Heart

Mortality risk of triglyceride levels in patients with coronary artery disease

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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

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ABSTRACT

Familial lecithin-cholesterol acyltransferase (LCAT) deficiency is a hereditary disease characterized by an abnormal lipid profile, corneal opacity, anemia and progressive renal disease. We report a patient with complete loss of LCAT activity due to a novel *lcat* gene mutation of Cys74Tyr in the lid region of LCAT protein. Esterification of cholesterol in this patient was disturbed by disruption of a substrate binding loop of Cys50-Cys74 in LCAT protein. She had progressive renal dysfunction, proteinuria, corneal opacity, anemia and an abnormal lipid profile. Her serum lipids showed a significant increase in abnormal lipoproteins at the original position in agarose gel electrophoresis and VLDL-cholesterol, and a severe decrease in serum HDL-cholesterol. Lipoprotein analyzes also revealed the presence of an abnormal midband lipoprotein, and a maturation disturbance of HDL particles. Renal function and proteinuria improved following the adoption of a fat-restricted diet and administration of an angiotensin II receptor blocker. The abnormal lipoproteins also decreased after this treatment.

1. Introduction

Lecithin-cholesterol acyltransferase (LCAT) deficiency is an uncommon autosomal recessive disorder which results from a gene mutation of LCAT. Since the first identification of LCAT as a unique plasma enzyme [1], 86 mutations in the LCAT gene have been described. Patients with LCAT deficiency show an abnormal circulating lipoprotein profile as a result of the disturbed esterification of free cholesterol incorporated into high-density lipoproteins. Increased plasma concentrations of unesterified cholesterol, triglyceride (TG) and phosphatidylcholine result in lipid deposition in the tissue. LCAT deficiency develops as two clinically distinct syndromes, familial LCAT deficiency (FLD) and fish eye disease (FED). Patients with FLD show corneal opacities, hemolytic anemia, and progressive renal disease [2]. Renal disease occurs as a result of the loss of enzyme activity against β-lipoproteins rather than against α-lipoproteins. Meanwhile, FED patients develop corneal opacities as a result of a partial deficiency in LCAT activity.

A number of approaches to the treatment of LCAT deficiency have been proposed. LCAT replacement therapy by plasma transfusion produced a marked improvement in the deranged composition of TG-rich lipoproteins and Apo-E concentrations [3,4]. Recent advances in gene therapy have allowed the transplantation of *ex vivo* lcat gene-transduced adipocytes and subsequent production of human LCAT protein in circulating plasma [5,6]. Further, a clinical trial of synthetic LCAT in patients with coronary arterial disease is also currently underway at NIH (NCT01554800). In contrast, a fat-restriction diet improves the hypertriglyceridemia in these patients by reducing TG-rich lipoproteins. The lipid-lowering drugs nicotinic acid and fenofibrate have been shown to ameliorate renal function and proteinuria [7], and corticosteroids and renin–angiotensin–aldosteron (RAA) system blockers such as ACE inhibitors and angiotensin II receptor blockers (ARB) also decreased proteinuria [8–10].

Here, we report a novel LCAT gene mutation in a patient which resulted in disruption of the disulfide bridges essential to the

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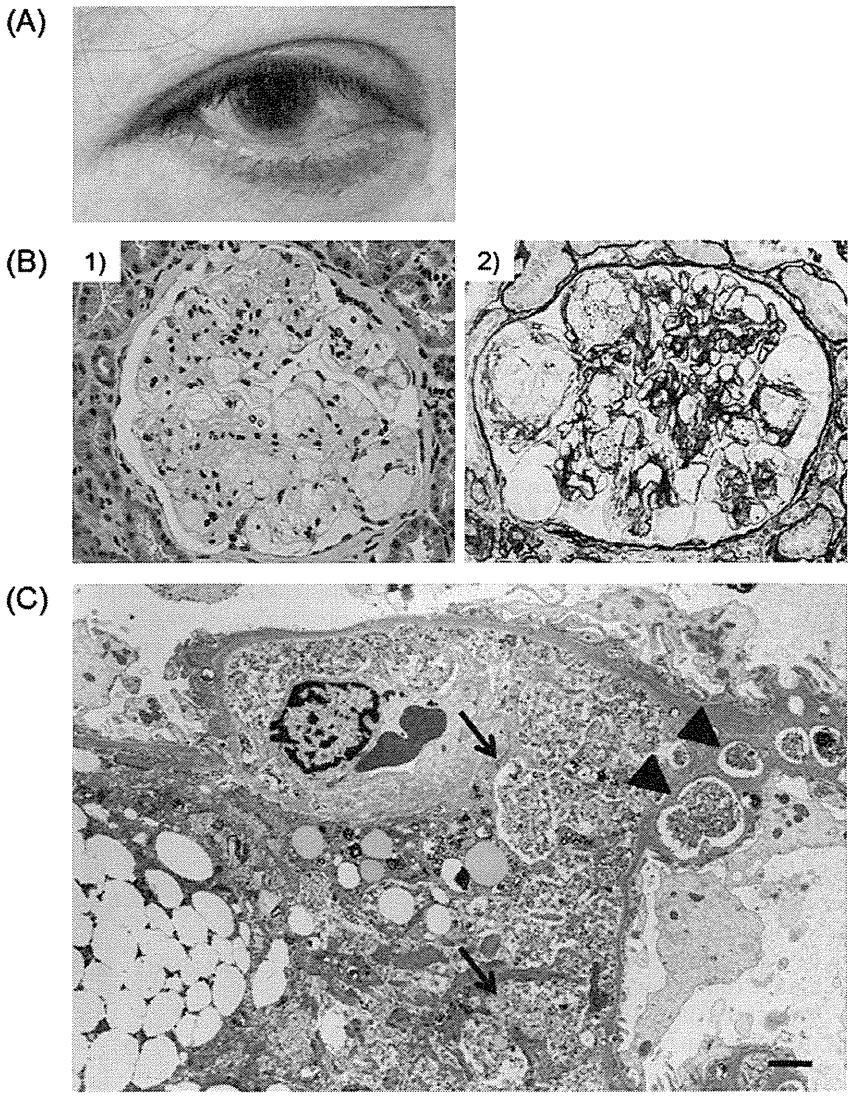

Fig. 1. Ocular and renal pathology (A) Corneal opacity in the patient. (B)-1) PAS staining of a renal biopsy specimen. 400×. An increase in cell number and matrix expansion are seen in the mesangial area of the glomerulus. Foam cell infiltration into capillary loops and the mesangial area are seen in the glomerulus. (B)-2) PAM staining shows irregular thickening and double contours in the glomerular basement membrane (GBM). Foam cells within granular structures are present in the capillary lumen. (C) Electron micrograph of the glomerulus shows the presence of clear vacuoles containing granular structures in the mesangium (↓) and within the GBM (▼). 3000×. Bar = 2 μm.

enzyme lid region. This patient had a complete deficiency in LCAT activity and renal insufficiency. We also investigated the effects of a fat-restriction diet and administration of an ARB on plasma lipoprotein profiles and proteinuria in this patient.

2. Materials and methods

2.1. Biochemical and genetic analysis

Biochemical and urine samples were analyzed by enzymatic methods using a chemical autoanalyzer (Hitachi Co., Tokyo, Japan). Esterified cholesterol concentrations were calculated as the difference between total and free cholesterol. LDL-cholesterol was determined a Determiner-L LDL-C (Kyowa Medex, Tokyo, Japan). LCAT activity in serum was measured using a colorimetric method for analyzing cholesterol esterification rate (CER) with synthetic dipalmitoyl lecithin sol [11]. Alpha-LCAT activity was also measured using Anasolb LCAT® (Sekisui Medical, Tokyo, Japan). Genomic DNA was purified from plasma with a QIAamp DNA kit (QIAGEN, Hilden, Germany). Genomic fragments were amplified by PCR, followed by agarose gel purification and direct sequencing. The entire sequence of the *lcat* gene locus thereby obtained was compared with a reference sequence (NM_000229) to identify nucleotide substitution. The study was approved by the Ethics Committee of Chiba University School of Medicine, and informed consent was obtained from the patient. Both parents were deceased, and informed consent for genetic analysis was not obtained from her younger sister, who shows corneal opacity.

2.2. Lipoprotein analysis

The serum lipoprotein profile was determined by polyacrylamide gel disc electrophoresis [12]. Two-dimensional electrophoresis was performed as described previously [13]. Lipoproteins were also evaluated by agarose gel electrophoresis using the rapid electrophoresis system [14–17]. After electrophoresis, cholesterol and triglycerides in the plates were separately stained and analyzed with a Cho/Trig COMBO kit according to the

manufacturer's instructions (Helena Laboratories, Saitama, Japan). Using the serum total cholesterol and TG concentrations in the blood samples, concentrations of cholesterol and TG in each fraction were determined using the detected ratio of cholesterol and TG after automatic densitometric analysis.

3. Results

3.1. Patient

A 61-year-old Japanese woman was transferred to the Department of Nephrology, Kitasato University Hospital. She complained of dyspnea on walking for the preceding five months, and eyelid and pretibial edema for one month. Family history showed her married parents were cousins, and that her younger sister had corneal opacity and mental retardation. She had anemia (Hemoglobin 9.5 g/dl) bilateral corneal opacities (Fig. 1A) and pitting edema. Urinalysis revealed proteinuria at 2 g/day, 1 + microscopic hematuria, and an N-acetyl-β-D-glucosaminidase (NAG) level of 29.1 unit/L (normal range: 1–4.2). Blood chemistry showed total protein 6.4 g/dL, albumin 3.4 g/dL, total cholesterol 235 mg/dL, TG 235 mg/dL, HDL-cholesterol 22 mg/dL, LDL-cholesterol 39 mg/dL, urea nitrogen 40 mg/dL, creatinine 1.83 mg/dL, and uric acid 8.2 mg/dL. CER of normal sera without heat inactivation was 96.3 ± 10.4 nmol/ml/h ($n = 3$), indicating normal LCAT activity. In contrast, CER of patient sera without heat-inactivation was 16.3 nmol/ml/h, which was below the CER of heat-inactivated normal sera (21.3 ± 1.8 nmol/ml/h, $n = 3$), indicating the total loss of LCAT activities in the patients. The 84 units of alpha-LCAT activity in patient serum measured by Anasorb LCAT® was also markedly low as compared with a standard level of 382–512 units. No abnormalities on chest X-ray, electrocardiography or echography findings were detected in either kidney. The renal biopsy specimen showed glomerular mesangial expansion, and foam cell infiltrates into glomerular tufts and mesangium. PAM staining revealed irregular thickening, double contouring, and vacuolation of the glomerular basement membrane (GBM) (Fig. 1B). Electron microscopic findings revealed numerous small vacuoles and granular structures within the vacuoles in the GBM and mesangial matrix (Fig. 1C). Immunofluorescence revealed negative staining for immunoglobulins of IgG, IgA and IgM, and for complement components of C1q, C4 and C3. These findings are consistent with the findings of LCAT deficiency.

3.2. Gene analysis

Direct sequencing of the *lcat* gene and comparison with a reference sequence (NM_000229) showed that the proband had a novel homozygous G to A nucleotide substitution in exon 2 resulting in Cys74Tyr [c.293 G > A (p.Cys74Tyr)]. The amino acid substitution was a novel mutation in the lid region of the LCAT protein. The substituted cysteine was one of four cysteine amino acid residues which formed the disulfide bonds in construction of the enzyme lid structure [23].

3.3. Lipid and lipoprotein profiles

Serum TG and free cholesterol values were higher than normal, while cholesterol ester, LDL-cholesterol and HDL-cholesterol values appeared lower (Table 1). Densitometric analysis for lipid staining of lipoproteins on disc polyacrylamide gel electrophoresis of serum showed a significant decrease in α and preβ-β positions, and a tiny abnormal "midband" localized on preβ-β position (Fig. 2A). Two-dimensional gel electrophoresis followed by immunodetection for apoprotein showed that distribution of apoprotein was shifted to the smaller HDL particles, indicating that the maturation of HDL particles was impaired (Fig. 2B). The production of LDL particles was thus severely disturbed, as evidenced by the presence of an abnormal "midband" lipoprotein and the disturbed maturation of HDL particles resulting in a severe decrease in HDL lipoprotein.

Table 1
Lipid profiles in a patient with LCAT deficiency following a fat-restriction diet and administration of losartan for 8 months. Lipid profiles at admission and after adoption of a fat-restriction diet consisting of 10 g fat, 45 g protein and 1570 kcal energy per day and administration of losartan 50 mg for 8 months on lipid profile in a patient with LCAT deficiency.

Lipid fraction		Normal value	At admission	At 8 months of treatment
Total cholesterol	(mg/dl)	120–220	235	80
Triglyceride	(mg/dl)	30–150	235	142
LDL-cholesterol	(mg/dL)	70–139	39	33
HDL-cholesterol	(mg/dL)	40–96	22	19
Free cholesterol	(mg/dL)	30–65	205	71
Cholesterol ester	(mg/dL)	90–200	30	9
Free/total cholesterol	(%)	70–80	87	88

Cholesterol and TG staining for lipoproteins which migrate to the α-position on agarose gel electrophoresis could not be seen in the serum at admission (Fig. 3A). Lipoproteins which migrate to the preβ-position were also decreased on both cholesterol and TG staining at admission (Fig. 3A), whereas lipoproteins which migrate to the β-position showed broad bands in both cholesterol and TG staining. The amount of cholesterol and TG in each lipoprotein fraction of serum at admission is shown in Table 2. Apolipoproteins AI and AII, which are predominantly contained in HDL-lipoprotein,

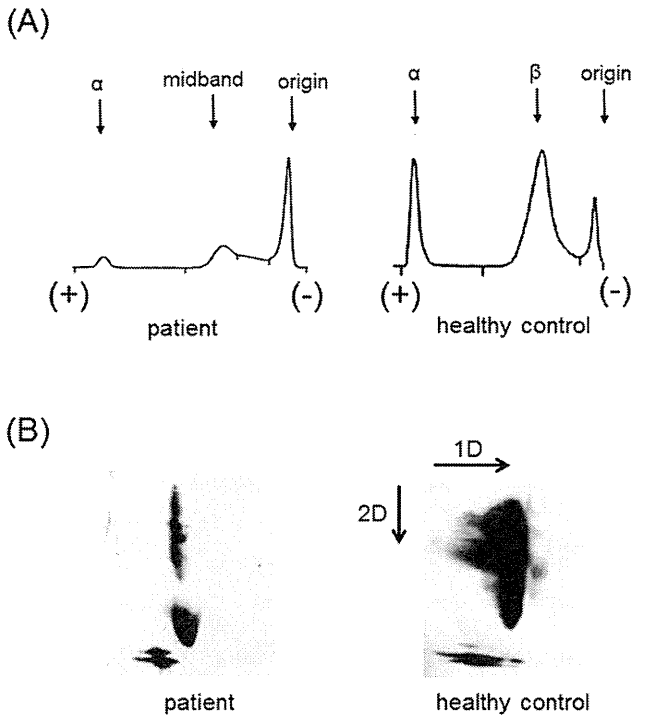

Fig. 2. Densitometric analysis of lipoproteins on disc polyacrylamide gel electrophoresis in the patient and a healthy control. (A) Serum at admission shows an increase in original position lipoproteins, a decrease in α-position lipoproteins, and the appearance of midband lipoproteins instead of β-position lipoproteins. (B) Two-dimensional disk electrophoresis consisting of charge separation for the first dimension and molecular weight separation for the second dimension followed by immunostaining for apolipoprotein. Apolipoprotein distribution was shifted towards the smaller HDL particles, indicating that the maturation of HDL particles in this patient was impaired.

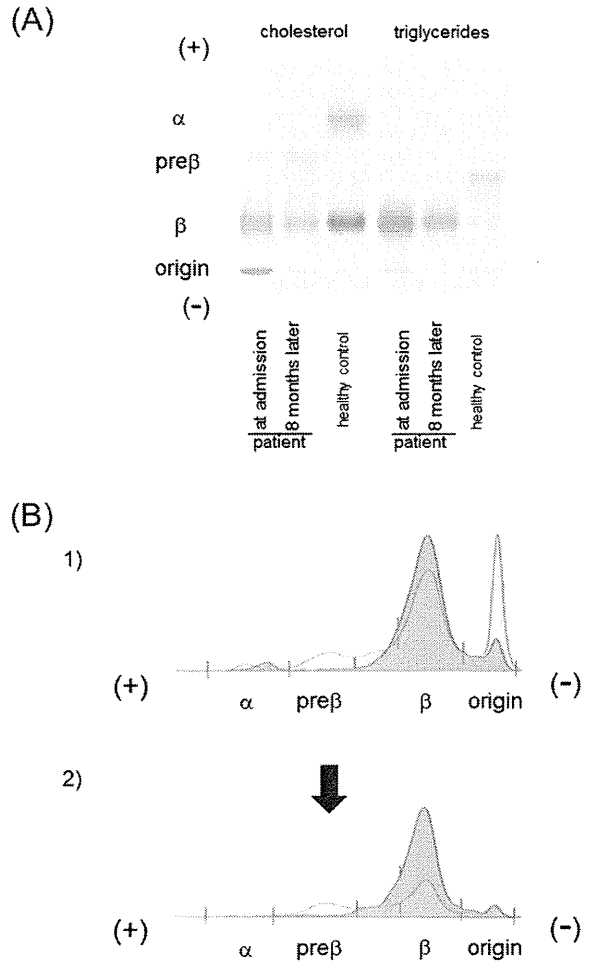

Fig. 3. Staining patterns for cholesterol and triglycerides in lipoproteins on agarose gel electrophoresis. (A) In cholesterol staining, the lipoproteins at original position increased, while the lipoproteins at α- and β-position decreased in the patient at admission compared with those of a healthy control. After 8 months on a fat-restriction diet and administration of losartan 50 mg, a decrease in original position lipoproteins is seen. In triglyceride staining, the serum sample at admission shows a decrease in preβ-position lipoproteins and increase in β-position lipoproteins compared with levels of a healthy control. (B) Densitometric analyzes for staining pattern. Cholesterol staining is shown as a red area, and triglycerides staining as a blue area. (B)-1) Staining patterns for lipoproteins in the patient's serum at admission. (B)-2) Staining patterns for lipoproteins in the patient's serum at 8 months after a fat-restriction diet and administration of losartan 50 mg. Original and β-position lipoproteins stained for cholesterol at admission have decreased after treatment for 8 months.

were decreased at admission (Table 2), whereas apolipoprotein CII and E were increased (Table 2).

3.4. Effects of a fat-restriction diet on LCAT deficiency-induced lipid profile and kidney disease

The patient was prescribed a fat-restricted diet consisting of meals containing 10 g of fat, 45 g of protein and 1570 kcal of energy during admission, and treatment with losartan, an ARB, was started by single daily administration at 50 mg from 14 days after admission. This treatment was continued following discharge, and follow-up at 8 months showed that compliance with both the diet and medication was good. Her body weight decreased from 64 to 52 kg at 6 month after the start of the fat-restricted diet, and was thereafter maintained. At one year of treatment, her proteinuria had decreased from 2.04 g/g·creatinine (Cr) to 0.62 g/g·Cr, and her serum Cr level had decreased from 1.83 mg/dl to 1.10 mg/dl.

Table 2
Apolipoprotein profile in a patient with LCAT deficiency before and after treatment with a fat-restriction diet and administration of losartan for 8 months. Apolipoprotein profile at admission, and effect of a fat-restriction diet consisting of 10 g fat, 45 g protein, and 1570 kcal energy per day for 8 months on apolipoproteins in a patient with LCAT deficiency.

Apolipoprotein fraction		Normal values	At admission	After 8 months of treatment
apoA-I	(mg/dL)	126–165	39	34
apoA-II	(mg/dL)	24–33.3	5.1	3.3
apoB	(mg/dL)	66–101	63	55
apoC-II	(mg/dL)	1.5–3.8	5.6	1.3
apoC-III	(mg/dL)	5.4–9.0	8.6	3.5
apoE	(mg/dL)	2.8–4.6	10.8	5.4

Changes in lipid and apolipoprotein fractions in sera samples collected at admission and 8 months of treatment are shown in Tables 1 and 2, respectively. Surprisingly, adoption of the fat-restriction diet resulted in a decrease in cholesterol, while TG contents and abnormal lipoproteins migrated to their original position (Table 2, Fig. 3A&B). The cholesterol content of lipoprotein at the β-position was also substantially decreased (Table 2, Fig. 3A&B). These data showed that the decrease in abnormal lipoproteins at the original position and change in lipid content in β-lipoproteins were the result of the fat-restriction diet and administration of an ARB.

4. Discussion

In this study, we report a patient with LCAT deficiency who experienced progressive renal dysfunction, proteinuria, anemia and corneal opacity. Her renal biopsy specimen showed many foam cell infiltrates into glomerular capillary tufts and mesangium, and numerous clear vacuoles containing granular structures within the GBM and mesangial matrix. Glomerular foam cells infiltrates are a characteristic feature of LCAT deficiency [18–20]. The numerous clear vacuoles within the GBM and mesangial matrix are also consistent with the findings of a previous study of LCAT deficiency [21]. However, the findings in our patient were not consistent with the structures of odd-shaped electron-dense materials with a membranous profile within clear vacuoles described in that study [21]. Differences in structure in clear vacuoles may be related to patient age or lipid composition in the vacuoles. It is conceivable that the glomerular lipid deposits are fully or partially composed of LpX, a cationized lipoprotein [22]. The glomerular charge barrier is composed of negatively-changed proteins and may be influenced by deposited cationized lipoproteins, resulting in the exacerbation of proteinuria. Indeed, agarose gel electrophoresis of serum from our patient at admission revealed a substantial amount of abnormal cationized lipoproteins suggestive of LpX at the original position (Fig. 3B-1). Proteinuria in this patient might have been induced by the deposition of this abnormal cationized lipoprotein into glomeruli.

Our patient had a novel mutation of Cys74Tyr in the lid region of LCAT protein. LCAT protein contains two functional disulfide bridges, Cys50-Cys74 and Cys313-Cys356 [23]. It appears likely that the Cys50-Cys74 bond was disrupted in our patient. In previous study, disruption of the Cys313-Cys356 bond by an amino acid substitution was shown to result in LCAT deficiency and early onset renal disease [10,24]. The former loop region in the LCAT protein, consisting of Tyr51-Asp73, has binding capacity for HDL- and LDL-cholesterol [25], and truncation of Lys53-Gly71 or Asp56-Leu68 from LCAT protein abolished the ability of LCAT protein to bind HDL- and LDL-cholesterol *in vitro* [26,27]. Our patient also showed a complete loss of LCAT activity, indicating that the former loop

region spanned by Cys50-Cys74 is essential for substrate recognition of LCAT in the esterification process [28]. Further *in vitro* and *in vivo* investigation of the effect of partial transformation of the Tyr51-Asp73 loop region on the cholesterol esterification process may contribute to our understanding of the biochemistry of enzyme–lipid interactions as well as the pathophysiology of LCAT deficiency.

Although sequential ultracentrifugation is a standard method in lipoprotein analysis, we applied agarose gel electrophoresis to detect abnormal LpX and LDL-like lipoproteins and for visualization of lipoproteins. Our patient had a significant increase in abnormal lipoproteins at the original position and a severe decrease in HDL-lipoprotein. Her serum also showed an abnormal midband lipoprotein and a disturbance in the maturation of HDL particles. We suggest that these lipoprotein abnormalities were due to the disturbance in esterification resulting from the loss of LCAT activity.

We also evaluated the effect of a fat-restriction diet and administration of an ARB on lipid profile, proteinuria and renal function in this patient. This treatment decreased proteinuria and resulted in a delay in the deterioration of renal function. A fat restriction diet obviously decreased her serum total-cholesterol and TG, except HDL-cholesterol and LDL-cholesterol at 8 months of treatment. The abnormal cationized lipoproteins at original positions, suggestive of LpX, disappeared after treatment. This disappearance of abnormal cationized lipoproteins in her serum after treatment may have induced a decreased in the amount of deposited cationized lipoproteins within the GBM, thereby resulting in restoration of the charge barrier in the GBM and decrease in proteinuria. The lipid content of lipoproteins at the β-position and the cholesterol content of the midband lipoprotein decreased also after treatment. Lipoproteins accumulating in the kidney are thought to be abnormal apoprotein E-rich lipoproteins which have migrated from the β-position [29], suggesting that the decrease in serum apoprotein E after treatment may be associated with the decrease in accumulated lipoproteins in the kidney. A fat-restriction diet in combination with ARB treatment may contribute to decrease in proteinuria and result in a delay in the deterioration of renal function in patients with LCAT deficiency.

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Original Article

Small Dense Low-Density Lipoproteins Cholesterol can Predict Incident Cardiovascular Disease in an Urban Japanese Cohort: The Suita Study

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Aim: **Several lines of evidence indicate that small dense low-density lipoproteins (sd-LDL) are more atherogenic than large buoyant LDL; however, few prospective studies have addressed the role of sd-LDL in cardiovascular disease (CVD). We therefore examined the association between sd-LDL cholesterol (sd-LDL-C) and CVD in a Japanese cohort.**

Methods: **An 11.7-year prospective study was performed using a general population aged 30-79 without a history of cardiovascular disease. Direct LDL-C and sd-LDL-C were measured in samples from 2034 participants (968 men and 1066 women).**

Results: **During the follow-up period, there were 116 incident cases of CVD. The multivariable-adjusted hazard ratios (HRs) of sd-LDL-C for CVD were calculated using a proportional hazards regression model after adjusting for age, hypertension, diabetes, use of lipid-lowering drugs, body mass index, and current smoking and alcohol drinking, and found that increasing quartiles of sd-LDL-C were associated with increased risk of CVD. We also determined that age and sex-adjusted HRs per 10 mg/dL of sd-LDL-C and HRs for CVD, stroke, cerebral infarction, and coronary artery disease were 1.21 (95% CI: 1.12-1.31), 1.17 (95% CI: 1.05-1.30), 1.15 (95% CI: 1.00-1.33), and 1.29 (95% CI: 1.14-1.45), respectively.**

Conclusions: **It was demonstrated that sd-LDL-C was significantly associated with CVD in a Japanese population, providing evidence of sd-LDL-C as an important biomarker to predict CVD.**

J Atheroscler Thromb, 2013; 20:195-203.

Key words; Cardiovascular disease, Lipoproteins, Lipids, Risk factors, Epidemiology

Introduction

The causal relationship between high levels of serum low-density lipoprotein cholesterol (LDL-C) and cardiovascular disease (CVD) has been well established in previous cohort studies[1-5]. Recent clinical trials have also indicated significant event reduction by statins in the primary and secondary prevention of CVD[6-8]; therefore, LDL-C is one of the most important risk factors of CVD and many guidelines, including ours, recommend certain target LDL-C goals for risk management to prevent the development of CVD[5].

Although we use LDL-C as the primary target for cholesterol-lowering therapy, LDL particles are heterogeneous with respect to size and density. Compared to large, buoyant LDL, small dense LDL (sd-LDL) particles exhibit a prolonged plasma residence time, increased penetration into the arterial wall, lower affinity for the LDL receptor, and increased sus-

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ceptibility to oxidation[9]. Thus, sd-LDL particles possess elevated atherogenic potential. Furthermore, elevated concentrations of sd-LDL can be found in patients with type 2 diabetes, metabolic syndrome, chronic kidney disease, and familial combined hyperlipidemia[10-14], all of which have been found as highly atherogenic conditions. Although Hirano *et al.* showed that sd-LDL-C is significantly higher in patients with coronary artery disease (CAD) in a cross-sectional study[14], no prospective study has addressed whether sd-LDL-C can predict a risk for CVD in non-Western populations. Recently, the Québec Cardiovascular Study has shown prospectively that men with an elevated proportion of LDL with a diameter less than 25.5 nm had a 3.6-fold increased risk of CAD compared with men with relatively normal LDL[15], indicating the strong link of sd-LDL to CVD as a biomarker of cardiovascular disease. Due to its atherogenic properties it is useful to measure sd-LDL for risk assessment; however, a reliable routine method is lacking.

sd-LDL has been measured by ultracentrifugation[16] or gradient gel electrophoresis[17]; however, these methods are both unsuitable for routine analysis, because each requires expensive equipment, complicated techniques, and long assay times. Hirano *et al.* have recently developed a simple precipitation method for sd-LDL-C quantification consisting of 2 steps: removal of apolipoprotein B-containing sd-LDL-free lipoproteins by precipitation with heparin and magnesium, followed by LDL-C measurement by the homogeneous method[18, 19]. This assay allowed us to screen sd-LDL-C in a large cohort. Using this assay, Ai *et al.* recently performed a case control study using samples from the Framingham Offspring Study and found significantly higher sd-LDL-C in women with CAD[20]. Koba *et al.* also showed that sd-LDL-C is more powerful than LDL-C for the determination of CAD[21]; however, these are cross-sectional studies and a prospective study is required to determine whether sd-LDL is an independent predictor of CVD. Therefore, the aim of this study was to address the role of sd-LDL-C for incident CVD in a large cohort study in Japan, the Suita study.

Methods

Population

The Suita study, a cohort study on CVD of urban residents, was established in 1989. The details of this study have been described elsewhere[22]. Briefly, 6485 men and women aged 30-79 years underwent a baseline survey at the National Cerebral and Cardiovascular Center between September 1989 and March 1994, and received medical examinations every 2 years. For these participants, we set the baseline of the present study at medical examinations held between April 1994 and February 1995, since at that time serum samples were collected and stored at −80℃. During this period, 2,437 participants attended the medical examination and were followed until the end of 2007. Of these, 403 participants were excluded due to the following reasons: history of CAD or stroke (n=106), lost to follow-up (n=132), and other reasons such as missing data (n=165). Data from the remaining 2,034 participants (968 men and 1,066 women) were included in the analysis. Informed consent was obtained from all participants. This cohort study was approved by the Institutional Review Board of the National Cerebral and Cardiovascular Center.

Baseline Examination

Blood samples were collected after the participants had fasted for at least 10 hours. The samples were centrifuged immediately. Blood pressure was measured in triplicate on the right arm after 5 min of rest by well-trained physicians using a standard mercury sphygmomanometer. The average of the second and third measurements was used for analysis. At baseline examination, subjects were classified into one of the 5 blood pressure categories based on the criteria of ESH-ESC 2007: optimal (SBP < 120 mmHg and DBP < 80 mmHg), normal (SBP 120-129 mmHg or DBP 80-84 mmHg), high-normal blood pressure (SBP 130-139 mmHg or DBP 85-89 mmHg), hypertension grade 1 (SBP 140-159 mmHg or DBP 90-99 mmHg), or hypertension grade ≥2 (SBP ≥160 mmHg or DBP ≥100 mmHg). Antihypertensive drug users were classified according to their blood pressure at the baseline survey. Diabetes was defined as fasting serum glucose ≥7.0 mmol/L (126 mg/dL) or current use of medications for diabetes. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. Well-trained health nurses obtained information on smoking, drinking, and medical histories.

Laboratory Measurements

Serum total cholesterol, triglyceride, and HDL cholesterol (HDL-C) were determined by standard enzymatic methods. Serum glucose was also measured. For the purposes of this study, we used archived plasma samples that had been frozen at −80℃ and never previously thawed for the assessment of direct LDL-C and sd-LDL-C by homogeneous methods on a Hitachi 7180 automated analyzer (Hitachi, Tokyo, Japan)[18, 19]. The kits used for these tests (LDL-C and sd-LDL-C) were provided by Denka Seiken (Tokyo, Japan). Assays

for direct LDL-C and sd-LDL-C were previously calibrated and directly compared with concentrations obtained after isolation of LDL and sd-LDL by ultracentrifugation.

Endpoint Determination

As previously reported, the endpoints of the present study were (1) date of first CAD or stroke event; (2) date of death; (3) date of leaving Suita city; and (4) the end of December 2007. The first step in the survey for CAD and stroke involved checking the health status of all participants by repeated clinical visits every two years and yearly questionnaires by mail or telephone. In the second step, in-hospital medical records of participants who were suspected of having CAD were reviewed by registered hospital physicians or research physicians who were blinded to the baseline information. The criteria for a diagnosis of CAD included first-ever acute myocardial infarction, sudden cardiac death within 24 h after the onset of acute illness, or coronary artery disease followed by coronary artery bypass surgery or angioplasty. The criteria for definite and probable MI were defined according to the criteria of the MONICA (Monitoring Trends and Determinants of Cardiovascular Disease) project[23]. The criteria for stroke were defined according to the US National Survey of Stroke criteria[24]. Classification of patients into stroke subtypes was based on examination of computed tomography, magnetic resonance imaging, or autopsy.

Statistical Analysis

Continuous variables between groups were compared by analysis of variance and categorical variables were compared by a chi-square test. Triglyceride levels were logarithmically transformed to improve the skewed distribution. The hazard ratio (HR) for MI or stroke was calculated using a proportional hazards model adjusted for age, sex, hypertension (dichotomous variable), diabetes, HDL-C, BMI, smoking (never-smoked; ex-smoker; current smoker) and drinking (never-drank; ex-drinker; regular drinker). All confidence intervals were estimated at the 95% level and significance was set at $p<0.05$. All statistical analyses were conducted using the SAS statistical software package (release version 8.2; SAS Institute, Cary, NC, USA).

Results

Baseline Clinical Characteristics According to sd-LDL-C Quartiles

To study the role of sd-LDL in the incidence of CVD, we divided the cohort into quartiles according to the basal level of sd-LDL-C. **Table 1** shows the clinical characteristics and cardiovascular risk factors of the study population according to the quartiles of sd-LDL-C. BMI, total cholesterol, LDL-C, and triglyceride significantly increased across the sd-LDL-C quartiles both in men and women, while HDL-C decreased in both genders. A significant trend was observed across the quartiles for the severity of high blood pressure, lipid-lowering drug use, and prevalence of diabetes at baseline both in men and women; however, a significant trend for age was only found in women, not in men.

Incidence of CVD According to sd-LDL-C Quartiles

To confirm our previous study, the association between LDL-C and CAD was examined by dividing the cohort into quartiles according to the baseline LDL-C. It was found that age- and multivariable-adjusted HRs for CAD were statistically significant only in men, not in women or the total cohort. The HR of the 4th quartile in men was 3.53 (95% confidence intervals (CIs): 1.31-9.54) in an age-adjusted model and 3.56 (95% CIs: 1.28-9.86) in a multivariable-adjusted model, consistent with our previous report[1]. We then performed analysis to examine the effect of sd-LDL-C. During the observation period, 116 cases of CVD, 53 cases of stroke, 36 cases of cerebral infarction, and 63 cases of CAD were reported. As shown in **Table 2**, increasing quartiles of sd-LDL-C were significantly associated with increased risks of CVD (stroke + CAD), stroke, cerebral infarction, and CAD after age and multivariable adjustment. Age and sex-adjusted HRs per 10 mg/dL of sd-LDL-C for CVD, stroke, cerebral infarction, and CAD were 1.21 (95% CI: 1.12-1.31), 1.17 (95% CI: 1.05-1.30), 1.15 (95% CI: 1.00-1.33), and 1.29 (95% CI: 1.14-1.45), respectively. HRs after multivariable adjustment were almost the same. When we analyzed each gender, age-adjusted HRs per 10 mg/dL of sd-LDL-C for CVD, stroke, cerebral infarction, and CAD were significant in women, while those for CVD and CAD were significant in men. HR for CAD of the fourth quartile was almost 4 after age and multivariable adjustment in men.

After putting LDL-C into the multivariable adjusted-models (Model A), sd-LDL-C was still associated with increased risk for CVD, stroke, cerebral infarction, and CAD in the total cohort, for CVD in men, and CVD, stroke, and cerebral infarction in women. After further putting logarithmically transformed triglyceride and HDL-C variables into Model A (Model B), sd-LDL-C was still associated with

Table 1. Baseline characteristics of cardiovascular risk factors according to small dense LDL cholesterol quartiles

	Small dense LDL Cholesterol				*p* value for Trend
	Q1	Q2	Q3	Q4	
Men					
Number of subjects	241	243	242	242	
Small dense LDL, range (mean), mg/dL	6.3-27.8 (21.1)	27.9-38.2 (32.7)	38.3-53.4 (45.3)	53.5-119.6 (67.3)	
Age, year	60.9 ± 13.1	59.7 ± 12.5	59.1 ± 12.3	59.4 ± 11.3	0.421
Body mass index, kg/m^2	21.5 ± 2.5	22.4 ± 2.8	23.4 ± 2.4	24.0 ± 2.7	<0.001
TC, mg/dL	170 ± 25	189 ± 24	199 ± 25	220 ± 27	<0.001
HDL-C, mg/dL	60 ± 15	57 ± 14	51 ± 11	48 ± 11	<0.001
LDL-C, mg/dL	86 ± 20	111 ± 21	124 ± 23	140 ± 26	<0.001
Triglyceride, (median) mg/dL	66	87	112	167	<0.001
Large-LDL-C, mg/dL	65 ± 17	78 ± 21	79 ± 22	72 ± 24	<0.001
Sd-LDL-C/LDL-C ratio	0.25 ± 0.05	0.31 ± 0.07	0.38 ± 0.08	0.50 ± 0.11	<0.001
Blood pressure category, %					0.002
Optimal blood pressure	31	26	25	19	
Normal blood pressure	30	24	19	26	
High-normal blood pressure	16	30	25	29	
Hypertension grade 1-3	19	26	29	28	
Antilipidemic drug use, %	1	4	5	8	0.003
Diabetes, %	3	5	7	9	0.023
Current Smoking, %	44	41	41	44	0.021
Current Drinking, %	66	71	72	74	0.577
Women					
Number of subjects	266	267	266	267	
Small dense LDL, range (mean), mg/dL	7.5-23.9 (18.7)	24.0-33.0 (28.6)	33.1-44.6 (38.5)	44.7-136.6 (59.7)	
Age, year	51.7 ± 13.0	57.3 ± 11.9	60.2 ± 11.2	60.4 ± 9.1	<0.001
Body mass index, kg/m^2	21.0 ± 2.5	21.8 ± 3.2	22.5 ± 3.1	23.2 ± 2.8	<0.001
TC, mg/dL	175 ± 23	200 ± 22	216 ± 25	234 ± 32	<0.001
HDL-C, mg/dL	67 ± 13	64 ± 12	60 ± 13	54 ± 12	<0.001
LDL-C, mg/dL	83 ± 17	109 ± 17	130 ± 18	153 ± 30	<0.001
Triglyceride, (median) mg/dL	61	78	97	140	<0.001
Large-LDL-C, mg/dL	64 ± 14	81 ± 15	92 ± 17	93 ± 25	<0.001
Sd-LDL-C/LDL-C ratio	0.23 ± 0.04	0.27 ± 0.04	0.30 ± 0.05	0.40 ± 0.08	<0.001
Blood pressure category, %					<0.001
Optimal blood pressure	34	27	22	17	
Normal blood pressure	25	24	26	25	
High-normal blood pressure	16	29	20	35	
Hypertension grade 1-3	16	21	31	32	
Antilipidemic drug use, %	4	5	6	12	0.002
Diabetes, %	0	1	3	6	<0.001
Current Smoking, %	13	10	6	7	0.056
Current Drinking, %	34	30	22	23	0.014

TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Large LDL-C, LDL-C-Sd-LDL-C. Hypertension was defined as described in methods. Diabetes was defined as fasting serum glucose ≥7.0mmol/L (126 mg/dL), the use of anti-diabetic agents, or both.

Table 2. Age- and multivariable-adjusted hazard ratios and 95% confidence intervals for the incidence of cardiovascular disease according to small dense LDL cholesterol quartiles

	Small dense LDL Cholesterol, mg/dL				
	Q1 (Lower)	Q2	Q3	Q4 (Higher)	per 10 mg/dL
Men and women, range (mean)	6.3-25.5 (19.7)	25.6-35.3 (30.5)	35.4-49.0 (41.4)	49.1-136.6 (63.9)	
Person-years	5,576	5,789	5,527	5,741	
Cardiovascular disease					
Case	21	23	29	43	
Age and sex-adjusted HR	1	0.75 (0.43-1.29)	1.11 (0.68-1.83)	**1.64 (1.04-2.60)**	**1.21 (1.12-1.31)**
Model 1-adjusted HR	1	0.81 (0.45-1.42)	1.08 (0.65-1.81)	1.60 (0.99-2.60)	**1.21 (1.11-1.32)**
Stroke					
Case	14	13	10	16	
Age and sex-adjusted HR	1	0.58 (0.30-1.14)	0.80 (0.43-1.48)	1.21 (0.69-2.12)	**1.17 (1.05-1.30)**
Model 1-adjusted HR	1	0.63 (0.32-1.23)	0.79 (0.41-1.50)	1.19 (0.65-2.16)	**1.18 (1.04-1.33)**
Cerebral infarction					
Case	8	10	6	12	
Age and sex-adjusted HR	1	1.08 (0.45-2.57)	1.14 (0.47-2.73)	1.74 (0.77-3.90)	**1.15 (1.00-1.33)**
Model 1-adjusted HR	1	1.18 (0.48-2.88)	1.16 (0.46-2.89)	1.85 (0.77-4.40)	**1.18 (1.00-1.39)**
Coronary artery disease					
Case	7	10	19	27	
Age and sex-adjusted HR	1	1.36 (0.49-3.77)	2.26 (0.89-5.73)	**3.35 (1.38-8.13)**	**1.29 (1.14-1.45)**
Model 1-adjusted HR	1	1.44 (0.51-4.08)	2.17 (0.83-5.66)	**3.26 (1.29-8.20)**	**1.28 (1.13-1.46)**
Men, range (mean)	6.3-27.8 (21.1)	27.9-38.2 (32.7)	38.3-53.4 (45.3)	53.5-119.6 (67.3)	
Person-years	2,499	2,615	2,519	2,608	
Cardiovascular disease					
Case	19	19	22	36	
Age-adjusted HR	1	1.06 (0.56-2.01)	1.31 (0.70-2.44)	**2.03 (1.16-3.57)**	**1.15 (1.04-1.28)**
Model 1-adjusted HR	1	1.17 (0.61-2.24)	1.36 (0.70-2.62)	**2.12 (1.16-3.86)**	**1.16 (1.04-1.30)**
Stroke					
Case	14	13	10	16	
Age-adjusted HR	1	1.03 (0.48-2.21)	0.87 (0.38-1.99)	1.43 (0.69-2.97)	1.06 (0.92-1.23)
Model 1-adjusted HR	1	1.13 (0.51-2.47)	0.98 (0.40-2.38)	1.55 (0.70-3.41)	1.08 (0.92-1.28)
Cerebral infarction					
Case	8	10	6	12	
Age-adjusted HR	1	1.33 (0.52-3.39)	0.85 (0.29-2.48)	1.81 (0.73-4.48)	1.08 (0.91-1.29)
Model 1-adjusted HR	1	1.43 (0.54-3.78)	0.90 (0.29-2.80)	1.93 (0.70-5.29)	1.10 (0.90-1.36)
Coronary artery disease					
Case	5	6	12	20	
Age-adjusted HR	1	1.24 (0.37-4.07)	2.48 (0.87-7.07)	**3.89 (1.45-10.42)**	**1.27 (1.10-1.47)**
Model 1-adjusted HR	1	1.27 (0.38-4.29)	2.34 (0.78-6.97)	**4.03 (1.42-11.40)**	**1.28 (1.09-1.50)**
Women, range (mean)	7.5-23.9 (18.7)	24.0-33.0 (28.6)	33.1-44.6 (38.5)	44.7-136.6 (59.7)	
Person-years	3,077	3,174	3,008	3,133	
Cardiovascular disease					
Case	7	12	13	23	
Age-adjusted HR	1	1.01 (0.39-2.60)	0.99 (0.39-2.50)	1.73 (0.74-4.06)	**1.31 (1.16-1.47)**
Model 1-adjusted HR	1	1.04 (0.40-2.72)	0.91 (0.35-2.35)	1.52 (0.63-3.68)	**1.29 (1.13-1.48)**
Stroke					
Case	5	8	6	16	
Age-adjusted HR	1	0.95 (0.30-2.94)	0.64 (0.19-2.11)	1.72 (0.62-4.74)	**1.31 (1.13-1.52)**
Model 1-adjusted HR	1	0.98 (0.31-3.14)	0.64 (0.18-2.19)	1.66 (0.58-4.76)	**1.33 (1.12-1.59)**

(Cont Table 2)

	Small dense LDL Cholesterol, mg/dL				
	Q1 (Lower)	Q2	Q3	Q4 (Higher)	per 10 mg/dL
Cerebral infarction					
Case	0	5	4	7	
Age-adjusted HR	1	–	–	–	**1.31 (1.05-1.63)**
Model 1-adjusted HR	1	–	–	–	**1.37 (1.05-1.80)**
Coronary artery disease					
Case	2	4	7	7	
Age-adjusted HR	1	1.22 (0.22-7.76)	1.90 (0.39-9.24)	1.84 (0.38-8.91)	**1.32 (1.08-1.61)**
Model 1-adjusted HR	1	1.27 (0.22-7.33)	1.83 (0.35-9.45)	1.54 (0.30-7.83)	1.23 (0.99-1.53)

Model 1: adjusted for age, (sex), body mass index, smoking, drinking, blood pressure category (optimal, normal, and high-normal blood pressure, hypertension grade 1 and 2 + 3), diabetes, and lipid-lowering drug user
Bold numbers: statistically significant

increased risks of CVD and stroke in the total cohort and in women, but not in men (**Table 3**).

Discussion

This study clearly indicates an increased risk of CVD, stroke, cerebral infarction, and CAD attributed to elevated sd-LDL-C concentrations in a Japanese population without a previous history of CVD. We also showed that HR was significant after multivariable adjustment and by analysis including LDL-C, log-transformed triglyceride, and HDL-C in the same model. Thus, sd-LDL-C measurement with the new test is promising as a new biomarker to predict the risk of CVD.

In addition to traditional risk factors for CVD, such as hypertension, diabetes, and dyslipidemia, other biomarkers are required to better define the risk and refine therapeutic decisions. There is scientific evidence that sd-LDL particles are highly atherogenic and can be a biomarker of CVD[15, 20, 21]. Our data provide additional evidence to show the role of sd-LDL-C as a CVD risk in the general population. Furthermore, measuring sd-LDL-C with this test has an advantage because it is more user-friendly and more applicable than specialized tests such as gradient gel electrophoresis, nuclear magnetic resonance, and gradient ultracentrifugation.

Until now, there have been no target goals of sd-LDL-C to prevent CAD. In this study the HR of the 4th quartile was statistically significant, suggesting that the cutoff of sd-LDL-C is approximately 50 mg/dL, although significance was not obtained in women probably due to the low event rate; therefore, a larger study should be performed to define an appropriate cutoff for sd-LDL-C. Because statins, fibrates, and ezetimibe have been shown to reduce the amount of sd-LDL[25-28], a randomized control study is required to address whether lowering sd-LDL-C to a certain goal by these drugs can prevent the development of CAD.

In this study we found that sd-LDL-C was significantly associated with traditional risk factors, such as hypertension and diabetes. BMI and the prevalence of diabetes increased and HDL-C decreased across the sd-LDL-C quartiles, and more hypertensive subjects were found in second to fourth quartiles than in the first quartile in both genders. We also found that age-adjusted partial correlation coefficients between sd-LDL-C and BMI, log-transformed triglyceride, LDL-C, and HDL-C (Pearson) were 0.305, 0.636, 0.554, and −0.346 ($p<0.0001$), respectively. Thus these data suggest that increased concentrations of sd-LDL-C may be associated with metabolic disorders and that lifestyle modification, such as exercise and weight control, would be effective to reduce sd-LDL in patients with diabetes and metabolic syndrome. Furthermore, we should address whether sd-LDL-C can be used to identify a very high-risk patient with type 2 diabetes, metabolic syndrome, and other metabolic disorders. In contrast to the association with metabolic disorders, an age-related change in sd-LDL-C was found only in women, consistent with the trend of increased atherogenic dyslipidemia in postmenopausal women. Ai *et al.* also found that postmenopausal women had higher levels of sd-LDL-C than premenopausal women in the Framingham Offspring Study[20].

In addition to type 2 diabetes and metabolic syn-

Table 3. Relationship between major lipid variables and cardiovascular disease

	Cardiovascular disase	Stroke	Cerebral infarction	Coronary artery disease
Men and women				
Age and sex-adjusted	**1.21 (1.12-1.31)**	**1.17 (1.05-1.30)**	**1.15 (1.00-1.33)**	**1.29 (1.14-1.45)**
Multivariable-adjusted Sd-LDL-C/10 mg/dL	**1.21 (1.11-1.32)**	**1.18 (1.04-1.33)**	**1.18 (1.00-1.39)**	**1.28 (1.13-1.46)**
Model A				
Sd-LDL-C/10 mg/dL	**1.26 (1.11-1.43)**	**1.26 (1.06-1.50)**	**1.29 (1.02-1.62)**	**1.29 (1.07-1.55)**
LDL-C/10 mg/dL	0.96 (0.89-1.04)	0.94 (0.85-1.04)	0.93 (0.81-1.06)	0.99 (0.88-1.11)
Model B				
Sd-LDL-C/10 mg/dL	**1.20 (1.01-1.42)**	**1.35 (1.07-1.71)**	1.31 (0.96-1.78)	1.05 (0.81-1.36)
LDL-C/10 mg/dL	0.98 (0.90-1.06)	0.93 (0.83-1.03)	0.92 (0.80-1.07)	1.05 (0.93-1.19)
ln_TG	1.15 (0.71-1.86)	0.76 (0.40-1.46)	0.86 (0.37-1.96)	1.82 (0.87-3.81)
HDL-C/10 mg/dL	0.94 (0.81-1.08)	1.00 (0.84-1.20)	0.93 (0.73-1.18)	0.80 (0.61-1.04)
Men				
Age-adjusted	**1.15 (1.04-1.28)**	1.06 (0.92-1.23)	1.08 (0.91-1.29)	**1.27 (1.10-1.47)**
Multivariable-adjusted Sd-LDL-C/10 mg/dL	**1.16 (1.04-1.30)**	1.08 (0.92-1.28)	1.10 (0.90-1.36)	**1.28 (1.09-1.50)**
Model A				
Sd-LDL-C/10 mg/dL	**1.17 (1.00-1.38)**	1.17 (0.92-1.48)	1.20 (0.90-1.60)	1.18 (0.94-1.48)
LDL-C/10 mg/dL	0.99 (0.89-1.09)	0.94 (0.82-1.08)	0.93 (0.79-1.09)	1.07 (0.93-1.24)
Model B				
Sd-LDL-C/10 mg/dL	1.10 (0.88-1.38)	1.28 (0.92-1.77)	1.28 (0.87-1.90)	0.96 (0.70-1.31)
LDL-C/10 mg/dL	1.01 (0.90-1.13)	0.92 (0.78-1.07)	0.91 (0.76-1.10)	1.14 (0.97-1.33)
ln_TG	1.23 (0.66-2.26)	0.75 (0.32-1.76)	0.86 (0.31-2.38)	1.87 (0.75-4.62)
HDL-C/10 mg/dL	0.96 (0.80-1.14)	1.05 (0.85-1.28)	1.08 (0.94-1.40)	0.72 (0.50-1.03)
Women				
Age-adjusted	**1.31 (1.16-1.47)**	**1.31 (1.13-1.52)**	**1.31 (1.05-1.63)**	**1.32 (1.08-1.61)**
Multivariable-adjusted Sd-LDL-C/10 mg/dL	**1.29 (1.13-1.48)**	**1.33 (1.12-1.59)**	**1.37 (1.05-1.80)**	1.23 (0.99-1.53)
Model A				
Sd-LDL-C/10 mg/dL	**1.44 (1.17-1.77)**	**1.48 (1.13-1.94)**	**1.62 (1.08-2.43)**	1.33 (0.94-1.89)
LDL-C/10 mg/dL	0.92 (0.81-1.04)	0.92 (0.79-1.08)	0.88 (0.69-1.11)	0.94 (0.75-1.16)
Model B				
Sd-LDL-C/10 mg/dL	**1.35 (1.03-1.77)**	**1.47 (1.04-2.08)**	1.33 (0.78-2.29)	1.12 (0.70-1.79)
LDL-C/10 mg/dL	0.93 (0.81-1.07)	0.92 (0.78-1.09)	0.92 (0.72-1.19)	0.98 (0.78-1.24)
ln_TG	1.19 (0.53-2.69)	0.91 (0.31-2.68)	0.86 (0.17-4.25)	1.84 (0.47-7.15)
HDL-C/10 mg/dL	0.92 (0.72-1.19)	0.92 (0.67-1.26)	0.56 (0.31-1.00)	0.92 (0.60-1.41)

Multivariable adjusted for age, sex, body mass index, smoking, drinking, blood pressure category (optimal, normal, and high-normal bloodpressure, hypertension grade 1 and 2 + 3), diabetes, and antilipidemic drug user
Model A: sd-LDL-C per 10 mg/dL and LDL-C per 10 mg/dL in the same model
Model B: sd-LDL-C per 10 mg/dL, LDL-C per 10 mg/dL, ln(TG), and HDL-C per 10 mg/dL in the same model
Sd-LDL-C, small dense LDL cholesterol; ln_TG, logarithmical transformed TG
Bold numbers: statistically significant

drome, sd-LDL-C is increased in familial combined hyperlipidemia and postprandial hyperlipidemia[29, 30]. Hirano *et al.* demonstrated that sd-LDL-C determined by this simple precipitation method is useful for screening familial combined hyperlipidemia in large populations[13]. Because the prevalence of familial combined hyperlipidemia is high in the general population and the increase of sd-LDL particles as well as large VLDL particles is a characteristic feature of familial combined hyperlipidemia, this assay would be quite useful for its diagnosis. Although sd-LDL is decreased by lipid-lowering drugs, such as statins and fibrates, the effect of adequate combination therapy on sd-LDL-C has not yet been confirmed; therefore, this assay would be also useful in determining the therapeutic strategy for patients with a high serum level of sd-LDL-C.

There are some limitations in our study. First, we

used plasma stored at −80℃, and there is no guarantee that we would have obtained the same results if we had used fresh serum; however, our results are consistent with those reported by Hirano *et al.*, who measured sd-LDL-C in a Japanese general population with the same method, and comparison studies performed in Japan indicate virtually identical results with the use of fresh vs. frozen plasma for sd-LDL-C[13]. Second, the single measurement of sd-LDL-C at the baseline survey and the fact we did not evaluate the longitudinal trend for each risk factor including lipid-lowering agents may have caused us to underestimate the relationship between these conditions and CAD due to regression dilution bias, although we statistically adjusted for the use of lipid-lowering agents at the baseline survey. Third, serum LDL-C was measured by the direct homogeneous assay, which failed to meet the National Cholesterol Education Program total error goals for diseased individuals, although it met these goals in non-diseased individuals[31]. However, the present study is a cohort study of community-dwelling citizens without a history of CVD. Furthermore, the serum levels of LDL-C determined by direct homogeneous assay are almost consistent with those calculated by the Friedewald formula in a large Japanese cohort.

Conclusions

In this large urban cohort study conducted in Japan, we demonstrated that sd-LDL-C is significantly associated with the development of CVD, providing evidence of sd-LDL-C as an important biomarker to predict CVD. A large intervention study is required to determine the appropriate target level of sd-LDL-C.

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Disclosures

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Comparison of Effects of Pitavastatin Versus Pravastatin on Serum Proprotein Convertase Subtilisin/Kexin Type 9 Levels in Statin-Naive Patients With Coronary Artery Disease

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Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a key regulator of serum low-density lipoprotein cholesterol levels. Although statins increase serum PCSK9 levels, the effects of different types of statins on the serum PCSK9 levels have not been examined in detail. The purpose of the present study was to compare the effects of pitavastatin versus pravastatin on the serum PCSK9 levels. A total of 164 patients with coronary artery disease who were not receiving lipid-lowering therapy were randomly assigned to receive either 4 mg/day of pitavastatin (intensive lipid-lowering therapy) or 20 mg/day of pravastatin (moderate lipid-lowering therapy). The serum PCSK9 levels were measured before statin treatment and 8 months after therapy. A significantly greater reduction in low-density lipoprotein cholesterol was observed in the pitavastatin group (−41% vs −28%, p = 0.0001). The serum levels of total PCSK9 and heterodimer PCSK9 significantly increased from 192 to 249 ng/ml (37%, p <0.0001) and 147 to 206 ng/ml (78%, p <0.0001) in the pitavastatin group and from 192 to 249 ng/ml (39%, p <0.0001) and 143 to 201 ng/ml (65%, p <0.0001) in the pravastatin group, respectively. The increase in total and heterodimer PSCK9 did not differ between the 2 groups. No significant correlations were found between the percentage of changes in heterodimer PCSK9 and changes in the various lipid parameters in either group. In conclusion, significant increases in the total and heterodimer PSCK9 levels were observed at 8 months after treatment with pitavastatin and pravastatin; however, these increases did not differ between the 2 statins. **(Am J Cardiol 2013;111:1415–1419)**

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a key regulator of serum low-density lipoprotein (LDL) cholesterol levels.[1–3] PCSK9 is secreted by the liver into the plasma and binds the hepatic LDL receptors, causing their subsequent degradation.[4–7] Although the mechanism by which PCSK9 degrades LDL receptors is extremely complex, this process reduces the capacity of the liver to bind LDL cholesterol and remove it, resulting in increased LDL cholesterol levels. It has been reported that statins upregulate PCSK9 mRNA expression[8–10] and increase circulating PCSK9 levels.[11–13] These observations explain the "rule of 6%" for statins, which indicates that each doubling of the statin dose results in only an approximate 6% additional reduction in LDL cholesterol levels. However, the effects of different types of statins on serum PCSK9 levels have not been examined in detail. Therefore, we compared the effects of pitavastatin versus pravastatin on the serum PCSK9 levels in statin-naive patients with coronary artery disease.

Methods

The present observational longitudinal study compared the intensive lipid-lowering effects of pitavastatin with the moderate lipid-lowering effects of pravastatin on serum PCSK9 levels. The results were obtained during the Treatment With Statin on Atheroma Regression Evaluated by Intravascular Ultrasound With Virtual Histology (TRUTH) study. The TRUTH study was a prospective, open-labeled, randomized, multicenter trial performed at 11 Japanese centers to compare the effects of 8 months of treatment with pitavastatin versus pravastatin on coronary atherosclerosis using virtual histology-intravascular ultrasound.[14] In brief, 164 patients with angina pectoris who were not receiving lipid-lowering therapy were randomly treated with either pitavastatin (4 mg/day, intensive lipid-lowering group) or pravastatin (20 mg/day, moderate lipid-lowering group).

The patients were included in the present study if they fulfilled the following criteria: the allocated statins were continued during the study period (8 months), and an adequate serum volume was available in frozen samples for

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See page 1418 for disclosure information.

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Table 1
Baseline characteristics

Variable	Pitavastatin (n = 51)	Pravastatin (n = 50)	p Value
Age (yrs)	66 ± 9	67 ± 10	0.59
Men	45 (88%)	39 (78%)	0.17
Body mass index (kg/m^2)	24.3 ± 3.6	24.3 ± 3.3	0.97
Status of coronary artery disease			0.88
Stable angina pectoris	36 (71%)	36 (72%)	
Unstable angina pectoris	15 (29%)	14 (28%)	
Hypertension	31 (61%)	35 (70%)	0.33
Diabetes mellitus	20 (39%)	25 (50%)	0.28
Family history of coronary artery disease	5 (10%)	5 (10%)	0.97
Smoker	18 (35%)	17 (34%)	0.73
Angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers	25 (49%)	30 (60%)	0.27
Calcium channel blockers	20 (39%)	34 (68%)	0.004
β blockers	6 (12%)	4 (8%)	0.53

Data are expressed as mean ± SD or n (%).

the required measurements. A total of 101 patients met the inclusion criteria.

The TRUTH study was conducted in accordance with the Declaration of Helsinki and with the approval of the ethical committees of the 11 participating institutions. Each patient enrolled in the present study provided written informed consent.

The serum lipid levels and inflammatory markers were measured before treatment (baseline) and at the 8-month follow-up point.[14] The serum PCSK9 levels in the conserved frozen samples obtained before treatment and at the 8-month follow-up point were annually measured by a central laboratory (BML, Kawagoe, Japan) using sandwich enzyme-linked immunosorbent assays.

Statistical analysis was performed using StatView, version 5.0 (SAS Institute, Cary, North Carolina). The results are expressed as the mean ± SD. Unpaired Student's *t* tests were used to compare the normally distributed continuous variables between the 2 groups, and Mann-Whitney *U* tests were used when normal distributions were not observed. Paired Student's *t* tests were also used to compare the normally distributed continuous variables within each group, and the Wilcoxon signed rank-sum tests were used when the variables were not normally distributed. The categorical variables between the 2 groups were compared using chi-square tests or Fisher's exact tests. Univariate regression analyses were performed to assess the relation between the percentage of changes in heterodimer PCSK9 and the various lipid parameters. Statistical significance was set at $p < 0.05$.

Results

The baseline characteristics of the subjects are listed in Table 1. Pitavastatin was used to treat 51 patients (50%) and pravastatin to treat 50 patients (50%). No differences in the baseline characteristics were found between the 2 groups, except for the frequency of calcium channel blocker use.

The serum LDL cholesterol levels were significantly decreased in both groups, with a significantly greater reduction observed in the pitavastatin group (−41% vs −28%, $p = 0.0001$). The mean LDL cholesterol levels at the 8-month follow-up point were significantly lower in the pitavastatin group. The serum levels of apolipoprotein B and small, dense LDL were significantly decreased in both groups. Furthermore, the high-density lipoprotein cholesterol levels and high-sensitivity C-reactive protein levels were significantly increased and decreased, respectively, in both groups. However, at the 8-month follow-up point, these levels did not differ between the 2 groups (Table 2).

The serum PCSK9 levels before treatment and at the 8-month follow-up point are listed in Table 3. Significant increases in the total and heterodimer PCSK9 levels were observed in both groups (pitavastatin 37%, $p < 0.0001$, and 78%, $p < 0.0001$; and pravastatin 39%, $p < 0.0001$ and 65%, $p < 0.0001$, respectively). The serum free-fragment PCSK9 levels showed no significant changes in either group. A slightly greater increase in the heterodimer PCSK9 levels was observed in the pitavastatin group; however, this increase was not statistically significant (Figure 1).

We assessed the relation between the changes in the heterodimer PSCK9 levels and the various lipid parameters (Table 4). We found no significant correlations between the changes in the PCSK9 levels and the LDL cholesterol levels or any other lipid parameters in either group.

Discussion

The major findings of the present study were as follows. First, the serum levels of total and heterodimer PCSK9 were significantly increased at 8 months after treatment with pitavastatin and pravastatin. Second, although a significantly greater reduction in LDL cholesterol was observed in the pitavastatin-treated patients, the increased levels of heterodimer PCSK9 were slightly greater in the pitavastatin-treated group. However, no significant difference was found between the 2 statins. Finally, the percentage of changes in PCSK9 did not correlate with the changes in the LDL cholesterol levels nor with any other measured lipid parameters.

PCSK9 is a protease that is produced by the liver and degrades hepatic LDL receptors and subsequently increases LDL cholesterol levels.[4–7] The mechanism by which PSCK9 degrades LDL receptors is extremely complex and is only beginning to be understood. Recently, it has been reported that PCSK9 binds to the LDL receptors and subsequently targets them for intracellular destruction within the hepatocyte.[15–17] Statins increase the nuclear translocation of sterol-regulatory element binding protein-2, which activates, not only the LDL receptors, but also PCSK9 gene expression,[8–10] and increases circulating PCSK9 levels.[11–13] As expected, the levels of PCSK9 and LDL receptors were increased by statin therapy. However, the amount of serum PCSK9 might not reflect the total amount of statin-induced increases in hepatic PCSK9 secretion, because the high levels of PCSK9 bind to hepatic LDL receptors and remove them from circulation. This explains why statin-induced increases in serum PCSK9 levels might not reflect the full effect to which statins modulate hepatic synthesis and secretion of the PCSK9 protein. The percentage of changes in PSCK9 levels did not correlate with those observed in the LDL cholesterol

Table 2
Blood parameters before treatment (baseline) and at follow-up

Variable	Pitavastatin (n = 51)			Pravastatin (n = 50)			p Value Between Groups	
	Baseline	Follow-Up	p Value	Baseline	Follow-Up	p Value	Baseline	Follow-Up
Total cholesterol (mg/dl)	196 ± 31	145 ± 26	<0.0001	207 ± 37	170 ± 23	<0.0001	0.11	<0.0001
Change (%)		−25 ± 13			−17 ± 11		—	0.0006
Low-density lipoprotein cholesterol (mg/dl)	123 ± 24	72 ± 21	<0.0001	135 ± 35	95 ± 23	<0.0001	0.047	<0.0001
Change (%)		−41 ± 15			−28 ± 15		—	0.0001
Triglycerides (mg/dl)	128 ± 75	106 ± 56	0.04	129 ± 56	125 ± 71	0.7	0.94	0.16
Change (%)		−9 ± 42			3 ± 48		—	0.18
High-density lipoprotein cholesterol (mg/dl)	47 ± 12	51 ± 14	0.007	46 ± 11	51 ± 12	0.01	0.83	0.85
Change (%)		10 ± 21			12 ± 27		—	0.72
Apolipoprotein A-I (mg/dl)	118 ± 21	131 ± 25	<0.0001	118 ± 20	132 ± 26	<0.0001	0.9	0.87
Change (%)		12 ± 17			13 ± 20		—	0.87
Apolipoprotein B (mg/dl)	99 ± 19	66 ± 17	<0.0001	107 ± 27	80 ± 16	<0.0001	0.06	<0.0001
Change (%)		−32 ± 15			−24 ± 13		—	0.004
Lipoprotein (a) (mg/dl)	19 ± 13	24 ± 26	0.02	19 ± 14	24 ± 25	0.01	0.94	0.96
Change (%)		27 ± 57			20 ± 49		—	0.5
High-sensitivity C-reactive protein (ng/ml)	15,859 ± 40,903	2,673 ± 5,147	0.02	9,367 ± 16,055	2,674 ± 5,710	0.001	0.3	0.99
Change (%)		−36 ± 120			−43 ± 83		—	0.74
Platelet-activating factor acetylhydrolase (μg/ml)	1.60 ± 0.49	1.09 ± 0.38	<0.0001	1.56 ± 0.59	1.24 ± 0.42	<0.0001	0.74	0.07
Change (%)		−30 ± 17			−18 ± 17		—	0.0005
High-density lipoprotein platelet-activating factor acetylhydrolase (ng/ml)	210 ± 92	144 ± 70	<0.0001	204 ± 108	155 ± 66	<0.0001	0.76	0.4
Change (%)		−27 ± 32			−15 ± 34		—	0.07
Oxidized low-density lipoprotein (U/ml)	12.0 ± 8.8	11.5 ± 10.7	0.7	11.6 ± 8.3	9.5 ± 6.2	0.04	0.8	0.28
Change (%)		15 ± 158			−7 ± 36		—	0.35
Small dense low-density lipoprotein (mg/dl)	23.4 ± 11.2	16.5 ± 8.3	0.0003	28.2 ± 15.9	21.0 ± 9.4	0.0002	0.08	0.01
Change (%)		−18 ± 54			−16 ± 34		—	0.84

Data are expressed as mean ± SD.

Table 3
Serum levels of proprotein convertase subtilisin/kexin type 9 before treatment (baseline) and at follow-up

Variable	Pitavastatin (n = 51)			Pravastatin (n = 50)			p Value Between Groups	
	Baseline	Follow-Up	p Value	Baseline	Follow-Up	p Value	Baseline	Follow-Up
Total (ng/ml)	192 ± 65	249 ± 84	<0.0001	192 ± 67	249 ± 81	<0.0001	0.99	0.99
Change (%)		37 ± 46			39 ± 51		—	0.84
Heterodimer (ng/ml)	147 ± 64	206 ± 85	<0.0001	143 ± 57	201 ± 75	<0.0001	0.73	0.75
Change (%)		78 ± 180			65 ± 115		—	0.66
Free fragment (ng/ml)	45 ± 29	43 ± 22	0.63	49 ± 34	47 ± 31	0.8	0.53	0.4
Change (%)		21 ± 82			24 ± 89		—	0.84

Data are expressed as mean ± SD.

levels in the present study. In addition, Cameron et al[18] reported that plasma PCSK9 is cleared by an LDL receptor-independent mechanism. We speculated that the mechanism for clearing PCSK9 from plasma would also partially explain the results obtained in the present study.

The effect of 4 mg of pitavastatin on the reduction of LDL cholesterol levels was significantly greater than that of 20 mg of pravastatin. However, no significant differences were found in the percentage of changes in the PCSK9 levels in the presence of the 2 statins. Although the differences in the precise effects of heterodimer and free-fragment PCSK9 were not fully evaluated, heterodimer PCSK 9 is considered to be the main protein responsible for the degradation of LDL receptors.[19] The increased levels of heterodimer PCSK9 were slightly greater in the pitavastatin group than in the pravastatin group, but these differences were not statistically significant. Careskey et al[11] reported that 10 mg of atorvastatin reduced the LDL cholesterol levels by 30% but did not affect the serum PCSK9 levels (1% decrease) after 16 weeks of treatment.

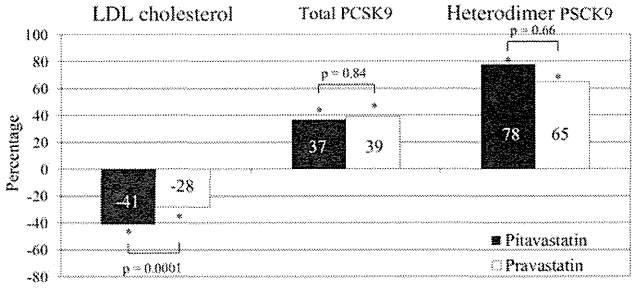

Figure 1. Percentage of changes in serum levels of LDL cholesterol and total and heterodimer PCSK9 levels at the 8-month follow-up point. *p <0.0001 compared to baseline.

Table 4
Univariate regression analyses of changes in heterodimer proprotein convertase subtilisin/kexin type 9

Variable	Pitavastatin		Pravastatin	
	r	p Value	r	p Value
Percentage of change in total cholesterol	−0.121	0.4	−0.127	0.38
Percentage of change in low-density lipoprotein cholesterol	−0.089	0.54	−0.125	0.39
Percentage of change in triglycerides	0.178	0.21	0.156	0.28
Percentage of change in high-density lipoprotein cholesterol	−0.103	0.47	−0.194	0.18
Percentage of change in apolipoprotein A-I	−0.061	0.67	−0.217	0.13
Percentage of change in apolipoprotein B	−0.029	0.84	−0.057	0.69
Percentage of change in lipoprotein (a)	−0.101	0.48	−0.172	0.24
Percentage of change in high-sensitivity C-reactive protein	−0.080	0.58	0.112	0.44
Percentage of change in platelet-activating factor acetylhydrolase	−0.160	0.26	0.024	0.87
Percentage of change in high-density lipoprotein platelet-activating factor acetylhydrolase	−0.159	0.27	−0.055	0.71
Percentage of change in oxidized low-density lipoprotein	−0.032	0.83	0.155	0.3
Percentage of change in small dense low-density lipoprotein	0.035	0.8	0.059	0.69

Comparatively, 16 weeks of treatment with 40 mg of atorvastatin reduced the LDL cholesterol levels by 42%, with a significant increase (34%) in the serum PCSK9 levels. Welder et al[20] reported that 80 mg of atorvastatin treatment increased the PCSK9 levels by 47% and decreased the LDL cholesterol levels by 55%. Furthermore, it has been reported that when the daily atorvastatin dose was increased from 5 to 80 mg, the plasma PCSK9 levels increased by an average of 30%.[17] These results suggest a clear dose–response effect for atorvastatin on PCSK9 levels, with increasing doses of atorvastatin causing greater increases in circulating PCSK9 levels. However, the effects of other types of statins on serum PCSK9 levels have not been well evaluated. Lakoski et al[21] recently demonstrated in a large trial that a low dose (10 mg) of simvastatin did not increase the circulating PSCK9 levels. It is not known whether statin-induced increases in serum PCSK9 levels would be class dependent, because most statin-induced increases in PCSK9 levels have been observed using atorvastatin. Importantly, the present study demonstrated that 8 months of treatment with 4 mg of pitavastatin or 20 mg of pravastatin significantly increased the serum PCSK9 levels.

It is important to understand the changes observed in the serum PCSK9 levels over time as a result of statin therapy. Welder et al[20] reported that the PCSK9 levels increased 47% after 4 weeks of 80 mg atorvastatin; and this increase was sustained at the 8-, 12-, and 16-week measurement points. Comparatively, Okada et al[22] recently reported that the plasma PCSK9 levels were significantly increased at 12 weeks after statin therapy; however, this increase was not sustained and was lower at 52 weeks. Therefore, we speculated that increases in serum PCSK9 levels caused by statin therapy will differ over short- and long-term periods. Huijgen et al[23] reported that the plasma PCSK9 levels at 1 year after treatment with atorvastatin did not significantly differ between the 10- and 80-mg treatment groups. Consistent with their report, the increased levels of PCSK9 at the 8-month follow-up point did not differ between the pitavastatin and pravastatin treatment groups. However, if the serum PCSK9 levels were measured within a shorter period, the increased serum PCSK9 levels might have been significantly greater in the pitavastatin group than in the pravastatin group.

The present study had several limitations. First, it was a post hoc analysis of the TRUTH trial. Second, the serum PCSK9 levels were measured using frozen samples at only 2 points, and previous studies have demonstrated the importance of evaluating changes in serum PCSK9 levels caused by statin therapy over time in detail. Finally, the small number of patients made the statistical power insufficient to compare the effects of statins on the serum PCSK9 levels. However, to our knowledge, this is the first study to compare the effects of 2 different types of statins on the serum PCSK9 levels.

A prospective, randomized study involving more patients is required to confirm our conclusions.

Disclosures

The authors have no conflicts of interest to disclose.

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