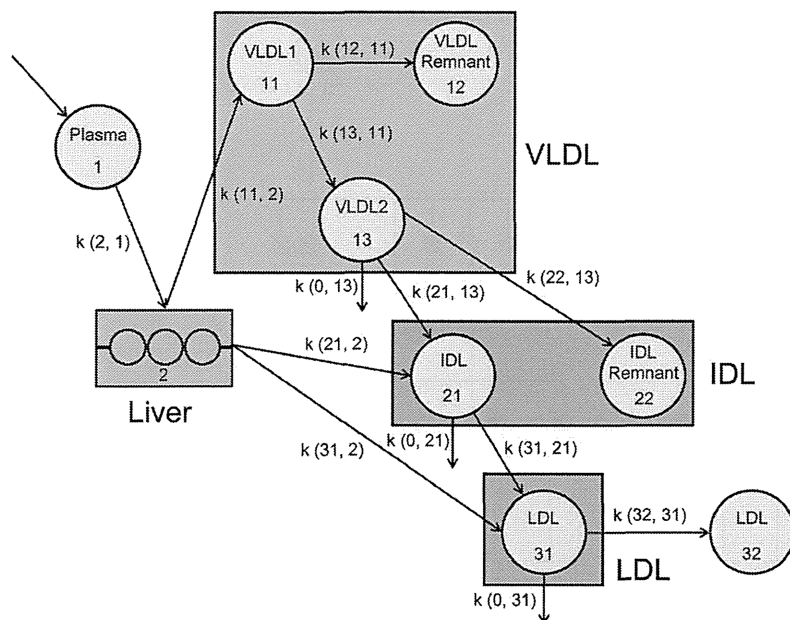


**Figure 1.** Multicompartmental model for apolipoprotein B–containing lipoproteins. Compartment 1 represents the intracellular amino acid pool and compartment 2 represents a delay for synthesis of lipoproteins. Very-low-density lipoprotein (VLDL) comprises 3 compartments: VLDL1, VLDL2, and VLDL remnant. Two compartments were allocated for intermediate-density lipoprotein (IDL): IDL and IDL remnant. LDL had intravascular and extravascular pools.

and a deficiency in this protein results in the decline of LDL-C catabolism, as seen with homozygous FH.[8] However, ARH differs from homozygous FH in the severity of the clinical phenotype and response to statins, the cause of which still remains unclear.[5]

One of the possible mechanisms of great responsiveness to statins was elucidated by a metabolic study using LDLRAP1 knockout mice that showed preserved ability for LDLR-dependent VLDL clearance.[9] However, few data exist regarding the metabolic basis of LDLRAP1 in clinical settings, especially, the metabolism of remnant lipoprotein fractions. Therefore, we examined lipoprotein kinetics in the homozygous ARH patient, using a stable isotope methodology with kinetic modeling including several remnant lipoprotein fractions, before and after atorvastatin therapy.

## Methods

### Study Subjects

This study was approved by the Ethics Committee of Kanazawa University, Suzu General Hospital, for the ARH patient and Jikei University School of Medicine for the control subjects. All study subjects gave their written informed consent to participate. We examined 8 subjects including 1 patient with suspected ARH without any evidence of chronic disease or malignancy and 7 normal control subjects (all men; age, 41±8 years). All lipid-lowering therapy had been strictly suspended for 3 months until the baseline study. We checked the lipid level of the patient suspected ARH 1 month before the baseline study as well as 1 week before the baseline study to confirm that his cholesterol level was appropriately elevated and reached plateau. Next, we reexamined ARH patient after treatment with atorvastatin of 20 mg/d for 3 months.

### Genetic Studies

Genomic DNA was isolated from peripheral blood white blood cells according to standard procedures and was used for PCR. We analyzed the coding regions of LDLR, PCSK9, and LDLRAP1 genes. Primers for the study were as used previously.[10,11] PCR products were purified by Microcon (Millipore Corp, Bedford, MA) and used as templates for direct sequencing. DNA sequencing was carried out according to the manufacturer's instructions, using a dye terminator method (ABI PRISM 310 Genetic Analyzer (PerkinElmer Biosystems, Waltham, MA).

### Biochemical Analysis and LDLR Activity

Serum concentrations of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically. LDL-C concentrations were derived by means of the Friedewald formula. Apolipoprotein E (apoE) phenotype was separated by isoelectric focusing and detected by Western blot with apoE polyclonal antibody (phenotyping apoE IEF system, JOKOH, Tokyo, Japan). Lipoprotein lipase (LPL) mass in postheparin plasma was measured according to the method we previously reported.[12]

LDLR activity was measured by 2 methods, both of which used peripheral lymphocytes; The first was commercially available binding assay and the second was our original assay, which was described in detail elsewhere.[13] Briefly, we could measure accurate LDLR activity by using heparin to exclude the overestimation signals only bound at the surfaces of lymphocytes, even in the case with internalization defective type of disease.

### Lipoprotein Kinetic Study

After an overnight fast, the study subjects were given a bolus injection (10 mg/kg) of [$^2H_3$]-leucine (Cambridge Isotope Laboratories, Woburn, MA). Blood samples were drawn periodically for 48 hours after the bolus injection.

### Determination of Isotopic Enrichment

Samples were prepared for GC-MS analysis as reported previously.[14,15] For detailed determination of isotopic enrichment, please see online-only Data Supplement Method I.

### Kinetic Modeling

Figure 1 shows the multicompartmental model used in this study, which was built using an interactive computer program (SAAM II, version 1.1; SAAM Institute Inc) to determine apoB kinetic parameters.[16,17] For detailed kinetic modeling, please see online-only Data Supplement Method 2.

### Changes in Lipoprotein Subfractions

Lipoproteins of ARH were separated by the method based on those sizes using HPLC (LipoSEARCH, Skylight Biotech, Akita, Japan).[18] Changes in cholesterol, triglyceride, free cholesterol, and phospholipids in each lipoprotein subfraction was assessed by HPLC.

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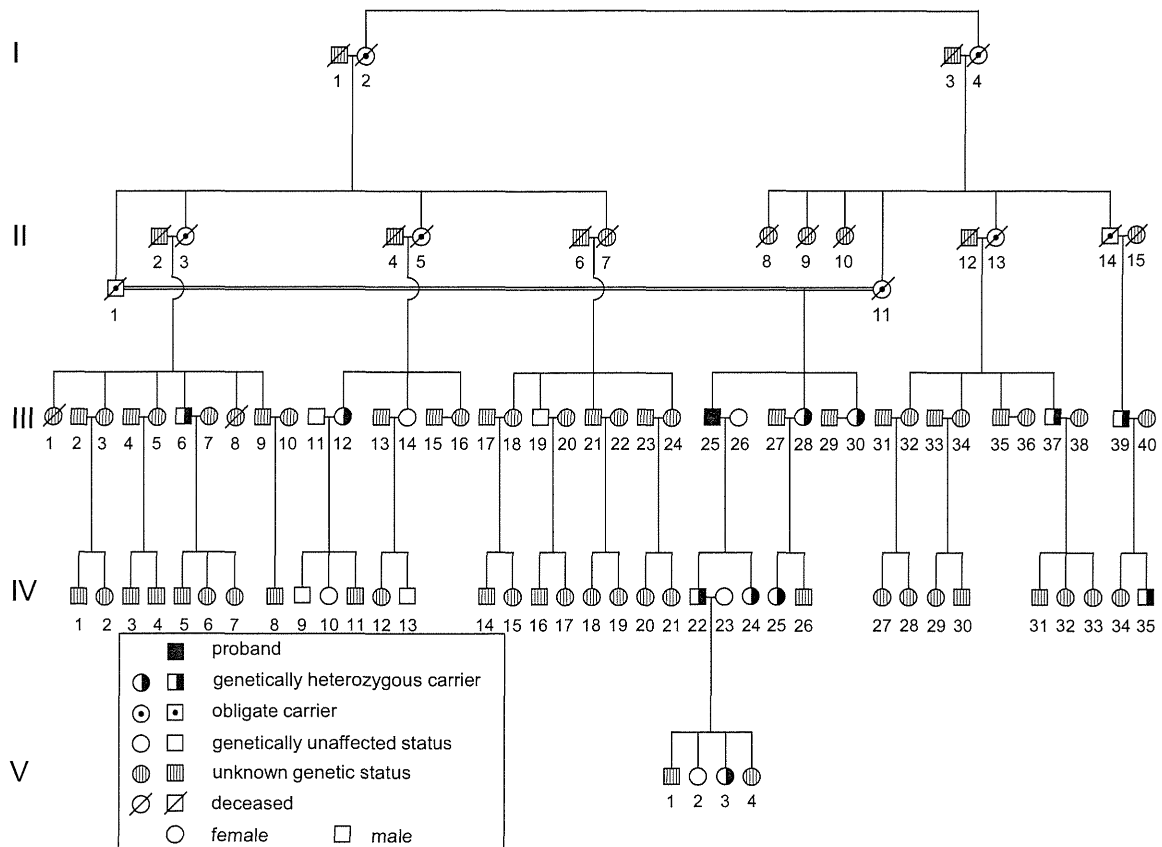


**Figure 2.** Pedigree of the autosomal recessive hypercholesterolemia patient. The proband was born to consanguineous parents (first cousins). The clinical data of the relatives, who were investigated further, are listed in online-only Data Supplement Table.

# Results

## Identification of ARH

A 68-year-old Japanese man presented at Kanazawa University Hospital for further examination of his hypercholesterolemia and severe tendon xanthomas (online-only Data Supplement Figure IA and IB). The proband was born to consanguineous parents (first cousins); neither parent had any signs of hypercholesterolemia or xanthomas. Large cutaneous and tendon xanthomas were identified on his fingers and foot, which had developed around 10 years of age. The thickness of his Achilles tendons reached 26 mm (online-only Data Supplement Figure IC). Initial serum TC and TG concentrations were high: 13.27 mmol/L and 3.39 mmol/L and were decreased to 5 mmol/L and 0.5 mmol/L after statin treatment for 8 years, respectively (online-only Data Supplement Figure ID). Several severe stenotic lesions including total occlusion of right common carotid artery were observed. Angiogram revealed total occlusion of bilateral external iliac arteries as well as left anterior descending artery (online-only Data Supplement Figure IE and IG). Bypass surgeries were conducted for both lesions (online-only Data Supplement Figure IF and IG). An abdominal aortic aneurysm, 33 mm in diameter, was observed. These extents of atherosclerosis are considered to be compatible with his high LDL-C level. Microscopic analysis revealed no specific findings in his liver (online-only Data Supplement Figure IH). Apo E phenotype of the ARH patient was E2/E3 in contrast to the result that those of control subjects were all E3/E3.

Although there was no mutation detected in LDLR and PCSK9 genes, homozygous mutation of an extra cytosine inserted into the region of the LDLRAP1 gene was found (c.606dup, previously described as ins $C_{599}$) in our proband (online-only Data Supplement Figure II), which is completely identical to that found in the first Japanese family identified with ARH.[19] An investigation, which extended back over 5 generations, failed to show any relationship between these 2 families, whose geographical origin were completely different. Using genetic analysis, we diagnosed 11 ARH heterozygous subjects and 6 normal subjects in the proband's family (Figure 2). Their lipid data and major clinical findings including the presence of coronary artery disease are listed in the online-only Data Supplement Table. As for LDLR activity, we found extremely accelerated LDLR activity (as much as 160% of normal control subjects) measured by the binding assay, using the measurement of 3,3′-dioctadecylindocarbocyanin (DiI)-labeled LDL uptake in blood peripheral lymphocytes (BML, Tokyo, Japan). In contrast, the value measured by our internalization assay using heparin showed that the activity was reduced to 14% of normal control subjects.

## Lipoprotein Kinetic Study

At the time of the kinetic study (Table 1), the ARH patient showed higher serum TC levels (10.26 versus 4.87±0.58 mmol/L) and higher LDL-C levels (8.63 versus 2.95±0.49 mmol/L) than those of the control subjects.

The VLDL apoB, IDL apoB, and LDL apoB tracer/tracee ratio curves at baseline and after atorvastatin therapy, as well

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**Table 1. Characteristics of ARH Patients and Control Subjects**

| Subjects | Sex | Age, y | BMI, kg/m$^2$ | TC, mmol/L | TG, mmol/L | LDL-C, mmol/L | HDL-C, mmol/L | ApoB, g/L | ApoB/LDL-C | Lathosterol, μg/mL | LPL, ng/mL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Baseline | Male | 68 | 26 | 10.26 | 1.26 | 8.63 | 1.06 | 1.90 | 0.56 | 6.3 | 324 |
| After statin therapy | | 68 | 26 | 6.02 | 1.06 | 4.22 | 1.32 | 1.13 | 0.69 | 1.2 | 401 |
| Control subjects (n=7) | All male | 41±8 | 22±1 | 4.87±0.58 | 1.08±0.24 | 2.95±0.49 | 1.38±0.13 | 0.89±0.12 | 0.78±0.24 | n.d. | n.d. |

Values of control subjects are shown as mean±SD.

ARH indicates autosomal recessive hypercholesterolemia; BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; apoB, apolipoprotein B; LPL, lipoprotein lipase; n.d., not determined.

as those for the mean of the control subjects, are shown in Figure 3. Kinetic parameter of apoB within each lipoprotein fraction is shown in Table 2. Fractional catabolic rates (FCRs) of VLDL, IDL and LDL apoB were markedly slower in the ARH patient at baseline (3.153 1/day for VLDL, 1.414 1/day for IDL, 0.109 1/day for LDL) compared with those of the control subjects (8.408±2.697 1/day for VLDL, 8.326±3.467 1/day for IDL, 0.450±0.122 1/day for LDL). Production rates (PRs) of the ARH patient of the 3 fractions were within the mean value ±2 SD of those of control subjects. Therefore, the markedly increased concentrations of IDL and LDL apoB were primarily due to the decreased catabolism rate in the ARH patient.

Surprisingly, the FCR of LDL apoB significantly increased to within the normal range after statin therapy in the ARH patient (0.109–0.464 1/day), resulting in a 70% reduction of LDL apoB concentration. This result was completely different from that seen with homozygous FH, where the FCR of LDL apoB was reported to be unchanged after statin therapy.[20] In addition to the response observed in FCR of LDL apoB, those of VLDL and IDL apoB also increased by the statin therapy in the ARH patient (3.153–7.881 1/day for VLDL, 1.414–2.525 1/day for IDL).

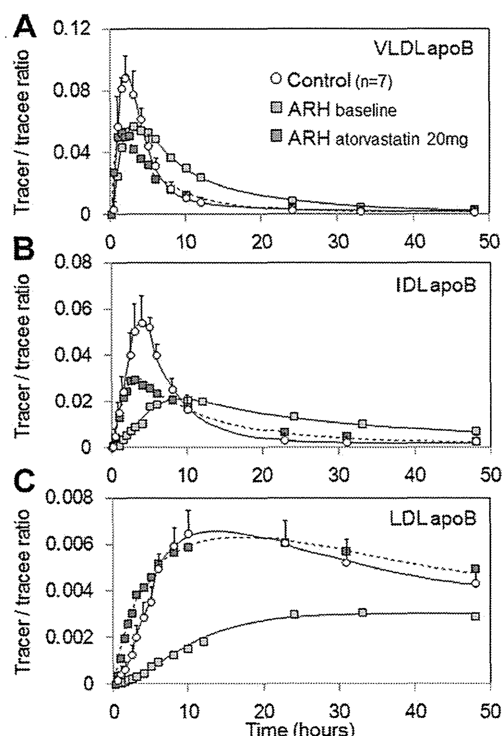


**Figure 3.** Tracer/trace ratios of apolipoprotein (apo)B-containing lipoproteins. Tracer/tracee ratios of very-low-density lipoprotein VLDL apoB (**A**), intermediate-density lipoprotein IDL apoB (**B**), and LDL apoB (**C**) in the autosomal recessive hypercholesterolemia patient at baseline (**blue squares**), on atorvastatin treatment (**pink squares with dotted line**), and in control subjects (**open circles**). Data were fitted by multicompartmental modeling using SAAMII. Bars represent standard error of the means.

## Remnant Fractions

Next, we investigated detailed metabolic channeling in the ARH patient (the results are summarized in Table 3). In the control subjects, the liver primarily secrets VLDL (87.0±11.0%), most of which (85.5±18.7%) was, in turn, converted to IDL by lipoprotein-mediated delipidation, thus leaving the VLDL remnant as a minor fraction (12.0±11.8% of total VLDL mass). Some crucial differences between the ARH patient and the control subjects were noted in VLDL metabolism. In the ARH patient: (1) only half of VLDL was converted to IDL (52.5%); (2) VLDL remnant mass comprised as much as 60.2%, resulting from an alteration in metabolic channeling in favor of the conversion to VLDL remnant (47.5% versus 1.8±2.1%, ARH versus control, respectively): (3) removal rate of VLDL remnant (k[0,12]) was increased (4.3 1/day) compared with that of the control subjects (1.3±0.9 1/day); and (4) direct removal of VLDL, including VLDL remnant, was much higher compared with that of the control subjects (47.5% versus 14.5±18.7%), a finding mirroring the decreased conversion to IDL as noted above. Furthermore, these tendencies were more pronounced after atorvastatin therapy. As shown in the middle panel of Table 3, most IDL was derived from VLDL and exclusively converted to LDL (97.8±3.1%) in the control subjects. In the ARH patient, however, about one-quarter of IDL was directly secreted from the liver and more IDL fractions were directed into remnant, again resulting in the increased remnant mass. These tendencies remained unchanged by atorvastatin therapy. Finally, the only notable difference in LDL metabolism was higher direct secretion of LDL with atorvastatin therapy, a finding consistent to higher tracer/tracee ratios during early time points (pink squares with dotted line in Figure 3C).

## Changes in Lipoprotein Subfractions

As shown in online-only Data Supplement Figure III, relatively wide range of apoB-containing lipoproteins, including large VLDL, could be reduced by atorvastatin therapy in all fractions of lipids (cholesterol, triglyceride, free-cholesterol, and phospholipids) in the ARH patient.

# Discussion

In this study, we performed an in vivo lipoprotein kinetic study, allowing us to assess detailed metabolic behavior of

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**Table 2. Kinetic Parameters of ApoB in the Study Subjects**

| Subjects | VLDL | | | IDL | | | LDL | | |
|---|---|---|---|---|---|---|---|---|---|
| | Conc, mmol/L | FCR, 1/Day | PR, mg/kg per Day | Conc, mmol/L | FCR, 1/Day | PR, mg/kg per Day | Conc, mmol/L | FCR, 1/Day | PR, mg/kg per Day |
| Baseline | 0.340 | 3.153 | 9.180 | 0.657 | 1.414 | 9.560 | 7.730 | 0.109 | 6.980 |
| After statin therapy | 0.248 | 7.881 | 3.026 | 0.341 | 2.525 | 13.335 | 2.333 | 0.464 | 16.756 |
| Control subjects (n=7) | 0.104±0.033 | 8.408±2.697 | 13.172±4.664 | 0.091±0.052 | 8.320±3.467 | 10.562±5.194 | 2.037±0.315 | 0.450±0.122 | 13.947±3.636 |

Values of control subjects are shown as mean±SD.
Apo indicates apolipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; Conc, concentration; FCR, fractional catabolic rate; PR, production rate.

apoB-containing lipoproteins in ARH. Our results demonstrated that in ARH there existed reduced LDL catabolism, which could be normalized by statin therapy and dramatically increased clearance of VLDL remnant as well as other remnant lipoprotein fractions in spite of the fact that our ARH patient has apoE2 isoform which could cause the disturbance in remnant clearance.[21] These unique metabolism of apoB-containing lipoprotein fractions, including VLDL and its remnant fractions were completely different from those reported in heterozygous/homozygous FH patients.[17,22]

One of the possible explanations for the paradoxical acceleration of remnant lipoprotein fractions in ARH is the existence of another pathway, which is independent from the FDNPVY internalization for VLDL and its remnants and does not require LDLRAP1 protein.[23] In addition, Altenburg et al[24] demonstrated that deficiency in the molecule which enhanced the affinity between ligands such as VLDL remnant and LDLR could accelerate the internalization of the remnants. This is consistent with the notion that remnants are passed from one cell surface molecule to the other before internalization.[24] If LDLRAP1 served as an anchor between VLDL remnant and LDLR, deficiency in this protein could result in the increased catabolism of VLDL remnant in ARH. Another possibility is that unknown pathways may exist that are inactivated in the presence of LDLRAP1. This hypothesis seems to be supported by the fact that the LDLR can transfer such remnants to an additional receptor for uptake by the liver when its internalization is impaired. These pathways are not always through LDLR, LDLR-related protein (LRP), and heparan sulfate proteoglycan.[25]

In contrast to homozygous FH patients, the ARH patient responded to statin therapy by an increasing rate of LDL apoB catabolism, resulting in about 70% reduction of LDL apoB pool size. Statin therapy also modulated LDL synthesis in favor of more direct secretion from the liver (11% at baseline to 16% with the treatment versus a mean of 7% for the control subjects). The rate of LDL catabolism is a function of LDLR activity or/and LDL particle affinity to the LDLR. Thus, our results indicate that atorvastatin upregulate LDLR activity in the absence of LDLRAP1. Another possibility for the increasing rate of LDL apoB catabolism seen in ARH is that directly secreted LDL may have a higher affinity for LDLR compared with LDL-processed delipidation/remodeling. Different ratio of apoB/LDL-C between the ARH patient and the control subjects suggest that different LDL processing occurred through delipidation/remodeling of LDL particles under the condition of the absence of this adaptor protein. We also provide additional information for the impact of atorvastatin on the distribution of lipoprotein subfractions in ARH. Relatively wide range of apoB-containing lipoproteins, including large VLDL, could be reduced by atorvastatin therapy. This may be explained by the statin-induced upregulation of possible pathway which could accelerate the clearance of remnant lipoprotein fractions in ARH.

As for the dramatic decrease in PR of VLDL apoB under atorvastatin therapy, one of the possible explanations is the upregulated activity of HMG-CoA reductase suggested by the relatively high level of lathosterol at baseline (Table 1). On

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**Table 3. Metabolic Channeling of ApoB in the Study Subjects**

| VLDL | Conversion to IDL, % | VLDL Direct Removal, % | Removal From Remnant, % | Remnant Mass, % |
|---|---|---|---|---|
| Baseline | 52.5 | 47.5 | 47.5 | 60.2 |
| After statin therapy | 28.1 | 71.9 | 42.8 | 82.3 |
| Control subjects (n=7) | 85.5±18.7 | 14.5±18.7 | 1.8±2.1 | 12.0±11.8 |

| IDL | Direct Production, % | Conversion to LDL, % | IDL Direct Removal, % | Removal From Remnant, % | Remnant Mass, % |
|---|---|---|---|---|---|
| Baseline | 8.6 | 56.4 | 77.2 | 77.2 | 80.8 |
| After statin therapy | 12.2 | 85.5 | 17.0 | 17.0 | 29.6 |
| Control subjects (n=7) | 5.9±7.7 | 97.8±3.1 | 2.3±3.3 | 2.3±3.3 | 17.4±14.4 |

| LDL | Direct Production, % | Via IDL, % |
|---|---|---|
| Baseline | 11.0 | 90.0 |
| After statin therapy | 16.2 | 85.8 |
| Control subjects (n=7) | 7.3±6.1 | 92.3±6.1 |

Values of control subjects are shown as mean±SD.

Apo indicates apolipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; and LDL, low-density lipoprotein.

the other hand, the increase in the PR of LDL apoB during atorvastatin therapy could be partially explained by the elevation of LPL mass (Table 1), in accordance with the previous report.[26] Also, another study has shown that atorvastatin therapy is associated with an increase in LPL activity.[27] These data suggest that atorvastatin treatment may cause an increase in the conversion of VLDL to LDL.

## Limitations

Our study has several limitations. First, only 1 ARH patient was included in this study because of the rarity of this disease, making it difficult to compare the results statistically. Also, the age of the control subjects were younger than the ARH patient, although all were male. Second, we did not measure apoE FCR in the ARH patient and thus could not draw any conclusion regarding the possibility of the clearance through VLDL receptor. However, the fact that the ARH patient has apoE2 isoform, which could cause the disturbance in remnant clearance, indicates the less influence of the apoE pathway on the catabolism of these lipoproteins. In this study, as much as 30% increase in HDL-C was achieved through atorvastatin therapy. Another kinetic study targeting apoA-I for the ARH patient may reveal the metabolic aspects about the increase in HDL-C.

Finally, it would be worthwhile to compare lipoprotein kinetics of ARH with that of FH directly. Although we cited previously published data on the apoB kinetics in FH patients to discuss the comparison between the kinetics of ARH and FH, further kinetic study comparing ARH and FH directly is needed to confirm this matter.

## Conclusion

In summary, the first detailed lipoprotein kinetic study including remnant lipoprotein fractions in ARH before and after statin therapy revealed 2 important aspects of the lipoprotein metabolic basis of this disease. First, FCR of LDL apoB in ARH was decreased by about 76% that of normal control subjects at baseline; however, the catabolic parameter was elevated to normal range after statin therapy (atorvastatin 20 mg). Second, and possibly the major finding from this investigation, is that the clearance of the VLDL remnant as well as other remnant fractions were dramatically increased compared with normal control subjects. We suggest that these results will provide new insights into the lipoprotein metabolism of ARH and the novel pharmacological target for LDLRAP1.

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## Disclosures

None.

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## CLINICAL PERSPECTIVE

Autosomal recessive hypercholesterolemia (ARH), which is due to mutations in an adaptor protein involved in low-density lipoprotein receptor internalization (LDLRAP1), is an extremely rare disorder, with only about 50 cases described in the literature. This defect appears to be a phenocopy of homozygous familial hypercholesterolemia; however, the clinical phenotype of ARH appears to be less severe and more responsive to statins—the mechanism for this observation still remains unknown. One of the possible mechanisms of great responsiveness of ARH to statins was elucidated by a metabolic study using LDLRAP1 knockout mice that showed a preserved ability for LDLR-dependent very-low-density lipoprotein (VLDL) clearance. However, few data exist regarding the metabolic basis of LDLRAP1 in clinical settings, especially the metabolism of remnant lipoprotein fractions. Therefore, we examined lipoprotein kinetics in the ARH patient by using a stable isotope methodology with kinetic modeling including several remnant lipoprotein fractions, before and after atorvastatin therapy. We demonstrate that ARH exhibits decreased LDL clearance associated with decreased fractional catabolic rates of LDL apoB and increased clearance for VLDL remnant; this observation indicates the lack of LDLRAP1-dependent modulation of VLDL metabolism, activating an alternate pathway that can remove VLDL remnant paradoxically. This preferred pathway could potentially contribute to the greater responsiveness of ARH to statins. Our results will provide new insights into the lipoprotein metabolism in ARH.

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## SUPPLEMENTAL MARERIAL

**online-only Data Supplement Method 1.** Determination of isotopic enrichment

Briefly, apoB isolated by isopropanol precipitation was hydrolyzed in 6N HCl (amino acid analysis grade, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 110°C for 24 hours. The protein hydrolysates were lyophilized in a Speed-Vac evaporator (Savant Instrument Inc., Farmingdale, NY). Free amino acids were purified from plasma or protein hydrolysates by cation exchange chromatography (AG-50W-X8, Bio-Rad Laboratories, Richmond, CA), and then derivatized to the N-heptafluorobutyryl isobutyl esters, and analyzed by GC-MS on a 6890 gas chromatograph connected to a 5973 quadruple mass spectrometer (Hewlett Packard, Palo Alta, CA) in the chemical ionization mode, using methane as the reagent gas. Selective ion monitoring at 365 m/z (M+2 isotopomer) for unlabeled leucine and 366 m/z (M+3 isotopomer) for labeled leucine was used to determine the tracer/tracee ratio by regression analysis of standards of known tracer/tracee ratios (0-10%) as reported previously. Each sample was analyzed at least 2 times.

**online-only Data Supplement Method 2.** Kinetic modeling used in this study

In this model, heterogeneity in VLDL is represented by large VLDL (VLDL1), small VLDL (VLDL2). For the VLDL1 fraction, supplementary heterogeneity was introduced in the model by 2 compartments: the first one, VLDL1 (compartment 11) was linked to VLDL2 (compartment 13) by delipidation cascade, and the second, VLDL remnants (compartment 12). This was performed to get a better fit of the data. ApoB-100 enters into plasma through VLDL secretion and direct production of IDL and LDL. ApoB-100 direct removal occurs from VLDL2 (k(0,13)), VLDL remnant (k(0,12)), IDL (k(0,21)), IDL remnant (k(0,22)), and LDL (k(0,31)). To make the model identifiable, the rate constant from VLDL1 to VLDL2 (k(13,11)), representing delipidation, was constrained to be equal to that from VLDL2 to IDL (k(21,13)). For comparison between 2 groups (ARH patient and controls) the VLDL1, VLDL2, and VLDL remnant data were presented as VLDL delipidation rate and VLDL FCR, which represents the sum of delipidation and direct removal rate. The VLDL conversion rate was calculated as VLDL2 delipidation flux divided by total VLDL mass. The VLDL direct removal was calculated as VLDL2 direct removal divided by total VLDL mass. The apoB100 PR in mg/kg per day represents the product of FCR and pool size of apoB100 in lipoprotein fractions assuming plasma volume equal to 4.5% of body weight.

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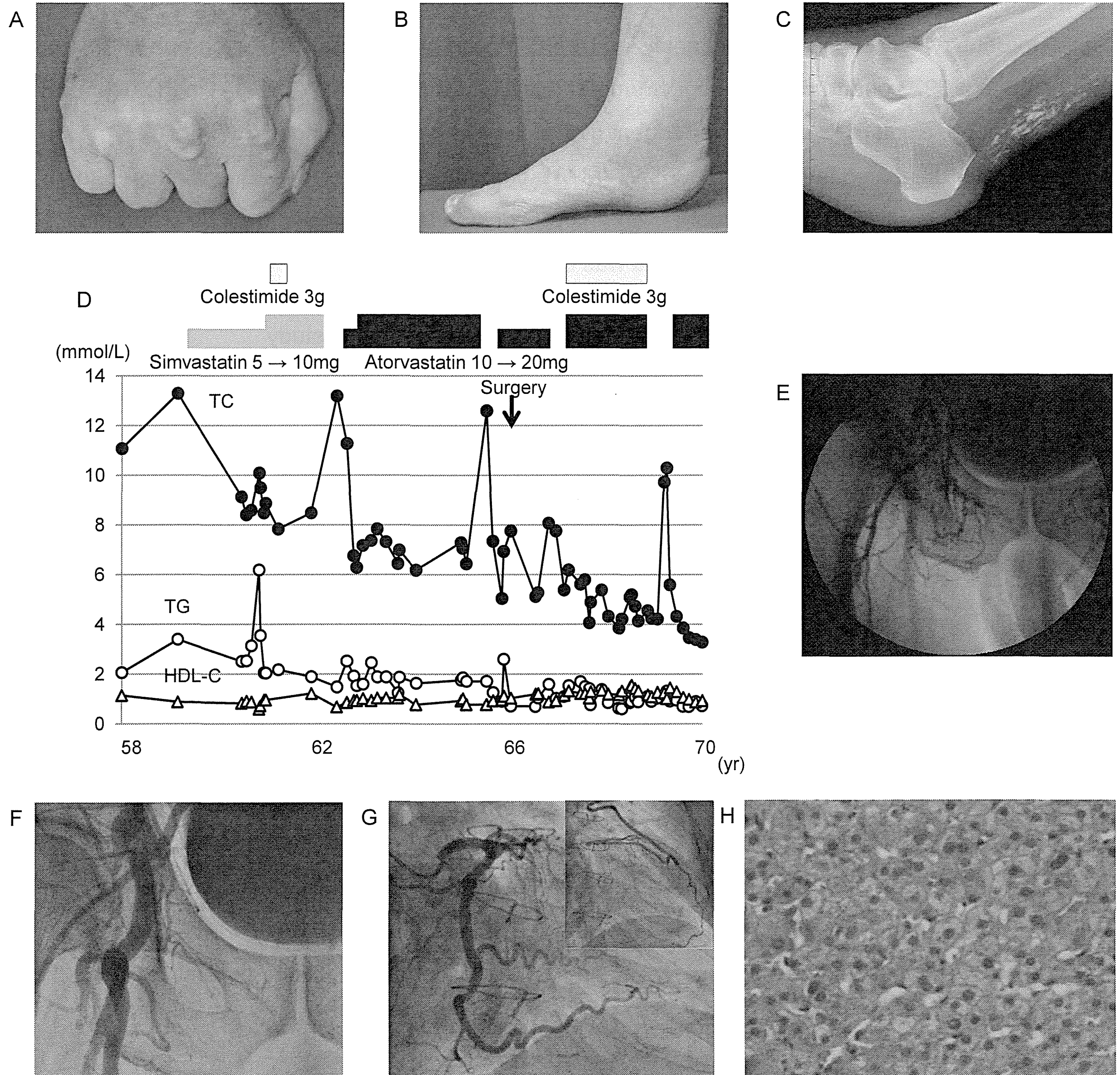
**online-only Data Supplement Figure 1**. Large cutaneous and tendinous xanthomas in the proband's hand (A) and foot (B). Achilles tendons show significant thickness (right = 26 mm) with calcification (C).Clinical course is shown, open circles indicate TC, closed circles indicate TG, and open triangles indicate HDL-C (D). Bypass surgery was carried out on the bilateral external iliac artery because of the complaint of intermittent claudication (E and F). And bypass surgery for the coronary artery was conducted (G). Microscopic findings of the proband's liver stained with hematoxylin and eosin (H). There was no evidence of fatty liver or any other specific findings.

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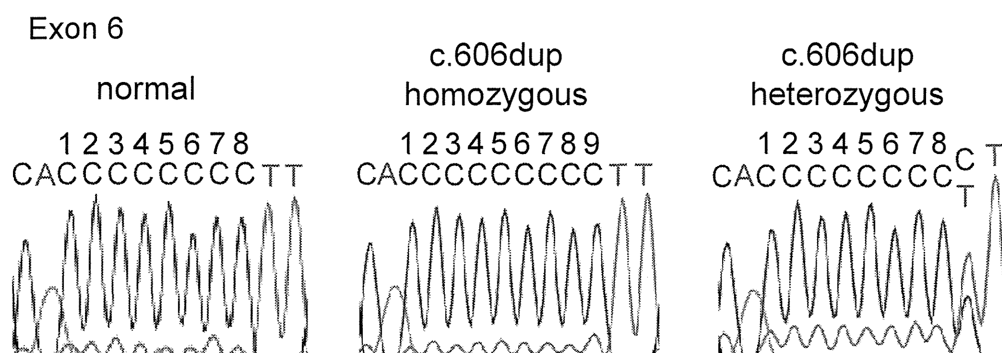
**online-only Data Supplement Figure 2.** DNA sequence data of the proband (central panel), his niece (right panel), and a control subject (left panel) for the LDLRAP1 gene exon 6.
Homozygosity for an extra cytosine insertion mutation in eight sequential cytosines between the nucleotide positions 599 and 606 (nucleotides are numbered from the first nucleotide that encodes the starting methyonine codon) was shown in the proband, with corresponding heterozygosity shown in his niece.




**online-only Data Supplement Table.** Clinical data of the proband's family

| Subjects† | Gender | Age (yr) | Condition | CAD | AT (mm) | TC (mmol/L) | TG (mmol/L) | LDL-C (mmol/L) | HDL-C (mmol/L) |
|---|---|---|---|---|---|---|---|---|---|
| III-6 | male | 70 | heterozygous | - | 6 | 6.13 | 0.94 | 4.73 | 0.96 |
| III-11 | male | 75 | normal | - | n.d. | 4.68 | 1.32 | 2.76 | 1.29 |
| III-12 | female | 72 | heterozygous | - | 6 | 7.32 | 1.88 | 5.04 | 1.40 |
| III-14 | female | 77 | normal | + | 6 | 5.64 | 1.55 | 3.49 | 1.42 |
| III-19 | male | 56 | normal | - | 8 | 5.90 | 1.95 | 3.57 | 1.42 |
| III-25* | male | 67 | homozygous | + | 26 | 13.27 | 3.39 | 10.84 | 0.88 |
| III-26 | female | 65 | normal | - | n.d. | 6.34 | 1.24 | 3.90 | 1.86 |
| III-28 | female | 60 | heterozygous | - | 6 | 6.28 | 2.16 | 3.85 | 1.42 |
| III-30 | female | 53 | heterozygous | - | 5 | 6.54 | 1.97 | 4.06 | 1.58 |
| III-37 | male | 55 | heterozygous | - | 8 | 7.37 | 1.05 | 4.89 | 1.99 |
| III-39 | male | 59 | heterozygous | - | 4 | 4.89 | 1.67 | 5.48 | 1.22 |
| IV-9 | male | 52 | normal | - | n.d. | 4.58 | 1.41 | 2.38 | 1.55 |
| IV-10 | female | 48 | normal | - | 5 | 6.23 | 1.04 | 3.70 | 2.04 |
| IV-13 | male | 53 | normal | - | 7 | 4.52 | 4.34 | 1.42 | 1.09 |
| IV-22 | male | 42 | heterozygous | - | n.d. | 5.48 | 2.03 | 3.36 | 1.19 |
| IV-24 | female | 39 | heterozygous | - | 5 | 4.60 | 1.41 | 2.51 | 1.45 |
| IV-25 | female | 38 | heterozygous | - | n.d. | 4.60 | 1.39 | 2.69 | 1.23 |
| IV-35 | male | 30 | heterozygous | - | 5 | 5.74 | 2.50 | 3.62 | 0.96 |
| V-2 | female | 16 | normal | - | n.d. | 5.07 | 0.91 | 3.21 | 1.45 |
| V-3 | female | 12 | heterozygous | - | n.d. | 4.60 | 0.78 | 2.46 | 1.79 |

CAD, coronary artery disease; AT, Achilles tendon; n.d., not done.
* Proband
† Generation-family member: see Supplemental Figure 1 for pedigree details of individual family members

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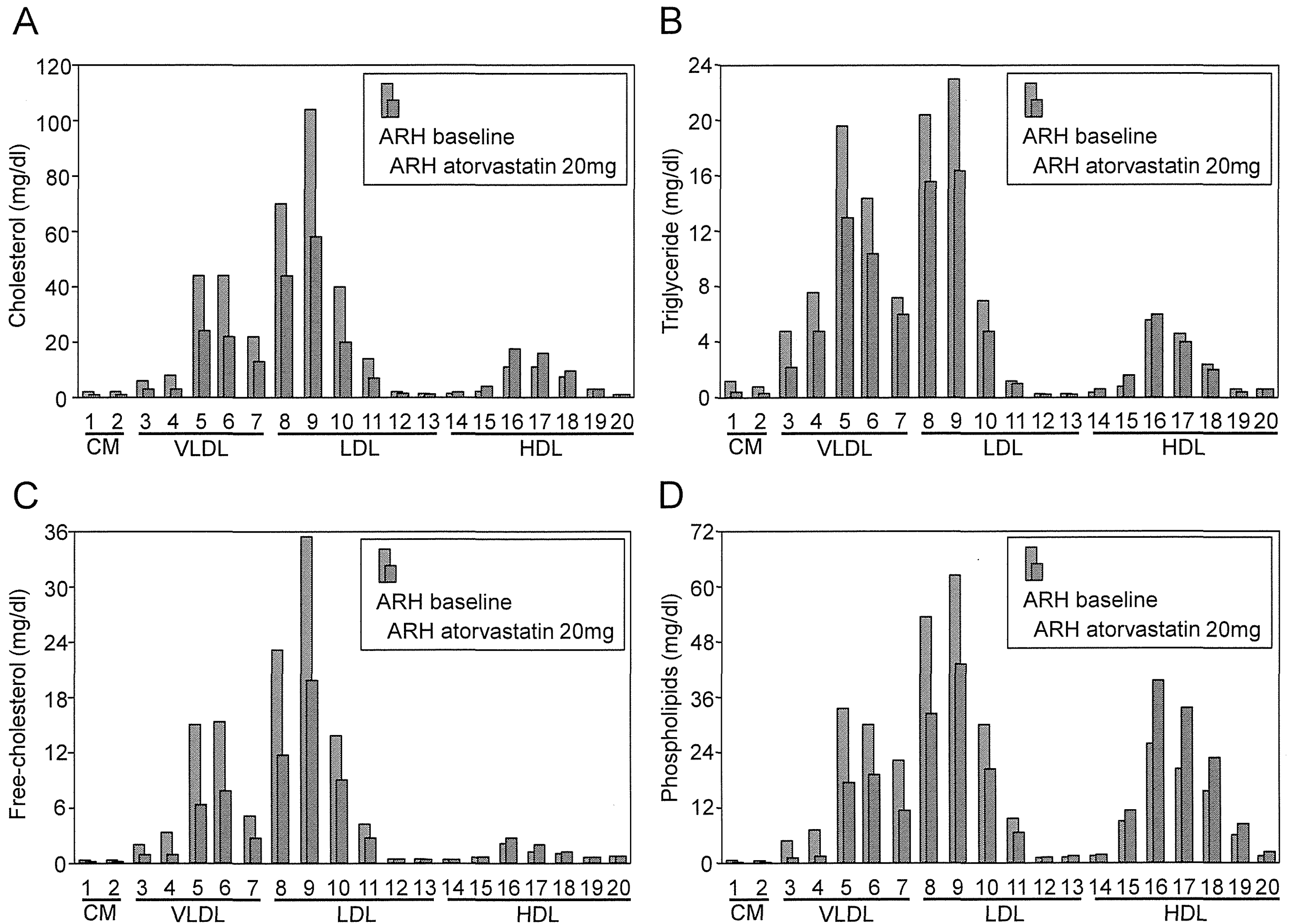
**online-only Data Supplement Figure 3.** Changes in lipoprotein subfractions.

Changes in cholesterol (A), triglyceride (B), free-cholesterol (C), and phospholipids (D) in each lipoprotein subfraction was assessed by HPLC. Blue bars indicate the baseline value of the ARH patient. Pink bars indicate the value of the ARH patient after statin therapy.

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Original Article

# Multicenter Study to Determine the Diagnosis Criteria of Heterozygous Familial Hypercholesterolemia in Japan

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***Aim***: Heterozygous patients of familial hypercholesterolemia (FH) are known to have a high risk of coronary artery disease (CAD). Early diagnosis and prompt treatment are necessary to prevent their CAD. In this study we tried to amend the Japanese diagnostic criteria of FH for general practitioners by examining each component of the current criteria.
***Methods***: A multicenter study was performed, which included 1356 dyslipidemic patients at 6 centers. Pretreatment demographic information including LDL-cholesterol (LDL-C), Achilles tendon thickness (ATT), family history of FH and premature CAD and the result of genetic analysis were analyzed.
***Results***: Of 1356 patients, 419 were diagnosed with FH by criteria in 1988, which were used as a golden standard. We tried to define FH according to 3 conventional major items, i.e., 1) LDL-C, 2) ATT and/or cutaneous nodular xanthomas (CX), 3) family history of FH and/or family history of premature CAD. We then determined the cutoff of LDL-C using the new criteria. When we used 180 mg/dL as the cutoff of LDL-C, 94.3% of FH patients and 0.85% of non-FH satisfied 2 or more criteria. When we used 190 mg/dL, 92.1% of FH and 0.85% of non-FH satisfied 2 or more criteria; therefore, we chose 180 mg/dL for the cutoff of LDL-C in the new criteria and proposed that the diagnosis of definite FH can be made if 2 or more criteria are satisfied.
***Conclusions***: We examined each component for the diagnosis of heterozygous FH in a multicenter study in Japan.

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**Key words;** Diagnosis criteria, Familial hypercholesterolemia, LDL cholesterol, Achilles tendon thickness, LDL receptor

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## Introduction

Familial hypercholesterolemia (FH) is a genetic disease caused by a mutation in genes related to low-density lipoprotein (LDL) metabolism. Heterozygous FH patients manifest high LDL cholesterol (LDL-C)

levels, skin and/or tendon xanthomas, and increased risk of premature coronary artery disease (CAD)[1]. High LDL-C levels are the first symptom that appears even from birth, while xanthomas on the Achilles tendon usually appear during or after the late 10s and CAD that determines the prognosis of FH patients appears during or after the third decade of life in men and the fifth decade in women[2-4]. Because morbidity and mortality of CAD in heterozygous FH are much higher than in the general population[1, 5-7], special attention should be paid to screen these patients and to prevent their atherosclerotic complications. For the diagnosis of FH, several criteria have been published throughout the world, including ours, reported in 1988[8]; however, appropriate diagnosis of FH by primary care physicians is not performed in general practice in Japan[9]. Therefore, it is very important to establish useful diagnostic criteria for primary care physicians to diagnose FH with high specificity and sensitivity.

Because FH patients are estimated to be more than 250,000, primary care physicians need to take care of most of them; therefore, the criteria should be as simple as possible for clinical usefulness and have high sensitivity and specificity. We have used diagnosis criteria for FH published in 1988 in Japan[8], which include hypercholesterolemia, presence of skin/tendon xanthoma and reduced LDL receptor activity as major items; however, it is difficult to measure LDL receptor activity in routine clinical practice and even lipid specialists do not measure its activity. Furthermore, it is not covered by Japan's health insurance system; therefore, it is necessary to make the current diagnostic criteria easy to use for general practitioners. Toward this end, we performed a multicenter collaborative study of 1397 patients with dyslipidemia.

## Methods

### Subjects

A total of 1397 patients with dyslipidemia, referred to the outpatient clinic of 6 hospitals (Kyoto University Hospital, Osaka University Hospital, Nippon Medical School Hospital, Chiba University Hospital, Kanazawa University Hospital, and National Cerebral and Cardiovascular Center Hospital), were the subjects to this study. Among these patients, 41 were excluded due to missing data. Consequently, 1356 patients with dyslipidemia were eligible for the present study. Most had been diagnosed with or without FH by lipid specialists at each hospital according to the criteria for FH reported in 1988, and genetic analysis was performed in 223 patients, some of whom were diagnosed with FH based only on mutations of the LDL receptor or PCSK9. The criteria were as follows: Major items included 3 items, (1) IIa or IIb phenotype at serum cholesterol level of 260 mg/dL or above; (2) Tendinous xanthoma or xanthoma tuberosum is present; (3) Reduced or abnormal receptor activity. Minor items included 3 items: (1) Xanthoma palpebratum; (2) Arcus juvenalis (<50 years); (3) Juvenile (<50 years) ischemic heart disease.

**Table 1.** Clinical characteristics of non-FH and FH patients in this cohort

| | non-FH | FH | $p$ |
|---|---|---|---|
| N | 937 | 419 | |
| Male (n, %) | 453 (48.3%) | 170 (42.7%) | <0.01 |
| Age (y.o.) | 58.3 ± 16.3 | 52.9 ± 18.6 | <0.01 |
| TC (mg/dL) | 236 ± 53 | 339 ± 72 | <0.01 |
| LDL-C (mg/dL) | 146 ± 46 | 257 ± 67 | <0.01 |

### Determination of Conventional Criteria for FH

In this study we tried to amend the current criteria. For the primary care setting, three major items, i.e. serum level of LDL-C, family history and specific physical findings of FH, were chosen as diagnostic items because all are easily assessed by general practitioners. Family history and specific physical findings were also separated in more detail. Finally, we set 5 items, (1) LDL-C, (2) specific physical findings: a) ATT, b) cutaneous nodular xanthomas (CX), (3) family history: a) family history of FH in 1st or 2nd degree relatives, b) family history of premature CAD in 1st or 2nd degree relatives. A family history of premature CAD was defined as having CAD before the age of 55 in males and 65 in females. First, we assessed the prediction for FH by the combination of physical findings and family history, and then we determined the cutoff point of LDL-C with the combination of the above-mentioned two items. LDL-C levels were calculated by the Friedewald formula. ATT levels were measured by X-ray according to the method previously described and determined as positive with 9 mm or more[10].

The data in the medical records of the patients were sent to the National Cerebral and Cardiovascular Center and examined. The study protocol was approved by the ethics committee of the National Cerebral and Cardiovascular Center (D#M20-25-2 for the multicenter trial and ID#M17-56-4 for the genetic analysis). The ethics committee of each hospital also approved the study protocol.

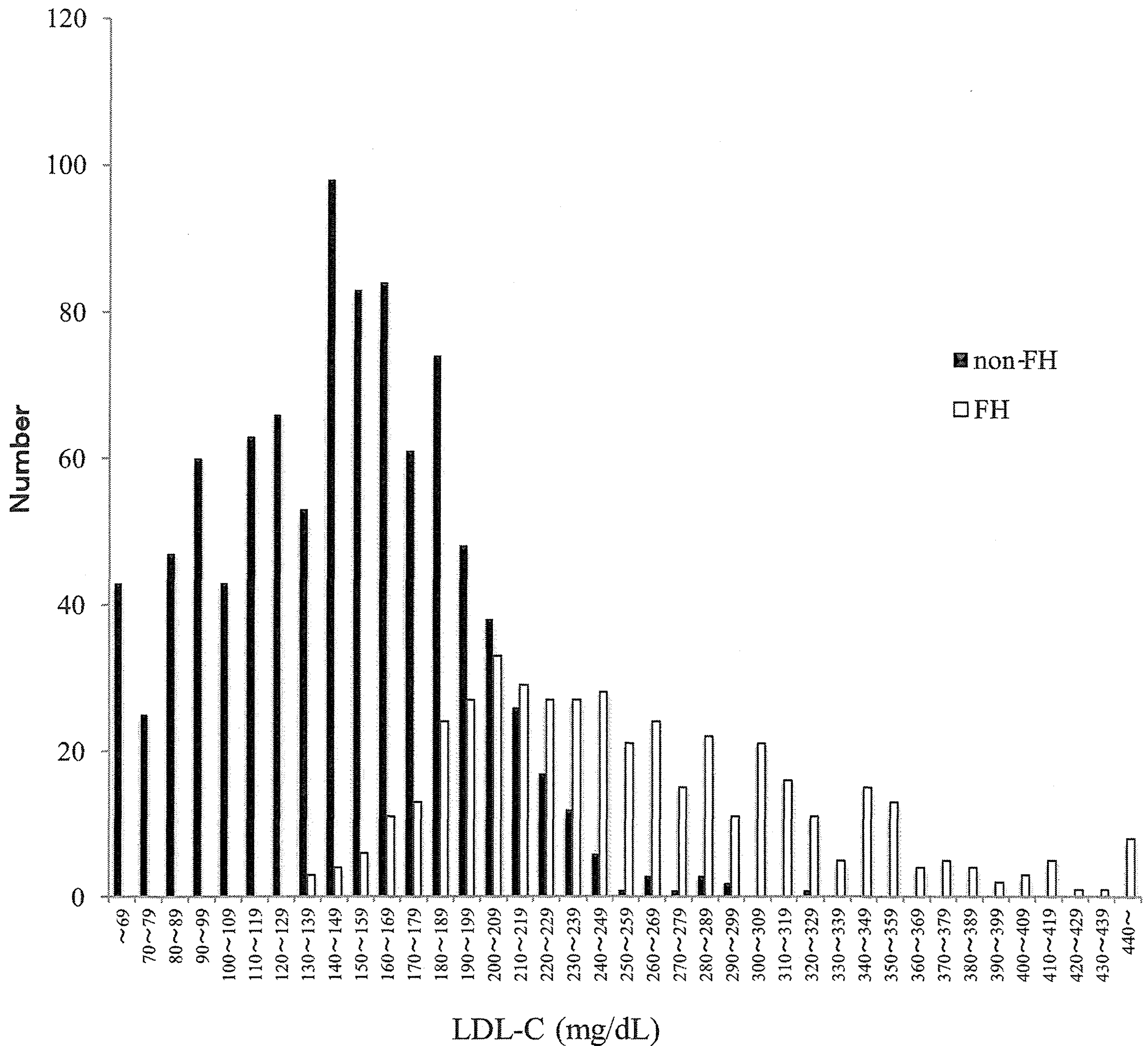


**Fig. 1.** Distribution of LDL-C levels before treatment in FH and non-FH patients. LDL-C levels were calculated by the Freidewald formula in patients with dyslipidemia diagnosed with FH or non-FH by specialists.

**Statistical Analyses**

Continuous variables are presented as the means ± SD. Categorical data are presented as numbers and percentages. Unpaired Student's $t$-test and one-way analysis of variance (ANOVA) were used to assess differences between groups in continuous variables. Differences in categorical variables were assessed by the $\chi^2$ test.

## Results

Among 1356 patients, 419 had been diagnosed with FH, while 937 with non-FH. Patient demographic data are shown in **Table 1**. FH patients were younger than non-FH patients. TC and LDL-C levels were 339 and 257 mg/dL in FH patients, respectively, and were significantly higher than in non-FH patients. The distribution of LDL-C levels in both groups is shown in **Fig. 1**. FH patients were divided into 3

**Table 2.** LDL-C levels in FH patients with or without genetic data

| LDL-C (mg/dL) | FH (Total) | FH (Mut+) | FH (Mut−) | FH (no genetic data) | $p$-value |
|---|---|---|---|---|---|
| N | 419 | 224 | 41 | 173 | |
| Mean | 257.4 | 266.2* | 229.0* | 252.9 | |
| SD | 67.39 | 69.85 | 60.14 | 63.70 | |
| MEDIAN | 244 | 253 | 216 | 241 | 0.003 |
| IQ | | | | | |
| 25% | 205 | 213 | 189 | 203 | |
| 75% | 300 | 308 | 244 | 295 | |

FH (Mut+): mutations in the LDL receptor or PCSK9, FH (Mut−): no mutations found, FH (no genetic data): no genetic analysis
*$p < 0.005$ by Bonferroni

**Table 3.** Sensitivity and specificity in screening FH by physical findings and family history

| | Specificity | Sensitivity |
|---|---|---|
| Physical findings | | |
| ATT (+) (%) | 98.6 | 64.1 |
| CX (+) (%) | 99.6 | 9.4 |
| ATT (+) or CX (+) (%) | 98.6 | 64.6 |
| ATT(+) and CX(−) | 99.6 | 11.7 |
| Family history | | |
| Family history of FH (+) (%) | 93.6 | 98.2 |
| Family history of CAD (+) (%) | 96.3 | 28.3 |
| Family history of FH (+) or CAD (+) (%) | 91.7 | 98.7 |
| Family history of FH (+) and CAD (+) (%) | 98.2 | 27.4 |

ATT: Achilles tendon thickness, CX: Cutaneous nodular xanthomas
FH ($n$=224) was diagnosed by mutations in the LDL receptor and/or PCSK9. Non-FH ($n$=937) was diagnosed by specialists.

groups depending on their genetic data: FH with mutation(s) in LDL receptor or PCSK9, FH with no mutation(s) and FH with no genetic data. The mean and median of LDL-C along with SD and interquartile range of each group are shown in **Table 2**. LDL-C levels in FH with mutations were higher than those in FH without mutations.

We tried to define FH according to the screening standards as 3 major items, i.e., 1) LDL-C, 2) ATT and/or cutaneous nodular xanthomas (CX), 3) family history of FH and/or family history of premature CAD. We used LDL-C instead of total cholesterol, because LDL-C should better reflect the activity of the LDL receptor and is used for the goal of lipid management in the current Japanese guideline[8]. We incorporated "family history" as a major item because general practitioners were able to find FH by a family history of FH and/or premature CAD instead of LDL receptor activity. Sensitivity and specificity in screening FH by physical findings and family history are listed in **Table 3**. Based on these data, we decided to use 1) ATT or CX, and 2) family history of FH or CAD as 2 major items in addition to high LDL-C levels.

Next we tried to determine the cutoff levels of LDL-C. The percentage of the patients who satisfied each criterion according to LDL-C levels is listed in **Table 4**. Levels of 180 or 190 mg/dL are suggested as candidate cutoff levels. Therefore, the criteria for model 1 were set as those who satisfy 2 or more of the 3 criteria: 1) LDL-C 180 mg/dL or higher, 2) ATT (+) or CX (+), 3) Family history of FH or CAD, and for model 2, for which the cutoff point of LDL-C was changed to 190 mg/dl or higher, their sensitivity, specificity, and false positive and false negative rates were compared (**Table 5**). When we compared model 1 with model 2, higher sensitivity in model 1 than model 2 was obtained without any change in specificity, suggesting that 180 mg/dL is a better cutoff for LDL-C. The percentages were quite similar in FH with mutation (s) in LDL receptor or PCSK9, FH with no mutation (s) and FH with no genetic data. The diagnostic criteria of FH were then determined

**Table 4.** Percent satisfying each LDL-C level in non-FH and FH patients

| | non FH | FH (All) | FH (Mut+) | FH (Mut−) | FH (No genetic data) |
|---|---|---|---|---|---|
| N | 937 | 419 | 223 | 41 | 155 |
| LDL-C ≥ 170 mg/dL (%) | 30.5 | 94.5 | 96.0 | 85.4 | 94.8 |
| LDL-C ≥ 180 mg/dL (%) | 24.3 | 94.3 | 94.6 | 82.9 | 92.9 |
| LDL-C ≥ 190 mg/dL (%) | 16.6 | 92.1 | 93.7 | 75.6 | 89.7 |
| LDL-C ≥ 200 mg/dL (%) | 11.6 | 80.0 | 84.3 | 63.4 | 78.1 |

FH(Mut+): mutations in the LDL receptor or PCSK9, FH(−): no mutations found, FH (no genetic data): no genetic analysis

**Table 5.** Accuracy metrics of FH criteria using LDL-C cutoff levels of 180 or 190 mg/dL

| | Sensitivity (%) | Specificity (%) | False positive (%) | False negative (%) |
|---|---|---|---|---|
| Model 1: Satisfying 2 or more of the following criteria: 1) LDL-C ≥ 180 mg/dL, 2) ATT(+) or CX(+), 3) Family history of FH or CAD | 94.5 | 99.1 | 0.85 | 5.5 |
| Model 2: Satisfying 2 or more of the following criteria: 1) LDL-C ≥ 190 mg/dL, 2) ATT(+) or CX(+), 3) Family history of FH or CAD | 92.1 | 99.1 | 0.85 | 7.9 |

**Table 6.** Diagnostic criteria for adult (15 years or older) heterozygous FH

| | |
|---|---|
| 1 | Hyper-LDL-cholesterolemia (LDL-C level before treatment: 180 mg/dL or more) |
| 2 | Tendon xanthoma (tendon xanthoma of the dorsal hands, elbows, and knees, or Achilles tendon thickening) or nodular xanthoma of the skin |
| 3 | Family history (relatives in the second degree): FH or premature CAD |

-A diagnosis should be made after ruling out the possibility of secondary hyperlipidemia.
-Patients meeting 2 criteria should be regarded as having FH. Concerning those meeting 1 criterion, refer to Fig. 4. When FH is suspected, gene tests should be conducted to make a diagnosis.
-Nodular xanthoma of the skin does not include palpebral xanthoma.
-Patients with Achilles tendon thickening (9 mm or more) on radiography should be regarded as having xanthoma.
-When the LDL-C level is 250 mg/dL or more, FH should be strongly suspected.
-During drug therapy, the pretreatment lipid level should be employed as a reference value.
- CAD in males younger than 55 years old and females younger than 65 years old is defined as premature CAD.
-When a diagnosis of FH is made, the patient's family should also be investigated.
- LDL-C may be decreased after surgery, myocardial infarction, severe inflammation and so on. In these cases, LDL-C values before the diseases should be requested to give a diagnosis.
- To diagnose patients who have already been treated with statins, pretreatment levels of LDL-C should be requested; however, termination of statin treatment is not recommended to obtain pretreatment levels of LDL-C, even if the data are not available.

and are shown in **Table 6**.

## Discussion

FH has the highest prevalence in genetic metabolic diseases, being heterozygous in one in 500 of the general population[1, 11]. Most young heterozygous FH patients have no symptoms other than high LDL-C levels, and those who have Achilles tendon thickness have no symptoms. The reason for undiagnosed FH patients to go to a clinic may be mainly divided into the following 4 situations: 1) a chance visit to a primary care physician due to flu or gastritis, etc., 2) recommendation of further medical examination due to high cholesterol at a health checkup, 3) transportation to the emergency room due to the development of acute coronary syndrome, 4) recommendation of medical consultation due to the presence of FH in his/

her family. The diagnostic criteria should be applied to these patients. Accordingly, conventional criteria are needed for the primary care setting.

Heterozygous FH patients show high levels of LDL-C, cutaneous and tendon xanthomas, and are complicated with myocardial infarction at young age by atherosclerosis due to intravascular exposure to high levels of LDL-C for many years. Because early diagnosis and treatment are recommended for these patients, the diagnostic criteria for FH have been reported in many countries including Japan[8, 12-17]. While some criteria give a satisfactory diagnosis of FH using specific items, others are adopting a scoring system. The Japanese criteria reported in 1988[8] were as follows. Major items included the following 3 items: (1) the patient shows the IIa or IIb phenotype at a serum cholesterol level of 260 mg/dL or above, in principle; (2) Tendinous xanthoma or xanthoma tuberosum is present; (3) Reduced or abnormal receptor activity is noted by LDL receptor analysis; however, for LDL receptor activity, even lipid specialists do not routinely measure activity. It would be even more difficult for primary care physicians to measure activity for the diagnosis of FH.

The cutoff level of serum cholesterol used in the first criterion in the criteria published in 1988 was 260 mg/dL; however, LDL-C is directly affected by dysfunction of the LDL receptor and is routinely measured in clinics by the direct method or Friedewald formula; therefore, we tried to use LDL-C as a cutoff level instead of total cholesterol. The presence of tendon and/or cutaneous nodular xanthomas was also used because of its convenience, high sensitivity and specificity. A family history of FH or premature CAD in 1st or 2nd degree relatives was proposed for the third criterion instead of measuring LDL receptor activity in the new diagnostic criteria. A family history of FH showed high sensitivity and specificity; however, primary care physicians may have difficulty obtaining this because it was not easy for them to reach a diagnosis of FH with the previous criteria. In the present study, accurate diagnosis of a family history of FH seemed to have been given because lipid specialists made the diagnosis at all the hospitals; however, the same result may not be applied to primary care physicians. Therefore, a family history of CAD, which may be easier to obtain, was added to the criteria. It should be noted that the sensitivity of a "family history of FH or CAD" was slightly higher than that of a "family history of FH". Accordingly, we chose a "family history of FH or premature CAD in 1st or 2nd degree relatives" as the third criterion.

The cutoff level of LDL-C for the diagnosis of FH should be set by its sensitivity and specificity in different cutoff points. The cutoff level of LDL-C for the diagnosis of FH was reported to be 190 mg/dL in Simon Broome[17], NICE[15] and 205 mg/dL in MEDPED[16]. In this study, 180 mg/dL was selected as the cutoff level together with the presence of xanthoma and the family history as the criteria for the diagnosis of FH because of its high sensitivity and specificity.

Reduced LDL receptor activity is direct evidence of FH and was used as one of the criteria in the previous version. Usually, LDL receptor activity is determined by the binding of fluorescent-labeled LDL to lymphocytes. The procedure of measuring LDLR activity is cumbersome and it is difficult to measure in routine clinical settings. Further, few companies can measure LDLR activity. Indeed, the specialists involved in this study measured LDLR activity only in 7 of 419 patients of FH, showing the sensitivity of the previous criteria as 60.9%. Therefore, in order to determine criteria sensitive enough to give a diagnosis of FH, the third item was changed from LDLR activity to family history.

There are some limitations in the present study. First, the patients analyzed in this study may have different characteristics from those followed by primary care physicians, because the physicians in this study are taking care of many FH patients and information about family history can be obtained more easily than by primary care physicians. Second, it is sometimes difficult for primary care physicians to take a complete family history, especially FH, and to diagnose ATT and/or the presence of CX, about which information can be missed in the primary care setting. Third, FH has been reported to have mutations in LDL receptor, PCSK9 and apolipoprotein B. Because mutations in PCSK9 may cause milder forms of FH, the sensitivity of the criteria may be reduced in these patients. Further study is required to address the applicability of the criteria for the primary care setting.

In conclusion, we have determined the cutoff of LDL-C for the diagnosis of FH by a multicenter study and proposed conventional diagnostic criteria by using high LDL-C, ATT and/or the presence of CX, and a family history of FH and/or CAD for primary care settings.

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## Disclosures

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# Comparison of Various Lipid Variables as Predictors of Coronary Heart Disease in Japanese Men and Women With Type 2 Diabetes

## Subanalysis of the Japan Diabetes Complications Study

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**OBJECTIVE**—To determine the best lipid variable to predict coronary heart disease (CHD) in Japanese patients with type 2 diabetes.

**RESEARCH DESIGN AND METHODS**—Eligible Japanese men and women (1,771) aged 40–70 years with type 2 diabetes from 59 institutes nationwide were followed for a planned 8-year period. The performance of eight conventional lipid variables, i.e., total cholesterol (TC), LDL-cholesterol (LDLC), HDL-cholesterol (HDLC), triglycerides (TGs), non-HDLC, TC/HDLC ratio, LDLC/HDLC ratio, and TG/HDLC ratio, as predictors of incident CHD were evaluated by four methods: hazard ratio (HR) per one SD increment by multivariate Cox analysis, $\chi^2$ likelihood ratio test, area under the receiver operating characteristic curve (AUC), and tertile analysis.

**RESULTS**—Although all variables significantly predicted CHD events in men, non-HDLC (HR per one SD 1.78 [95% CI 1.43–2.21]; AUC 0.726) and TC/HDLC (HR 1.63 [1.36–1.95]; AUC 0.718) had the better predictive performances among the variables, including LDLC. In women, TGs (log-transformed; HR 1.72 [1.21–2.43]; AUC 0.708) were the best predictor according to results of tertile analysis (HR of the top tertile versus the bottom tertile 4.31 [1.53–12.16]). The associations with incident CHD were linear and continuous.

**CONCLUSIONS**—For Japanese diabetic men, non-HDLC and TC/HDLC were the best predictors, whereas TGs were most predictive for women. These findings, which included prominent sex differences, should be considered among clinical approaches to risk reduction among East Asians with diabetes.

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Type 2 diabetes is characterized by an excessive incidence of coronary heart disease (CHD), and serum lipid values are among the strongest predictors of CHD (1,2). Although serum LDL-cholesterol (LDLC) has been conventionally used as a therapeutic marker and/or target in many guidelines based on trials using statins (1,2), characteristic features of diabetic dyslipidemia, which are closely associated with insulin resistance, are elevated levels of triglycerides (TGs) and small, dense LDLC (independent of LDLC level) as well as decreased levels of HDL-cholesterol (HDLC) (1,2). The use of LDLC alone for assessment of cardiovascular risk would ignore these TG-rich lipoproteins (TRLs, i.e., VLDL and intermediate-density lipoprotein) and low HDLC, all of which affect the risk of a CHD event independently of LDLC (1–4). Moreover, LDLC values, as estimated by the Friedewald formula, become progressively less accurate as the TG level increases.

Based on this background, it has been established that other lipid parameters, typically non-HDLC (determined by subtracting the HDLC concentration from the total cholesterol [TC] concentration in plasma) or apolipoprotein B (apoB), both of which reflect TRLs and small, dense LDLC, can be considered better predictors of CHD than LDLC and have been introduced into some guidelines as a secondary target for therapy (5–7). Furthermore, the ratios of TC to HDLC (TC/HDLC), which has clinical significance equivalent to non-HDLC/HDLC, LDLC to HDLC (LDLC/HDLC), and TGs to HDLC (TG/HDLC) are also used for assessing cardiovascular risk (3,4). It should be mentioned that non-HDL/HDL is always one unit lower than TC/HDLC.

Despite these considerations, these fundamental lipid measures (TC, HDLC, and TGs) and their calculated indices (LDLC, non-HDLC, TC/HDLC, LDLC/HDLC, and TG/HDLC) have not been completely and directly compared as predictors of CHD by

multiple analytical methods in past prospective studies in diabetic subjects (8–19). Results obtained have been inconsistent, and only one study (19) analyzed men and women separately. Therefore, whether LDLC performs better than the other indices or, if not, which variable is the best predictor of a CHD event has not been fully determined in diabetic subjects. Furthermore, all previous examinations of the performance of lipid variables as predictors of CHD in diabetic subjects (8–19) were performed in Western countries or in Caucasians. It is uncertain whether their results can be extrapolated to East Asian diabetic subjects, who have substantially different profiles regarding CHD and its risk factors, including a much lower incidence of CHD and degree of obesity (20–22).

In this analysis of data from a long-term follow-up of Japanese patients with type 2 diabetes, we compared eight conventional lipid variables, all of which are routinely measured or can be easily calculated in clinical care settings, as predictors of CHD events. To directly and quantitatively compare variables having different average values as well as variations in quantities and ratios, we used four different analytical methods to determine the best predictor of CHD. These were the multivariate-adjusted hazard ratio (HR) per one SD increment in the Cox hazard model, $\chi^2$ likelihood ratio test, area under the receiver operating characteristic (ROC) curve (AUC), and tertile analysis.

## RESEARCH DESIGN AND METHODS

### Recruitment of patients

The present analysis was conducted as part of the Japan Diabetes Complications Study, a multicenter prospective study of the incidence of and risk factors for macro- and microvascular complications among 2,033 Japanese patients with type 2 diabetes aged 40–70 years with $HbA_{1c}$ levels >6.5% who were registered from January 1995 to March 1996 from outpatient clinics in 59 university and general hospitals nationwide that specialize in diabetes care. For this analysis of macrovascular complications, of those 2,033 individuals, 940 men (mean age 57.8 ± 7.1 years) and 831 women (mean age 58.7 ± 6.8 years) were selected for the current study after consideration of the exclusion criteria prespecified in the study protocol (23). Excluded were patients with impaired glucose tolerance, a history of angina pectoris, myocardial infarction, stroke, peripheral artery disease, familial hypercholesterolemia, type III hyperlipidemia (diagnosed by broad β band on electrophoresis), nephrotic syndrome (urine protein >3.5 g per day and serum total protein <6.0 mg/dL), and serum creatinine levels >1.3 mg/dL (120 μmol/L). In the 8-year planned observation period, the median follow-up for the 1,771 patients was 7.86 years (final follow-up rate was 75%; 1,332/1,771 patients). The total person-years studied was 11,743 (6,106 for men and 5,637 for women). Diabetes and impaired glucose tolerance were diagnosed according to the Report of the Committee of the Japan Diabetes Society on the Classification and Diagnostic Criteria of Diabetes Mellitus, which is almost identical in terms of thresholds for glucose levels to those of the World Health Organization. The study protocol, which is in accordance with the Declaration of Helsinki and the Ethical Guidelines for Clinical/Epidemiological Studies of the Japanese Ministry of Health, Labor, and Welfare, received ethical approval from the institutional review boards of all participating institutes. All enrolled patients provided written informed consent.

### Clinical and laboratory measurements

Patients were assessed yearly after the baseline evaluation. Mean values of at least two measurements each year were obtained for $HbA_{1c}$, fasting plasma glucose, and fasting serum lipids. $HbA_{1c}$ assays were performed according to procedures outlined by the Laboratory Test Committee of the Japan Diabetes Society (JDS), which is known to be converted by the formula $HbA_{1c}$ (JDS)(%) = $HbA_{1c}$ (National Glycohemoglobin Standardization Program [NGSP])(%) − 0.4%. All other laboratory tests were performed at each participating institute. Serum LDLC was calculated using the Friedewald equation, except where TGs exceeded 400 mg/dL, in which case LDLC data were treated as "missing". This was applicable to 20 subjects. All other measurements, including those for body weight, blood pressure, and a 12-lead electrocardiogram, were performed at least once yearly. A baseline dietary survey, which was validated and is widely used in Japan (24) and was comprised of food records and a food frequency questionnaire that included alcohol consumption, was undertaken. Information on cigarette smoking was collected using a self-administered questionnaire. Smoking status was classified into one of three categories: current smokers, ex-smokers, and never smokers (25).

### Outcome measures

The outcomes analyzed were a fatal or first nonfatal manifestation of CHD comprised of angina pectoris and myocardial infarction, both of which were diagnosed according to criteria defined by the Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA; World Health Organization) project. A patient with a first percutaneous coronary intervention or coronary artery bypass graft was also counted as having a CHD event. Information regarding primary outcome and other clinical variables for each subject was collected through an annual report that included detailed findings at the time of the event from each participating diabetologist who was providing care to those patients. The adjudication of end points was performed by central committees comprised of experts who were masked to risk factor status and was based on additional data such as a detailed history, sequential changes in electrocardiogram and serum cardiac biomarkers, and results of coronary angiography. The rate of concordance in diagnosis between participating diabetologists and committee experts was 93%.

### Statistical analysis

All statistical analyses and data management were conducted at the central data center. Patient characteristics were described as mean ± SD, median and interquartile range, or percentage. We compared a CHD group with a no-CHD group by Student *t* test and Fisher exact test for numerical and categorical variables, respectively. Multivariate Cox regression analysis was used to calculate the adjusted HRs and 95% CIs for risk factors. The strength of associations of each lipid variable was assessed using the $\chi^2$ likelihood ratio test, and the corresponding *P* values were estimated from the regression coefficient based on the Cox proportional hazards model. In addition, the relationships between tertiles of each baseline lipid variable and HR for CHD risks were assessed by the Cox proportional hazards model using the first tertile of each variable as the reference group. The discriminatory powers for CHD of the lipid variables were also compared by ROC curve analysis with application of various thresholds to the predicted probability obtained from the logistic regression model. The AUC was calculated by integrating the area between the ROC curve and the diagonal line where sensitivity