Author Contributions

M. Nishida, T. Arai, H. Yoshida, K. Yamauchi-Takihara, T. Moriyama, N. Tada and S. Yamashita supervised the progress of the clinical trial and D. Masuda, H. Hanada, N. Tada and S. Yamashita undertook the examination of the data and the preparation of this article.

References

1) Iso H, Naito Y, Sato S, Kitamura A, Okamura T, Sankai T, Shimamoto T, Iida M, Komachi Y: Serum triglycerides and risk of coronary heart disease among Japanese men and women. Am J Epidemiol, 2001; 153: 490-499
2) Zilversmit DB: Atherogenesis: a postprandial phenomenon. Circulation, 1979; 60: 473-485
3) Karpe F: Postprandial lipoprotein metabolism and atherosclerosis. J Intern Med, 1999; 246: 341-355
4) Hussain MM: A proposed model for the assembly of chylomicrons. Atherosclerosis, 2000; 148: 1-15
5) Fujioka Y, Ishikawa Y: Remnant lipoproteins as strong key particles to atherogenesis. J Atheroscler Thromb, 2009; 16: 145-154
6) Nakajima K, Saito T, Tamura A, Suzuki M, Nakano T, Adachi M, Tanaka A, Tada N, Nakamura H, Campos E, Havel RJ: Cholesterol in remnant-like lipoproteins in human serum using monoclonal anti apo B-100 and anti apo A-I immunoaffinity mixed gel. Clin Chim Acta, 1993; 223: 53-71
7) Miyauchi K, Kayahara N, Ishigami M, Kuwata H, Mori H, Sugiuchi H, Irie T, Tanaka A, Yamashita S, Yamamura T: Development of a homogeneous assay to measure remnant lipoprotein cholesterol. Clin Chem, 2007; 53: 2128-2135
8) Kugiyama K, Doi H, Takazoe K, Kawano H, Soejima H, Mizuno Y, Tsunoda R, Sakamoto T, Nakano T, Nakajima K, Ogawa H, Sugiyama S, Yoshimura M, Yasue H: Remnant lipoprotein levels in fasting serum predict coronary events in patients with coronary artery disease. Circulation, 1999; 99: 2858-2860
9) Sakai N, Uchida Y, Ohashi K, Hibuse T, Saika Y, Tomari Y, Kihara S, Hiraoka H, Nakamura T, Ito S, Yamashita S, Matsuzawa Y: Measurement of fasting serum apoB-48 levels in normolipidemic and hyperlipidemic subjects by ELISA. J Lipid Res, 2003; 44: 1256-1262
10) Hanada H, Mugii S, Okubo M, Maeda I, Kuwayama K, Hidaka Y, Kitazume-Taneike R, Yamashita T, Kawase R, Nakaoka H, Inagaki M, Yuasa-Kawase M, Nakatani K, Tsubakio-Yamamoto K, Masuda D, Ohama T, Matsuyama A, Ishigami M, Nishida M, Komuro I, Yamashita S: Establishment of chemiluminescence enzyme immunoassay for apolipoprotein B-48 and its clinical applications for evaluation of impaired chylomicron remnant metabolism. Clin Chim Acta, 2012; 413: 160-165
11) Masuda D, Sakai N, Sugimoto T, Kitazume-Taneike R, Yamashita T, Kawase R, Nakaoka H, Inagaki M, Nakatani K, Yuasa-Kawase M, Tsubakio-Yamamoto K, Ohama T, Nakagawa-Toyama Y, Nishida M, Ishigami M, Masuda Y, Matsuyama A, Komuro I, Yamashita S: Fasting serum apolipoprotein B-48 can be a marker of postprandial hyperlipidemia. J Atheroscler Thromb, 2011; 18: 1062-1070
12) Kinoshita M, Ohnishi H, Maeda T, Yoshimura N, Takeoka Y, Yasuda D, Kusano J, Mashimo Y, Saito S, Shimamoto K, Teramoto T: Increased serum apolipoprotein B48 concentration in patients with metabolic syndrome. J Atheroscler Thromb, 2009; 16: 517-522
13) Masuda D, Nakagawa-Toyama Y, Nakatani K, Inagaki M, Tsubakio-Yamamoto K, Sandoval JC, Ohama T, Nishida M, Ishigami M, Yamashita S: Ezetimibe improves postprandial hyperlipidaemia in patients with type IIb hyperlipidaemia. Eur J Clin Invest, 2009; 39: 689-698
14) Masuda D, Hirano K, Oku H, Sandoval JC, Kawase R, Yuasa-Kawase M, Yamashita Y, Takada M, Tsubakio-Yamamoto K, Tochino Y, Koseki M, Matsuura F, Nishida M, Kawamoto T, Ishigami M, Hori M, Shimomura I, Yamashita S: Chylomicron remnants are increased in the postprandial state in CD36 deficiency. J Lipid Res, 2009; 50: 999-1011
15) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirose K, Funahashi T, Yokote K, Yokode M: Japan Atherosclerosis Society (JAS) Committee for Epidemiology and Clinical Management of Atherosclerosis. Diagnostic criteria for dyslipidemia. Executive summary of Japan Atherosclerosis Society (JAS)'s Guidelines for diagnosis and prevention of atherosclerotic cardiovascular diseases for the Japanese. J Atheroscler Thromb, 2007; 14: 155-158
16) Matsuzawa Y: Metabolic syndrome: Definition and diagnostic criteria in Japan. J Jpn Soc IntMed, 2005; 94: 188-203
17) Teramoto T, Sasaki J, Ishibashi S, Birou S, Daida H, Dohi S, Egusa G, Hiro T, Hirobe K, Iida M, Kihara S, Kinoshita M, Maruyama C, Ohta T, Okamura T, Yamashita S, Yokode M, Yokote K: Executive Summary of the Japan Atherosclerosis Society (JAS) Guidelines for the Diagnosis and Prevention of Atherosclerotic Cardiovascular Diseases in Japan -2012 Version. J Atheroscler Thromb, 2013; 20: 517-523
18) Nakajima K, Saito T, Tamura A, Suzuki M, Nakano T, Adachi M, Tanaka A, Tada N, Nakamura H, Campos E, Havel RJ: Cholesterol in remnant-like lipoproteins in human serum using monoclonal anti apo B-100 and anti apo A-I immunoaffinity mixed gel. Clin Chim Acta, 1993; 223: 53-71
19) Lemieux S, Fontani R, Uffelman KD, Lewis GF, Steiner G: Apolipoprotein B-48 and retinyl palmitate are not equivalent markers of postprandial intestinal lipoproteins. J Lipid Res, 1998; 39: 1964-1971
20) Schneeman BO, Kotite L, Todd KM, Havel RJ: Relationships between the responses of triglyceride-rich lipoproteins in blood plasma containing apolipoproteins B-48 and B-100 to a fat-containing meal in normolipidemic humans. Proc Natl Acad Sci USA, 1993; 90: 2069-2073
21) Lovegrove JA, Isherwood SG, Jackson KG, Williams CM, Gould BJ: Quantitation of apolipoprotein B-48 in triacylglycerol-rich lipoproteins by a specific enzyme-linked immu-

nosorbent assay. Biochim Biophys Acta, 1996; 1301: 221-229
22) Lorec AM, Juhel C, Pafumi Y, Portugal H, Pauli AM, Lairon D, Defoort C: Determination of apolipoprotein B-48 in plasma by a competitive ELISA. Clin Chem, 2000; 46: 1638-1642
23) Uchida Y, Kurano Y, Ito S: Establishment of monoclonal antibody against human apo B-48 and measurement of apo B-48 in serum by ELISA method. J Clin Lab Anal, 1998; 12: 289-292
24) Yuasa-Kawase M, Masuda D, Kitazume-Taneike R, Yamashita T, Kawase R, Nakaoka H, Inagaki M, Nakatani K, Tsubakio-Yamamoto K, Ohama T, Toyama-Nakagawa Y, Nishida M, Ishigami M, Saito M, Eto M, Matsuyama A, Komuro I, Yamashita S: Apolipoprotein B-48 to triglyceride ratio is a novel and useful marker for detection of type III hyperlipidemia after antihyperlipidemic intervention. J Atheroscler Thromb, 2012; 19: 862-871
25) Tanimura K, Nakajima Y, Nagao M, Ishizaki A, Kano T, Harada T, Okajima F, Sudo M, Tamura H, Ishii S, Sugihara H, Yamashita S, Asai A, Oikawa S: Association of serum apolipoprotein B48 level with the presence of carotid plaque in type 2 diabetes mellitus. Diabetes Res Clin Pract, 2008; 81: 338-344
26) Nakatani K, Sugimoto T, Masuda D, Okano R, Oya T, Monden Y, Yamashita T, Kawase R, Nakaoka H, Inagaki M, Yuasa-Kawase M, Tsubakio-Yamamoto K, Ohama T, Nishida M, Ishigami M, Komuro I, Yamashita S: Serum apolipoprotein B-48 levels are correlated with carotid intima-media thickness in subjects with normal serum triglyceride levels. Atherosclerosis, 2011; 218: 226-232
27) Keane WF, Tomassini JE, Neff DR: Lipid abnormalities in patients with chronic kidney disease: Implications for the pathophysiology of atherosclerosis. J Atheroscler Thromb, 2013; 20: 123-133
28) Masuda D, Sugimoto T, Tsujii K, Inagaki M, Nakatani K, Yuasa-Kawase M, Tsubakio-Yamamoto K, Ohama T, Nishida M, Ishigami M, Kawamoto T, Matsuyama A, Sakai N, Komuro I, Yamashita S: Correlation of fasting serum apolipoprotein B-48 with coronary artery disease prevalence. Euro J Clin Invest, 2012; 42: 992-999

Original Article

Serum Apolipoprotein B-48 Concentration Is Associated with a Reduced Estimated Glomerular Filtration Rate and Increased Proteinuria

Manabu Okubo[1], Hiroyuki Hanada[1], Masahiko Matsui[1], Yoh Hidaka[2], Daisaku Masuda[3], Yasushi Sakata[3] and Shizuya Yamashita[4]

[1]Division of Laboratory for Clinical Investigation, Department of Medical Technology, Osaka University Hospital, Suita, Osaka, Japan
[2]Department of Laboratory Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[3]Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[4]Department of Community Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

Aim: Apolipoprotein B-48 (apoB-48) is a constituent of chylomicrons and their remnants (chylomicron remnants). A high concentration of serum apoB-48 is suspected to be a major risk factor for the development of atherosclerotic cardiovascular disease. Proteinuria and a reduced estimated glomerular filtration rate (eGFR) are independent risk factors for cardiovascular events and renal dysfunction. In the present study, we examined whether the serum apoB-48 concentration is associated with renal dysfunction.

Methods: A total of 264 patients was enrolled and classified into four groups according to the eGFR and level of proteinuria: a high eGFR (>60 mL/min/1.73 m^2) without proteinuria (≥1 + by urine dipstick) (n=50); a high eGFR with proteinuria (n=75); a low eGFR (>60 mL/min/1.73 m^2) without proteinuria (n=74); and a low eGFR with proteinuria (n=65). Biochemical markers of lipid metabolism, including the fasting serum apoB-48 concentration, were compared between the four groups.

Results: The serum log-apoB-48 and log-apoB-48/TG levels were significantly higher in the patients with a high eGFR with proteinuria, low eGFR with proteinuria and low eGFR without proteinuria than in those with a high eGFR without proteinuria, with the most significant differences for these parameters. The eGFR was found to be significantly correlated with the log-apoB-48 and log-apoB-48/TG levels, whereas urinary protein was found to be significantly correlated with the log-apoB-48 level only. A multiple regression analysis indicated that the log-apoB-48/TG level was a significant determinant of a reduced eGFR.

Conclusions: Both a low eGFR (<60) and proteinuria (≥1 +) are independent determinants of a high apoB-48 concentration. Taken together, the present results suggest that an increased serum apoB-48 concentration contributes to an increased risk of cardiovascular events.

J Atheroscler Thromb, 2014; 21:974-982.

Key words: Apolipoprotein B-48, Chylomicron remnants, Chronic kidney disease, eGFR, Proteinuria

Introduction

Proteinuria is an independent risk factor for the progression of chronic kidney disease (CKD) and the development of cardiovascular disease (CVD)[1, 2]. Patients with CKD are at a substantially increased risk of developing atherosclerosis, and approximately 40% of CKD patients have CVD even before they reach end-stage renal failure requiring hemodialysis[3]. The cardiovascular mortality is 30 times higher among hemodialysis patients than individuals from the general population[4]. In an effort to standardize the stage

Address for correspondence: Daisaku Masuda, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: masuda@cardiology.med.osaka-u.ac.jp
Received: January 6, 2014
Accepted for publication: March 17, 2014

of CKD, a system was recently developed based on the calculation of the estimated glomerular filtration rate (eGFR) using the serum creatinine level and age[5]. Recent data have demonstrated the impact of albuminuria, as well as eGFR, on the incidence of CVD, and it is evident that both measurements together confer an enhanced risk of CVD[6]. Recently, the prevalence of CKD has been increasing, approximately 13.3 million adults with CKD living in Japan[7]. Therefore, CKD is a major health problem worldwide, and its prevention is an urgent issue.

Dyslipidemia associated with CKD is characterized by an increased level of serum triglycerides (TG) and a decreased level of high-density lipoprotein (HDL) cholesterol (HDL-C)[8]. A high level of low-density lipoprotein (LDL) cholesterol (LDL-C) has been established as a strong risk factor for CVD; however, it is not consistently observed among patients with CKD[9]. It has also been reported that individuals with CKD have elevated levels of TG-rich lipoproteins (TRL), including chylomicrons (CM), very-low-density lipoproteins (VLDL) and their remnant lipoproteins[5]. Furthermore, many basic studies have suggested that remnant lipoproteins are associated with an increased risk of cardiovascular disease[10]. The accumulation of CM remnant (CM-R) particles, the hydrolyzed products of CMs produced by lipoprotein lipase (LPL), are related to foam cell formation among macrophages and may promote the development of atherosclerosis in the arterial wall[11, 12]. It is presumed that measurements of the serum apoB-48 concentration can be used to evaluate the synthesis and metabolism of CM and CM-R, as each contains one apolipoprotein B-48 (apoB-48) molecule per particle[13]. We previously developed a novel enzyme-linked immunosorbent assay (ELISA)[14, 15] and chemiluminescent enzyme immunoassay (CLEIA)[16] for conveniently determining the serum apoB-48 concentration. In our previous study, we reported that the serum apoB-48 concentrations are substantially elevated in patients with CKD of stage 4 (eGFR: 15-29 mL/min/1.73 m^2) and 5 (eGFR: <15 mL/min/1.73 m^2)[17]. Recently, Japanese guidelines for the diagnosis and treatment of CKD[18] were released that indicate that both an increased GFR and the existence of proteinuria may exacerbate the pathogenesis of CKD. However, few studies conducted to date have investigated the association between the fasting serum apoB-48 concentration and proteinuria and/or eGFR.

In the current study, we examined whether the serum apoB-48 concentration is associated with renal dysfunction in patients with a low eGFR and/or proteinuria.

Materials and Methods

Subjects

The study subjects included 264 patients who attended the outpatient clinic of Osaka University Hospital (Osaka, Japan) or who were admitted to the hospital. Subjects taking lipid-lowering medications and those with heart, liver or thyroid disease were excluded. All subjects provided their informed consent, and the research protocol was approved by the Ethics Committee of Osaka University Hospital.

Measurements of the Renal Function, Serum Lipids and Apolipoproteins

In each subject, a sample of blood was drawn from the cubital vein after an overnight fast (over 12 hours), and the serum was collected following brief centrifugation. The basal performance of the recently developed CLEIA for apoB-48 measurement kit (Fujirebio Inc., Tokyo, Japan) used in this study has been previously reported[16], and the assay was carried out on the Lumipulse *f* fully automated immunoassay analyzer (Fujirebio, Inc.). The serum total cholesterol (TC), TG and HDL-C levels were determined according to enzymatic methods, while the serum LDL-C levels were determined using the direct method (Sekisui Medical Co., Ltd., Tokyo, Japan). The non-HDL-cholesterol levels were calculated by subtracting the value of HDL-C from that of serum TC. The serum creatinine levels were measured using a photometric assay (Wako, Osaka, Japan). The urine test results were classified as (−), (±), (+), (2+) or (3+).

The eGFR values were subsequently estimated using the new equation proposed by the Japanese Society of Nephrology[19]: eGFR (mL/min/1.73 m^2) = $194 \times \text{S-Cr}^{-1.094} \times \text{Age}^{-0.287}$ (for female patients, the total value was multiplied by 0.739). An eGFR of < 60 mL/min/1.73 m^2 is generally considered to be indicative of CKD; therefore, subjects with an eGFR of <60 mL/min/1.73 m^2 (eGFR <60) were defined as having a low eGFR. As for proteinuria, (−) and (±) urine test results were defined as normal; all other results [(≥1+);(+), (2+) or (3+)] were defined as indicating proteinuria. All samples were treated in accordance with the Helsinki Declaration.

Statistical Analysis

Before the analysis, skewed variables (TG, apoB-48 and apoB-48/TG ratio) were logarithmically transformed to improve data normalization. The data are expressed as the mean ± SD for continuous variables and percentages for categorical variables. The significance of differences in the systolic BP, diastolic BP,

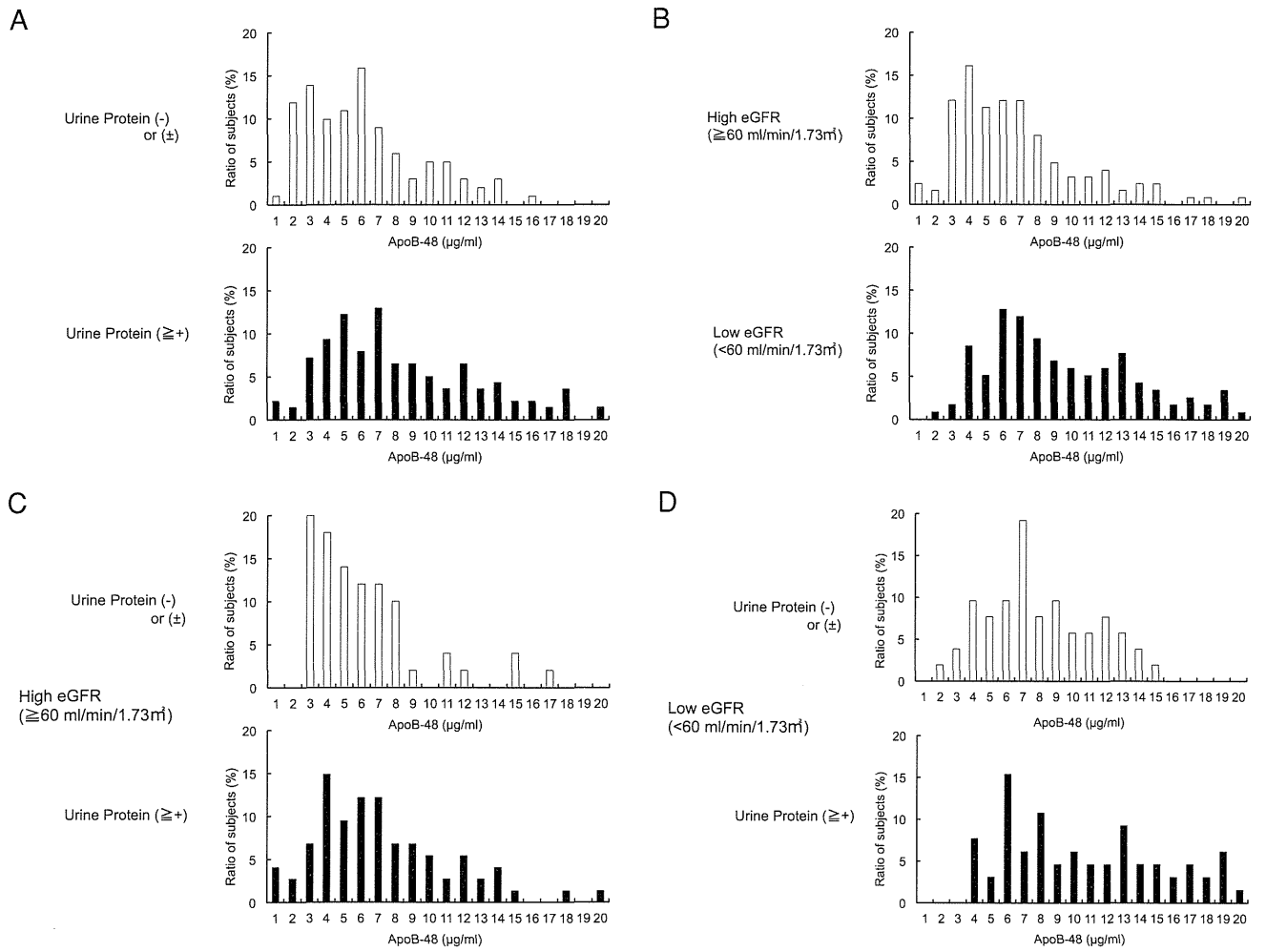

Fig. 1. Distribution of the fasting serum apoB-48 concentrations in the subjects with and without urinary protein, the subjects with a low and high eGFR and the subjects with both eGFR and proteinuria.

Serum apoB-48 concentration = 1 represents a concentration between 0.0 and 1.0 μg/mL.
A) Fasting serum apoB-48 concentrations in the subjects without urinary protein (open squares, $n = 124$) and with urinary protein (closed squares, $n = 140$).
B) Fasting serum apoB-48 concentrations in the subjects with a high eGFR (open squares, $n = 125$) and a low eGFR (closed squares, $n = 139$).
C) Fasting serum apoB-48 concentrations in the subjects with a combination of a high eGFR without urinary protein (open squares, $n = 50$) and a high eGFR with urinary protein (closed squares, $n = 75$).
D) Fasting serum apoB-48 concentrations in the subjects with a combination of a low eGFR without urinary protein (open squares, $n = 74$) and a high eGFR with urinary protein (closed squares, $n = 65$).

UA, TC, TG, HDL-C, LDL-C, non-HDL-C, log-apoB-48 and log-apoB-48/TG values between the four groups was tested using an analysis of variance (ANOVA) with the least significant difference (LSD) test as a post hoc test and the Bonferroni correction for multiple comparisons. The significance of differences with respect to the prevalence of diabetes and drug treatment were tested using the χ^2 test. Correlations between the kidney function parameters (eGFR and urinary protein) and various other parameters were analyzed using Spearman's rank correlation coefficient, and a stepwise multiple logistic regression analysis was performed to determine independent predictors of CKD. The statistical analyses were conducted using the JMP8 software program (SAS Institute, Cary, NC). Statistical significance was established at a p value of < 0.05.

Table 1. Clinical characteristics of the four groups based on eGFR and proteinuria

	High eGFR (≧60 mL/min/1.73 m²)		Low eGFR (<60 mL/min/1.73 m²)		*p* values[a]
	without proteinuria	with proteinuria	without proteinuria	with proteinuria	
Age (year)	60±15	61±17	65±12	63±13	0.215
Sex (m/f)	26/24	38/37	39/35	35/30	0.88
BMI (kg/m²)	22±4	22±3	23±3	23±4	0.362
Diabetes (%)	16 (32)	27 (36)	21 (28)	24 (37)	0.581
Prevalence of drug-treated patients (%)	7 (14)	10 (14)	10 (14)	13 (20)	0.666
Systolic BP (mmHg)	123±24	122±18	122±21	136±23[bcd]	<0.001
Diastolic BP (mmHg)	76±13	74±11	72±10	77±14	0.052
Prevalence of drug-treated patients (%)	10 (20)	26 (35)	30 (41)	43 (66)[bcd]	<0.001
eGFR (mL/min/1.73 m²)	80±18	79±16	36±16[c]	34±17[c]	<0.001
UA (mg/dL)	5.3±1.2	5.3±1.5	6.8±1.3[bc]	7.0±1.6[bc]	<0.001
Prevalence of drug-treated patients (%)	8 (16)	7 (9)	10 (14)	25 (38)[cd]	<0.001
TC (mg/dL)	194±42	205±58	296±47	205±70	0.648
Log-TG	2.0±0.2	2.2±0.2[b]	2.1±0.2[b]	2.1±0.2[b]	<0.001
HDL-C (mg/dL)	62±16	55±19	57±16	60±26	0.209
LDL-C (mg/dL)	109±44	121±49	114±41	117±48	0.495
Non-HDL-C (mg/dL)	133±40	150±55	139±33	145±63	0.301
Log-ApoB-48	0.57±0.28	0.74±0.29[b]	0.78±0.32[b]	0.89±0.26[bc]	<0.001
Log-ApoB-48/TG	0.28±0.13	0.35±0.13[b]	0.35±0.16[b]	0.41±0.11[bc]	<0.001

Data represent the means±S.D. Comparisons between patients were divided into 4 groups based on eGFR and proteinuria. We divided all subjects (n=264) into low (<60 mL/min/1.73 m²) and high (≧60 mL/min/1.73 m²) eGFR levels; these two groups were also divided into those without or with proteinuria (by dipstick). *P* values refer to the results after analysis after adjusting for age, gender, and BMI. BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; UA, uric acid; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

[a] *p* values for ANOVA test or χ^2 test.

[b] Bonferroni's post-hoc analysis <0.05 vs High eGFR without proteinuria.

[c] Bonferroni's post-hoc analysis <0.05 vs High eGFR with proteinuria

[d] Bonferroni's post-hoc analysis <0.05 vs Low eGFR without proteinuria

Results

Distribution of the Serum ApoB-48 Concentrations in the Patients with Proteinuria and a Low eGFR

The distribution of the serum apoB-48 concentrations in the patients with proteinuria was significantly shifted toward higher values as compared with that observed in the patients without proteinuria (**Fig. 1-A**). Meanwhile, in the patients with a low eGFR, the distribution was significantly shifted toward higher values as compared with that observed in the patients with a high eGFR (**Fig. 1-B**).

Comparison of Clinical Profiles between the High eGFR Subjects without Proteinuria, High eGFR Subjects with Proteinuria, Low eGFR Subjects without Proteinuria and Low eGFR Subjects with Proteinuria

Table 1 shows the characteristics of the four groups of subjects: those with a high eGFR without proteinuria (n=50), high eGFR with proteinuria (n=75), low eGFR without proteinuria (n=74) and low eGFR with proteinuria (n=65). The serum uric acid (UA) levels were significantly higher in the subjects with a low eGFR with and without proteinuria than in the age-, sex- and body mass index (BMI)-matched subjects with a high eGFR with and without proteinuria (**Table 1**). The fasting serum apoB-48 concentrations ranged from 0 to 17.0 μg/mL in the subjects with a high eGFR with or without proteinuria (**Fig. 1-C**), from 0 to 15.0 μg/mL in those with a low GFR without proteinuria and from 0 to 20.0 μg/mL in those with a low GFR without proteinuria (**Fig. 1-D**). The serum log-apoB-48 and log-apoB-48/TG levels were significantly higher in the patients with a high eGFR with proteinuria, low eGFR without proteinuria and low eGFR with proteinuria than in those with a high eGFR without proteinuria. The

Table 2. Correlation coefficients (r) between the urine protein levels and various parameters in all subjects

	Coefficient	Univariate *p* value
Age	−0.1123	0.0826
BMI	−0.0195	0.7653
Systolic BP	−0.2034	0.0017
Diastolic BP	−0.1151	0.0782
eGFR	−0.0240	0.7057
TC	0.0515	0.4262
Log-TG	0.2876	<0.0001
HDL-C	−0.1475	0.0220
LDL-C	0.0954	0.1398
Non-HDL-C	0.0930	0.1502
Log-ApoB-48	0.2622	<0.0001
Log-ApoB-48/TG	0.0876	0.1751

A univariate analysis was performed using Pearson's correlation analysis. Abbreviations; BMI, body mass index; BP; eGFR, estimated glomerular filtration rate; BP, blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

Table 3. Correlation coefficients (r) between eGFR and various parameters in all subjects

	Coefficient	Univariate *p* value
Age	−0.3938	<0.0001
BMI	−0.1121	0.0843
Systolic BP	−0.1208	0.0646
Diastolic BP	−0.0223	0.7341
Urin protein	−0.0244	0.7057
TC	0.0489	0.4498
Log-TG	−0.0218	0.7359
HDL-C	−0.0270	0.6764
LDL-C	−0.0106	0.8704
Non-HDL-C	−0.2693	<0.0001
Log-ApoB-48	−0.3090	<0.0001
Log-ApoB-48/TG	−0.3161	<0.0001

A univariate analysis was performed using Pearson's correlation analysis. Abbreviations; BMI, body mass index; BP, blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

average levels of serum log-apoB-48 and log-apoB-48/TG were higher in the patients with a low eGFR with proteinuria than in any of the other groups (**Table 1**).

Correlations between the Kidney Function and Various Parameters

The correlations between the renal function parameters (urinary protein and eGFR) and various other parameters were analyzed using a logistic regression analysis. According to Pearson's correlation analysis, significant correlations with urinary protein were observed for systolic blood pressure (BP), log-TG, HDL-C and log-apoB-48 (**Table 2**), while significant correlations with eGFR were observed for age, non-HDL-C, log-apoB-48 and log-apoB-48/TG (**Table 3**). A multiple regression analysis indicated that the log-apo-B-48/TG levels were a significant determinant of a reduced eGFR among the various lipid parameters, with the most significant differences observed for these parameters (**Table 4**).

Discussion

Dyslipidemia in patients with CKD is characterized by increased levels of TRL and TG and a decreased level of HDL-C[8]. However, it is interesting to note that the serum LDL-C level is usually normal or slightly reduced in CKD patients[9]. In order to evaluate the accumulation of CM and CM-R quantitatively, we developed the system to measure the apoB-48 concentration. Both an eGFR of <75 mL/min/1.73 m^2 and the presence of trace urinary protein or more on a dipstick test are independently associated with all-cause mortality, cardiovascular mortality and kidney events in the general population[20]. In the current study, we measured the fasting apoB-48 concentrations in CKD patients. As in our former study[17], the apoB-48 concentrations were significantly higher in the patients with CKD of Stage 4 (eGFR: 15-25 mL/min/1.73 m^2) and CKD of Stage 5 (eGFR: <15 mL/min/1.73 m^2) than in those with CKD of Stage 1 (>90 mL/min/1.73 m^2). In accordance with the new Japanese CKD guidelines both a decreased GFR and the existence of proteinuria may exacerbate the pathogenesis of CKD and induce the development of end-stage renal disease[18]. In the current study, we found that the apoB-48 concentrations were higher in the patients with proteinuria (≥1 +) than in those without proteinuria, and the existence of proteinuria increased the apoB-48 concentrations in patients with both a high and low eGFR (**Table 1**). Recently, many studies have shown that proteinuria is clustered with a number of risk factors, including hypertension, dyslipidemia, renal dysfunction, hyperhomocysteinemia and various inflammatory and oxidative stress markers, and this parameter has been demonstrated to be an independent predictor of adverse cardiovascular events, even after adjusting for these factors[20-22]. These results indicate that the existence of proteinuria impairs remnant lipoprotein metabolism and enhances

Table 4. A stepwise multiple regression analysis of the eGFR and various parameters

	Model 1		Model 2	
	F value	*p* value	*F* value	*p* value
Age	37.38	<0.001	Not included	
BMI	Not remain		Not included	
Systolic BP	Not remain		Not included	
Diastolic BP	Not remain		Not included	
HDL-C	Not remain		Not remain	
Non-HDL-C	Not remain		3.48	0.063
Log-ApoB-48/TG	17.13	<0.001	28.19	<0.001

A stepwise multiple regression analysis was used to determine eGFR with the *p* value-to-enter and *p* value-to-remain set at 0.20. Abbreviations; BMI, body mass index; BP, blood pressure; HDL-C, high density lipoprotein-cholesterol.

the accumulation of CM-R and that both proteinuria and accumulated CM-R deteriorate the renal function in CKD patients, synergistically leading to the development of end-stage renal disease.

We also calculated the apoB-48/TG ratio in the current study. In the postprandial serum, the TG moiety of CMs is promptly hydrolyzed by LPL, resulting in the subsequent production of CM-R of various sizes. Small CM-R particles contain a smaller amount of TG than do large CM-R particles; however, both sizes of CM-R contain one molecule of apoB-48. Therefore, it has been suggested that a high apoB-48 concentration is related to the accumulation of CM-R of all sizes, while a high apoB-48/TG ratio is related to the accumulation of small CM-R particles. Indeed, we previously found that the apoB-48/TG ratios are significantly higher in patients with type Ⅲ hyperlipidemia, whose remnant lipoproteins accumulate due to a genetic abnormality in apolipoprotein E, than in those with other types of HL, although the subjects received lipid-lowering medications[23]. In the current study, the eGFR values were found to be significantly correlated with the log-apoB-48 and log-apoB-48/TG levels, whereas urinary protein was found to be correlated with the log-apoB-48 level only. These results suggest that the number of small CM-R particles is increased in patients with a low eGFR, while the number of large CM-R particles is increased in patients high a eGFR and proteinuria. Apolipoprotein C-Ⅲ (ApoC-Ⅲ) has been shown to inhibit the LPL and hepatic triglyceride lipase (HTGL) activity as well as the uptake of TRLs and CM-Rs by hepatic lipoprotein receptors[24]. The plasma apoC-Ⅲ concentrations are significantly elevated in patients with CKD[25]. Unfortunately, we did not measure other apolipoproteins, although it is suspected that these changes may hydrolyze CM particles insufficiently, resulting in an increased level of large-sized CM-Rs. Remnant particles are taken up by the liver via LDL receptors and LDL receptor-related proteins (LRPs)[26, 27]. A previous study demonstrated a decrease in the LDL-receptor mRNA levels in rats with chronic renal failure[28]. Therefore, the clearance of hydrolyzed small-sized CM-Rs by LDL-receptors may be delayed. Therefore, the decreased activity of LPL and HTGL, as well as the downregulation of LDL-receptors, may be attributed to the increase in large- and small-sized CM-Rs in subjects with a low eGFR. The urinary loss of apolipoprotein C-Ⅱ (apoC-Ⅱ), an activator of LPL, in the setting of proteinuria-dominant CKD, may decrease the LPL activity[29]. A recent study demonstrated the downregulation of LDL-receptors in the liver in nephrotic rats[30]. However, patients with nephrotic syndrome do not exhibit a decreased LDL apoB fractional catabolic rate[31]. Therefore, the decreased activity of LPL, but not decreased catabolism of CM-Rs, may be associated with the increased level of large CM-R particles observed in subjects with a high eGFR and proteinuria.

A number of studies have suggested that CM-Rs have highly atherogenic properties and that CM-R accumulation correlates with the development of atherosclerotic cardiovascular disease[12], whereas a reduced eGFR and proteinuria are independent risk factors for the development of cardiovascular disease. According to our multiple regression analysis, the log-apoB-48/TG level was found to be a significant determinant of a reduced eGFR. Therefore, an increased serum CM-R level may exacerbate atherosclerotic cardiovascular disease in patients with a reduced eGFR and/or proteinuria.

The present study is associated with some limitations. First, the subjects had already been treated with antidiabetic drugs, antihypertensive drugs and antihy-

peruricemic drugs. In particular, the subjects with a low eGFR and proteinuria were treated with antihypertensive and antihyperuricemic drugs at a high frequency. However, it has not been reported whether the serum apoB-48 concentration is affected by treatment with any antihypertensive or antihyperuricemic agents. Second, we did not measure the levels of other apolipoproteins, which play a key role in regulating CM catabolism. Finally, it was not possible to clarify whether an increased serum apoB-48 concentration contributes to atherosclerosis and CHD in patients with CKD. In our recent study, a high concentration of fasting apoB-48 was found to be significantly correlated with the intima-media thickness in subjects with normal but relatively high TG levels ($100 < TG \leq 150$ mg/dL) and the fasting serum apoB-48 concentration was found to be significantly correlated with the prevalence of coronary artery disease[32, 33].

In conclusion, both a low eGFR (<60) and proteinuria (≥1 +) are independent determinants of a high apoB-48 concentration. Taken together, the present results suggest that an increased serum apoB-48 concentration contributes to an increased risk of cardiovascular events.

Acknowledgements

The authors gratefully acknowledge FUJIREBIO, INC. for measuring the samples using high-quality standards. The authors also gratefully acknowledge Kaori Hizu-Shioyama, Risa Wada and Kyoko Ozawa for their excellent clerical and technical assistance.

Funding

This work was supported by the Japan Heart Foundation and an Astellas/Pfizer Grant for Research on Atherosclerosis Update (to D. Masuda) and Health and Labour Sciences Research Grants for Research on rare and intractable disease (to S. Yamashita).

Disclosures

S. Yamashita and D. Masuda received lecture fees in 2010 from FUJIREBIO, INC. FUJIREBIO, INC. shared the cost for the apoB-48 measurements as a joint research. The other authors have nothing to disclose.

Conflicts of Interest

There is nothing to disclose, except that FUJIREBIO, INC. shared the cost for the apo B-48 measurements as a joint research.

List of Abbreviations

apo: apolipoprotein
BMI: body mass index
CKD: chronic kidney disease
CLEIA: chemiluminescence enzyme immunoassay
CM: chylomicron
CM-R: chylomicron remnant
CVD: cardiovascular disease
eGFR: estimated glomerular filtration rate
ELISA: enzyme-linked immunosorbent assay
HDL-C: high-density lipoprotein cholesterol
HTGL: hepatic triglyceride lipase
LDL-C: low-density lipoprotein cholesterol
LPL: lipoprotein lipase
LRP: LDL receptor-related protein
TC: total cholesterol
TG: triglyceride
TRL: TG-rich lipoprotein
VLDL: very-low-density lipoprotein
VLDL-R: very-low-density lipoprotein remnant
UA: uric acid

References

1) Keith DS, Nichols GA, Gullion CM: Longitudinal follow-up and outcomes among a population with chronic kidney disease in a large managed care organization. Arch Intern Med, 2004; 164: 659-663
2) Menon V, Wang X, Sarnak MJ, Hunsicker LH, Madero M, Beck GJ, Collins AJ, Kusek JW, Levey AS, Greene T: Long-term outcomes in nondiabetic chronic kidney disease . Kidney Int, 2008; 73: 1310-1315
3) Foley RN, Parfrey PS, Samak MJ: Epidemiology of cardiovascular disease in chronic renal disease. J Am Soc Nephrol, 1998; 9: 16-23
4) Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, Mccullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW: Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation, 2003; 108: 2154-2169
5) Kidney Disease Outcomes Quality Initiative (K/DOQI) Group: K/DOQI clinical practice guidelines for management of dyslipidemias in patients with kidney disease. Am J Kidney Dis, 2003; 41: I-IV, S1-91
6) Levey AS, de Jong PE, Coresh J, El Nahas M, Astor BC, Matsushita K, Gansevoort RT, Kasiske BL, Eckardt KU: The definition, classification, and prognosis of chronic kidney disease: a KDIGO Controversies Conference

report. Kidney Int, 2011; 80: 17-28
7) Imai E, Horio M, Watanabe T, Iseki K, Yamagata K, Hara S, Ura N, Kiyohara Y, Moriyama T, Ando Y, Fujimoto S, Konta T, Yokoyama H, Makino H, Hishida A, Matsuo S: Prevalence of chronic kidney disease in the Japanese general population. Clin Exp Nephrol, 2009; 13: 621-630
8) Farbakhsh K, Kasiske BL: Dyslipidemias in patients who have chronic kidney disease. Med Clin North Am, 2005; 89: 689-699
9) Deighan CJ, Caslake MJ, McConnell M, Boulton-Jones JM, Packard CJ: Athergenic lipoprotein phenotype in end-stage renal failure: origin and extent of small dense low density lipoprotein formation. Am J Kidney Dis, 2000; 35: 852-862
10) Karpe F, Boquist S, Tang R, Bond GM, de Faire U, Hamsten A: Remnant lipoproteins are related to intima-media thickness of the carotid artery independently of LDL cholesterol and plasma triglycerides. J Lipid Res, 2001; 42: 17-21
11) Wilhelm MG, Cooper AD: Induction of atherosclerosis by human chylomicron remnants: a hypothesis. J Atheroscler Thromb, 2003; 10: 132-139
12) Fujioka Y, Ishikawa Y: Remnant lipoproteins as strong key particles to atherogenesis. J Atheroscler Thromb, 2009; 16: 145-154
13) Phillips ML, Pullinger C, Kroes I, Kroes J, Hardman DA, Chen G, Curtiss LK, Gutierrez MM, Kane JP, Schumaker VN: A single copy of apolipoprotein B-48 is present on the human chylomicron remnant. J Lipid Res, 1997; 38: 1170-1177
14) Uchida Y, Kurano Y, Ito S: Establishment of monoclonal antibody against human ApoB-48 and measurement of ApoB-48 in serum by ELISA method. J Clin Lab Anal, 1998; 12: 289-292
15) Sakai N, Uchida Y, Ohashi K, Hibuse T, Saika Y, Tomari Y, Kihara S, Hiraoka H, Nakamura T, Ito S, Yamashita S, Matsuzawa Y: Measurement of fasting serum apoB-48 concentrations in normolipidemic and hyperlipidemic subjects by ELISA. J Lipid Res, 2003; 44: 1256-1262
16) Hanada H, Mugii S, Okubo M, Maeda I, Kuwayama K, Hidaka Y, Kitazume-Taneike R, Yamashita T, Kawase R, Nakaoka H, Inagaki M, Yuasa-Kawase M, Nakatani K, Tsubakio-Yamamoto K, Masuda D, Ohama T, Matsuyama A, Ishigami M, Nishida M, Komuro I, Yamashita S: Establishment of chemiluminescence enzyme immunoassay for apolipoprotein B-48 and its clinical applications for evaluation of impaired chylomicron remnant metabolism. Clin Chim Acta, 2012; 413: 160-165
17) Okubo M, Hanada H, Mastui M, Hidaka Y, Masuda D, Yamashita S: Clinical significance of apolipoprotein B-48 (apoB-48) in chronic kidney disease patients. Rinsho Byori, 2010; 58: 878-883 in Japanese
18) Japan Nephrology Society. Special issue: Clinical practice guidebook for diagnosis and treatment of chronic kidney disease 2012. Nihon Jinzo Gakkai Shi, 2012; 54: 1034-1191 in Japanese
19) Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A: Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis, 2009; 53: 982-992
20) Oh SW, Baek SH, Kim YC, Goo HS, Heo NJ, Na KY, Chae DW, Kim S, Chin HJ: Mild decrease in estimated glomerular filtration rate and proteinuria are associated with all-cause and cardiovascular mortality in the general population. Nephrol Dial Transplant, 2012; 27: 2284-2290
21) Fliser D, Buchholz K, Haller H: EUropean Trial on Olmesartan and Pravastatin in Inflammation and Atherosclerosis (EUTOPIA) Investigators: antiinflammatory effects of angiotensin II subtype 1 receptor blockade in hypertensive patients with microinflammation. Circulation, 2004; 110: 1103-1107
22) Ogawa S, Mori T, Nako K, Kato T, Takeuchi K, Ito S: Angiotensin II type 1 receptor blockers reduce urinary oxidative stress markers in hypertensive diabetic nephropathy. Hypertension, 2006; 47: 699-705
23) Yuasa-Kawase M, Masuda D, Kitazume-Taneike R, Yamashita T, Kawase R, Nakaoka H, Inagaki M, Nakatani K, Tsubakio-Yamamoto K, Ohama T, Toyama-Nakagawa Y, Nishida M, Ishigami M, Saito M, Eto M, Matsuyama A, Komuro I, Yamashita S: Apolipoprotein B-48 to triglyceride ratio is a novel and useful marker for detection of type III hyperlipidemia after antihyperlipidemic intervention. J Atheroscler Thromb, 2012; 19: 862-871
24) Ooi EM, Barrett PH, Chan DC, Watts GF: Apolipoprotein C-III: understanding an emerging cardiovascular risk factor. Clin Sci (Lond), 2008; 114 (10): 611-624
25) Chan DT, Dogra GK, Irish AB, Ooi EM, Barrett PH, Chan DC, Watts GF: Chronic kidney disease delay VLDL-apoB-100 particle catabolism: potential role of apolipoprotein C-III. J Lipid Res, 2009; 50: 2524-2531
26) Fujioka Y, Cooper AD, Fong LG: Multiple processes are involved in the uptake of chylomicron remnants by mouse peritoneal macrophages. J Lipid Res, 1998; 39: 2339-2349
27) Cooper AD: Hepatic uptake of chylomicron remnants. J Lipid Res, 1997; 38: 2173-2192
28) Chmielewski M, Sucajtys-Szulc E, Kossowska E, Swierczynski J, Rutkowski B, Boguslawski W: Increased gene expression of liver SREBP-2 in experimental chronic renal failure. Atherosclerosis, 2007; 191: 326-332
29) Shoji T, Abe T, Matsuo H, Egusa G, Yamasaki Y, Kashihara N, Shirai K, Kashiwagi A: Chronic kidney disease, dyslipidemia, and atherosclerosis. J Atheroscler Thromb, 2012; 19: 299-315
30) Wang L, Shearer GC, Budamagunta MS, Voss JC, Molfino A, Kaysen GA: Proteinuria decreases tissue lipoprotein receptor levels resulting in altered lipoprotein structure and increasing lipid levels. Kidney Int, 2012; 82: 990-999
31) Stenvinkel P, Berglund L, Ericsson S, Alvestrand A, Angelin B, Eriksson M: Low-density lipoprotein metabolism and its association to plasma lipoprotein(a) in the nephrotic syndrome. Eur J Clin Invest, 1997; 27: 169-177
32) Nakatani K, Sugimoto T, Masuda D, Okano R, Oya T, Monden Y, Yamashita T, Kawase R, Nakaoka H, Inagaki M, Yuasa-Kawase M, Tsubakio-Yamamoto K, Ohama T, Nishida M, Ishigami M, Komuro I, Yamashita S: Serum apolipoprotein B-48 levels are correlated with carotid

intima-media thickness in subjects with normal serum triglyceride levels. Atherosclerosis, 2011; 218: 226-232
33) Masuda D, Sugimoto T, Tsujii K, Inagaki M, Nakatani K, Yuasa-Kawase M, Tsubakio-Yamamoto K, Ohama T, Nishida M, Ishigami M, Kawamoto T, Matsuyama A, Sakai N, Komuro I, Yamashita S: Correlation of fasting serum apolipoprotein B-48 with coronary artery disease prevalence. Eur J Clin Invest, 2012; 42: 992-999

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association.

Lipoprotein Subfractions Highly Associated With Renal Damage in Familial Lecithin:Cholesterol Acyltransferase Deficiency

Masayuki Kuroda, Adriaan G. Holleboom, Erik S.G. Stroes, Sakiyo Asada, Yasuyuki Aoyagi, Kouju Kamata, Shizuya Yamashita, Shun Ishibashi, Yasushi Saito and Hideaki Bujo

Arterioscler Thromb Vasc Biol. 2014;34:1756-1762; originally published online May 29, 2014;
doi: 10.1161/ATVBAHA.114.303420
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

Lipoprotein Subfractions Highly Associated With Renal Damage in Familial Lecithin:Cholesterol Acyltransferase Deficiency

Masayuki Kuroda, Adriaan G. Holleboom, Erik S.G. Stroes, Sakiyo Asada, Yasuyuki Aoyagi, Kouju Kamata, Shizuya Yamashita, Shun Ishibashi, Yasushi Saito, Hideaki Bujo

Objective—In familial lecithin:cholesterol acyltransferase (LCAT) deficiency (FLD), deposition of abnormal lipoproteins in the renal stroma ultimately leads to renal failure. However, fish-eye disease (FED) does not lead to renal damage although the causative mutations for both FLD and FED lie within the same *LCAT* gene. This study was performed to identify the lipoproteins important for the development of renal failure in genetically diagnosed FLD in comparison with FED, using high-performance liquid chromatography with a gel filtration column.

Approach and Results—Lipoprotein profiles of 9 patients with LCAT deficiency were examined. Four lipoprotein fractions specific to both FLD and FED were identified: (1) large lipoproteins (>80 nm), (2) lipoproteins corresponding to large low-density lipoprotein (LDL), (3) lipoproteins corresponding to small LDL to large high-density lipoprotein, and (4) to small high-density lipoprotein. Contents of cholesteryl ester and triglyceride of the large LDL in FLD (below detection limit and 45.8±3.8%) and FED (20.7±6.4% and 28.0±6.5%) were significantly different, respectively. On in vitro incubation with recombinant LCAT, content of cholesteryl ester in the large LDL in FLD, but not in FED, was significantly increased (to 4.2±1.4%), whereas dysfunctional high-density lipoprotein was diminished in both FLD and FED.

Conclusions—Our novel analytic approach using high-performance liquid chromatography with a gel filtration column identified large LDL and high-density lipoprotein with a composition specific to FLD, but not to FED. The abnormal lipoproteins were sensitive to treatment with recombinant LCAT and thus may play a causal role in the renal pathology of FLD. (***Arterioscler Thromb Vasc Biol.* 2014;34:1756-1762.**)

Key Words: chromatography, gel ■ LDL ■ lecithin acyltransferase deficiency ■ renal insufficiency

Lecithin:cholesterol acyltransferase (LCAT)–deficiency syndromes are rare autosomal recessive diseases, characterized by hypo-α-lipoproteinemia and corneal opacity.[1,2] They are caused by mutations in the *LCAT* gene, of which 88 have been reported to date.[3] Severe mutations lead to familial LCAT deficiency (FLD), mild mutations lead to fish-eye disease (FED). In FLD, the mutant LCAT enzyme is either absent in plasma (not secreted from the hepatocyte or rapidly degraded on secretion) or exhibits no catalytic activity on any lipoprotein; in FED, LCAT cannot esterify cholesterol on high-density lipoprotein (HDL; loss of α-activity) but retains its activity on lipoproteins containing apolipoprotein B (β-activity).[1,2] Likely, the molecular difference is causal to the major clinical difference between FLD and FED: patients with FLD develop renal failure, whereas patients with FED do not.[2,4]

To prevent renal failure in patients with FLD, replacement therapy with recombinant enzyme is currently being developed.[5–8] Alternatively, we are developing a long-lasting gene therapy by transplantation of human *LCAT* gene-transduced autologous adipocytes.[7,9] Recombinant LCAT (rLCAT) secreted by the *LCAT* gene-transduced adipocytes corrected abnormal HDL subpopulations in sera of FED patients in vitro.[10]

LCAT catalyzes the esterification of cholesterol with acyl groups hydrolyzed from phospholipids, predominantly on HDL particles. This leads to mature lipoproteins with cores filled with cholesterol ester. LCAT dysfunction leads to decreased maturation of the HDL particle and to increased levels of both its substrates: unesterified cholesterol and phosphatidylcholine. In the absence of LCAT activity, abnormal lipid particles have been observed throughout lipoprotein fractions.[11–14] The HDL fraction contains disk-shaped particles in rouleaux and small spherical particles. Density-gradient ultracentrifugation followed by electron microscopy

Received on: August 9, 2013; final version accepted on: May 14, 2014.

From the Department of Genome Research and Clinical Application, Graduate School of Medicine (M.K., S.A., Y.A., H.B.) and Center for Advanced Medicine, Chiba University Hospital (M.K.), Chiba University, Chiba, Japan; Department of Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands (A.G.H., E.S.G.S.); Department of Nephrology in Internal Medicine, Kitasato University Hospital, Sagamihara, Japan (K.K.); Department of Internal Medicine and Molecular Science, Osaka University Graduate School of Medicine, Suita, Japan (S.Y.); Division of Endocrinology and Metabolism, Department of Medicine, Diabetes Center, Jichi Medical University, Shimotsuke, Japan (S.I.); Chiba University, Chiba, Japan (Y.S.); and Department of Clinical-Laboratory and Experimental-Research Medicine, Toho University Sakura Medical Center, Sakura, Japan (H.B.).

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.303420/-/DC1.

Correspondence to Hideaki Bujo, MD, PhD, Department of Clinical-Laboratory and Experimental-Research Medicine, Toho University Sakura Medical Center, 564-1 Shimoshizu, Sakura, 285-8741, Japan. E-mail hideaki.bujo@med.toho-u.ac.jp

Nonstandard Abbreviations and Acronyms	
CE	cholesteryl ester
FC	free cholesterol
FED	fish-eye disease
FLD	familial lecithin:cholesterol acyltransferase deficiency
GFC	gel filtration column
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
Lp	lipoprotein
LpX	lipoprotein-X
rLCAT	recombinant LCAT

revealed that the low-density lipoprotein (LDL) fraction contains 3 abnormal particles with different sizes, lipid composition, and associated apolipoproteins,[11,12] which were proposed to be important in the pathogenesis of renal manifestation in patients with FLD.[15–18] Of these, lipoprotein-X (LpX)[19,20] have been postulated to accumulate in glomeruli, potentially causing the renal damage observed in patients with FLD.[16–18] In 1 patient with FLD, lipid-lowering therapy led to a reduction of LpX and a concomitant reduction in proteinuria.[21] LpX is phospholipid (PL)-rich and free cholesterol (FC)–rich but triglyceride (TG)-poor particle without apolipoproteins, ranging in size between very low density lipoprotein and large LDL.[22]

To characterize the abnormal lipoproteins associated with the renal pathology of FLD, we characterized lipoprotein fractions by analyzing patients with different mutations and manifestations in comparison with another LCAT-deficiency syndrome, FED. We applied high-performance liquid chromatography with a gel filtration column (HPLC-GFC) for the first time to characterize the above abnormal lipoproteins and in fact identified lipoprotein subfractions specific to FLD. The lipid contents and particle size were biochemically determined, and the responsiveness of the lipoproteins against incubation with rLCAT was investigated in vitro.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Lipoprotein Subfractions Specific to LCAT-Deficiency Syndromes

Five patients with FLD (1–5) and 4 patients with FED (6–9) were compared with 4 nonaffected normolipidemic controls. Clinical and molecular characteristics and lipid profiles of the patients are given in Tables 1 and 2, respectively. Ultracentrifugation fractionation followed by determination of lipid contents was performed in patients 1, 2, and 5 (Table I in the online-only Data Supplement). LCAT α-activities in the patients' sera were all <2% of reference. As expected in LCAT deficiency, mature HDL particles found at fraction (Fr.) 16 and 17 of unaffected controls were absent in the 9 patients (Figure 1). Although the lipid profiles of patients were heterogeneous, HPLC-GFC showed 4 lipoprotein fractions in sera of patients with FLD and FED that were not present in sera of unaffected controls: large lipoproteins (>80 nm) in Fr. 1 (Lp1), lipoproteins corresponding to large LDL in Fr. 8 (or Fr. 7–10; Lp8), lipoproteins corresponding to very small LDL and large HDL in Fr. 12 to 16 (Lp12–16), and lipoproteins corresponding to small HDL in Fr. 18 to 20 (Lp18–20). The levels of cholesterol, TG, and PL in these specific fractions varied among the 9 patients (Figure 1). Serum apolipoprotein analyses of Fr. 7 to 10, Fr. 13 to 15, and Fr. 18 to 20 in 3 patients (1, 2, and 5) showed that Fr. 13 to 15 and Fr. 18 to 20 were rich in apolipoprotein A as normolipidemic control although varied among patients (Figure I in the online-only Data Supplement). Apolipoprotein Cs were also rich in Fr. 18 to 20 but not in Fr. 13 to 15. Apolipoprotein B was mostly distributed in Fr. 8 to 10 among the 3 fraction categories. Apolipoprotein E was abundant in all 3 fraction categories when compared with that in the control.

Abnormal Lipoproteins Are Present in FLD Regardless of Degree of Proteinuria

To study the relationship between lipoproteins and the degree of proteinuria in patients with FLD, lipoproteins between 2 sibling patients with FLD homozygous for the C337Y mutation in LCAT were compared (Figure 1, patients 1 and 3). Patient 1 had proteinuria in the nephrotic range (6 g/24 h), whereas patient 3 had only mild proteinuria (0.45 g/L).[23] All 4 abnormal lipoproteins were present in both patients (Figure 2A), although 3 lipoproteins (Lp1, Lp8, and Lp18-20) were lower in the younger patient.

Table 1. Clinical and Molecular Characteristics of Patients With Lecithin:Cholesterol Acyltransferase Deficiency

Patient	Sex	Age, Y	Race	Renal Failure/Proteinuria	Corneal Opacity	Anemia	CAD	Phenotype	AA Substitution	References
1	F	17	White (Morocco)	6 g/24 h	+	11.4 g/dL	–	FLD	C337Y	23
2	F	61	Japanese	2 g/24 h	+	9.5 g/dL	–	FLD	C98Y	24
3	F	12	White (Morocco)	0.45 g/L	+	9.2 g/dL	–	FLD	C337Y	23
4	F	63	Japanese	0.23 g/24 h	+	10.3 g/dL	–	FLD	G203R	25
5	M	68	Japanese	0.5 g/L	+	6.6 g/dL	–	FLD	G54S	26
6	M	38	Japanese	–	+	–	–	FED	T147I	10
7	M	58	White (Dutch)	–	+	–	–	FED	T147I	None
8	M	36	White (Dutch)	–	+	–	–	FED	W99S/T147I	27
9	F	30	White (Dutch)	–	+	–	–	FED	T147I/V333M	28

Patients 8 and 9 are compound heterozygotes; others are homozygotes for the indicated mutations. AA indicates amino acid; CAD, coronary artery disease; F, female; FED, fish-eye disease; FLD, familial lecithin:cholesterol acyltransferase deficiency; and M, male.

Table 2. Lipid Profiles of Patients With Lecithin:Cholesterol Acyltransferase Deficiency

Patients	TC	TG	HDL-C	LDL-C	CE/TC
1	109	179	5.8	67	0
2	123	307	9.3	52	0.13
3	47	56	10.1	26	0
4	47	89	6.3	23	0.13
5	56	59	2.0	42	0
6	85	120	4.0	57	0.57
7	133	120	4.7	104	0.54
8	144	205	3.9	99	0.57
9	98	118	4.9	70	0.39

Values for LDL-C were calculated according to Friedewald et al.[29] CE/TC indicates cholesteryl ester/total cholesterol ratio; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; and TG, triglyceride.

Next, lipoprotein profiles of a patient with FLD with homozygous for the C98Y[24] mutation before and after a fat-restricted diet, which led to a reduction of proteinuria from 2.0 g/gCr to 0.6 g/gCr, were compared (Figure 1, patient 2). All 4 lipoproteins remained present after the diet although Lp1 and Lp8 were decreased to some extent (Figure 2B).

Lp8 and Lp12 to 16 Are Specific to FLD and Not to FED

Next, composition of the 4 Lps was analyzed (Figure II in the online-only Data Supplement). In all lipoproteins, cholesteryl ester (CE) was absent in FLD and low in Lp1, Lp12 to 16, and Lp18 to 20 in FED (panel A). PL in Lp8 was significantly lower in FLD when compared with that in FED (panel D). PL and FC were increased in Lp12 to 16 in FLD when compared with that in FED (panels B and D). FC, TG, and PL in both Lp1 and Lp18 to 20 did not differ between FLD and FED.

Lp8 Is a Large LDL, Rich in FC, PL, and TG, and Different From LpX

In comparison with unaffected controls and to patients with FED, CE in the LDL fractions of FLD sera was significantly decreased, whereas TG was increased (Figure 3A). In patients with both FLD and FED, FC, TG, and PL in Fr. 8 were significantly higher than in Fr. 9, whereas in controls, FC, TG, and PL in Fr. 8 were significantly lower than in Fr. 9 (Figure 3B). As a result, average sizes of Lp8 (Fr. 7–10) in FLD were significantly increased when compared with normal, whereas averaged particle size in FLD was lower than those in FED because of the severe deficiency of CE (Figure 3C). The composition of Lp8 in our patients with FLD is consistent with the previously reported FLD-LDL, and not consistent with the lipid characteristics of LpX.

Abnormal Lipid Compositions of FLD-Specific Lps Are Ameliorated by In Vitro Incubation With rLCAT

In vitro rLCAT incubation was performed followed by HPLC-GFC analyses (Figure III in the online-only Data Supplement). Incubation of patients' sera with rLCAT increased CE, TG, and PL in Fr. 16 to 18 in both FLD and FED (Figure IV in

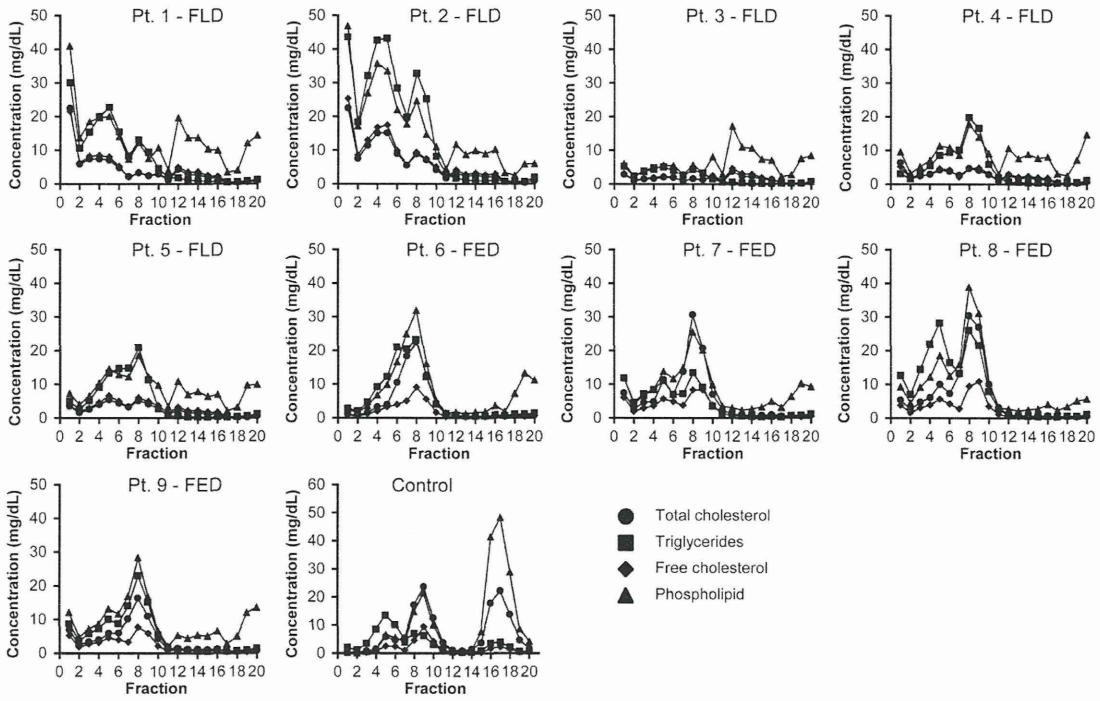

Figure 1. Lipoprotein profiles in patients with familial lecithin:cholesterol acyltransferase deficiency (FLD) by high performance liquid chromatography (HPLC) with gel filtration column (GFC). Sera from patients with 5 FLD (patients [Pts.] 1–5) and 4 Fish-eye disease (FED; Pts. 6–9) were subjected to lipoprotein size fractionation with concomitant determination of lipid concentrations in each fraction by high-performance liquid chromatography-GFC analyses. Representative result is shown for normolipidemic subjects. Concentrations of total cholesterol (●), triglyceride (■), free cholesterol (♦), and phospholipid (▲; *y* axis) in each fraction (*x* axis) are shown.

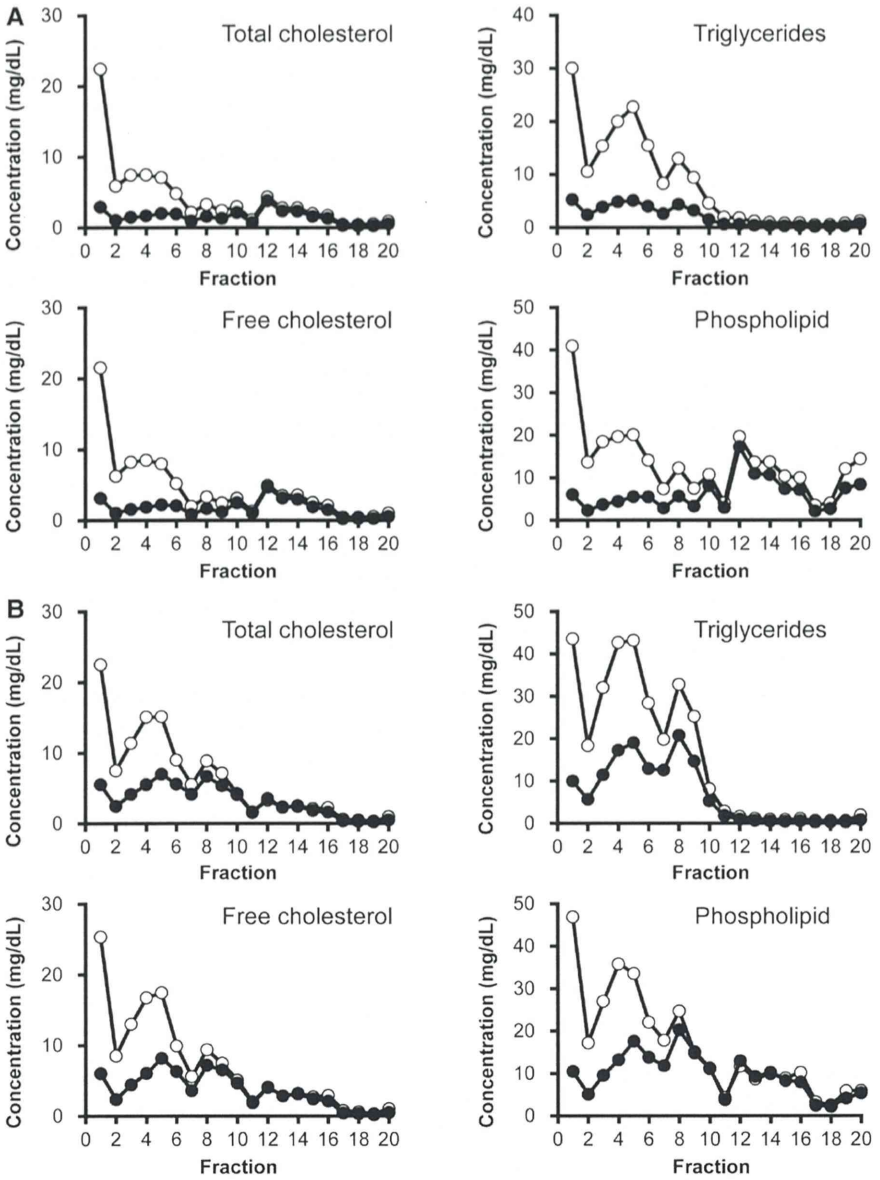

Figure 2. Differences in lipoproteins in patients with familial lecithin:cholesterol acyltransferase deficiency (FLD) with or without renal insufficiency. **A**, Lipoprotein profiles were compared between a patient with FLD with nephrotic range proteinuria (patient 1, ○) and patient 3 with mild proteinuria (●). **B**, Lipoprotein profiles were compared between before (○) and after (●) fat-restricted diet.

the online-only Data Supplement), indicating LCAT-mediated maturation of HDL. CE and PL contents of Lp8 were significantly increased and decreased, respectively, in FLD after incubation with rLCAT, whereas TG content was not significantly altered (Figure 4A and 4B). In FED, composition of Lp8 was not significantly altered by the treatment (Figure 4A and 4B). On incubation with rLCAT, Lp8 increased in size in FLD and it decreased in size in FED (Figure 4C). However, FC and PL in Lp12 to 16 decreased on incubation (Figure 4D).

Discussion

In this study, 4 lipoprotein fractions specific to LCAT-deficiency syndromes were identified by the HPLC-GFC analysis of samples from genetically diagnosed patients with different mutations and manifestations. Two of these had lipid compositions that were specific to FLD and thus may be involved in causing the renal damage that characterizes FLD. In vitro incubation with rLCAT corrected the abnormal fractions.

Lp1, one of the abnormal lipoproteins characteristic to LCAT-deficiency syndrome, was rich in TG and PL, and associated with the degree of proteinuria in 2 siblings with FLD, and was decreased on fat restriction in another patient with FLD (Figure 2). Indeed, abnormal lipoproteins with size of ≈100 nm corresponding to Lp1 have been identified in patients with LCAT deficiency with renal failure.[2,11,12,15] The lipid composition of Lp1 did not change on incubation with rLCAT (data not shown). Together, this suggests that Lp1 is most likely secondary to renal failure rather than directly caused by LCAT deficiency.

As opposed to controls, Fr. 8 was richer in total cholesterol, TG, FC, and PL than Fr. 9 in the patients with LCAT

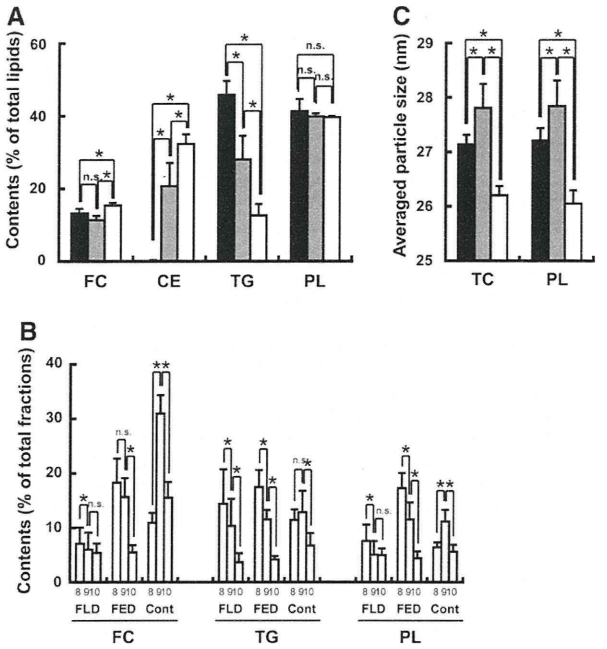

Figure 3. Characterization of lipid profiles in Lp8 of familial lecithin:cholesterol acyltransferase deficiency (FLD) and Fish-eye disease (FED). **A**, Lipid compositions of Fr. 7 to 10 fractions (Lp8) were compared among FLD (closed column), FED (gray column), and normal (open column). *P<0.05. **B**, Lipid concentrations of fractions 8, 9, and 10 were compared in FLD (n=5), FED (n=4), and controls (n=4). *P<0.05. Cholesteryl ester (CE) concentrations in FLD are not shown because levels were undetectable. **C**, Size distribution of lipoproteins in Lp8 (Fr. 7–10) was compared among FLD (closed column), FED (gray column), and normal (open column) based on total cholesterol (TC) and phospholipid (PL) concentrations. *P<0.05. FC indicates free cholesterol; and TG, triglyceride.

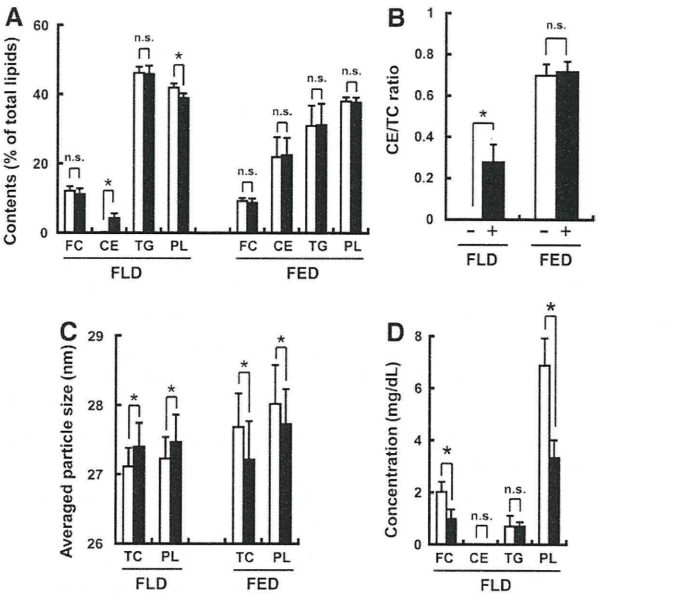

Figure 4. Effects of in vitro familial lecithin:cholesterol acyltransferase (LCAT) supplementation on the lipid profiles of abnormal lipoproteins in LCAT-deficiency syndrome. After analyses described in Figure II in the online-only Data Supplement, lipid composition (**A**), cholesteryl ester (CE)/TC ratio (**B**), averaged particle size based on total cholesterol (TC) and phospholipid (PL) concentrations (**C**), in Lp8, and lipid concentrations in Lp12 to 16 (**D**), were compared between culture media containing recombinant LCAT (rLCAT; closed column) and media without rLCAT (open column). *P<0.05. FC indicates free cholesterol; FED, Fish-eye disease; and TG, triglyceride.

deficiency (Figures 1 and 4B). Lp8 also differed in composition between FLD and FED: in FLD, it contained increased TG and decreased CE in comparison with FED (Figure 3A). Importantly, although the levels varied with the severity of renal damage as did those in Lp1, the buoyance of the peak at Fr. 8 did not vary with severity of renal damage (Figure 2), strongly suggesting that Lp8 directly results from a lack of LCAT and not from metabolic disturbances that occur during proteinuria and progressive renal failure.

In addition to the above-mentioned characteristics for Lp8 in LCAT-deficiency syndrome, HPLC-GFC analyses clarified novel unique lipid properties of Lp8 in FLD in comparison with that in FED; the averaged sizes of Lp8 are smaller in FLD than those in FED (Figure 3C). The lipid compositions of Lp8 in FLD were, in part, ameliorated by rLCAT incubation (Figure 4A). The averaged sizes of the Lp8 increased in FLD, whereas those in FED decreased (Figure 4C). rLCAT increased the CE formation in both LDL and HDL fractions in FLD sera. Thus, these findings indicated that the abnormal compositions were most likely caused primarily by the dysfunction of LCAT in the patients, and that the abnormal characteristics of Lp8 were not because of metabolic disturbances that occur during proteinuria and progressive loss of kidney function.

Previous extensive analyses using electron microscopy have identified 3 abnormal lipoproteins in the LDL fraction of FLD[12]: TG-rich and CE-poor particles of sizes similar to normal LDL (FLD-LDL); FC- and PL-containing particles of sizes distributing from 40 to 60 nm (LpX-like particle)[2]; particles with a diameter of 100 nm (designated as LM-LDL)[17,30] that were later reported to be identical to LpX.[15] LpX is FC- and PL-rich but TG-poor lipid particles (30%, 60%, and 2%, respectively)[22] without apolipoproteins, which range from very low density lipoprotein to large LDL fractions in fast performance liquid chromatography analysis.[31] The abnormal particles have been shown to be decreased by lipid-lowering therapy in a patient with FLD.[21] Lipoproteins in Lp8 were different from LpX in the lipid contents; the fractions were rich in FC and PL and also rich in TG (13.2±1.3%, 41.4±3.3%, and 45.8±3.8%, respectively). The composition analyses suggested that Lp8 corresponds to FLD-LDL, but the calculated sizes of Lp8 were larger than normal LDL using the data obtained by size fractionation with HPLC-GPC in the present study. Thus, the identified Lp8 in LCAT-deficiency syndrome was most likely not identical to LpX in the characteristics.

There is a limitation for the interpretation of the quantitative measurement of LpX in the frozen samples collected in our study because the abnormal lipoproteins were known to be labile to freezing-and-thawing treatment. In this context, fresh sera were collected from patients 2 and 4 and analyzed by agarose gel electrophoresis. The lipid staining of lipoproteins electrophoresed in agarose gel detected the abnormally slowly migrating TG-poor lipoproteins, LpX, at the expectedly migrating position, as well as TG-rich abnormal β-lipoproteins (LDL) in the once-frozen sample, as well as the fresh sample

in patient 4, although the staining intensity tended to decrease in comparison with the fresh counterpart. However, LpX was not detected in either sample with or without freeze-and-thaw treatment from patient 2. Thus, LpX was indeed labile to freeze/thawing, and the frozen samples were not adequate for the quantitative measurement. However, the presence was still able to be evaluated after once-freezing treatment. On the basis of background data, HPLC-GFC analysis showed that lipid contents in Lp8 were not largely affected by once-freezing treatment in both patients 2 and 4: in contrast, the contents of TG and PL were slightly decreased in lipoproteins with peak of Fr. 5 (data not shown). Additional studies using fresh samples of patients with distinct mutations and manifestations are needed to interpret the significance of novel lipoproteins in comparison with LpX for the development of renal insufficiency in LCAT0deficiency syndrome quantitatively.

In FLD but not in FED, Lp12 to 16 were heterogeneous in size and rich in PL. rLCAT decreased PL in these fractions specifically (Figure 5D; Figure II in the online-only Data Supplement). This may suggest that the heterogeneous-sized PL-rich particles in Fr. 12 to 16 converge to normal-sized HDL (Fr. 16–18) on incubation with rLCAT, with concomitant esterification of FC.

In conclusion, 4 lipoprotein fractions specific to LCAT-deficiency syndromes were identified by the HPLC-GFC analysis of samples from genetically diagnosed patients with different mutations and manifestations. The composition of 2 of these was unique to only FLD; these were not likely compatible with the previously reported LpX. These abnormal lipoproteins may be causal to the renal pathology in FLD, the main cause of increased morbidity and mortality in this condition. The regular evaluation of these specific lipid fractions during LCAT enzyme replacement therapy in patients with LCAT deficiency may provide guidance for success of the intervention. The value of these lipid fractions for risk of future renal disease needs to be addressed in prospective follow-up studies in patients with FLD with various mutations in the *LCAT* gene before the onset of proteinuria.

Sources of Funding

This study was supported, in part, by Health and Labour Sciences Research grants for translational research and for research on rare and intractable diseases, Japan (Hideaki Bujo). A.G. Holleboom is supported by a Veni grant (project number 91613031) from the Netherlands Organization of Research NWO.

Disclosures

None.

References

1. Glomset JA, Assmann G, Gjone E, Norum KR. Lecithin cholesterol acyltransferase deficiency and fish eye disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Stanbury JB, Wyngaarden JB, Fredrickson DS, eds. *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed. New York, NY: McGraw-Hill Inc; 1995:1933–1951.
2. Santamarina-Fojo S, Hoeg JM, Assman G, Brewer HB Jr. Lecithin cholesterol acyltransferase deficiency and fish eye disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*, 8th ed. New York, NY: McGraw-Hill Inc;2001:2817–2833.
3. Human Gene Mutation Database. 2013. *Human Gene Mutation Database*. http://www.hgmd.org.
4. *The Online Metabolic and Molecular Bases of Inherited Diseases (OMMBID), part 12: LIPIDS*, http://www.ommbid.com/OMMBID/the_online_metabolic_and_molecular_bases_of_inherited_disease/b/abstract/part12/ch118.
5. Séguret-Macé S, Latta-Mahieu M, Castro G, Luc G, Fruchart JC, Rubin E, Denèfle P, Duverger N. Potential gene therapy for lecithin-cholesterol acyltransferase (LCAT)-deficient and hypoalphalipoproteinemic patients with adenovirus-mediated transfer of human LCAT gene. *Circulation*. 1996;94:2177–2184.
6. Rousset X, Vaisman B, Auerbach B, Krause BR, Homan R, Stonik J, Csako G, Shamburek R, Remaley AT. Effect of recombinant human lecithin cholesterol acyltransferase infusion on lipoprotein metabolism in mice. *J Pharmacol Exp Ther*. 2010;335:140–148.
7. Kuroda M, Bujo H, Aso M, Saito Y. Adipocytes as a vehicle for ex vivo gene therapy: novel replacement therapy for diabetes and other metabolic diseases. *J Diabetes Invest*. 2011;2:333–340.
8. Chen Z, Chu D, Castro-Perez JM, et al. AAV8-mediated long-term expression of human LCAT significantly improves lipid profiles in hCETP;Ldlr(+/-) mice. *J Cardiovasc Transl Res*. 2011;4:801–810.
9. Kuroda M, Aoyagi Y, Asada S, Bujo H, Tanaka S, Konno S, Tanio M, Ishii I, Machida K, Matsumoto F, Satoh K, Aso M, Saito Y. Ceiling culture-derived proliferative adipocytes are a possible delivery vehicle for enzyme replacement therapy in lecithin: cholesterol acyltransferase deficiency. *Open Gene Ther J*. 2011;4:1–10.
10. Asada S, Kuroda M, Aoyagi Y, Bujo H, Tanaka S, Konno S, Tanio M, Ishii I, Aso M, Saito Y. Disturbed apolipoprotein A-I-containing lipoproteins in fish-eye disease are improved by the lecithin:cholesterol acyltransferase produced by gene-transduced adipocytes *in vitro*. *Mol Genet Metab*. 2011;102:229–231.
11. Glomset JA, Nichols AV, Norum KR, King W, Forte T. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency. Further studies of very low and low density lipoprotein abnormalities. *J Clin Invest*. 1973;52:1078–1092.
12. Forte T, Nichols A, Glomset J, Norum K. The ultrastructure of plasma lipoproteins in lecithin:cholesterol acyltransferase deficiency. *Scand J Clin Lab Invest Suppl*. 1974;137:121–132.
13. Glomset JA, Norum KR, King W. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: lipid composition and reactivity *in vitro*. *J Clin Invest*. 1970;49:1827–1837.
14. Norum KR, Glomset JA, Nichols AV, Forte T. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: physical and chemical studies of low and high density lipoproteins. *J Clin Invest*. 1971;50:1131–1140.
15. Guérin M, Dolphin PJ, Chapman MJ. Familial lecithin:cholesterol acyltransferase deficiency: further resolution of lipoprotein particle heterogeneity in the low density interval. *Atherosclerosis*. 1993;104:195–212.
16. Gjone E. Familial lecithin:cholesterol acyltransferase deficiency—a clinical survey. *Scand J Clin Lab Invest Suppl*. 1974;137:73–82.
17. Gjone E, Blomhoff JP, Skarbovik AJ. Possible association between an abnormal low density lipoprotein and nephropathy in lecithin: cholesterol acyltransferase deficiency. *Clin Chim Acta*. 1974;54:11–18.
18. Guerin M, Dachet C, Goulinet S, Chevet D, Dolphin PJ, Chapman MJ, Rouis M. Familial lecithin:cholesterol acyltransferase deficiency: molecular analysis of a compound heterozygote: LCAT (Arg147 –> Trp) and LCAT (Tyr171 –> Stop). *Atherosclerosis*. 1997;131:85–95.
19. Lambert G, Sakai N, Vaisman BL, et al. Analysis of glomerulosclerosis and atherosclerosis in lecithin cholesterol acyltransferase-deficient mice. *J Biol Chem*. 2001;276:15090–15098.
20. Zhu X, Herzenberg AM, Eskandarian M, Maguire GF, Scholey JW, Connelly PW, Ng DS. A novel *in vivo* lecithin-cholesterol acyltransferase (LCAT)-deficient mouse expressing predominantly LpX is associated with spontaneous glomerulopathy. *Am J Pathol*. 2004;165:1269–1278.
21. Yee MS, Pavitt DV, Richmond W, Cook HT, McLean AG, Valabhji J, Elkeles RS. Changes in lipoprotein profile and urinary albumin excretion in familial LCAT deficiency with lipid lowering therapy. *Atherosclerosis*. 2009;205:528–532.
22. Lynn EG, Choy PC, Magil A, O K. Uptake and metabolism of lipoprotein-X in mesangial cells. *Mol Cell Biochem*. 1997;175:187–194.
23. Holleboom AG, Kuivenhoven JA, van Olden CC, Peter J, Schimmel AW, Levels JH, Valentijn RM, Vos P, Defesche JC, Kastelein JJ, Hovingh GK, Stroes ES, Hollak CE. Proteinuria in early childhood due to familial LCAT deficiency caused by loss of a disulfide bond in lecithin:cholesterol acyl transferase. *Atherosclerosis*. 2011;216:161–165.
24. Naito S, Kamata M, Furuya M, Hayashi M, Kuroda M, Bujo H, Kamata K. Amelioration of circulating lipoprotein profile and proteinuria in a patient

with LCAT deficiency due to a novel mutation (Cys74Tyr) in the lid region of LCAT under a fat-restricted diet and ARB treatment. *Atherosclerosis*. 2013;228:193–197.
25. Wang XL, Osuga J, Tazoe F, Okada K, Nagashima S, Takahashi M, Ohshiro T, Bayasgalan T, Yagyu H, Okada K, Ishibashi S. Molecular analysis of a novel LCAT mutation (Gly179 → Arg) found in a patient with complete LCAT deficiency. *J Atheroscler Thromb*. 2011;18:713–719.
26. Asztalos BF, Schaefer EJ, Horvath KV, Yamashita S, Miller M, Franceschini G, Calabresi L. Role of LCAT in HDL remodeling: investigation of LCAT deficiency states. *J Lipid Res*. 2007;48:592–599.
27. Holleboom AG, Kuivenhoven JA, Peelman F, Schimmel AW, Peter J, Defesche JC, Kastelein JJ, Hovingh GK, Stroes ES, Motazacker MM. High prevalence of mutations in LCAT in patients with low HDL cholesterol levels in The Netherlands: identification and characterization of eight novel mutations. *Hum Mutat*. 2011;32:1290–1298.
28. Hovingh GK, Hutten BA, Holleboom AG, Petersen W, Rol P, Stalenhoef A, Zwinderman AH, de Groot E, Kastelein JJ, Kuivenhoven JA. Compromised LCAT function is associated with increased atherosclerosis. *Circulation*. 2005;112:879–884.
29. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499–502.
30. Glomset JA, Norum KR, Nichols AV, King WC, Mitchell CD, Applegate KR, Gong EL, Gjone E. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: effects of dietary manipulation. *Scand J Clin Lab Invest Suppl*. 1975;142:3–30.
31. Nishiwaki M, Ikewaki K, Bader G, Nazih H, Hannuksela M, Remaley AT, Shamburek RD, Brewer HB Jr. Human lecithin:cholesterol acyltransferase deficiency: *in vivo* kinetics of low-density lipoprotein and lipoprotein-X. *Arterioscler Thromb Vasc Biol*. 2006;26:1370–1375.

Significance

Lecithin:cholesterol acyltransferase-deficiency syndromes are classified into 2 forms: familial lecithin:cholesterol acyltransferase deficiency and fish-eye disease. Patients with familial lecithin:cholesterol acyltransferase deficiency develop renal failure, whereas fish-eye disease patients do not. This study was performed to identify abnormal lipoproteins associated with the renal damage of patients with different mutations and manifestations. Size fractionation with gel filtration of patients' sera and in vitro incubation experiments with recombinant lecithin:cholesterol acyltransferase showed abnormal lipoproteins associated with the renal damage. Thus, our novel analytic approach identified large low-density lipoprotein and high-density lipoprotein with a composition specific to familial lecithin:cholesterol acyltransferase deficiency but not to fish-eye disease. The identification of abnormal lipoproteins may shed light on the clarification of renal pathology and the development of treatment for the patients with familial lecithin:cholesterol acyltransferase deficiency.

Materials and methods

Identification of FLD and FED patients

Patients were referred because of a clinical suspicion of LCAT deficiency due to the presence of corneal opacification and HDL deficiency, with or without proteinuria and/or renal insufficiency (Table 1). Lipid profiles of the patients are also given in Table 2. In our clinics, the definitive molecular diagnoses were established for all patients. The renal biopsy analyses were performed to make a diagnosis for renal damage in some patients. Patient(Pt)s 1 and 3 are Morroccan sister[24]. Other patients are unrelated. Pt.2 was treated with a fat-restricted diet (1570 kcal; fat 10 g and protein 45 g) during admission, with the prescription of losartan 50 mg/day for 8 months. Younger sister of Pt. 2 also shows corneal opacification. Informed consent from her was not obtained for genetic analysis and current study. Proteinuria for FLD patients were 6g/24h (Pt. 1), 2 g/24h (Pt. 2), 0.45 g/l (Pt. 3), 0.23g/24h (Pt. 4), and 0.5 g/l (Pt. 5).

Analysis of patient samples

This study was approved by the Ethics Committees of Chiba University School of Medicine and Academic Medical Center, University of Amsterdam, and informed consent was obtained from the participants including unaffected normolipidemic controls. Blood samples were obtained from participants, and serum was prepared and stored at -80 °C until use. LCAT activity (α-activity) was measured using artificial proteoliposomes as substrate[1]. Serum lipoproteins were fractionated by high-performance liquid chromatography with gel filtration column (HPLC-GFC)[2,3] and analyzed simultaneously by online enzymatic method to quantify total cholesterol (TC), free cholesterol (FC), triglyceride (TG), and phospholipid (PL) (Skylight Biotech, Akita, Japan). The resulting raw chromatograms (elution time versus lipid concentration) were further processed by computer program with the modified Gaussian curve fitting for resolving the overlapping peaks by mathematical treatment. Finally, the system subdivided the lipoprotein particles of normal subjects by size into the following 20 subclasses: chylomicron (CM, >80 nm, fractions 1-2), very low density lipoprotein (VLDL, 30-80 nm, fractions 3-7), low density lipoprotein (LDL, 16-30 nm, fractions 8-13), and high density lipoprotein (HDL, 8-16 nm, fractions 14-20). In this fractionation analysis, standard particle diameters have been reported to be >90, 75, 64, 53.6, 44.5, 36.8, 31.3, 28.6, 25.5, 23.0, 20.7, 18.6, 16.7, 15.0, 13.5, 12.1, 10.9, 9.8, 8.8, 7.6 nm for fraction 1 through 20, respectively[2]. Average sizes of lipoprotein