Detection, Quantification, and Characterization of Potentially Atherogenic Triglyceride-Rich Remnant Lipoproteins

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Abstract—Triglyceride-rich lipoprotein (TRL) remnants are formed in the circulation when apolipoprotein (apo) B-48—containing chylomicrons of intestinal origin or apoB-100—containing VLDL of hepatic origin are converted by lipoprotein lipase, and to a lesser extent by hepatic lipase, into smaller and more dense particles. Compared with their nascent precursors, TRL remnants are depleted of triglyceride, phospholipid, and C apolipoproteins and are enriched in cholesteryl esters and apoE. They can thus be identified, separated, and/or quantified in plasma according to their density, charge, size, specific lipid components, apolipoprotein composition, and/or apolipoprotein immunospecificity. Each of these approaches has contributed to our current understanding of the compositional characteristics of TRL remnants and their potential to promote atherosclerosis. An ongoing search is nevertheless under way for more accurate and clinically applicable remnant lipoprotein assays that will be able to better define coronary artery disease risk in patients with hypertriglyceridemia. (Arterioscler Thromb Vasc Biol. 1999;19:2474-2486.)

Key Words: atherogenesis ■ triglycerides ■ apolipoproteins ■ lipoproteins ■ assay

Increasing experimental and clinical evidence suggests that triglyceride-rich lipoproteins (TRL) play a significant role in the pathogenesis of atherosclerosis.1–3 The value of measuring plasma triglyceride concentration and of treating patients with hypertriglyceridemia is therefore becoming more widely accepted.4–7 Epidemiological studies, however, have often failed to identify plasma triglyceride concentration as an independent risk factor for coronary artery disease (CAD).8 Despite a strong univariate association between plasma triglyceride levels and risk of CAD, this relationship often fails to reach statistical significance when other lipid risk factors, such as HDL cholesterol, are taken into account. Several reasons can be put forth to explain this phenomenon. First, plasma triglyceride concentration increases and decreases throughout the day in response to the ingestion of frequent meals. Even if measured after a 10- to 12-hour overnight fast (as is normal clinical practice), triglyceride levels vary considerably more than LDL and HDL cholesterol levels, with day-to-day biological variability being 23%, 9.5%, and 7% for triglyceride, LDL, and HDL cholesterol levels, respectively.9 Second, there is a strong metabolic interdependence between the levels of different plasma lipids and lipoproteins, and a significant mathematical correlation consequently exists between different lipoprotein parameters. This is particularly exemplified by the strong inverse correlation between levels of total triglyceride and HDL cholesterol,10 which thwarts attempts to assign statistically significant independence for these parameters. Third, plasma triglyceride is carried in a number of different lipoproteins (eg, chylomicrons, large and small VLDL, and chylomicron remnants), and the ability of different triglyceride-rich lipoproteins to promote atherosclerosis is not the same.

Differences in the potential atherogenicity associated with different types of TRL is best illustrated by the prevalence of premature CAD in patients with different inherited forms of hypertriglyceridemia. Type I or type V hyperlipoproteinemic patients have extremely high triglyceride levels caused most commonly by lipoprotein lipase or apolipoprotein (apo) C-II deficiency, and they have a significant increase in circulating levels of very large TRL. They are not, however, at greatly increased risk of CAD.11 In contrast, type III hyperlipoproteinemic patients have more moderate hypertriglyceridemia caused by reduced hepatic uptake of TRL remnants and subsequent accumulation in plasma of β-VLDL, and they do have an increased risk of CAD and peripheral vascular disease.12 Smaller, partially catabolized TRL (TRL remnants) are thus believed to be more atherogenic or thrombogenic than larger, newly-secreted TRL. This is supported by experimental data showing the following: (1) smaller remnant particles can diffuse into the arterial intima, whereas large chylomicrons and VLDL (diameter >75 nm) are excluded from entering the vessel wall13; (2) due to their reduced size, increased apoE content, and association with lipoprotein lipase, TRL remnants are more likely to be retained by heparan sulfate proteoglycans within the arterial intima14;
Biochemical Criteria Used to Separate and Quantify TRL Remnants and Reference to Studies Linking These Remnant Parameters to CAD

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IDL indicates intermediate density lipoproteins; RE, retinal ester; ISL, intermediate-sized lipoprotein; RLP, remnant-like particle.

(3) TRL-induced cholesteryl ester accumulation by macrophages is dependent on the exposure of apoE epitopes through lipolysis of TRL.15; (4) lipoprotein lipase–mediated generation of TRL remnants results in the formation of lipolytic products, which are cytotoxic to macrophages16 and which can increase endothelial cell layer permeability17; and (5) increased activity of coagulation factor XII in patients with hypertriglyceridemia is dependent on TRL lipolysis.18

Difficulties in Detecting and Isolating TRL Remnants

TRL remnants are formed in the circulation when apoB-48–containing chylomicrons of intestinal origin or apoB-100–containing VLDL of hepatic origin are converted by lipoprotein lipase (and to a lesser extent by hepatic lipase) into smaller and more dense particles.19 Compared with their nascent precursors, TRL remnants are depleted of triglyceride, phospholipid, and apoCs (and apoA-I and apoA-IV in the case of chylomicrons) and are enriched in cholesterol esters and apoE.20,21 They can thus be identified, separated, or quantified in plasma on the basis of their density, charge, size, specific lipid components, apolipoprotein composition, or apolipoprotein immunospecificity22 (Table). Each of these approaches has provided useful information about the structure and function of remnant lipoproteins and has helped to establish the role of TRL remnants in the pathogenesis of atherosclerosis. Accurate measurement and characterization of plasma remnant lipoproteins, however, has proven to be difficult for the following reasons: (1) despite their reduced size and triglyceride content, they are difficult to differentiate from their triglyceride-rich precursors; (2) due to their rapid plasma catabolism, they exist in plasma at relatively low concentrations; and (3) because remnants are at different stages of catabolism, they are very heterogeneous in size and composition. This latter characteristic is illustrated in Figure 1, in which TRL become progressively smaller, more dense, and less negatively charged as they are converted to TRL remnants. They gradually lose triglyceride and, in relative terms, become enriched in cholesteryl ester. They also lose their complement of C apolipoproteins (apoC-I, apoC-II, and apoC-III), which are replaced by apoE. At any given time, there is a continuous spectrum of different-sized remnants in the blood. Some of these particles are of intestinal origin. They contain apoB-48 and are more numerous after a fat-rich meal. The majority, however (in both the fed and fasted state), contain apoB-100 and are derived from the liver. Depending on the extent to which they have been lipolyzed, both species of TRL contain different proportions of triglyceride and cholesterol and may or may not contain apoCs or apoE. Remnant lipoproteins are thus structurally and compositionally diverse, which has made it necessary to use different biochemical techniques for the detection, quantification, and characterization of these lipoproteins.

Remnant Lipoproteins Separated According to Density

The traditional method for isolating TRL remnants is by ultracentrifugation, whereby analytical, sequential, or density gradient ultracentrifugation has been used to isolate lipoproteins intermediate in density (1.006 < d < 1.019 g/mL; Sf 12 to 20) between VLDL and LDL.23 IDL concentration in plasma has been measured in terms of IDL total mass, cholesterol, triglyceride, or apoB. In normolipidemic subjects, the plasma concentration of IDL cholesterol is 5 to 15 mg/dL, and the total mass of IDL is 10 to 30 mg/dL.24–26 Under normal circumstances, 3% to 10% of total plasma cholesterol is thus isolated as IDL cholesterol, whereas in patients with significant plasma remnant lipoprotein accumulation (type III hyperlipoproteinemia), 15% to 20% of total plasma cholesterol is isolated as IDL.27 Individuals with combined hyperlipidemia tend to have higher levels of IDL cholesterol than those with hypertriglyceridemia or hypercholesterolemia alone (18.8 ± 4.7 versus 15.6 ± 4.7 versus 11.4 ± 7.9 mg/dL, respectively),28 and patients with familial hypercholesterolemia (FH) have significantly higher IDL cholesterol levels than control subjects, although there is no significant difference between IDL levels in FH heterozygotes and homozygotes.24 In normolipidemic subjects, lipoproteins in the IDL fraction have a hydrated particle diameter of 27.5 to 30 nm and contain 10% to 20% triglyceride, 40% to 50% cholesterol, 21% phospholipid, and 18% protein.28 The majority of IDLs have a charge similar to that of LDL. More than 90% of IDL protein is composed of apoB-100, and in relative terms, this fraction contains only small amounts of apoE and apoCs.

Although IDL cholesterol concentration is the parameter most widely used in research laboratories to assess plasma
remnant lipoprotein concentration, there is no standardized clinical procedure for its measurement. Probably the most accurate approach is to simultaneously centrifuge a sample of serum or plasma at d=1.006 g/mL and a second aliquot at d=1.019 g/mL. IDL cholesterol can then be calculated as the difference between cholesterol in the d=1.006 and d=1.019 fractions. Unfortunately, this means that 2 relatively large, experimentally-determined numbers are subtracted to obtain a considerably smaller number, which then has an inherently large experimental error. The precision of this assay is therefore less than optimal, and variability between laboratories can be high. Ultracentrifugation also requires costly and specialized equipment that is not readily available in all clinical laboratories. It also must be recognized that the IDL fraction represents a collection of cholesterol-enriched, triglyceride-depleted remnant particles that does not include the triglyceride-rich, less completely catabolized remnants having a density <1.006 g/mL. Small VLDL (S, 20 to 60) have been shown in vivo kinetic studies to be derived from large VLDL, 29 and to be of similar, if not greater, pathophysiological significance than IDLs. 30,31 For this reason, IDLs have sometimes been defined as lipoproteins in the S, 12 to 60 range.

Numerous cross-sectional studies have demonstrated that patients with CAD tend to have increased plasma IDL levels. 25,30,32–35 The first of these studies was published in 1950 and showed that the incidence of measurable concentrations of lipoproteins in the S, 10 to 20 range was significantly higher in 20- to 40-year-old men compared with females of the same age, subjects >40 years of age compared with younger subjects, diabetic versus non-diabetic subjects, and patients proven to have had a myocardial infarction (MI) compared with control subjects. 32 Japanese male and female survivors of acute MI (n=97) similarly had increased IDL (1.006<d<1.019 g/mL) triglyceride and cholesterol levels, 33 and Canadian men with objectively documented CAD were found to have significantly higher IDL (S, 12 to 60) triglyceride and apoB levels than subjects free of disease. 30 Multivariate analysis demonstrated that CAD was independently related to smoking and IDL levels. Individuals who smoked had higher IDL levels than nonsmokers, although the association between IDL levels and CAD did not appear to depend on smoking. 30

Plasma concentrations of IDL have been related to the extent and severity of angiographically-assessed coronary artery atherosclerosis in both cross-sectional 34,35 and longitudinal 31,36–38 studies. Serum total IDL (S, 12 to 20) mass concentrations were measured by analytical ultracentrifugation in a subset of 57 male subjects in the National Heart, Lung, and Blood Institute Type II Coronary Intervention Study, 36 in which treatment of hypercholesterolemic subjects with diet and cholestyramine resin for 5 years resulted in reduced progression of CAD, as assessed by angiography. 39 Changes in IDL levels measured over 2 years were strongly associated with the extent of progression of CAD in both drug- and placebo-treated subjects. Changes in IDL mass and ratios of HDL to total cholesterol or HDL to LDL cholesterol were inversely correlated and had a similar ability to predict disease progression. 36 IDL cholesterol levels were also measured at repeated intervals during the 3 year duration of the St. Thomas’ Atherosclerosis Regression Study (STARS). 40 Seventy-four hypercholesterolemic men with CAD completed treatment with placebo, diet alone, or diet plus cholestyramine. Mean absolute width of angiographically assessed coronary segments increased significantly in the active-treatment groups. Improvement in CAD was inversely related to in-trial IDL and LDL cholesterol levels and positively related to in-trial HDL cholesterol levels, although multiple linear regression analysis did not identify IDL as a significant independent predictor of disease. 37 In a somewhat larger study 38 (272 men and 63 women) with a follow-up period of 4 to 6 years, IDL cholesterol was found to have a significant positive correlation and HDL cholesterol a significant negative correlation with 2-year difference in mean percentage diameter of coronary artery stenoses. Remnant lipoprotein cholesterol concentration (defined as the sum of cholesterol in IDL plus estimated remnant cholesterol in VLDL) was found to be independently associated with progression of CAD, as well as with ischemic cardiovascular events; a 1 mg/dL increase in remnant cholesterol concentration was associated with a 2% increase in the possibility of a cardiovascular event. These findings are in turn supported by results from the Monitored Atherosclerosis Regression Study (MARS), in which middle-aged men and women were randomized to treatment with lovastatin or placebo. Coronary artery lesion progression was found to be independently correlated with plasma concentrations of small VLDL (S, 20 to 60) (as well as inversely with HDL₃), 31 whereas the increase in carotid intima-media thickness was independently correlated with IDL levels (S, 12 to 20). 41
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Separation of VLDL remnant lipoproteins by agarose gel electrophoresis. Total plasma, d<1.006 g/mL (VLDL) and d>1.006 g/mL (LDL+HDL) fractions are shown for a healthy subject, a patient with double pre-beta-lipoproteinemia (DPBL), and a patient with type III hyperlipoproteinemia. The healthy subject was a 42-year-old male patient with a plasma cholesterol concentration of 6.23 mmol/L, a plasma triglyceride level of 2.02 mmol/L, and an HDL cholesterol level of 1.11 mmol/L. The DPBL patient was a 58-year-old female with a plasma cholesterol concentration of 6.49 mmol/L, a plasma triglyceride level of 5.56 mmol/L, and an HDL cholesterol level of 1.06 mmol/L. The type III patient was a 43-year-old male with a plasma cholesterol concentration of 9.83 mmol/L, a plasma triglyceride level of 7.00 mmol/L, and an HDL cholesterol level of 0.78 mmol/L. The point of sample application (origin) is indicated. Lipoprotein bands were lipid stained with Sudan black. The α-, pre-β-, and β-migrating regions of the gel are also labeled. In the total plasma of the normolipemic subject, these bands correspond to HDL, VLDL, and LDL, respectively. The band above the α-migrating HDL represents free fatty acids (FFA) bound to albumin. Two VLDL bands (pre-β and slow pre-β) are identifiable in the d<1.006 g/mL fraction of the DPBL patient. The slow pre-β-VLDL (remnant) band is indicated by an arrow and is also visible in total plasma. The slow pre-β band of the DPBL patient migrates further than the β-migrating remnant VLDL of the type III patient (also indicated by an arrow). Taken from Reference 43.

Remnant Lipoproteins Separated According to Their Charge

Plasma lipoproteins have routinely been separated according to their charge by agarose gel electrophoresis. Large TRL of intestinal origin (chylomicrons) remain at the origin of the gel, while ultracentrifugally-isolated VLDL (d<1.006 g/mL) normally migrate as a single band with pre-β mobility (Figure 2). Smaller, less-triglyceride-rich VLDL are less negatively charged and migrate with slower mobility.12,42 They often appear as a diffuse smear of lipid-stained material at the trailing edge of the pre-β-migrating band. In some individuals, particularly those with combined hyperlipidemia and an apoE 3/2 or 4/2 genotype,43 these remnants form a second distinct, slow pre-β-migrating band, this characteristic being referred to as “double pre-β lipoproteinemia” (DPBL).21 In extreme cases, exemplified by patients with type III hyperlipoproteinemia, remnant lipoproteins are significantly enriched in apoE and cholesteryl ester, and they migrate (like LDL) with β-mobility (ie, β-VLDL; Figure 2).44 Slow pre-β VLDL have a particle diameter of 33 to 38 nm and are composed of 41% triglyceride, 26% cholesterol, 18% phospholipid, and 15% protein. Approximately 60% percent of this protein is apoB-100, 8% is apoE, and the remainder (≈30%) is composed of apoCs.21 The majority of β-VLDL have a particle diameter of 35 to 110 nm. On average, they are more cholesterol rich than slow pre-β VLDL, containing an average of 38% triglyceride, 34% cholesterol, 18% phospholipid, and 10% protein. They also differ from slow pre-β VLDL by containing significant amounts of chylomicron remnants, as evidenced by the presence of apoB-48 (representing as much as one third of total β-VLDL apoB).43 From a clinical perspective, the measurement of plasma β-VLDL (or slow pre-β VLDL) is of limited diagnostic value, because time and expense are required to perform the ultracentrifugal isolation of VLDL fractions, subjective judgment is required to identify slow-migrating VLDL, and although densitometric scanning of lipid-stained lipoprotein bands or calculation of β-VLDL cholesterol concentration with a formula46 can be used to estimate the presence of remnant lipoproteins, slow-migrating VLDL are difficult to quantify accurately and objectively. Separation of remnant lipoproteins according to their charge, therefore, remains a qualitative rather than quantitative assessment of plasma remnant lipoprotein levels.

The best clinical evidence for the ability of β-VLDL to promote atherosclerosis is provided by patients with type III hyperlipoproteinemia,12 who have greatly increased plasma levels of β-VLDL and increased risk of coronary and carotid artery atherosclerosis, as well as peripheral vascular disease.47 Experimental animals fed diets containing large amounts of cholesterol also have increased circulating levels of cholesteryl ester–rich β-VLDL. These lipoproteins are believed to be directly responsible for development of atherosclerosis because of their ability to induce lipid accumulation in cultured macrophages.48 β-VLDL isolated from dyslipidemic patients also cause macrophages in culture to take on the morphological characteristics of atherosclerotic foam cells, provided that these β-VLDL contain apoE with normal receptor-binding characteristics.49,50 This is supported by results of experiments with J774 mouse macrophages showing that cholesteryl ester accumulation induced by VLDL (S< sub-B> to 400) from hypertriglyceridemic (type IV) patients is substantially inhibited by the presence of anti-apoE monoclonal antibody.51 Most patients with elevated levels of β-VLDL and type III hyperlipoproteinemia, however, are homozygous for a variant form of apoE (apoE2), which binds poorly to lipoprotein receptors owing to a single amino acid substitution (Arg158→Cys).12 Transgenic mice or rabbits expressing this form of apoE have increased levels of β-VLDL and develop spontaneous atherosclerosis.52,53 Paradoxically, impaired binding of apoE2 to apoB/E receptors leads to poor macrophage recognition and uptake of apoE2-containing β-VLDL and minimal cellular cholesterol accumulation.51 Consequently, it has been proposed that the potential atherogenicity of these lipoproteins resides in their susceptibility to oxidation, which promotes foam cell formation by a mechanism analogous to that which occurs with oxidized LDL.54

Separation of Remnant Lipoproteins According to Their Size

TRL remnants are intermediate in size between VLDL and LDLs and have been separated as “midband lipoproteins” (between bands of VLDL and LDL) by 3% polyacrylamide gel electrophoresis.55 A similar separation can be achieved with 2% to 16% gradient gel electrophoresis.28 Increased
incidence of midband lipoproteins has been demonstrated in MI patients, FH patients with angiographic evidence of CAD, and FH patients with diabetes or impaired glucose tolerance. More directly, these lipoproteins have been shown to be potentially atherogenic because they promote foam cell formation in cultured macrophages. Preparative isolation of midband lipoproteins has not been performed, and therefore their biochemical characteristics have not been determined. It is nevertheless well accepted that IDLs, β-VLDL, and lipoprotein(a) [Lp(a)] are intermediate in size between VLDL and LDLs and are thus isolated as midband lipoproteins. Because an increased Lp(a) concentration is associated with increased risk of atherosclerosis and thrombosis, its inclusion within midband lipoproteins may be advantageous from a diagnostic standpoint. On the other hand, this assay represents a nonspecific measure of remnant lipoproteins and, like agarose gel electrophoresis, is a means for detecting rather than accurately measuring TRL remnants.

Identification of Remnant Lipoproteins

According to Lipid Composition

Although TRL remnants cannot be isolated on the basis of their lipid composition, their presence in plasma can be estimated according to certain of their lipid components. For example, retinyl esters (RE) in the blood after the ingestion of a vitamin A–containing fat-rich meal have been used as markers for the presence of apoB-48–containing TRL of intestinal origin (chylomicrons and their remnants). The rationale for this approach is based on the concept that dietary vitamin A is esterified in the intestine and is incorporated into the core of chylomicron particles. These lipoproteins are secreted into intestinal lymph, and their component triglycerides are hydrolyzed by lipoprotein lipase. It is assumed that the majority of RE remain associated with chylomicrons during lipolysis and are taken up within chylomicron remnants via hepatic receptor–mediated processes. The liver does not resecrete RE, and they are either stored or resecreted as unesterified retinol molecules bound to retinol binding protein. The measurement of plasma RE concentration in the fed state has provided important insights into dietary lipid and plasma chylomicron clearance; however, this approach cannot be considered an ideal measurement of circulating chylomicron remnants. First, the assumption that RE are always associated with apoB-48–containing lipoproteins is not totally accurate, because RE can be detected in apoB-100–containing TRL and in LDL and HDL at later postprandial time points. Second, it is difficult to distinguish newly synthesized chylomicrons from chylomicron remnants, and even though ultracentrifugation has been used to separate larger, less-dense RE-containing lipoproteins from smaller remnant-like particles, this separation on the basis of density is somewhat arbitrary. Despite these limitations, several studies have demonstrated that patients with CAD, or at increased risk of CAD, have increased postprandial levels of RE.

A second approach has been to estimate the presence of remnant lipoproteins in the VLDL (d<1.006 g/mL) fraction of plasma by calculating the VLDL cholesterol-to-triglyceride ratio or, alternatively, the ratio of VLDL cholesterol to total plasma triglyceride. The rationale behind this approach is that the presence of cholesteryl ester–enriched remnants in the VLDL fraction ought to be reflected by an increase in the ratio of VLDL cholesterol to triglyceride. The ratio of VLDL cholesterol to total plasma triglyceride has routinely been used as a diagnostic criteria for defining patients with type III hyperlipoproteinemia (ie, VLDL cholesterol/total triglyceride >0.3 for measurements in mg/dL or >0.7 for measurements in mmol/L). Patients with combined hyperlipidemia and DPBL also have elevated VLDL cholesterol/total triglyceride molar ratios (0.57±0.11) compared with combined hyperlipidemia patients without DPBL (0.47±0.10), and these ratios are both significantly less than in patients with β-VLDL (0.90±0.24). Studies have shown that VLDL cholesterol–to-triglyceride ratios are significantly higher in patients with CAD and are related to the progression of coronary artery atherosclerosis and to clinical events related to CAD.

Quantification of Remnant Lipoproteins

According to Apolipoprotein Composition

ApoE plays a central role in controlling TRL metabolism by participating in the lipolytic conversion of TRL remnants to LDL and by acting as a specific ligand for receptor-mediated uptake of TRL remnants by the liver. ApoE is consequently an important determinant of plasma remnant lipoprotein concentration, as illustrated by the pronounced accumulation of TRL remnants in patients with variant forms of apoE. Although it cannot be considered to be a specific marker of plasma remnants, owing to its presence on nascent hepatic VLDL, apoE is (under normal circumstances) a characteristic feature of TRL remnants. Different assays have therefore measured the concentration of TRL containing apoE or the concentration of apoE associated with TRL. In the former case, 2 site-differential enzyme-linked immunosorbent assays (ELISAs) have been used to measure the amount of apoB associated with plasma lipoproteins containing apoE. In normolipidemic subjects, ~20% of total plasma apoB is associated with apoE, decreasing to ~15% in hypercholesterolemic subjects and increasing to as much as 30% in hypertriglyceridemic and 85% in type III hyperlipoproteinemic subjects. Higher levels of apoB associated with apoE (LpE:B) occur in VLDL than in IDL or LDL, and hypertriglyceridemic subjects have LpE:B levels 2-fold higher than control subjects. LpE:B levels have been found to be significantly elevated in CAD case subjects compared with control subjects and to be higher in populations at greater risk of CAD. Two-site ELISAs have similarly been used to measure apoE in apoB-containing lipoproteins (LpB:E). ApoE has also been measured in TRL fractions isolated by ultracentrifugation, gel filtration chromatography, or plasma precipitation. ApoE has been determined in remnant-like intermediate-sized lipoproteins (ISL) separated by gel filtration chromatography. ISL apoE concentrations are particularly elevated in patients with combined hyperlipidemia or in patients with type III hyperlipoproteinemia. ISL apoE levels are significantly correlated with total triglyceride, cholesterol, and apoB and inversely related to HDL cholesterol levels. Plasma apoE-LpB concentration (measured as the difference between total plasma apoE and apoE in plasma made devoid of apoB-containing lipoproteins by immunoprecipitation) is also positively correlated with
total cholesterol, VLDL cholesterol, and total triglyceride levels and inversely correlated with HDL cholesterol levels.\textsuperscript{96} ApoE-LpB levels were found to be significantly higher in Irish but not in French MI survivors and to be higher in Irish than in French control subjects (Irish subjects being at significantly increased risk of MI). ApoE-LpB, however, was not found to be a statistically significant independent predictor of disease.\textsuperscript{96} Sequential immunofluorescence chromatography or immunoprecipitation has revealed the existence of 3 major species of plasma lipoproteins containing both apoE and apoB\textsuperscript{97}: LpB:C:E particles (ie, TRL containing apoC-I, C-II, C-III, and apoE); LpA-II:B:C:D:E particles (ie, TRL containing several apolipoproteins including apoA-II and apoE, characteristic of patients with Tangier disease or type V hyperlipoproteinemia\textsuperscript{98}); and LpB:E particles (ie, cholesteryl ester–enriched lipoproteins resembling LDL with apoE).\textsuperscript{99} Two additional apoB-containing lipoproteins in human plasma are LpB:C particles (ie, TRL containing only C apolipoproteins) and LpB (ie, LDL containing only apoB\textsuperscript{100}). It remains to be determined which of these lipoprotein species best represents TRL remnants. One can argue that each of them has certain remnant characteristics, some being more indicative of newly formed remnants (eg, LpB:C) and others being more characteristic of end products of TRL catabolism (eg, LpB:E).\textsuperscript{97} Ultimately, the final product of TRL catabolism is LDL or low-density LpB particles (lipoproteins with apoB-100 as their sole apolipoprotein and cholesteryl ester as their dominant neutral lipid), although these lipoproteins are not usually considered as remnants because they are not “intermediate” in their lipolytic conversion. The relative atherogenicity of different apoB-containing lipoproteins has not been completely elucidated,\textsuperscript{101} although elevated levels of all 3 TRL species (LpB:C, LpB:C:E, and LpA-II:B:C:D:E) have been linked to either the presence or severity of CAD.\textsuperscript{102,103}

Remnant lipoprotein accumulation can also occur in the absence of increased remnant apoE levels, as exemplified by the increased remnant levels of apoE-deficient individuals\textsuperscript{104} and of apoE gene–knockout mice.\textsuperscript{105,106} The latter animals have provided significant evidence for a link between apoE, remnant lipoproteins, and atherogenesis.\textsuperscript{107} Under normal circumstances, mice have low levels of VLDL and LDL cholesterol and carry the majority of their plasma cholesterol in HDL. They thus lack atherogenic lipoproteins and are relatively resistant to the development of atherosclerosis. Mice lacking apoE, however, are severely hypercholesterolemic, with average plasma cholesterol levels of 400 to 800 mg/dL on a regular chow diet. A large proportion of this cholesterol is carried in the VLDL plus IDL lipoprotein fractions. When fed a Western-type diet containing moderate amounts of cholesterol (0.15%) and fat (20%), they respond with even higher levels of VLDL and IDL and cholesterol levels of \(\sim1800\) mg/dL. TRL and TRL-remnant clearance are severely impaired, consistent with the well-recognized function of apoE as a ligand for lipoprotein receptors. Atherosclerosis develops spontaneously in apoE-deficient animals, with the appearance of foam cell lesions as early as 8 weeks and more advanced, complex lesions (resembling those in human disease) after 15 weeks.\textsuperscript{108,109} Although it has been proposed that apoE deficiency can lead to foam cell formation through impaired apoE-mediated cellular cholesterol efflux,\textsuperscript{110,111} accumulating evidence suggests that atherogenesis is a result of increased oxidation of VLDL and IDL remnant particles.\textsuperscript{112–114}

ApoC-III is a second plasma apolipoprotein that plays an important role in TRL metabolism and whose plasma concentration and lipoprotein distribution have been used to assess the extent of plasma TRL catabolism.\textsuperscript{115} Plasma apoC-III concentration is strongly correlated with that of total plasma triglyceride,\textsuperscript{116–118} which reflects the ability of apoC-III to inhibit the activity of lipoprotein lipase\textsuperscript{119–121} and to inhibit the recognition and uptake of TRL remnants by the liver.\textsuperscript{122–125} ApoC-III also has the potential to inhibit hepatic lipase,\textsuperscript{126} an enzyme that plays a critical role in the catabolism of both intestinal and hepatic TRL remnants.\textsuperscript{127} Like apoE, TRL apoC-III has been quantified in terms of the concentration of TRL containing apoC-III\textsuperscript{90,101} or in terms of the concentration of apoC-III associated with TRL.\textsuperscript{84,96,116–118,128,129} Angiographic studies have provided evidence that under certain circumstances, levels of apoC-III in whole plasma or in TRL are associated with increased coronary\textsuperscript{130,131} or carotid\textsuperscript{132,133} artery atherosclerosis. Plasma lipoprotein distribution of apoC-III between TRL and HDL has also been independently related to the severity of CAD in normotensive, nondiabetic subjects\textsuperscript{102} and to the presence of CAD in subjects from France and Northern Ireland participating in the ECTIM study.\textsuperscript{96} It must be remembered, however, that apoC-III can only be considered as a nonspecific marker of remnant lipoproteins, because it is unclear to what extent apoC-III in TRL represents the presence of large, innocuous TRL and to what extent it reflects the presence of smaller, potentially atherogenic TRL remnants. The lack of increase in atherosclerosis associated with plasma accumulation of large TRL in apoE gene–knockout mice overexpressing human apoC-III\textsuperscript{134} and the only modest increase in extent of atherosclerosis in dietary cholesterol-fed mice overexpressing human apoC-III\textsuperscript{135} provide experimental evidence for the association of apoC-III with larger, less-atherogenic TRL. In contrast, overexpression of human apoC-III in mice deficient in the LDL receptor results in the accumulation in plasma of smaller LDL-like (remnant) particles, which are in turn associated with increased development of atherosclerosis.\textsuperscript{136}

ApoB-48 is the major structural protein of chylomicrons secreted by the human small intestine and is an additional example of an apolipoprotein that has been used to measure the concentration of TRL and their remnants in circulating blood.\textsuperscript{137} ApoB-48 is not produced by the human liver\textsuperscript{138} and is therefore a specific marker for plasma chylomicrons (ie, TRL of intestinal origin). Thus, in normolipidemic subjects after an overnight fast, the plasma concentration of apoB-48 is very low (ie, 0.6±0.4 mg/L\textsuperscript{140} or 2.5±1.2 mg/L\textsuperscript{140} as measured by staining of apoB-48 separated by gradient slab gel electrophoresis; 0.46±0.27 mg/L as measured by ELISA\textsuperscript{141}). This reflects a low basal rate of intestinal production of apoB-48–containing TRL, maintaining the enterohepatic transport of lipids. Within 3 hours after the ingestion of a fat-rich meal, plasma concentration of apoB-48 increases 5-fold and remains elevated for 6 to 8 hours after the meal.\textsuperscript{142,143} Fasting and postprandial levels of apoB-48 are 2- to 3-fold higher in patients with endogenous hypertriglyceri-
Remnant Lipoproteins Separated According to Their Apolipoprotein Immunospecificity

A system based on recognizing TRL remnants according to their apolipoprotein content and immunospecificity has been developed recently that provides a quantitative and clinically applicable approach to the measurement of plasma remnant lipoproteins. In this assay, remnant-like particles (RLP) are separated from plasma by immunoaffinity chromatography with a gel containing an anti–apoA-I and a specific apoB-100 monoclonal antibody (JI-H).\(^{147,148}\) The former antibody recognizes all HDL and any newly synthesized chylomicrons containing apoA-I, whereas the latter antibody recognizes all apoB-100–containing lipoproteins, except for certain particles enriched in apoE. The reason the anti–apoB-100 antibody does not recognize these latter apoE-enriched remnant-like lipoproteins is not entirely clear, although the amino acid sequence of the epitope region of the apoB-100 antibody is homologous to an amphipathic helical region of apoE, which suggests that apoE can compete for binding of the antibody to its epitope on apoB-100.\(^{148}\) HDL, LDL, large chylomicrons, and the majority of VLDL are thus retained by the gel. The unbound RLP are made up of remnant-like VLDL containing apoB-100 and TRL containing apoB-48, which are routinely measured in terms of cholesterol, although they can also be quantified in terms of triglyceride or specific apolipoproteins (ie, apoB, apoC-III, or apoE\(^{149}\)).

Plasma concentration of RLP cholesterol has been shown to be significantly correlated with the plasma concentration of total triglyceride, VLDL triglyceride, and VLDL cholesterol. It is not strongly correlated with LDL cholesterol or LDL apoB.\(^{140,150}\) Median concentration of RLP cholesterol is 5.9 mg/dL (0.15 mmol/L) in 35- to 54-year-old American men and 4.6 mg/dL (0.12 mmol/L) in similarly-aged women.\(^{151}\) RLP cholesterol is higher in older versus younger subjects.\(^{147,150}\) men versus women,\(^{150,151}\) postmenopausal versus premenopausal women,\(^{150}\) the fed versus the fasted state,\(^{152,153}\) and patients with a familial dysbetalipoproteinemia,\(^{147,148}\) hemo dialysis patients,\(^{156,157}\) and patients with coronary artery restenosis after angioplasty.\(^{158}\) It has been demonstrated that RLP cholesterol concentration is significantly higher in patients with CAD than in control subjects.\(^{147,151,159–166}\) The potential atherogenicity of RLP is supported by the observation that RLP can promote lipid accumulation by mouse peritoneal macrophages,\(^{163}\) stimulate whole-blood platelet aggregation,\(^{164,165}\) and impair endothelium-dependent vasorelaxation.\(^{166}\)

Although certain physiological and pathophysiological aspects of the RLP fraction have been investigated, relatively little is known about the lipoprotein composition of this fraction and to what extent this composition varies from one individual to another. The physical and chemical properties of lipoproteins not recognized by the apoB-100 monoclonal antibody JI-H, subsequently isolated by ultracentrifugation at a density <1.006 g/mL, have been described, however.\(^{167}\) These lipoproteins contained more molecules of apoE and cholesteryl esters than those that were bound, consistent with them being remnant-like lipoproteins. They had slow pre-\(\beta\) electrophoretic mobility compared with the bound VLDL fraction and ranged in size from 25 to 80 nm. Other lipoproteins, however, may be present when the JI-H monoclonal antibody (together with an anti–apoA-I antibody) is used to isolate RLP by immunoaffinity chromatography from total plasma (in the absence of ultracentrifugation). High-performance liquid chromatographic analysis of RLP fractions isolated in this way from normolipidemic and diabetic subjects\(^{148}\) and fast protein liquid chromatographic analysis of RLP from type III and type IV patients\(^{168}\) have revealed considerable size heterogeneity in RLP, with particles ranging in size from VLDL to HDL. The relative amount of lipid and apolipoprotein in RLP can also vary considerably from one individual to another, with hypertriglyceridemic patients having more triglyceride and apoC-III and less apoE relative to apoB in RLP than normolipidemic subjects.\(^{149}\) Hypertriglyceridemic patients invariably have elevated levels of RLP cholesterol, and the clinical usefulness of this assay will depend on future studies being able to show that RLP cholesterol concentration can predict the presence of coronary or carotid atherosclerosis independently of plasma triglyceride level.\(^{160,169}\)

Physical or Biochemical Determinants of Remnant Atherogenicity

Despite the large number of clinical and experimental studies linking remnant lipoproteins and CAD, the question remains: what physical and/or biochemical characteristics are responsible for making these lipoproteins potentially atherogenic? Possible determinants of remnant atherogenicity have been mentioned in previous sections, but they can be summarized as follows.

Size and Number

In the initial stages of atherosclerosis, lipid accumulation in the artery wall is dependent on sub endothelial entry and retention of lipid-rich lipoproteins.\(^{170}\) The influx of lipoproteins into the intima increases directly with increasing lipoprotein concentration in plasma and decreases inversely with increasing lipoprotein diameter.\(^{171}\) Very large TRL (diameter >75 nm) thus appear to be too large to enter the vessel wall, whereas smaller, partially lipolyzed TRL have greater access.\(^{172}\) Fractional loss of lipoproteins from the intima is also dependent on size, meaning that VLDL, IDLs, and LDLs are retained in the intima to a greater extent than HDL or albumin.\(^{173}\) It is significant that in both fasting diabetic and nondiabetic individuals, smaller TRL (Sf 12 to 60) are 4 to 6 times more prevalent in plasma than larger TRL
(S, 60 to 400) and that ≈70% of interindividual differences in plasma triglyceride concentration are due to differences in the number rather than the size of TRL.\textsuperscript{124,175}

**ApoE**

Cholesterol-loaded macrophages are a characteristic feature of developing atherosclerotic lesions and, as mentioned before, can be produced in vitro by incubating cultured macrophages with TRL and their remnants.\textsuperscript{49} This has been shown to be a 2-step process\textsuperscript{51} whereby lipoprotein lipase secreted by macrophages hydrolizes lipoprotein triglycerides and liberates free fatty acids, which are taken up and reesterified into triglycerides. The cholesteryl ester–enriched remnants are then taken up by a receptor-mediated mechanism mediated by apoE. Functional apoE is critical to this process, because cellular cholesteryl ester accumulation can be blocked with anti-apoE monoclonal antibody and does not occur with apoE2-containing β-VLDL from type III hyperlipoproteinemic patients.\textsuperscript{50,51} The absolute amount of apoE on each remnant particle, the conformation of individual apoE molecules, and the presence of other remnant apolipoproteins may be important determinants. The pathophysiological relevance of apoE-mediated remnant uptake by macrophages in vivo, however, needs to be established, particularly because apoE is regarded as having antiatherogenic rather than proatherogenic properties.\textsuperscript{176}

**Oxidizability**

Evidence has been presented demonstrating that macrophage lipid accumulation can occur in the absence of apoE. For example, cellular cholesterol esterification and cholesteryl ester mass increase when macrophages are incubated with the VLDL/LDL fraction isolated from apoE gene–knockout mice.\textsuperscript{177,178} This effect may be due to apoE-independent binding of TRL to specific macrophage membrane-binding proteins\textsuperscript{179,180} or may be the result of cellular recognition and uptake of oxidized remnant lipoproteins. It has been shown that macrophage uptake of VLDL from type III and type IV patients is significantly enhanced by oxidation.\textsuperscript{54,181} Partial in vitro lipolysis of these VLDL caused increased susceptibility to oxidation and increased cellular cholesteryl ester accumulation. In vivo evidence for a link between remnant lipoprotein oxidation and atherosclerosis is provided by apoE gene–knockout mice, which have very high remnant levels, and very high autoantibody titers to epitopes of oxidized lipoproteins, such as malondialdehyde-lysine.\textsuperscript{112,182} Treatment of these animals with antioxidants, which had little effect on their plasma lipoprotein levels, was found to significantly reduce the extent of their atherosclerosis.\textsuperscript{113,114} Oxidized lipids may particularly be an atherogenic determinant of chylomicron remnants, on the basis of the hypothesis that oxidized lipids in the circulation can be derived from oxidized lipids in the diet. In rats, the level of oxidized lipids in mesenteric lymph chylomicrons and in the serum VLDL plus LDL fraction has been directly correlated with the quantity of oxidized lipids in the diet.\textsuperscript{183} Similarly, higher levels of oxidized lipids have been found in serum chylomicrons of humans consuming diets high in oxidized fat.\textsuperscript{184} Additional support for this concept is derived from rabbits fed an oxidized lipid (0.25% cholesterol) diet for 12 to 14 weeks, which were found to have increased oxidized lipid levels in their β-VLDL (but not LDLs) and which had a 100% increase in aortic fatty streak lesions compared with rabbits fed a control (0.25% cholesterol) diet.\textsuperscript{185}

**Conclusions**

In summary, plasma remnant lipoproteins of both hepatic and intestinal origin have been isolated and measured by use of a number of different biochemical methods and procedures. Each of these methods has contributed to our current understanding of the compositional characteristics of TRL remnants and their potential to promote atherosclerosis. Remnant lipoprotein parameters, however, cannot be considered to be equivalent, because some reflect the plasma concentration of larger, less completely catabolized TRL (eg, β-VLDL), whereas others reflect smaller, more cholesterol-rich remnant lipoproteins (eg, IDLs). The relative atherogenicity of these different remnant species and the physical or biochemical characteristics that determine their atherogenicity have not been clearly defined. These topics need to be addressed by future studies. There is also an ongoing search for more accurate and clinically-applicable remnant lipoprotein assays, which will be able to better define the risk of CAD in patients with hypertriglyceridemia.

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