**Development and Clinical Application of an Enzyme-Linked Immunosorbent Assay for Oxidized High-Density Lipoprotein**

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**Aims:** HDL particles have various anti-atherogenic functions, whereas HDL from atherosclerotic patients was demonstrated to be dysfunctional. One possible mechanism for the formation of dysfunctional HDL is the oxidation of its components. However, oxidized HDLs (Ox-HDLs) remain to be well investigated due to lack of reliable assay systems.

**Methods:** We have developed a novel sandwich enzyme-linked immunosorbent assay (ELISA) for Ox-HDL by using the FOH1a/DLH3 antibody, which can specifically recognize oxidized phosphatidylcholine, a major component of HDL phospholipid (HDL-PL). We defined forced oxidation of 1 mg/L HDL-PL as 1 U/L Ox-HDL. We assessed serum Ox-HDL levels of normolipidemic healthy subjects (n=94) and dyslipidemic patients (n=177).

**Results:** The coefficients of variation of within-run and between-run assays were 12.5% and 13.5%. In healthy subjects, serum Ox-HDL levels were 28.5±5.0 (mean±SD) U/L. As Ox-HDL levels were moderately correlated with HDL-PL (r=0.59), we also evaluated the Ox-HDL/HDL-PL ratio, which represents the proportion of oxidized phospholipids in HDL particles. In dyslipidemic patients, Ox-HDL levels were highly variable and ranged from 7.2 to 62.1 U/L, and were extremely high (50.4±13.3 U/L) especially in patients with hyperalphalipoproteinemia due to cholesteryl ester transfer protein deficiency. Regarding patients with familial hypercholesterolemia, those treated with probucol, which is a potent anti-oxidative and anti-hyperlipidemic drug, showed significantly lower Ox-HDL (16.2±5.8 vs. 30.2±5.4, p<0.001) and Ox-HDL/HDL-PL ratios (0.200±0.035 vs. 0.229±0.031, p=0.015) than those without probucol.

**Conclusion:** We have established a novel sandwich ELISA for Ox-HDL, which might be a useful and easy strategy to evaluate HDL functionality, although the comparison study between this Ox-HDL ELISA and the assay of HDL cholesterol efflux capacity remains to be done. Our results indicated that probucol treatment may be associated with lower Ox-HDL levels.

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**Key words:** Apolipoproteins, HDL, Oxidized lipids, Phospholipids, Familial hypercholesterolemia

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**Introduction**

Epidemiological studies have shown that serum HDL cholesterol (HDL-C) levels are negatively correlated with the incidence of atherosclerotic cardiovascular diseases (ASCVD)¹¹. However, HDL-C raising...
therapy such as niacin or cholesteryl ester transfer protein (CETP) inhibitors have failed in clinical trials\(^2,3\). HDL particles have various kinds of anti-atherogenic functions. The major functions of HDL are the efflux of cholesterol from lipid-laden macrophages and its transport to the liver, a process called “reverse cholesterol transport.” Cholesterol efflux capacity was demonstrated to be inversely associated with the prevalence and incidence of ASCVD\(^4,5\). HDL also facilitates a variety of atheroprotective functions such as anti-oxidative, anti-inflammatory, and anti-apoptotic functions, among others\(^6\). Conversely, under particular circumstances, HDL loses its atheroprotective properties, resulting in the formation of dysfunctional or even proinflammatory HDL\(^6,7\). Evaluation of HDL dysfunctionality could lead to better understanding and new diagnostic approach to atherosclerosis in clinical practice.

Recent proteomic studies have elucidated that HDL has more than approximately 90 HDL-associated proteins, which may exert these anti-atherogenic functions\(^8\). The function of HDL from ASCVD patients is impaired, and the proteome of which is altered when compared to that of healthy subjects\(^9\), indicating that these proteomic changes might attribute to HDL dysfunctionality. Another possible mechanism for the formation of dysfunctional HDL is the oxidation of the HDL component. ApolipoproteinA-I (apoA-I), the main protein of HDL, is easily oxidized, and oxidized apoA-I demonstrated a proinflammatory activity on endothelial cells\(^10\). Regarding HDL lipids, cholesterol is the most characteristic component. However, it is actually phospholipids (PLs) that quantitatively predominate in HDL lipidome, accounting for approximately 50% of total lipids\(^11\). Importantly, HDL PLs can be oxidized in vivo with the formation of biologically active oxidized compounds\(^12,13\).

Although the oxidation of HDL PLs is vitally important for HDL function, these oxidized HDLs (Ox-HDLs) remain to be investigated due to the lack of a standard and reliable assay system. Therefore, in the present study, we have attempted to establish a novel sandwich ELISA for detecting Ox-HDL using FOH1a/DLH3 antibody, the epitope of which resides in oxidized products of phosphatidylcholine (PC)\(^14\). Using this ELISA for Ox-HDL, we have evaluated serum Ox-HDL levels in normolipidemic healthy subjects and dyslipidemic patients.

### Materials and Methods

#### Materials and Chemicals

Reagents were obtained from the following sources: 3,3’,5,5’-tetramethylbenzidine (TMBZ) solution (TMBBlue, TSI Co., Milford, USA); anti-human apo A-I mouse IgG (Abnova Corp, Taipei, Taiwan); polyethylene glycol 6000 (Wako Chemicals, Osaka, Japan); sodium hyaluronate (Acros Organics, New Jersey, USA); Tween20 (Sigma-Aldrich Co. LLC., St Louis, USA); and Bioace (K.I Chemical Industry Co., Ltd., Shizuoka, Japan). All other reagents were of analytical grade.

#### Preparation and Modification of Lipoproteins

LDL and HDL were separated from human serum by sodium bromide stepwise density gradient ultracentrifugation\(^15\). After ultracentrifugation, the fractions with a density of 1.006–1.063 g/L were pooled as LDL, and the fractions with a density of 1.063–1.21 g/L were pooled as HDL. Each fraction was desalted using an ultrafiltration membrane (Amicon Ultra-15 Ultracel-50k, Merck Millipore, Darmstadt, Germany).

#### Establishment of the Sandwich ELISA for Ox-HDL

The buffers used regularly were as follows: coating buffer: 50 mmol/L tris (hydroxymethyl) aminomethane (Tris)/HCl buffer (pH 8.0) containing 500 mmol/L NaCl and 0.01% (w/v) sodium hyaluronate; blocking buffer: 50 mmol/L Tris/HCl buffer (pH 8.0) containing 100 mmol/L NaCl, 5% (w/v) trehalose, 1% (w/v) BSA, and 0.01% (w/v) sodium hyaluronate; sample storage buffer: 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl, 1% (w/v) BSA, 0.1% (w/v) and 40% (w/v) glucose; reaction buffer: 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl, 1% (w/v) BSA, 0.1% (w/v) Bioace, 4.8% (w/v) polyethylene glycol 6000, and 0.01% (w/v) Tween 20; washing buffer: 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl and 0.05% (w/v) Tween 20; antibody dilution buffer: 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl, 1% (w/v) BSÅ, 0.1% (w/v) Bioace, and 0.05% (w/v) Tween 20.

Monoclonal antibodies were prepared according to the method described previously\(^16\). A hybridoma cell line, which produced FOH1a/DLH3 mouse MAb, was acclimatized for culture in a serum-free culture medium, ASF-104 (Ajinomoto Co. Inc., Tokyo, Japan), and the MAb were purified from the serum-free culture supernatant by hydrophobic chromatography and gel filtration chromatography. The acclimatized cells were cultured in the serum-free culture medium to confluence, and the culture supernatant was separated from the insoluble matter (cells and other particulates) by centrifugation at 3000 rpm for 10 min.
10 min, and was then concentrated to 1/10 with a diaflow membrane. Ammonium sulfate was added to the concentrated supernatant to 1.5 mol/L, and the mixture was applied to a phenylsepharose column equilibrated with 50 mmol/L Tris/HCl buffer (pH 8.0) containing 1.5 mol/L ammonium sulfate and eluted with a 1.5–0.0 mol/L ammonium sulfate linear gradient. The fractions containing IgM were collected, concentrated, and applied to a Sephacryl S-300 (Pharmacia) column (2.5 cm × 3100 cm) equilibrated with 10 mmol/L phosphate buffer (pH 7.4) containing 0.5 mol/L NaCl. The fractions containing IgM were collected, and the antibody was confirmed to be monomeric (not containing aggregated IgM) and homogenous by HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively.

Polystyrene 96 well microtiter plates (Maxisorp Immunoplates. NUNC, Roskilde, Denmark) were sensitized by coating them with 100 µL/well of FOH1a/DLH3 antibody (30 µg/mL in coating buffer) for 24 h at room temperature. After aspirating the coating buffer, the plates were blocked with a blocking buffer for 3 h at room temperature. After aspirating the blocking buffer, the blocked plates were allowed to dry in a vacuum desiccator and were sealed in an aluminum-coated pack with a drying agent (Hi-sheet dry, Marutani Chemical Plant & Engineering Co., Ltd., Tokyo, Japan) and stored 4°C until use. Under these storage conditions, the sensitized plates were stable for at least 1 year. Anti-apoA-I mouse IgG-HRP conjugate was prepared using Peroxidase Labeling Kit-NH2 (Dojindo Laboratories Co., Ltd., Kumamoto, Japan) and stored 4°C for 24 h at room temperature. After aspiration and washing buffer, the blocked plates were allowed to dry in a vacuum desiccator and were sealed in an aluminum-coated pack with a drying agent (Hi-sheet dry, Marutani Chemical Plant & Engineering Co., Ltd., Tokyo, Japan) and stored 4°C until use. Under these storage conditions, the sensitized plates were stable for at least 1 year. Anti-apoA-I mouse IgG-HRP conjugate was prepared using Peroxidase Labeling Kit-NH2 (Dojindo Laboratories Co., Ltd., Kumamoto, Japan). The conjugate produced was diluted to 200 ng/mL with an antibody dilution buffer when used.

For the calibrator in the ELISA, HDL fraction was prepared from pooled sera freshly collected from normal subjects, as described above. To generate copper (Cu)-oxidized HDL, the HDL fraction was incubated with 10 µmol/L CuSO4 at 37°C for 16 h under air. The reaction was stopped by the addition of EDTA (final 0.25 mM). When used as the calibrator in the ELISA, the Cu-oxidized HDL was diluted with reaction buffer. We defined Ox-HDL obtained by forced oxidation of 1 mg/L HDL phospholipids (HDL-PL) as 1 U/L Ox-HDL. The concentration of HDL-PL in the Ox-HDL was determined by an enzymatic method with Determiner L PL Kit (Hitachi Chemical Diagnostics Systems Co., Ltd, Tokyo, Japan).

Before assay, calibrator and samples were diluted with reaction buffer as described above. These diluted samples and calibrator (100 µL) were added to each well of the FOH1a/DLH3-coated plate, and the plate was incubated for 2 h at 37°C. After washing the plate four times with washing buffer, HRP-labeled anti-human apoA-I mouse IgG conjugate (100 µL) was added to each well, and the plate was incubated for 1 h at 37°C. The usual antibodies against apoA-I can bind modified apoA-I of Ox-HDL as reported previously17, 18. After washing the plate four times with washing buffer, 100 µL of TMBlue solution was added to each well, and the plate was then incubated for 30 min at 37°C. To stop the enzyme reaction, 50 µL of 0.5 mol/L H2SO4 was added, and the absorbance at 450 nm was measured with an Emax plate reader (Molecular Devices, LLC., Sunnyvale, USA).

Specificity and Precision Studies
Specificity was checked using Cu-oxidized and non-oxidized lipoprotein fractions. Each LDL and HDL fraction was prepared from pooled serum freshly collected from normal subjects, as described above. Cu-oxidized lipoprotein fraction was prepared by incubation with 10 µmol/L CuSO4, at 37°C for 16 h. The reaction was stopped by the addition of EDTA (final 0.25 mM). Each fraction diluted 1/600–1/4000 with reaction buffer when assayed.

Within-run (n=20) and between-run (n=5 or n=10) imprecisions were evaluated with a quality control (QC) and some human serum samples. Lyophilized pooled serum was used as a QC. Reconstituted QC by distilled water was further diluted 1/8000 with a dilution buffer for use. Serum samples were mixed with an equal amount of sample storage buffer and then stored at −80°C until use.

Interference Studies
Interference of unoxidized PC was examined by the addition of some purified phospholipid samples (Sigma-Aldrich Co. LLC., St Louis, USA) to aliquots of serum. Approximately 2–2000 µg/mL of unoxidized PC (natural or synthetic) or lypo-PC was added to Cu-oxidized HDL containing 2 µg/mL of oxidized PL, and these mixtures were added to each well of the FOH1a/DLH3-coated plate. The FOH1a/DLH3 antibody has an affinity for lyso-PC besides PC hydroperoxide and PC aldehyde ester, as reported previously19.

Subjects
We enrolled 94 normolipidemic healthy controls collected from Health and Counseling Center Osaka University and 177 dyslipidemic patients, including subjects with familial hypercholesterolemia (FH) and CETP deficiency diagnosed without genetic testing, consecutively attending to Osaka University Hospital Lipid Clinic, respectively. The inclusion criteria for normolipidemic healthy controls were 1) HDL-C
Results

Specificity, Imprecision, and Interferences of the Ox-HDL ELISA

This sandwich ELISA for serum Ox-HDL was conducted with a 96-well microtiter plate as the solid phase, with FOH1a/DLH3 antibody as the capture antibody, anti-human Apo-A-I mouse IgG-HRP conjugate as the second enzyme-labeled antibody, and TMBZ solution as the enzyme substrate (Fig. 1). The specificity of this assay to various native and modified forms of lipoproteins is shown in Fig. 2A. In this ELISA, Cu-oxidized HDL was specifically detected and neither Cu-oxidized LDL nor native LDL was detected. Native HDL was also recognized but the signal was low, suggesting that a small amount of Ox-HDL contained in native HDL may respond.

Imprecision was evaluated as the coefficient of variation (CV). Within-run and between-run variations were examined using a QC and some healthy serum samples. The within-run imprecision exhibited CVs of 12.5% in the QC and 13.9% in the healthy serum sample \( (n = 10) \). The between-run imprecision exhibited CVs of 13.5% in the QC \( (n = 10) \) and 5.6% and 7.8% in the healthy serum samples \( (n = 5) \) (Table 1). Therefore, we considered that this ELISA system would be reliable from the standpoint of imprecision.

Next, we evaluated the interference of unoxidized lipoproteins ranging from 40 to 90 mg/dL; 2) LDL-C less than 140 mg/dL; 3) TG less than 150 mg/dL; and 4) no medical history including dyslipidemia, diabetes mellitus, and hypertension. The investigation conformed to the principles outlined in the Declaration of Helsinki. All patients provided written informed consent, and the Ethics Committee of Osaka University Hospital approved the research protocol. Venous blood was drawn after overnight fasting for 12 h. Serum was separated by low-speed centrifugation (3000 rpm, 15 min, 4°C). Serum samples thus obtained were immediately mixed with an equal amount of sample storage buffer and stored at \(-80°C\) until use. When used in the analysis, these samples were diluted 1/4000 with reaction buffer. Under these storage conditions, Ox-HDL in these samples was stable for at least 6 months.

Statistical Analysis

We conducted a statistical analysis with the Graphpad Prism Ver.7.01 software program (GraphPad Software, San Diego, CA). Results are expressed as mean \( \pm \) SD. Spearman correlation coefficient analysis was used to evaluate associations between measured parameters. Two-group comparison was performed with the Student \( t \)-test or Mann–Whitney \( U \) test, as appropriate. Statistical significance was established at a \( p \) value of \( < 0.05 \).
Fig. 2.

A: Reactivity of the sandwich ELISA of Ox-HDL to various modified lipoproteins. Native lipoproteins (HDL and LDL) and Cu-oxidized lipoproteins (Ox-HDL and Ox-LDL) (about 100 mg/dL as cholesterol) were diluted 1/600~1/4800 with reaction buffer.

B: Interference of unoxidized phosphatidylcholine (PC). To Cu-oxidized HDL sample (diluted with reaction buffer to 2 µg/mL as HDL-PL), 2~2000 µg/mL of unoxidized PC (natural or synthetic) or lyso-PC were added, and these mixtures were added to each well of the FOH1a/DLH3-coated plate.

Table 1. Within- and between-run imprecision

|                  | Measured value (U/mL) | SD (U/mL) | CV (%) | n  |
|------------------|-----------------------|-----------|--------|----|
| Within-run imprecision |                       |           |        |    |
| QC 1 human serum | 38.3                  | 4.8       | 12.5   | 20 |
| Between-run imprecision |                   |           |        |    |
| QC 2 human serum 1| 26.3                  | 3.6       | 13.5   | 10 |
| QC 2 human serum 2| 30.1                  | 3.6       | 13.5   | 10 |
|                  |                       |           |        |    |
| Within-run imprecision were evaluated with a quality control (QC) sample and a human serum (n=20). Between-run imprecision were evaluated with a QC and 2 human serum (n=5). SD, standard deviation; CV, coefficient of variation. |
In the present study, we have developed a novel sandwich ELISA for Ox-HDL using the FOH1a/DLH3 antibody. This antibody was previously reported to recognize oxidized products of PC but not of other lipid species. We also performed inhibition assays with various types of PLs, showing that PC by inhibition assay. The assay with various types of PLs revealed that lysophosphatidylcholine (lyso-PC) showed concentration-dependent inhibition, but no inhibition by unoxidized PC was observed even in the condition of excess concentration (Fig. 2B). These results strongly suggested that oxidized PLs in HDL are specifically recognized in this ELISA.

**Healthy Reference Value of Serum Ox-HDL Levels**

The normolipidemic healthy subjects (n = 94) characteristics are shown in Table 2. The mean age was 39.9 years old, and 44 (44.5%) of healthy subjects were men. The correlation of serum Ox-HDL levels and lipid parameters in healthy subjects are shown in Fig. 3. Serum Ox-HDL levels were moderately correlated with HDL-C, HDL-PL, and apo A-I levels, and mildly correlated with HDL-TG levels (Fig. 3A-3D). Conversely, they were not correlated with LDL-C or TG levels (Fig. 3E-3F). As this Ox-HDL ELISA recognizes oxidized PC, a major component of HDL-PL, we also evaluated Ox-HDL/HDL-PL ratio to evaluate what proportion of PLs is oxidized in the HDL particles. There was no significant relationship between Ox-HDL/HDL-PL and lipid parameters including HDL-PL (Supplementary Fig. 1). Distribution of Ox-HDL and Ox-HDL/HDL-PL ratio in healthy subjects is shown in Fig. 4A. The mean Ox-HDL was 28.5 U/L, and the Ox-HDL/HDL-PL ratio was 0.232 U dL/mg L.

**Serum Ox-HDL Levels in Dyslipidemic Patients**

The characteristics of dyslipidemic patients (n = 177) in the present study are summarized in Table 3 and Table 4. The mean age was 63.8 years old, and 91 (51.4%) of patients were men. In terms of comorbidities, 40 (22.6%) patients were FH, and 15 (8.5%) patients were hyperalphalipoproteinemia (HALP) including five CETP-D patients. Compared with healthy subjects, Ox-HDL levels of dyslipidemic patients displayed quite a high variability that ranges from 7.2 to 62.1 U/L. Distribution of Ox-HDL and Ox-HDL/HDL-PL ratio in dyslipidemic patients was shown in Fig. 4B. The mean Ox-HDL level was 27.7 U/L, and the Ox-HDL/HDL-PL ratio was 0.220 U dL/mg L. These parameters were not correlated with both serum aspartic aminotransferase (AST) or alanine aminotransferase (ALT) levels and creatinine (Cre) levels (data not shown). We have compared the Ox-HDL levels or Ox-HDL/HDL-PL ratio in dyslipidemic patients treated with or without probucol and whose serum HDL-C levels were less than 40 mg/dL. The Ox-HDL levels and Ox-HDL/HDL-PL ratio in patients whose HDL-C was less than 40 mg/dL and who were treated with probucol (n = 26) were significantly lower than those in patients whose HDL-C was less than 40 mg/dL and who were not treated with probucol (n = 13) (Ox-HDL: 15.2 ± 4.1 vs. 22.2 ± 6.4 U/L, p < 0.001; Ox-HDL/HDL-PL: 0.194 ± 0.033 vs. 0.249 ± 0.100 U dL/mg L, p = 0.015).

Of these dyslipidemic patients, patients with CETP-D showed extremely high Ox-HDL levels (50.4 ± 13.3 U/L) (Fig. 5A and Table 4). Conversely, patients treated with probucol showed lower Ox-HDL and Ox-HDL/HDL-PL ratio (Fig. 5B and Table 4). Regarding FH, patients treated with probucol showed significantly lower Ox-HDL levels and Ox-HDL/HDL-PL ratio compared with those without probucol (16.2 ± 5.8 vs. 30.2 ± 5.4 U/L, p < 0.001, and 0.200 ± 0.035 vs. 0.229 ± 0.031 U dL/mg L, p = 0.015, respectively) (Fig. 5C-5D), indicating that probucol may prevent the formation of these Ox-HDLs in FH patients.

**Discussion**

In the present study, we have developed a novel sandwich ELISA for Ox-HDL using the FOH1a/DLH3 antibody. This antibody was previously reported to recognize oxidized products of PC but not of other lipid species. We also performed inhibition assays with various types of PLs, showing that this ELISA specifically recognized oxidized PLs in...
changes in the cohort of Omagari area, where marked HALP due to CETP-D are very frequent. Recent epidemiological studies also elucidated that the association between HDL-C and all-cause and cardiovascular mortality was U-shaped, with extremely high HDL-C levels being associated with high all-cause and cardiovascular mortality risk. Several possible mechanisms have been suggested regarding this. We have previously reported that large and CE-rich HDL2 in patients with CETP-D showed impaired cholesterol efflux capacity, which cannot prevent from

HDL particles. HDL phospholipid moiety can be easily oxidized in vivo with the formation of biochemically oxidized compounds, which leads to HDL dysfunctionality. Importantly, we have first established this sandwich ELISA, which can detect oxidized phospholipid but not oxidized apo A-I.

An important finding in the present study is that the Ox-HDL levels of HALP patients due to CETP-D were extremely high. We previously demonstrated that U-shaped relationship was observed between serum HDL-C and the incidence of ischemic ECG

Fig. 3. Scatter plots of Ox-HDL against A: HDL-C, B: HDL-PL, C: HDL-TG, D: ApoA1, E: LDL-C, and F: TG in normolipidemic healthy subjects (n=94).

Dashed lines represent a linear regression between Ox-HDL and lipid parameters. Correlation coefficients and P-values are also presented for each lipid parameter.
Fig. 4. Histogram of serum Ox-HDL levels and Ox-HDL/HDL-PL ratio in normolipidemic healthy subjects (n=94) and dyslipidemic patients (n=177).

A: Histogram of serum Ox-HDL levels and Ox-HDL/HDL-PL ratio in normolipidemic healthy subjects
B: Histogram of serum Ox-HDL levels and Ox-HDL/HDL-PL ratio in dyslipidemic patients

Table 3. Clinical characteristics of dyslipidemic patients

|                      | dyslipidemic patients | Mediations - no. (%) |
|----------------------|-----------------------|----------------------|
| Age (year)           | 63.8 ± 14.9           |                      |
| Number (male/female) | 177 (91/86)           | Statin 130 (73.4)    |
| BMI                  | 22.6 ± 3.3            | EPA 56 (31.6)        |
| waist circumference  | 81.8 ± 10.6           | Probucol 38 (21.5)   |
| Current smoker       | 12 (6.8)              | Fibrate 28 (15.8)    |
| Presenting history   |                      | Ezetimibe 50 (28.2)  |
| or diagnosis - no. (%)|                      |                      |
| Hypertension         | 63 (35.6)             |                      |
| Diabetes Mellitus    | 21 (11.9)             |                      |
| Familial Hypercholesterolemia | 40 (22.6) |                      |
| Coronary artery disease | 35 (19.8)      |                      |
| Hyperalphacholesterolemia | 15 (8.5)     |                      |
| CETP deficiency      | 5 (2.8)               |                      |

BMI, body mass index; CETP deficiency, cholesteryl ester transfer deficiency; EPA, eicosapentaenoic acid; Data are shown as the mean ± SD.
Lipid profile of dyslipidemic patients was decreased in CETP-D28). Additionally, our shot-gun proteomic analysis demonstrated that several atherogenic proteins such as apo C-III, complement C3, and ANGPTL3 were increased in HDL of CETP-D29). Besides these, we have demonstrated increased Ox-HDL levels in CETP-D in the present study, which might be a new mechanism for the atherogenicity of HALP due to CETP-D.

Another important finding is that patients treated with probucol showed lower Ox-HDL levels than those without probucol. Furthermore, this probucol effect was also seen in FH patients in the present study. FH patients are at very high risk for premature ASCVD, even treated with LDL-C lowering drugs such as statins or ezetimibe. In terms of risk stratification in FH, HDL functionality may be important beyond LDL. Actually, a recent study has shown that the cholesterol efflux capacity of HDL is independently and inversely associated with the presence of ASCVD in FH patients30). Conversely, we focused on Ox-HDL of FH patients treated with and without probucol in the present study. We previously reported that probucol treatment decreased the Achilles tendon thickness of FH patients31). We also reported that long-term treatment with probucol prevents secondary cardiovascular events in a cohort study of FH patients in Japan32). Furthermore, recent studies have shown that probucol treatment also improved the outcomes in patients with a history of coronary revascularization33) or ischemic stroke34). This Ox-HDL ELISA assay might be useful for patient selection of probucol treatment at high risk including FH. We have compared the Ox-HDL levels or Ox-HDL/HDL-PL ratio in dyslipidemic patients treated with or without probucol, and whose serum HDL-C levels were less than 40 mg/dL. As both these patients are assumed to be enriched with HDL3 particles, we supposed that lower Ox-HDL levels and Ox-HDL/HDL-PL ratio in patients treated with probucol are not due to the shift of HDL size but due to the anti-oxidant effect of probucol.

There are several limitations in this study. First, as this is a cross-sectional study, we cannot conclude that Ox-HDL levels are associated with the incidence of ASCVD. Further prospective studies are warranted. Second, data on cholesterol efflux capacity were not

| Table 4. Lipid profile of dyslipidemic patients |
|-----------------------------------------------|
|                                                |
| all dyslipidemic patients | patients treated with probucol | patients with CETP deficiency |
|--------------------------|-------------------------------|-------------------------------|
| Age (year)               | 63.8 ± 14.9                  | 67.3 ± 12.4                   | 64.8 ± 12.0                   |
| Number (male/female)     | 177 (91/86)                  | 39 (25/14)                    | 5 (3/2)                       |
| BMI                      | 22.6 ± 3.3                   | 22.4 ± 3.3                    | 21.8 ± 4.2                    |
| waist circumference (cm) | 81.8 ± 10.6                  | 81.8 ± 10.0                   | 78.9 ± 16.4                   |
| Current smoker - no. (%) | 12 (6.8)                     | 2 (5.1)                       | 0 (0)                         |
| Lipid Profile            |                               |                               |                               |
| TC (mg/dL)               | 192.6 ± 7.1                  | 159.6 ± 30.6                  | 362. ± 185.6                  |
| TG (mg/dL)               | 120.9 ± 47.0                 | 86.3 ± 69.3                   | 123.3 ± 96.8                  |
| HDL-C (mg/dL)            | 58.1 ± 10.5                  | 36.6 ± 13.1                   | 230.6 ± 86.8                  |
| LDL-C (mg/dL)            | 111.7 ± 6.8                  | 103.8 ± 28.6                  | 111.7 ± 54.9                  |
| apo A-I (mg/dL)          | 143.3 ± 23.8                 | 106.0 ± 29.8                  | 262.1 ± 77.7                  |
| HDL-PL (mg/dL)           | 120.9 ± 7.3                  | 89.0 ± 24.0                   | 306.6 ± 86.8                  |
| Ox-HDL (U/L)             | 27.1 ± 6.7                   | 18.7 ± 6.6                    | 50.4 ± 13.3                   |
| Ox-HDL/HDL-PL (U dL/mg L)| 0.228 ± 0.054                | 0.209 ± 0.041                 | 0.166 ± 0.023                 |

BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; apo A-I, apolipoprotein A-I; HDL-PL, high-density lipoprotein phospholipid; Ox-HDL, oxidized high-density lipoprotein

Data are shown as the mean ± SD.
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research.

available in this study. Third, we could not evaluate
the Ox-HDL levels of dyslipidemic patients not
treated with anti-lipidemic drugs. To evaluate the
impacts on Ox-HDL levels of these drugs, serial time
course assessment of Ox-HDL may also be required.

In conclusion, we have established a novel sand-
wich ELISA for Ox-HDL, which elucidated that Ox-
HDL levels are substantially high in CETP-D
patients. FH patients treated with probucol showed
lower Ox-HDL and Ox-HDL/HDL-PL ratio than
those without probucol, indicating that probucol may
prevent the formation of these Ox-HDLs.
Conflict of Interest

D.M. declares honoraria from Hitachi Chemical Diagnostics Systems Co., Ltd. and Daiichi-Sankyo Company, Ltd.; and grants or funds from Otsuka Pharmaceutical Co., Ltd. and Daiichi-Sankyo Company, Ltd. Y.S. declares honoraria from Otsuka Pharmaceutical Co., Ltd. and Daiichi-Sankyo Company, Ltd.; and grants or funds from Otsuka Pharmaceutical Co., Ltd. and Daiichi-Sankyo Company, Ltd. S.Y. declares grants or funds from Hitachi Chemical Diagnostics Systems Co., Ltd.

The other authors declare they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Supplementary Fig. 1. Scatter plots of Ox-HDL/HDL-PL ratio against A: HDL-C, B: HDL-PL, C: HDL-TG, D: ApoA1, E: LDL-C, and F: TG in normolipidemic healthy subjects (n=94)