Delayed In Vivo Catabolism of Intermediate-Density Lipoprotein and Low-Density Lipoprotein in Hemodialysis Patients as Potential Cause of Premature Atherosclerosis

Katsunori Ikewaki, Juergen R. Schaefer, Michael E. Frischmann, Keio Okubo, Tatsuo Hosoya, Seibu Mochizuki, Benjamin Dieplinger, Evi Trenkwalder, Horst Schweer, Florian Kronenberg, Paul Koenig, Hans Dieplinger

Objective—Premature cardiovascular disease is the leading cause of death in patients with end-stage renal disease treated by hemodialysis (HD). Low-density lipoprotein (LDL) levels are not generally increased in HD patients, but their LDL metabolism is still poorly understood. We therefore investigated the in vivo metabolism of apoB-containing lipoproteins in two different ethnic populations of HD patients and controls.

Methods and Results—We performed stable isotope kinetic studies using a primed constant infusion of deuterated leucine in 12 HD patients and 13 healthy controls. Tracer/tracee ratio of apoB was determined by means of gas chromatography/mass spectrometry, and the modeling program SAAMII was used to estimate the fractional catabolic rate (FCR) of apoB. Mean LDL-apoB plasma concentrations were almost identical in both groups (HD: 95±30 mg/dL, controls: 91±40 mg/dL), whereas LDL-apoB FCR was 50% lower in HD patients as compared with controls (0.22±0.12 days⁻¹ versus 0.46±0.20 days⁻¹, P=0.001) with concomitantly decreased production rates of LDL. Compared with controls, intermediate-density lipoprotein (IDL)-apoB FCR was 65% lower (2.87±1.02 days⁻¹ versus 8.89±4.94 days⁻¹, P=0.014), accompanied by 1.5-fold higher IDL-apoB levels in HD. Very low-density lipoprotein metabolism was similar in both study groups.

Conclusions—In vivo catabolism of LDL and IDL is severely impaired in HD patients but misleadingly masked by normal plasma cholesterol levels. The resulting markedly prolonged residence times of both IDL and LDL particles might thus significantly contribute to the well-documented high risk for premature cardiovascular disease in HD patients. (Arterioscler Thromb Vasc Biol. 2005;25:2615-2622.)

Key Words: cardiovascular diseases ■ isotopes ■ kidney ■ lipoproteins ■ metabolism

Thirty years ago, Lindner and colleagues recognized in their seminal report the excessive risk of cardiovascular disease for hemodialysis (HD) patients. The prevalence and incidence of cardiovascular disease is much higher in HD patients, and current mortality rates are 10 to 20 times greater than the general population with rates even higher at young ages. A remarkable number of factors, including dyslipoproteinemia, chronic inflammation, hypertension, oxidative stress, elevated homocysteine, and anemia, that may contribute to this increased frequency of atherosclerotic complications have been identified.

HD patients are characterized by a complex plasma dyslipoproteinemic profile. The most notable quantitative abnormalities are elevated plasma triglyceride and very low-density lipoprotein (VLDL) levels with a prevalence of 25% to 75%, increased levels of atherogenic intermediate density lipoprotein (IDL) and lipoprotein(a) particles, and decreased high-density lipoprotein (HDL) levels. Interest-ingly, total and low-density lipoprotein (LDL) cholesterol plasma levels are usually normal or even subnormal in HD patients as compared with healthy controls.

In addition to quantitative changes in lipoprotein particles, numerous compositional and qualitative lipoprotein changes have been demonstrated as well. These include accumulation of small dense LDL as well as oxidation, glycation, and carbamylation of LDL. The association of small dense LDL...
with increased risk for cardiovascular disease in the general population has been controversially discussed.\textsuperscript{14} The abnormal lipid composition of all lipoprotein classes has been reported to be caused mainly by a combination of an impaired reversed cholesterol transport\textsuperscript{11} and lipolytic cascade.\textsuperscript{18}

To date, only 2 apoB kinetic studies have been reported in HD patients: Chan et al injected radio-labeled VLDL into HD patients with or without hyperlipidemia and found decreased fractional catabolic rates (FCR) of VLDL- and LDL-apoB (the latter only in hyperlipidemic patients).\textsuperscript{16} Unfortunately, the LDL turnover was not investigated in this study. Hörkkö et al injected radio-labeled LDL and observed a decreased LDL-apoB clearance in renal patients treated with peritoneal dialysis but not in HD patients.\textsuperscript{17} Overall, the exact underlying metabolic abnormalities of apoB-containing lipoproteins in HD patients are far from clear. LDL-apoB kinetic studies in predialysis patients with chronic kidney disease have yielded controversial results; radiotracer studies reported decreased LDL clearance rates,\textsuperscript{18} whereas more recent studies using stable isotopes found unchanged FCR values for LDL-apoB in these patients.\textsuperscript{19}

To resolve the apparent discrepancy between an obviously impaired lipoprotein metabolism and normal LDL plasma concentrations in HD patients, we independently studied the in vivo kinetics of apoB-containing particles in Austrian and Japanese HD patients and healthy controls using stable isotope technology. This study demonstrates for the first time significantly increased residence times of the most atherogenic lipoproteins IDL and LDL (despite normal levels of the latter) and might help explain the extremely high prevalence of cardiovascular disease in these patients.

**Subjects and Methods**

**Study Design**

The two kinetic studies followed corresponding protocols previously described\textsuperscript{20–22} and approved by the Internal Review Boards of the Philippus University of Marburg, Germany, the Innsbruck Medical University, Austria, and the Jikei University School of Medicine, Tokyo, Japan. Informed, written consent was obtained from each study participant before the study. The study was performed in all HD patients 1 day after dialysis. For 3 days preceding the study, all study participants received a standardized isocaloric diet composed of both types of leucine (second MS). In the Japanese study, isotopic enrichment was calculated to be equal to the fractional synthetic rate. Residence time equals 1/FCR. Fractional standard deviations, which equal the coefficient of variation of the respective parameter, for apoB FCR provided reasonable levels; the mean fractional standard deviation was 4.8\% for VLDL-apoB, 3.7\% for LDL-apoB, and 3.6\% for LDL-apoA1.

**Determination of Isotopic Enrichment**

Apolipoprotein bands were excised from gels, hydrolyzed in 6 mol/L HCl at 110°C for 24 hours under nitrogen and lyophilized. Free amino acids were purified from plasma or protein hydrolysates by cation exchange chromatography (AG-50W-X6; Bio-Rad Laboratories) and then derivatized to n-heptafluorobutyrylisobutyl esters and, after derivatization, analyzed by gas chromatography/two-stage quadrupole mass spectrometry in the chemical ionization and selected ion-monitoring mode, as previously reported.\textsuperscript{23} The ions monitored were 363.1 (mass-to-charge ratio for unlabelled L-leucine) and 366.1 m/z for labeled [2H3] L-leucine as parent ions (first mass spectrometry [MS]) and 280.1 m/z for the daughter ions of both types of leucine (second MS). In the Japanese study, isotopic enrichment was analyzed by gas chromatography (GC)-MS on a 6890 gas chromatograph connected to a 5973 quadrupole mass spectrometer (Hewlett Packard). Tracer enrichment was calculated as the tracer-to-tracer ratio, which is equivalent to the specific activity in radiotracer studies.\textsuperscript{24}

**Kinetic Modeling**

A multicompartmental model was built using an interactive computer program (SAAMII, version 1.1; SAAM Institute Inc)\textsuperscript{25} to determine apoB kinetic parameters. A previously published compartmental model\textsuperscript{26} was used as the template for this study. Briefly, the plasma amino acid pool (compartment 1) was used as a forcing function, followed by a delay compartment (compartment 2) for lipidoprotein assembly and subsequent secretion of lipoproteins from the liver. A single compartment was allocated for VLDL (compartment 3), IDL (compartment 4), and LDL (compartment 5). In the Austrian study group, the LDL compartment was excluded attributable to the lack of LDL data.

Individual percentage changes in plasma concentrations of VLDL-, IDL-, and LDL-apoB, triglycerides, total and HDL-cholesterol were within 5\% throughout the study period (data not shown), indicating steady state conditions. Therefore, the FCR was assumed to be equal to the fractional synthetic rate. Residence time equals 1/FCR. Fractional standard deviations, which equal the coefficient of variation of the respective parameter, for apoB FCR provided reasonable levels; the mean fractional standard deviation was 11.4\% for VLDL-apoB, 18.3\% for LDL-apoB, and 7.4\% for LDL-apoA1.

Because plasma volume (PV) has been shown to be increased in HD patients,\textsuperscript{25–29} we adjusted PV values by hematocrit (Hct) using a recently reported formula by Mitra et al\textsuperscript{30} (PV = blood volume [BV]/[1 + 0.86\%Hct]). The factor 0.86 corrects for the difference between Hct levels in the systemic circulation and whole-body Hct staff were studied. The mean age of HD patients was 51.4 ± 13 years, whereas the age of healthy control subjects was 35.6 ± 10 years. Dialysis was performed 3 times weekly for 4 hours on average. Dialysis adequacy was monitored monthly by calculating Kt/V values. In case of falling below a value of 1.2, dialysis session length was increased accordingly resulting in a value of 1.3 in 10 and 1.2 in 2 patients. HD patients had been dialyzed for an average of 66 months before the study. None of the study subjects had diabetes mellitus or any history of cardiovascular disease, familial hyperlipidemia, and nephrotic syndrome affecting the lipid metabolism at the time of this study. HD patients took vitamins, erythropoietin, and bicarbonate but had never received any lipid-lowering medication. The primary reason for developing ESRD as well as further clinical characteristics of all subjects are summarized in Table 1.

**Preparation of Lipoproteins and Apolipoproteins**

VLDL (d < 1.006 g/mL), IDL (d = 1.006 to 1.019 g/mL), and LDL (d = 1.019 to 1.063 g/mL) were isolated by sequential preparative ultracentrifugation from 5 mL of plasma (50.3 Ti rotor, L-70K centrifuge; Beckman Instruments). VLDL-apoB, IDL-apoB, and LDL-apoB were isolated by preparative 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. IDL were not collected from the Austrian study group.

**Study Participants**

A total of 12 male end-stage renal disease (ESRD) patients treated with HD (7 Austrians and 5 Japanese) and 13 male healthy controls (9 Austrians and 4 Japanese, selected from university and hospital
ELISA.34 An affinity-purified polyclonal antibody against apoB concentrations of plasma and fractions thereof were measured by LDL cholesterol was calculated with the Friedewald formula. ApoB with commercially available kits from Roche Diagnostics GmbH. Triglycerides, total cholesterol, and HDL cholesterol were measured Routine Laboratory Parameters Quantification of Lipids, Apolipoproteins, and Table 1. Clinical Characteristics of HD Patients and Control Subjects

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GP indicates Goodpasture syndrome; RAS, renal arterial stenosis; IgA, IgA nephritis; SK, small kidney; PN, polycystic kidney disease; CRP, C-reactive protein; Alb, albumin; BMI, body mass index; Chol, cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TG, triglycerides; ESRD, end-stage renal disease; Creat, creatinine.

Controls: subjects 1 to 9 (whites), subjects 10 to 13 (Japanese); Patients: subjects 1 to 7 (whites), subjects 8 to 12 (Japanese)

level as well as trapped plasma (~4%). BV is similar between HD patients and controls when normotensive patients are selected.31,32 Because patients in the present study were all normotensive, they were considered to have no intravascular fluid expansion. We therefore used 7% as the percentage BV (% BV/body weight) in both HD patients and control subjects.33 Taken together, production rate (PR, mg/kg per day) was calculated by the formula PR=FCR·apolipoprotein concentration, a plasma, / (1 to 0.86 · Hct) · 0.07.

Quantification of Lipids, Apolipoproteins, and Routine Laboratory Parameters

Triglycerides, total cholesterol, and HDL cholesterol were measured with commercially available kits from Roche Diagnostics GmbH. LDL cholesterol was calculated with the Friedewald formula. ApoB concentrations of plasma and fractions thereof were measured by ELISA. An affinity-purified polyclonal antibody against apoB (produced in our laboratory by immunizing rabbits with purified apoB) was used for coating and the same antibody, labeled with horseradish peroxidase, for detection. A calibrated standard (Apo-proteins Human ApoB; Technoclone) served as a secondary standard. ApoE phenotyping was performed on delipidated plasma by isoelectric focusing. Urea, creatinine, total protein, albumin, and C-reactive protein were determined using standard assays on a COBAS INTEGRA analyzer (Roche Diagnostics).

Statistical Analysis

Data are presented as mean±SD, except as noted. For statistical analysis we used the nonparametric Mann-Whitney U test to test for significant differences between groups. Nonparametric correlations were calculated according to Spearman test. Significance is defined as P<0.05.
Results
Clinical Characteristics of HD Patients and Controls
Lipids and lipoprotein profiles of the 12 HD patients and of 13 healthy controls are summarized in Table 1. Total cholesterol, LDL cholesterol, and apoB concentrations were almost identical in both groups. Triglycerides were significantly higher (137 mg/dL versus 100 mg/dL) and HDL cholesterol significantly lower (32 mg/dL versus 45 mg/dL) as compared with controls, which is in accordance with previous findings.12

Kinetics of ApoB-Containing Lipoproteins
Figure 1 illustrates the mean tracer/tracee curves of apoB from the Austrian and Japanese studies, respectively. In both study groups, VLDL curves did not differ between HD patients and controls (not shown). In contrast, the LDL tracer/tracee curve increased more slowly in HD patients than in control subjects. In the Japanese study, the tracer/tracee curve of IDL-apoB from HD patients increased slowly, reached a peak around 20 hours, and then slowly decreased. This was in contrast to the control IDL-apoB curve, which peaked around 10 hours and thus closely followed the VLDL apoB tracer/tracee curve, particularly toward the second half of the study period. In line with the results from the Austrian study, the LDL apoB tracer/tracee curve was also slower than the control’s counterpart and kept rising during the full study period of 48 hours.

Despite the fact that total cholesterol, LDL cholesterol, and LDL apoB levels were almost identical in HD patients and controls, we found dramatic differences in the in vivo kinetic parameters of IDL- and LDL-apoB between both groups, whereas the kinetic parameters of VLDL were not significantly different (Table 2 and Figure 2). Compared with controls, IDL-apoB FCR was one-third as high (2.87±1.02 versus 8.89±4.94 pools/d, \( P = 0.014 \)) in HD patients, accompanied by a 24% decrease in PR (9.05±3.08 versus 11.84±4.42 mg/kg-day) which did not, however, reach statistical significance (\( P = 0.221 \)). This resulted in a 60% increase in IDL-apoB levels in HD patients (6.2±1.8 versus 3.9±2.7 mg/dL). LDL-apoB FCR was significantly decreased to 50% in HD patients as compared with controls (0.22±0.12 versus 0.46±0.20 pools/d), which corresponded to a severely prolonged residence time of 4.6 days in HD versus 2.2 days in healthy controls. Furthermore, the LDL-apoB PR was significantly decreased in HD as compared with controls (9.8±4.9 versus 18.4±13.3 mg/kg-day).

Discussion
The major outcome of this study is the elucidation of an impaired metabolism of atherogenic lipoproteins which might significantly contribute to the high rate of cardiovascular disease in HD. We studied for the first time the in vivo kinetics of the atherogenic lipoproteins VLDL, IDL, and LDL in HD patients using stable isotope-labeling technology. Interestingly, the FCRs of IDL and LDL apoB were severely decreased in HD patients as compared with controls, whereas the in vivo kinetics of VLDL did not change significantly.

We did not investigate the metabolism of VLDL subfractions separately because it was previously shown in healthy subjects that FCR values of VLDL1 and VLDL2 did not differ from those of total VLDL.35,36 We cannot, however, exclude that changes in the subfraction distribution in HD patients could be different compared with controls. Another limitation of the study is the assumption of comparable BVs
between normotensive HD patients and controls, which is the prerequisite for calculating PV using the formula of Mitra et al. Previous investigations, however, have found very minor differences in the relative BV between HD patients and controls. HD results in a relative BV reduction in the range of up to 15% per ultrafiltration cycle. Even when suspecting a persistently reduced BV on the interdialytic day (which was our day of investigation), this effect is very unlikely having caused the large difference in PRs of LDL-apoB between patients and controls. The two other kinetic parameters (FCRs and residence times) are independent of parameters’ blood concentrations.

Markers for malnutrition and inflammation are widely recognized as predictors for cardiovascular disease in chronic kidney disease. Our HD patients did not show signs of inflammation or malnutrition: although their plasma albumin, total protein, and C-reactive protein plasma levels differed significantly from those of controls, they were within normal range. In fact, plasma levels in HD patients should be corrected for their Hct levels to be accurately comparable to those of healthy controls. This calculation would result in even higher mean values for total protein and albumin in HD patients compared with controls. Resulting C-reactive protein values would then still be within normal range (except patient #4 whose kinetic parameters were, nevertheless, all close to the mean levels of the whole patient group).

A decreased FCR for IDL and LDL apoB is identical to an extended residence time of these highly atherogenic particles. The longer residence time of these lipoprotein fractions results in an extended oxidation time of IDL and LDL in a highly oxidative environment. This was in fact experimentally shown by a highly significant correlation of 5-hydroxy-2-aminovaleric acid (HAVA) in LDL, an oxidation product of apoB-100, with the LDL residence time in normolipidemic controls. In line with these results, two recent randomized placebo-controlled studies revealed a significant reduction in

### Table 2. Plasma Concentrations, FCRs, and PRs of VLDL-apoB, IDL-apoB, and LDL-apoB From HD Patients and Control Subjects

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<th>VLDL-apoB-PR, mg/kg/day</th>
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<th>IDL-apoB-PR, mg/kg/day</th>
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</table>

Controls, Mean ± SD 3.7±3.5 14.38±12.18 21.90±24.91 3.9±2.7 8.89±4.94 11.84±4.42 90.5±39.8 0.462±0.197 18.44±13.30

Patients, Mean ± SD 5.3±4.0 9.26±8.87 17.51±15.51 6.2±1.8 2.87±1.02 9.05±3.08 94.6±29.7 0.216±0.123 9.77±4.91

P Value 0.221 0.157 0.663 0.327 0.014 0.221 0.550 0.001 0.026

n.d. indicates not determined.

See Table 1 for patient and control subject specifications.
significant difference in LDL-apoB FCR between their HD patients with chronic kidney failure, they could not find a difference to the above-mentioned study is an age difference between study groups. Finally, when we reanalyzed the data from the Finnish study, we found 2 HD patients (subjects 10 and 11) who had substantially higher LDL-apoB FCR (0.451 and 0.472 pools/d) than did the remaining HD patients. Indeed, when compared without these 2 outliers, LDL-apoB FCR was found also to be significantly decreased in the HD group (0.306 pools/day) as compared with the control group (0.376 pools/day, $P=0.0008$) indicating agreement with our results.

Several mechanisms might contribute to our observations. First, the diminished LDL catabolism in HD patients might be explained by a possible contribution of LDL uptake by the healthy human kidney, which does not function appropriately in chronic kidney failure. In fact, glomerular cells like mesangial or epithelial cells have been shown in vitro to express lipoprotein receptors and to take up LDL comparably to fibroblasts and hepatocytes. It is, however, completely unclear whether the kidney plays a significant role in LDL catabolism in vivo. Perfusion studies in rat kidneys indicated that virtually no intact LDL is cleared from the circulation by the kidney. Second, the impaired lipolytic cascade in HD patients most likely also contributes to our results. The relatively normal VLDL levels and kinetic parameters and the corresponding impaired IDL parameters are in good accordance with previous findings of normal lipoprotein lipase masses but significantly decreased activities of hepatic triacylglyceride lipase (HTGL) in HD patients. Because HTGL promotes the conversion of IDL to LDL, a decrease in HTGL activity might contribute to the accumulation of IDL and reduced PRs of LDL (without accumulating small dense LDL) in HD patients. In fact, analysis of the Japanese subjects of this study showed the conversion rate from IDL to LDL (k[5,4]) to be significantly decreased by 68% to 2.87±1.02 pools per day in HD patients as compared with 8.89±4.94 pools per day in control subjects ($P=0.014$), which is consistent with the previously reported 47% decrease in HTGL activity in HD patients.

Disorders in the metabolism of LDL with normal circulating plasma LDL levels have been reported to result from overproduction and increased clearance of LDL (reviewed by Grundy et al). Several impairments in LDL metabolism, including reduced clearance and increased PRs, have been described in various renal diseases. In contrast to the composite cardiovascular disease end points when HD patients were treated for two years with supplementation of antioxidants like vitamin E or acetylcysteine.

Most remarkably, the observed substantially impaired metabolism of apoB-100—containing lipoproteins is accompanied by normal levels of LDL-apoB and elevated levels of IDL-apoB (Table 2, Figure 2), in line with previous reports which found increased levels of IDL as an independent risk factor for atherosclerosis in HD patients.

Our kinetic data seem to contrast with a previously published turnover study in Finnish HD patients performed with conventional radiotracer techniques. Although the authors found decreased LDL clearance rates in predialysis patients with chronic kidney failure, they could not find a significant difference in LDL-apoB FCR between their HD patients and controls. The reason for this discrepancy is not clear. There might be ethnic differences in the lipoprotein metabolism between Finns, Austrians, and Japanese although we could not detect substantial differences in our data when stratified into Japanese and Austrian subgroups (Figure 2). FCR and residence time values of LDL-apoB differed significantly between HD patients and controls in both ethnic groups. The differences in PR of LDL-apoB between cases and controls did only reach significance when calculated in the total groups and showed only borderline significance when considered separately for each ethnic group, suggesting limited statistical power with respect to PR values. One major difference to the above-mentioned study is an age difference between patients and controls in our study but not in the Finnish study. Our control subjects were considerably younger than the HD patients (35 versus 51 years). At first glance, this age difference might explain to some extent the dramatic differences found in our study because LDL clearance rates have been repeatedly described to decrease with age presumably attributable to downregulated hepatic LDL receptor expression in the elderly. Based on the results of these studies, an age difference of 15 years (as observed in our work) would result in an $\approx10\%$ change in FCR values and could not therefore explain the more than 2-fold difference in our study. Comparative analysis of an age-matched subsample of five patients (subjects 1, 3, 5, 10, and 11, mean age 38.8 years) and controls (subjects 2, 3, 4, 5, and 11, mean age 38.2 years) revealed very similar mean LDL-FCR values (0.225 and 0.458 pools/d, respectively) as compared with the whole study collective. In addition, LDL-apoB FCR did not correlate with age in our study (HD patients: $r=-0.112$, $P=0.728$; controls: $r=0.165$, $P=0.590$), no matter whether the total group or Japanese and Austrian subjects were calculated separately. The observed differences in kinetic parameters therefore cannot be explained by age differences between study groups.

Disorders in the metabolism of LDL with normal circulating plasma LDL levels have been reported to result from overproduction and increased clearance of LDL (reviewed by Grundy et al). Several impairments in LDL metabolism, including reduced clearance and increased PRs, have been described in various renal diseases. In contrast to the
results shown in this study in HD patients, they all, however, result in elevated LDL plasma levels. Because lipoprotein metabolism substantially differs between the various stages and treatment modalities of chronic kidney disease (reviewed in6), it is not surprising to find the respective kinetic parameters of VLDL-, IDL-, LDL-apoB differently reported between predialysis, HD, and peritoneal dialysis.17–19

The implications of our study are far-reaching and not restricted to the investigated patient group. To the best of our knowledge, this is the first described example of a clinical condition in which reduced synthesis and clearance rates of an atherogenic lipoprotein are masked by normal plasma concentrations. The obtained results therefore demonstrate the need for in vivo kinetic studies to understand complex metabolic systems in humans, even in situations where the snap-shot ex vivo values seem to be almost normal. In particular, the observed alterations in lipoprotein metabolism put HD patients at high risk for developing atherosclerotic disease despite their normal cholesterol and LDL cholesterol plasma levels. These patients should therefore be identified and given appropriate therapy. In fact, recent studies using lipid-lowering therapy of ESRD (including HD) demonstrated a substantial normalization of the dyslipidemic plasma profile and reduced progression of renal disease51,52 and in one study also showed reduced mortality53 in these patients. Because most lipid-lowering drugs act by “normalizing” the residential times of the major atherogenic lipoproteins IDL and LDL,54 these drugs are expected to correct some of the basic defects of the severely disturbed lipoprotein metabolism in HD patients. Kinetic studies of the impact of lipid-lowering medication on the lipoprotein metabolism of ESRD (including HD) patients are therefore urgently required.

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Delayed In Vivo Catabolism of Intermediate-Density Lipoprotein and Low-Density Lipoprotein in Hemodialysis Patients as Potential Cause of Premature Atherosclerosis
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