Altered Metabolism of Low-Density Lipoprotein and Very-Low-Density Lipoprotein Remnant in Autosomal Recessive Hypercholesterolemia

Results From Stable Isotope Kinetic Study In Vivo

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**Background**—Autosomal recessive hypercholesterolemia (ARH) exhibits different responsiveness to statins compared with that in homozygous familial hypercholesterolemia (FH). However, few data exist regarding lipoprotein metabolism of ARH. Therefore, we aimed to clarify lipoprotein metabolism, especially the remnant lipoprotein fractions of ARH before and after statin therapy.

**Methods and Results**—We performed a lipoprotein kinetic study in an ARH patient and 7 normal control subjects, using stable isotope methodology (10 mg/kg of \([\text{H}_3]\)-leucine). These studies were performed at baseline and after the 20 mg daily dose of atorvastatin. Tracer/tracee ratio of apolipoprotein B (apoB) was determined by gas chromatography/mass spectrometry and fractional catabolic rates (FCR) were determined by multicomartmental modeling, including remnant lipoprotein fractions. FCR of low-density lipoprotein (LDL) apoB of ARH was significantly lower than those of control subjects (0.109 versus 0.450±0.122 1/day). In contrast, the direct removal of very-low-density lipoprotein remnant was significantly greater in ARH than those in control subjects (47.5 versus 2±2%). Interestingly, FCR of LDL apoB in ARH dramatically increased to 0.464 1/day, accompanying reduction of LDL cholesterol levels from 8.63 to 4.22 mmol/L, after treatment with atorvastatin of 20 mg/d for 3 months.

**Conclusions**—These results demonstrate that ARH exhibits decreased LDL clearance associated with decreased FCR of LDL apoB and increased clearance for very-low-density lipoprotein remnant. We suggest that increased clearance of remnant lipoprotein fractions could contribute to the great responsiveness to statins, providing new insights into the lipoprotein metabolism of ARH and the novel pharmacological target for LDLRAP1. (Circ Cardiovasc Genet. 2012;5:35-41.)

**Key Words:** lipoproteins ■ ARH ■ genetics ■ metabolism ■ LDLRAP1

Familial hypercholesterolemia (FH) is a common inherited disorder of plasma lipoprotein metabolism, characterized by an elevated level of low-density lipoprotein cholesterol (LDL-C), tendon xanthomas, and premature coronary artery disease. Genetic causes of FH involve gene mutations such as LDL receptor (LDLR), apolipoprotein B-100 (apoB-100), and proprotein convertase subtilisin/kexin type 9 (PCSK9). In contrast, there was a report of autosomal recessive inherited cases, who showed elevation of LDL-C, large xanthomas, and premature coronary artery disease typical of homozygous FH but in whom the fibroblasts had normal LDLR function. Subsequently, Garcia et al showed that this disorder was caused by a recessive form of null mutations in the LDLR adaptor protein 1 (LDLRAP1).

**Clinical Perspective on p 41**

Since then, evidence has been accumulating that it was not linked to mutations in the LDLR gene. The N-terminal domain of LDLRAP1 contains a phosphotyrosine-binding (PTB) domain, which binds to the internalization sequence (FDNPVY) in the cytoplasmic tail of the LDLR. LDLRAP1 protein serves as an adaptor for LDLR endocytosis in the liver.

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

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. 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. 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. 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. 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 [2H3]-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. 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. 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). Changes in cholesterol, triglyceride, free cholesterol, and phospholipids in each lipoprotein subfraction was assessed by HPLC.
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). 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 C599) 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’-dioctadecyldimcarbocyanin (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/tracce ratio curves at baseline and after atorvastatin therapy, as well
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 ± 0.697 1/day for VLDL, 0.109 ± 0.067 1/day for LDL) compared with those of the control subjects (8.408 ± 2.697 1/day for VLDL, 3.153 ± 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.

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
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. 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. 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. In addition, Altenburg et al. 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. 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.

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 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
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.

References
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 novel method for determining functional LDL receptor activity in familial hypercholesterolemia: application of the CD3/CD28 assay in lymphocytes. 

**CLINICAL PERSPECTIVE**

Only about 50 cases of autosomal recessive hypercholesterolemia (ARH) have been described in the literature. This condition is due to mutations in the LDLRAP1 gene, which encodes an adaptor protein that interacts with the LDL receptor. ARH is characterized by less severe phenotype compared to homozygous familial hypercholesterolemia and greater responsiveness to statins. One mechanism for this greater responsiveness is the LDLRAP1-dependent modulation of VLDL metabolism, activating an alternate pathway that can remove VLDL remnant paradoxically. This pathway could potentially contribute to the greater responsiveness of ARH to statins.
Altered Metabolism of Low-Density Lipoprotein and Very-Low-Density Lipoprotein Remnant in Autosomal Recessive Hypercholesterolemia: Results From Stable Isotope Kinetic Study In Vivo

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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.

**SUPPLEMENTAL MATERIAL**

**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 110ºC 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.
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.
**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 methionine codon) was shown in the proband, with corresponding heterozygosity shown in his niece.

```
Exon 6

normal  c.606dup  c.606dup

1 2 3 4 5 6 7 8
CACCCCCCCC T T
CACC

3 2 1
```

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

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<td>52</td>
<td>normal</td>
<td>-</td>
<td>n.d.</td>
<td>4.58</td>
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</tr>
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<td>IV-10</td>
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<td>-</td>
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<td>-</td>
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<td>4.34</td>
<td>1.42</td>
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<td>2.03</td>
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<td>39</td>
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<td>-</td>
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<td>4.60</td>
<td>1.41</td>
<td>2.51</td>
<td>1.45</td>
</tr>
<tr>
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<td>38</td>
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<td>V-3</td>
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<td>2.46</td>
<td>1.79</td>
</tr>
</tbody>
</table>

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
**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.