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 antihyperuricemic 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.

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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

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Original Article

Reference Interval for the Apolipoprotein B-48 Concentration in Healthy Japanese Individuals

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Aim: Small intestine-derived chylomicrons and chylomicron remnants, which are predominant in patients with postprandial hypertriglyceridemia, chylomicron syndrome and/or familial dyslipidemia, carry one molecule of apolipoprotein B-48 (apo B-48) per lipoprotein particle. We investigated the reference interval for the apo B-48 concentration.

Methods: We studied 516 individuals who provided written informed consent and confirmed that they were not taking any medications. BMI, waist circumference, blood pressure and the fasting serum concentrations of LDL-C, triglyceride (TG), HDL-C and apo B-48 were measured. The Apo B-48 concentrations were compared according to sex, a pre- or postmenopausal status, dyslipidemia (LDL-C $\geq$ 140 mg/dL, TG $\geq$ 150 mg/dL, HDL-C $<$ 40 mg/dL), metabolic syndrome (MetS) and the number of risk factors.

Results: The fasting apo B-48 concentrations (mean $\pm$ SD) were significantly higher in men than in women (3.8 ± 3.3 μg/mL vs 2.4 ± 1.9 μg/mL, $p<0.001$), subjects with a BMI of ≥ 25 kg/m^2 versus a BMI of <25 kg/m^2 (4.4 ± 3.7 μg/mL vs 2.8 ± 2.4 μg/mL, $p<0.001$) and those with versus without MetS (6.5 ± 4.3 μg/mL vs 3.0 ± 2.6 μg/mL, $p<0.001$). High apo B-48 concentrations were also observed in correlation with the number of risk factors for the MetS. The upper reference limit of apo B-48 was estimated to be 5.7 μg/mL among the 332 patients with normolipidemia, excluding those exhibiting a mean value above ± 2.58 standard deviations (SDs), as the mean and range of mean ± 1.96 SD were calculated to be 2.04 μg/mL (reference value) and 0.74 to 5.65 μg/mL (reference interval), respectively.

Conclusions: Based on our study of normolipidemic patients, the upper reference limit for the fasting apo B-48 concentration is estimated to be 5.7 μg/mL.

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Key words: Apolipoprotein B-48 (apo B-48), Chylomicrons, Chylomicron remnants, Reference interval

Introduction

Fasting and postprandial hypertriglyceridemia are serious causative factors of cardiovascular events and sudden cardiac death[1]. An increased serum triglyceride (TG) concentration results from the accumulation of TG-rich lipoproteins (TRLs), particularly after a meal. Postprandial hyperlipidemia refers to the occurrence of a high TG concentration after a meal, which is known to be significantly associated with the development of atherosclerotic cardiovascular disease[2, 3]. TRL contain two types of apolipoprotein

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(apo) B, apo B-100 derived from the liver and apo B-48 derived from the small intestine[4].

Chylomicrons (CMs) are synthesized from apo B-48, TG and cholesterol ester in small intestinal cells following the ingestion of lipid-rich foods. After being released into the peripheral blood, CMs are metabolized into smaller remnant particles, CM-remnants, by lipoprotein lipase (LPL) attached to the peripheral vascular wall and taken up by the liver. Apo B-100, a major component of very-low-density lipoprotein (VLDL) is produced in the liver. VLDL is also reduced to smaller VLDL-remnants (or intermediate-density lipoprotein, IDL) by the actions of LPL in the peripheral blood. These remnant particles (CM-remnants and VLDL-remnants) directly infiltrate the vascular wall, subsequently triggering the development of atherosclerotic disease via accelerated macrophage foam cell formation, platelet coagulation and small dense LDL accumulation, as well as the induction of a low concentration of high-density lipoprotein (HDL) cholesterol (HDL-C)[5].

A number of remnant cholesterol assays have been developed and are currently being used to evaluate the risks of atherosclerotic diseases, such as cardiovascular disease (CAD)[6-8]. However, these methods cannot be used to accurately discriminate small intestine-derived CM-remnants from liver-derived VLDL-remnants; therefore, the development of a new assay system is required in order to quantitatively measure the CM-remnant concentration independently. Since one CM-remnant particle contains one apo B-48 molecule and the concentration of apo B-48 is equivalent to that of CM-remnants, we developed a new assay system for measuring the apo B-48 concentration. First, we prepared an enzyme-linked immunosorbent assay (ELISA)[9] for use on a fully automated analyzer system based on the chemiluminescent enzyme immunoassay (CLEIA)[10]. Remnants are usually metabolized immediately; however, the apo B-48 concentration remains elevated due to increased food-derived lipid intake, accelerated TRL synthesis and/or delayed TRL catabolism.

The half-life of the CM particles produced following the ingestion of fat is approximately 30 minutes in the peripheral blood, although the measurable concentration of apo B-48 proteins remains under a fasting condition due to the large amount of lipid absorption and CM production in the small intestine. Therefore, the fasting apoB-48 concentration is correlated with an increase in the TG level following the consumption of a high-fat meal, implying that the fasting apo B-48 concentration is a marker of postprandial hyperlipidemia[11]. High apo B-48 concentrations are usually observed in patients with type III hyperlipidemia[9], metabolic syndrome (MetS)[12], type IIb hyperlipidemia[13] or CD36 deficiency[14]. However, the reference interval for the apo B-48 concentration in healthy fasting individuals has not yet been established.

Aim

In this study, we attempted to establish the upper reference limit and reference interval for the fasting apo B-48 concentration in individuals with normolipidemia.

Subjects and Methods

Subjects

The subjects of this study included 516 individuals who received their annual health checkup and were not taking any medications. The study was carried out under the approval of the Osaka University Health Care Center and Saint (St.) Marguerite Hospital, and all participants provided their written informed consent. The institutional ethics committees of both facilities approved the research protocol. After confirming the lack of a significant adverse medical history known to affect lipoprotein or carbohydrate metabolism, various anthropometric parameters, including height, body weight and waist circumference were obtained and the body mass index (BMI, body weight [kg]/height [m]^2) was calculated. Blood samples were collected in the morning after overnight fasting. The serum samples were then separated via low-speed centrifugation and stocked at −80℃ until the analyses. All specimens were handled according to the protocols of the Helsinki Declaration.

Measurements

Blood pressure (BP) was measured in the sitting position. Hypertension was diagnosed based on a systolic BP of ≥140 mmHg and/or a diastolic BP of ≥90 mmHg. A high BP status was determined based on a systolic BP of ≥130 mmHg and/or a diastolic BP of ≥85 mmHg (according to the guidelines for the management of hypertension issued by the Japanese Society of Hypertension). The serum TG concentration was measured according to an enzymatic method, and the LDL-cholesterol (LDL-C) and HDL-C levels were measured using direct methods. We identified cases of dyslipidemia and normolipidemia based on the diagnostic criteria for dyslipidemia of the Japan Atherosclerosis Society: (a) an LDL-C level of ≥140 mg/dL, (b) a TG level of ≥150 mg/dL, (c) an HDL-C level of

< 40 mg/dL (according to the guidelines for the diagnosis and prevention of atherosclerotic cardiovascular disease for the Japanese)[15]. Abnormal factors were summarized in the patients with dyslipidemia. The fasting plasma glucose (FPG) concentration was measured according to the hexokinase UV method, and the hemoglobin A1c (HbA1c) (JDS) level was measured according to the latex agglutination method. A high fasting glucose level was defined as an FPG of ≥ 110 mg/dL, according to the criteria of the Japan Diabetes Society. MetS was diagnosed based on the criteria of the Japanese Society of Internal Medicine[16], namely, a waist circumference of ≥ 85 cm in men and ≥ 90 cm in women combined with at least two of the following factors: (a) a high BP status and hypertension (a systolic BP of ≥ 130 mmHg and/or a diastolic BP of ≥ 85 mmHg), (b) abnormal lipid metabolism (a TG level of ≥ 150 mg/dL and/or an HDL-C level of < 40 mg/dL), (c) high fasting glucose (an FPG level of ≥ 110 mg/dL). Cardiac risk factors were summarized in cases of MetS.

The serum apo B-48 concentration was determined using the CLEIA system (Fujirebio, Inc., Tokyo, Japan)[10]. Briefly, serum samples were incubated with treatment buffer solution supplemented with surfactant in order to separate apo B-48 from CMs and CM-remnants. The pre-treated samples were incubated with ferrite particles coupled with murine monoclonal antibodies against apo B-48 in the solid phase. After washing, further incubation was carried out with alkaline phosphatase-conjugated anti-apo B monoclonal antibodies as a second antibody. After further washing, a chemiluminescent substrate was added to the test cartridge, after which the relative chemiluminescent intensity was measured and the serum apo B-48 concentration was calculated according to a standard curve.

Statistical Analysis

The statistical analysis was performed using the non-parametric Mann-Whitney U test according to F-study with the Stat Flex software program (ver. 6, Artec Inc., Osaka, Japan) after confirming the distribution. The level of significance was assumed to be 95%. The upper reference limit and reference interval for the apo B-48 concentration were estimated according to the methods recommended by CLSI (Clinical and Laboratory Standards Institute). Briefly, after normalizing all data using logarithm conversion, the mean and standard deviation (SD) were calculated and patients exhibiting a mean value above ± 2.58 SD were eliminated. This process was repeated until no exception data were calculated. Subsequently, the value was returned to the integer, and the upper reference limit and reference interval were defined.

Table 1. Clinical and Laboratory Data

	Mean ± SD	95% Confidence Interval
Men/Women	284/232	
Age (year)	42 ± 10/42 ± 11	
Post-menopausal	48/232	
BMI (kg/m²)	22.4 ± 3.3	22.0-22.7
Waist circ. (cm)	91.1 ± 5.6	90.0-92.2
sBP (mmHg)	115.9 ± 14.6	114.6-117.2
dBP (mmHg)	73.2 ± 11.3	72.2-74.2
TC (mg/dL)	199 ± 31	196.0-201.6
TG (mg/dL)	94 ± 69	87.7-99.7
HDL-C (mg/dL)	65 ± 15	63.1-65.9
LDL-C (mg/dL)	121 ± 29	118.1-123.3
FPG (mg/dL)	87 ± 13	86.2-88.5
HbA1c (JDS) (%)	5.0 ± 0.5	4.9-5.1
	Number of Patients	
BMI ≥ 25 kg/m²	111 (21.5%)	
BMI < 25 kg/m²	405 (78.5%)	
Hypertension	47 (9.1%)	
High BP status	103 (20.0%)	
High FPG	10 (1.9%)	
Number of abnormal factors for dyslipidemia		
0	337 (65.3%)	
1	138 (26.7%)	
2	37 (7.2%)	
3	4 (0.8%)	
Number of risk factors for metabolic syndrome (MetS)		
0	303 (58.7%)	
1	135 (26.2%)	
2	53 (10.3%)	
3	24 (4.6%)	
4	1 (0.2%)	

The abbreviations used in this Table are as follows.
dBP: diastolic blood pressure, sBP: systolic blood pressure, BMI: body mass index, FPG: fasting plasma glucose, HbA1c: hemoglobin A1c, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, TC: total cholesterol, TG: triglycerides

Results

Background Characteristics of the Subjects

The total number of registered subjects was 516 (284 men and 232 women: 183 premenopausal patients, 48 postmenopausal patients and one unknown patient) at two hospitals. The assay data and classification of the subjects are summarized in **Table**

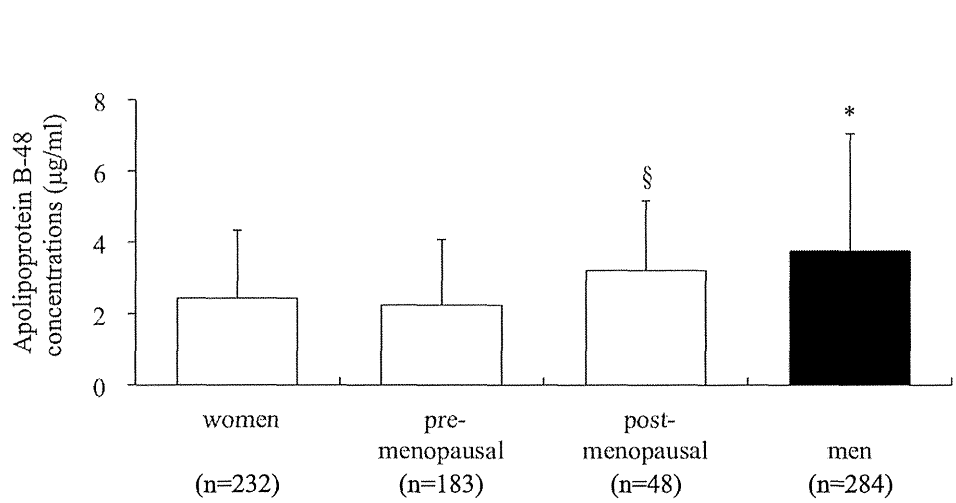

Fig. 1A. Comparison of the apolipoprotein B-48 concentrations in all cases.

The apolipoprotein B-48 concentrations in 284 men and 232 women (183 premenopausal patients, 48 postmenopausal patients and one unknown patient) were compared. The values indicate the mean ± standard deviation as follows: women = 2.4 ± 1.9 μg/mL, premenopausal women = 2.2 ± 1.8 μg/mL, menopausal women= 3.2 ± 2.0 μg/mL, men = 3.8 ± 3.3 μg/mL. Statistical significance was assessed using the Mann-Whitney *U* test. $^{*}p<0.001$ against women, $^{\S}p<0.001$ against premenopausal women.

1. A total of 111 patients had a BMI of ≥25 kg/m^2, while a waist circumference beyond the standard range (indicating abdominal obesity) was observed in 114 cases (one-fifth of all cases). Regarding abnormal factors related to dyslipidemia (a high LDL-C concentration, high TG concentration or low HDL-C concentration), two-thirds of the subjects (337 patients, 161 men and 176 women: 152 premenopausal patients and 24 postmenopausal patients) were classified as having no abnormal factors for dyslipidemia; these patients were classified into the normolipidemic group. One-third of the patients exhibited more than one abnormal factor for dyslipidemia. Twenty-four patients, or one-fifth of those with a high BMI (≥25 kg/m^2), were diagnosed with MetS, as their waist circumference was beyond the standard range and they exhibited two of three risk factors, including BP, FPG and abnormal lipid metabolism. Most of the patients exhibited either no risk factors (58.7%, 303 patients) or one risk factor (26.2%, 135 patients) for MetS, including hypertension (or a high BP status), hypertriglyceridemia, low HDL-cholesterolemia and a high FPG level.

Apo B-48 Concentrations and their Distribution in Several Classifications

We examined the fasting apo B-48 concentrations after classifying the patients into various groups. First, a sex difference was observed, namely, the mean apo B-48 concentration in men (284 patients) was higher than that observed in women (232 patients)

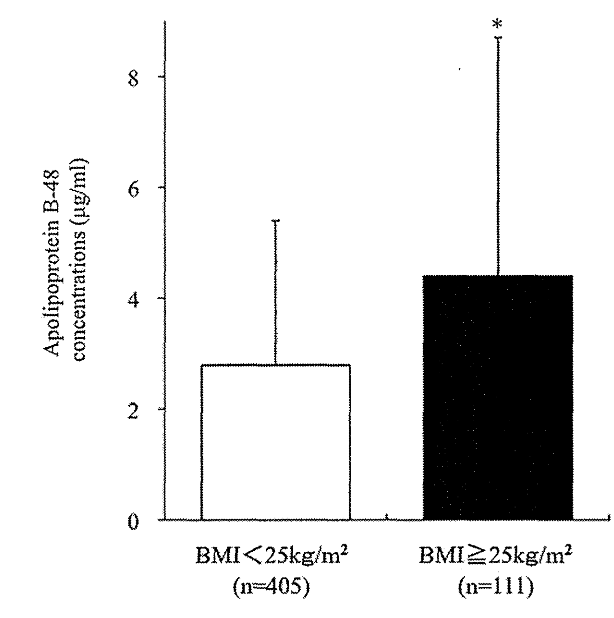

Fig. 1B. Comparison of the apolipoprotein B-48 concentrations in the subjects with a BMI of <25 kg/m^2 and those with a BMI of ≥25 kg/m^2.

The values indicate the mean ± standard deviation, as follows: BMI <25 kg/m^2 = 2.8 ± 2.4 μg/mL (*n* = 405), BMI ≥25 kg/m^2 = 4.4 ± 3.7 μg/mL (*n* = 111). The number of subjects is shown in brackets. Statistical significance was assessed using the Mann-Whitney *U* test. $^{*}p<0.001$

(3.8 ± 3.3 μg/mL vs 2.4 ± 1.9 μg/mL, $p<0.001$, Mann-Whitney *U* test) (**Fig. 1A**). A significant difference was also observed between the pre- and postmenopausal

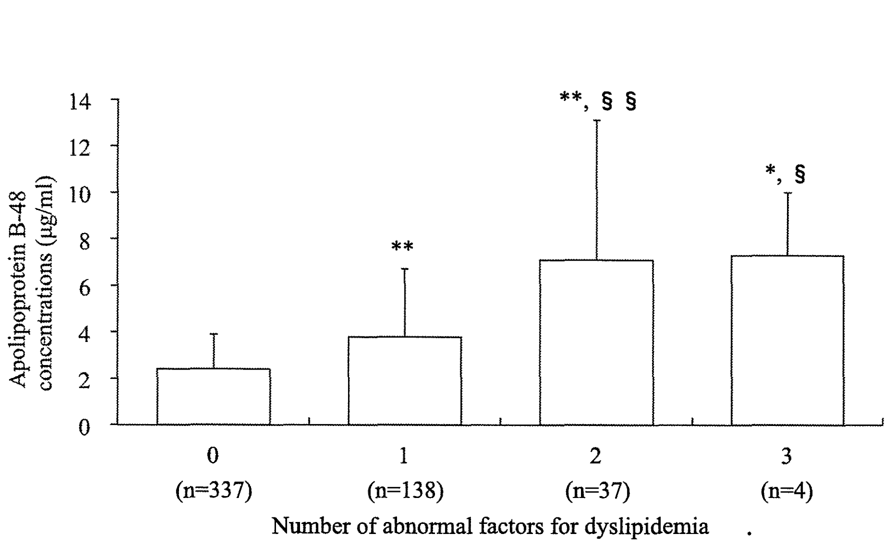

Fig. 2. Comparison of the apolipoprotein B-48 concentrations according to the cumulative number of abnormal factors for dyslipidemia.

The number of abnormal factors for dyslipidemia (a high LDL-C concentration [LDL-C ≥140 mg/dL], high TG concentration [TG ≥150 mg/dL] or low HDL-C concentration [HDL-C <40 mg/dL]) was counted in all patients. The apo B-48 concentrations were compared between four groups: patients with no abnormal factors (n=337) and those with one (n=138), two (n=37) and three abnormal factors (n=4). The values indicate the mean ± standard deviation, as follows: no abnormal factors = 2.4 ± 1.5 μg/mL, one abnormal factor = 3.8 ± 2.9 μg/mL, two abnormal factors = 7.1 ± 6.0 μg/mL, three abnormal factors = 7.3 ± 2.7 μg/mL. Statistical significance was assessed using the Mann-Whitney U test. $^{*}p<0.01$, $^{**}p<0.001$ against patients with no abnormal factors, $^{\S}p<0.05$, $^{\S\S}p<0.001$ against patients with one abnormal factor.

women: the apo B-48 concentrations of the 48 postmenopausal patients were higher than those of the 183 premenopausal patients, while the mean value of the postmenopausal patients was increased, drawing near the average observed in men (3.2 ± 2.0 μg/mL vs 2.2 ± 1.8 μg/mL, $p<0.001$). When all subjects were classified according to BMI, 111 patients with a BMI of ≥25 kg/m^2 were found to exhibit a statistically significantly high apo B-48 concentration in comparison with that observed in the 405 patients with a BMI of <25 kg/m^2 (4.4 ± 3.7 μg/mL vs 2.8 ± 2.4 μg/mL, $p<0.001$, Mann-Whitney U test) (**Fig. 1B**). The number of abnormal factors for dyslipidemia (a high LDL-C concentration [LDL-C ≥140 mg/dL], high TG concentration [TG ≥150 mg/dL] or low HDL-C concentration [HDL-C <40 mg/dL]) was counted in all patients. The apo B-48 concentrations in the patients with one (n=138), two (n=37) or three (n=4) abnormal factors for dyslipidemia were significantly higher than those observed in the patients with no abnormal factors for dyslipidemia (n=337) (**Fig. 2**). The 24 patients with MetS displayed significantly higher apo B-48 concentrations than the 492 patients without MetS (6.5 ± 4.3 μg/mL vs 3.0 ± 2.6 μg/mL, $p<0.001$, Mann-Whitney U test) (**Fig. 3A**)[16]. In addition, a positive correlation was observed between the apo B-48

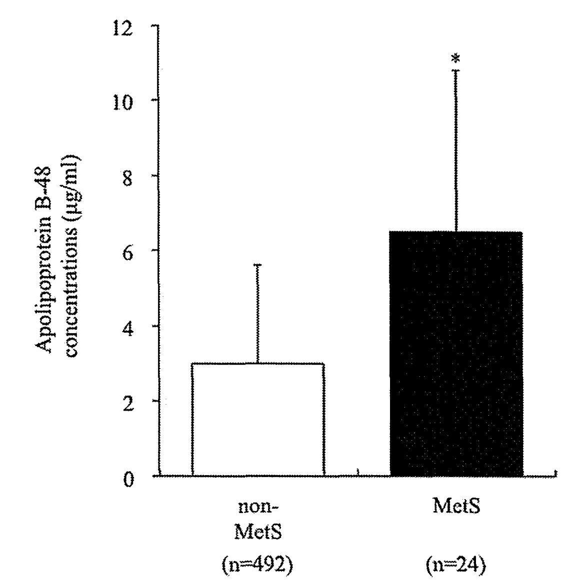

Fig. 3A. Comparison of the apolipoprotein B-48 concentrations in the subjects with or without metabolic syndrome (MetS).

The subjects were divided into two groups, MetS (n=24) and non-MetS (n=492), according to the criteria of the Japanese Society of Internal Medicine. The values indicate the mean ± standard deviation, as follows: non-MetS = 3.0 ± 2.6 μg/mL and MetS = 6.5 ± 4.3 μg/mL. Statistical significance was assessed using the Mann-Whitney U test. $^{*}p<0.001$

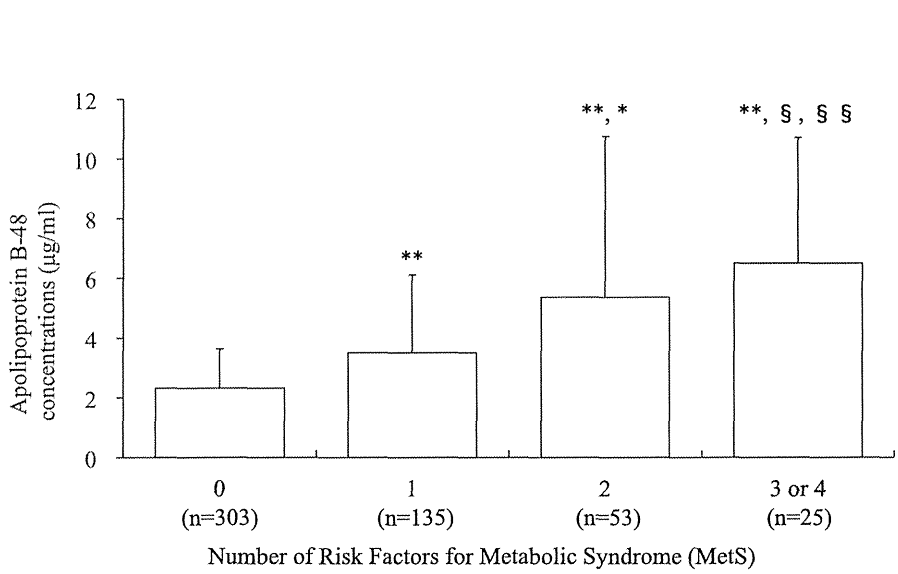

Fig. 3B. Comparison of the apolipoprotein B-48 concentrations according to the cumulative number of risk factors for metabolic syndrome (MetS).

The subjects were divided into four groups: patients with no risk factors ($n=303$) and those with one ($n=135$), two ($n=53$) and three or four risk factors ($n=25$), according to the number of abnormal factors for MetS (waist circumference, a high BP status, high TG/low HDL-C concentrations, a high FPG concentration). The values indicate the mean ± standard deviation, as follows: no risk factors = 2.3 ± 1.3 μg/mL, one risk factor = 3.5 ± 2.6 μg/mL, two risk factors = 5.4 ± 5.4 μg/mL, three or four risk factors = 6.5 ± 4.2 μg/mL. Statistical significance was assessed using the Mann-Whitney *U* test. $^{**}p<0.001$ against patients with no risk factors, $^{*}p<0.01$, $^{\S}p<0.001$ against patients with one risk factor, $^{\S\S}p<0.05$ against patients with two risk factors.

concentration and the number of risk factors for the components of MetS (hypertension, including a high BP status, hypertriglyceridemia, low HDL-cholesterolemia and a high fasting glucose level) (**Fig. 3B**).

Calculation of the Upper Reference Limit for the Apo B-48 Concentration in the Patients with Normolipidemia

The upper reference limit and reference interval for the apo B-48 concentration were calculated in 337 patients without parameters of abnormal lipid metabolism, as no differences in data were observed between the 152 pre- and 24 postmenopausal normolipidemic patients, as shown in **Fig. 4**; namely, the mean value among the postmenopausal patients increased (2.1 ± 1.2 μg/mL vs 2.6 ± 1.8 μg/mL, not statistically significant) approaching the average observed in the 161 men (2.7 ± 1.7 μg/mL vs 2.6 ± 1.8 μg/mL, not statistically significant). We estimated the upper reference limit for the apo B-48 concentration in 332 normolipidemic patients, excluding those with a mean value of ± 2.58 SD. The calculated mean value and range of mean ± 1.96 SD were 2.04 μg/mL (reference value) and 0.74 to 5.65 μg/mL (reference interval), respectively. Based on these results, we consider 5.7 μg/mL to be the optimum apo B-48 upper reference limit (**Fig. 5**). The reference interval and upper reference limit for the apo B-48 concentration were determined according to the results obtained with the CLEIA system (Fujirebio, Inc., Tokyo, Japan).

Discussion

The occurrence of a high TG concentration after a meal, or postprandial hypertriglyceridemia, is a risk factor for atherosclerosis. Meal-derived TG elevation results from the assembly of CMs, which contain a large quantity of TG in each particle in comparison with VLDL. CMs are immediately hydrolyzed to CM-remnants in patients with normolipidemia, whereas an abnormally high concentration of CM-remnants is observed six hours after meal intake in those with postprandial hypertriglyceridemia. Therefore, the accumulation of CM-remnants due to postprandial hypertriglyceridemia is one of the most serious risk factors for the development of arteriosclerosis-related diseases[17]. Several CM-remnant assay methods have been reported, including the retinyl palmitate method, the combination method employing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Western blotting and the remnant-like particle-cholesterol assay method[18]. However, these

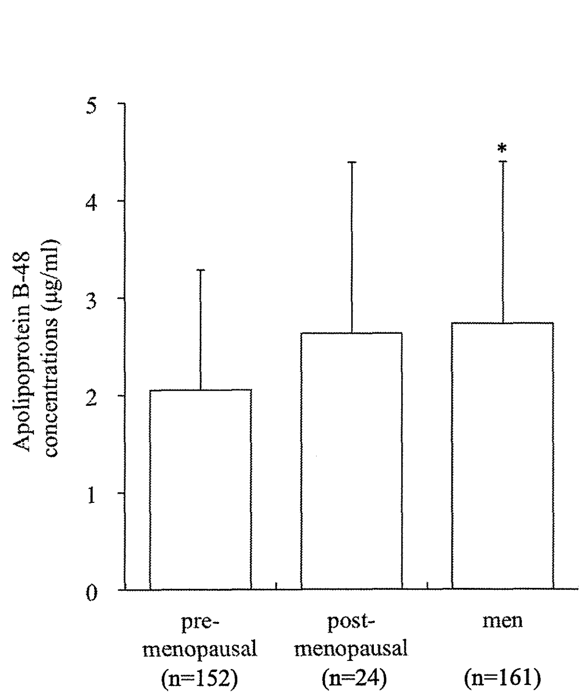

Fig. 4. Comparison of the apolipoprotein B-48 concentrations in the patients with normolipidemia.

The apolipoprotein B-48 concentrations in 161 men and 176 women (152 premenopausal patients and 24 postmenopausal patients) were compared. The values indicate the mean ± standard deviation, as follows: premenopausal = 2.1 ± 1.2 µg/mL, postmenopausal = 2.6 ± 1.8 µg/mL, men = 2.7 ± 1.7 µg/mL. Statistical significance was assessed using the Mann-Whitney *U* test. $^*p<0.001$ against premenopausal women.

methods are associated with problems related to instability, complexity, reproducibility and inaccuracy regarding the assay target[19, 20]. In contrast, apo B-48 is a component of CMs and CM-remnants; therefore, the apo B-48 concentration is a direct marker of alteration of the meal-derived TG concentration, although the apo B-48 concentration in the peripheral blood is approximately one-fiftieth or one-hundredth of the apo B-100 concentration. Several assay methods for measuring the apo B-48 concentration using polyclonal antibodies and/or monoclonal antibodies have been reported[21, 22]. However, as the amino acid sequence of apoB-48 is completely identical to the N-terminal side of apoB-100, it is very difficult to prepare monoclonal and polyclonal antibodies. As a result, the accuracy of these ELISA methods is insufficient for the measurement of apo B-48. On the other hand, an accurate ELISA method was recently developed with the cooperation of Sakai *et al.*[9] using a highly specific monoclonal antibody to the C-terminal of apo B-48 established by Uchida *et al.*[23]. This ELISA system was subsequently improved to create a fully-automated assay system based on CLEIA[10].

In this study, we determined the reference level for the apoB-48 concentration using serum samples obtained from healthy individuals with normolipidemia. Namely, normolipidemic patients were selected by applying the diagnostic criteria for dyslipidemia of the Japan Atherosclerosis Society: (a) an LDL-C level of ≥ 140 mg/dL, (b) a TG level of ≥ 150 mg/dL and (c) an HDL-C level of < 40 mg/dL (Guidelines for the diagnosis and prevention of atherosclerotic cardiovascular disease for the Japanese)[15]. We then used the CLSI recommended method to calculate the reference level. Briefly, we estimated the upper reference limit and reference interval for the apo B-48 concentration in 332 normolipidemic patients, excluding those with a mean value above ± 2.58 SD. We thus determined the reference level for the apo B-48 concentration to be 2.04 µg/mL, the reference interval to range from 0.74 to 5.64 µg/mL and the upper reference limit to be 5.7 µg/mL. Incidentally, a different apo B-48 measuring kit (Human apo B-48 ELISA, Shibayagi, Gunma, Japan) is currently available in Japan. Therefore, the upper reference limit and reference interval for the apo B-48 concentration determined in this study should be restricted to the results obtained using the CLEIA system (Fujirebio, Inc., Tokyo, Japan). We then attempted to determine whether abnormal CM-remnant metabolism was present in the normolipidemia group. When the apo B-48 concentrations of all health checkup patients were measured, a high apo B-48 concentration was observed in the following order: men, postmenopausal women and premenopausal women. The apo B-48 concentrations also differed according to the presence or absence of obesity or MetS. The TG and LDL-C concentrations, which are affected by the apo B-48 concentrations, also differed between men and women and between pre- and postmenopausal women. The upper reference limit and reference interval for the apo B-48 concentration were estimated in patients with normolipidemia; this group also contained patients with hypertension, obesity and hyperglycemia, all of which may affect lipoprotein metabolism. In this study, we examined patients who received their annual health checkup; it was not assumed that these patients had severe metabolic disorders. Therefore, it is necessary to conduct separate studies of different patient groups, including those with relatively severe metabolic disorders.

Recent reports have highlighted the clinical usefulness of the apo B-48 concentration as a screening marker of type Ⅲ hyperlipidemia in patients with accumulated CM-remnants[9, 24] and parameter of the CM-remnants status in those with diabetes mellitus (DM) exhibiting carotid artery plaque[25]. Additionally, correlations have been reported between the apo B-48

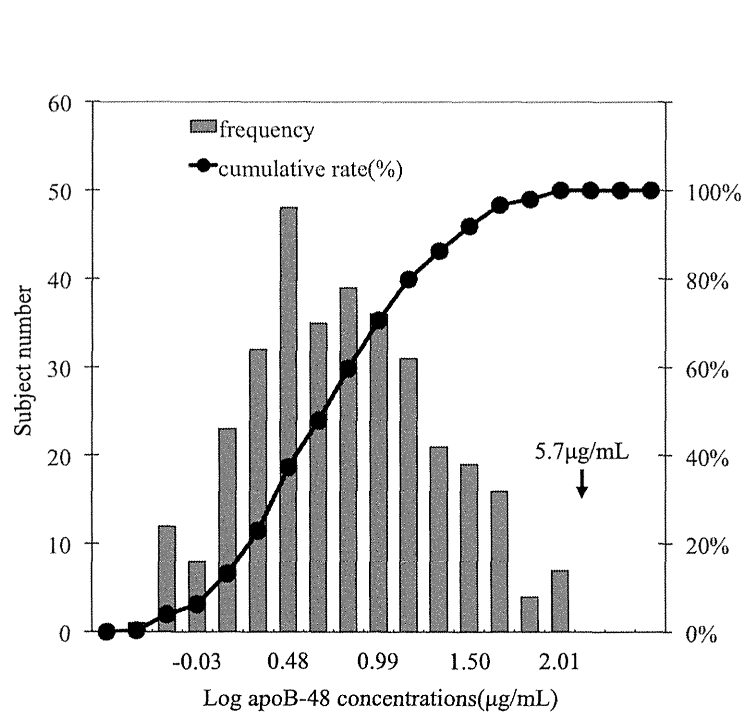

Fig. 5. Distribution of the apolipoprotein B-48 concentrations in the patients with normolipidemia.

The apolipoprotein B-48 concentration is expressed as the log concentration. The upper limit among the 332 patients with normolipidemia was found to be 5.7 μg/mL.

concentration and the carotid intima-media thickness in normotriglyceridemic (100 < TG < 150 mg/dL) subjects[26] as well as the status of kidney dysfunction in DM patients[27] and the incidence of CAD in ischemic heart disease patients in comparison with other risk factors, such as hypertriglyceridemia, low HDL-cholesterolemia, hypertension and/or hypoadiponectinemia[28]. Furthermore, an elevated incidence of CAD is observed in patients with a high apo B-48 concentration and the risk factors described above. Ultimately, this apo B-48 assay may have numerous applications in future studies.

Conclusion

Based on the results of this multicenter study of Japanese normolipidemic patients not taking any medications, the upper reference limit for the apo B-48 concentration in a fasting state is 5.7 μg/mL, as the mean value was found to be 2.04 μg/mL (reference value) and the mean ± 1.96 SD ranged from 0.74 to 5.65 μg/mL (reference interval).

Study Limitations

The limited number of subjects treated at two clinical facilities likely affected the results of this study.

Acknowledgements

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Conflicts of Interest

Fujirebio, Inc. shared the costs of apo B-48 measurement. All authors have no other conflicts of interest to disclose.

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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.

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

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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

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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

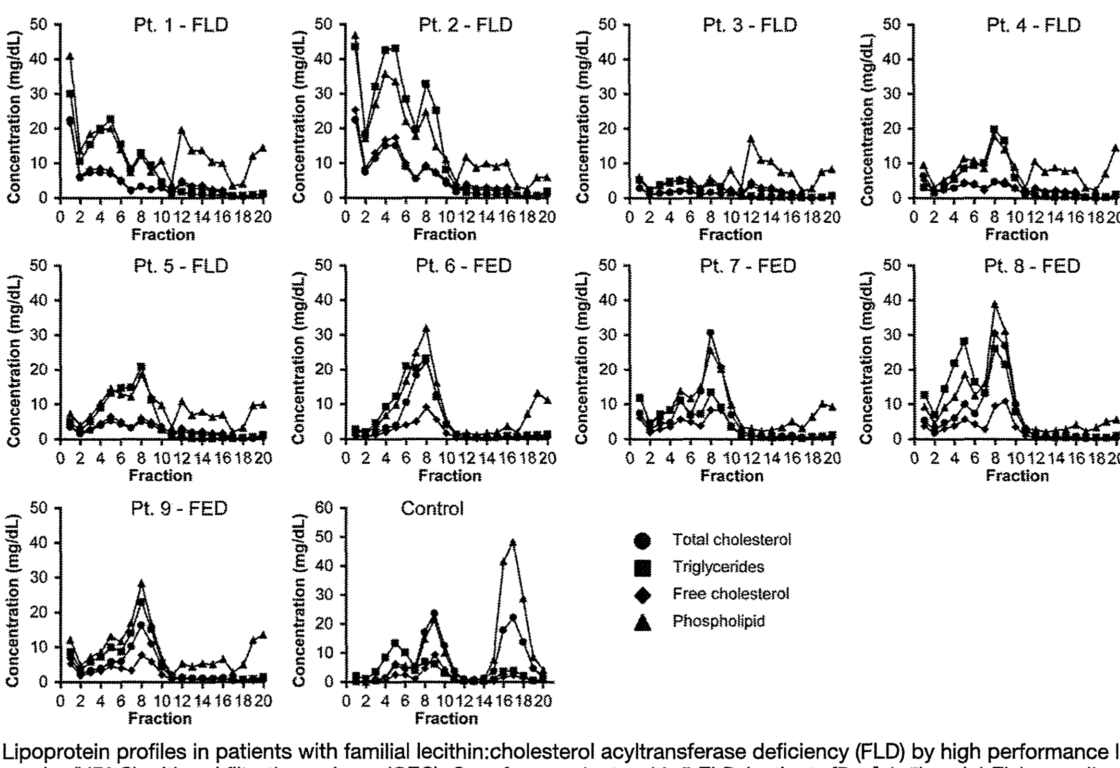

Figure 1. Lipoprotein profiles in patients with familial lecithin:cholesterol acyltransferase deficiency (FLD) by high performance liquid chromatography (HPLC) with gel filtration column (GFC). Sera from patients with 5 FLD (patients [Pts.] 1–5) and 4 Fish-eye disease (FED; Pts. 6–9) were subjected to 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.

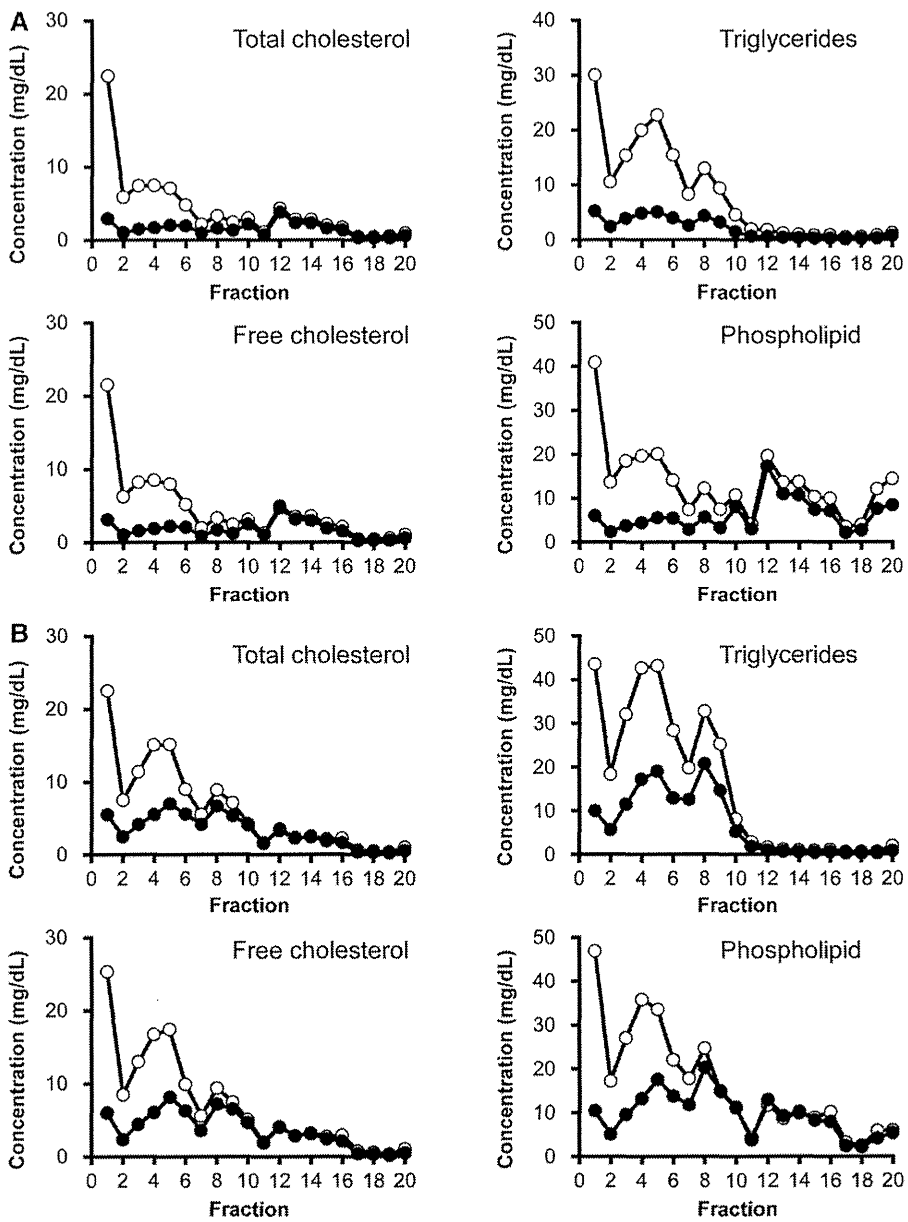

Figure 2. Differences in lipoproteins in patients with familial lecithin:cholesterol acyltransferase deficiency (FLD) with or without renal insufficiency. **A**, Lipoprotein profiles were compared between a patient with FLD with nephrotic range proteinuria (patient 1, ○) and patient 3 with mild proteinuria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