact between the acceptor and the cell (429). In the other, an apoA-I-containing acceptor interacts with the exporting cell surface in a process that markedly increases cholesterol desorption from the cell membrane. Evidence suggests that HDL

subpopulations differ in terms of their effectiveness as acceptors of cell cholesterol (430,431,210).

Lipid-free apolipoproteins may also be involved in the initial step of RCT. ApoA-II, apoA-IV, apoE, as well as apoA-I have been shown to accept cell phospholipids and cholesterol (432,433). The small molecular size of these lipid-free molecules facilitates their transfer from plasma to the interstitial space where they may be enriched with exported cholesterol and phospholipids and subsequently transformed into lipid-poor small HDL. In a study by Czarnecka and colleagues (434), incubation of lipid-free apoA-I and apoA-II with cells led to the formation of HDL particles. Lipid-free apoA-I can thus be a very nascent form of HDL, but it can also be formed by lipid unloading during HDL remodeling (the third step in RCT). In addition to being an initial acceptor of cholesterol and phospholipids from cellular efflux, lipid-free apoA-I may be metabolically channeled in several ways: 1) it may be reincorporated into pre-existing HDL particles which increase in size as a result of accumulating CE formed in the LCAT-mediated reaction (the second step in RCT) (435), 2) it may interact with lipids released from TRL undergoing lipolysis to form new HDL (202), 3) or it may be cleared from plasma through glomerular filtration and tubular reabsorption in the kidney (Fig 1.12) (436).

Secondly, LCAT activity catalyzes the esterification of free cholesterol in HDL. LCAT-mediated enrichment of discoidal preβ particles with CE results in the formation of larger, α-migrating, spherical HDL. The change in shape, size and electrophoretic mobility of HDL during this process of maturation is also

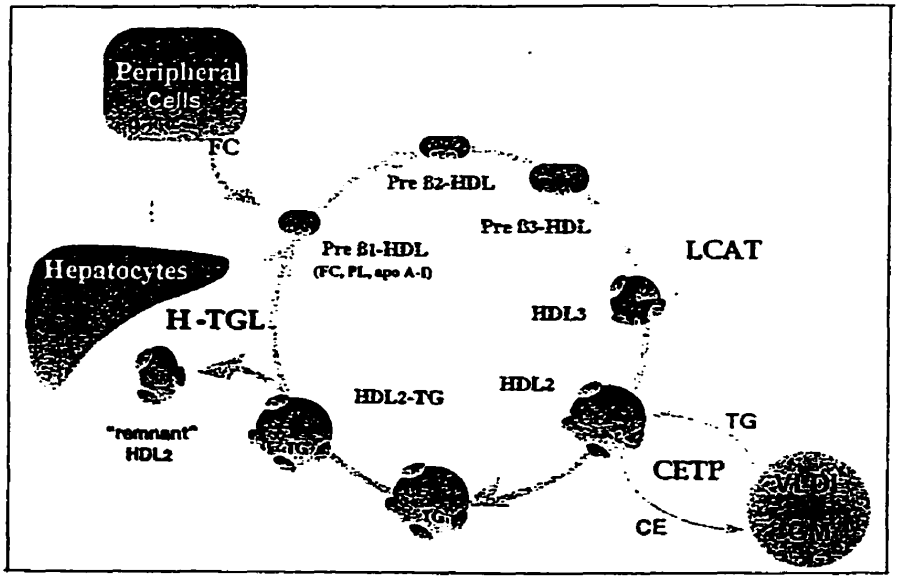
accompanied by a change in the conformation of apoA-I on the particle (94,96,99). In contrast, little change in the conformation of apoA-I has been observed during cycles of expansion and shrinkage (HDL_3 to HDL_2, and HDL_2 to HDL_3, respectively) which characterize remodeling of spherical HDL (100,436). In addition, large spherical HDL have apoA-II as a second structural protein (438).

Thirdly, CE are delivered to the liver and steroidogenic tissues. This delivery process is thought to occur both directly and indirectly. The direct pathway involves selective CE uptake by steroidogenic tissues and the liver. The indirect pathway, which is quantitatively more important, involves CETP-mediated transfer to TRL and their remnants and their subsequent uptake by the liver.

HDL CE have been shown to be cleared by steroidogenic tissues and the liver without parallel uptake of the protein moiety of the HDL particle (253). Although the mechanism of this selective uptake is poorly-defined, the scavenger receptor SR-B1 has been implicated in the process (251-253). HDL supposedly docks to the cell membrane, delivers some CE (and perhaps other lipids) and then dissociates from the cell surface and continues to circulate in the blood, now as a partially lipid-depleted particle.

In the indirect pathway, CETP-mediates the exchange of CE on HDL with TG. Subsequently, HL hydrolyzes TG on HDL in a process that is thought to be responsible for 1) the formation of HDL3 from HDL2, and 2) the generation of preβ1 and possibly the shedding of lipid-free apoA-I from α-HDL (57,58). An

A



B

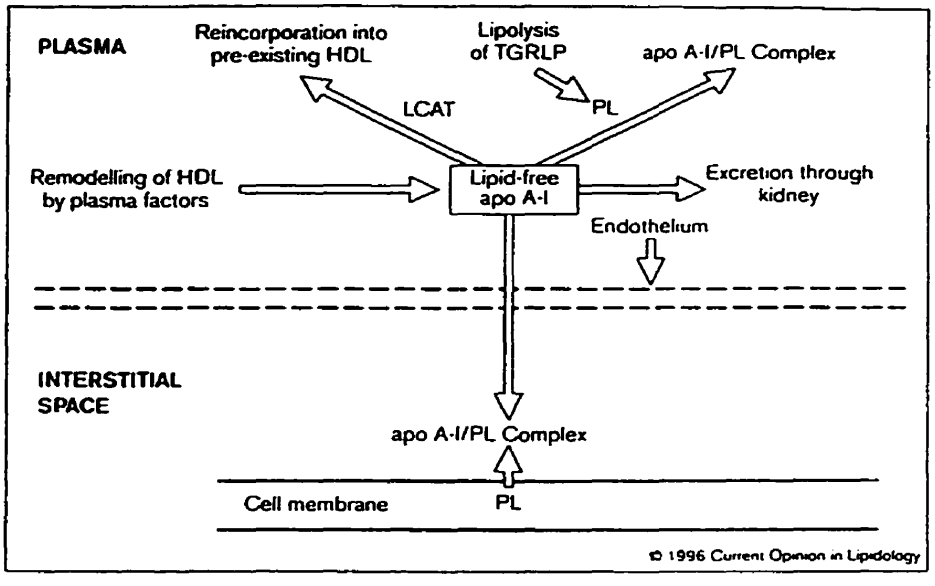


Figure 1.12. Reverse cholesterol transport (A), and metabolic fates of apoA-I (B) (ref. 437 and 57, respectively).

increase in pre-β HDL postprandially has been reported, particularly after heparin injection (439) as plasma activities of HL and CETP increased (440).

In conclusion, the processes of RCT, HDL maturation and HDL remodeling in plasma are interrelated. The net effect of these events is a transfer of cholesterol from peripheral tissues mainly to the liver, its site of clearance, and to steroidogenic tissues. These events are also associated with a net transport of apoA-I and apoA-II in plasma. ApoA-I is produced by the liver and intestine, probably as a lipid-free molecule, and is subsequently channeled into the HDL metabolic cycle, as described previously. ApoA-I ultimately gets cleared from plasma either as a lipid-free or lipid-poor particle by glomerular filtration and incomplete tubular reabsorption in the kidney. Similarly, apoA-II is produced mainly in the liver, but little is known about its catabolic fate. The *in vivo* kinetics of apoA-I and apoA-II reflect their net transport in plasma.

1.9.3. HDL Deficiency

Human HDL deficiency (hypoalphalipoproteinemia) defines a group of dyslipidemias characterized by an HDL cholesterol level below the 10th percentile for age- and sex-matched subjects (441). Several primary disorders have been found to be associated with HDL deficiency. The list of these disorders includes: 1) apoA-I/CIII or apoA-I/C-III/A-IV deficiency, 2) the presence of apoA-I structural variants, 3) apoA-II deficiency, 4) LCAT deficiency, 5) severe hypertriglyceridemia and 6) Tangier disease or familial HDL deficiency (reviewed in 442-445). HDL deficiency can also be secondary to: 1) obesity, a low total fat

diet, or a diet enriched with polyunsaturated fat, 2) drugs (probucol, antihypertensive agents, anabolic steroids, and cigarette smoking), and 3) certain diseases (liver and kidney failure, diabetes, and acute inflammation) (442,443). These conditions are not by themselves sufficient to induce HDL deficiency, and a genetic predisposition is probably necessary for the expression of the dyslipidemia. Finally, HDL deficiency is more prevalent in men than in women (442). The increase in endogenous androgens at puberty in males is thought to lead to a reduction in the HDL level. Exogenous estrogens increase HDL-C while exogenous androgens have the opposite effect (446).

Reduction in plasma HDL-C in patients with HDL deficiency is usually associated with a reduction in the plasma concentration of apoA-I and apoA-II. Numerous *in vivo* kinetic studies have been carried out in an attempt to identify the cause of variation in apoA-I and apoA-II levels in the general population, and the cause of their markedly low levels in patients with HDL deficiency. These studies have been conducted using exogenous or endogenous labeling techniques. In experiments involving exogenous labeling (447), HDL were separated from human plasma and apoA-I and/or apoA-II were isolated. HDL, or the individual apolipoproteins (in one case, different isoforms of apoA-I (448)), were labeled with ^{125}I or ^{131}I, and reinjected into plasma of studied subjects. In endogenous labeling studies (449,450), a stable-isotopically labeled amino acid was introduced into studied subjects and its rate of appearance in apoA-I or apoA-II was measured.

1.9.4. Plasma Kinetics of ApoA-I and ApoA-II

1.9.4.1. Kinetics of ApoA-I and ApoA-II in Normolipidemic Subjects

Most studies have shown that variation in plasma apoA-I level in the general population is determined by the rate of catabolism of apoA-I (451,452), and that reduced apoA-I catabolism is responsible for higher plasma levels in women compared to men (453). In contrast, variations in plasma apoA-II levels, as well as the distribution of apoA-I between LpA-I and LpAI:AII particles is determined by the production rate of apoA-II (454). At the same time, FCRs of both apoA-I and apoA-II have been found to be inversely correlated with HDL-C levels (453).

Studies have also shown that apoA-I is catabolized at a significantly faster rate than apoA-II (447). Ikewaki and colleagues (449,450) compared the plasma kinetics of apoA-I and apoA-II in a group of normolipidemic subjects through the simultaneous use of exogenous and endogenous labeling techniques. Using exogenous labeling, the mean RT for apoA-I was found to be 4.80 days in a group of five subjects, compared to 5.19 days for apoA-II. Also, by using endogenous labeling, the mean RT for apoA-I was found to be 5.14 days, and that for apoA-II was 6.09 days. The difference in RTs obtained using the two methods could be attributed to protein modification in exogenous labeling, which could take place during isolation and radioactive labeling (449,450) (see section 1.10). In another exogenous labeling study by the same group (454), mean PR of apoA-I for a group of 50 normolipidemic subjects was measured to be 11.8 mg/kg.day, whereas the mean PR of apoA-II for 35 of these subjects was 2.68

mg/kg.day. In another study, apoA-I was found to have shorter RT if injected on LpA-I into normolipidemic subjects compared to LpAI:AII (mean 4.39 vs. 5.17 days, n=3) (114), suggesting that apoA-I on LpAI is catabolized faster than apoA-I on LpAI:AII.

Finally, proapoA-I, the precursor of apoA-I which constitutes 2-3% of the plasma apoA-I pool in normolipidemic subjects (92,455), has a markedly shorter RT compared to that of the mature form. Using exogenous labeling, Bojanovski and his colleagues (448) found the RT of plasma proapoA-I to be 0.19 and 0.27 days in two normolipidemic subjects. The corresponding RTs for mature apoA-I were 5.2 and 7.7 days, respectively. The presence of a plasma apoA-I pool with a fast turnover rate (RT less than 1 day) was also suggested by a study of Fisher and colleagues (456), in which a multicompartmental model was developed to fit kinetic data obtained by the simultaneous use of tritium- and deuterium-labeled leucine as tracers.

1.9.4.2. Kinetics of ApoA-I and ApoA-II in Patients with HDL Deficiency

1.9.4.2.1. ApoA-I Deficiency and apoA-I Variants

The marked reduction in plasma levels of apoA-I and apoA-II in patients with HDL deficiency has been attributed mainly to increased rates of plasma catabolism rather than decreased rates of production. Nevertheless, apoA-I synthesis and secretion into plasma has been shown to be absent in patients with apoA-I/CIII or apoA-I/C-III/A-IV deficiency. In this form of HDL deficiency, several mutations in the apoA-I/C-III/A-IV gene locus have been identified (442),

including: 1) apoA-I/C-III inversion, 2) apoA-I/C-III/A-IV deletion, 3) nonsense point mutations in the apoA-I gene, 4) insertion mutation in the apoA-I gene leading to a frame shift and a subsequent premature termination of translation, 5) point deletion in the apoA-I gene leading to a frame shift and a subsequent premature termination of translation, and 6) a 45-base pair deletion in the apoA-I gene.

Synthesis and secretion into plasma of genetically-determined variants of apoA-I have been found to be associated with increased catabolism of apoA-I and HDL (442,444). Many structural mutations in apoA-I have been described, though not all lead to HDL deficiency. Two different mutations in apoA-I are associated, not only with low levels of HDL-C, but also with systemic amyloidosis: $Gly^{26}\rightarrow Arg$ (apoA-I_{Iowa}) (457,458) and $leu^{60}\rightarrow Arg$ (459). Increased catabolism of structural variants of apoA-I has been demonstrated in several studies (460-463). Kinetic analysis of plasma radioactivity curves demonstrated that clearance of the mutant apoA-I_{Iowa} from plasma was at least two fold faster compared to normal apoA-I (460). ApoA-I_{Milano}, which is another mutant form of apoA-I ($Arg^{173}\rightarrow$Cysteine) was also cleared faster from plasma compared to apoA-I in normolipidemic subjects (461). Furthermore, in patients with HDL deficiency related to the presence of apoA-I_{Milano}, normal apoA-I was also catabolized faster compared to apoA-I in control subjects. The metabolism of apoA-I and apoA-II was examined in a Finnish kindred with a 3-bp deletion in the apoA-I gene resulting in a deletion of Lys^{107} in the mature apoA-I protein ($Lys^{107}\rightarrow 0$) (463). The affected family members had a reduction in LpAI:AII but

not LpAI plasma levels, due to a shorter RT of both apoA-I and apoA-II, and an increased production rate of only apoA-I.

1.9.4.2.2. ApoA-II Deficiency

There is only one report that describes clinical cases of apoA-II deficiency (109). Two Japanese siblings with apoA-II deficiency had normal HDL-C levels but apoA-I levels that were 25% and 40% lower than normal, suggesting that the absence of apoA-II may result in increased catabolism of apoA-I, and that the presence of apoA-II stabilizes apoA-I on the HDL particle. A study of two inbred mouse strains with 10-fold differences in apoA-II levels showed that there was no difference in hepatic mRNA levels or apoA-II synthesis rates between the two strains, suggesting that the underlying factor behind the reduction in apoA-II level in this case was increased catabolism (464).

1.9.4.2.3. LCAT Deficiency

Both classical LCAT deficiency and fish eye disease are associated with markedly reduced levels of HDL-C, apoA-I and apoA-II (212,444). *In vivo* kinetic studies of apoA-I and apoA-II were performed in five patients with classic LCAT deficiency or fish eye disease using both exogenous and endogenous labeling (465). The FCRs of apoA-I and apoA-II were 2.2 and 3 fold higher than those in control subjects, suggesting that cholesterol esterification is necessary for HDL maturation and that in its absence apoA-I, and even more so apoA-II, are highly susceptible to hypercatabolism.

1.9.4.2.4. Hypertriglyceridemia

Several forms of hypertriglyceridemia are associated with low levels of HDL-C and HDL apolipoproteins. Furman et al. (466) were the first to show increased catabolism of the protein moiety of HDL in two patients with endogenous hypertriglyceridemia, one of whom had excessive alcohol intake, and two patients with LPL deficiency. In contrast, Fidge et al. (467) found the FCRs of apoA-I and apoA-II to be similar in both normal and hypertriglyceridemic patients, while Saku et al. (468) found that in a series of patients with endogenous hypertriglyceridemia and HDL deficiency, the low plasma HDL-C, apoA-I and apoA-II levels resulted from decreased synthesis and increased FCR of apoA-I and apoA-II. In more recent studies, Brinton et al. (469) investigated the kinetics of apoA-I and apoA-II in 15 human subjects with low HDL-C, six with normal plasma TG and nine with high TG, and 13 control subjects. They found FCR of apoA-I to be equally elevated in both groups of hypoalphalipoproteinemic subjects compared to controls, whereas PR for apoA-I was normal and PR for apoA-II was slightly higher. Finally, Goldberg et al. (470) showed that the FCR of homologous monkey HDL apolipoproteins injected into LPL-inhibited animals was more than double that of normal animals, and that a greater proportion of HDL apolipoprotein degradation occurred in the kidneys of hypertriglyceridemic animals compared to controls.

1.9.4.2.5. Tangier Disease and Familial HDL Deficiency

1.9.4.2.5.1. Clinical Findings and Plasma Lipoprotein Profile

Tangier disease (TD) is a rare autosomal disorder characterized by severe deficiency or absence of normal HDL in plasma and by the accumulation of CE in many tissues including tonsils, peripheral nerves, liver, spleen, lymph nodes, thymus, intestinal mucosa, skin, bone marrow, and cornea (471). The major clinical signs in TD patients are hyperplastic orange tonsils, splenomegaly, and relapsing neuropathy, probably related to lipid deposition in Schwann cells. TD patients are severely hypoalphalipoproteinemic. HDL-C levels in homozygotes are virtually reduced to zero and their apoA-I and apoA-II plasma levels are extremely low (1-3 and 5-10% of normal, respectively) (472,473). A subpopulation of HDL particles contain only apoA-II without apoA-I, and some apoA-II is found associated with VLDL (474-476). Most apoA-I in TD patients is found on pre-β particles (473,477), and the relative distribution of apoA-I between proapoA-I and mature apoA-I isoforms, as shown by isoelectric focusing gels, is markedly shifted towards higher concentrations of proapoA-I (478). Further, plasma TG levels in TD patients are either normal or elevated (471).

Despite the atherogenic plasma lipoprotein profile in patients with TD, the association with premature CHD has not been fully established. In a fairly recent review by Serfaty-Lacrosniere et al. (479), CHD was observed in 20% of TD patients vs. 5% of controls ($p<0.05$), and in those that were between 35 and 65 years of age, 44% had evidence of CHD vs. 6.5% in controls ($P<0.01$). The

authors concluded that coronary artery disease is a significant clinical problem in middle-aged and elderly TD homozygotes.

1.9.4.2.5.2. Etiology of TD

The Tangier phenotype is not associated with complete absence of apoA-I or apoA-II or with the presence of structural variants of either apolipoprotein (471,475,480). Furthermore, there is no evidence to suggest that a primary impairment in the activity of a lipoprotein processing enzyme is responsible for the occurrence of the disease (471). Partial LCAT deficiency has been reported in TD patients, however this deficiency is probably secondary to the absence of LCAT substrates in HDL and its co-factor, apoA-I (481). In fact the only common clinical feature in TD and LCAT deficiency is the accumulation of cholesterol in the cornea, which may reflect disturbed RCT.

One theory has suggested that TD is a disorder of intracellular processing and subsequent export of cholesterol and phospholipids (482-486). The cellular interaction of Tangier monocytes with normal HDL was shown to be markedly different from control monocytes. HDL binding to Tangier monocytes was moderately increased, but the bulk of the internalized HDL was detected in secondary lysosomes and was not resecreted (482). Cholesterol efflux, mediated by HDL3 binding to the plasma membrane, was 50% less in Tangier fibroblasts compared to control fibroblasts (483). Further experiments showed that this reduction in efflux of cholesterol from Tangier fibroblasts was due to reduced efflux of newly synthesized sterol and was possibly related to an impairment in the activation of protein kinase C (PKC). A subsequent study by

Walter et al. (484) showed that: 1) HDL3- and apoA-I-induced hydrolysis of phosphatidylcholine via phosphatidylcholine-specific phospholipase D (PC-PLD) was markedly reduced (60-80%) in Tangier fibroblasts compared to normal fibroblasts, whereas the formation of diacylglycerol via PC-specific phospholipase C (PC-PLC) was two to threefold enhanced, and 2) impaired mobilization of cholesterol in Tangier fibroblasts could be completely overcome by increasing phosphatidic acid levels. These results suggested that the molecular defect in TD is upstream of PKC and affects G-protein-dependent regulation of PC-specific phopholipases. Two independent studies have demonstrated that apoA-I has an impaired ability to remove cholesterol from Tangier fibroblasts (484-486). It has also been shown that Tangier cells are characterized by an impaired removal of phospholipids and a defective interaction of apoA-I with cell-surface binding sites (486).

Alternatively, TD may be caused by an impairment in HDL maturation in plasma. Huang et al. (487) has suggested that TD is associated with the absence of a factor in plasma that converts preβ1-LpA-I into α-LpA-I. Finally, in a recent breakthrough, Rust et al. (488) reported the localization of the genetic defect in TD to chromosome 9q31, and suggested that TD may be due to a loss-of-function defect.

1.9.4.2.5.3. *In vivo* Kinetics of Plasma apoA-I and apoA-II in TD

Several *in vivo* studies of the plasma kinetics of apoA-I and apoA-II in TD patients have been carried out using exogenous labeling. The aim of these

studies was to determine whether the reduction in plasma levels of apoA-I and apoA-II in TD was due to decreased production or increased catabolism (hypercatabolism). Both apoA-I and apoA-II were shown to be catabolized markedly faster in TD homozygotes and heterozygotes, compared to normolipidemic subjects (448,489,490). These kinetic data are consistent with reports showing no evidence of apoA-I or apoA-II impaired production in TD patients (471,475,480), and suggest that the reduction in apoA-I and apoA-II levels, as well as HDL-C concentration, is due to hypercatabolism of HDL. Interestingly, TD is characterized by a relative increase in plasma proapoA-I level compared to mature apoA-I (478). This relative increase is not due to defective conversion of proapoA-I to mature apoA-I as was shown *in vitro* (491) and *in vivo* (448). The relative increase in proapoA-I, and equally the relative decrease in mature apoA-I, is rather due to a milder hypercatabolism of proapoA-I compared to the one affecting mature apoA-I. It is also due to the fact that direct loss from the proapoA-I pool decreases the transport into the mature apoA-I pool, thus contributing to its reduction (448). In a kinetic study carried out in 2 TD homozygotes and 2 control subjects, using radiolabeled apoA-I isoforms, RT of proapoA-I was found to be almost two-fold less in TD patients compared to control subjects (mean: 0.09 vs. 0.23 days, respectively) whereas the RT of mature apoA-I in TD patients was only 4% that in control subjects (mean: 0.22 vs. 6.45 days, respectively) (448). These data suggest that in TD patients all isoforms of apoA-I are hypercatabolized, though mature apoA-I is more affected than proapoA-I.

1.9.4.2.5.4. FHD with no Clinical Manifestations of Tangier Disease

Several published reports have presented cases of non-Tangier FHD (492-496). Patients studied in these reports had plasma lipoprotein profiles similar to that in TD but did not show clinical manifestations of TD.

In one study, Marcil et al. (492), described "severe" familial HDL deficiency in 3 French Canadian kindreds, as a trait with autosomal codominant inheritance. Two members of one kindred, who were extensively investigated, had normal fasting TG concentrations with HDL-cholesterol levels below the 5th percentile. One of them had evidence of CHD. Both patients had a 50-80% reduction in plasma apoA-I concentration, a decrease in average HDL particle size, and a relative increase in plasma proapoA-I levels. No evidence was obtained for the presence of an apoA-I or apoA-II gene abnormality, and these patients had none of the LPL gene mutations commonly found in French Canadians (497). LCAT activity was also normal. Finally, none of the patients had clinical manifestations of TD. In a subsequent study in these patients (493), HDL3- and apoA-I-mediated cellular cholesterol and phospholipid efflux was investigated. Fibroblasts from patients displayed an almost 25% reduction in HDL3-mediated cholesterol efflux and 50-80% reduction in apoA-I-mediated C and PL efflux, compared with normal cells. CE mobilization was also significantly reduced in patients' fibroblasts. Finally, the reduction in cholesterol and phopholipid efflux was not found to be associated with abnormal cellular HDL3 binding.

In a separate study, Eberhart et al. (496) investigated cellular cholesterol transport in a case of FHD without clinical evidence of TD. ApoA-I-mediated efflux of cholesterol from fibroblasts of the 51-yr-old woman was reduced by 50%, as was efflux to HDL. Interestingly, the woman's fibroblasts synthesized greater amounts of lecithin than did normal or Tangier fibroblasts, while they did not differ in their sterol synthesis rates, cellular cholesterol and CE content, or incorporation of oleate into CE.

Finally, there have been a couple of reports that investigated the plasma kinetics of apoA-I and apoA-II in cases of FHD without clinical evidence of TD (494,495). Emmerich et al. (494) described a 46-year-old man with coronary artery disease, who had severely reduced levels of plasma HDL-C and total plasma apoA-I. His apoA-I was structurally normal and he had no clinical features of TD. Using endogenous labeling, apoA-I was found to be hypercatabolized from plasma of this patient, while production was normal. Rader et al. (496) similarly identified two male and three female probands with very low HDL levels (apparently of familial origin), who had no evidence of premature CHD. They had no clinical or biochemical characteristics typical of known HDL deficiency states, and all five individuals had increased catabolism of HDL apoA-I and apoA-II.

The originality of the work presented in this thesis is due in part to the use of endogenous labeling with stable isotopes in the study of plasma kinetics of apolipoproteins. Section 1.10 discusses this method, its advantages and

disadvantages, and compares it to the more conventional method of exogenous labeling with radioactive isotopes.

1.10 Stable Isotopes in the Investigation of Plasma Apolipoprotein Metabolism

The use of stable isotopes in metabolic studies in humans was pioneered by Schoenheimer and Rittenberg in the 1930's, prior to the advent of radiotracer methodology (498-500). However, due to technical difficulties in the production and detection of stable isotopes, radiotracer methodology took over as the method of choice for *in vivo* kinetic experiments.

Exogenous radiolabeling experiments have contributed significantly to our understanding of the plasma kinetics of apolipoproteins in normolipidemic subjects and in patients with several dyslipidemias. This method however is not without its limitations. In addition to the issue of safety, related to the use of radiotracers in human studies, there is a concern regarding the validity of the "tracer assumption", which is essential for meaningful interpretation of kinetic data (501). The tracer assumption simply states that the labeled material (tracer) behaves physiologically like the unlabeled counterpart (tracee), thus the kinetics of the "experiment" describe those of the "system". Isolation and exogenous radiolabeling may modify the protein under investigation and the tracer may be introduced in a non-physiological fashion, or even associate differently with physiological compartments upon its re-introduction (498). For instance, Patterson and Lee (501) have presented evidence which suggests that self-

association, as well as affinity, capacity, and kinetics of apoA-I binding to small unilamellar lipid vesicles (EYPC), were significantly altered following radioiodination. Furthermore, Ikewaki et al. (449,450) studied the plasma kinetics of apoA-I and apoA-II using simultaneous endogenous stable isotope and exogenous radiotracer methods, and reported shorter RT for both proteins using exogenous labeling. The authors suggested that, at least in the case of apoA-II, the difference may be attributed to protein modifications during purification and radioiodination in the exogenous labeling protocol (450).

Recent advances in the synthesis of compounds labeled with stable isotopes and improvements in computer-supported mass spectrometry have permitted the revival of Schoenheimer and Rittenberg's concept of using stable isotopes and endogenous labeling in human kinetic studies, including those of plasma apolipoproteins (498,502). The use of endogenous labeling methods in general have the advantage of allowing for a "physiological" introduction of the tracer through tissue-mediated incorporation of labeled amino acid into proteins. The tracer in this case is not subjected to method-related modification and is more likely to resemble the studied tracee. Moreover, endogenous labeling allows for experiments with shorter periods of time and under more constrained conditions, such as fasting. This is because most endogenous labeling experiments are designed with the aim of directly measuring the rate of production of apolipoproteins. The latter can be derived from the rate of incorporation of newly-synthesized (labeled) protein in the physiological pool of interest within a few hours from the start of labeled amino acid infusion. Finally,

endogenous labeling allows for simultaneous study of several proteins at the same time, due to the fact that what is being introduced to the system in these experiments is a labeled universal precursor (amino acid) and not product (protein). The use of stable isotopes in particular has the obvious advantage of safety and allows for multiple studies in the same subject and studies in special groups such as children and pregnant women (498,502,503).

On the other hand, endogenous labeling with stable isotopes has its limitations. Firstly, because proteins are labeled simultaneously, endogenous labeling is not optimal for the study of interconversions. Secondly, deriving meaningful data from the later phase of the experiment, i.e. the decay phase following termination of labeled precursor infusion, is complicated by the effect of tracer recycling. Thirdly, little information, if any, can be obtained from the "excretion data", which can be an important source of information in exogenous radiotracer labeling experiments. Fourthly, the introduction of a labeled precursor, as opposed to a labeled product in exogenous labeling procedure, complicates the multicompartmental modeling, which is now the method of choice for interpreting kinetic data (504,505); the model will have to account for several precursor pools as well as product pools. Finally, and perhaps most importantly, it is difficult in endogenous labeling to determine the precursor enrichment, a parameter which is critical for data interpretation in the majority of applications of this method. This is because the true precursor for an amino acid incorporated in an apolipoprotein is the amino acyl-transfer RNA (tRNA) in the liver and other sites of synthesis (506,507). An acceptable solution for this

problem is to measure a parameter that may closely resemble the precursor enrichment. In several endogenous labeling studies, the precursor enrichment was estimated from: 1) tracer enrichment of the plasma amino acid pool (508), 2) enrichment of the amino acid metabolites (e.g. hippurate from glycine, and α-ketoisocaproate from leucine) (509,510) isolated from plasma or urine, on the basis that they are likely to be produced from the same intracellular compartment which charges tRNA, and 3) plateau enrichment of an apolipoprotein pool with a fast turnover, e.g. VLDL apoB-100 pool (511). The values obtained by the second and third approach are probably more representative of the true precursor enrichment, as they are a function of intracellular enrichment. Interestingly, Mass Isotopomer Distribution Analysis (MIDA), which is a technique for measuring biosynthesis and turnover of polymers *in vivo*, provides an accurate mathematical solution for measuring the precursor enrichment of polymers labeled with stable isotopes (512). This approach has been applied to fatty acids, cholesterol, and glucose, and is perhaps applicable to proteins.

Other shortcomings of the endogenous labeling method which are related to the use of stable isotopes are: 1) the relative lack of sensitivity in the detection of stable isotopes, compared to radiotracers, which requires the use of larger doses of the stable isotope, 2) the use of fairly sophisticated and expensive computer-supported mass spectrometers to measure stable isotope enrichment, and 3) the natural presence of stable isotopes in physiological systems, which mandates data corrections prior to interpretation (513). Conversion of raw mass

spectrometry data, and data interpretation in stable isotope experiments are further discussed in the methods section.

In conclusion, endogenous labeling with stable isotopes has been revived recently as an alternative for the use of exogenous radiolabeling in the study of plasma kinetics of apolipoproteins. This method offers several important advantages and is appropriate for the study of the plasma kinetics of apolipoproteins. In chapter two, work related to setting up this method in our laboratory, and customizing it to allow for the study of plasma kinetics of apoE and apoC-III is presented.

Chapter 2

Methodology

This chapter describes the methods and procedures used in the current project, as well as the results of experiments carried out to optimize and standardize these techniques.

2.1. Stable-Isotope Infusion Protocol

The infusion protocol was based on the method by Cohn and his colleagues, published in the *Journal of Clinical Investigation* in 1990 (511). Primed constant infusions of a stable isotopically-labeled amino acid were carried out in healthy subjects and dyslipidemic patients in the lipid clinic of the Clinical Research Institute of Montreal (CRIM), with the help of the medical and nursing staff. Subjects were asked to fast for 12 hours (h) overnight prior to the start of the infusion. On the day of the experiment, each subject arrived at the lipid clinic at 7:00 am. The subject was asked to lie down and a catheter was inserted into a forearm vein in the left arm. At 8:00 am (0 h of the infusion), a bolus injection of 10μmol per kg of body weight (10 μmol/kg) of 5′,5′,5′ tri-deuterium L-leucine ([D_3]L-leucine, C[H^2]$_3$-CH(CH_3)-CH_2-CH(NH_2)COOH, MW: 134.18, 97% pure, Cambridge Isotope Laboratories, MA) dissolved in physiological saline (0.9 % NaCl) was given to the subject through the catheter. At the same time, a blood sample (20 ml) was collected from an antecubital vein in the right arm through a second intravenous line. Following the bolus injection, a constant infusion of 10μmol per kg per h of [D_3]L-leucine dissolved in physiological saline was started. The aim of the primed constant infusion was to

attain a certain concentration of [D_3]L-leucine in plasma with the bolus injection and to maintain this concentration with the constant infusion. The infusion was carried out for 12 h using a peristaltic pump (Life Care Pump Model 3, Abbott) which was set to deliver 48 ml of infusate per h. Subjects were not given food during the time course of the infusion but had free access to drinking water. They were encouraged to move around to maintain good blood circulation. Blood samples (20 ml) were collected during the infusion from the intravenous line in the right arm at regular intervals (15 min, 30 min, and 45 min, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h) into tubes containing EDTA to a final concentration of 0.1 %. Samples were kept on ice, and plasma was immediately separated by centrifugation at 3,500 rpm for 15 min at 4^0C. An antimicrobial agent (sodium azide) and a protease inhibitor (aprotinin) were added to plasma to give a final concentration of 0.02 % and 1.67 μg/ml, respectively.

2.2. Isolation of Lipoproteins and Apolipoproteins

Very low density lipoproteins (VLDL)($d < 1.006$ g/ml), intermediate plus low density lipoproteins (IDL + LDL)($1.006 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml), were isolated from 5 ml plasma by sequential ultracentrifugation with an XL-90 ultracentrifuge using a 50.4 Ti rotor (Beckman) at 50,000 rpm for 10 h. Total lipoproteins were isolated from plasma by ultracentrifugation (50,000 rpm, 10 h) of 1 ml of plasma, adjusted to a density of 1.25 g/ml with KBr. Lipoproteins were recovered in the supernate by tube-slicing. VLDL apoB-100 was isolated by preparative SDS-polyacrylamide gel electrophoresis (SDS-

PAGE) with a 4-22.5 % gradient gel (511). VLDL and HDL apoC-III and apoE, as well as total plasma apoA-I, A-II, C-III and E were isolated by preparative isoelectric focusing (IEF) on 7.5 % polyacrylamide-urea (8M) gels (pH gradient 4-7) (514) (Fig 2.1). Protein bands were identified in gels with Coumassie blue staining. Total plasma and HDL fractions were dialyzed against 10mM ammonium bicarbonate, preincubated with cysteamine (β-mercaptoethylamine, Sigma-Aldrich) at a ratio of 6 mg cysteamine for every mg of protein for 4 h at 37°C, and then delipidated. VLDL fractions were not treated with cysteamine. Cysteamine introduces an amino group to the single cysteine residues of apoE3 (515) and apoA-II, but does not react with proapoA-I and apoC-III_0 since these proteins do not contain cysteine. Cysteamine-modified apoE3 and apoA-II migrate to higher positions in the IEF gel, due to their increased positive charge (see Fig 1 in chapter 4). They are thus separated from proapoA-I and asialylated apoC-III (apoC-III_0) which normally co-migrate to the same positions.

2.3. Quantitation of Plasma Lipids and Apolipoproteins

Plasma and lipoprotein fractions were assayed for total (free and esterified) cholesterol and triglyceride with a COBAS MIRA-S automated analyzer (Hoffman-LaRoche) using enzymatic reagents. Plasma apoB concentration was measured by non-competitive ELISA using an immuno-purified goat anti-human apoB antibody and horseradish peroxidase-conjugated monoclonal antibody (516). Plasma apoE and apoC-III concentrations were measured by ELISAs developed in our laboratory (517,518). ApoE phenotype

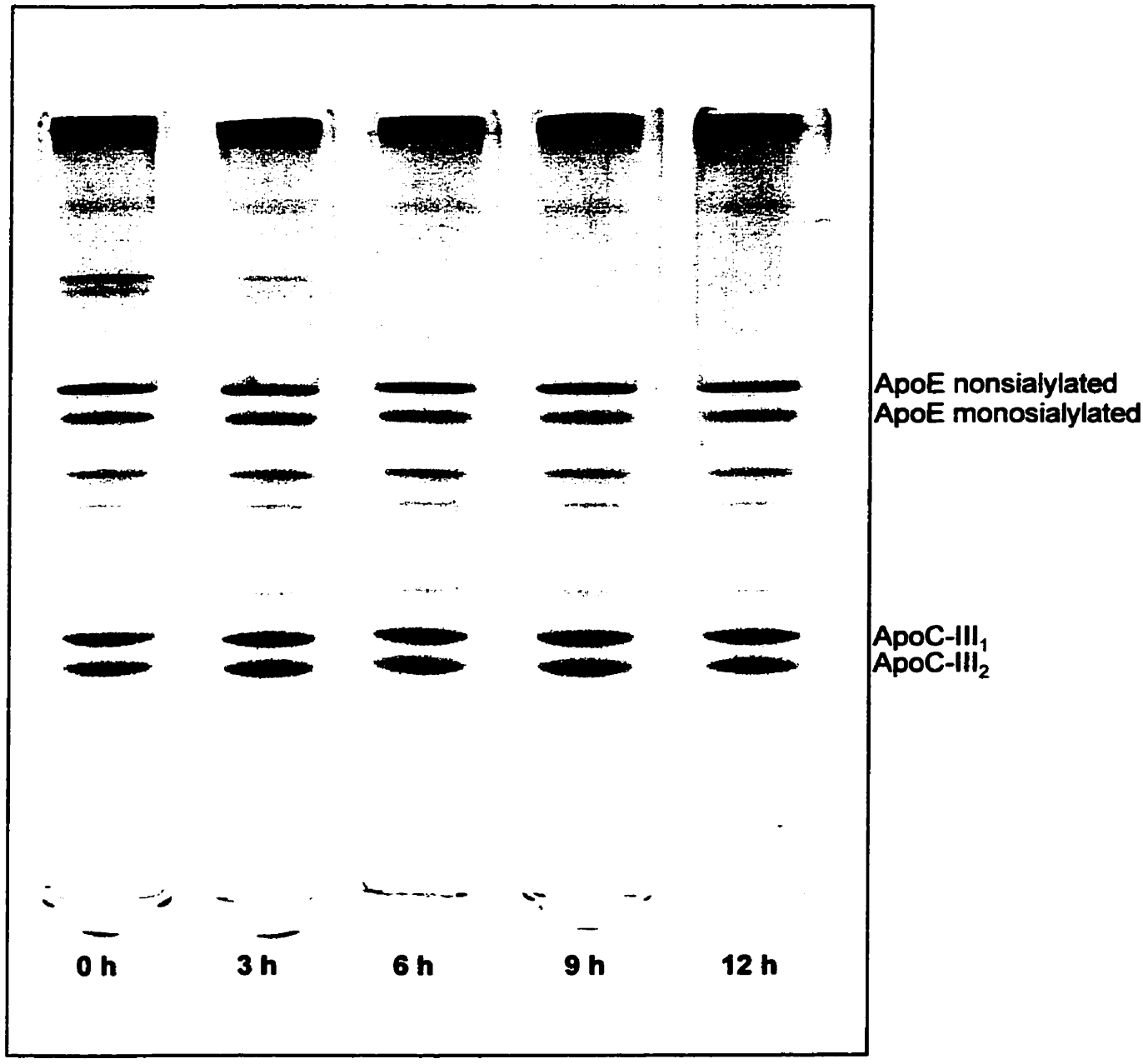


Figure 2.1. Isoelectric focusing gel electrophoresis (IEF) for preparative isolation of VLDL apo-E and C-III. VLDL were separated from plasma of a single subject obtained at time points 0, 3, 6, 9 and 12 h of stable isotope infusion experiment.

was determined by isoelectric focusing of delipidated VLDL (514). Plasma apoA-I concentration was measured by nephelometry on a Behring Nephelometer 100 (Behring) using Behring protocol and reagents. Plasma apoA-II was measured by nephelometry in the laboratory of Dr. Linda Bausserman (Meriam Hospital, Brown University, RI, USA) (519).

2.3.1. Quantitation of ProapoA-I

Mature apoA-I and proapoA-I concentrations in plasma of normolipidemic subjects and patients with familial HDL deficiency (chapter 4) were derived from total plasma apoA-I concentrations measured by nephelometry. The proportion of each apoA-I isoform contributing to total plasma apoA-I was determined by gas chromatography-mass spectroscopy (GC-MS) and densitometric scanning of IEF gels. The amount of leucine associated with the major mature apoA-I IEF band (isoform apoA-I_0) and the major proapoA-I band (isoform apoA-I_{+2}) (520) was determined by comparing the areas under the peaks of leucine and norleucine (an internal standard - see the following section), separated by GC-MS. The amount of apoA-I protein present in each band was then calculated as:

$$\text{apoA-I } (\mu g) = \frac{\text{leucine } (\mu g) \times \text{mol. wt. of apoA-I isoform (daltons)}}{\text{total wt. of leucine residues in apoA-I isoform (daltons)}}$$

Amount of protein in minor mature apoA-I bands (isoforms apoA-I_{-1} and apoA-I_{-2}) and the minor proapoA-I band (isoform apoA-I_{+1}) (520) was then estimated by

measuring the relative amounts of these isoforms by IEF gel scanning densitometry. Plasma concentration of proapoA-I was calculated as:

$$\text{proapoA-I (mg/dl)} = \text{apoA-I (mg/dl)} \times \frac{\text{apoA-I}_{+2} + \text{apoA-I}_{+1}\ (\mu g)}{\text{apoA-I}_{+2} + \text{apoA-I}_{+1} + \text{apoA-I}_{0} + \text{apoA-I}_{-1} + \text{apoA-I}_{-2}\ (\mu g)}$$

2.4 Determination of Isotopic Enrichment of Apolipoproteins

Figure 2.2 is a flow chart describing the steps involved in determining isotopic enrichment of apolipoproteins. Apolipoprotein bands, as well as blank (non-protein-containing) gel slices were excised from polyacrylamide gels (VLDL B-100 from SDS-PAGE gels and apoE, apoC-III, apoA-I and apoA-II from IEF gels). Each slice was added to a borosilicate 2 ml sample vial containing 600 µl of 6N HCL, and an internal standard of 250 ng norleucine (Sigma-Aldrich) dissolved in 50 µl double distilled water. Each vial was tightly capped with a teflon/silicon cap. Gel slices were hydrolyzed at 110°C for 24 h, cooled to -20°C for 20 min, and centrifuged at 3,500 rpm for 5 min. Free amino acids in the hydrolysate were aspirated from the polyacrylamide pellet. HCL was evaporated from the samples, while being centrifuged in a SpeedVac SC100 apparatus (Savant). The residue was resuspended in 2 ml of 1 N Acetic acid, and amino acids were purified by cation exchange chromatography using AG 50 W-X8 resin (BioRad) (Fig 2.3.). Amino acids were then derivatized by treatment with 200 µl of acetyl chloride-acidified 1-propanol (1:5 V/V) for 1 h at 100°C, followed by 50 µl of heptaflurobutyric anhydride (HFBA)(Supelco) for 20 min at 60°C. Derivatization served to mask the polar groups on the amino acids, making them

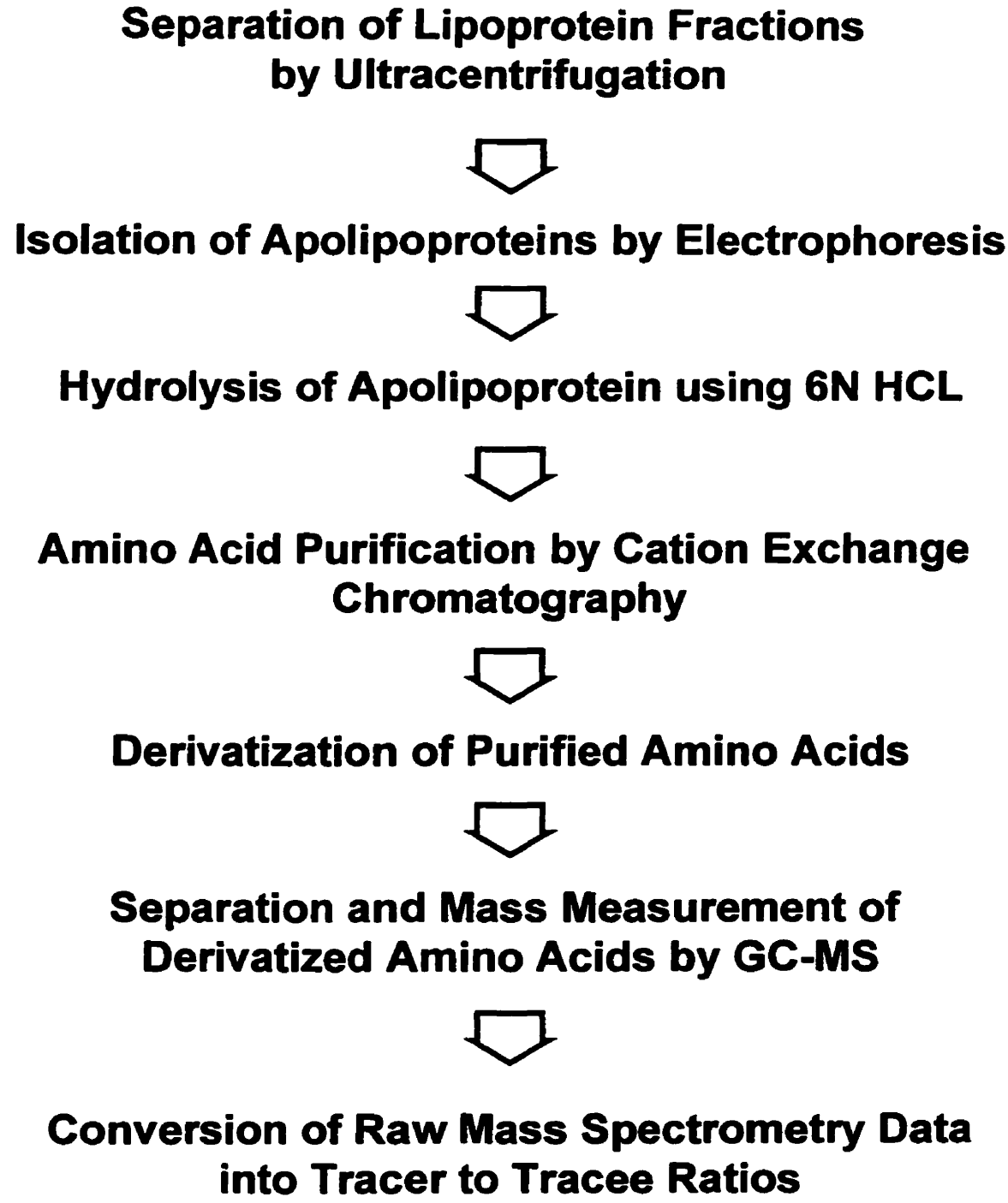


Figure 2.2. Steps involved in the determination of isotopic enrichment of apolipoproteins in total plasma and in lipoprotein fractions.

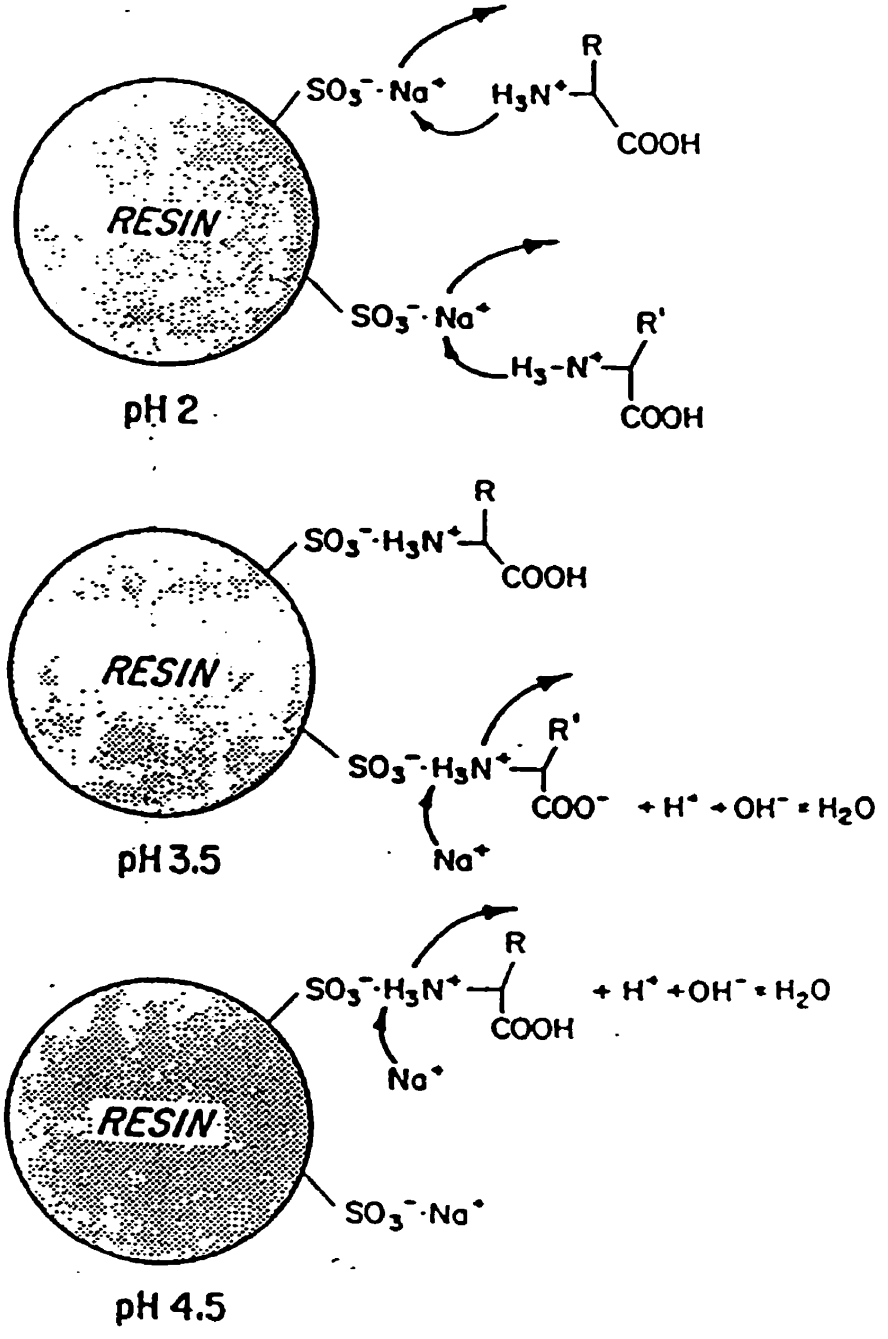
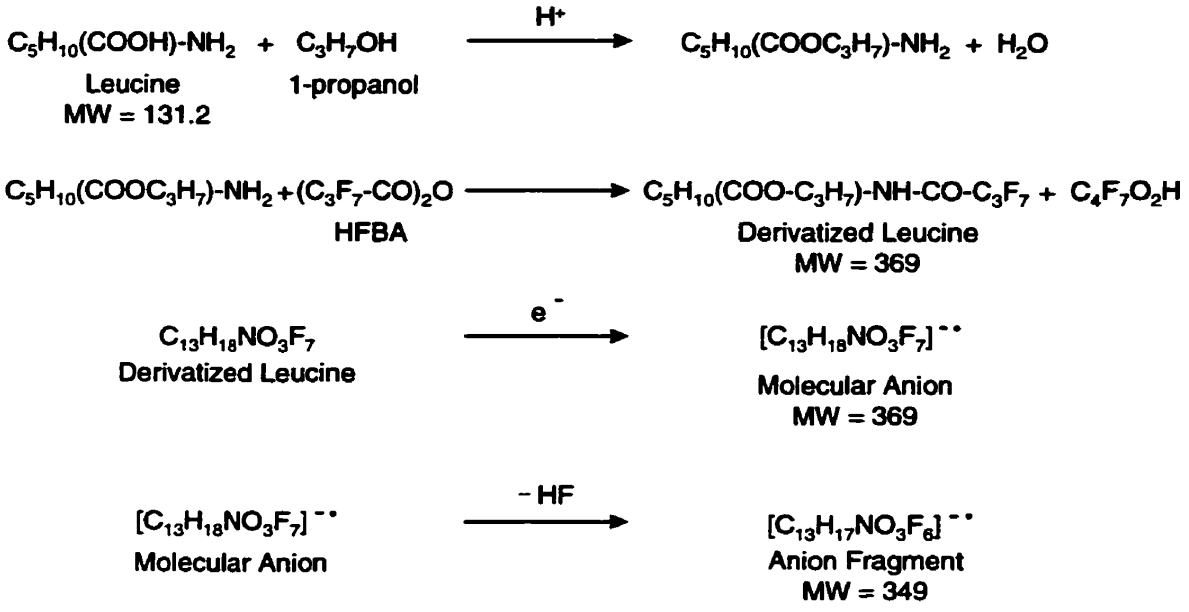


Figure 2.3. Schematic representation of the purification of amino acids by cation exchange chromatography. At a low pH (acidic), amino acids bind to the resin. When the pH is increased, amino acids elute as a function of the dissociation constant of their ionizable groups (521).

volatile and thermally stable, which was necessary for their separation and subsequent mass measurement by gas chromatography-mass spectrometry (GC-MS). Finally, the derivatized amino acids were resuspended in 200µl of 1N sodium bicarbonate and extracted into 200µl of ethyl acetate, which was the form of the sample injected into the GC-MS. The extraction step served to prolong the life of the capillary column of the GC-MS by reducing the amount of corroding HFBA in the final sample.

Enrichment of samples with ([D_3] L-leucine) was determined by GC-MS (Hewlett-Packard, 5988 GC-MS) using negative chemical ionization and methane as the reagent gas. Briefly, 1 µl of the final ethyl acetate solution, which contained derivatized amino acids, was injected into a preheated capillary column (80 °C) in a temperature-programmable oven. The sample was evaporated in the heated injector (180 °C), and an inert gas (helium) passing through the injector swept it through the column. The initial temperature of the oven was held for 1 min, then increased 20 °C per min up to 210 °C and then held again for 1 min. Subsequently, the oven was baked out at 280 °C for 2 min and reset back to 80 °C for the next sample. Retention time of each derivatized amino acid species in the capillary column was a function of its partition coefficient (the ratio of the weight of solute per ml stationary phase, which was a non-volatile liquid coated on the internal sides of the capillary column, to the weight of solute per ml of helium, the mobile phase). The partition coefficient was inversely related to the vapor pressure of the derivatized amino acid, i.e. the derivatized amino acid with the highest vapor pressure eluted first off the column.

After elution from the capillary column, molecules were forced to the GC-MS interface (set to a temperature of 250 °C) and from there to the ion source (set to a temperature of 100 °C). In the ion source, molecules were ionized by capturing thermal electrons (electrons having lost a significant amount of their energy due to collision with the reagent gas (methane) in the ion source). Subsequently, the formed negative ions released an HF molecule leading to a reduction in mass of 20 units. Compared with electron impact which fragments the molecule into smaller molecular weight ions, this "gentle" negative chemical ionization keeps molecules almost intact and allows for higher sensitivity in measuring enrichment with stable isotopes. Such sensitivity is critical for measuring slight changes in apolipoprotein enrichment *in vivo* (less than 0.5%). In fact, due to certain inconveniences related to the use of negative chemical ionization, such as the corroding effect of HFBA and the safety concern when using methane as a reagent gas in overnight runs, we initially attempted to ionize derivatized molecules using electron impact instead of negative chemical ionization. These attempts were not however successful since an insufficient level of sensitivity was attained. The following chemical formulas summarize the chemical and mass modifications which occur to leucine due to derivatization and the subsequent negative chemical ionization:



Ions formed in the ion source entered the quadrupole, which consisted of 4 electromagnetic field - radiating parallel circular rods. The quadrupole filtered ions with masses resonating with the frequency and amplitude of its voltage. All other ions which did not have the desired mass were pumped away. Thus, the quadrupole selectively allowed for the passage and subsequent detection of ions with desired masses, a process known as Selected Ion Monitoring (SIM). In the described method, the quadrupole was set to filter ions with masses 349 and 352 (masses of derivatized $[D_0]$L-leucine and derivatized $[D_3]$L-leucine ions, respectively), each for a period of 100 millisecond. Finally, the amount of the monitored ion was determined by the intensity of the electronic signal which it released upon reaching the detector. This signal was amplified multiple times depending on the voltage to which the multiplier was set (1400-2400 mV).

2.5. Verification of Chromatographic Resolution

The first question that was addressed was whether the GC-MS could adequately separate leucine from other apolipoprotein amino acids. In theory, the GC separation along with SIM at mass 349 ought to have allowed for separation of leucine from other amino acids with differed molecular weights (based on the fact that the quadrupole would filter only ions with mass 349). The more challenging aspect was to separate derivatized leucine from derivatized isoleucine. The latter had the same molecular weight as leucine (isoleucine is a structural isomer of leucine) and produced an ion with mass 349. Separation of leucine and isoleucine thus relied solely on separation by GC in the capillary column where each ion was retained differently depending on its partition coeffecient.

In order to test the resolving power of the GC, a mixture of amino acids, including glycine, phenylalanine, tryptophan, leucine, isoleucine and alloisoleucine was prepared and derivatized according to the described method (section 2.4). The reason for adding alloisoleucine, which is not a physiological amino acid, to the mixture, was to verify the ability of the GC to separate amino acids with very similar structures. Alloisoleucine is a stereoisomer of isoleucine (but not a mirror image) and the 2 amino acids differ only in the arrangement of chemical groups around their 2 achiral carbon atoms. The GC-MS print-out of SIM at mass 349 showed successful exclusion of derivatized amino acid ions with masses different from 349 (glycine, phenylalanine, and tryptophan) (Fig 2.4). Furthermore, it showed a good separation of leucine from isoleucine, and

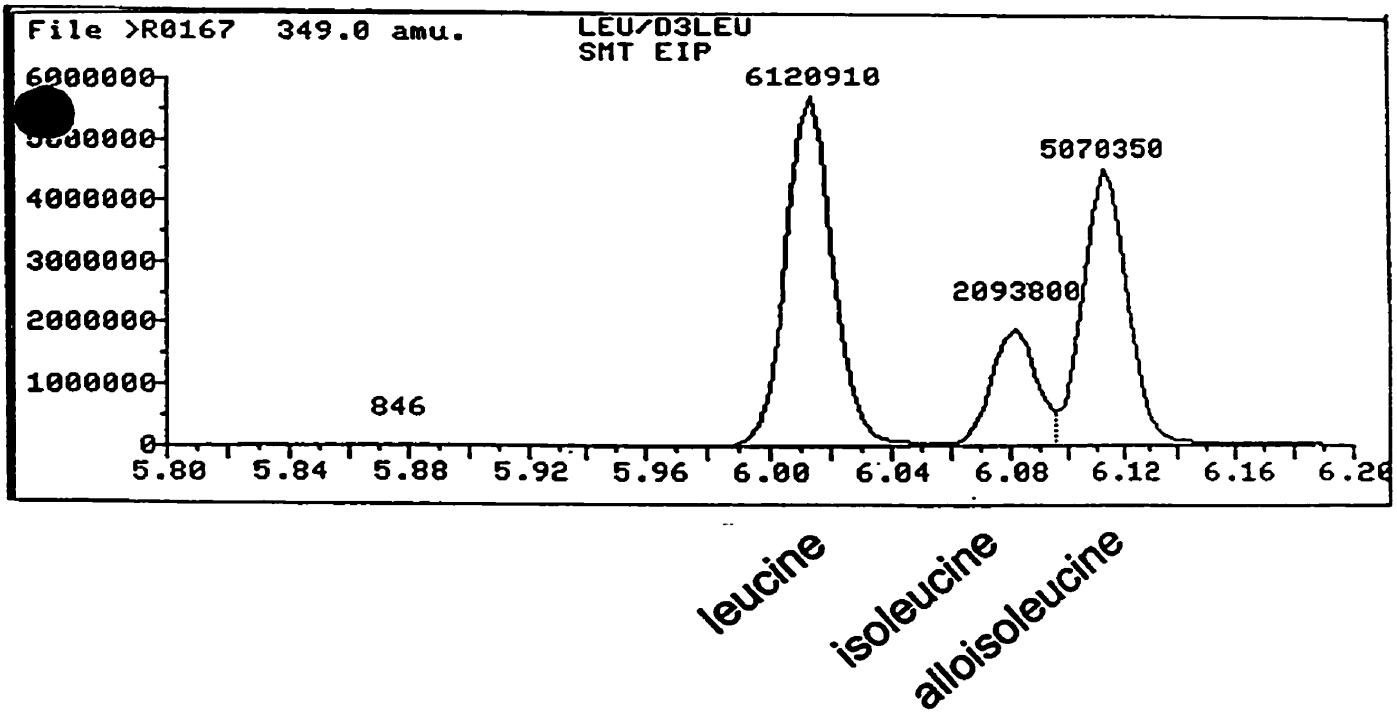


Figure 2.4. Separation of the structural isomers leucine, isoleucine and alloisoleucine, by gas chromatography-mass spectrometry (GC-MS). A sample containing a mixture of aminoacids (1 ng of glycine, phenylalanine, tryptophan, leucine, isoleucine, and alloisoleucine) was separated by GC and monitored by MS at mass 349. The vertical scale shows the intensity of the electronic signal released when ions hit the detector of the MS and the time values on the horizontal line reflect the retention time (min) in the capillary column of the GC. Values above peaks represent their integrated areas.

even isoleucine from alloisoleucine. Leucine had the smallest partition coefficient and eluted first, and was followed by isoleucine and alloisoleucine. The good separation of isomeric amino acids showed that isoleucine did not interfere with leucine mass measurements in the described method.

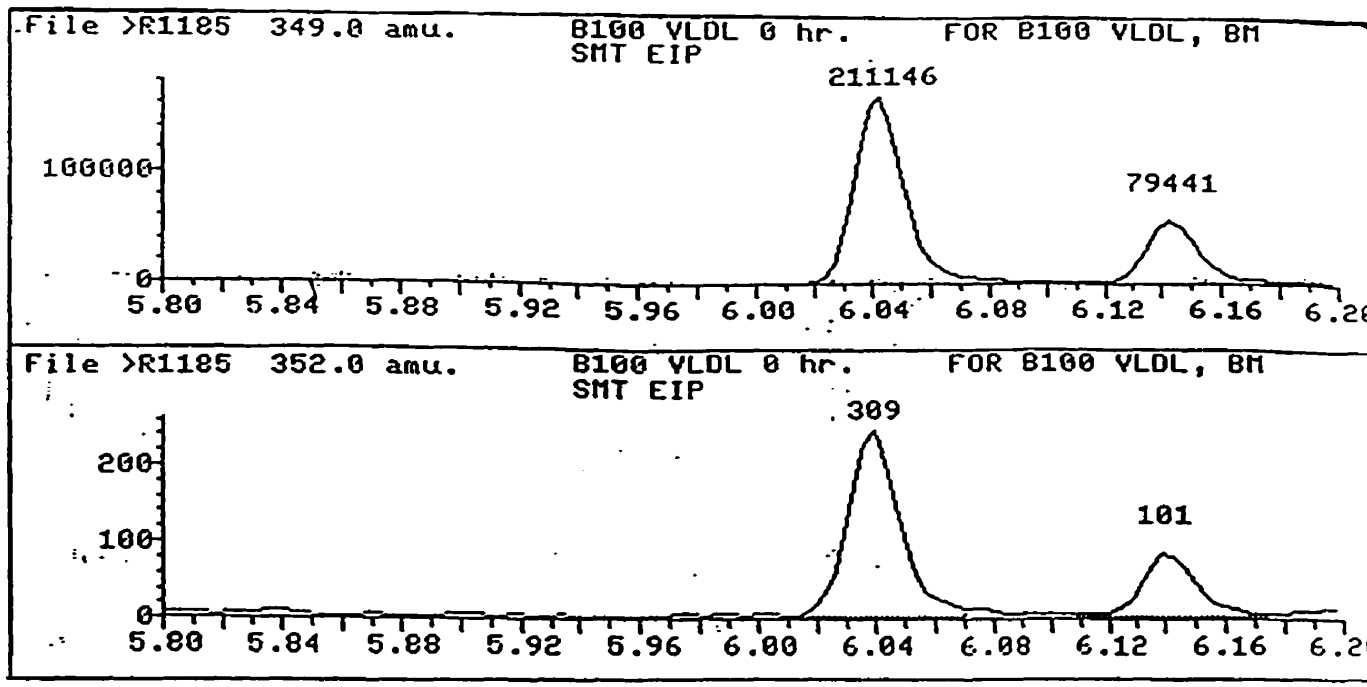
2.6. Accuracy and Precision of [D_3]L-leucine Enrichment Measurements

Ratio of areas under the peaks of derivatized leucine at masses 352 and 349 (AUPs 352/349) represented levels of apolipoprotein enrichment with [D_3]L-leucine at different time points during stable isotope infusion experiments. Measuring a ratio, as opposed to an absolute mass, was advantageous because it was not dependent on the isolated amount of apolipoprotein, or the recovery of the measured samples. Thus, although an effort was made to isolate similar amounts of protein at different time points and to subject samples to the same processing conditions, AUPs 352/349 were a function of the ratios of [D_3]L-leucine to [D_0]L-leucine in the protein sample, and were not dependent on sample recovery. Figure 2.5 shows selected ion monitoring at masses 349 and 352 for VLDL apoB-100 hydrolysates separated from plasma of a single subject at time 0 h and 9 h during a [D_3]L-leucine infusion experiment. A marked increase in AUPs 352/349 of leucine, but not isoleucine, is evident at time 9 h vs time 0 h (i.e. 15784/163112 vs. 309/211146; 9.68 % vs. 0.15 %)

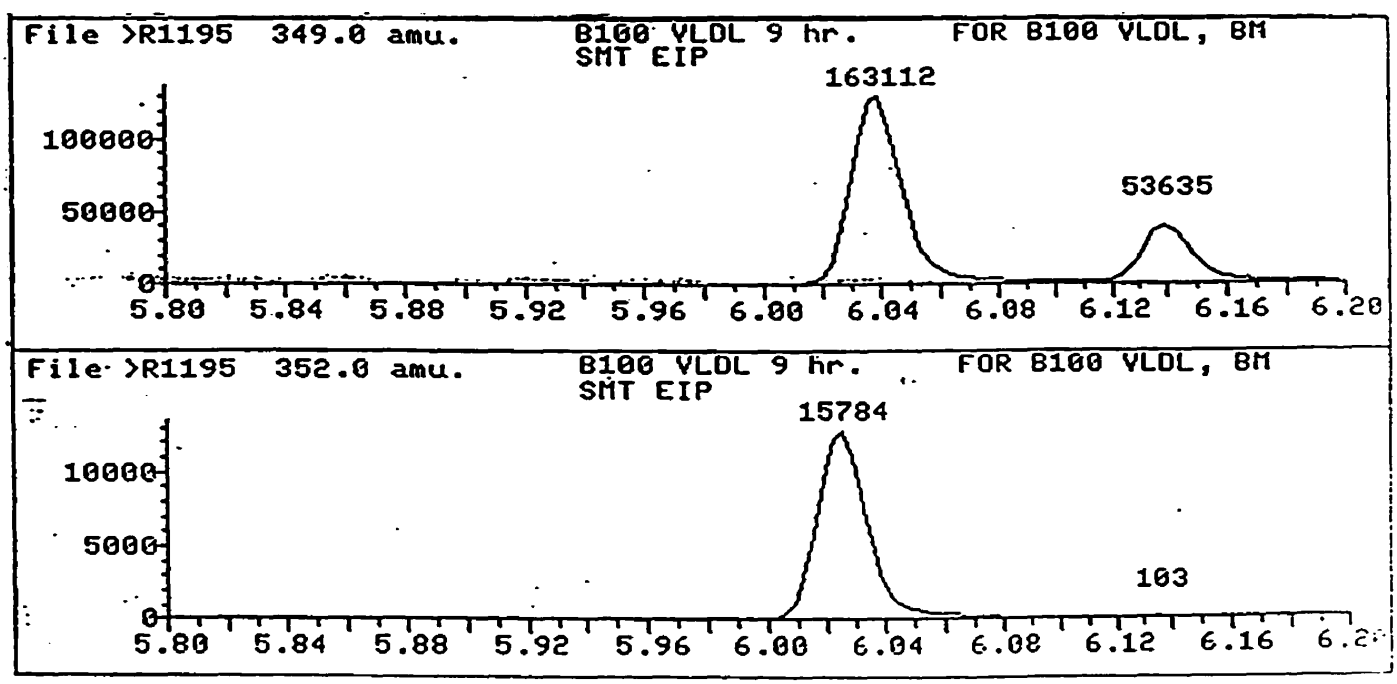
In order to verify the accuracy of enrichment measurements, increasing amounts of [D_3] leucine (0-90 picogram) were added to 50 ng of [D_0] leucine to

Figure 2.5. Selected ion monitoring at masses = 349 and 352 (top & bottom panels, respectively) of VLDL apoB-100 amino acid hydrolysates. VLDL was separated from plasma of a single subject obtained at time points 0 h (A) and 9 h (B) of a stable isotope infusion experiment. The print-out shows peaks corresponding to leucine and isoleucine ions. VLDL apoB-100 was enriched with [D_3] leucine at 9 h (AUPs 352/349 : 9.68% vs 0.15% at 0 h).

A



B



leucine

isoleucine

give samples with calculated $[D_3]/[D_0]$ leucine ranging from 0 to 0.18 %. The resulting mixtures were derivatized and 1µl of each sample was injected into the GC-MS. The measured AUPs 352/349 correlated well (r^2 = 0.99) with the calculated $[D_3]/[D_0]$ leucine, and the line connecting the two sets of values did not differ significantly from the line of unity (Fig 2.6, the ratios were adjusted for natural background as explained in section 2.7.2). In a subsequent experiment, it was shown that incremental increases of less than 0.0025 % (up to 0.02%) in sample enrichment could not be accurately reflected in the enrichment measurements (data not shown). Hence, the margin of error in enrichment measurement was determined to be 0.02%.

Finally, variability in AUPs 352/349 should only be a function of the physiological change in the enrichment of the studied apolipoprotein with $[D_3]$ leucine. In order to test the precision of the GC-MS measurements, 10 consecutive injections (1 µl each) of a single sample were made into the GC-MS. The sample consisted of ethyl acetate-solubilized derivatized amino acids of SDS-PAGE-separated VLDL apoB-100 hydrolysate. The AUP 352/349 measurements had a mean of 0.135 % (background AUP 352/349 measurement due to natural abundance of stable isotopes was 0.14 %, see section 2.7.2) (Fig 2.7) with a standard deviation of 0.002 (coefficient of variation was 1.5 %). This represented a good level of within-run precision.

Figure 2.6. Accuracy of stable isotope enrichment measurements. Increasing amounts of [D_3]L-leucine (0-90 picogram) were added to 50 ng of [D_0]L-leucine. The resulting mixtures were derivatized and 1 μl of each sample was injected into the GC-MS according to the described method. Measured ratios of areas under the peaks 352/349 are shown in comparison with calculated D_3/D_0 leucine ratios (the measured ratios were adjusted for background enrichment, as described in text). The line of unity (dotted) is also shown.

Sample	Amount of D_3 Leucine (ng)	Amount of D_0 Leucine (pg)	Calculated D_3/D_0 Leucine (x100)	Measured AUPs 352/349 (x100)
1	0	50	0	0
2	15	50	0.03	0.03
3	30	50	0.06	0.06
4	45	50	0.09	0.09
5	60	50	0.12	0.11
6	75	50	0.15	0.13
7	90	50	0.18	0.16

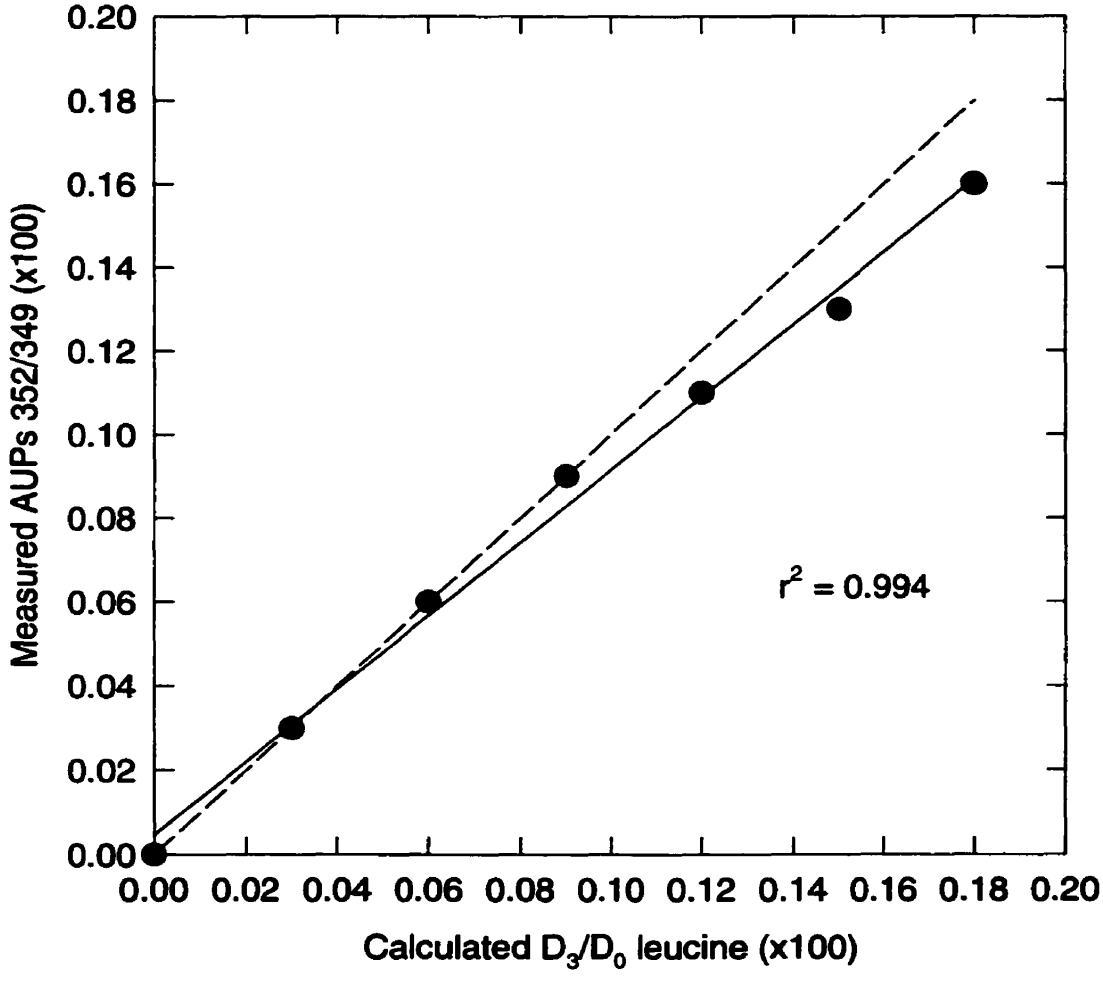
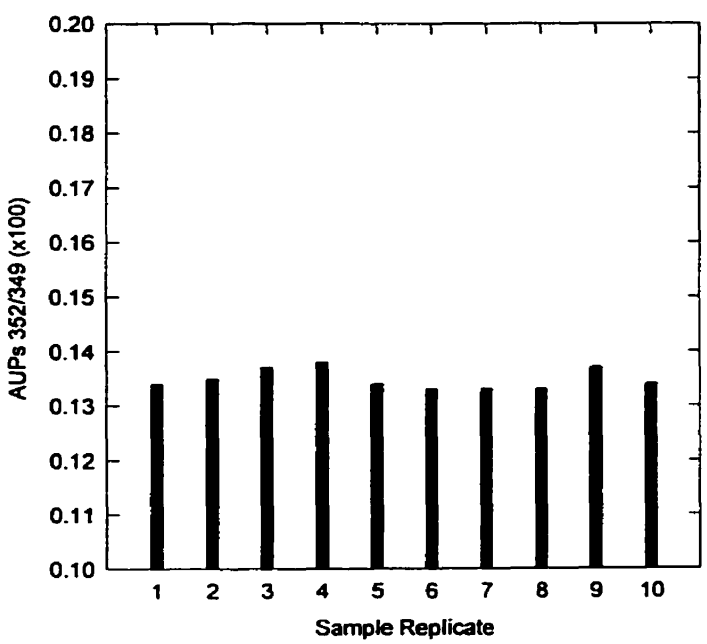


Figure 2.7. Precision of stable isotope enrichment measurements. VLDL apoB-100 (8 μg) was separated by SDS-PAGE and hydrolyzed. The amino acids of the hydrolysate were purified and derivatized, according to the described method. Ten consecutive injections, each of 1 μl, from the final 200 μl ethyl acetate solution of the sample were made into the GC-MS. The figure at the bottom of the page shows graphically the AUPs 352/349 for the ten replicates. The with-in run coefficient of variation was 1.5%.

Sample replicate	AUP[a] 352	AUP[b] 349	AUPs[c] 352/349 (x100)
1	8000	5956440	0.134
2	8014	5925790	0.135
3	8468	6181820	0.137
4	8557	6192960	0.138
5	8812	6602550	0.134
6	8975	6758730	0.133
7	8958	6750470	0.133
8	8751	6605010	0.133
9	9381	6865330	0.137
10	8792	6579160	0.134
Mean ± SD			0.135 ± 0.002
C.V.			(1.5%)

[a] Area under the peak for derivatized leucine ion at mass = 352
[b] Area under the peak for derivatized leucine ion at mass = 349
[c] Ratio of areas under the peaks of leucine at masses 352 and 349



2.7. Conversion of Raw Mass Spectrometry Data to Tracer to Tracee Ratio

2.7.1. Method-Introduced Leucine

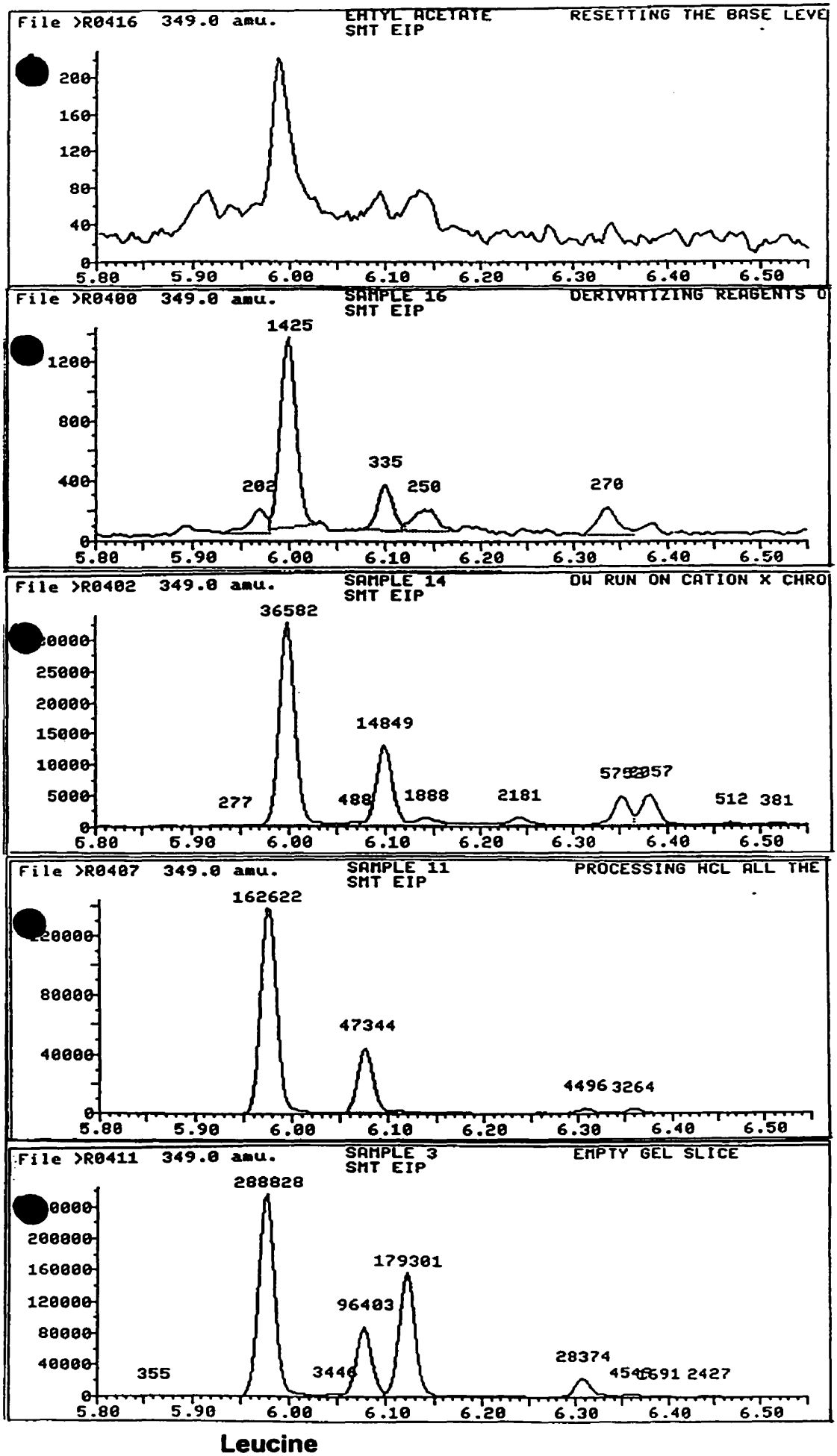
The ratio of the areas under GC-MS separated peaks, which is measured by mass spectrometry, is a function of the amount of both [D_3]L-leucine and [D_0]L-leucine in the injected sample. In order for this ratio to be physiologically meaningful, the relative distribution of leucine between the two mass species in the sample should accurately reflect its relative distribution in the physiological compartment from which the sample was taken (e.g. apoB-100 in VLDL). It is evident then that any deviation in the ratio of [D_3]/[D_0] leucine in the sample from the physiological one will compromise the physiological accuracy of the MS measurement, and one possible reason for this deviation is dilution of the protein-derived leucine in the sample with method-introduced leucine.

The presence of method-introduced leucine in samples was noticed in the early stages of setting up the present method in our laboratory. Significant amounts of leucine were routinely detected in non-protein-containing samples. Considerable work was carried out in an attempt to: 1) identify the source(s) of this leucine contamination, 2) reduce or eliminate the contamination, and 3) quantify and correct for the contamination if it could not be eliminated. Several sample processing steps were identified as being responsible for introducing the leucine contamination. Measures were taken to reduce it significantly, though it was not eliminated totally, and raw mass spectrometry data were subsequently corrected for the persisting low level of contamination.

Figure 2.8 shows the contribution of different methodological steps to the introduction of "contaminating" leucine. Neither the GC-MS run nor the derivatization process contributed to contamination. In contrast, amino acid purification by cation exchange chromatography, the acid hydrolysis step, as well as the separation of apolipoproteins on polyacrylamide gels by electrophoresis were responsible for introducing leucine. The relative contribution of each of these 3 steps alone could not be accurately determined, but together they introduced 200-400 ng of leucine to each sample (quantity determined using an internal standard (norleucine) as explained in section 2.4). This amount of leucine represented that which would be expected from the hydrolysis of 2-4 μg of apoE or apoC-III (the amount of E or C-III normally isolated for $[D_3]$L-leucine enrichment measurement).

Amount of contaminating leucine was found to be a function of the purity of reagents and the cleanness of glassware. Two of these steps (electrophoresis and cation exchange chromatography) involved the use of big volumes of liquid reagents, and the third one (hydrolysis) involved a long and vigorous interaction between a concentrated acid (HCl 6N) and a big glass surface (recyclable 15 ml glass tubes capped with a Teflon cap). In order to reduce the contamination, the purity of reagents used in these steps was upgraded to the highest commercially available grade. The water used was also upgraded to Nanopure grade (Barnstead). The surface area of the used glassware was significantly reduced by switching to disposable 2 ml borosilicate vials capped with Teflon/Silicon. Finally, extra attention was given to sample

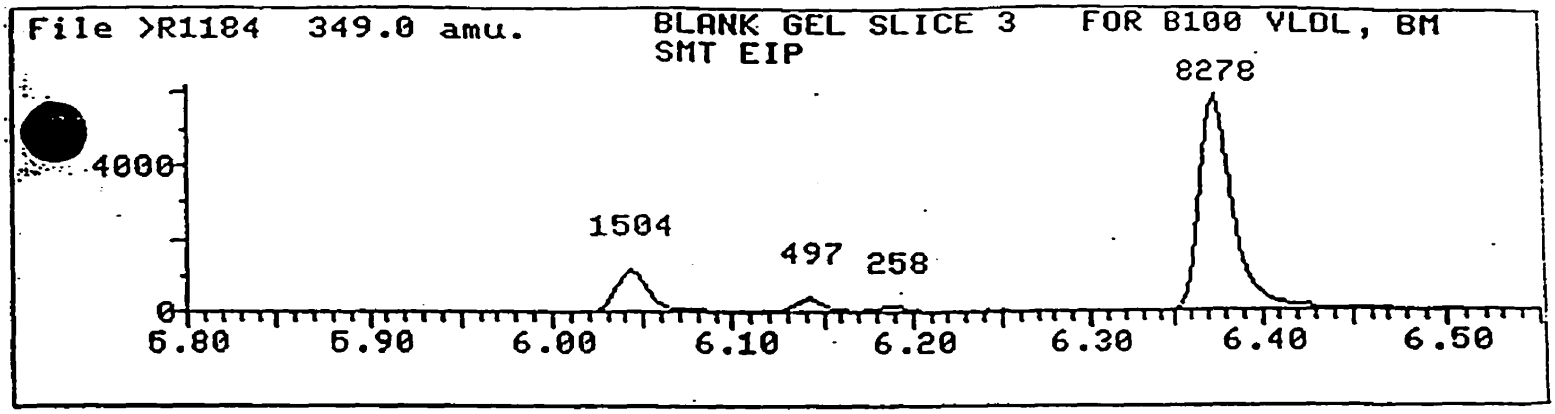
Figure 2.8. Contribution of different methodological steps to contamination with leucine: A) Ethyl acetate (1 μl) was injected into the GC-MS to verify the contribution of gas chromatography and mass spectrometry steps in introducing contamination), B) derivatization reactions were carried out in an empty borosilicate vial (no amino acids) and sample was then injected into the GC-MS, C) cation exchange chromatography was applied to 2 ml of 1N acetic acid and sample was then derivatized and injected into the GC-MS, D) HCl 6N (600 μl) was incubated at 110 °C in a borosilicate vial for 24 h. The sample was then subjected to cation exchange chromatography, derivatized, and injected into the GC-MS, E) a non protein-containing SDS-PAGE gel slice was HCl-hydrolyzed. The sample was then subjected to cation exchange chromatography, derivatized, and injected into the GC-MS. Value above the derivatized leucine peak (eluting at 5.98 - 6 min) is representative of amount of leucine in each sample. The scale on the vertical line adjusts according to the intensity of the ion signal, thus it is not similar in the five panels.

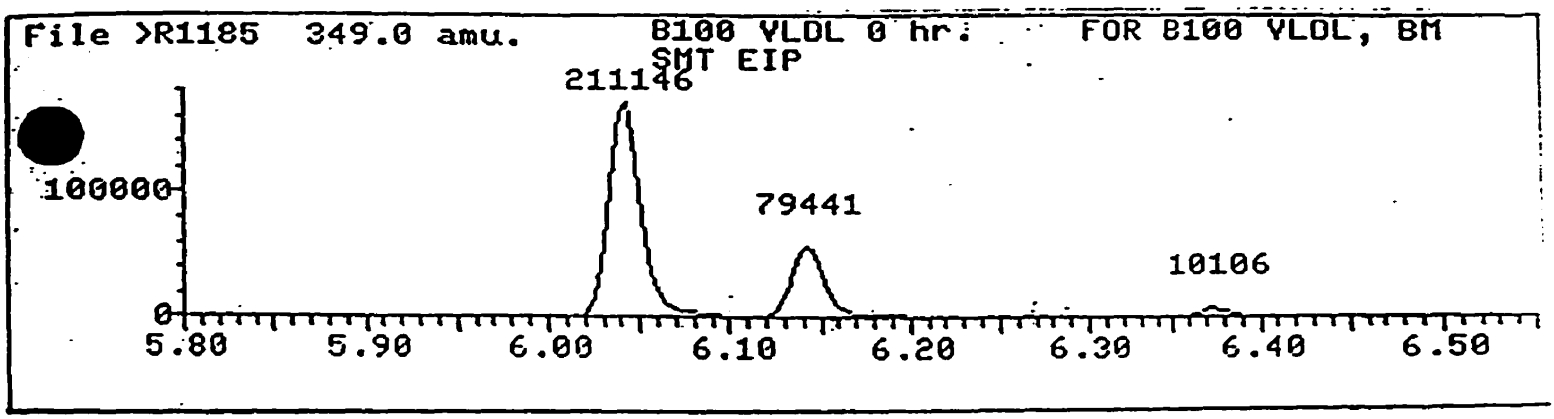

A
File >R0416 349.0 amu.
EHIYL ACETATE
SMT EIP
RESETTING THE BASE LEVE
200
160
120
80
40
0
5.80 5.90 6.00 6.10 6.20 6.30 6.40 6.50
B
File >R0400 349.0 amu.
SAMPLE 16
SMT EIP
DERIVATIZING REAGENTS O
1425
1200
800
400
0
335
250
270
5.80 5.90 6.00 6.10 6.20 6.30 6.40 6.50
C
File >R0402 349.0 amu.
SAMPLE 14
SMT EIP
OW RUN ON CATION X CHRO
36582
25000
20000
15000
10000
5000
0
14849
277
488
1888
2181
512 381
5.80 5.90 6.00 6.10 6.20 6.30 6.40 6.50
D
File >R0407 349.0 amu.
SAMPLE 11
SMT EIP
PROCESSING HCL ALL THE
162622
80000
40000
0
47344
4496 3264
5.80 5.90 6.00 6.10 6.20 6.30 6.40 6.50
E
File >R0411 349.0 amu.
SAMPLE 3
SMT EIP
EMPTY GEL SLICE
288828
200000
160000
120000
80000
40000
0
179301
96403
355
28374
2427
5.80 5.90 6.00 6.10 6.20 6.30 6.40 6.50
Leucine

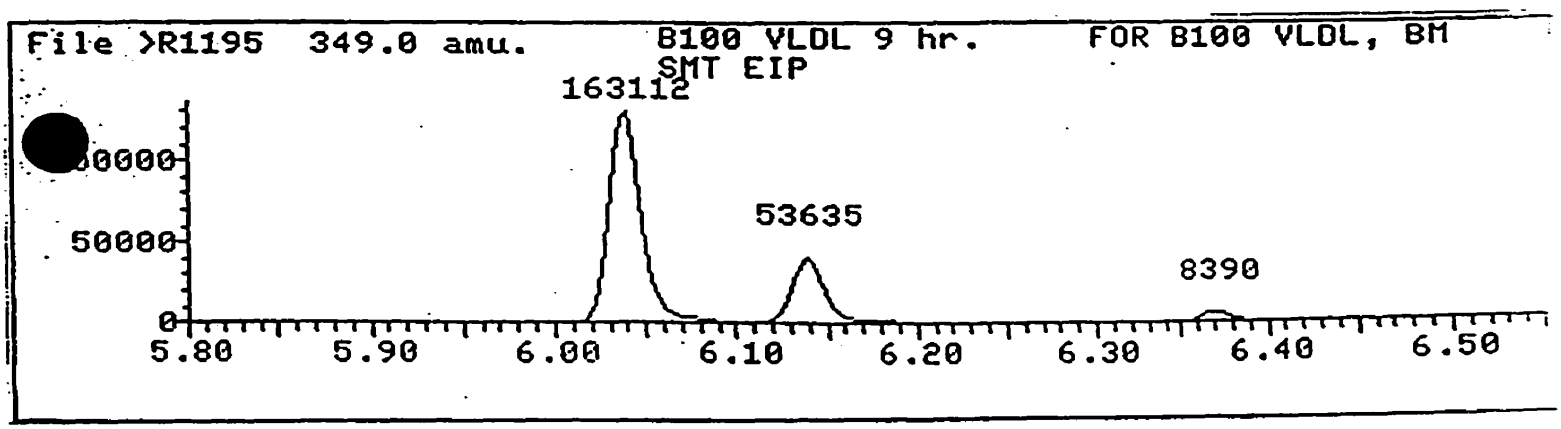
handling, in and between the different steps of the process. These measures led to a significant reduction in the amount of method-introduced leucine in each sample (down to less than 100 ng as determined by the internal standard). The contamination, however, could not be totally eliminated. Electro-elution of the protein out of the gel slice into a membrane trap (Amicon) followed by vigorous washing was not found to provide additional benefit.

Since the amount of "contaminating" leucine could not be totally eliminated, measures were taken to quantify it in each sample and to correct raw mass spectrometry data accordingly. In order to do this, 250 ng of norleucine (Sigma-Aldrich) dissolved in 50 µl of double distilled water was added to each sample immediately following the addition of 6N HCL (the hydrolysis step). Norleucine is an isomer of leucine (norleucine, MW = 131.2: CH_3-CH_2-CH_2-CH_2-CH(COOH)-NH_2) with a higher partition coefficient than both leucine and isoleucine (retained longer in the capillary column). The ratio of areas under the peaks of leucine and norleucine (AUPs leucine/norleucine) in a blank (non-protein-containing) sample, together with that in protein-containing samples, served to estimate the amount of method-introduced leucine in the latter (Fig 2.9). The amount of norleucine added (250 ng) was chosen to be within the range of the expected amounts of leucine produced by the hydrolysis of isolated apoE or apoC-III (100-500 ng), according to the described method. The amount of method-introduced leucine in each apolipoprotein sample was then subtracted from total leucine (from both AUP 349 and AUP 352) prior to subsequent conversions of the raw mass spectrometry data, as discussed later.

Figure 2.9. Addition of an internal standard (norleucine 250 ng) to samples, in order to quantify method-introduced leucine. Top panel shows the area under the peak of leucine in comparison to norleucine in a blank (non-protein-containing) sample. Leucine/norleucine ratio X 250 ng allowed for calculation of the leucine amount in the blank sample. This amount of leucine, which was method-introduced, was small compared to the amount of leucine found in the VLDL apoB-100 samples, as shown in bottom two panels.







leucine

isoleucine

norleucine

In order for the described correction to be valid, two assumptions had to be made: 1) that measured leucine/norleucine ratios were an accurate representation of calculated leucine/norleucine ratio in samples, and 2) the amount of method-introduced leucine in protein samples was similar to that in blank samples processed in parallel. To test the first assumption, increasing amounts of leucine (0-500 ng) were added to 250 ng norleucine. The resulting mixtures were derivatized, and 1 µl of each sample was injected into the GC-MS. AUPs leucine/norleucine correlated well with the calculated leucine/norleucine ratios (Fig 2.10). It was concluded that the GC-MS measurements accurately reflected leucine/norleucine ratio in samples. The second assumption was harder to validate; no direct measurement could be taken to show that the amount of method-introduced leucine was similar in protein-containing samples to that in blank samples processed in parallel. However, the reproducibility of the amount of method-introduced leucine in blank samples between runs (as shown for blank samples processed over a one year period of time (Table 2.1.) allowed us to safely assume that the amount of "contaminating" leucine was the same in blank and protein-containing samples processed within the same run.

2.7.2. Conversion of AUPs 352/349 to Isotopic Ratio

Further adjustments were needed to obtain isotopic ratios and finally tracer to tracee mass ratios. The nature of these adjustments is complex, and the need for making them is intrinsic to the use of stable isotopes as tracers.

Figure 2.10. Calibration of the internal standard (norleucine) with leucine. Increasing amounts of leucine (0-500 ng) were added to 250 ng norleucine. The resulting mixtures were derivatized and 1 μl of each sample was injected into the GC-MS. The measured ratios of areas under the peaks of leucine and norleucine at mass 349 are shown in comparison to calculated leucine/norleucine ratios.

Sample	Leucine (ng)	Norleucine (ng)	Calculated leucine/norleucine (x100)	Measured AUPs leucine/norleucine (x100)
1	0	250	0.00	0.0
2	100	250	0.40	0.41
3	250	250	1.00	1.03
4	500	250	2.00	2.28

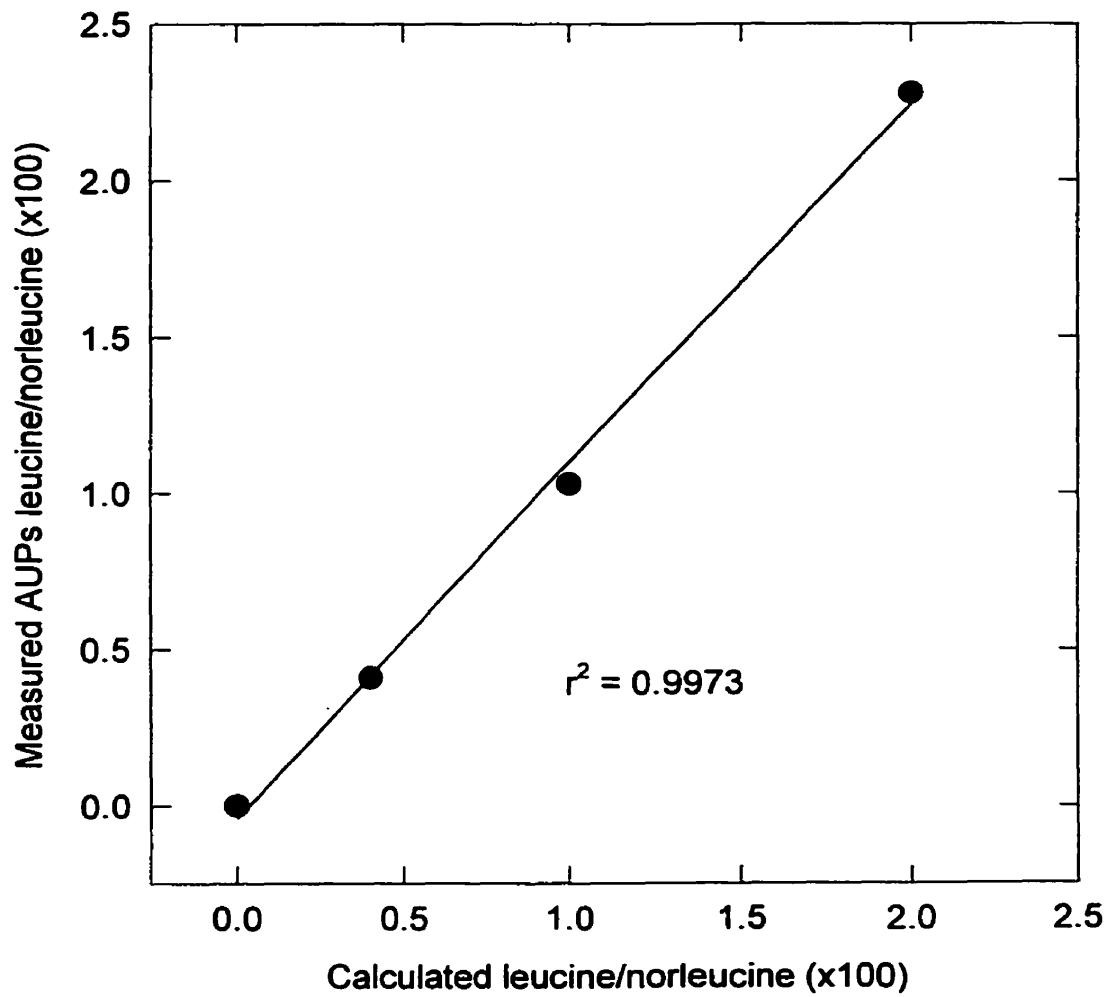


Table 2.1. Amount (ng) of leucine in blank (non-protein-containing) gel slice samples processed in parallel to protein samples (1 blank sample per protein) over a period of one year. Norleucine (250 ng) was added to each sample prior to acid hydrolysis as described in the method.

Date of Analysis	Blank Sample Number	AUP 349 Leucine	AUP 349 Norleucine	Leucine (ng)
February 1996	1	368303	3333270	27.6
	2	315703	2218630	35.6
	3	43666	362930	30.1
	4	46892	368039	31.9
	5	32345	377079	21.4
	6	80106	768560	26.1
	7	271238	2253900	30.1
	8	280738	2555330	27.5
	9	109207	400171	68.2
May 1996	10	81393	245259	83.0
	11	279192	932255	74.9
	12	3951	24549	40.2
	13	308455	1380780	55.9
	14	58145	351763	41.3
	15	52986	229212	57.8
	16	26407	149150	44.3
	17	287793	1172230	61.4
	18	142927	745392	47.9
	19	26363	174707	37.7
October 1996	20	78486	502810	39.0
	21	82313	348492	59.1
	22	74017	354893	52.1
	23	18471	187264	24.7
	24	1824	11205	40.7
	25	1504	8278	45.4
	26	8806	35858	61.4
	27	3650	11845	77.0
	28	3661	16235	56.4
	29	21176	54822	96.6
	30	2180	9695	56.2
	31	1316	5841	56.3
	32	2339	6417	91.1
	33	10453	89261	29.3
	34	10789	48764	55.3
	35	10701	43522	61.5
	36	18334	46480	98.6
	37	17875	71033	62.9
	38	8618	43844	49.1
	39	19987	78032	64.0
February 1997	40	19092	65537	72.8
	41	15220	55094	69.1
	42	15551	65227	59.6
	43	7764	53391	36.4
	44	10344	68680	37.7
	45	5057	48553	26.0
	46	8973	62804	35.7
	47	25385	253324	25.1
	48	10729	101644	26.4
	49	128838	419109	76.9
	50	2308	10413	55.4
	Mean			50.8
	SD			19.9

The isotopic ratio (r(t)) is the ratio of the mass of the "labeled" species (present in both the tracer and the tracee) to the mass of the "unlabeled" species (present in both the tracer and the tracee) (513). The AUPs 352/349 in the described method was not equivalent to r(t) because of the presence of a heterogeneous population of derivatized leucine ionic species which had a mass of 352 but were not derivatized 5´,5´,5´ [D_3]L-leucine ions. The combined mass of this population overestimated r(t) and had to be subtracted from AUP of derivatized leucine at mass 352. The presence of this population of ionic species was a result of the natural abundance of stable isotopes (see Table 2.2.). In the described method, the population of derivatized leucine ions with an atomic mass of 352 consisted mainly of artificially-enriched 5´,5´,5´ [D_3]L-leucine ($C_{13}H_{14}D_3NO_3F_6$). However, it also included ions which carried less naturally abundant stable isotopes of different elements at different positions with a combined increase in mass of 3 atomic units. For example, a derivatized leucine ion with a mass of 352 might carry no deuterium at all. It might carry the 3 stable isotopes ^{13}C, ^{15}N and ^{17}O, instead of ^{12}C, ^{14}N and ^{16}O. Thus, although this ion is not derivatized 5´,5´,5´ [D3]L-leucine, its mass will be part of the leucine mass at 352.

The sum of the probabilities of combinations resulting in a natural increase of 3 units in the mass of derivatized leucine was dependent on: 1) the molecule's elementary composition, 2) the number of atoms it carried of each element, and 3) the natural abundance of stable isotopes for each composing element. Using computing power ("Isotope" program module in operating

Table 2.2. Partial list of stable isotopes and their natural abundance

Element	Stable Isotope	Natural Abundance %	Element	Stable Isotope	Natural Abundance %
H	1	99.985	Fe	54	5.82
	2	0.015		56	91.66
				57	2.19
C	12	98.89		58	0.33
	13	1.11			
			Zn	64	48.89
N	14	99.63		66	27.81
	15	0.37		67	4.11
				68	18.57
O	16	99.760		70	0.62
	17	0.037			
	18	0.204	Se	74	0.87
				76	9.02
S	32	95.00		77	7.58
	33	0.76		78	23.52
	34	4.22		80	49.82
				82	9.19
			Si	28	92.21
				29	4.90
				30	3.09

system Opus V3.1X by Micromass Instruments of Manchester, UK) , the probability of having derivatized non-artificially stable isotope-enriched leucine ions with a mass of 352 units was calculated to be 0.0014 (Fig 2.11). Empirical data confirmed this calculation. An AUPs 352/349 = 0.14% was measured for samples which only contained non-artificially stable isotope-enriched leucine, for example those of apolipoproteins separated from plasma attained at time point 0 h of the constant infusion (see AUPs 352/349 for VLDL apoB-100 at time point 0 h in Figure 2.5).

2.7.3. Conversion of Isotopic Ratio to Tracer to Tracee Ratio

Tracer to tracee ratio (z(t)) is the stable isotope equivalent to specific activity in radioactive kinetic formalism and is often the ratio that is used to derive kinetic parameters. To arrive at z(t) from r(t), the latter had to be adjusted for the presence of unlabeled species in the tracer (the infusate) and labeled species in the physiological system (tracee). Discrepancy between r(t) and z(t) was due to 1) the impurity in the stable isotope infusate (5′,5′,5′ [D_3]L-leucine provided by Cambridge Isotope was 97.3% pure with impurities being 2.6 % of 5′,5′ [D_2]L-leucine, and 0.1 % of 5′ [D_1]L-leucine, according to the manufacturer), and 2) the natural abundance of deuterium which resulted in the presence of 5′,5′,5′ [D3]L-leucine in the physiological system (tracee). Figure 2.12 (513) schematizes the relative contribution of labeled and unlabeled species to tracee, to stable or radioactive tracer, and to a generic sample taken during experiment (tracer and tracee simultaneously present). The scheme is additionally useful because it

Mass	Fractional Abundance	Relative Abundance
349.111	85.45	**100.00**
350.111	12.99	15.21
351.111	1.43	1.68
352.111	0.12	**0.14**
353.111	0.01	0.01
354.111	0.00	0.00
355.111	0.00	0.00
356.111	0.00	0.00
357.111	0.00	0.00
358.111	0.00	0.00
359.111	0.00	0.00

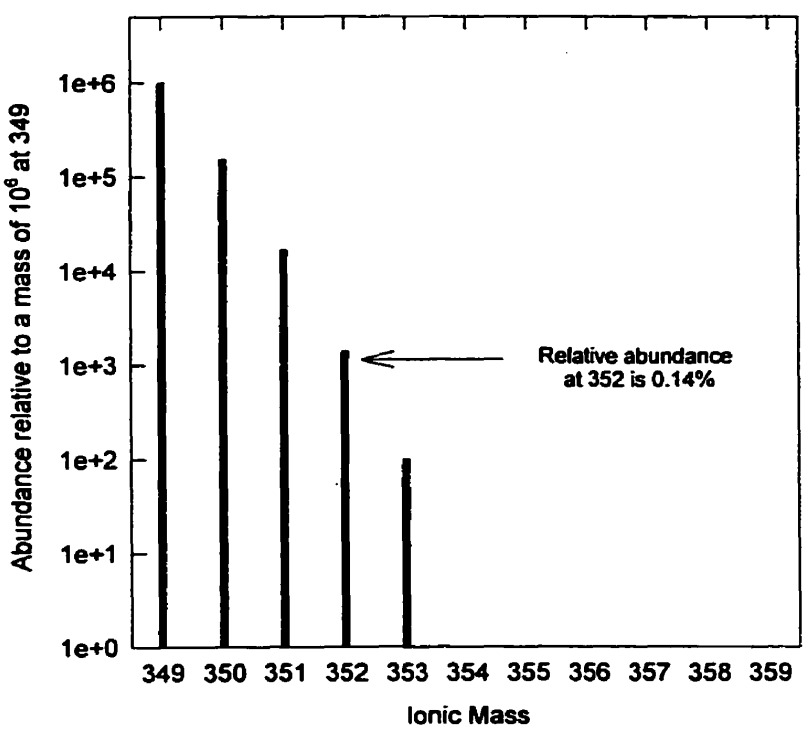


Figure 2.11. The fractional and relative abundance of ionic species accompanying derivatized leucine fragmented anion ($C_{13}H_{17}NO_3F_6$). The presence of these populations is a result of the inclusion of natural abundance of the stable isotopes ^{2}H, ^{13}C, ^{15}N, ^{17}O, and ^{18}O.

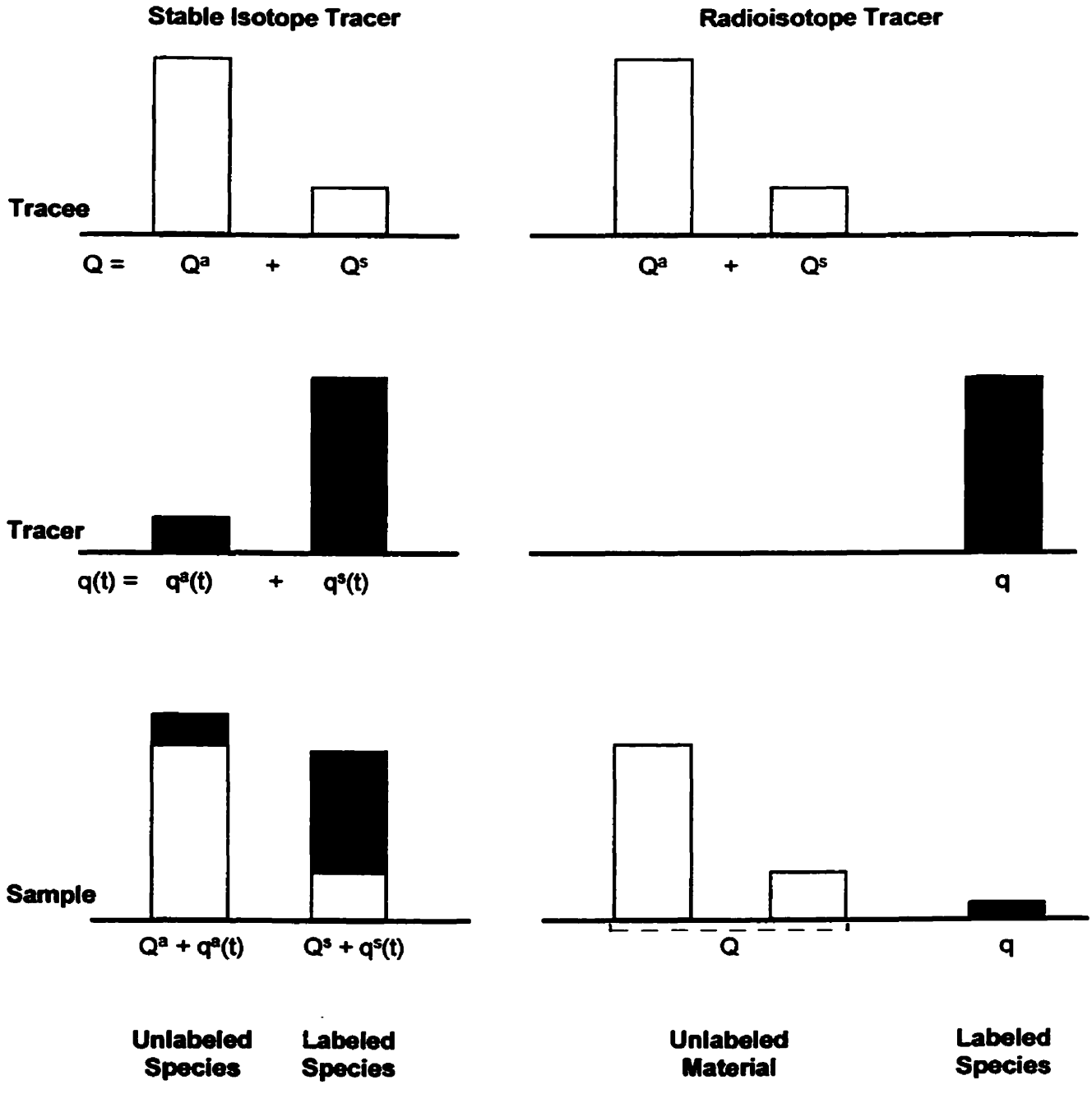


Figure 2.12. Bar graph representing relative contribution of labeled and unlabeled species to tracee, to stable or radioactive tracer, and to a generic sample taken during experiment. Q is tracee mass, Q^a is the mass of unlabeled tracee, Q^s is the mass of labeled tracee, q(t) is tracer mass, $q(t)^a$ is the mass of unlabeled tracer, and $q(t)^s$ is the mass of labeled tracer (513).

highlights an important advantage of radiolabeling over stable isotope labeling, which is the complete absence of labeled species in the tracee in the former. Z(t) is calculated from r(t) using a formula which was derived by Cobelli and his colleagues (513), in which:

$$z(t) = \left[\frac{r(t) - r_N}{r_I - r(t)} \right] \left[\frac{1 + r_I}{1 + r_N} \right]$$

where, r_N and r_I are naturally occurring and infusate isotopic ratios, respectively.

Among the 3 adjustments of the raw mass spectrometry data (correction for method-introduced leucine, adjustment for the presence of a derivatized non 5´,5´,5´ [D3] L-leucine population with mass 352 units, and the presence of unlabeled species in the tracer and labeled species in the tracee), the correction for contamination was the one which adjusted the data "disproportionately" (the magnitude of correction was different from one time point to the other and from one apolipoprotein to the other), with each measurement being corrected according to the relative contribution of method-introduced leucine to total leucine in the sample. It is also worth mentioning that the quantitative impact of this correction was more significant at late-time-points when proteins were highly enriched than in early time points (the higher the enrichment of leucine at a time point, the more the impact of contamination with non-enriched leucine will be on the value of z(t)). It was also more significant in proteins with faster turnover such as VLDL apoE, than in ones with slow turnover such as apoC-III (proteins with faster turnover attain higher z(t) during the 12 h infusion period, their z(t)s are thus subjected to more significant corrections). Finally, the correction was

more quantitatively significant in apoE, apoC-III, and proapoA-I than apoB-100, mature apoA-I, and apoA-II; the isolated amounts of the former proteins were less, making the relative contribution of method-introduced leucine to total leucine in their samples higher. Tables 2.3a and 2.3b show the calculated relative contribution of method-introduced leucine to total leucine in samples of 6 apolipoproteins (data for a single subject). Figure 2.13 shows the effect of correction for leucine contamination on tracer to tracee ratios measured for 6 apolipoproteins (data for a single subject).

The precision of our method as a whole (including protein isolation from electrophoretic gels, protein hydrolysis, amino acid purification by cation exchange chromatography, derivatizations, GC-MS analysis, and subsequent corrections of raw mass spectrometry data) was tested recently (December 1997) for the purpose of deriving a variance parameter necessary for the multicompartmental modeling of the kinetic data (see section 2.8). The results are shown in Table 2.4. Tracer to tracee ratios measured for VLDL apoB-100 from a single subject at 3 time points (30 min, 5 h, and 10 h) were shown to be reproducible in several aliquots. Furthermore, the recently obtained tracer to tracee ratios were comparable to those measured a year earlier (October 1996) during the initial analysis of VLDL apoB-100 in that subject (z(t)s measured in October 1996 were 0.05%, 6.65% and 9.87% for time points 30 min, 5h and 10h, respectively).

Tables 2.3a and 2.3b. Contribution of method-introduced leucine to total leucine in apolipoprotein samples in a single subject. Amount of contaminating leucine, which is used to correct raw mass spectrometry data, is calculated from AUPs leucine/norleucine in blank samples, as described in the text.

VLDL apoB-100		VLDL apoE		VLDL apoC-III	
Time point (h)	contamination %	Time point (h)	contamination %	Time point (h)	contaminatio %
0.00	0.87	2.00	4.11	1	0.62
0.25	0.75	4.00	3.60	2	0.87
0.50	0.66	5.00	4.81	4	0.86
1.00	0.58	6.00	3.02	5	0.84
2.00	0.76	7.00	2.89	6	0.80
3.00	0.77	9.00	4.71	7	0.89
4.00	0.81	10.00	1.50	9	0.88
5.00	1.62	11.00	2.97	10	0.88
6.00	0.81	12.00	1.38	11	2.72
7.00	1.29			12	0.92
9.00	0.94				
10.00	1.83				
11.00	1.58				
12.00	1.16				
Mean	1.03		2.98		0.94
SD	0.40		1.40		0.65

Plasma mature apoA-I		Plasma proapoA-I		Plasma apoA-II	
Time point (h)	contamination %	Time point (h)	contamination %	Time point (h)	contaminatio %
2.00	0.28	2.00	6.38	2.00	1.08
3.00	0.31	3.00	6.13	3.00	1.03
4.00	0.27	4.00	6.54	4.00	1.01
5.00	0.28	5.00	6.99	5.00	1.21
6.00	0.26	6.00	5.73	6.00	1.45
7.00	0.27	7.00	5.73	7.00	1.13
9.00	0.30	9.00	6.61	9.00	2.11
10.00	0.31	10.00	7.47	10.00	1.35
11.00	0.32	11.00	8.33	11.00	1.26
12.00	0.30	12.00	7.26	12.00	3.65
Mean	0.29		6.14		1.53
SD	0.02		2.06		0.81

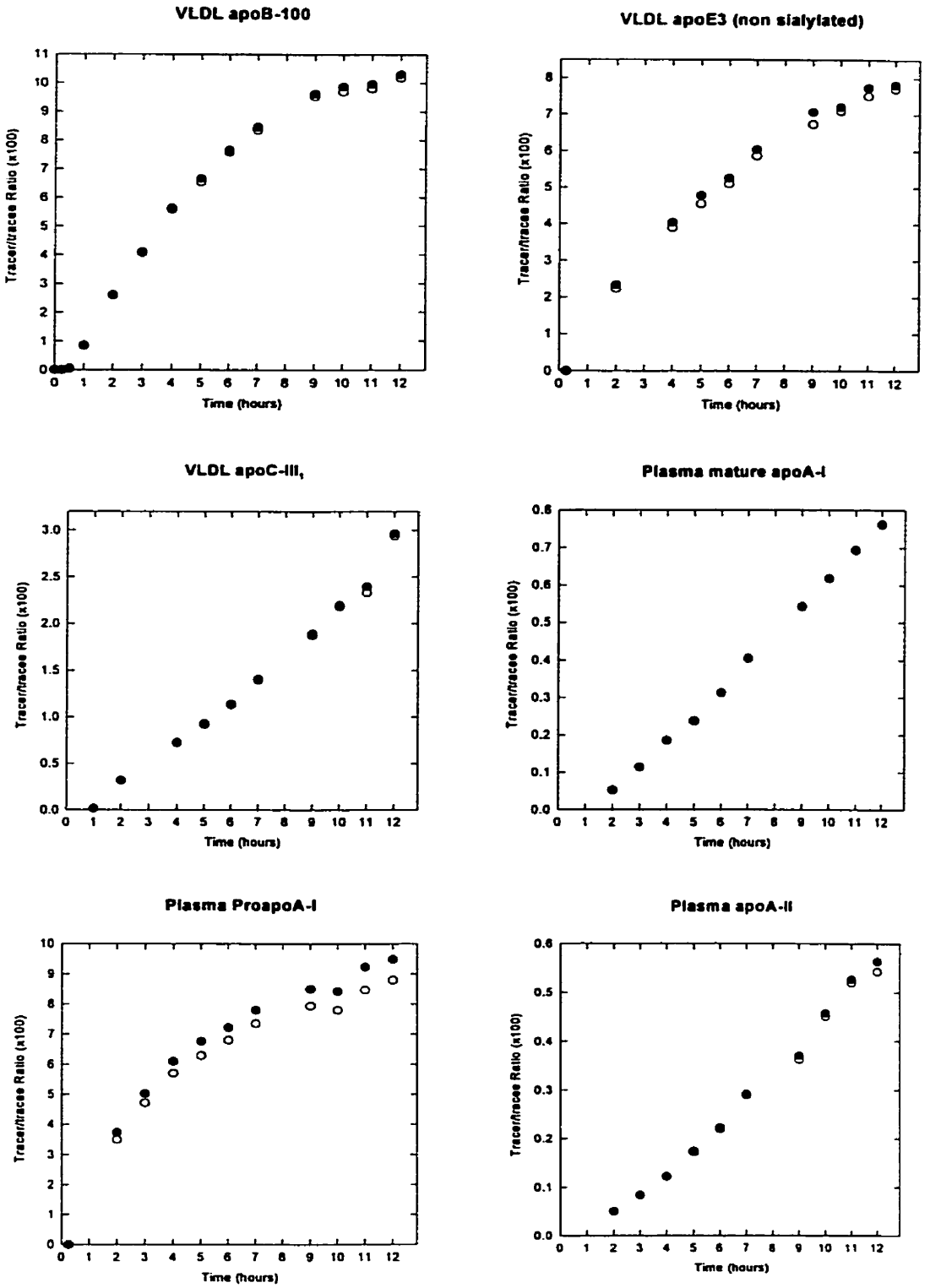


Figure 2.13. Tracer to tracee ratio (z(t)) vs. infusion time for six apolipoproteins in a single subject. Z(t)s corrected for method-introduced leucine are represented by solid symbols. Non-corrected z(t)s are represented by open symbols.

Table 2.4. Precision of measurements of tracer/tracee ratios obtained by the developed method. VLDL apoB-100 was isolated from plasma obtained at times 5 h, 30 min and 10 h during a primed constant infusion of [D_3]L-leucine in a single individual. VLDL from plasma attained at 5 h were separated on 3 polyacrylamide gel lanes to obtain 3 apoB-100-containing gel slices. After hydrolysis, each of the 3 samples was divided into 3 aliquots and amino acids of each aliquot were cation exchange chromatography-purified and derivatized separately. VLDL from plasma obtained at times 30 min and 10 h were separated on polyacrylamide gel lanes. After hydrolysis, each of the 2 samples was divided into 6 aliquots and these aliquots were processed. The tracer to tracee ratio obtained for each aliquot is given, and a coefficient of variation based on the mean ± SD is shown.

Time point: 30 min		Time point: 5 h		Time point: 10 h	
Aliquot number	corrected z(t) %	Aliquot number	corrected z(t) %	Aliquot number	corrected z(t) %
1	0.041	7	6.802	16	10.154
2	0.033	8	6.853	17	9.901
3	0.041	9	6.915	18	9.989
4	0.034			19	9.847
5	0.052	10	6.958	20	9.900
6	0.038	11	6.971	21	9.845
		12	6.964		
		13	6.983		
		14	6.807		
		15	6.997		
Mean	0.040		6.917		9.939
SD	0.007		0.077		0.118
CV%	17.5		1.11		1.19

2.8 Deriving Kinetic Parameters from Tracer to Tracee Ratios

The rate of change in the tracer to tracee ratio was used to calculate kinetic parameters, namely 1) the fractional production rate (FPR) (or fractional transport rate (FTR)) which is equivalent to FCR in radioactive formalism, 2) the RT, and 3) the PR (or transport rate (TR)). Additionally, the delay time which accounted for the synthesis and intracellular processing of newly-synthesized protein molecules was also obtained.

FPR (FTR) is the fraction of a pool (e.g. the VLDL apoB-100 pool) which is produced (transported) per unit of time, and was expressed in pools/hour or pools/day. RT (expressed in days) is the average time a protein molecule (e.g. an apoB-100 molecule) resides in a pool, and was calculated as the inverse of FPR. Finally, PR (TR) is the flux of protein through a pool per unit of time (usually expressed in mg/day per kg of body weight, i.e. mg/kg.day). PR was calculated from FPR and the pool size (mg) as :

$$PR = \frac{FPR \times Pool\ Size}{body\ weight}$$

Where pool size = plasma concentration (mg/dl) X plasma volume (0.045 L/kg)

To obtain FPR, tracer to tracee ratios of mature apoA-I, proapoA-I and apoA-II isolated from plasma of patients with FHD and control subjects (chapter 4) were fitted to a monoexponential function using SAAM II computer software (SAAM II Institute, WA). The function was defined as $z(t) = Zp\{1-e^{[-k(t-d)]}\}$ where z(t) was the tracer to tracee ratio at time t, Zp was the tracer to tracee ratio of the tissue precursor amino acid pool from which the protein in question was derived (the precursor enrichment, see section 1.10). Zp of the intestinal and hepatic

precursor amino acid pools from which proapoA-I and hence apoA-I were derived were taken to be the enrichment of proapoA-I at plateau. Since apoA-II, like apoB-100, is predominantly of hepatic origin, the enrichment of the apoA-II precursor pool was taken to be the enrichment of VLDL apoB-100 at plateau. Finally, d was the delay time in hours and k was FPR (pools/hour).

Subsequently, multicompartmental modeling was used to derive kinetic parameters for VLDL apoB-100, plasma apoC-III and plasma apoE in normolipidemic and hypertriglyceridemic subjects (chapter 3). A Multicompartmental model is a mathematical expression of the rate of flow of traced material in a compartmentalized system. To derive FPR and PR from physiological data (i.e. rate of change in tracer to tracee ratio, or specific activity in radioactive formalism, and pool size), several compartments, which may or may not have physiological analogs, are created. These compartments are linked with a set of differential equations that describe the flow of material in the studied system. The modeling software (e.g. SAAM II) fits the model to the data and solves the differential equations simultaneously to come out with transfer rates (k) and absolute fluxes. K from one compartment to another represents the fractional transport of traced material from the first compartment to the second, while k from a compartment to the "outside world" is actually the FPR (FCR in radioactive formalism). Similarly, the calculated flux is the absolute transport rate of traced material through a compartment and is equal to the PR if this compartment initiates the entry of material into the studied system. In addition, a multicompartmental model often includes delay compartments which

account for delay events. The delay times can also be obtained from the mathematical solution of the model.

The multicompartmental model which was applied in the study of the plasma kinetics of apoE and apoC-III (chapter 3) has been described earlier (522). It consists of 3 compartments (Fig 2.14). Compartment 1 is a precursor compartment (leucine compartment). Compartment 2 is a delay compartment, and compartment 3 is a plasma protein compartment (e.g. VLDL apoB-100 or HDL apoC-III).

An estimate for the precursor enrichment was derived from plasma leucine tracer to tracee ratio over the 12 h infusion period, and was used as a forcing function in compartment 1. The role of the forcing function was to decouple the kinetics of the precursor system (leucine) from that of the product (protein) by forcing the contents of compartment 1 to equal the estimate of the precursor enrichment. As was discussed in section 1.10, multicompartmental modelling of endogenous labeling experiments is complicated by the need to account for precursor kinetic compartments. Using a forcing function is one way to simplify the modeling process.

Alternatively, a more complex multicompartmental model which simultaneously describes the plasma kinetics of apoB-100, apoA-I, apoE and apoC-III is currently being developed by Dr. Hugh Barrett from the SAAM II Institute (Fig 2.15). In this model, several assumptions are made to link the metabolic pathways of the four apolipoproteins in lipoprotein fractions.

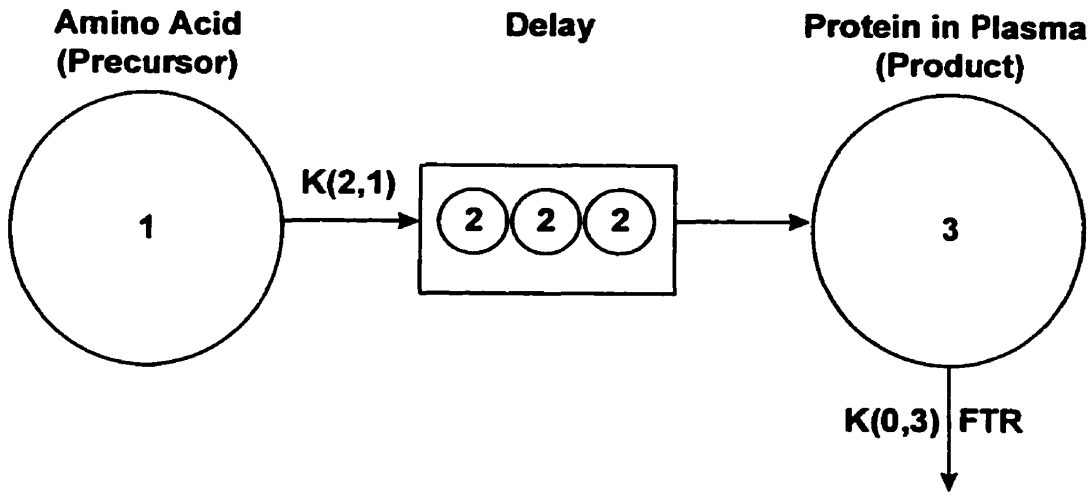


Figure 2.14. The multicompartmental model which was used to derive kinetic parameters for VLDL apoB-100, apoE and apoC-III, as described in the text.

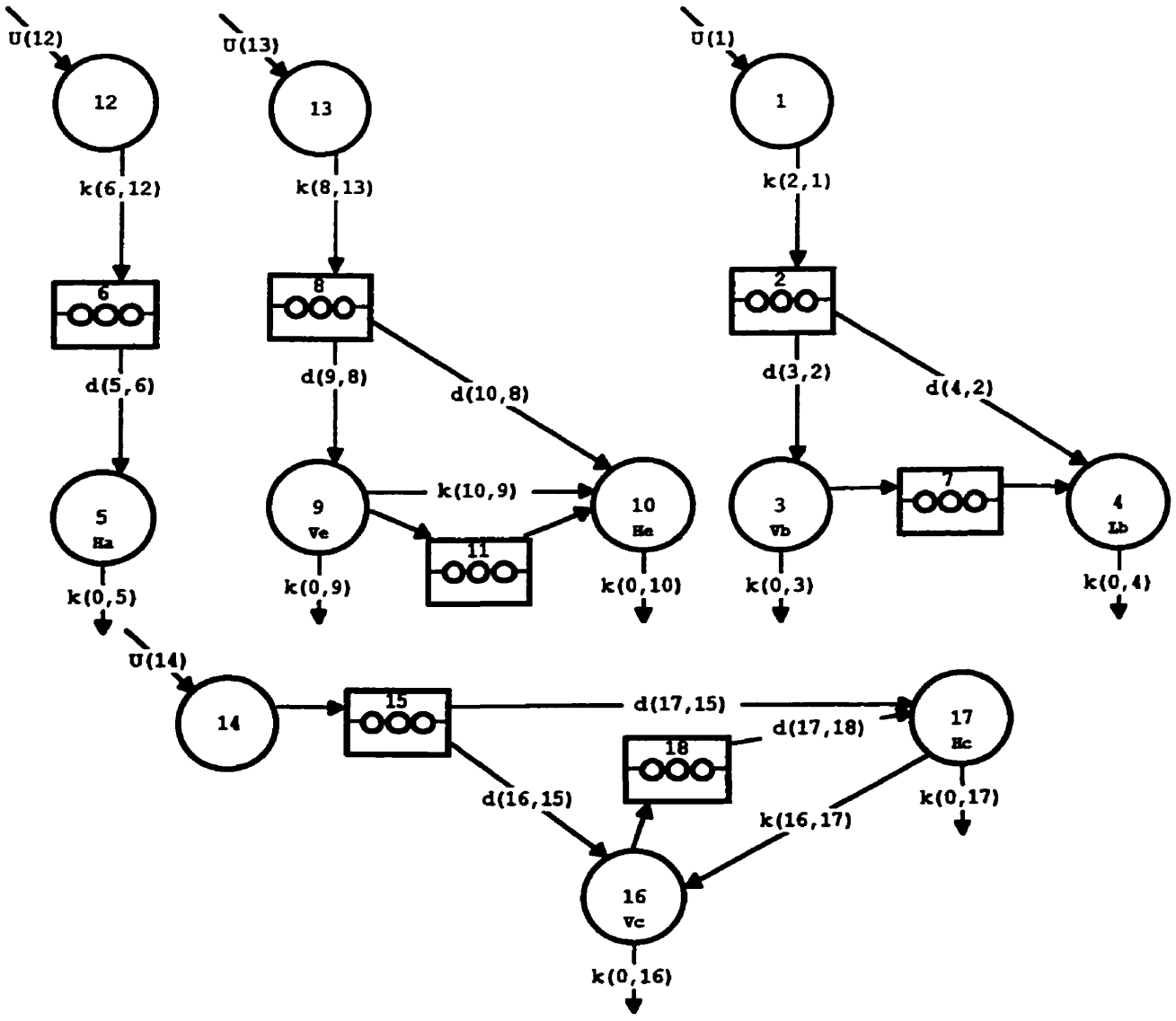


Figure 2.15. A multicompartmental model which integrates the kinetic data obtained for plasma apoA-I, apoE, apoC-III and apoB in one normolipidemic subject. Compartments 12 (precursor), 6 and 5 (HDL) describe the kinetics of apoA-I. Similarly, compartments 13 (precursor), 8, 9 (VLDL), 11 and 10 (HDL), describe apoE. Compartments 14 (precursor), 15, 16 (VLDL), 18 and 17 (HDL) describe apoC-III. Finally, compartments 1 (precursor), 2, 3 (VLDL), 7 and 4 (LDL) describe apoB-100. A forcing function was applied to the precursor compartments (12, 13, 14 and 1) to decouple the precursor system from that of the product. Several assumptions were made to link the metabolic pathways of the four apolipoproteins (Hugh Barrett, SAAM II Institute).

In conclusion, a method was established in our laboratory, in collaboration with the Biomedical Mass Spectrometry Unit of McGill University (Montreal) and the SAAM II Institute (WA), to study the *in vivo* kinetics of plasma apoE, apoC-III, apoA-I, and apoB-100 using endogenous labeling with stable isotopes. The method was tested for accuracy and precision. Controls were developed to correct for method-introduced variations, and proper procedures were followed to convert raw mass spectrometry data to tracer to tracee ratios. Numerical monoexponential fitting and subsequently multicompartmental modeling were applied to derive FPR, RT and PR from obtained tracer to tracee ratios data.

Chapter 3

Plasma Kinetics of ApoC-III and ApoE in Normolipidemic and Hypertriglyceridemic Subjects

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Contribution of Co-authors

1. Mr. Michel Tremblay isolated apoC-III and apoE by peprarative IEF gels. He also participated in measuring apoC-III and apoE plasma concentrations by ELISAs.

2. Dr. Hugh Barrtett helped derive kinetic parameters from tracer to tracee ratio data using the SAAM II computer software.

3. Mme. Hélène Jacques measured apoB, apoC-III and apoE plasma concentrations by ELISAs. She also helped in the preparation and GC-MS analysis of leucine samples for the second type III hyperlipoproteinemic patient.

4. Dr. Alexandre Fredenrich helped set up the ELISA used in our laboratory for measuring plasma concentration of apoC-III.

5. Dr. Orval Mamer supervised the GC-MS analysis of leucine samples which was carried out in the Mass Spectrometry Unit of McGill University.

6. Dr. Jean Davignon and Dr. Jeffrey Cohn supervised the study.

Plasma Kinetics of ApoC-III and ApoE in Normolipidemic and Hypertriglyceridemic Subjects

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Supplementary key words: cholesterol, atherosclerosis, type III hyperlipoproteinemia, stable isotope.

Short title: Plasma apoC-III and apoE kinetics

Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; GC-MS, gas chromatography - mass spectrometry; FTR, fractional transport rate; VLDL, very low-density lipoprotein; HDL, high-density lipoprotein; IEF, isoelectric focusing; TR, transport rate; RT, residence time; NL, normolipidemic; HTG, hypertriglyceridemic; TypIII, type III hyperlipoproteinemia; pts., patients; conc., concentration.

Abstract

Apolipoprotein (apo) C-III and apoE play a central role in controlling plasma lipoprotein metabolism, and in regulating plasma levels of potentially atherogenic triglyceride-rich lipoproteins (TRL) and their remnants. In order to determine whether increased levels of plasma apoC-III and apoE in hypertriglyceridemic subjects are due to increased production or reduced catabolism of these proteins, we have investigated the plasma kinetics of total, very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) apoC-III and apoE in normolipidemic (NL) subjects (n = 5), hypertriglyceridemic (HTG) patients (pts., n = 5) and in pts. (n = 2) with type III (TypIII) hyperlipoproteinemia (both groups of pts. having reduced rates of VLDL apoB-100 catabolism and no evidence of VLDL apoB-100 overproduction). Kinetic parameters were determined using a primed constant (12 h) infusion of deuterium-labeled leucine, followed by multicompartmental analysis of plasma apolipoprotein enrichment curves. Elevated (3- to 12-fold) total plasma and VLDL apoC-III concentrations (concs). in HTG and TypIII pts., although associated with lower rates of apoC-III catabolism (i.e., increased residence times (RTs)), were mainly due to significantly increased rates of apoC-III production (plasma apoC-III transport rates (TRs, means ± SEM): (NL) 2.05 ± 0.22, (HTG) 4.90 ± 0.81 ($P < 0.01$), and (TypIII) 8.78 mg. $kg^{-1}.d^{-1}$; VLDL apoC-III TRs: (NL) 1.35 ± 0.23, (HTG) 5.35 ± 0.85 ($P < 0.01$), and (TypIII) 7.40 mg. $kg^{-1}.d^{-1}$). Elevated total plasma and VLDL apoE concs. in HTG (2- and 6-fold, resp.) and particularly in TypIII (9- and 43-

fold) pts. were associated with increased VLDL apoE RTs (0.21 ± 0.02, 0.46 ± 0.05 ($P < 0.01$), and 1.21 days, NL vs. HTG vs. TypIII, resp.), as well as significantly increased apoE TRs (plasma: (NL) 2.94 ± 0.78, (HTG) 5.80 ± 0.59 ($P < 0.01$) and (TypIII) 11.80 $mg.kg^{-1}.d^{-1}$; VLDL: (NL) 1.59 ± 0.18, (HTG) 4.52 ± 0.61 ($P < 0.01$) and (TypIII) 11.95 $mg.kg^{-1}.d^{-1}$). These results demonstrate that hypertriglyceridemic patients, having reduced rates of VLDL apoB-100 catabolism (including patients with type III hyperlipoproteinemia) are characterized by overproduction of plasma and VLDL apoC-III and apoE.

Introduction

ApoC-III and apoE are proteins, which play a central role in controlling the plasma metabolism of triglyceride-rich lipoproteins (TRL). ApoC-III is a 8.8 kD glycoprotein, synthesized by the liver and intestine (1,2). It is associated in circulating blood with all major classes of lipoproteins and has the ability to exchange between TRL and high-density lipoproteins (HDL) (3). In normolipidemic (NL) subjects, the majority of apoC-III is bound to HDL, while in hypertriglyceridemic (HTG) subjects, the majority is bound to TRL (4-6). The importance of apoC-III in regulating plasma TRL metabolism is demonstrated by the fact that: 1) in the general population, total plasma triglyceride levels are strongly correlated with the concentration of total plasma and TRL apoC-III (7,8); 2) individuals with certain apoC-III gene polymorphisms have increased susceptibility to hypertriglyceridemia (9-11); 3) patients with an inherited deficiency of apoC-III have low plasma triglyceride levels (12,13); 4) overexpression of the human apoC-III gene in transgenic mice results in hypertriglyceridemia (14), whereas mice lacking apoC-III through gene targeting are hypotriglyceridemic (15); and 5) *in vitro* evidence demonstrates that apoC-III has the capacity to inhibit: a) the activity of lipoprotein lipase (LPL) (16,17), b) the capacity of TRL to bind to LPL (18), and c) the uptake of TRL by the liver (19,20) through reduced binding of TRL and their remnants to the LDL receptor (LDL-R) (21), the lipolysis-stimulated receptor (LSR) (22), and cell-surface glycosaminoglycans (23), (though not the LDL receptor-related protein (LRP)

(24)). Together, these results demonstrate that increased plasma and TRL levels of apoC-III contribute to the reduced lipolysis and receptor-mediated clearance of TRL in HTG individuals.

ApoE, on the other hand, is a 34.2 kD glycoprotein, synthesized by the liver and to a lesser extent by peripheral tissues (25). It is polymorphic in humans and three different alleles (ε2, ε3, ε4) at a single gene locus are responsible for three major apoE isoforms (E2,E3, E4), of which apoE3 is the most common. ApoE can readily transfer between plasma lipoproteins and, like apoC-III, its concentration in total plasma and TRL is significantly correlated with the level of plasma triglyceride (26-28). ApoE plays a pivotal role in mediating the hepatic recognition and uptake of TRL, by serving as a ligand for lipoprotein binding to the LDL-R (29), LRP (30) and glycosaminoglycans (31). It is also necessary for the normal conversion of VLDL to LDL (32, 33). This is best exemplified by patients with dysbetalipoproteinemia or type III (TypIII) hyperlipoproteinemia, who have functionally impaired apoE, reduced clearance and catabolism of plasma TRL, and a pronounced increase in the plasma concentration of apoE-containing TRL remnants. The majority of TypIII patients are homozygous for the apoE2 isoform and are characterized by an increase in plasma levels of triglyceride, cholesterol and apoE, the presence of skin xanthomas, and the development of premature cardiovascular disease (34).

Although previous *in vivo* studies have investigated the plasma kinetics of apoC-III and apoE in HTG and TypIII patients, the etiology of their elevated plasma and TRL apoC-III and apoE levels has not been completely elucidated.

Plasma apoC-III kinetics have been studied in NL and HTG patients (35-39), and those of apoE have been determined in NL and TypIII patients (40-45). In the case of apoC-III, HTG patients were found in one study (39) to have increased rates of apoC-III production and relatively normal rates of apoC-III catabolism. However, in two other studies, HTG patients were characterized by significantly decreased rates of apoC-III catabolism (35,37). The kinetics of apoE have not been investigated in HTG patients, and those of apoC-III have not been investigated in TypIII patients. Furthermore, the kinetics of these proteins have not been studied simultaneously in the same individuals. We have therefore carried out the present study, using an endogenous-labeling primed constant (stable isotope) infusion technique to simultaneously investigate the plasma kinetics of apoC-III and apoE. Our objective was to determine whether increased levels of plasma apoC-III and apoE in HTG and TypIII patients (both having increased levels of very low-density lipoprotein (VLDL) apoB-100, due to reduced rates of VLDL apoB-100 catabolism) were due to an increase in their rates of apoC-III and apoE production or a decrease in their rates of catabolism.

Methods

Study Subjects. A total of 12 subjects (11 males and 1 female) were investigated in the present study. Five of these individuals were normolipidemic. They were apparently healthy male subjects, who were selected because they had a fasting plasma triglyceride concentration < 2.2 mmol/l, a total plasma cholesterol concentration < 5.2 mmol/l, and were within 10% of desirable body weight. Four of them had an apoE 3/3 phenotype and 1 had an apoE 3/2 phenotype. They had no evidence nor history of dyslipidemia, diabetes mellitus, nor other metabolic disorder, and were not taking medications known to affect plasma lipid metabolism. Five individuals, including the female, were hypertriglyceridemic (HTG) patients recruited from the lipid clinic of the Clinical Research Institute of Montreal. They had plasma triglyceride concentrations > 2.2 mmol/l. Four of them were classified as having type IV hyperlipoproteinemia, since their LDL cholesterol levels were < 3.4 mmol/l. One HTG patient also had an elevated LDL cholesterol level (> 4.0 mmol/l) and was classified as having type IIb hyperlipoproteinemia. Two HTG patients had an apoE 3/3 phenotype, one was apoE 4/3, one was apoE 3/2 and one was apoE 4/2. The latter two patients had a familial form of dyslipidemia, characterized by plasma TRL remnant accumulation, as evidenced by the presence in plasma of VLDL particles with slow pre-β agarose gel electrophoretic mobility. These patients have been previously described by our laboratory (46). An additional two male patients were studied, who had type III (TypIII) hyperlipoproteinemia. They were

selected on the basis that they were hyperlipidemic, had an apoE 2/2 phenotype, and had β-migrating VLDL (34), as determined by agarose gel electrophoresis. Patients taking lipid-lowering medications (statins or fibrates) were asked to stop their medications 30 days prior to their infusion experiments. All twelve subjects gave informed consent to the study protocol, which was approved by the ethics committee of the Clinical Research Institute of Montreal.

Stable Isotope Infusion Protocol. After a 12-h overnight fast, study subjects were given a primed constant intravenous infusion of deuterium-labeled leucine ([D_3]L-leucine 98%, Cambridge Isotope Laboratories, MA), as previously described (47,48). They were injected via a needle attached to a left forearm vein with 10 μmol per kg body weight of [D_3]L-leucine, dissolved in physiological saline, followed by a 12-h constant infusion (given by peristaltic pump) of 10 μmol [D_3]L-leucine per kg per h. Subjects remained fasted during the infusion but had free access to drinking water. Blood samples (20 ml) were collected from an antecubital vein of the right arm at regular intervals (0, 15, 30, and 45 min, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h) in tubes containing EDTA to a final concentration of 0.1%. Plasma was immediately separated by centrifugation at 3,500 rpm for 15 min at 4°C. An antimicrobial agent (sodium azide) and a protease inhibitor (aprotinin) were added to plasma samples to give a final concentration of 0.02 % and 1.67 μg/ml, respectively.

Isolation of Lipoproteins and Apolipoproteins. VLDL, IDL together with LDL, and HDL, were isolated from 5 ml plasma by sequential ultracentrifugation in an XL-90 ultracentrifuge using a 50.4 Ti rotor (Beckman) at 50,000 rpm for 10

h, at densities (d) of 1.006, 1.063 and 1.21 g/ml, respectively. Total lipoproteins were isolated from plasma by ultracentrifugation (50,000 rpm, 10 h) of 1 ml of plasma, adjusted to d of 1.25 g/ml with KBr. Lipoproteins were recovered in the supernate by tube-slicing. VLDL apoB-100 was isolated by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-22.5 % gradient gels (48). ApoC-III and apoE were isolated from VLDL, HDL and total plasma lipoproteins (d < 1.25 g/ml fractions) by preparative isoelectric focusing (IEF) on 7.5 % polyacrylamide-urea (8M) gels (pH gradient 4-7) (49). For three subjects, apoE was also isolated from total plasma (1 ml) by immunoaffinity chromatography (50). HDL and d < 1.25 g/ml fractions were dialyzed against 10mM ammonium bicarbonate, preincubated with cysteamine (β-mercaptoethylamine, Sigma-Aldrich) in a ratio of 6 mg for every mg of protein for 4 h at 37°C, and then delipidated. The aim of cysteamine treatment was to separate apoE and apoA-I isoforms, which normally co-migrate to the same position on IEF gels. Cysteamine treatment caused an amino group to bind to the single cysteine residue of apoE3. Cysteamine treatment resulted in two amino groups to be introduced into apoE2, which contained two cysteine residues (51). ApoA-I and apoE4 were not affected since they did not contain cysteine. Cysteamine-modified apoE2 and apoE3 consequently migrated to a higher position in IEF gels, due to their increased positive charge (48). VLDL samples were delipidated, but were not treated with cysteamine, prior to electrophoresis. Coumassie blue staining was used to identify the position of apolipoproteins in gels after electrophoresis.

Plasma lipids and apolipoproteins. Plasma and lipoprotein fractions were assayed for total (free and esterified) cholesterol and triglyceride with a COBAS MIRA-S automated analyzer (Hoffman-LaRoche) using enzymatic reagents. Plasma and VLDL apoB concentrations were measured with a non-competitive ELISA using immunopurified goat anti-human apoB antibody and horse radish peroxidase-conjugated monoclonal anti-apoB antibody (52). Plasma apoA-I concentrations were measured by nephelometry on a Behring Nephelometer 100 (Behring) using Behring protocol and reagents. Plasma and lipoprotein apoC-III and apoE concentrations were measured with ELISAs developed in our laboratory (6,28). ApoE phenotypes were determined by isoelectric focusing of delipidated VLDL (49). Total recovery (mean ± SD) of apoC-III and apoE in lipoprotein fractions separated by ultracentrifugation was 89 ± 9 % and 63 ± 12 %, resp. ApoC-III and apoE in the bottom fractions, which represented 0.5 ± 0.4 % and 13.5 ± 3.1 %, resp. (of total plasma apolipoproteins), were considered to be predominantly HDL apolipoproteins and were added to the HDL pool. Lipoprotein apoC-III and apoE concentrations were then corrected for non-recovered apolipoprotein by increasing VLDL, IDL/LDL and HDL levels by a factor (on average) of 1.1 and 1.25, respectively.

Determination of isotopic enrichment. Apolipoprotein bands, as well as blank (non-protein containing) gel slices were excised from polyacrylamide gels (VLDL B-100 from SDS-PAGE; apoC-III and apoE from IEF gels), as described previously (47,48). The band corresponding to the major isoform of apoC-III, monosialylated apoC-III (apoC-III_1), was excised and analysed in all cases. For

apoE, the non-sialylated form of apoE3 was analyzed. In subjects with 2 apoE isoforms, both VLDL apoE bands were analyzed (only one apoE band was present in HDL and d < 1.25 g/ml fractions of these subjects due to the effect of cysteamine treatment). Each slice was added to a borosilicate sample vial containing 600 µl of 6N HCL, and an internal standard of 250 ng norleucine (Sigma-Aldrich) dissolved in 50 µl double distilled water. Gel slices were hydrolyzed at 110°C for 24 h, cooled to -20°C for 20 min, and centrifuged at 3,500 rpm for 5 min. Free amino acids in the hydrolysate were separated from precipitated polyacrylamide, purified by cation exchange chromatography using AG 50 W-X8 resin (BioRad), and derivatized by treatment with 200 µl of acetyl chloride-acidified 1-propanol (1:5 V/V) for 1 h at 100°C, and 50 µl of heptaflurobutyric anhydride (Supelco) for 20 min at 60°C (47). Plasma amino acids were also separated by cation exchange chromatography and derivatized to allow for the analysis of plasma leucine isotopic enrichment. Enrichment of samples with deuterium-labeled leucine was determined by GC-MS (Hewlett-Packard, 5988 GC-MS) using negative chemical ionization and methane as the reagent gas. Selective ion monitoring at m/z =352 and 349 (ionic species corresponding to derivatized deuterium-labeled and derivatized non-deuterium-labeled leucine, respectively) was performed, and tracer to tracee ratios were derived from isotopic ratios for each sample according to the formula derived by Cobelli et al. (53). Tracer to tracee ratios were corrected for background leucine in gel slices (and for leucine introduced during the amino acid purification and derivitization procedures) by estimating the amount of leucine in processed blank

gel slices in relation to the norleucine internal standard. Background leucine represented (mean ± SD) 2.7 ± 0.8 % of total leucine recovered for apoB-100 samples and 9.2 ± 3.9 % of total leucine recovered for apoC-III and apoE samples.

Kinetic analysis. SAAM II computer software (SAAM II institute, WA) was used to fit a three compartment model to the tracer to tracee data (54). The first compartment represented the tissue amino acid precursor pool. The second compartment was a delay compartment, which accounted for the synthesis, assembly and secretion of apolipoproteins. The third compartment was the plasma protein compartment. The enrichment of plasma with deuterium-labeled leucine was used as the forcing function for precursor pool enrichment. Computer modeling of tracer to tracee ratio data for apoB-100, apoC-III and apoE allowed for the determination of their fractional transport rates (FTR) (i.e., the fraction of protein pools being renewed per day). Residence time (RT) was calculated as the reciprocal of FTR (1/FTR), and transport rate (TR) was calculated (in mg. $kg^{-1}.day^{-1}$) as:

$$TR = \frac{\text{FTR (pools/day) X apolipoprotein pool size (mg)}}{\text{body weight (kg)}}$$

where:pool size = plasma concentration (mg/dl) X plasma volume (0.045 liter/kg).

Transport rates were also expressed in molar units (nmol. kg^{-1}. day^{-1}) using a molecular weight of 8,746 daltons for apoC-III, 34,200 daltons for apoE, and 549,000 daltons for apoB-100. Kinetic parameters for total plasma, VLDL and HDL apolipoproteins were determined independently. Transport rates for total plasma apoC-III and apoE, and VLDL apoB-100, represented their rates of total plasma production. This was not however the case for VLDL and HDL apoC-III and apoE, where their transport rates represented direct apolipoprotein production, as well as apolipoprotein transfer from one lipoprotein pool to another.

Statistical analysis. The statistical significance of differences between mean values was assessed by paired and unpaired t-tests using SigmaStat software (Jandel Scientific, CA). Pearson correlation coefficients (r) were calculated to describe the correlation between different kinetic and mass parameters.

Results

Characteristics of study subjects. Plasma lipid and lipoprotein characteristics of the study subjects are shown in Table I. Total plasma triglyceride concentrations were on average 5-fold higher in HTG patients compared to NL subjects. VLDL-TG, VLDL-C, IDL/LDL-TG, but not IDL/LDL-C, concentrations were also significantly increased, and plasma HDL-C, but not apoA-I, concentrations were significantly reduced in HTG patients. TypIII patients were severely hypertriglyceridemic and hypercholesterolemic, and had higher levels of VLDL-TG, VLDL-C, IDL/LDL-TG (though not IDL/LDL cholesterol) compared to HTG and NL subjects.

Kinetics of plasma VLDL apoB-100. HTG and TypIII patients had significantly elevated levels of VLDL apoB-100 (4-fold and 10-fold, resp.) (Table II). These patients had impaired VLDL catabolism rather than VLDL overproduction, which was determined by measuring the plasma kinetics of their VLDL apoB-100. Deuterium-labeled leucine enrichment of VLDL apoB-100 from NL, HTG and TypIII subjects is shown in Figure 1, and kinetic parameters derived by multicompartmental analysis of enrichment curves are shown in Table II. Delayed catabolism of VLDL apoB-100 was reflected by higher VLDL apoB-100 residence times (RTs) in HTG and TypIII patients (5- and 13-fold, resp.) compared to NL subjects. VLDL apoB-100 transport rates (TRs), on the other hand, were not significantly different between the three groups.

Table I. Characteristics of Subjects

Subjects	Age *y*	BMI *kg/m²*	TG *mmol/l*	TC *mmol/l*	VLDL-TG *mmol/l*	VLDL-C *mmol/l*	IDL/LDL-TG *mmol/l*	IDL/LDL-C *mmol/l*	HDL-C *mmol/l*	ApoB *mg/dl*	ApoA-I *mg/dl*
Normolipidemic	31	24.9	0.89	4.00	0.74	0.19	0.12	2.55	1.27	79.3	125.0
(*n*=5)	± 2	± 0.5	± 0.17	± 0.33	± 0.16	± 0.07	± 0.02	± 0.27	± 0.15	± 5.9	± 10.1
Hypertriglyceridemic	50*	25.8	4.36‡	5.22	3.81‡	1.72*	0.42‡	2.80	0.70*	129.4§	118.5
(*n*=5)	± 6	± 1.0	± 0.77	± 0.58	± 0.78	± 0.56	± 0.07	± 0.38	± 0.09	± 7.4	± 5.4
Type III	44	28.3	12.02	14.79	11.22	12.35	0.58	1.96	0.49	139.3	126.4
(*n*=2)											

Values are means ± SEM for (*n*) subjects in each group, except those for type III patients which are means only. Mean concentrations were calculated from a single value for each subject, which was the average of five measurements made at 3-hour intervals during the stable-isotope infusion experiment.

BMI is body mass index; TG, triglyceride; TC, total cholesterol; VLDL, very low -density lipoprotein; -C, cholesterol; IDL/LDL, intermediate-density and low-density lipoproteins in the $1.006 < d < 1.063$ g/ml fraction; and HDL, high-density lipoprotein.

Significantly different from normolipidemic subjects by unpaired t-test: *$P < 0.05$, ‡ $P < 0.01$, § $P < 0.001$.

Table II. Kinetic Parameters for VLDL apoB-100

	VLDL apoB-100		
Subjects	Concentration *mg/dl*	RT *days*	TR *mg. $kg^{-1}.d^{1}$*
Normolipidemic (*n*=5)	5.9 ± 1.7	0.07 ± 0.02	39.5 ± 5.8
Hypertriglyceridemic (*n*=5)	24.9* ± 4.4	0.33* ± 0.06	36.3 ± 6.5
Type III (*n*=2)	61.6	0.91	31.4

Values are means ± SEM for (*n*) subjects in each group, except those for type III patients which are means only. Mean concentrations were calculated from a single value for each subject, which was the average of five measurements made at 3-hour intervals during the stable-isotope infusion experiment.
RT is residence time; and TR, transport rate.
Significantly different from normolipidemic subjects by unpaired t-test: *$P < 0.01$.

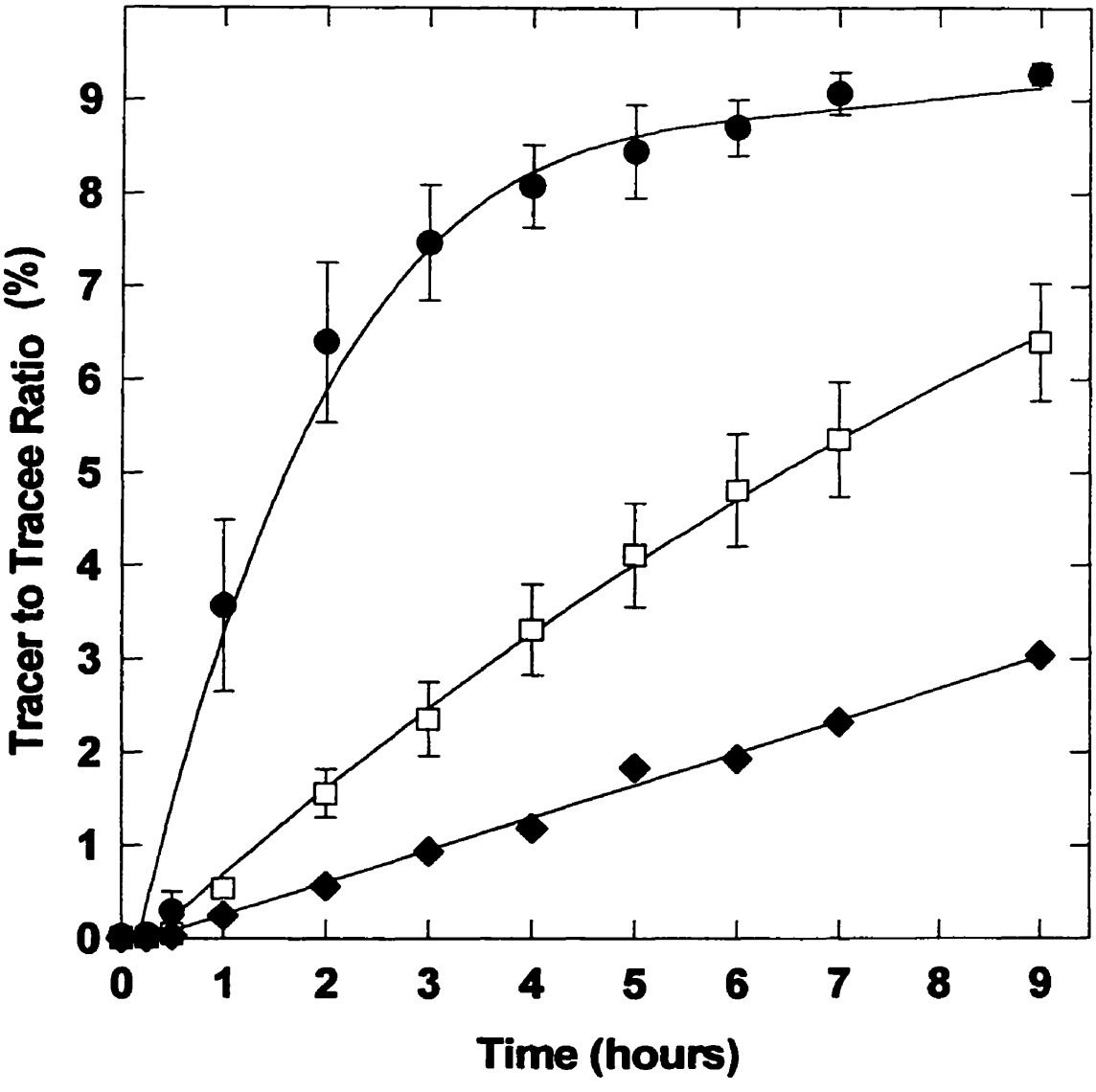

Figure 1. Enrichment of VLDL apoB-100 with deuterium-labeled leucine in normolipidemic (n = 5) (● - ●), hypertriglyceridemic (n = 5) (□-□) and type III (n = 2) (◆ - ◆) subjects. Error bars represent SEM. Tracer to tracee ratios were normalized to the same level of plateau enrichment to allow for groups to be visually compared.

Kinetics of plasma, VLDL and HDL apoC-III. Plasma and VLDL levels of apoC-III were significantly elevated in HTG and TypIII pts. compared to NL subjects (Table III). Plasma apoC-III levels were elevated 3- and 6-fold, while VLDL apoC-III levels were elevated 6- and 12-fold in HTG and TypIII pts., resp. HDL apoC-III concentrations were not significantly different. Mean data for deuterium-labeled leucine enrichment of apoC-III in VLDL, HDL and total plasma are shown for NL, HTG and TypIII pts. in Figure 2. Linear enrichment was observed for all three fractions in all 12 subjects during the time course of the experiment. Fractional rates of appearance of newly-synthesized apoC-III, reflected by the slopes of enrichment lines, were (without exception) higher in VLDL than in HDL. VLDL and HDL apoC-III enrichment lines were thus significantly different in all cases, providing *in vivo* evidence for the presence of apoC-III, which did not exchange and equilibrate between VLDL and HDL. Enrichment of apoC-III in total plasma was intermediate between that of VLDL and HDL (Figure 2). In NL subjects, where apoC-III was predominantly associated with HDL, plasma apoC-III enrichment was more similar to that of HDL than to that of VLDL. Conversely, in HTG and TypIII pts., where the majority of apoC-III was associated with VLDL, plasma apoC-III enrichment resembled that of VLDL rather than HDL. Kinetic parameters for apoC-III, derived by compartmental analysis, are shown in Table III. ApoC-III RT in VLDL was somewhat higher in HTG (1.7-fold) and TypIII pts. (2.6-fold) compared to NL subjects. In contrast, TRs for apoC-III in total plasma and VLDL were significantly higher in both HTG (2.4- and 4-fold) and TypIII pts. (4.3- and 5.5-

fold, resp.) compared to NL subjects. HDL apoC-III RTs and TRs were not significantly different (Table III). Elevated levels of plasma and VLDL apoC-III in HTG and TypIII pts. were thus found to be predominantly due to overproduction of VLDL apoC-III.

Kinetics of plasma, VLDL and HDL apoE. Plasma and VLDL levels of apoE were also significantly elevated in HTG and TypIII patients compared to NL subjects (Table IV). Plasma apoE levels were elevated 2- and 9-fold, while VLDL apoE levels were elevated 6- and 43-fold in HTG and TypIII patients, resp. HDL apoE concentrations were not significantly different in HTG compared to NL subjects, but were more than 2-fold higher in Typ III pts. Mean data for deuterium-labeled leucine enrichment of apoE in VLDL, HDL and total plasma are shown for NL, HTG and TypIII pts. in Figure 3. VLDL apoE enrichment curves were curvilinear in NL and HTG subjects, but were relatively linear in TypIII pts., reflecting a markedly reduced rate of VLDL apoE catabolism in these latter pts. As was the case for apoC-III, fractional rates of appearance of newly-synthesized apoE were higher in VLDL than in HDL in all subjects, and the dissimilarity in the slopes of these curves provided evidence for the existence of non-exchangeable pools of apoE in human plasma. Rate of enrichment of apoE in total plasma was (like apoC-III) intermediate between that of VLDL and HDL, resembling that of HDL in NL subjects and that of VLDL in TypIII pts. (Figure 3). Moreover, enrichment curves for apoE isolated by immunoaffinity chromatography from the plasma of 3 individuals were not different from those of total plasma apoE isolated by ultracentrifugation at d < 1.25 g/ml (Figure 4),

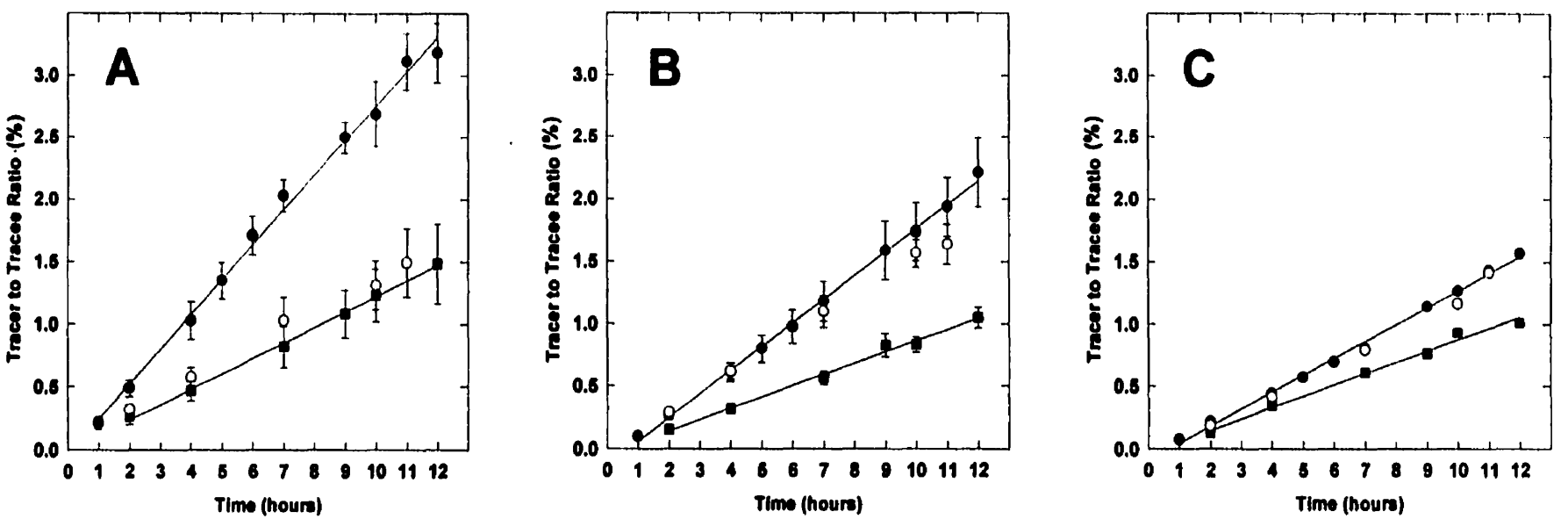

Figure 2. Enrichment of VLDL (● - ●), HDL (■ - ■) and total plasma apoC-III (O - O) with deuterium-labeled leucine in normolipidemic (n = 5) (**A**), hypertriglyceridemic (n = 5) (**B**) and type III (n = 2) (**C**) subjects. Error bars represent SEM. Tracer to tracee ratios were normalized to the same level of plateau enrichment to allow for groups to be compared visually.

Table III. Kinetic Parameters for apoC-III

Subjects	VLDL apoC-III			HDL apoC-III			Plasma apoC-III		
	Concentration *mg/dl*	RT *days*	TR *mg. $kg^{-1}.d^{1}$*	Concentration *mg/dl*	RT *days*	TR *mg. $Kg^{-1}.d^{1}$*	Concentration *mg/dl*	RT *days*	TR *mg. $kg^{-1}.d^{1}$*
Normolipidemic (*n=5*)	3.59 ± 0.80	1.17 ± 0.11	1.35 ± 0.23	5.34 ± 1.16	3.51 ± 0.62	0.80 ± 0.21	9.47 ± 0.84	2.16 ± 0.26	2.05 ± 0.22
Hypertriglyceridemic (*n=5*)	22.36* ± 4.38	1.97 ± 0.45	5.35* ± 0.85	3.69 ± 0.52	3.80 ± 0.50	0.47 ± 0.09	28.71* ± 5.12	2.66 ± 0.31	4.90* ± 0.81
Type III (*n=2*)	44.35	2.99	7.40	6.10	4.38	0.73	52.99	2.90	8.78

Values are means ± SEM for (*n*) subjects in each group, except those for type III patients which are means only. Mean concentrations were calculated from a single value for each subject, which was the average of five measurements made at 3-hour intervals during the stable-isotope infusion experiment.
RT is residence time; and TR, transport rate.
Significantly different from normolipidemic subjects by unpaired t-test: *$P < 0.01$.

confirming that apoE isolated by ultracentrifugation was representative of plasma apoE, despite the stripping of apoE during ultracentrifugal isolation (26,27). Kinetic parameters for apoE, derived by compartmental analysis, are shown in Table IV. VLDL apoE RTs, but not HDL or total plasma apoE RTs, were significantly ($P < 0.01$) higher in HTG pts. compared to NL subjects. Total plasma and VLDL apoE TRs were also significantly increased (2- and 3-fold, resp.), whereas HDL apoE TRs were reduced by about 50% ($P < 0.05$), in HTG pts. RTs of VLDL, HDL and plasma apoE were markedly higher (6-, 4-, and 2-fold, resp.) in Typ III pts. compared to NL subjects. TRs of apoE in total plasma and VLDL, but not HDL, were also significantly increased in Typ III pts., demonstrating that increased production and delayed catabolism contributed to the increase in VLDL and plasma apoE levels in HTG and TypIII pts.

In order to summarize and compare the plasma kinetics of apoB-100, apoC-III and apoE in VLDL, TRs were expressed in terms of the number of molecules transported per unit time (i.e., in molar units: $nmol.kg^{-1}.\ day^{-1}$). These data are presented, together with VLDL apolipoprotein residence times, in Figure 5. In NL subjects, VLDL apoB-100, apoE and apoC-III TRs were 72 ± 11, 47 ± 5, and 155 ± 26 $nmol.kg^{-1}.\ day^{-1}$, resp., demonstrating that, on average, for every 2 molecules of VLDL apoB-100 produced by the liver, there was the appearance in VLDL of 1 molecule of newly-synthesized apoE and 4 molecules of newly-synthesized apoC-III. In contrast, in HTG pts., in whom apoC-III and apoE TRs were significantly increased, 2 molecules of newly-synthesized apoE and 10 molecules of newly-synthesized apoC-III appeared in VLDL for every

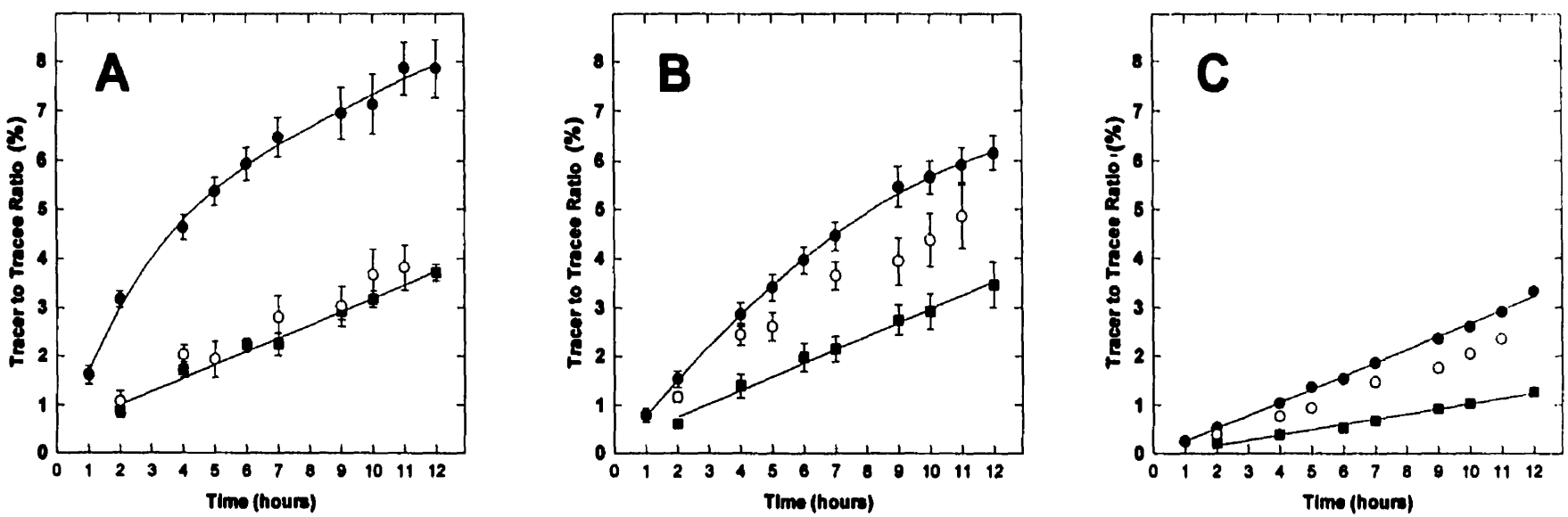

Figure 3. Enrichment of VLDL (● - ●), HDL (■ - ■) and total plasma apoE (○ - ○) with deuterium-labeled leucine in normolipidemic (n = 5) (**A**), hypertriglyceridemic (n = 5) (**B**) and type III (n = 2) (**C**) subjects. Error bars represent SEM. Tracer to tracee ratios were normalized to the same level of plateau enrichment to allow for groups to be compared visually.

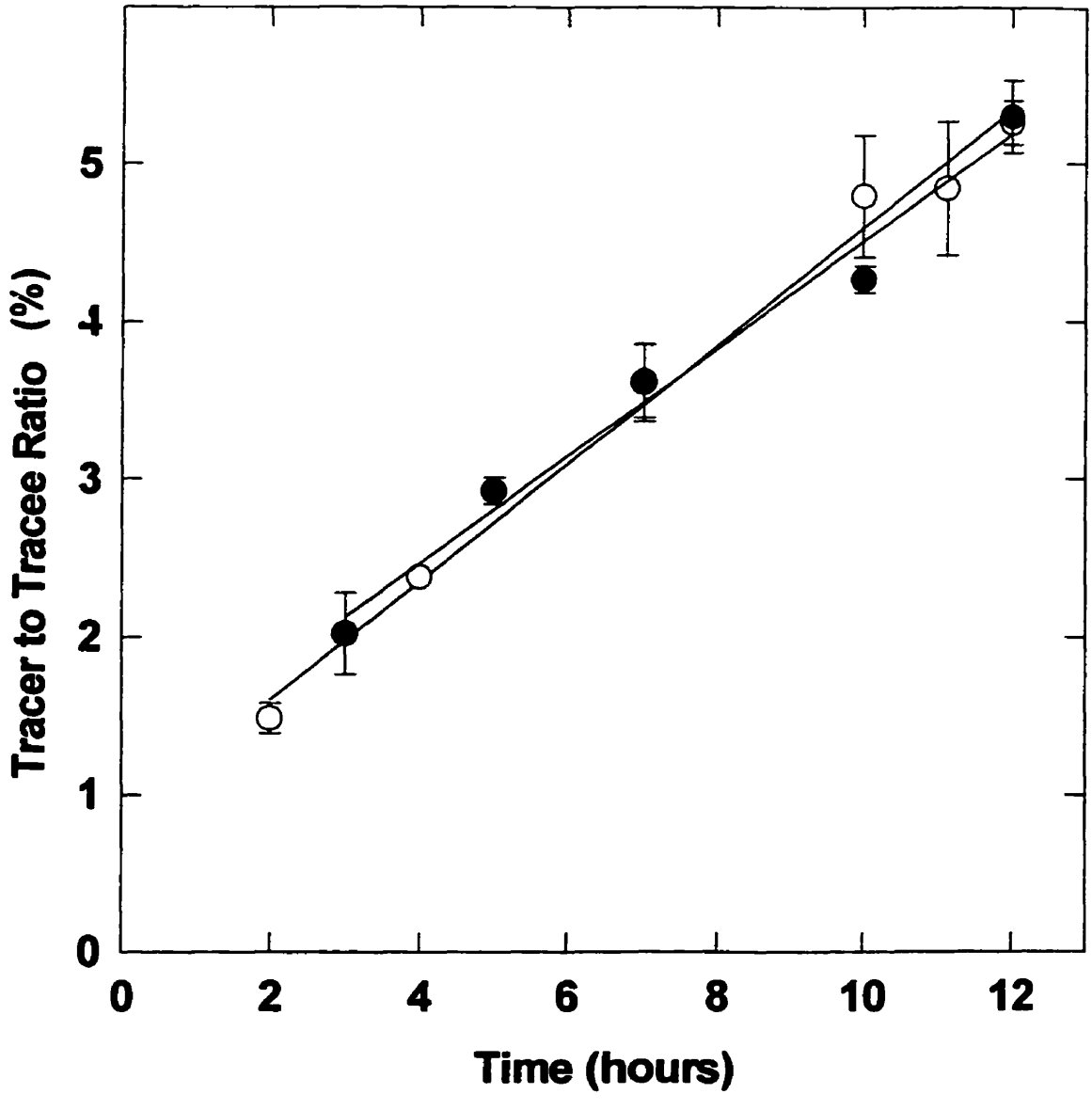

Figure 4. Deuterium-labeled leucine enrichment of total plasma apoE isolated by ultracentrifugation at d < 1.25 g/ml (O - O) or by immunoaffinity chromatography (● - ●) (see Methods). Results (± SEM) are shown for 3 selected subjects. Tracer to tracee ratios were normalized to the same level of plateau enrichment, as in previous figures.

molecule of newly-synthesized apoB-100. In the case of TypIII pts., 6 molecules of newly-synthesized apoE and 15 molecules of newly-synthesized apoC-III appeared in VLDL for every molecule of newly-synthesized apoB-100. In all individuals, VLDL apoC-III RTs were higher than VLDL apoE RTs (NL: $P < 0.001$; HTG: $P < 0.05$), which were in turn higher than VLDL apoB-100 RTs (NL: $P < 0.01$; HTG: $P < 0.01$, Figure 5B).

Table IV. Kinetic Parameters for apoE

Subjects	VLDL apoE			HDL apoE			Plasma apoE		
	Concentration *mg/dl*	RT* *days*	TR *mg. $kg^{-1}.d^{-1}$*	Concentration *mg/dl*	RT *days*	TR *mg. $Kg^{-1}.d^{-1}$*	Concentration *mg/dl*	RT *days*	TR *mg. $kg^{-1}.d^{-1}$*
Normolipidemic (*n*=5)	0.72 ± 0.08	0.21 ± 0.02	1.59 ± 0.18	2.99 ± 0.26	0.91 ± 0.09	1.56 ± 0.24	4.28 ± 0.31	0.85 ± 0.19	2.94 ± 0.78
Hypertriglyceridemic (*n*=5)	4.63‡ ± 0.79	0.46‡ ± 0.05	4.52‡ ± 0.61	2.22 ± 0.35	1.14 ± 0.18	0.87§ ± 0.06	8.03‡ ± 0.83	0.65 ± 0.09	5.80§ ± 0.59
Type III (*n*=2)	30.97	1.21	11.95	7.07	3.34	0.95	40.40	1.55	11.80

Values are means ± SEM for (*n*) subjects in each group, except those for type III patients which are means only. Mean concentrations were calculated from a single value for each subject, which was the average of five measurements made at 3-hour intervals during the stable-isotope infusion experiment.

RT is residence time; and TR, transport rate.

*RT of VLDL apoE in subjects heterozygous for apoE was calculated as the mean of RT values obtained for the two isoforms, and that value was used in calculating TR.

Significantly different from normolipidemic subjects by unpaired t-test: §$P < 0.05$, ‡$P < 0.01$.

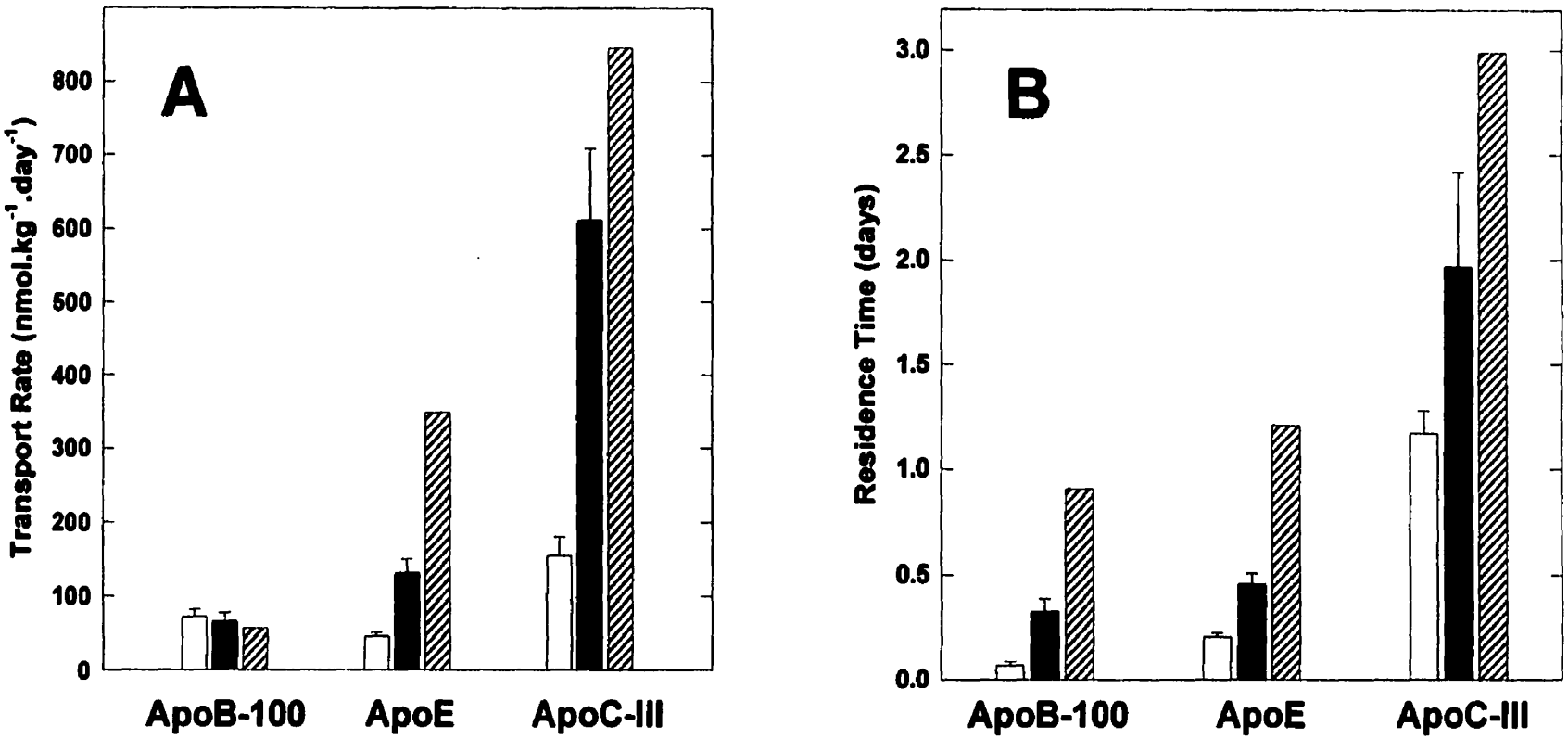

Figure 5. Comparison of VLDL apolipoprotein (apoB-100, apoC-III, apoE) transport rates (A) and residence times (B) in normolipidemic (open bars), hypertriglyceridemic (filled bars) and Type III subjects (hatched bars). Transport rates are expressed in molar units, in order to compare them in terms of numbers of molecules newly-synthesized. Error bars represent SEMs (n = 5).

Discussion

Kinetics of total plasma apoC-III and apoE have been investigated in previous studies by monitoring the plasma disappearance of exogenously-labeled apolipoproteins. ApoC-III has been investigated after intravenous injection of ^{125}I-labeled VLDL (35-37), ^{125}I-labeled HDL (37), ^{125}I-apoC-III incorporated into HDL (38), or ^{125}I-apoC-III incorporated into total plasma lipoproteins ($d < 1.25$ g/ml) (39). ApoE has been investigated after intravenous injection of free ^{125}I-apoE (44), or ^{125}I-apoE incorporated into $d < 1.21$ g/ml plasma lipoproteins (40-43).

Irrespective of the form in which radiolabeled apoC-III or apoE have been administered, they have been found to exchange between different lipoproteins in the circulation. In some cases, this exchange was complete, suggesting kinetic homogeneity of plasma apoC-III and apoE (35). In others, radiolabeled apoC-III or apoE were not fully exchangeable or had different catabolic rates on different lipoproteins, suggesting the presence of kinetically distinct pools on these lipoproteins. For example, Bukberg et al. found that when ^{125}I-labeled VLDL was injected intravenously, the specific radioactivity of apoC-III in VLDL remained higher than in HDL, and conversely, when ^{125}I-labeled HDL was injected, apoC-III specific activity remained higher in HDL than in VLDL (37). Malmendier et al., and based on the bi- or triphasic nature of plasma and urine ^{125}I-apoC-III decay curves, proposed the presence in plasma of at least 2 kinetically distinct populations of apoC-III; a "quickly metabolized" population

associated with apoB-containing particles, and a slowly metabolized one associated with apoA-I-containing particles (38). In the case of apoE, Gregg et al. found that immediately after injection of ^{125}I-apoE-labeled lipoproteins, the specific activity of apoE in different lipoprotein fractions was equal. However, the rate of disappearance of ^{125}I-apoE from these lipoproteins was dissimilar, being highest from VLDL and lowest from HDL (41,42). The implication of this kinetic heterogeneity is that exogenous labeling methods, although useful for measuring transport rates of total plasma apoC-III and apoE, may not be ideal for investigating the kinetics of these apolipoproteins in different lipoprotein fractions. In contrast, an endogenous labeling procedure, such as the one applied in the present study, introduces a label physiologically and simultaneously into different plasma lipoprotein fractions, through direct production and subsequent exchange. Consequently, this approach allows for measurement of kinetic activity in these fractions.

Results of the present study confirmed the existence of kinetically distinct (i.e., non-exchangeable) pools of apoC-III and apoE on VLDL and HDL. As depicted in Figures 2 and 3, fractional rates of appearance of newly-synthesized apoC-III and apoE were higher in VLDL compared to HDL in the three study groups. If VLDL and HDL apolipoprotein pools had been fully exchangeable and kinetically homogenous, enrichment curves would have been superimposable. In fact, simple mathematical analysis combining total plasma, VLDL and HDL transport rates suggests that only a small proportion of VLDL and HDL apoC-III and apoE was exchangeable (Figures 6 and 7). Contribution of

exchangeability to the transport of apoC-III and apoE was minimal compared to direct production in the case of VLDL pools. It is important to note that these findings, as well as those of studies mentioned previously, describe plasma apolipoprotein kinetics under a steady state assumption. This does not mean that exchange of apoC-III or apoE between plasma lipoproteins is not quantitatively important in non-steady state situations. For example, apoE can markedly redistribute from HDL to VLDL during alimentary lipemia (57) and from TRL to HDL following heparin-induced lipolysis (58). An additional conclusion which can be derived from the analyses presented in Figures 6 and 7, is that in both normolipidemic and hypertriglyceridemic subjects, a significant proportion of total plasma apoC-III and apoE is produced on VLDL. Hepatic synthesis and secretion of apoC-III and apoE on nascent VLDL and HDL has been demonstrated by studies with perfused rat livers and isolated human hepatocytes (59-62). The relative importance of different lipoproteins in the tissue output of these apolipoproteins has not however been clearly defined. Evidence has been presented showing that apoC-III and apoE are almost completely associated with nascent VLDL in highly purified Golgi fractions of rat hepatocytes (63). Fazio et al. have shown that stimulation of lipogenesis and increased production of apoB-containing lipoproteins in HepG2 cells cause increased intracellular association of apoE with VLDL and decreased association with HDL, but does not result in an increase in total apoE production (64). Millar et al. have in turn demonstrated that apoE is predominantly produced on VLDL in the fed state (65). In general, our results, and those of others, support the notion that kinetics of apoC-III and

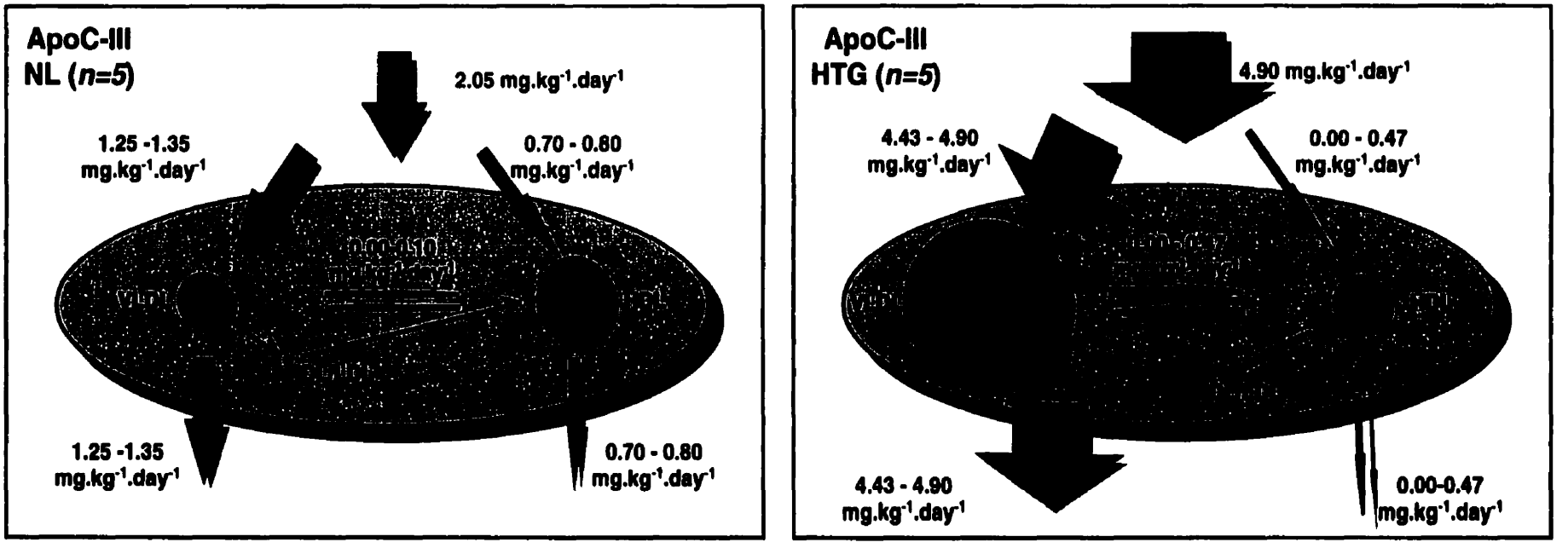

Figure 6. Schematic representation of plasma apoC-III metabolism in normolipidemic (NL) and hypertriglyceridemic (HTG) subjects. Elliptical areas represent plasma. Filled circles represent lipoprotein pools of apoC-III (their size being proportional to the relative mass of apoC-III). Transport of apoC-III is depicted by arrows (their size being proportional to the mass of apoC-III transported per kg per day). Minimum and maximum possible rates of transport are shown for each pathway. A maximum value in one pathway can be associated with minimum values in others. These values were determined by solving a set of simultaneous equations, in which: a) total plasma apoC-III transport rate was assumed to be equal to the sum of direct production on VLDL and HDL (VLDL apoC-III transport rate in HTG patients was reduced by 8% to allow for this equality), b) VLDL apoC-III transport rate was equal to the sum of direct production on VLDL and transfer to VLDL from HDL. and c) HDL apoC-III transport rate was equal to the sum of direct production on HDL and transfer to HDL from VLDL. ApoC-III transport into the IDL/LDL pool was assumed to be negligible. (IDL/LDL apoC-III: 6% and 9% of total plasma apoC-III in NL and HTG, respectively).

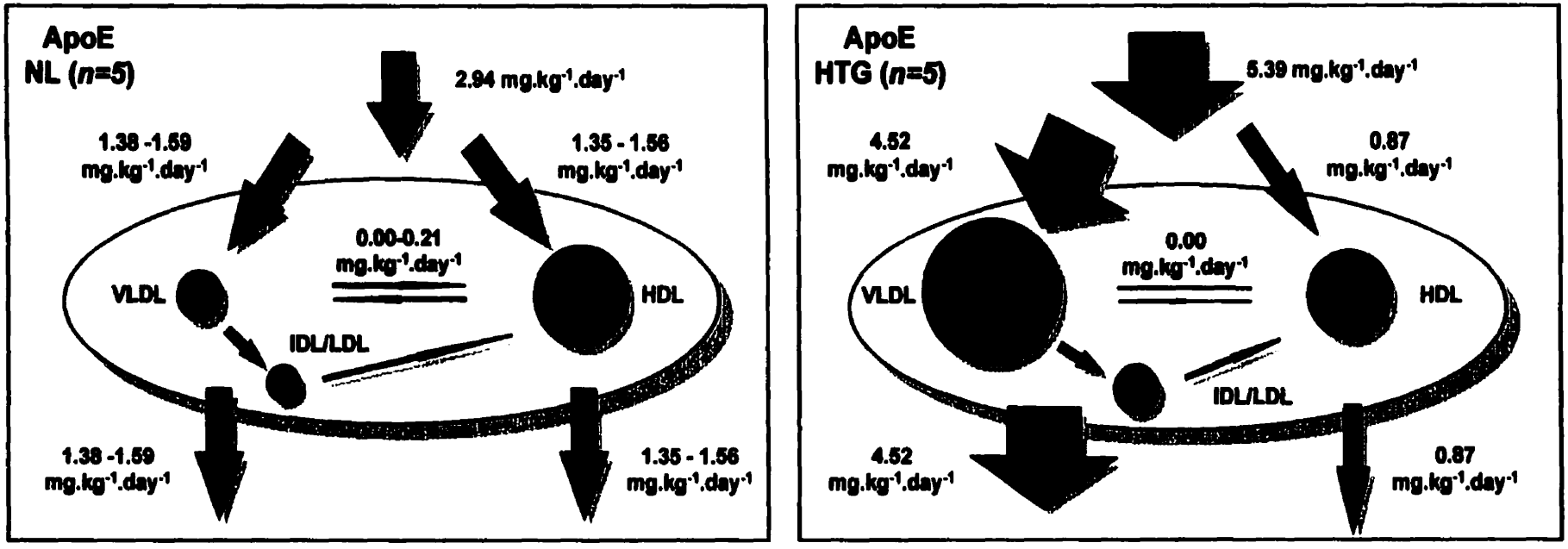

Figure 7. Schematic representation of plasma apoE metabolism in normolipidemic (NL) and hypertriglyceridemic (HTG) subjects. Elliptical areas represent plasma. Filled circles represent lipoprotein pools of apoE (their size being proportional to the relative mass of apoE). Transport of apoE is depicted by arrows (their size being proportional to the mass of apoE transported per kg per day). Minimum and maximum possible rates of transport are shown for each pathway. A maximum value in one pathway can be associated with minimum values in others. These values were determined by solving a set of simultaneous equations, in which: a) total plasma apoE transport rate was assumed to be equal to the sum of direct production on VLDL and HDL (total plasma apoE transport rate in HTG patients was reduced by 7% to allow for this equality), b) VLDL apoE transport rate was equal to the sum of direct production on VLDL and transfer to VLDL from HDL. and c) HDL apoE transport rate was equal to the sum of direct production on HDL and transfer to HDL from VLDL. ApoE transport into the IDL/LDL pool was assumed to be negligible (IDL/LDL apoE: 13% and 15% of total plasma apoE in NL and HTG, respectively).

apoE on VLDL are quite distinct from HDL. They depict VLDL pools as kinetically more active, even in normolipidemic subjects having relatively small VLDL pools (i.e. less apoC-III and apoE on VLDL), compared to HDL.

In order to learn more about these VLDL pools, transport rates and residence times of VLDL apoC-III, apoE, and apoB-100 were directly compared (Figure 5). In NL subjects, The transport rate of VLDL apoC-III, expressed in molar terms, was higher than VLDL apoB-100, which in turn was higher than VLDL apoE. This indicates that, on average, a newly-synthesized VLDL particle was secreted into plasma associated with several apoC-III molecules but not all the time associated with apoE. In contrast, in HTG and TypIII pts., a newly-synthesized VLDL particle was secreted into plasma significantly enriched with both apoC-III and apoE. The increase in VLDL C-III/B and E/B molar ratios in hypertriglyceridemic patients has been previously documented (6). In the present study however we show this to be due to an enrichment of nascent plasma VLDL particles with newly-synthesized apoC-III and apoE molecules. Interestingly, residence times of VLDL apoC-III and E were higher than VLDL apoB-100, even in the case of HTG and TypIII pts. who have significantly reduced catabolism of VLDL apoB-100 (Figure 5B). This suggests that catabolism of VLDL apoC-III and apoE, particularly apoC-III, was at least partially independent of VLDL apoB-100. Furthermore, it indicates that a transfer of apoC-III and apoE molecules took place between VLDL particles. The majority of apoC-III, and a significant proportion of apoE, abandoned VLDL particles during catabolism and replenished newly-synthesized ones.

Our data show that elevation in total plasma and VLDL apoC-III levels in HTG patients was mainly due to overproduction of plasma and VLDL apoC-III (Table III). Residence times of plasma and VLDL apoC-III also tended to be higher. Overproduction of plasma and VLDL apoC-III was somewhat unexpected, in view of the fact that this group of HTG pts. had increased VLDL levels, resulting from reduced catabolism rather than overproduction of VLDL apoB-100. VLDL apoC-III overproduction therefore existed in the absence of VLDL apoB-100 overproduction. Kinetics of total plasma apoC-III in HTG patients have been previously investigated. In two studies (35,37), mean residence times of plasma apoC-III were higher in HTG patients compared to NL subjects (1.54 and 1.60 vs. 3.21 and 2.98 days for apoC-III_1 and apoC-III_2; and 0.83 vs. 2.41 days for apoC-III, NL vs. HTG, respectively). In a third study (39), the transport rate was higher in HTG patients compared to NL subjects (2.3 vs. 6.1 $mg.kg^{-1}.day^{-1}$, NL vs. HTG, respectively). Expression of human apoC-III gene, which is found in the apoA-I/C-III/A-IV gene cluster on chromosome 11, is regulated at the level of transcription (54). Transcriptional activity of the apoC-III gene is modulated by a variety of agents, including insulin (68). The hypotriglyceridemic action of fibrates, activators of peroxisome proliferator activated receptors (PPAR), is primarily due to decreased hepatic apoC-III gene expression and thus plasma apoC-III levels (69). This action of fibrates was shown in chow-fed rats to occur without a concomitant decrease in apoB or VLDL production (77) supporting the notion that VLDL apoC-III production is independent of VLDL apoB-100. Moreover, although apoC-III and apoA-I genes

fall within the same gene cluster, production of plasma apoA-I was not significantly increased in HTG patients, compared to NL subjects (data not shown). It nevertheless tended to be higher. The present results, together with those from studies of: a) apoC-III transgenic and gene knockout mice (14,15,23,66,67), b) apoC-III human gene polymorphisms and deficiency states (9-13), and c) hormonal and drug effects on apoC-III gene expression (68-70) suggest that apoC-III production is not just linked, but is a primary determinant of circulating TRL levels, and hence also the concentration of total plasma triglyceride.

Similar to the case of apoC-III, elevated plasma and VLDL concentrations of apoE in HTG patients were associated with increased production of plasma and VLDL apoE (Table IV). However, residence time of VLDL apoE was significantly higher, and HDL transport rate was significantly lower, compared to NL subjects. Residence times and production rates (PR) of total plasma apoE in normolipidemic subjects, homozygous for apoE3 isoform, were measured previously using exogenous labeling techniques (RT: 0.73 ± 0.18 days; PR: 3.4 ± 1.5 $mg.kg^{-1}.day^{-1}$, mean ± SD) (41). Residence times of TRL and HDL apoE were also measured in normolipidemic subjects using an endogenous labeling procedure (TRL: 0.11 ± 0.05 days; HDL: 2.95 ± 0.99 days, mean ± SD) (65). Radiolabeled apoE4 was shown to be cleared faster than apoE3 from plasma of normolipidemic subjects homozygous for apoE3 isoform (42), whereas apoE2 was cleared slower (40). Four of the subjects in the present study were heterozygous for the apoE phenotype. However, VLDL residence times of their

apoE isoforms were not found to be markedly different (data not shown). The similarity in this case may be due to the fact that we only compared residence times of these isoforms on VLDL (methods). Total plasma kinetic measurements in normolipidemic subjects in previous studies could have reflected the preferential association of apoE4 isoform with VLDL, and apoE3 (and possibly apoE2) with HDL (42,56). Reduced catabolism of VLDL apoE in plasma of HTG patients in the present study could have been a result of slower catabolism of VLDL particles. The changes in apoE production on VLDL and HDL however are more difficult to explain. We postulate that these changes were part of a compensatory mechanism, whereby the liver synthesized and secreted increased amounts of apoE on VLDL in response to reduced hepatic uptake of remnant lipoproteins and reduced flux of lipids into the liver. Nevertheless, the possibility that these changes were primary in nature cannot be ruled out.

Plasma kinetics of apoC-III and apoE in TypIII patients deserve special attention. It is well accepted that the massive accumulation of TRL in the plasma of these patients is primarily due to defective receptor binding and impaired clearance of several dysfunctional apoE isoforms, mainly apoE2 (71,72). Indeed, our data show a marked increase in the residence time of plasma, VLDL, and HDL apoE in TypIII patients, compared to NL subjects (2-, 6-, and 4-fold, respectively) (Table IV). Similarly, there was an increase in residence time of VLDL apoC-III, which could be the result of delayed catabolism of the VLDL particle (i.e. VLDL apoB-100). Transport rates of plasma and VLDL apoE however were also higher in TypIII patients, compared to NL subjects (4- and 8-

fold, respectively). In fact, increased production of plasma and VLDL apoE contributed more to apoE accumulation than did reduced catabolism. Overproduction of plasma apoE (associated with delayed catabolism) has been demonstrated previously in one TypIII patient (55). He had a dominant form of Type III hyperlipoproteinemia caused by the presence of an apoE mutant isoform (Lys146 →Glu). We now show this to be also true in the more common case of TypIII patients homozygous for apoE2. Interestingly, TypIII patients in the present study had increased production of plasma and VLDL apoC-III (plasma apoC-III TR: 8.78 vs. 2.05; VLDL apoC-III TR: 7.40 vs.1.17 $mg.kg^{-1}.day^{-1}$, TypIII vs. NL, respectively) (Table III). This increase is most likely to be independent of the series of metabolic events induced by the presence in plasma of dysfunctional apoE. It is well known that homozygosity for the apoE2 isoform is not sufficient to cause overt type-III hyperlipoproteinemia (73,74). Development of this lipid phenotype is dependent on the presence of other genetic or environmental factors, such as hypothyroidism, pregnancy, diabetes, estrogen withdrawal, or obesity (34). Simultaneous occurrence of apoC-III gene polymorphism (associated with increased plasma triglyceride concentration) and an apoE 2/2 phenotype may be responsible for the expression of type III hyperlipoproteinemia in some individuals. This is analogous to the co-occurrence of LDL receptor defect (75) or apoB defect (76) with an apoE2/2 phenotype. Overproduction of apoC-III may also be a biochemical mechanism by which some factors, such as diabetes and obesity precipitate the hyperlipidemic phenotype. Based on our findings in hypertriglyceridemic

patients, including those with type III hyperlipoproteinemia, we propose the following sequence of events: 1) an increase in apoC-III production on VLDL resulted in elevation in its VLDL concentration; 2) this in turn caused: a) reduction in lipolytic processing and clearance of the VLDL particle, which affected apoB-100 and VLDL-associated apoC-III and apoE, and b) compensatory increase in VLDL apoE production. In the particular case of TypIII patients, overproduction of VLDL apoC-III stressed a system which was already burdened by a dysfunctional apoE. Consequently, plasma TRL catabolism in their case was further impaired.

In conclusion, the present results have shown that: 1) apoC-III and apoE are not fully exchangeable *in vivo* between VLDL and HDL, and thus represent kinetically distinct pools of apolipoprotein; 2) in both normolipidemic and hypertriglyceridemic subjects, a significant proportion (50% or more) of total plasma apoC-III and apoE production is accounted for by apolipoprotein production into VLDL; 3) increased levels of plasma and VLDL apoC-III in hypertriglyceridemic subjects, having decreased VLDL apoB-100 catabolism, are mainly the result of an increase in apoC-III production rather than a decrease in apoC-III catabolism; 4) increased levels of plasma and VLDL apoE in hypertriglyceridemic patients are associated with increased VLDL apoE residence times, as well as significantly increased rates of apoE production; 6) apoE overproduction, and not just reduced apoE catabolism, is an important factor contributing to increased plasma and VLDL apoE levels in type III patients and 7) plasma and VLDL apoC-III production is increased in patients with type III

hyperlipoproteinemia, which may be an important biochemical mechanism responsible for overt hyperlipidemia in individuals with an apoE 2/2 phenotype. These results provide evidence that normal levels of plasma apoC-III and apoE production are crucial for maintaining a normal plasma lipid profile, and that therapeutic intervention aimed at reducing apoC-III production is a logical strategy for the treatment of hypertriglyceridemia.

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Chapter 4

Familial HDL Deficiency Characterized by Hypercatabolism of Mature ApoA-I but Not ProapoA-I

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Contribution of Co-authors

1. Mr. Michel Tremblay isolated apoA-I and apA-II by peprarative IEF gels.

2. Mr. Larbi Krimbou separated lipoproteins by Two-dimensional gel electrophoresis and immunolocalized apoA-I- and apoA-II- containing lipoproteins.

3. Dr. Orval Mamer supervised the GC-MS analysis of leucine samples which was carried out in the Mass Spectrometry Unit of McGill University.

4. Dr. Jacques Genest, Jr. provided access to the FHD patients in the Lipid Clinic of the Clinical Research Institute of Montreal.

5. Dr. Jean Davignon and Dr. Jeffrey Cohn supervised the study.

Familial HDL Deficiency Characterized by Hypercatabolism of Mature ApoA-I but not ProapoA-I

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Short title: Plasma apoA-I kinetics in FHD

Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; GC-MS, gas chromatography - mass spectrometry; FCR, fractional catabolic rate; FPR, fractional production rate; FHD, familial high-density lipoprotein deficiency; HDL, high-density lipoprotein; IEF, isoelectric focusing; LCAT, lecithin cholesterol acyltransferase; PR, production rate; RCT, reverse cholesterol transport; RT, residence time.

ABSTRACT

We have previously described patients with familial high-density lipoprotein (HDL) deficiency (FHD), having a marked reduction in the plasma concentration of HDL-cholesterol and apolipoprotein (apo) A-I, but lacking clinical manifestations of Tangier disease or evidence of other known causes of HDL deficiency. In order to determine whether FHD in these individuals was associated with impaired HDL production or increased HDL catabolism, we have investigated the kinetics of plasma apoA-I and apoA-II in 2 related FHD patients (plasma apoA-I: 17 and 37 mg/dl) and 4 control subjects (apoA-I: 126 ± 18 mg/dl, mean ± SD), using a primed constant infusion of deuterated leucine. Kinetic analysis of plasma apolipoprotein enrichment curves demonstrated that mature plasma apoA-I production rates (PRs) were similar in patients and controls (7.9 and 9.1 vs 10.5 ± 1.7 mg/kg.day). Residence times (RTs) of mature apoA-I were however significantly less in FHD patients (0.79 and 1.66 days) compared to controls (5.32 ± 1.05 days). Essentially normal levels of plasma proapoA-I (the precursor protein of apoA-I) in FHD patients were associated with normal plasma proapoA-I PRs (7.8 and 10.4 vs 10.9 ± 2.6 mg/kg.day), and proapoA-I RTs (0.18 and 0.15 vs 0.16 ± 0.03 days). The RTs of apoA-II were however less in patients (3.17 and 2.92 days) vs controls (7.24 ± 0.71 days), while PRs of apoA-II were similar (1.8 and 1.9 vs 1.7 ± 0.2 mg/kg.day). Increased plasma catabolism of apoA-II in FHD patients was associated with the presence in plasma of abnormal apoA-II-HDL without apoA-I. These results demonstrate that FHD in our patients

is characterized, like Tangier disease, by hypercatabolism of mature apoA-I and apoA-II, but unlike Tangier disease, by essentially normal plasma catabolism and concentration of proapoA-I.

INTRODUCTION

Epidemiological studies have consistently demonstrated that low plasma high-density lipoprotein (HDL) levels are associated with the presence of coronary artery disease (CAD) (1,2). This association has been attributed to a role of HDL in mediating reverse cholesterol transport (RCT) (3). HDL has other potentially anti-atherogenic functions however, such as inhibiting low-density lipoprotein (LDL) oxidation (4), reducing the expression of endothelial cell adhesion molecules (5), and inhibiting platelet aggregation (6).

Apolipoprotein (apo) A-I and apoA-II are the major structural proteins of HDL (3). ApoA-I (28 kDa) is synthesized in the liver and intestine as a prepropeptide (7). It is processed co-translationally to form proapoA-I, which is secreted into the circulation. A metalloenzyme rapidly cleaves 6 amino acids from its amino-terminus, leading to the formation of mature apoA-I (243 amino acids) (8), which represents the majority (>95%) of total plasma apoA-I. ApoA-I is believed to be cleared from the circulation, in an almost lipid-free form, by glomerular filtration and tubular reabsorption in the kidney (9). ApoA-II (17kDa) is synthesized predominantly by the liver (10) and is secreted in plasma either as a propeptide, which is readily cleaved, or directly as mature apoA-II (11). ApoA-II is found in association with apoA-I in the plasma of normolipidemic subjects (on particles designated LpAI:A-II), while apoA-I is also present in particles not containing apoA-II (LpAI) (12). Kinetic studies in humans have shown that variation in plasma apoA-I levels is determined by the fractional catabolic rate (FCR) of apoA-I (13,14), whereas variation in plasma apoA-II levels, as well as

distribution of apoA-I between LpAI and LpAI:AII particles (15), is determined by the rate of production of apoA-II. At the same time, both FCR of apoA-I and apoA-II have been found to be inversely correlated with HDL cholesterol levels (16).

Human HDL deficiency (hypoalphalipoproteinemia) defines a group of dyslipidemias having an HDL cholesterol level below the 10th percentile for age- and gender- matched subjects (17). HDL deficiency has been shown to be the result of: 1) apoA-I gene abnormalities caused by deletion, inversion, insertion, nonsense or missense mutations, 2) apoA-II deficiency, 3) complete or partial absence of lecithin cholesterol acyltransferase (LCAT) activity, as seen in classical LCAT deficiency or Fish Eye disease, 4) increase in cholesteryl ester transfer protein (CETP) activity, or 5) severe hypertriglyceridemia (reviewed in refs. 18 and 19). HDL deficiency is also a major characteristic of Tangier disease, where an abnormality in HDL-mediated cholesterol and phospholipid efflux from peripheral cells (20,21), and/or a defect in HDL particle interconversion (22) have been suggested as possible metabolic causes.

Severe familial HDL deficiency (FHD) has recently been described in 3 French Canadian kindreds, as a trait with autosomal codominant inheritance (23). Two members of one particular kindred, who were extensively investigated, had normal fasting triglyceride concentrations with HDL-cholesterol levels below the 5th percentile. One of them had evidence of CAD. Both patients had a 50-80% reduction in plasma apoA-I concentration, a decrease in average HDL particle size, and a relative increase in plasma proapoA-I levels. No evidence

was obtained for the presence of an apoA-I or apoA-II gene abnormality, and FHD patients had none of the lipoprotein lipase gene mutations commonly found in French Canadians (24). LCAT activity was normal in FHD patients. Finally, none of the patients had clinical manifestations of Tangier disease (23), e.g., cholesteryl ester deposition in reticuloendothelial tissues, hyperplastic orange tonsils, splenomegaly or relapsing neuropathy.

In order to investigate the plasma kinetics of HDL apolipoproteins (proapoA-I, mature apoA-I and apoA-II) in the aforementioned FHD patients, we have carried out a stable-isotope kinetic study in 2 FHD and 4 normolipidemic control subjects. Our aim was to determine whether catabolism of apoA-I was greatly increased in these individuals, as previously documented for Tangier disease patients (25) and other HDL deficiency states (26-32), or whether production of apoA-I was impaired.

METHODS

Subjects

Six male subjects were investigated in the present study: 2 brothers with FHD and 4 healthy control subjects (Table 1). The medical history of the two affected brothers has been described previously (23). Briefly, patient 1 (proband 24430-301) was diagnosed with CAD at the age of 42. When diagnosed, he had a history of high blood pressure and he had been a smoker. His HDL-cholesterol concentration had been known to be low, but he had no signs of abnormal liver function nor clinical manifestations of Tangier disease. He underwent percutaneous transluminal coronary angioplasty at the age of 42 years and subsequently had coronary bypass surgery at the age of 48. Patient 2 (24430-313) did not have evidence of CAD nor did he show clinical signs of Tangier disease. HDL-cholesterol concentrations in the 2 patients, measured during routine visits to the lipid clinic of the Clinical Research Institute of Montreal (CRIM), were consistently found to be lower than 0.25 mmol/l. Both patients were not on medications known to affect plasma lipid levels, and their HDL deficiency was not due to known causes (23). Four healthy male subjects acted as controls. They had no evidence or history of dyslipidemia, diabetes mellitus, or any other metabolic disorder, and were not on medications known to affect plasma lipid levels. Only one of them, subject 4, was a smoker (less than one pack a day). All six subjects gave informed consent to the study protocol, which was approved by the ethics committee of the CRIM.

Infusion Protocol

The *in vivo* measurement of plasma apolipoprotein kinetics was carried out as described previously (33). After a 12-hour overnight fast, subjects were given an injection of 10 μmol per kg of body weight (10 μmol/kg) of [D_3]L-leucine ([D_3]L-leucine 98%, Cambridge Isotope Laboratories, MA) dissolved in physiological saline (0.9% NaCl), via an intravenous line attached to a left forearm vein. Following the bolus injection, subjects were infused for 12 h with 10 μmol/kg per h of [D_3]L-leucine dissolved in physiological saline. The infusion was carried out using a volumetric pump (Life Care Pump Model 3, Abbott) set to deliver 48 ml of infusate per hour. Subjects were not given food during the time course of the infusion but had free access to drinking water. They were encouraged to move around to maintain good blood circulation. Blood samples (20 ml) were collected from an antecubital vein at regular intervals (0, 15, 30, and 45 min, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h) in tubes containing EDTA to a final concentration of 0.1%. Samples were kept on ice, and plasma was immediately separated by centrifugation at 3,500 rpm for 15 min at 4°C. An antimicrobial agent (sodium azide) and a protease inhibitor (aprotinin) were added to plasma to give a final concentration of 0.02 % and 1.67 μg/ml, respectively.

Isolation of Lipoproteins and Apolipoproteins

Very low density lipoproteins (VLDL), intermediate plus low density lipoproteins (IDL + LDL) and HDL, were isolated from 5 ml plasma by sequential

ultracentrifugation in an XL-90 ultracentrifuge using a 50.4 Ti rotor (Beckman) at 50,000 rpm for 10 h, at densities (d) of 1.006, 1.019 and 1.21 g/ml, respectively. Total lipoproteins were isolated from plasma by ultracentrifugation (50,000 rpm, 10 h) of 1 ml of plasma, adjusted to d of 1.25 g/ml with KBr. Lipoproteins were recovered in the supernate by tube-slicing. VLDL apoB-100 was isolated by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-22.5 % gradient gel (33). Plasma apoA-I and apoA-II were isolated by preparative isoelectric focusing (IEF) on 7.5 % polyacrylamide-urea (8M) gels (pH gradient 4-7) of apolipoproteins in total plasma lipoproteins (d < 1.25 g/ml) (34). Fractions were dialyzed against 10mM ammonium bicarbonate, preincubated with cysteamine (• -mercaptoethylamine, Sigma-Aldrich) in a ratio of 6 mg for every mg of protein for 4 h at 37°C, and then delipidated. Protein bands were identified on gels by Coumassie blue staining. The aim of cysteamine treatment was to separate proapoA-I from apoE3, and apoA-II from asialylated apoC-III ($apoC\text{-}III_0$), which normally co-migrate to the same position on IEF gels. Cysteamine introduces an amino group to the single cysteine residues of apoE3 (35) and apoA-II, but does not affect proapoA-I and $apoC\text{-}III_0$ since these proteins do not contain cysteine. Cysteamine-modified apoE3 and apoA-II thus migrated to a higher position in the gel, due to their increased positive charge (Fig 1).

Separation of Lipoproteins by Two-Dimensional Gel Electrophoresis

Plasma lipoproteins were separated by two-dimensional non-denaturing gel electrophoresis, essentially as described previously (36). Agarose strips (approximately 7.5 cm in length) containing electrophoretically-separated lipoproteins were positioned at the top of 3-24% non-denaturing concave gradient polyacrylamide gels and sealed with agarose. Molecular size markers (Pharmacia, Piscataway, NJ), labeled with radioiodine, were also separated on each gel. They were equilibrated by a pre-run for 20 min at a constant voltage of 125 V. Samples were pre-electrophoresed at 70 V for 1 h and then separated for 24 h at 125 V. After electrophoresis, lipoproteins were transferred (180 mA, 24 h) to a nitrocellulose membrane (0.4 μm pore size). Fixing, blocking, and immunolocalization of apoA-I- and apoA-II-containing lipoproteins were performed as described (36), using immunopurified polyclonal anti-human apoA-I antibody, and monoclonal anti-apoA-II antibody (^{125}I-labeled). Plasma without apoA-I-containing lipoproteins was prepared by immunoaffinity chromatography using anti-apoA-I latex (Genzyme, MA). Plasma (200 μl) was added to 1 ml of apoA-I latex suspension, gently mixed for 15 min at room temperature and then centrifuged at 12,000 rpm for 10 min. The infranate, which contained non latex-bound plasma, devoid of apoA-I, was concentrated using Centricon-10 concentrators (Amicon, MA), before being separated by electrophoresis.

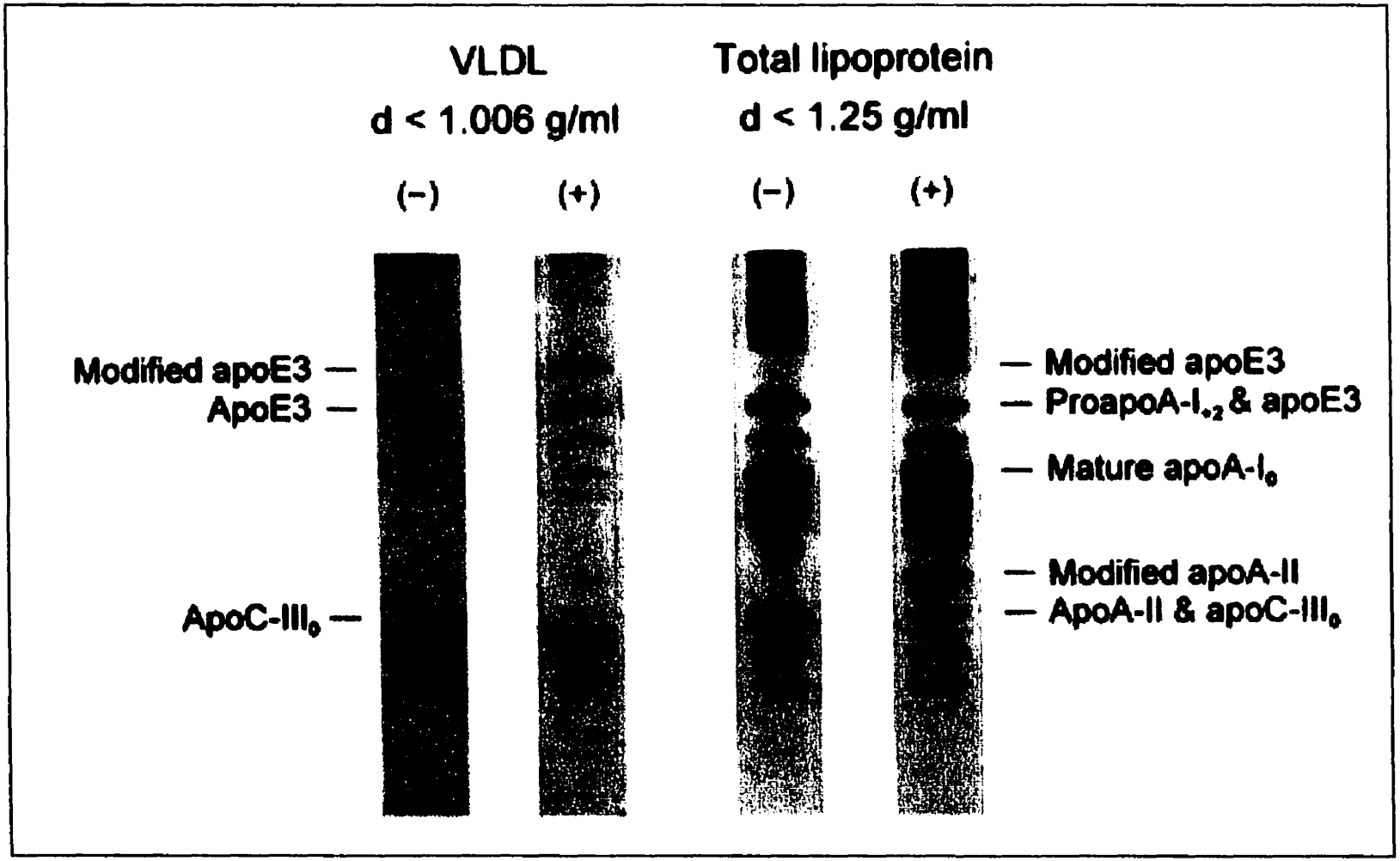

FIG 1. Separation by isoelectric focusing (IEF) gel electrophoresis of apolipoproteins in cysteamine-treated (+) and non-treated (-) VLDL (d < 1.006 g/ml) and total plasma lipoprotein (d < 1.25 g/ml) fractions. Chemical modification by cysteamine treatment (ref. 34) caused amino groups to be added to cysteine residues in apoE3 and apoA-II, resulting in a single positive charge increase, and migration of these proteins to a higher position in IEF gels. Cysteamine-treatment of apoE3 and apoA-II in d < 1.25 g/ml fractions, allowed these proteins to be separated from proapoA-I and apoC-III, as indicated in the right-hand gels. The major isoform of mature apoA-I (apoA-I$_0$), which was excised for stable-isotope analysis, is also indicated.

Plasma Lipids and Apolipoproteins

Plasma and lipoprotein fractions were assayed for total (free and esterified) cholesterol and triglyceride with a COBAS MIRA-S automated analyzer (Hoffman-LaRoche) using enzymatic reagents. Plasma apoB concentration was measured by non-competitive ELISA using an immuno-purified goat anti-human apoB antibody and horse radish peroxidase-conjugated monoclonal antibody (37). Plasma apoA-I concentration was measured by nephelometry on a Behring Nephelometer 100 (Behring) using Behring protocol and reagents. Plasma apoA-II was measured by nephelometry in the laboratory of Dr. Linda Bausserman (Meriam Hospital, Brown University, RI, USA) (38). Plasma apoE and apoC-III concentrations were measured by ELISAs developed in our laboratory (39,40). ApoE phenotype was determined by isoelectric focusing of delipidated VLDL (34).

Quantitation of ProapoA-I

Mature apoA-I and proapoA-I concentrations in plasma of normolipidemic and FHD subjects were derived from total plasma apoA-I concentrations measured by nephelometry. The proportion of each apoA-I isoform contributing to total plasma apoA-I was determined by gas chromatography-mass spectroscopy (GC-MS) and densitometric scanning of IEF gels. The amount of leucine associated with the major mature apoA-I IEF band (apoA-I_0) and the major proapoA-I band (isoform apoA-I_{+2}) (41) was determined by comparing the areas under the peaks of leucine and norleucine (an internal standard - see

following section), separated by GC-MS. The amount of apoA-I protein present in each band was then calculated as:

$$\text{ApoA-I protein (μg)} = \frac{\text{leucine (μg) X mol. wt. of apoA-I isoform (daltons)}}{\text{total wt. of leucine residues in apoA-I isoform (daltons)}}$$

Amount of protein in minor mature apoA-I bands (isoforms apoA-I_{-1} and apoA-I_{-2}) and the minor proapoA-I band (isoform apoA-I_{+1}) (41) was then estimated by measuring the relative amounts of these isoforms by IEF gel scanning densitometry. Plasma concentration of proapoA-I was then calculated as:

$$\text{proapoA-I (mg/dl)} = \text{apoA-I (mg/dl) X } \frac{\text{apoA-}I_{+2} + \text{apoA-}I_{+1}\ (\mu g)}{\text{apoA-}I_{+2} + \text{apoA-}I_{+1} + \text{apoA-}I_{0} + \text{apoA-}I_{-1} + \text{apoA-}I_{-2}\ (\mu g)}$$

Determination of Isotopic Enrichment in Isolated Apolipoproteins

Apolipoprotein bands, as well as blank (non-protein containing) gel slices were excised from polyacrylamide gels (VLDL B-100 from SDS-PAGE and apoA-I and apoA-II from IEF) (33). Each slice was added to a borosilicate sample vial containing 600 μl of 6N HCL, and an internal standard of 250 ng norleucine (Sigma-Aldrich) dissolved in 50 μl double distilled water. Gel slices were hydrolyzed at 110°C for 24 h, cooled to -20°C for 20 min, and centrifuged at 3,500 rpm for 5 min. Free amino acids in the hydrolysate were separated from precipitated polyacrylamide, purified by cation exchange chromatography using AG 50 W-X8 resin (BioRad), and derivatized by treatment with 200 μl of acetyl chloride-acidified 1-propanol (1:5 V/V) for 1 h at 100°C, and 50 μl of

heptaflurobutyric anhydride (Supelco) for 20 min at 60°C (33). Enrichment with deuterated leucine was determined by GC-MS (Hewlett-Packard, 5988 GC-MS) using negative chemical ionization and methane as the reagent gas. Selective ion monitoring at m/z =352 and 349 (ionic species corresponding to derivatized deuterated and derivatized non-deuterated leucine, respectively) was performed, and tracer to tracee ratio was calculated from the isotopic ratio in each sample according to the formula derived by Cobelli et al. (42):

$$z(t) = \frac{r(t) - rN}{rI - r(t)} \times \frac{1 + rI}{1 + rN}$$

where, z(t) is tracer to tracee ratio at time t, r(t) is isotopic ratio at time t, and rN and rI are naturally-occuring and infusate isotopic ratios respectively. r(t) was obtained from the ratio of areas under the peak (rAUP) of leucine ionic species m/z = 352 and 349. rAUP of the ionic species m/z = 349 of leucine and the internal standard (norleucine) detected in blank samples were used to correct for background (method-introduced) leucine, which was 0.44 ± 0.27, 6.04 ± 2.06, and 4.12 ± 2.96% (mean ± SD) of total leucine in mature apoA-I, proapoA-I and apoA-II samples, respectively.

Kinetic Analysis

Tracer to tracee ratios of VLDL apoB-100, plasma mature apoA-I, proapoA-I, and apoA-II were fitted to a monoexponential function using SAAM II computer software (SAAM II institute, WA). The function was defined as: $z(t) = Zp(1 - e^{(-k(t-d))})$, where z(t) was tracer to tracee ratio at time t, Zp was the tracer

to tracee ratio of the tissue precursor amino acid pool from which the protein in question was derived (estimated from VLDL apoB-100 and proapoA-I enrichment curves at plateau, see below), d was the delay time (h), and k was the fractional production rate (FPR) (pools/hour). Residence time (RT) was calculated as the reciprocal of FPR (1/FPR), and absolute production rate (PR) in mg/kg.day was calculated as:

$$PR = \frac{\text{FPR (pools/day) X apolipoprotein pool size (mg)}}{\text{body weight (kg)}}$$

where:Pool size = plasma concentration (mg/dl) X plasma volume (0.045 liter/kg) The tracer to tracee ratio (Zp) of the intestinal and hepatic precursor amino acid pools from which proapoA-I (and hence apoA-I) were derived, was taken to be the enrichment of proapoA-I at plateau. Since apoA-II is predominantly, like apoB-100, of hepatic origin, the enrichment of the apoA-II precursor pool was taken to be the enrichment of VLDL apoB-100 at plateau.

Results

Plasma Lipids and Apolipoproteins

Plasma concentration of lipids and apolipoproteins in subjects on the day of the infusion are shown in Table 1. The two brothers with FHD had plasma HDL-cholesterol concentrations of 0.19 and 0.27 mmol/l, which were on average 18% and 26% of HDL-cholesterol concentrations in control subjects. Their plasma concentrations of apoA-I and apoA-II were 14% and 29% (for apoA-I) , and 46 % and 44% (for apoA-II) of controls, respectively. Total plasma triglyceride, VLDL-triglyceride and apoB concentrations were slightly higher in FHD patients compared to controls, while plasma apoE levels were slightly lower. Total plasma cholesterol and apoC-III levels were within a normal range.

The separation by IEF gel electrophoresis of proapoA-I isoforms, mature apoA-I isoforms, and apoA-II from total plasma lipoproteins (d<1.25 g/ml) of 2 control subjects and 2 FHD patients is shown in Fig 2. ApoA-I_{+2} and apoA-I_{+1} correspond to proapoA-I, while apoA-I_0, apoA-I_{-1}, and apoA-I_{-2} correspond to mature apoA-I (41). The increased positive charge on the two proapoA-I isoforms is due to the additional six amino acid amino terminal tail of proapoA-I. The minor mature apoA-I isoform (apoA-I_{-2}) is a deamidation product of apoA-I_0 and apoA-I_{-1} (41). The same amount of protein (600 µg) was loaded onto each gel. In relative terms, samples from FHD patients contained considerably less mature apoA-I, but similar amounts of proapoA-I (apoA-I_{+2} isoform). As is evident from Fig. 2, the minor proapoA-I isoform (apoA-I_{+1} isoform) was somewhat reduced in FHD patients compared to controls 1 and 2, however

Table 1. Characteristics of FHD Patients and Control Subjects

Subjects	Age yr	BMI kg/m²	apoE phenotype	TC mmol/l	HDL-C mmol/l	TG mmol/l	VLDL-TG mmol/l	apoA-I† mg/dl	apoA-II mg/dl	apoB mg/dl	apoE mg/dl	apoC-III mg/dl
FHD												
1	52	23.0	3/3	4.14	0.19	2.54	1.08	17	12.8	177	2.9	12.8
2	42	26.3	3/2	3.54	0.27	2.10	1.12	37	12.2	134	3.7	13.2
Control												
1	36	24.5	3/3	4.78	1.26	0.67	0.22	149	25.5	114	4.1	9.7
2	26	24.4	3/2	3.18	0.94	0.72	0.36	122	26.2	65	5.3	7.2
3	35	26.1	3/3	4.75	0.94	1.56	1.02	105	28.4	98	4.7	9.8
4	41	24.1	3/3	5.79	1.03	2.22	1.30	129	30.7	142	6.1	19.8
Mean ± SD‡	35 ± 6	24.8 ± 0.9		4.63 ± 1.08	1.04 ± 0.15	1.29 ± 0.74	0.73 ± 0.52	126 ± 18	27.7 ± 2.4	105 ± 32	5.1 ± 0.9	11.6 ± 5.6

Plasma concentrations for individual subjects represent the average of 5 measurements made at 3-hourly intervals during the stable isotope infusion.
†Includes all apo A-I isoforms.
‡Mean data for control subjects ± standard deviation.
Abbreviations: BMI, body mass index; TC, total cholesterol; HDL-C, HDL-cholesterol; TG, triglyceride; VLDL-TG, VLDL-triglyceride; apo, apolipoprotein.

quantitation of total plasma mature apoA-I and total proapoA-I revealed that decrease in plasma apoA-I concentration in patients was due to a decrease in mature apoA-I and not proapoA-I concentration (Table 2). The relative contribution of proapoA-I to total plasma apoA-I was thus higher in FHD patients (18.2 and 9.5% for patients 1 and 2 respectively) compared to control subjects (2.9 ± 0.3%, mean ± SD for 4 subjects).

Analysis of ApoA-I- and ApoA-II-Containing HDL

Plasma lipoproteins of control and FHD patients were separated according to their charge and size by two-dimensional gradient gel electrophoresis. ApoA-I-containing HDL subfractions for two control and two FHD patients are shown in Fig 3. Considerably less apoA-I was detected in plasma samples from FHD patients, due to a marked reduction in apoA-I associated with • -migrating HDL (i.e., • -LpA-I). The amount of apoA-I associated with pre-• -migrating HDL (pre-• $_1$- and pre• • $_2$-LpA-I) was however normal. The average particle size of • -LpA-I in FHD patients tended to be smaller than that of control subjects.

Electrophoretically-separated HDL of plasma from FHD patients was also found to contain less apoA-II compared to controls (Fig 4), which corresponded to a two-fold reduction in total plasma apoA-II concentration (measured by nephelometry) (Table 2). Larger-sized • -migrating HDL containing apoA-II were virtually absent from the plasma of FHD patients, which meant that the average particle size of • -LpA-II in FHD patients was smaller than that of controls. As

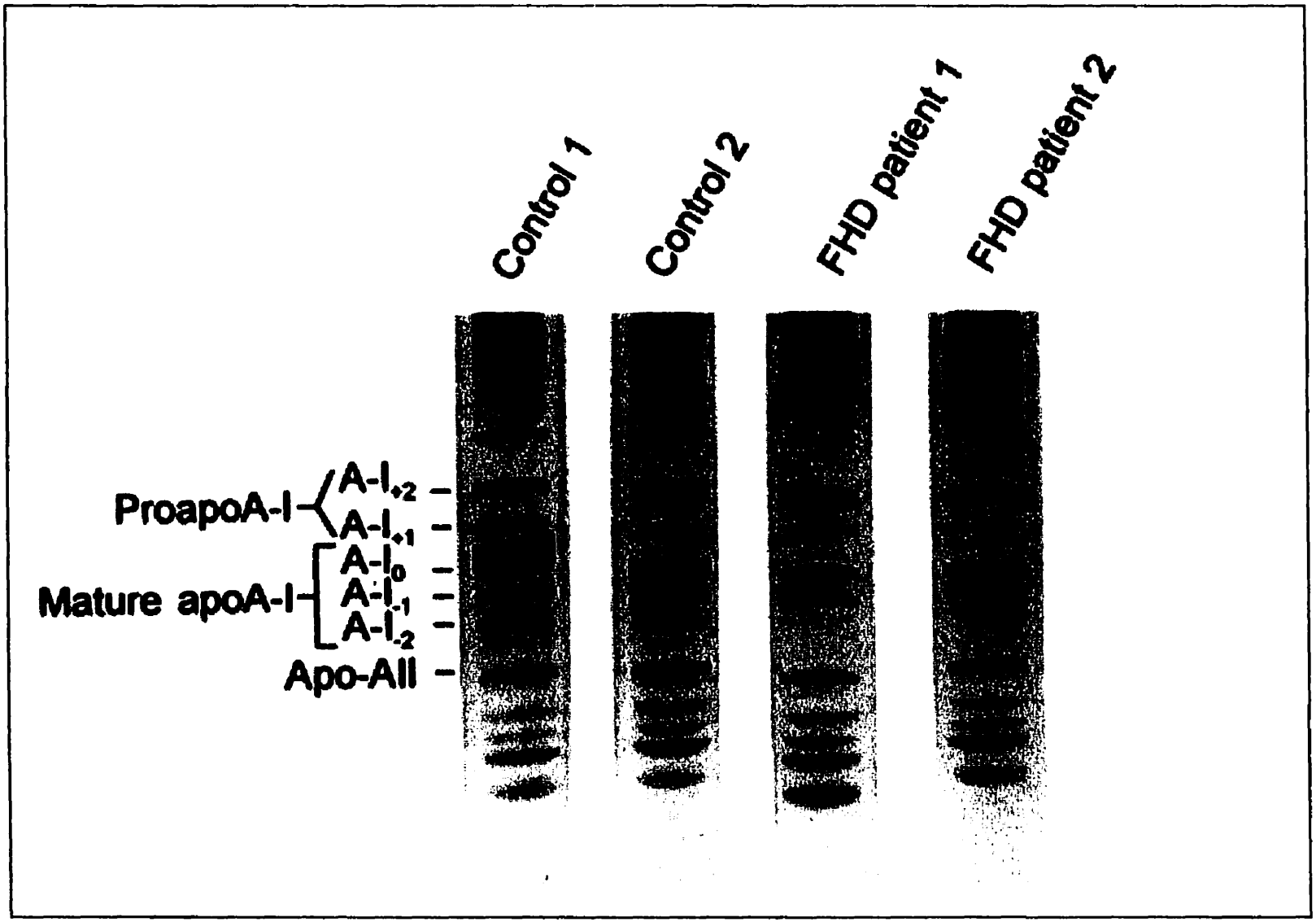

FIG 2. Comparison of the relative amounts of proapoA-I, mature apoA-I and apoA-II in total plasma lipoproteins (d < 1.25 g/ml) of control and FHD subjects, separated by IEF gel electrophoresis. Gels are shown for two control and two FHD patients, corresponding to control subjects 1 and 2, and FHD patients 1 and 2 in Table 1. Different isoforms of proapoA-I and mature apoA-I are indicated. Similar amounts of total protein (600 μg) were separated on each gel.

shown in gels C and D in Fig 4, these apoA-II-containing HDL were unique in that they did not contain apoA-I, unlike the majority of apoA-II-HDL in control subjects, which were removed by apoA-I affinity chromatography; efficiency of removal of apoA-I-containing lipoproteins was greater than 97% for patients and controls, as assessed by essentially complete absence of apoA-I in electrophoretically-separated samples after affinity chromatography. This is consistent with the observation that all HDL apoA-II is (under normal circumstances) bound to apoA-I in LpA-I:A-II particles in normolipidemic subjects (12).

Stable Isotope Enrichment of Plasma ApoA-I

Newly synthesized mature apoA-I, enriched with deuterated leucine, was detected in the plasma of all subjects within 2 h of the start of the stable-isotope infusion experiment (Fig 5). The rate of appearance of deuterium-labeled mature apoA-I - measured as a percentage tracer (deuterated leucine) to tracee (non-deuterated leucine) ratio - was linear for the 12 h duration of the study in all 6 subjects. Fractional rate of appearance of labeled mature apoA-I (the slope of the enrichment curves) was 7-fold and 3-fold higher in the two FHD patients, compared to controls, corresponding to significantly increased fractional production rates (measured in pools per day) (Table 2). Since mature apoA-I pool sizes were proportionately decreased, absolute apoA-I production rates were similar in patients and controls (Table 2), ranging between 7.9 and 12.9 mg/kg.day. Residence time (RT) of mature apoA-I in plasma was however

Table 2. Kinetic Parameters for Mature ApoA-I, ProapoA-I, and ApoA-II

	Mature apoA-I				ProapoA-I				ApoA-II			
Subjects	Plasma conc.* mg/dl	FPR pools/day	RT days	PR mg/kg.day	Plasma conc. † mg/dl	FPR pools/day	RT days	PR mg/kg.day	Plasma conc. mg/dl	FPR pools/day	RT days	PR mg/kg.day
FHD												
1	13.9	1.266	0.79	7.9	3.1	5.6	0.18	7.8	12.8	0.316	3.17	1.8
2	33.5	0.602	1.66	9.1	3.5	6.6	0.15	10.4	12.2	0.343	2.92	1.9
Control												
1	144.4	0.151	6.64	9.8	4.6	5.6	0.18	11.6	25.5	0.132	7.58	1.5
2	118.3	0.243	4.11	12.9	3.7	6.6	0.12	14.3	26.2	0.152	6.58	1.8
3	102.0	0.197	5.07	9.0	3.0	6.6	0.15	8.9	28.4	0.149	6.71	1.9
4	125.7	0.183	5.46	10.4	3.3	6.0	0.17	8.9	30.7	0.124	8.07	1.7
Mean ± SD‡	122.6 ± 17.6	0.194 ± 0.04	5.32 ± 1.05	10.5 ± 1.7	3.7 ± 0.7	6.7 ± 1.3	0.16 ± 0.03	10.9 ± 2.6	27.7 ± 2.4	0.139 ± 0.014	7.24 ± 0.71	1.7 ± 0.2

*Includes isoforms apoA-I_0, apoA-I_{-1},and apoA-I_{-2}.
†Includes isoforms apoA-I_{+1} and apoA-I_{+2}.
‡Mean data for control subjects ± standard deviation.
Abbreviations: apo, apolipoprotein. FPR, fractional production rate. RT, residence time. PR, absolute production rate.

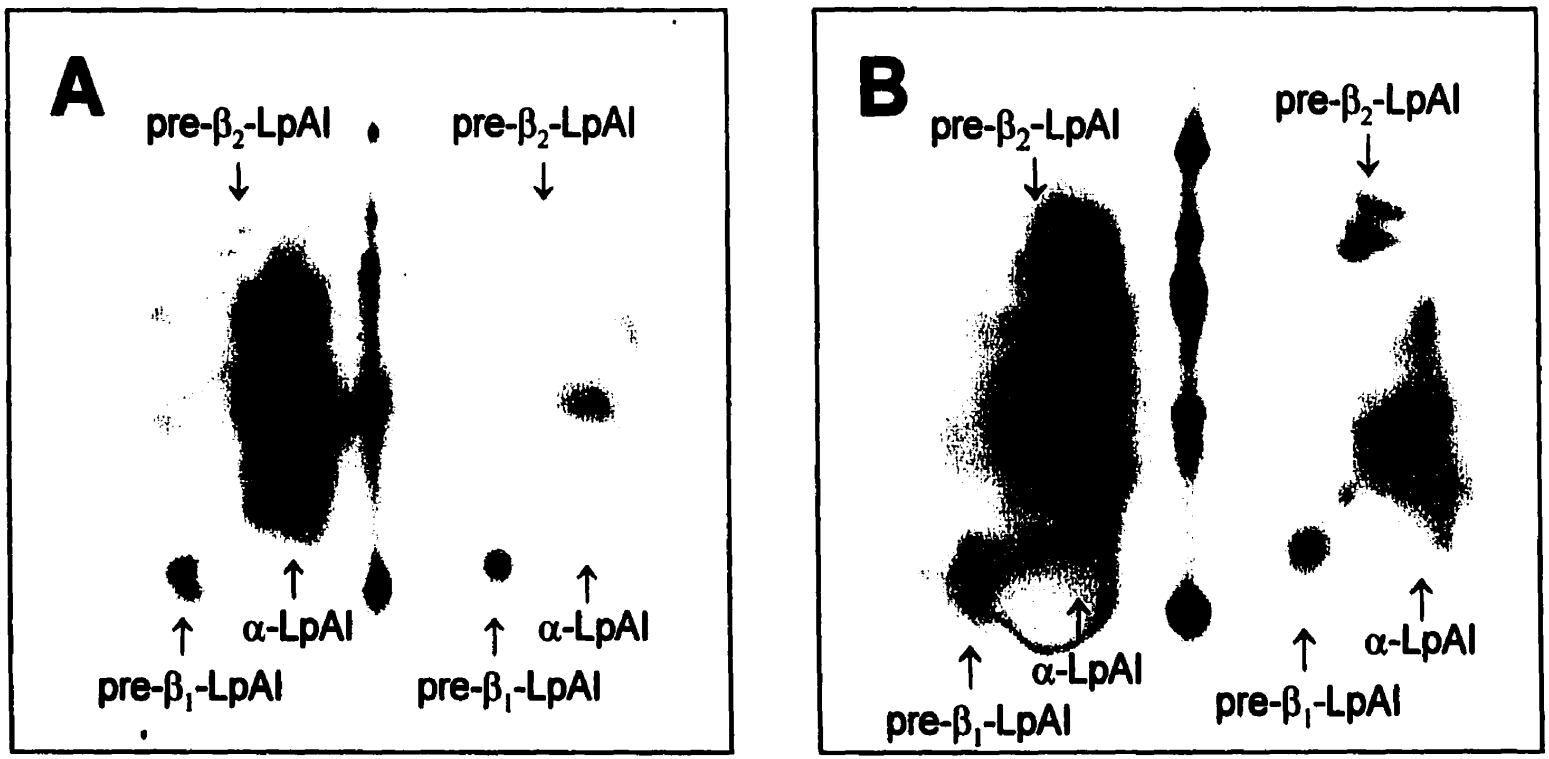

FIG 3. Two-dimensional gel electrophoretic separation of apoA-I-containing lipoproteins from the plasma of control and FHD subjects. Gels (A and B) are shown for two control and two FHD patients, corresponding to control subjects 1 and 2, and FHD patients 1 and 2 in Table 1. Plasma (200µl) was separated in the first dimension (left to right) by agarose gel electrophoresis, and in the second dimension (top to bottom) by 3-24% polyacrylamide gradient gel electrophoresis. Lipoproteins containing apoA-I were detected with ^{125}I-labeled polyclonal anti-human apoA-I antibody, after electrotransfer onto nitrocellulose membranes. Different apoA-I-containing HDL subpopulations are indicated with vertical arrows. Molecular size markers were separated between plasma samples (in the center of each gel): thyroglobulin (17nm), ferritin (12.2 nm), catalase (9.5 nm), lactate dehydrogenase (8.2 nm), and albumin (7.1 nm), from top to bottom.

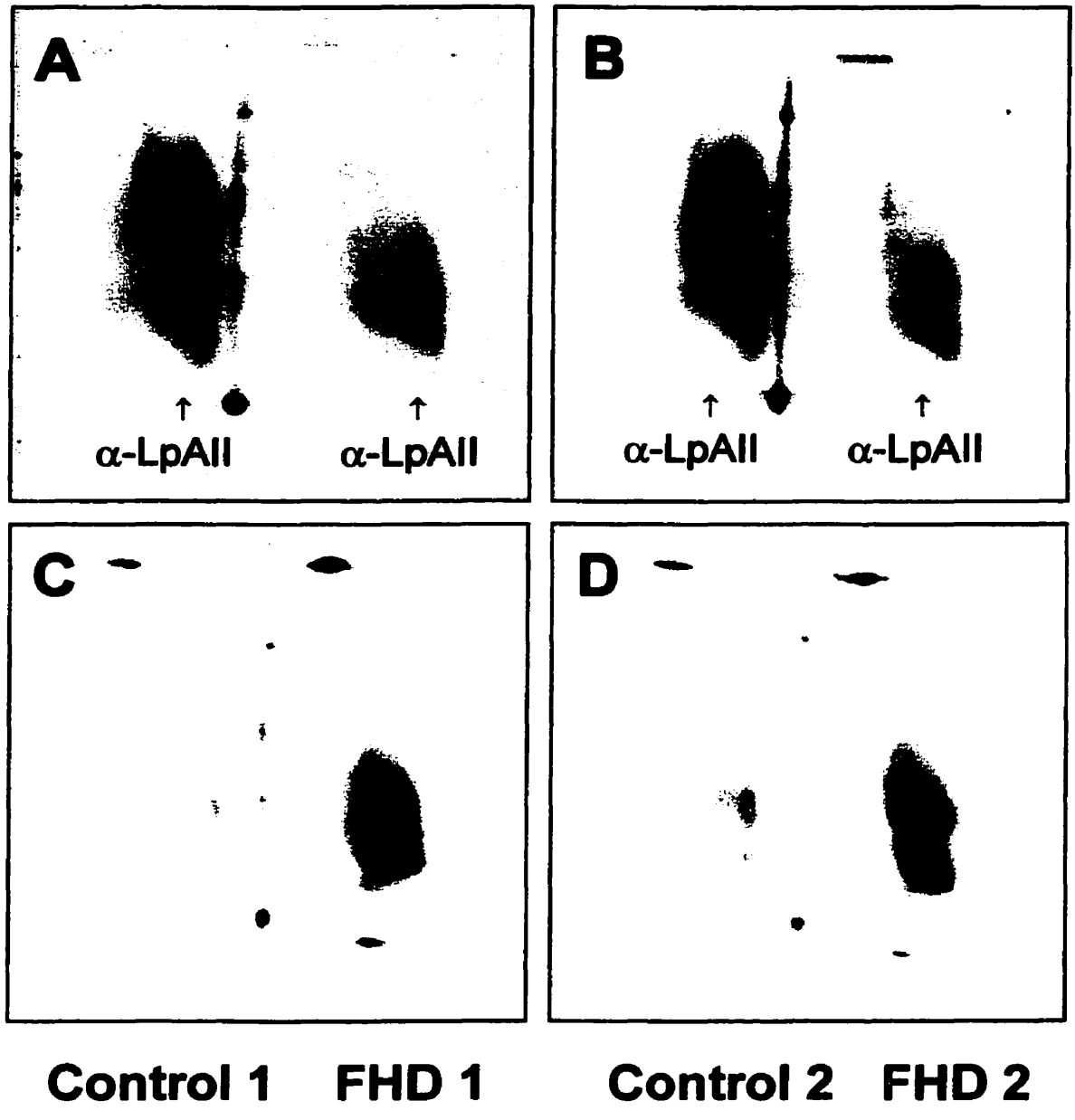

FIG 4. Separation of apoA-II-containing lipoproteins, in the absence and presence of apoA-I, from the plasma of control and FHD subjects. Gels (A and B) show the presence of apoA-II-containing lipoproteins in the plasma (200 µl) of two control and two FHD patients, corresponding to control subjects 1 and 2, and FHD patients 1 and 2 in Table 1. Lipoproteins containing apoA-II were detected with ^{125}I-labeled polyclonal anti-human apoA-II antibody, after electrotransfer of lipoproteins separated by two-dimensional gel electrophoresis. Gels (C and D) show the presence of apoA-II-containing lipoproteins in the plasma from the same subjects after apoA-I-containing lipoproteins were removed by affinity chromatography (see Methods). ApoA-II-containing HDL in control plasmas were almost completely removed by this procedure, in contrast to plasma from FHD patients, where a large proportion of apoA-II was not bound to apoA-I. Molecular size markers were separated between plasma samples (in the center of each gel): thyroglobulin (17nm), ferritin (12.2 nm), catalase (9.5 nm), lactate dehydrogenase (8.2 nm), and albumin (7.1 nm), from top to bottom.

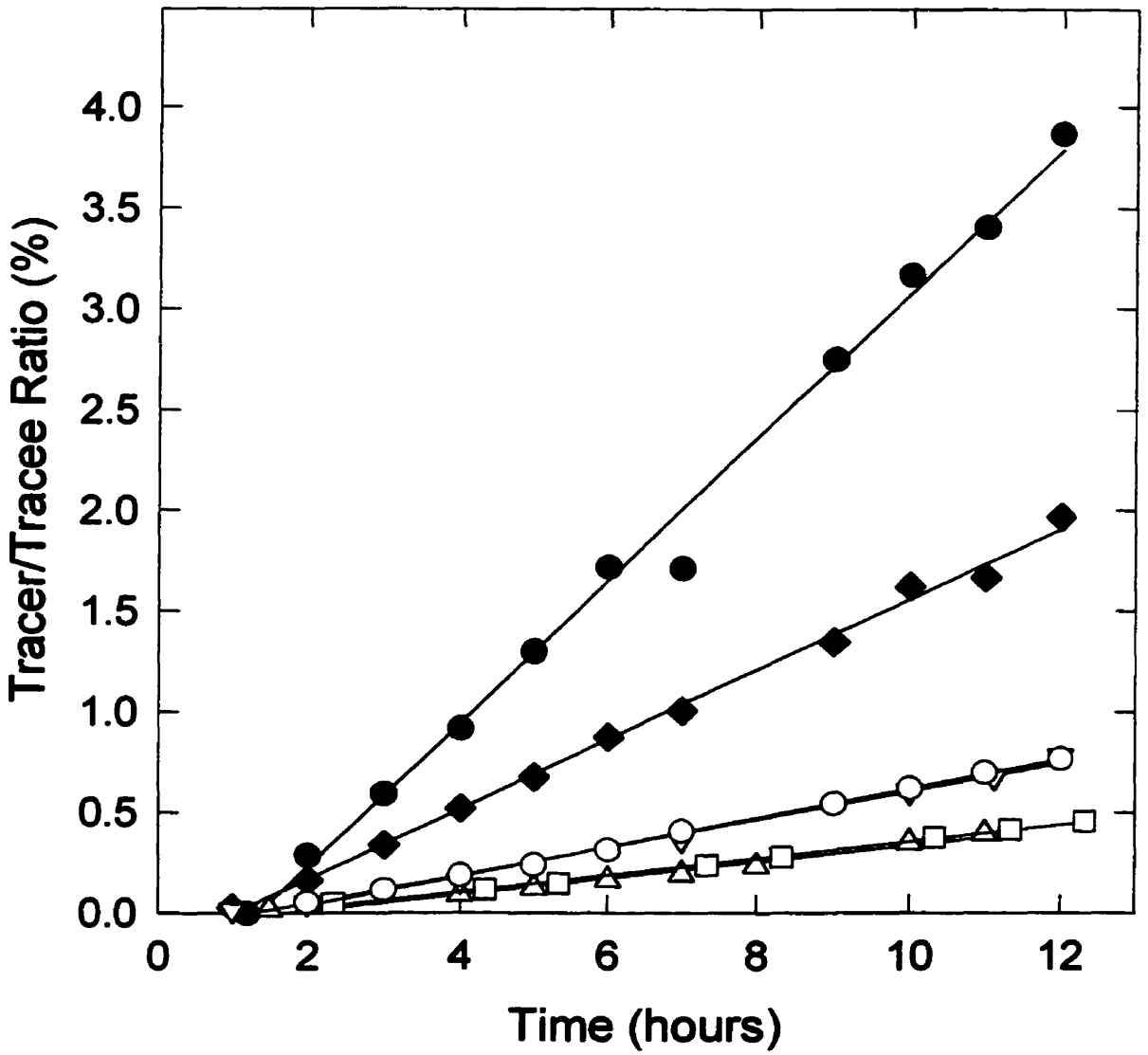

FIG 5. Rate of appearance of newly-synthesized mature apoA-I, enriched with deuterated leucine, in the plasma of 2 FHD patients: ● - ●, and ◆ - ◆ (patients 1 and 2, respectively, Table 1) and 4 control subjects: Δ - Δ, □ - □, ∇ - ∇ and O - O (subjects 1 to 4, respectively, Table 1). The ratio of deuterated to non-deuterated leucine in mature apoA-I (apoA-I_O) isolated by IEF gel electrophoresis from d < 1.25 g/ml lipoproteins obtained at regular intervals, was determined by gas chromatography/ mass spectrometry and expressed as a tracer to tracee ratio (see Methods).

significantly shorter in patients (0.79 and 1.66 days) compared to controls (5.32 ± 1.05 days), demonstrating that mature apoA-I catabolism was significantly increased in FHD patients. Thus, in the case of FHD patient 1, each molecule of mature apoA-I remained in plasma for a time period which was 6.7 times less than that of control subjects, corresponding to a plasma apoA-I concentration which was 8.8 times lower than controls. In the case of FHD patient 2, each molecule of mature apoA-I remained in plasma 3.2 times less than that of control subjects, corresponding to a plasma apoA-I concentration which was 3.7 times lower than controls.

The time-course of enrichment of plasma proapoA-I for the four control subjects (A) and 2 FHD patients (B) is shown in Fig 6. The tracer to tracee ratio of plasma proapoA-I increased monoexponentially and reached a plateau level during the time course of the infusion. Mean level of proapoA-I enrichment at plateau for the control subjects was 6.7 ± 2.4 %, compared to 8.2 ± 2.2 % for VLDL apoB-100. Since the proapoA-I plateau varied somewhat from one individual to another (being a function of the amount of deuterated leucine reaching the proapoA-I precursor amino acid pool, and the level of unlabeled leucine in this pool), curves in Fig 6 have been normalized to allow for data from individual subjects to be visually compared (this was unnecessary for slower-turning over mature apoA-I). ProapoA-I ratios have thus been expressed as a percentage of the tracer to tracee ratio of proapoA-I at plateau in each individual. As shown in Fig 6B, the time-course of enrichment of proapoA-I for the two FHD patients was not different from that of the control subjects; mean data (± SD) for

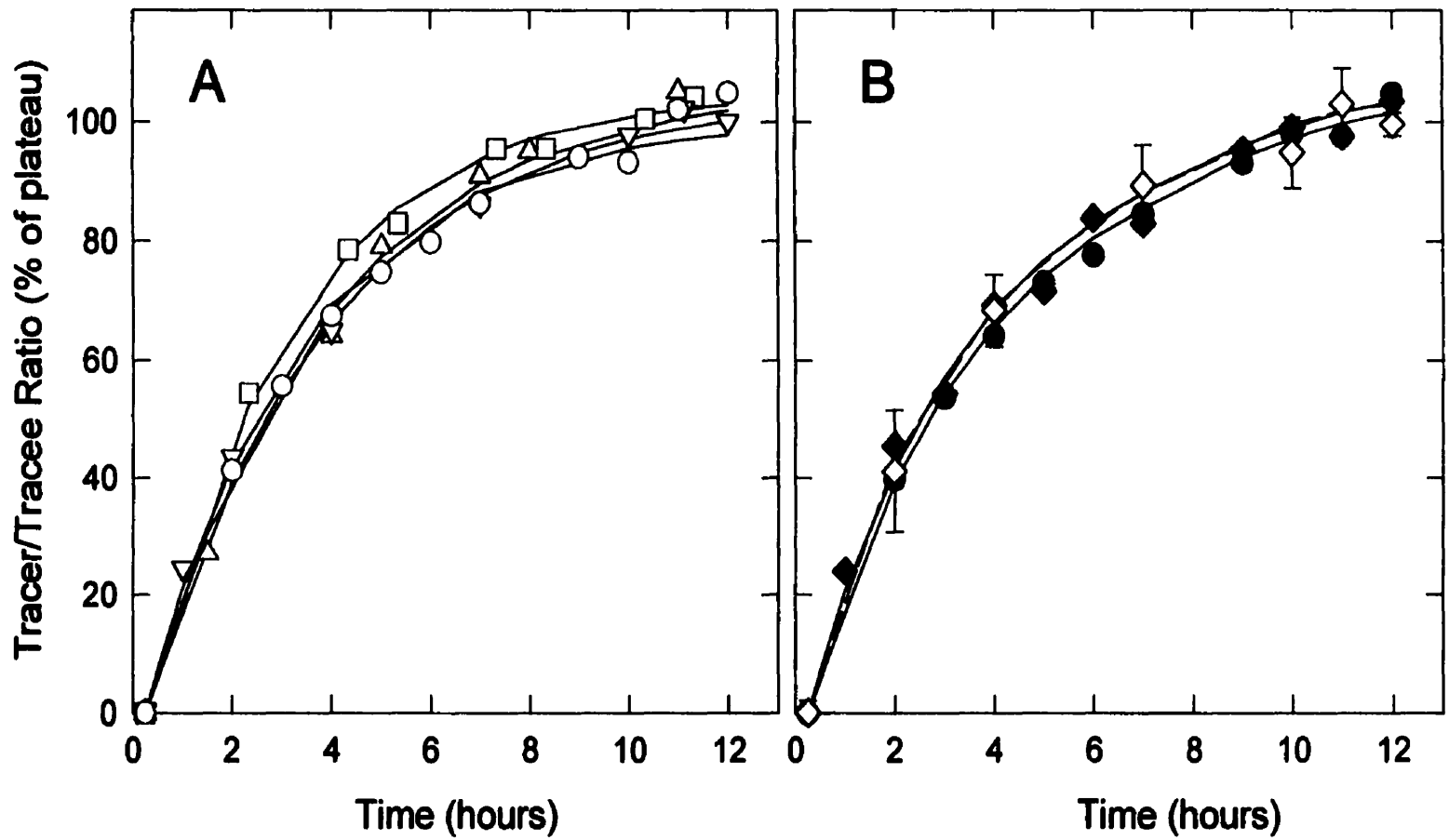

FIG 6. Rate of appearance in plasma of newly synthesized proapoA-I enriched with deuterated leucine. Results for 4 control subjects are shown in A: Δ - Δ, □ - □, ∇ - ∇ and O - O (subjects 1 to 4, respectively, Table 1). Data for 2 FHD patients are shown in B: ● - ●, and ◆ - ◆ (patients 1 and 2, respectively, Table 1), together with mean data for the control subjects ◊ - ◊ (error bars are standard deviations). Tracer/tracee ratios were expressed as percentages of those at plateau to allow for direct comparison between subjects.

the 4 controls are shown with open diamond symbols. Kinetic analyses of these curves demonstrated that neither the residence time nor the production rate of proapoA-I was different in FHD patients compared to controls (Table 2).

Stable-Isotope Enrichment of Plasma ApoA-II

The time-course of plasma apoA-II enrichment with deuterated leucine for the four control subjects and two FHD patients is shown in Fig 7. As was the case for mature apoA-I, the tracer to tracee ratio of apoA-II increased linearly and the fractional rate of appearance of newly synthesized apoA-II was about two-fold higher in patients compared to controls. Since plasma apoA-II concentration (and hence apoA-II pool size) was approximately two times less, apoA-II production rates in FHD patients (1.8 and 1.9 mg/kg.day) were not significantly different from that of controls (1.7 ± 0.2 mg/kg.day). ApoA-II residence times on the other hand (3.17 and 2.92 days) were significantly less compared to controls (7.24 ± 0.71 days), demonstrating that the fractional rate of plasma apoA-II catabolism was significantly increased in FHD patients.

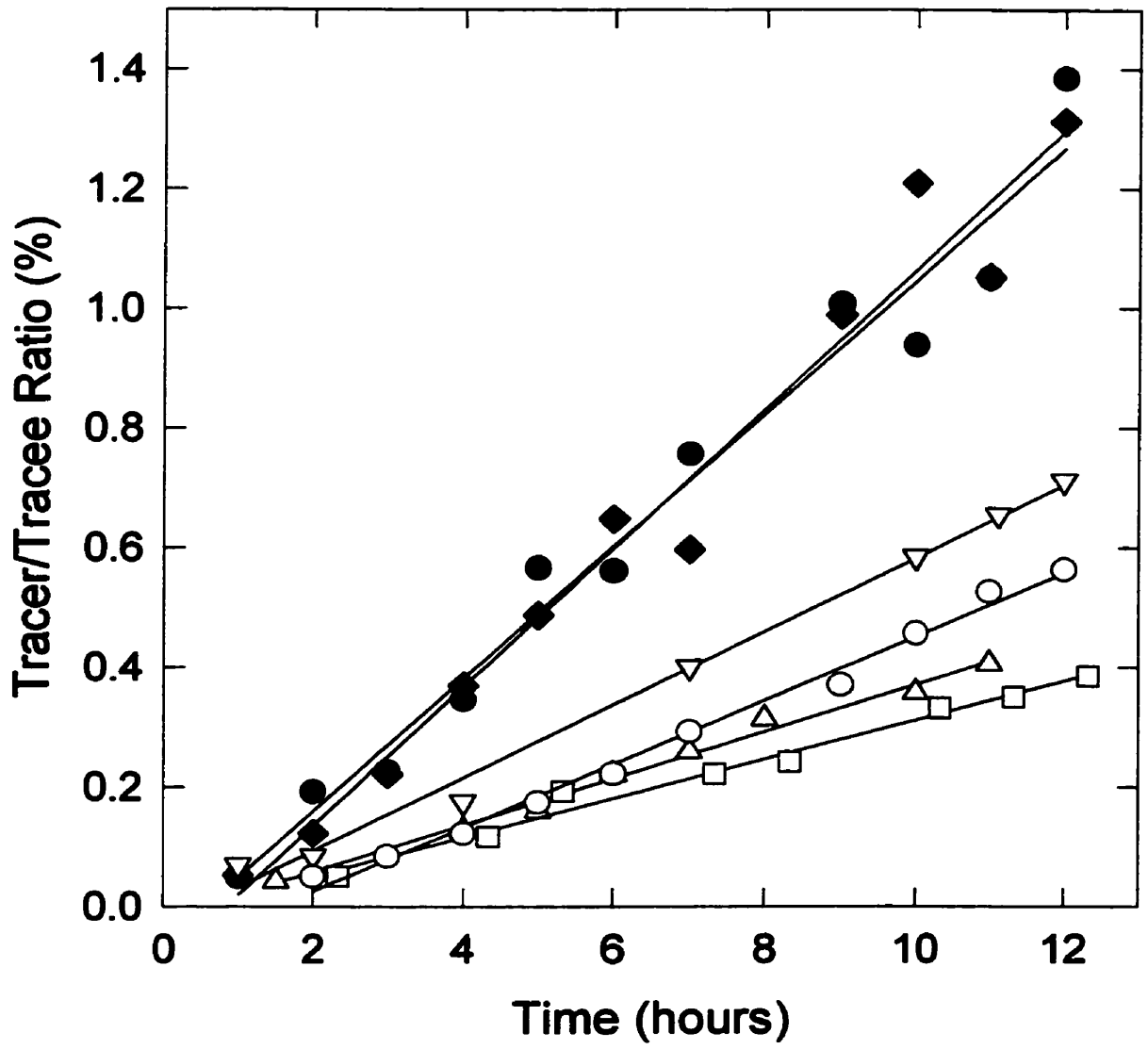

FIG 7. Rate of appearance in plasma of newly synthesized apoA-II enriched with deuterated leucine. Results are shown for 2 FHD patients: ● - ●, and ◆ - ◆ (patients 1 and 2, respectively, Table 1) and 4 control subjects: Δ - Δ, □ - □, ∇ - ∇ and O - O (subjects 1 to 4, respectively, Table 1).

Discussion

Our results have demonstrated that FHD patients in the present study (with no clinical symptoms of Tangier disease, nor evidence of other known causes of HDL deficiency) had significantly reduced levels of plasma apoA-I and apoA-II, which were not caused by reduced apoA-I or apoA-II production. Reduced HDL levels were instead associated with increased plasma catabolism of mature apoA-I and to a lesser extent apoA-II, but not increased catabolism of proapoA-I. We have therefore concluded that hypercatabolism of mature plasma apoA-I, but not proapoA-I, was responsible for HDL deficiency in these patients.

Two previous studies have investigated the plasma kinetics of HDL apolipoproteins in patients with severe genetic HDL deficiency of unknown origin. Emmerich et al. (31) described a 46-year-old man with coronary artery disease, who had severely reduced levels of plasma HDL-cholesterol (5.0 mg/dl) and total plasma apoA-I (4.5 mg/dl). His brother and two children had reduced HDL levels, suggesting codominant inheritance of the abnormality. His apoA-I was structurally normal, he had no clinical features of Tangier disease, and he was found to have marked hypercatabolism of apoA-I. Rader et al. (29) similarly identified two male and three female probands with very low HDL levels (of apparently familial origin), who had no evidence of premature coronary heart disease. They had no clinical or biochemical characteristics typical of known HDL deficiency states, and all five individuals had increased catabolism of HDL apoA-I and apoA-II. These reported cases of severe HDL deficiency of unknown etiology resemble those described in the present study, in that an increase in

plasma apoA-I catabolism, rather than a reduction in apoA-I production, was responsible for reduced HDL levels. This is analogous to the situation in patients with less severe reductions in HDL, in whom the fractional catabolic rate of plasma apoA-I has consistently been shown to be the primary metabolic predictor of intersubject variability in plasma apoA-I and HDL-cholesterol concentrations (13,14,16). It has been hypothesized that increased fractional catabolism of apoA-I in these individuals is caused by triglyceride enrichment and cholesterol depletion of HDL particles, the formation of smaller HDL, increased interaction of HDL with lipoprotein and hepatic lipases, greater dissociation of apoA-I from HDL, and subsequent clearance of "free" apoA-I by the kidney (9). Increased renal clearance of apoA-I in a lipid-free or greatly lipid-depleted form may also be responsible for apoA-I hypercatabolism in our more severely affected FHD patients. This may be the result of impaired HDL_3- and apoA-I-mediated cellular cholesterol and phospholipid efflux, which we have found to be a characteristic of fibroblasts from FHD patients (data not shown), similar to that of fibroblasts from Tangier patients (20,21). Decrease in availability of tissue-derived cholesterol in precursor HDL particles could reduce cholesteryl ester formation in HDL (catalyzed by LCAT), resulting in impaired maturation of these particles into larger cholesteryl ester-rich HDL and formation of relatively small lipid-depleted apoA-I particles, which are rapidly cleared. Support for this concept is provided by our two-dimensional electrophoretic analysis of apoA-I- and apoA-II-containing HDL-sized lipoproteins (Fig 3 and 4), showing that larger alpha-migrating HDL, particularly those containing apoA-II,

were greatly reduced in FHD patients, whereas small pre-• $_1$-LpA-I, representing lipid-poor (43) or lipid-free apoA-I particles (22), were present in similar amounts compared to controls, and at significantly elevated levels relative to other apoA-I subfractions in FHD plasma.

A distinguishing feature of the present investigation is the measurement (for the first time) of the plasma kinetics of proapoA-I with an endogenous, stable-isotope labeling technique, and the finding that plasma proapoA-I production rate and residence time were essentially normal in FHD patients compared to controls (Table 2). The *in vivo* kinetics of plasma proapoA-I have previously been studied in two normolipidemic and two Tangier disease patients by Bojanovski et al. (44, 45), using purified and radioactively labeled proapoA-I. The two normolipidemic subjects with plasma proapoA-I concentrations of 5.5 and 6.0 mg/dl, had proapoA-I residence times of 0.19 and 0.27 days, and proapoA-I production rates of 11.6 and 8.8 mg/kg.day, respectively (45). These values are similar to those obtained in the present study for control subjects (RT: 0.16 ± 0.03 days, PR: 10.9 ± 2.6 mg/kg.day, Table 2), providing evidence that exogenous and endogenous tracer techniques give comparable results.

In view of this similarity, we have made a direct comparison between kinetic parameters obtained previously for Tangier patients (45), and those obtained for our control and FHD patients (Fig 8) (average results for two subjects are shown in the case of FHD and Tangier patients). As depicted by the relative size of shaded circles in Fig. 8, FHD patients had mean plasma mature apoA-I concentrations of 24 mg/dl, compared to 1.0 mg/dl in Tangier

patients and 123 mg/dl in control subjects. Plasma pools of mature apoA-I were thus 5 times smaller on average in FHD patients compared to control subjects, and were more than one hundred times smaller in Tangier patients (reflecting the greater severity of HDL deficiency in Tangier patients). In relative terms, plasma concentrations of proapoA-I were increased in FHD and Tangier patients (i.e., proapoA-I represented 14% of total apoA-I in FHD patients and 60% in Tangier patients, compared to 3% in controls). In absolute terms, however, average plasma concentrations of proapoA-I were 3.3, 1.5 and 3.7 mg/dl, respectively, and proapoA-I pool sizes were thus reduced in Tangier patients, though not in patients with FHD (Fig 8). It is significant that these relatively normal levels of proapoA-I in FHD patients were associated with essentially normal rates of proapoA-I production and fractional catabolism, unlike Tangier patients, who were characterized by slightly reduced rates of proapoA-I production and significantly increased rates of proapoA-I fractional clearance from plasma (9.3 pools/day vs 0.3 and 0.3 pools/day, in FHD and control subjects, respectively). Thus, as depicted by the relative widths of arrows in Fig. 8, 3-7% of proapoA-I is cleared from plasma and escapes conversion to mature apoA-I in control and FHD patients, whereas a significant proportion (~70%) of proapoA-I in Tangier patients is removed in unconverted form from plasma. Mature apoA-I is subsequently catabolized at an increased fractional rate in both FHD and Tangier patients, with hypercatabolism being 6 times greater in Tangier than FHD patients. ApoA-II fractional catabolism is also increased in FHD patients (Table 2), though not to the same extent as in Tangier patients (plasma apoA-II

RT in FHD vs Tangier: 3.0 vs 0.8 days) (25). These data taken together demonstrate that, from an HDL kinetic perspective, FHD patients are clearly dissimilar from control subjects. Furthermore, they are distinguishable from patients with Tangier disease, since: a) reduction in plasma apoA-I and hypercatabolism of plasma apoA-I are significantly more severe in Tangier patients, b) increase in fractional catabolism of plasma apoA-II is also more severe in Tangier patients, and c) plasma catabolism and concentration of proapoA-I are essentially normal in FHD, but not in Tangier patients. The reason for this latter difference in proapoA-I metabolism, and its pathophysiological significance, deserves further investigation.

As shown by the data in Fig 4, and as demonstrated previously (12), nearly all apoA-II in the plasma of normolipidemic subjects is associated with HDL containing apoA-I. We have found however that our patients with FHD had abnormal apoA-II-containing HDL, which did not contain apoA-I. It is significant that similar particles have been detected in the plasma of patients with Tangier disease (46-48). These lipoproteins, designated Lp(AII), were found to be equally as effective as Lp(AI) in promoting cholesterol efflux from cholesterol-loaded human fibroblasts (48), and therefore no evidence has been obtained suggesting that these lipoproteins are of particular atherogenic potential. Nevertheless, they represent a characteristic feature of HDL deficiency states, and rapid turnover of plasma apoA-I is perhaps responsible for abnormal conversion of LpAI:AII into LpAII, or simply prevents the normal formation of LpAI:A-II particles. Alternatively, impaired HDL_3- and apoA-I-mediated cellular

cholesterol and phospholipid efflux, shown to be a characteristic of our FHD patients (as mentioned previously), leads to the formation of abnormal apoA-II-only containing HDL. A third possibility is that absence or partial absence (as shown for Tangier patients (22)) of a plasma factor, responsible for the formation of mature HDL by converting pre• $_1$-LpA-I into • -Lp-A-I, results in the abnormal formation of LpAII. The reason for the existence of LpAII thus remains speculative, though we have assumed that these lipoproteins represent a consequence rather than a cause of HDL deficiency.

We have found in the present study that the tracer to tracee ratio of plasma proapoA-I reaches a plateau during the time course of a 12-hour infusion experiment (Fig 6). This is of methodological significance, since the tracer to tracer ratio of proapoA-I at plateau can be assumed to represent the enrichment of the intestinal and hepatic precursor amino acid pools from which proapoA-I is derived, in the same way as the plateau enrichment of VLDL apoB-100 represents the enrichment of hepatic precursor amino acid pools (33). We have found that the level of plasma proapoA-I enrichment at plateau was less than the enrichment of VLDL apoB-100, in FHD patients as well as in all four control subjects (proapoA-I plateau enrichment: 6.7 ± 2.4% vs VLDL apoB-100: 8.2 ± 2.2%), suggesting that intestinal precursor leucine pools were enriched with deuterated leucine to a lesser extent than hepatic leucine pools. This is consistent with previous data showing that VLDL apoB-48 (of intestinal origin) plateaus at a lower enrichment than VLDL apoB-100 of hepatic origin (49, 50). We therefore contend that using proapoA-I enrichment at plateau rather than

VLDL apoB-100 enrichment at plateau to calculate apoA-I kinetic parameters is more accurate, and results in an average residence time of mature apoA-I in plasma of 5.3 days for control subjects, compared to values of 3.4 days (12), and 4.5 days (13) for total apoA-I, and 6.5 days for mature apoA-I (44) obtained in exogenous apoA-I radioiodination studies. A somewhat higher residence time is expected for mature apoA-I compared to total apoA-I, since total apoA-I includes both mature and proapoA-I (the latter having a faster rate of turnover, i.e., shorter residence time).

In conclusion, although a specific gene defect has not as yet been identified as the cause of HDL deficiency in our FHD patients, the present results demonstrate that these patients are kinetically distinguishable from control subjects and also patients with Tangier disease. The gene responsible for low HDL levels and associated hypercatabolism of HDL in FHD patients may therefore be different from that of Tangier disease or may represent a less severe abnormality of the same gene. Further investigation of this kindred is thus warranted, since it has the potential to provide new insight into genetic factors affecting HDL metabolism.

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Chapter 5

Supplementary Data and General Discussion

Table 5.1. Characteristics of Study Subjects

Subjects	Age y	BMI kg/m^2	ApoE	TG mmol/l	TC mmol/l	VLDL-TG mmol/l	VLDL-C mmol/l	IDL/LDL-TG mmol/l	IDL/LDL-C mmol/l	HDL-C mmol/l	ApoB mg/dl	ApoA-I mg/dl
Normal												
1	36	24.5	3/3	0.67	4.78	0.55	0.20	0.10	2.92	1.66	84.9	148.8
2	30	23.5	3/3	0.69	3.76	0.53	ND	0.12	2.19	1.57	67.4	147.4
3	26	24.4	3/2	0.72	3.18	0.64	0.11	0.06	1.88	1.19	65.4	122.2
4	26	25.9	3/3	0.80	3.55	0.62	0.21	0.15	2.36	0.98	81.2	101.2
5	35	26.1	3/3	1.56	4.75	1.35	0.43	0.18	3.38	0.94	97.7	105.4
Mean	31	24.9		0.89	4.00	0.74	0.19	0.12	2.55	1.27	79.3	125.0
SE†	± 2	± 0.5		± 0.17	± 0.33	± 0.16	± 0.07	± 0.02	± 0.27	± 0.15	± 5.9	± 10.1
HTG												
6	41	24.1	3/3	2.22	5.79	1.78	0.67	0.37	4.09	1.03	142.3	129.0
7	40	29.3	3/3	3.14	3.57	2.67	0.98	0.29	1.81	0.78	102.5	101.6
8	63	26.6	3/2	4.30	4.13	3.38	0.88	0.68	2.69	0.56	131.1	129.8
9	38	24.7	4/3	5.87	6.15	5.37	2.54	0.37	3.02	0.59	143.4	111.4
10	67	24.1	4/2	6.25	6.46	5.85	3.52	0.37	2.38	0.56	127.9	120.8
Mean	50*	25.8		4.36**	5.22	3.81**	1.72*	0.42**	2.80	0.70*	129.4***	118.5
SE†	± 6	± 1.0		± 0.77	± 0.58	± 0.78	± 0.56	± 0.07	± 0.38	± 0.09	± 7.4	± 5.4
Type-III												
11	31	26.9	2/2	11.48	15.59	10.64	13.21	0.61	1.95	0.43	145.2	124.8
12	56	29.6	2/2	12.56	13.99	11.80	11.48	0.54	1.96	0.55	133.4	128.0
Mean	44	28.3		12.02	14.79	11.22	12.35	0.58	1.96	0.49	139.3	126.4

BMI is body mass index; TG, triglyceride; TC, total cholesterol; and -C, cholesterol. Plasma concentrations for individual subjects represent the average of five measurements made at 3-hour intervals during the stable-isotope infusion.
†Mean data ± standard error of the mean.
*, ** and *** indicate statistically significant differences between normolipidemic and hypertriglyceridemic subjects with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Table 5.2. Kinetic Parameters for apoC-III

Subjects	VLDL apoC-III			HDL apoC-III			Plasma apoC-III		
	Concentration mg/dl	RT days	TR mg. $kg^{-1}.d^{-1}$	Concentration mg/dl	RT days	TR mg. $Kg^{-1}.d^{-1}$	Concentration mg/dl	RT days	TR mg. $kg^{-1}.d^{-1}$
Normal									
1	1.53	0.89	0.77	7.82	4.70	0.75	9.69	1.98	2.20
2	3.68	1.32	1.26	8.10	2.48	1.47	12.24	2.16	2.55
3	3.23	0.91	1.60	3.72	1.66	1.01	7.15	1.55	2.08
4	3.07	1.34	1.03	4.92	4.00	0.55	8.46	3.10	1.23
5	6.45	1.38	2.10	2.13	4.72	0.20	9.79	2.02	2.18
Mean	3.59	1.17	1.35	5.34	3.51	0.80	9.47	2.16	2.05
SE†	± 0.80	± 0.11	± 0.23	± 1.16	± 0.62	± 0.21	± 0.84	± 0.26	± 0.22
HTG									
6	13.08	1.60	3.68	4.37	3.39	0.58	19.79	2.46	3.62
7	15.62	1.38	5.09	2.15	4.35	0.22	18.60	2.15	3.89
8	17.55	1.52	5.20	3.03	3.94	0.35	23.59	2.78	3.82
9	30.23	1.59	8.56	3.74	2.18	0.77	37.16	2.11	7.93
10	35.34	3.76	4.23	5.17	5.16	0.45	44.39	3.82	5.23
Mean	22.36**	1.97	5.35**	3.69	3.80	0.47	28.71**	2.66	4.90**
SE†	± 4.38	± 0.45	± 0.85	± 0.52	± 0.50	± 0.09	± 5.12	± 0.31	± 0.81
Type-III									
11	37.64	1.83	9.26	6.96	2.95	1.06	47.37	2.01	10.61
12	51.05	4.15	5.54	5.24	5.80	0.41	58.61	3.79	6.96
Mean	44.35	2.99	7.40	6.10	4.38	0.73	52.99	2.90	8.78

RT is residence time; and TR, transport rate. Concentrations for individual subjects represent the average of five measurements made at 3-hour intervals during the stable-isotope infusion.
†Mean data ± standard error of the mean.
** indicates statistically significant difference between normolipidemic and hypertriglyceridemic subjects with $p < 0.01$

Table 5.3. Kinetic Parameters for VLDL apoB-100

	VLDL apoB-100		
Subjects	Concentration mg/dl	RT days	TR mg. $kg^{-1}.d^{-1}$
Normal			
1	3.0	0.03	45.0
2	4.4	0.05	39.6
3	4.0	0.05	36.0
4	5.5	0.12	20.6
5	12.5	0.10	56.3
Mean	5.9	0.07	39.5
SE†	± 1.7	± 0.02	± 5.8
HTG			
6	20.0	0.20	45.0
7	15.5	0.31	22.5
8	18.5	0.27	30.8
9	38.2	0.30	57.3
10	32.2	0.56	25.9
Mean	24.9**	0.33**	36.3
SE†	± 4.4	± 0.06	± 6.5
Type-III			
11	64.0	0.77	37.4
12	59.2	1.05	25.4
Mean	61.6	0.91	31.4

RT is residence time; and TR, transport rate. Concentrations for individual subjects represent the average of five measurements made at 3-hour intervals during the stable-isotope infusion.
†Mean data ± standard error of the mean.
**indicates statistically significant difference between normolipidemic and hypertriglyceridemic subjects with $p < 0.01$

Table 5.4. Kinetic Parameters for apoE

Subjects	VLDL apoE			HDL apoE			Plasma apoE		
	Concentration mg/dl	RT days	TR mg. $kg^{-1}.d^{-1}$	Concentration mg/dl	RT days	TR mg. $Kg^{-1}.d^{-1}$	Concentration mg/dl	RT days	TR mg. $kg^{-1}.d^{-1}$
Normal									
1	0.76	0.19	1.78	2.81	1.13	1.12	4.08	1.30	1.41
2	0.51	0.25	0.90	2.56	0.91	1.27	3.44	1.19	1.30
3	0.83	0.20	1.90	3.96	0.85	2.10	5.27	0.93	2.56
4	0.55	0.16	1.59	2.99	0.62	2.16	3.96	0.33	5.35
5	0.94	0.24	1.78	2.63	1.04	1.14	4.67	0.51	4.09
Mean	0.72	0.21	1.59	2.99	0.91	1.56	4.28	0.85	2.94
SE†	± 0.08	± 0.02	± 0.18	± 0.26	± 0.09	± 0.24	± 0.31	± 0.19	± 0.78
HTG									
6	2.11	0.40	2.35	2.56	1.23	0.94	6.13	0.56	4.90
7	4.35	0.44	4.42	1.62	0.79	0.93	6.78	0.63	4.83
8	4.25	0.38	5.00	2.85	1.23	1.05	8.64	0.54	7.19
9	5.50	0.41	6.06	1.14	0.73	0.70	7.76	0.48	7.29
10	6.92	0.65	4.78	2.91	1.74	0.75	10.66	1.02	4.81
Mean	4.63**	0.46**	4.52**	2.22	1.14	0.87	8.03**	0.65	5.80*
SE†	± 0.79	± 0.05	± 0.61	± 0.35	± 0.18	± 0.06	± 0.83	± 0.09	± 0.59
Type-III									
11	32.08	0.99	14.55	7.00	3.21	0.98	41.21	1.44	12.91
12	29.85	1.44	9.35	7.13	3.47	0.92	39.58	1.67	10.69
Mean	30.97	1.21	11.95	7.07	3.34	0.95	40.40	1.55	11.80

RT is residence time; and TR, transport rate. Concentrations for individual subjects represent the average of five measurements made at 3-hour intervals during the stable-isotope infusion.

†Mean data ± standard error of the mean.

* and ** indicate statistically significant differences between normolipidemic and hypertriglyceridemic subjects with $p < 0.05$ and $p < 0.01$, respectively.

In chapter 3, I presented and discussed results pertaining to the plasma kinetics of apolipoprotein (apo)C-III and apoE in normolipidemic subjects and hypertriglyceridemic patients, having reduced catabolism of VLDL apoB-100. Characteristics of study subjects and kinetic parameters for VLDL apoB-100, apoC-III, and apoE were reported as mean values (± SE) due to the constraints of the manuscript format. In this chapter, values for individual subjects are included (tables 5.1-5.4).

The main goal of the study in chapter 3 was to determine whether elevated levels of plasma and VLDL apo(s) C-III and E in hypertriglyceridemic patients, including those with type III hyperlipoproteinemia, are due to overproduction or delayed catabolism. Plasma kinetics of apo(s) C-III and E in hypertriglyceridemic patients were therefore the primary focus. Despite this, issues like: 1) exchangeability between lipoprotein fractions, namely VLDL and HDL, 2) kinetic homogeneity vs. heterogeneity of lipoprotein fraction pools and the related notion of similar vs. different catabolic rates, and 3) first appearance of apo(s) C-III and E in plasma were extensively discussed.

The first reason for doing this was the originality of the findings related to these topics. By introducing a labeled precursor (amino acid), an endogenous labeling procedure captures protein tissue production and subsequent secretion into plasma. It can therefore be useful in monitoring the first appearance of protein into plasma. For example, apoC-III was previously suggested to appear on HDL first, and then to transfer to nascent VLDL (25). The fact that stable isotope-enriched newly-synthesized apoC-III appeared simultaneously on VLDL

and HDL did not seem to support this notion; there was no evidence for a precursor-product relationship between the two fractional pools. Furthermore, it is unlikely that a pool with slow turnover like HDL apoC-III was solely capable of supplying VLDL, which had a markedly faster turnover. Endogenous labeling is also useful for exposing kinetic heterogeneity, provided that several plasma pools are sampled. This was evident in the case of apo(s) C-III and E on VLDL vs. HDL. Enrichment curves of VLDL and HDL apoC-III were quite different, and so were those of VLDL and HDL apoE. A homogenous plasma pool, by definition, shows full exchangeability between protein molecules on lipoprotein fractions (e.g. VLDL and HDL), as well as identical catabolic rates on each. Had this been the case, enrichment curves for apo(s) C-III and E on VLDL and HDL would have been superimposable. Clearly they were not (Figures 2 and 3, chapter 3).

The second reason for discussing these basic issues was wanting to establish that VLDL apo(s) C-III and E pools are kinetically more active than HDL. The idea was to focus on VLDL, and not total plasma, as the pool that matters most. Total plasma measurements were reflective of kinetically dormant HDL pools as well as active VLDL. Their values in hypertriglyceridemic patients did not fully expose the drastic changes which were taking place in VLDL. For example, table IV of chapter 3 shows a less than 2-fold increase in plasma residence time (RT) of apoE in patients with type III hyperlipoproteinemia (TypIII), compared to normolipidemic (NL) subjects. This change fades in comparison with the 6-fold increase in the RT of VLDL apoE measured in the

patients. Compromised value of total plasma measurement is due to the fact that it accounts for both severely-impaired VLDL apoE and less affected HDL apoE. Furthermore, I was not convinced that RTs of total plasma apoC-III or apoE are meaningful parameters. Each is a weighted average of the RTs of protein molecules in several plasma pools (e.g. VLDL- and HDL apoC-III). Because RT of VLDL apoC-III, for example, is very different from HDL apoC-III, the weighted average does not resemble any of the two. Using the jargon of statistics, RT of total plasma apoC-III is an arithmetic mean. In the case of a normal distribution, it can be taken as a proxy for the mode (the value of the RT with the highest occurrence) which is indeed an informative parameter. Unfortunately, the population of apoC-III in plasma is not normally distributed. It probably assumes a binomial distribution, at least in NL subjects, with two quite distinct values for the random variable (RTs of VLDL- and HDL apoC-III). The arithmetic mean in this case will still fall between the two values of the random variable but will be far from the mode. The same logic of course applies to apoE. To illustrate further, I will use RTs of VLDL-, HDL-, and total plasma apoC-III in NL subjects, reported in table III of chapter 3 (1.17, 3.51, and 2.16 days, respectively). If one assumes that: 1) the exchange of apoC-III between VLDL and HDL is minimal, which seems to be the case in fasted NL subjects, and 2) the contribution of the IDL/LDL apoC-III pool is negligible, then RT of VLDL apoC-III will become its plasma residence time. Similarly, RT of HDL apoC-III will become its plasma residence time. One immediately realizes that apoC-III molecules are either residing in plasma for 1.17 days or 3.51 days.

Because the majority of apoC-III molecules are associated with HDL in NL subjects (5.34 vs. 3.59 mg/dl, HDL apoC-III vs. VLDL apoC-III), the most frequent RT (mode) is 3.51 days, not 2.16 days. RT of total plasma apoC-III, which is around the arithmetic mean (2.57 days), has little, if any, physiological meaning.

Plasma apoB-100 kinetic data were only briefly discussed in chapter 3 due to the fact that they were not the focus of that study. I will therefore use some of the space here to further discuss them and also report supplemental data. In all study subject, RT of VLDL apoB-100 determined VLDL apoB-100 concentration. Pearson product moment correlation coefficient between RT of VLDL apoB-100 and its concentration was 0.93 ($P < 0.0001$) in the 12 subjects (Table 5.5). RT of VLDL apoB-100 explained the concentration variations between the three groups, NL, HTG, and TypIII, as well as within each group. In contrast, TR of VLDL apoB-100 was not correlated with its concentration. Evidence from *in vitro* studies and work in transgenic mice suggest that apo(s) C-III and E determine plasma TRL levels by regulating: 1) TRL lipolytic processing (conversion to LDL), and 2) TRL receptor-mediated uptake. Both of these events are catabolic in nature. Nevertheless, *in vivo* kinetic studies rarely explained VLDL apoB-100 accumulation in plasma of hypertriglyceridemic patients (which often have elevated levels of plasma apo(s) C-III and E) on the basis of reduced catabolism (325). Even in the case of Type III hyperlipoproteinemia, where there is certainly an element of reduced catabolism, VLDL apoB overproduction was suggested to be a primary cause for its

Table 5.5. Pearson Product Moment Correlation of Kinetic Parameters for VLDL apolipoproteins

	B-100 RT	B-100 TR	C-III Conc.	C-III RT	C-III TR	E Conc.	E RT	E TR
B-100 Conc.	0.93* 0.000013†	-0.07 0.838000	0.95 0.000003	0.67 0.016800	0.85 0.000500	0.93 0.000016	0.91 0.000047	0.94 0.000007
B-100 RT	—	-0.36 0.255000	0.96 0.000001	0.84 0.000733	0.68 0.016800	0.93 0.000019	0.98 0.000000	0.86 0.000427
B-100 TR	—	—	-0.20 0.530000	-0.39 0.211000	0.08 0.812000	-0.22 0.489000	-0.30 0.341000	-0.14 0.709000
C-III Conc.	—	—	—	0.84 0.000701	0.78 0.002640	0.84 0.000669	0.92 0.000020	0.83 0.000847
C-III RT	—	—	—	—	0.34 0.282000	0.60 0.039100	0.81 0.001400	0.47 0.128000
C-III TR	—	—	—	—	—	0.68 0.015600	0.62 0.033400	0.86 0.000366
E Conc.	—	—	—	—	—	—	0.93 0.000009	0.94 0.000007
E RT	—	—	—	—	—	—	—	0.82 0.001060

B-100, VLDL apoB-100; C-III, VLDL apoC-III; E, VLDL apoE; conc., concentration; RT, residence time; and TR, transport rate. The upper (*) and lower (†) figures in each cell are the correlation coefficient and P value for each correlation, respectively.

accumulation in plasma (282,523). Some investigators have discussed the notion that undercatabolism of VLDL apoB in dyslipidemias can be more common than what kinetic studies suggest. Grundy and Vega (524) argued that slower removal of large VLDL particles, which are usually produced and cleared fast, leads to their channeling into "visible" (sampled) kinetic compartments. This creates the perception that VLDL apoB is overproduced. Williams et al. (525) proposed that incomplete remodeling of nascent lipoprotein particles in or near the space of Dissé, which is caused by LPL deficiency, impairs their clearance. This in turn can appear in kinetic studies as overproduction.

The observation that production rate of VLDL apoB-100 did not differ between NL subjects and HTG and TypIII patients (chapter 3) is important. It suggests that increased production of VLDL apoC-III and apoE in these cases was not a function of increased number of nascent VLDL particles. Admittedly, the measured production rates in all study subjects, normolipidemic and hypertriglyceridemic, were higher than what was reported in most previous studies. This discrepancy was not a function of pools sizes, labeling procedure, or data modeling. At this stage, the reason for it remains unknown. For the sake of this study, however, emphasis was on the comparative value of production rate measurements. I therefore accepted them as evidence for similarity between study groups.

I also had the opportunity to sample apoB-100 IDL and LDL pools in order to examine their kinetics in NL subjects and HTG patients. The endogenous labeling procedure allowed me to monitor changes in the speed of catabolic

events along the VLDL lipolytic cascade pathway. I was able to estimate the time period needed to: 1) synthesize, assemble and secrete VLDL particles into plasma, 2) convert VLDL to IDL, and 3) convert IDL to LDL. This was done simply by detecting the first appearance of newly-synthesized, stable isotope-enriched, apoB-100 molecule in VLDL, IDL and LDL pools (fig 5.1-5.4). In NL subjects, newly-synthesized apoB-100 appeared in VLDL within 30 min from the start of stable isotope infusion. Newly-synthesized apoB-100 appeared in IDL after 45 min, and in LDL after 120 min. In HTG patients, newly-synthesized apoB-100 also appeared in VLDL within 30 min. The lipolytic conversion process on the other hand was markedly slower, particularly in HTG patients with TG > 5.87 mmol/l. Newly-synthesized apoB-100 appeared in IDL after 75 min or 120 min (in HTG patients with lower and higher plasma TG, respectively, fig 5.4). Furthermore, it appeared in LDL after 300 min. Data in one TypIII patient were similar to HTG patients with the highest plasma TG (data in the second TypIII patient were not collected). Impaired conversion of VLDL to LDL, via IDL, thus contributed to the accumulation of VLDL and IDL apoB-100 in plasma of HTG and one TypIII patient. The magnitude of this contribution (vs. impaired receptor-mediated clearance) is still to be determined by an ongoing attempt to integrate all kinetic data in a single multicompartmental model (section 2.8).

The main focus of the study presented in chapter 3 was plasma kinetics of apo(s) C-III and E in HTG and TypIII patients. Consequently, kinetic parameters for apo(s) C-III and E in HTG and TypIII patients were reported and discussed extensively (chapter 3). The main conclusions were that: 1) HTG and TypIII

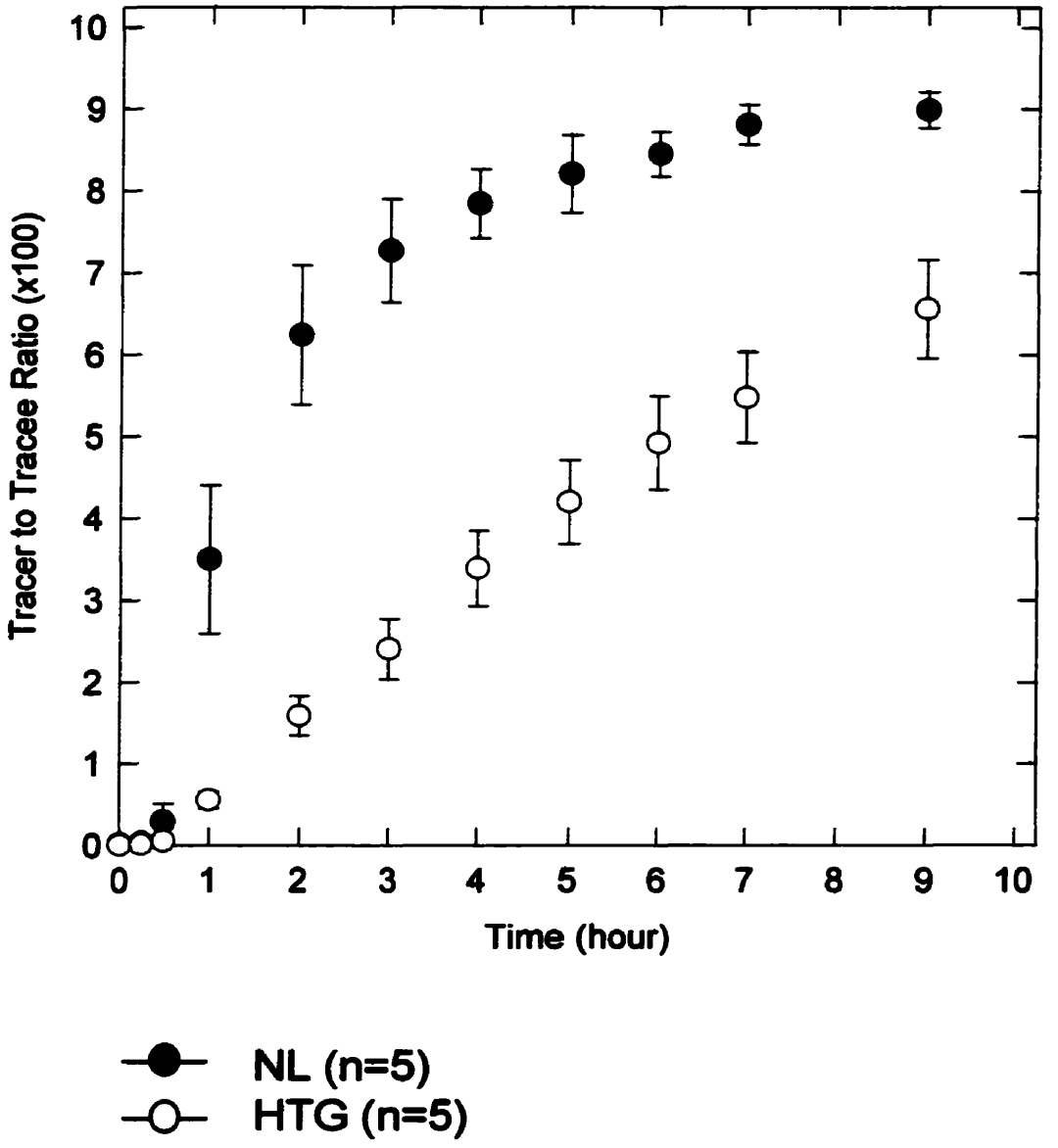

Figure 5.1. Fractional rate of appearance of newly-synthesized apoB-100 in VLDL of NL subjects and HTG patients. Data are mean values. Error bars are SE.

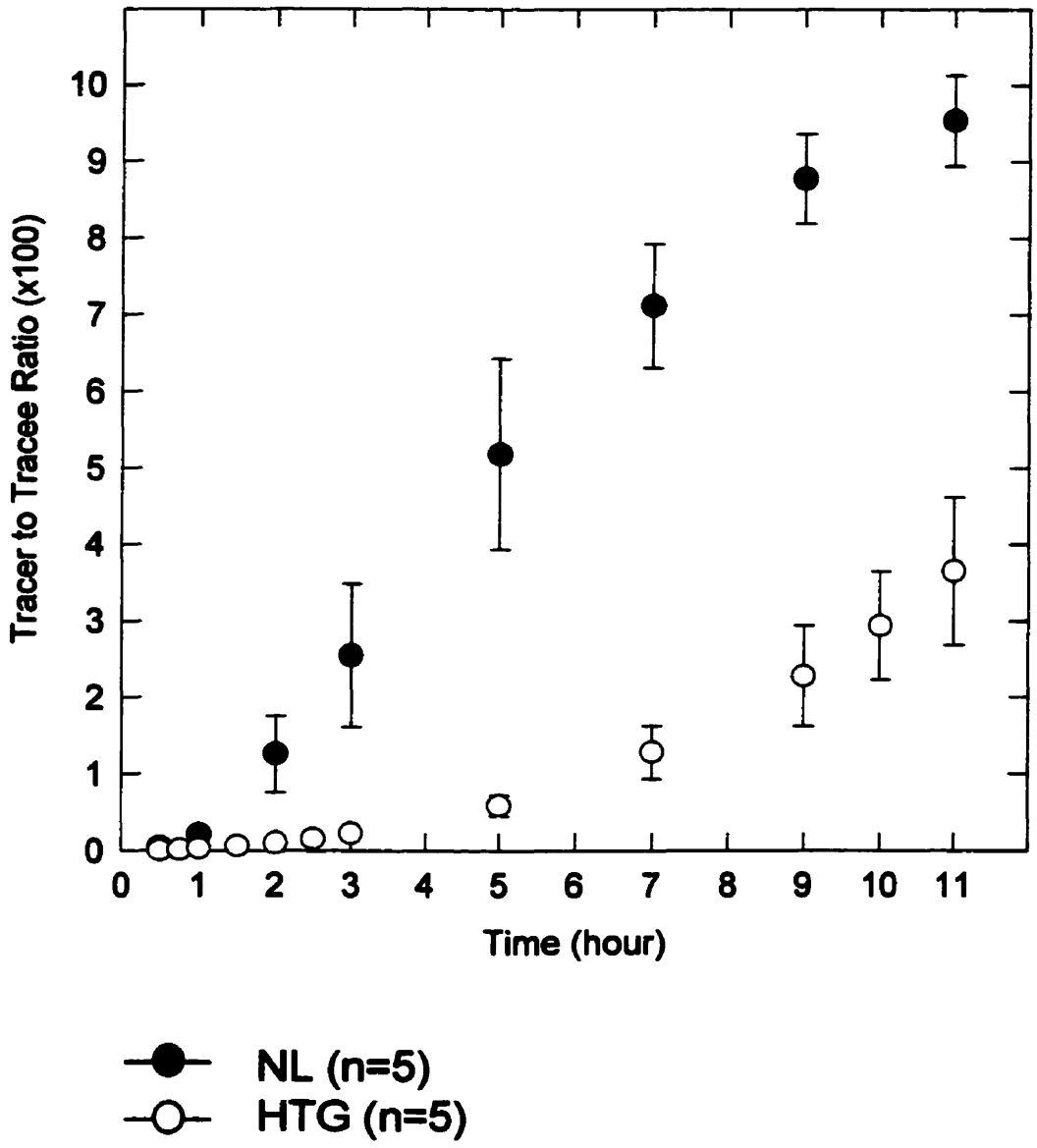

Figure 5.2. Fractional rate of appearance of newly-synthesized apoB-100 in IDL of NL subjects and HTG patients. Data are mean values. Error bars are SE.

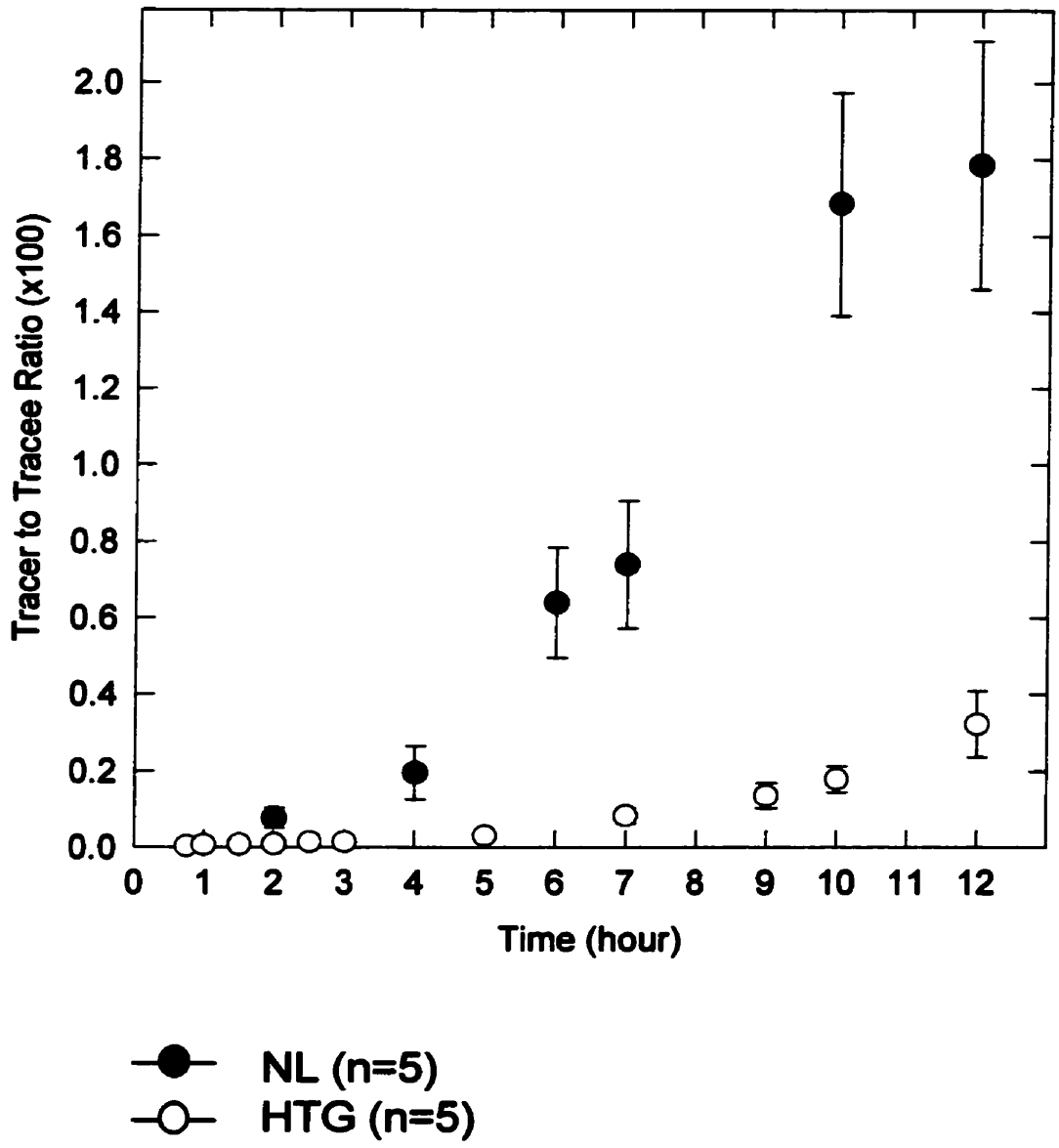

Figure 5.3. Fractional rate of appearance of newly-synthesized apoB-100 in LDL of NL subjects and HTG patients. Data are mean values. Error bars are SE.

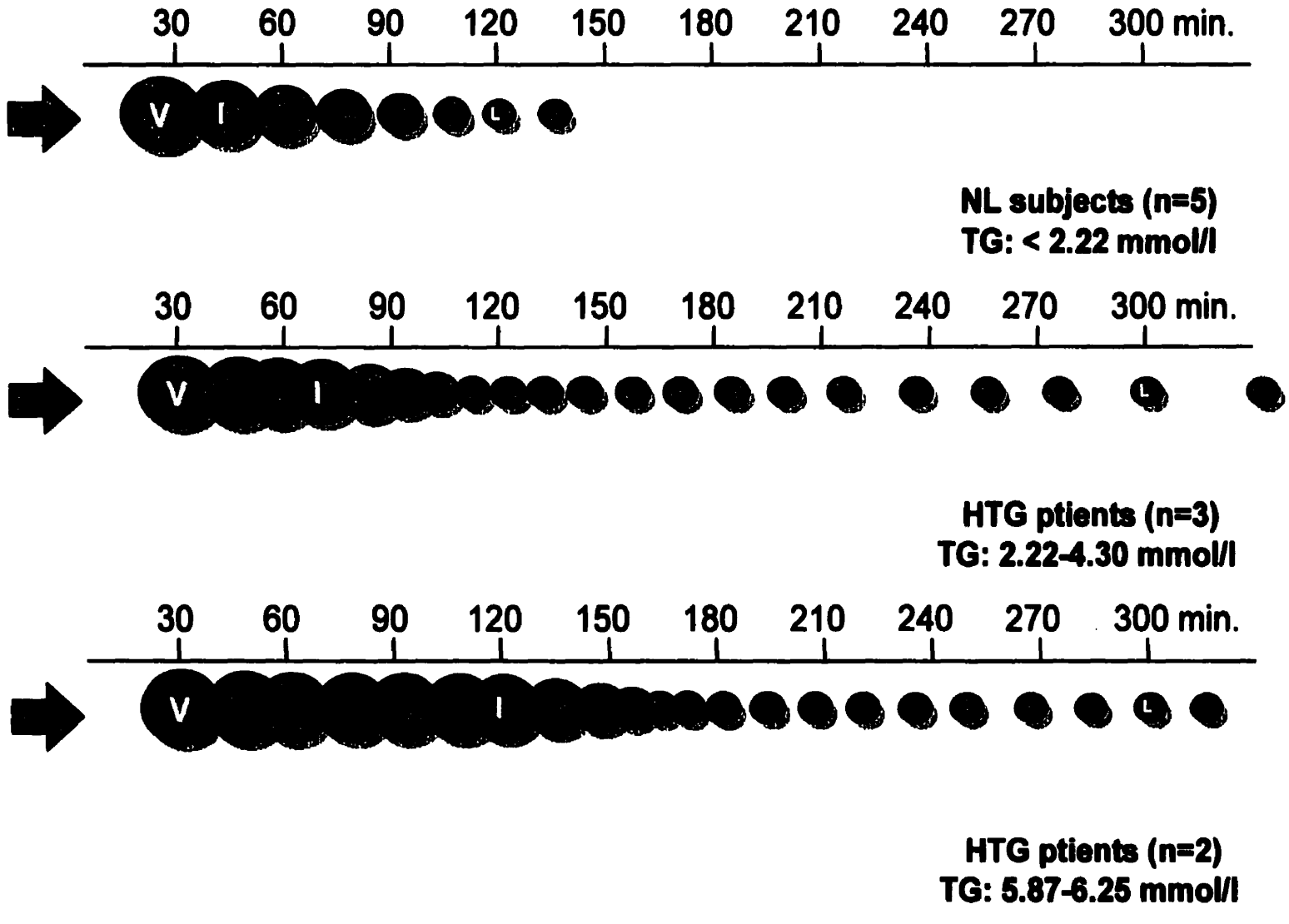

Figure 5.4. First appearance of newly-synthesized apoB-100 in VLDL (V), IDL (I) and LDL (L) of NL subjects and HTG patients

patients had an overproduction of VLDL apoC-III, 2) they also had an overproduction of VLDL apoE, 3) RT of VLDL apoE increased, particularly in TypIII patients, and 4) RT of VLDL apoC-III increased only mildly (not significant in the case of HTG patients).

Data related to plasma kinetics of apoB-100 and apoC-III fit together well. ApoC-III reduces TRL catabolism (conversion and clearance) *in vitro.* Its expression in transgenic mice results in hypertriglyceridemia and marked reduction in VLDL fractional catabolic rate. Elevated plasma and VLDL levels of apoC-III are associated with TRL accumulation in hypertriglyceridemic patients. It is likely then that abnormalities in TRL and apoC-III metabolism in hypertriglyceridemic patients are interrelated. The study in chapter 3 has taken this argument one step further. It proposed that overproduction of VLDL apoC-III is primary, and is inhibiting plasma TRL catabolism in HTG and TypIII patients. Consequently, increased RT of VLDL apoC-III in this scenario is secondary to reduced catabolism of the whole VLDL particle, which in turn is a function of increased VLDL apoC-III production. Increased RT of VLDL apoE in HTG patients can also be due to reduced VLDL catabolism. In contrast, that in TypIII patients is a primary defect caused by defective binding to hepatic receptors. This explains why the increase is markedly higher in the their case, compared to HTG patients (6-fold and 2-fold increase in RT of apoE , TypIII vs. HTG, respectively).

But what about increased production of VLDL apoE in HTG and TypIII patients ? In the article, I proposed two scenarios to explain it: 1) it is parallel to

increased production of VLDL apoC-III, i.e. it is primary in nature; 2) it is induced by TRL accumulation, i.e. it is a compensatory mechanism. Based on the present data, it is difficult to weight one possibility over the other. Nevertheless, the first scenario implies the presence of two independent mechanisms occurring concomitantly in HTG patients. The second scenario only accounts for one, whereby overproduction of VLDL apoC-III is the only primary event.

Regardless of which of the two scenarios better reflects what is happening, two questions come to mind: 1) why is increased production of VLDL apoE, and subsequent increase in its plasma level, not correcting the hypertriglyceridemia? and 2) could it actually be exacerbating it?

In the case of TypIII patients, increased production of apoE2 is unlikely to improve TRL accumulation. In addition to being defective in receptor binding, apoE2 impairs lipolytic processing and conversion of VLDL to LDL in a dose-dependent manner. This is possibly due to the fact that high concentrations of apoE2 displace apoC-II, the cofactor of LPL, from the TRL particle (360). In fact, whereas transgenic mice expressing low and intermediate levels (< 10 and 10-30 mg/dl, respectively) of human apoE2 are hypolipidemic, those expressing high levels (>50 mg/dl) are hyperlipidemic and have an accumulation of plasma TRL (358). Increased production of apoE2 in TypIII patients may therefore be worsening TRL accumulation.

Unlike TypIII patients, HTG patients are not homozygous for the apoE2 isoform. Overproduction of apoE in their case results in an increased number of functional apoE molecules on TRL. Despite this, the hypertriglyceridemia in

these patients persists. One explanation for this paradox is that apoE is actually correcting the hypertriglyceridemia, albeit partially. Several studies (152,526), but not all (155), have shown that enriching TRL with exogenous apoE is not enough to totally overcome the inhibitory effect of apoC-III on their clearance. Furthermore, in a case where increased production of VLDL apoE is secondary to that of apoC-III, the newly-synthesized apoE molecules may not be capable of displacing apoC-III. It is possible that only a limited number of apolipoprotein molecules can be accommodated on the surface of a lipoprotein particle. Additional apoE molecules may be attaching to each other in a protein-protein interaction instead of being properly positioned through a protein-lipid interaction. Granted, the stoichiometry caused by apoC-III and apoE overproduction in HTG patients is not in favor of apoE. The molar increase in apoC-III production in HTG patients is several folds higher than apoE (chapter 3, Figure 5). This suggests that excessive apoE molecules on the VLDL particle are grossly outnumbered by those of apoC-III.

Alternatively, increased production of apoE in HTG patients can be aggravating plasma TRL accumulation. Despite a considerable body of evidence supporting a favorable role for functional apoE in increasing TRL catabolism, there are suggestions that high levels of apoE may have an opposing effect. One study has shown that, *in vitro*, all apoE isoforms inhibit VLDL conversion to LDL to a similar extent (527). Evidence showing that a high level of apoE2 impairs VLDL conversion by depleting the particle of apoC-II (360)

implies that this happens by stearic hindrance. There is no reason why other apoE isoforms cannot exert the same effect when excessively present on VLDL.

Is increased production of VLDL apoC-III a second hit precipitating overt type III hyperlipoproteinemia? It certainly qualifies as such. VLDL apoC-III overproduction is upstream from apoE2-related catabolic impairment, and can be mediating the involvement of several metabolic disorders. For example, in animals and in cultured cells, apoC-III gene is transcriptionally downregulated by insulin (179). It is not unreasonable therefore to hypothesize that the association of diabetes with overt type III hyperlipoproteinemia is due to increased apoC-III production. Furthermore, although overproduction of VLDL apoC-III in the study presented in chapter 3 was not associated with increased number of newly-synthesized VLDL particles, this need not be the case all the time. Increased production of VLDL apoC-III in other incidences may well be a part of a general overproduction event affecting all VLDL components. Obesity, another metabolic disorder which precipitates type III hyperlipoproteinemia, is characterized by overproduction of VLDL particles. Whether there is increased production of VLDL apoC-III in obese subjects is yet to be established. When Demant et al. (340) compared plasma apoB turnover in normolipidemic E2 subjects and TypIII patients before and during bezafibrate therapy, they concluded that: "There is a remarkable similarity between treated type III and normolipidemic E2 subjects in both the apoB content of lipoprotein fractions and metabolic behavior indicating that bezafibrate removes the hyperlipidemia component from the type III pattern but does not correct the abnormality owing to

E2 genetic variant". As was mentioned previously, one effect of fibrates is to downregulate hepatic apoC-III gene expression.

Evidently, more work is needed to confirm the involvement of increased VLDL apoC-III production in TRL accumulation in hypertriglyceridemic patients, particularly those with type III hyperlipoproteinemia. For one, a transgenic mouse model is critical to decouple increased production of apoC-III from that of apoE. Huang and coworkers (360) have generated elegant transgenic mice models. Some of these animals were expressing human apoE2 in the absence of endogenous mouse apoE. It will be interesting to see whether cross breading an animal expressing low levels of human apoE2 with another overexpressing apoC-III will produce a model for type III hyperlipoproteinemia.

Here in our laboratory we need to recruit more TypIII patients to confirm the association of the type III phenotype with increased VLDL apoC-III production. More importantly, we still need to investigate the kinetics of apoC-III in normolipidemic subjects homozygous for the apoE2 isoform. If production of apoC-III was found to be normal in these patients then that would considerably strengthen our theory. Preliminary data in our laboratory suggest that these subjects have almost a 2-fold increase in plasma apoE concentration (vs. 10-fold in TypIII patients), compared to normolipoidemic subjects. Moreover, these subjects have normal concentrations of plasma apoC-III, suggesting that plasma and VLDL apoC-III production in these subjects may well be normal. It will also be interesting to subject the previously studied TypIII patients to a fibrate regimen and re-measure their VLDL apo(s) C-III and E production under

treatment. We will be able to determine whether or not normalization of the lipid profile in these patients will be associated with normalization of VLDL apoC-III production.

Finally, although genetic variation(s) causing familial combined hyperlipidemia (FCH) has not yet been identified, the locus with the greatest involvement in FCH is that of the apoA-I/C-III/A-IV gene complex (section 1.8.3). One important study that will be carried out in our laboratory in the near future will look at plasma kinetics of apoC-III in families with FCH. The objective will be to determine whether or not affected members of these families have increased production of VLDL apoC-III. If there is indeed an overproduction, with or without that of VLDL apoB-100, that will strengthen the argument for the involvement of the apoA-I/C-III/A-IV gene locus in FCH.

In the remaining pages of this chapter, I will further discuss the findings presented in chapter 4. Reduced plasma levels of apoA-I and apoA-II in patients with familial HDL deficiency (FHD) were found to be due to hypercatabolism of mature apoA-I and apoA-II but not proapoA-I. Accordingly, FHD patients differ from those with Tangier Disease (TD) by having a less severe form of HDL deficiency. The fractional turnover of mature apoA-I and apoA-II in FHD patients is less, compared to TD patients, and catabolism of proapoA-I is normal.

Are FHD and TD two different dyslipidemias ? The answer is probably no. Both FHD and TD were shown to be associated with impaired cellular cholesterol and phospholipid efflux from peripheral cells. It is possible that Tangier

manifestations are dose-dependent and that FHD is a heterozygous form of the disease. Alternatively, FHD can be caused by only one of several defects necessary for the expression of the severe TD phenotype. In both cases, FHD is a useful model to study the plasma kinetics of HDL apo(s) A-I and A-II in cases of HDL deficiency associated with impaired cholesterol and phospholipid efflux. This is regardless of whether these cases have clinical manifestations of TD or not.

Except for apoA-I/C-III/A-IV genetic deficiencies, all forms of primary HDL deficiency are characterized by increased catabolism of plasma apoA-I. ApoA-I hypercatabolism in turn is associated with: 1) presence of apoA-I structural variants, 2) apoA-II deficiency, 3) total or partial absence of CE from the core of the HDL particle and/or its replacement with TG in cases like: a) LCAT deficiency, b) severe hypertriglyceridemia, and c) TD and FHD. A common denominator between these forms of HDL deficiency may well be an abnormal interaction of the apoA-I molecule with the CE core of HDL. The carboxy-terminal region of apoA-I plays an important role in lipid binding (528). Carboxy-terminal domain truncations were shown to be defective in their ability to bind lipids. Similar to the structural variants of apoA-I, their presence is associated with rapid clearance of plasma apoA-I. TG does not seem to be able to play an equivalent role to CE in HDL protein-lipid interaction. On the contrary, enrichment of the HDL particle with TG destabilizes it and increases the shedding of apoA-I, either by displacing CE or by making HDL more susceptible to the lipolytic action of HL (201,202). Increased catabolism of apoA-I in severe

hypertriglyceridemia could therefore be due to absolute and relative decrease of the CE content of HDL. This in turn could be due to : 1) decrease in the flow of cholesterol from TRL to HDL resulting from impaired lipolysis of the former, and 2) increase in the enrichment of HDL with TG originating from accumulated TRL.

ApoA-II could be stabilizing the interaction of apoA-I with CE. It is well known that apoA-I is catabolized faster on LpAI compared to LpA-I:A-II (114). This however could also be explained on the basis of LpA-I:A-II particles being more enriched with CE, compared to LpA-I. It is likely that the association of high levels of HDL-CE with increased concentrations of HDL apoA-I and its reduced FCR is causal rather than only correlative (529). Several studies have shown that lipid-depleted apoA-I particles resulting from CE reverse transport are highly susceptible to clearance by the kidney. Lipid-poor apoA-I either re-associates with CE or gets catabolized. Glass et al. (530) have demonstrated that apoA-I is constantly being filtered, reabsorbed and degraded in the kidney. In contrast, glomerular filtration of lipid-containing HDL is unlikely because of their high molecular weight. Moreover, core lipids of HDL have not been detected in the kidney (530,531).

In the case of TD and FHD, it is possible that lipid-poor apoA-I does not associate with sufficient amount of peripheral cell-derived cholesterol due to impaired efflux of the latter. Consequently, CE is not formed and apoA-I gets rapidly catabolized. In TD, hypercatabolism of plasma apoA-I is markedly higher, compared to FHD, which could reflect the different degrees of impairment of cholesterol efflux in the two states. Moreover, apoA-II is rapidly catabolized in

both cases, but to a lesser extent than apoA-I. One can argue that apoA-II is more robust than apoA-I and is less dependent on the presence of CE for its association with HDL particles. Consequently, apoA-II without apoA-I particles are formed in both FHD and TD.

One feature distinguishing FHD from TD is unequal susceptibility of different apoA-I isoforms to hypercatabolism. In FHD patients, there is an increase in direct clearance of mature apoA-I, but not proapoA-I. In contrast, in patients with TD, both mature apoA-I and proapoA-I are hypercatabolized to the same extent. In fact, increased direct clearance of proapoA-I is an important contributor to the severe reduction of mature apoA-I pool size in TD. Unlike in FHD, mature apoA-I pool suffers from increased catabolic pressures as well as reduced transport of protein molecules from the proapoA-I pool (FIG 8, chapter 4). It is unlikely that different susceptibility of proapoA-I and mature apoA-I to hypercatabolism is due to the presence of six amino acids on the precursor protein. There is no evidence for a conformational change. What could actually be different between the two isoforms is their association with different HDL populations. The precursor protein is likely to be found on nascent (newly-synthesized) lipid-poor HDL particles. In plasma, proapoA-I gets rapidly converted to mature apoA-I (within 2 h from the moment of its synthesis, according to my data). The probability of it being present on nascent HDL particles is therefore higher than on mature HDL particles. In fact, the relative increase in proapoA-I isoforms in FHD patients is associated with a relative increase in preβ HDL, particularly preβ-1. Admittedly, not all plasma preβ-1 HDL

are newly-synthesized particles. They can be formed as a result of HDL remodeling (HDL_2 to HDL_3) and CE reverse transport, as discussed previously (section 1.9.2). Unlike newly-synthesized preβ-1 HDL however, these HDL remnants will probably not carry proapoA-I. Conversely, one report has shown: 1) the presence of traces of proapoA-I in HDL_2, and 2) their absence from preβ-1 HDL (532). While the first observation can be explained on the basis of direct incorporation of newly-synthesized apoA-I into mature HDL particles as part of the particle enrichment with CE and protein, the second observation is difficult to reconcile with the presented logic.

I propose a model whereby susceptibility of apoA-I to hypercatabolism in FHD is a function of its association with different populations of HDL particles. Being newly-synthesized, the majority of proapoA-I molecules assume a conformation which is less dependent on the presence of lipids to preserve their stability. This conformation is typical of apoA-I on preβ-1 HDL. As apoA-I "ages" (one clear sign being the conversion to mature apoA-I), it assumes a different conformation. This conformation is typical of apoA-I on α-HDL. Its stability is more dependent on binding to lipids, particularly CE, and is further enhanced by the presence of apoA-II. Consequently, impairment of cholesterol efflux from peripheral cells and the resulting thin lipid content of HDL particles is detrimental to apoA-I in the mature conformation. It is however less relevant for apoA-I in the nascent conformation. This could explain why mature apoA-I but not proapoA-I is hypercatabolized in FHD. Cholesterol and phospholipid efflux is enough to stabilize the nascent apoA-I conformation but not enough to protect

apoA-I in the mature conformation from shedding. In TD, and because cholesterol and phospholipid efflux is further impaired, the lipid content cannot even support apoA-I in its robust nascent conformation. This leads to severe hypercatabolism of proapoA-I as well as mature apoA-I. It is known that apoA-I assumes different conformations on preβ-1 HDL and α-HDL (58). It has also been suggested that the conformational change of preβ-1 HDL to α-HDL is induced by particle enrichment with lipids, particularly CE formed by LCAT activity. Some CE may be sufficient to induce conformational change but not enough to protect apoA-I in the new conformation from shedding.

Evidently, more experimental work is needed to support this model. First of all, the question of which HDL populations carry proapoA-I needs to be answered. One can simply separate lipoproteins from plasma of a normolipidemic subject by two-dimensional gel electrophoresis (methods, chapter 4). Subsequently, proteins can be electroeluted from gel slices containing preβ-1, preβ-2 and α-HDL and isolated by IEF gel electrophoresis. For this model to be correct, preβ-1- and preβ-2 HDL would have to be relatively enriched with proapoA-I, whereas little proapoA-I would be found on α-HDL. Furthermore, when comparing the plasma kinetics of apoA-I on preβ-1 HDL in FHD patients with those in normolipidemic subjects, one would expect to see no difference in the fractional turnover of apoA-I between the two groups. In contrast, apoA-I on α-HDL would be hypercatabolized in FHD patients compared to normolipidemic subjects.

Combining the two techniques of two dimensional gel electrophoresis and endogenous labeling is also useful in addressing a broader question; that related to the precursor-product relationship in the HDL maturation cycle. Plasma samples collected at early time points from normolipidemic subjects can be used to determine the first appearance of newly-synthesized, stable isotope-enriched, apoA-I in different HDL populations. According to the traditional model of HDL maturation (58), apoA-I will first form a lipid-poor apoA-I particle (preβ-1 HDL), then discoidal preβ-2 HDL, large spherical HDL (HDL_2), and finally HDL_3. Appearance of newly-synthesized apoA-I, enriched with stable isotope, in different HDL pools is supposed to reflect this sequence of events. A more informative experiment would be to deliver a bolus dose of stable isotopically-labeled amino acid without subsequent constant infusion. This *in vivo* pulse-chase experiment will allow for determination of the dynamics of apoA-I transfer between HDL particles. For example, if apoA-I released during HDL remodeling (conversion of HDL_2 to HDL_3) does indeed form preβ-1 HDL, then one will expect to find evidence for a second input of stable isotope-enriched apoA-I into preβ-1 HDL. This input will be detected in the form of an increase in tracer to tracee ratio following the die-out of the primary signal. Similarly, if apoA-I released by HDL remodeling does re-associate with lipids and regenerates HDL_2, then there will be evidence for a second input into HDL_2 after the die-out of the primary signal.

Finally, further work will be needed to identify the genetic defect(s) responsible for TD and FHD, and to determine whether or not these two forms of HDL deficiency are indeed related.

In addition to these current and future endeavors, there will be plenty of other opportunities where the expertise and knowledge which our group has developed will come to be useful. Identifying these opportunities and capitalizing on them will be up to fellow investigators in our laboratory, and elsewhere. My hope is that the demanding nature of kinetic endogenous labeling studies will not discourage them from realizing their great potential.

Conclusion and Summary

1. In order to investigate the plasma kinetics of apolipoproteins in normolipidemic and dyslipidemic individuals, an endogenous labeling procedure was set up, in which a primed constant (12 h) infusion of deuterium-labeled leucine was administered to fasted study subjects. Fractional rate of appearance of newly-synthesized, stable isotope-enriched, apolipoprotein in plasma pools was determined by GC-MS. RT was derived from tracer to tracee ratios, and TRs were calculated from residence times and pool sizes.

2. Plasma kinetics of apoC-III and apoE were simultaneously investigated in normolipidemic subjects, hypertriglyceridemic patients and patients with type III hyperlipoproteinemia (both groups of patients having reduced catabolism of VLDL apoB-100. Elevated total plasma and VLDL apoC-III concentrations in hypertriglyceridemic and type III hyperlipoproteinemic patients were mainly due to significantly increased rates of apoC-III production. Elevated total plasma and VLDL apoE concentrations in hypertriglyceridemic patients and particularly in patients with type III hyperlipoproteinemia were associated with increased VLDL apoE RT, as well as significantly increased apoE TR. These results demonstrate that hypertriglyceridemic patients, having reduced rates of VLDL apoB-100 catabolism (including patients with type III hyperlipoproteinemia) are characterized by overproduction of plasma and VLDL apoC-III and apoE.

3. Plasma kinetics of apoA-I and apoA-II were also investigated in control subjects and patients with Familial HDL Deficiency. These patients had a marked reduction in the plasma concentration of HDL-C and apoA-I but lacked clinical manifestations of Tangier Disease. Mature plasma apoA-I TRs were similar in the patients and controls but RTs of mature apoA-I were significantly less in the patients. Normal levels of plasma proapoA-I in the patients were associated with normal plasma proapoA-I TRs and RTs, whereas the RTs of apoA-II were less in the patients vs. controls, and TRs of apoA-II were similar. Increased plasma catabolism of apoA-II in the patients was associated with the presence in plasma of abnormal apoA-II-HDL without apoA-I. These results demonstrate that HDL deficiency in these patients is characterized, like Tangier Disease, by hypercatabolism of mature apoA-I and apoA-II, but unlike Tangier Disease, by essentially normal plasma catabolism and concentration of proapoA-I.

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