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Review

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Innovatively Established Analysis Method for Lipoprotein Profiles Based on High-Performance Anion-Exchange Liquid Chromatography

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Separation analysis of lipoprotein classes have various methods, including ultracentrifugation, electrophoresis, and gel permeation chromatography (GPC). All major lipoprotein classes can be separated via ultracentrifugation, but performing the analysis takes a long time. Low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and very low-density lipoprotein (VLDL) in patient samples cannot be sufficiently separated via electrophoresis or GPC. Thus, we established a new method [anion-exchange high-performance liquid chromatography (AEX-HPLC)] by using HPLC with an AEX column containing nonporous gel and an eluent containing chaotropic ions. AEX-HPLC can separate five lipoprotein fractions of high-density lipoprotein (HDL), LDL, IDL, VLDL, and others in human serum, which can be used in substitution for ultracentrifugation method. The method was also approved for clinical use in the public health-care insurance in Japan in 2014. Furthermore, we developed an additional method to measure cholesterol levels of the four leading lipoprotein fractions and two subsequent fractions (i.e., chylomicron and lipoprotein(a)). We evaluated the clinical usefulness of AEX-HPLC in patients with coronary heart disease (CHD), diabetes, and kidney disease and in healthy volunteers. Results indicate that the cholesterol levels in IDL and VLDL measured by AEX-HPLC may be useful risk markers of CHD or diabetes. Furthermore, we developed another new method for the determination of alpha-tocopherol (AT) in lipoprotein classes, and this method is composed of AEX-HPLC for the separation of lipoprotein classes and reverse-phase chromatography to separate AT in each lipoprotein class. The AT levels in LDL were significantly correlated with the lag time to copper ion-induced LDL oxidation, which is an index of oxidation resistance. The application of AEX-HPLC to measure various substances in lipoproteins will be clinically expected in the future.

Key words: **Lipoprotein, Anion-exchange chromatography, Alpha-tocopherol**

Introduction

The initially discovered lipoprotein was chylomicron. In 1924, Gage and Fish showed particles with approximately 1 μm diameter in blood taken from humans after a fatty meal, and they named such particles as chylomicrons[1]. Poulletier de la Salle, a French doctor and chemist, first identified solid-form cholesterol from gallstones in 1769, and the compound was named "cholesterine" by Dr. Michel Eugène Chevreul in 1815[2]. Cholesterol is present in lipoproteins, which are measured by a variety of methods. In 1946, Cohn *et al.* isolated a variety of proteins from human plasma and fractionated five major protein families using gradual changes in pH, ionic strength, and ethanol concentration[3]. Fractions III and IV contained lipids. In 1950, Oncley *et al.* isolated β-lipoprotein from fraction III via flotation at a density of 1.035 g/mL with ultracentrifuge[4], and high-density α-lipoprotein was found in fraction IV.

In 1963, Lees and Hatch separated four lipoprotein classes, namely, chylomicron, β-lipoprotein [low-density lipoprotein (LDL); density: 1.006–1.063 g/mL], pre-β-lipoprotein [very low-density lipoprotein (VLDL); density: <1.006 g/mL], and α-lipoprotein [high-density lipoprotein (HDL), density >1.063 g/mL], using paper electrophoresis[5]. In 1965, Fredrickson and Lees reported a system for phenotyping hyperlipoproteinemia with paper electrophoresis[6], and the classification was later adopted by the World

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Table 1. Types of hyperlipoproteinemia by Fredrickson classification

	Plasma	Mainly increased lipids	Increased lipoprotein
Type I	Creamy top layer	Triglyceride	Chylomicron
Type IIA	Clear	Cholesterol	β-lipoprotein (LDL)
Type IIB	Cloudy	Cholesterol, triglyceride	β-lipoprotein (LDL), pre-β-lipoprotein (VLDL)
Type III	Cloudy	Cholesterol, triglyceride	Intermediate-density lipoprotein (IDL), Floating β-lipoproteins (VLDL remnant, chylomicron remnant)
Type IV	Cloudy	Triglyceride	Pre-β-lipoprotein (VLDL)
Type V	Creamy top layer and cloudy bottom	Triglyceride	Chylomicron, pre-β-lipoprotein (VLDL)

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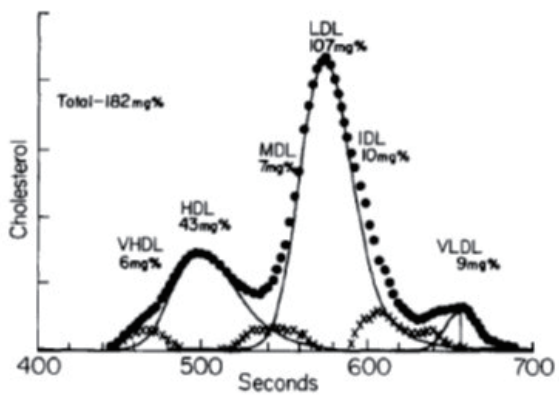

Fig. 1. Computer graphics of density gradient ultracentrifugation method for the quantification of cholesterol in lipoproteins with Gaussian distribution

This figure is referred to Reference #11.

Health Organization[7]. **Table 1** shows the six types of hyperlipoproteinemia in accordance with the Fredrickson classification[7].

In 1949, Gofman *et al.* reported a density gradient ultracentrifugation method for the analysis of lipoprotein classes[8] and showed that LDL was positively associated with cardiovascular disease (CVD)[9]. In 1981, Chung *et al.* developed a density gradient ultracentrifugation method with a vertical rotor[10], and the cholesterol levels in six lipoprotein classes, namely, very high-density lipoprotein (VHDL), HDL, medium-density lipoprotein, like lipoprotein(a) (Lp(a)) with intermediate-density between HDL and LDL, LDL, IDL, and VLDL, can be measured by assuming lipoprotein peaks as Gaussian distribution (**Fig. 1**)[11]. In 1955, Havel *et al.* established a sequential flotation ultracentrifugation and separated three major lipoprotein classes, namely, density <1.019 g/mL (VLDL and IDL), density of 1.019–1.063 g/mL (LDL), and density >1.063 (HDL), from 43 healthy human sera[12]. In 1960, Baxter, Goodman, and Havel isolated density <1.006 g/mL (VLDL) and density 1.006–1.019 g/mL (IDL) from the sera of 44 patients with nephrotic syndrome[13].

Epidemiologic studies play an important role in elucidating the risk factor of coronary heart disease (CHD). The Framingham Heart Study (FHS) was started in 1948 under the direction of the National Heart, Lung, and Blood Institute. In the town of Framingham, Massachusetts, 5,209 people (male/female: 45%/55%), who were aged 30–62 years and had not yet developed overt symptoms of CVD or suffered a heart attack or stroke, were recruited for an original cohort. In FHS, CHD risk factors included hypertension, hypercholesterolemia, and diabetes mellitus[14], and LDL cholesterol (LDL-C) was a predictive factor of the progression of CHD[15]. In 1977, Gordon et al. reported an inverse relationship between HDL cholesterol (HDL-C) and CHD incidence, in contrast to the positive association between LDL-C and CHD risk[16]. FHS group also reported that the increased VLDL cholesterol (VLDL-C), measured by an ultracentrifugation method, is a predictive factor of CHD independently of LDL-C[17].

The ultracentrifugation methods have a high ability for the separation of lipoprotein classes but takes a long time without convenience. At present, homogeneous methods are used for the measurement of lipoprotein classes in clinical practice, but they are only applied for the determination of HDL-C and LDL-C. Therefore, we sought to establish a convenient method for the separation of lipoprotein classes as a substitute for the ultracentrifugation method. We

have invented a new convenience method (anion-exchange high-performance liquid chromatography: AEX-HPLC) by using AEX chromatography with a column composed of nonporous gel and eluent containing chaotropic ions. We applied for a patent in the Japan Patent Office in 2002. We started to evaluate the clinical usefulness of AEX-HPLC with blood samples of patients with CHD, diabetes, and kidney disease and of healthy volunteers. In this review article, we show the principle of the new method to measure lipoprotein cholesterol concentrations using AEX-HPLC and the overview of several clinical study results reported so far.

Principle of Analysis Method for the Lipoprotein Classes by AEX-HPLC

A separation analysis of lipoprotein classes by GPC and HPLC was initiated in the 1960s. Foldin and Killander reported that human serum proteins were separated into three major peaks in accordance with the molecular size by using a dextran gel (Sephadex G-200), with the absorbance detection at 280 nm, and the first peak contained LDL[18]. Franzini carried out the separation of lipoproteins in human serum by Sephadex G-200 via cholesterol monitoring[19]. Two peaks of lipoprotein cholesterol were detected, and the first and second peaks were LDL and HDL, respectively. Sata *et al.* reported that lipoproteins in human plasma were separated by a column composed of 2% agarose gel (Bio-Gel A), and two VLDL peaks in addition to LDL and HDL were detected[20]. One VLDL peak was detected in the void volume of the gel. In 1980, Okazaki *et al.* reported an application of high-performance aqueous GPC where VLDL, LDL, HDL, and albumin were separated by a column of hydroxylated methacrylate gel (TSKgel G5000PW)[21], or the lipoproteins in serum were separated into chylomicron, VLDL, LDL, HDL2, and HDL3 by two columns of silica gel (TSKgel G4000SW + TSKgel G3000SW), and the cholesterol level in lipoproteins were measured by post-column reaction[22]. Then, they showed that cholesterol levels in VLDL, LDL, HDL2, and HDL3 measured by the two columns correlated well with those by ultracentrifugation method (correlation coefficient = 0.835–0.997)[23]. In 1990s, the separation method for three lipoprotein classes, namely, VLDL, LDL, and HDL, with a column of agarose gel (Superose 6B), and post-column derivatization of an enzymatic cholesterol reagent was reported[24, 25]. In 2002, Usui *et al.* showed the chromatograms of human serum lipoproteins detected by columns of TSKgel LipopropakXL (hydroxylated methacrylate gel) or Superose 6HR (agarose gel) with post-column dual enzymatic reactions for cholesterol and triglyceride (TG)[26]. In the chromatogram of TSKgel LipopropakXL and Superose 6HR, four lipoprotein peaks (chylomicron, VLDL, LDL, and HDL) and three lipoprotein peaks (chylomicron + VLDL, LDL, and HDL) appeared, respectively. The chylomicron peak in the chromatogram of TSKgel LipopropakXL and the chylomicron + VLDL peak in the chromatogram of Superose 6HR seemed to be eluted in the void volume of each column. In 2005, Okazaki et al. reported a method for the measurement of cholesterol levels in four major lipoproteins and the subclasses by using TSKgel LipopropakXL and Gaussian curve fitting for resolving the overlapping peaks (**Fig. 2**)[27].

The improvement of the separation of lipoproteins with GPC has been required to make large-sized exclusion limit of the gel for separating all lipoproteins and increase the column size for separating lipoproteins, such as IDL, which has difficulty in separation. However, increasing size exclusion limit weakens the gel strength, and a large-sized column extends analysis time. Therefore, we started a study of a new separation method for lipoprotein classes by using AEX-HPLC because lipoproteins are negatively charged in neutral pH. The diameter of LDL is smaller than that of VLDL, and the eluted time of LDL is later than that of VLDL in GPC. The charge of VLDL is more negative than that of LDL, and the eluted time of VLDL may be later than that of LDL in AEX-HPLC with the eluent of neutral pH. We decided to use the AEX column composed of the nonporous gel. We also thought that the hydrophobic interaction between the column gel surface and lipoproteins may be a cause for the decreased separation ability, and then we decided to use eluent with sodium perchlorate. Chaotropic ions, such as perchlorate and thiocyanate, are known to disrupt and decrease hydrophobic band[28].

First, we tried the separation of lipoprotein classes in human serum by AEX-HPLC with a linear gradient and identified the peaks in the chromatogram by analyzing the lipoprotein samples separated by a sequential flotation ultracentrifugation method[29]. We found out two HDL peaks, a broad LDL peak, an IDL peak, and a broad VLDL peak (**Fig. 3A**). Second, we tried the separation of four major lipoprotein classes with a step gradient, and the chromatogram represented four sharp peaks of HDL, LDL, IDL, and VLDL (**Fig. 3B**)[30]. These linear and step gradient methods are similar to density gradient and sequential flotation methods with ultracentrifugation, respectively. The last peak contained chylomicrons, including chylomicron remnant, and Lp(a). **Fig. 4** shows the chromatogram of serum from an untreated patient with type III dyslipidemia[31]. The level of IDL-C was

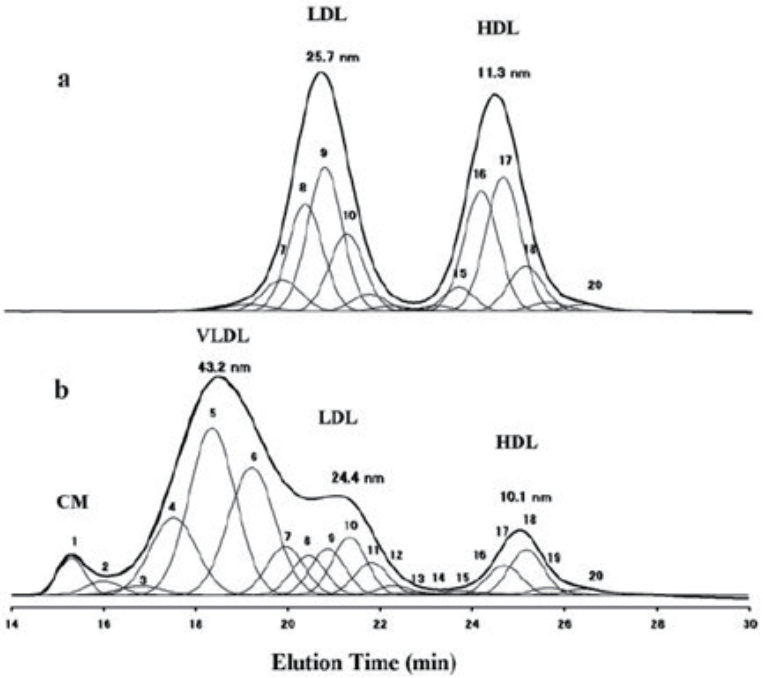

Fig. 2. Chromatograms of a healthy woman (a) and a patient with lipoprotein lipase deficiency (b) with TSKgel LipopropakXL and Gaussian curve fitting

Peaks 1 and 2, peaks 3–7, peaks 8–13, and peaks 14–20 are chylomicrons, VLDL, LDL, and HDL, respectively.
This figure is referred to Reference #27.

1.06 mmol/L, accounting for 18.4% of the total cholesterol. The levels of healthy subjects were 0.38 ± 0.11 mmol/L, accounting for 7.5 ± 1.9% of the total cholesterol peaks. We tried the further separation of chylomicrons and Lp(a) and established the separation method for HDL, LDL, IDL, VLDL, chylomicrons, and Lp(a) with a step gradient of sodium perchlorate in 26 min for the assay of one sample (**Fig. 3C**)[32]. In addition, a separation method for subfractions of HDL and LDL was established (**Fig. 3D**)[33]. The faster- and slower-eluting HDL fractions contained HDL3 and HDL2, respectively. The faster-eluting LDL fraction was changed into the slower-eluting LDL fraction by oxidation with copper ion. Therefore, we thought that a major component of slower-eluting LDL fraction was circulating oxidized LDL. The separation method for the four major lipoprotein classes (**Fig. 3B**) was processed in 20 min for the assay of one sample. We improved the separation method with the downsized column, and consequently the measurement time was shortened to 5.2 min/test[34]. In Japan, the diagnostic system with the improved separation method has been recently approved for clinical use in the public health-care insurance.

CVD

In FHS, a risk sore was established to estimate a 10-year individual risk of developing CHD[35]. The FHS risk score was calculated by data on gender, age, blood pressure, diabetes, smoking, LDL-C or total cholesterol, and HDL-C. We compared lipoprotein profiles measured by AEX-HPLC to FHS risk scores in 487 Japanese men, enrolled from subjects who underwent medical check-ups, and patients with drug therapy for hypertension, diabetes, and dyslipidemia also were included[36]. IDL-C was positively correlated with FHS risk score in multiple stepwise regression analysis ($p < 0.0005$). After FHS began, various epidemiological studies in many places were performed. The Hisayama Study started in Hisayama, a country town in Fukuoka, Japan, in 1961. Hisayama risk score was established to estimate a 10-year individual risk of developing CVD (stroke and CHD) and was calculated by data of gender, age, blood pressure, smoking, LDL-C, and HDL-C[37]. The Suita Study started in Suita, an urban town in Osaka, Japan, in 1989. The Suita score was established to estimate a 10-year individual risk of developing CHD and was calculated by data of gender, age, blood pressure, diabetes, smoking, LDL-C, HDL-C, and the stage classification of

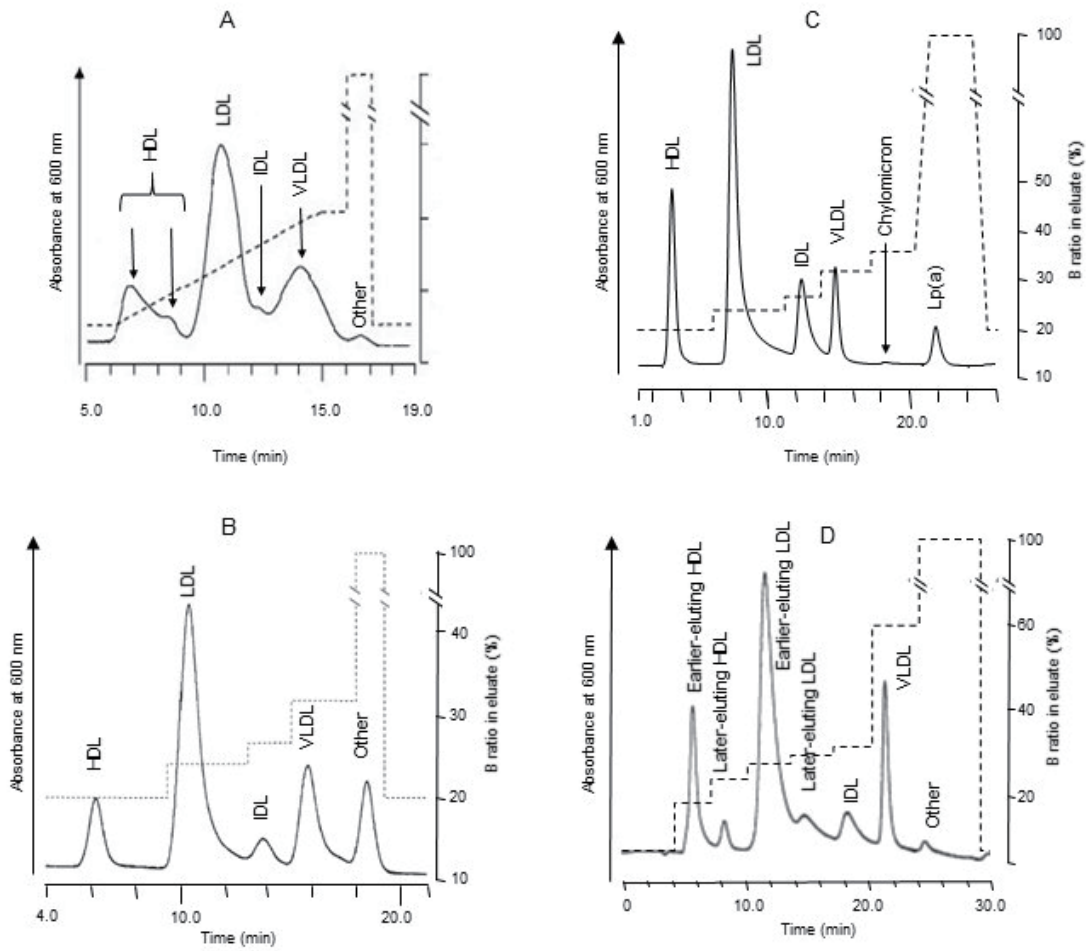

Fig. 3. Chromatograms and gradient patterns

The patterns of lipoprotein profile and gradient are indicated by a solid line and dotted line, respectively. The two solutions are used for separation of lipoproteins, eluent A (50 mM Tris-HCl + 1 mM ethylenediaminetetraacetic acid, disodium salt, dihydrate, pH 7.5) and eluent B (50 mM Tris-HCl + 500 mM sodium perchlorate + 1 mM ethylenediaminetetraacetic acid, disodium salt, dihydrate, pH 7.5). Eluents A and B were mixed on-line, and they were flowed into the column. The separated lipoproteins are detected by a post-column reaction with enzymatic cholesterol reagent. A: Chromatogram of four major lipoprotein classes in human serum with a linear gradient. The data of a serum are as follows: total cholesterol (TC) = 7.24 mmol/L, triglyceride (TG) = 5.14 mmol/L. The last lipoprotein peak is indicated by the composition of other fractions. The last peak contains chylomicron, its remnant, and Lp(a). B: Chromatogram of four major lipoprotein classes in human serum with step gradient. The data of a serum are as follows: TC = 7.25 mmol/L, TG = 6.14 mmol/L, HDL-C = 0.68 mmol/L, LDL-C = 3.76 mmol/L, IDL-C = 0.45 mmol/L, VLDL-C = 1.47 mmol/L, Other-C = 0.89 mmol/L. C: Chromatogram of five lipoprotein classes and Lp(a) in human serum. The data of a serum are as follows: TC = 5.28 mmol/L, TG = 2.01 mmol/L, HDL-C = 0.83 mmol/L, LDL-C = 3.11 mmol/L, IDL-C = 0.65 mmol/L, VLDL-C = 0.42 mmol/L, chylomicron-C = 0.03 mmol/L, Lp(a)-C = 0.24 mmol/L, Lp(a)-protein = 0.84 mg/mL. D: Chromatogram of subfactions of HDL and LDL and three lipoprotein classes in human serum. The data of a serum are as follows: TC = 5.66 mmol/L, TG = 1.92 mmol/L, earlier-eluting HDL-C = 0.64 mmol/L, later-eluting HDL-C = 0.37 mmol/L, earlier-eluting LDL-C = 3.18 mmol/L, later-eluting LDL-C = 0.66 mmol/L, IDL-C = 0.34 mmol/L, VLDL-C = 0.41 mmol/L, Other-C = 0.07 mmol/L.
Panels A, B, C, and D are referred to References #29, 30, 32, and 33, respectively.

chronic kidney disease (CKD)[38]. We compared lipoprotein profiles measured by AEX-HPLC separating six lipoprotein fractions to the Hisayama risk scores and Suita scores in Japanese healthy men[39]. Chylomicron-C levels, most likely reflecting cholesterol of chylomicron remnant lipoprotein (RLP), were positively correlated with the Hisayama risk scores and Suita scores in rank correlation analysis ($p < 0.005$), whereas Lp(a)-C levels were not correlated with these scores.

Non-HDL-C, a good marker for CVD risk, is composed of the sum of cholesterol of atherogenic lipoproteins (LDL, Lp(a), IDL, VLDL, and chylomicron remnant) and chylomicron. Lipid Research Clinics Program Follow-up study for an average of 19 years with 2,406 men and 2,056 women at entry showed that non-HDL-C was a better predictor of CVD mortality than LDL-C[40]. Therefore, the determination of non-HDL-C is useful for CVD risk assessment.

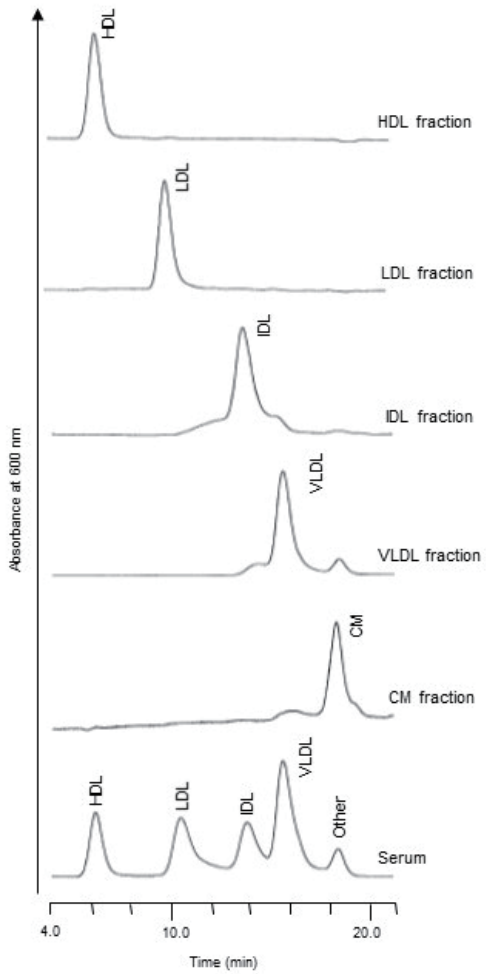

Fig. 4. Chromatograms of serum lipoprotein fractions obtained by an ultracentrifugation method and whole serum

A human serum with type III dyslipidemia was used. Lipoprotein fractions of the serum were separated by ultracentrifugation. The flotation rates of chylomicrons and VLDL were set at >400 and 20–400, respectively, in a solution of 1.745 mol/L sodium chloride (d=1.063 g/mL). Densities of IDL, LDL, and HDL were set as follows: 1.006 <d<1.019 g/mL, 1.019<d<1.063 g/mL, and 1.063 <d g/mL, respectively. The data of human serum with a patient with type III dyslipidemia were as follows: TC=5.82 mmol/L, TG=4.16 mmol/L, HDL-C=0.88 mmol/L, LDL-C=1.34 mmol/L, IDL-C=1.06 mmol/L, VLDL-C=2.12 mmol/L, Other-C=0.37 mmol/L.
This figure is referred to Reference #31.

Bypass Angioplasty Revascularization Investigation (BARI) study followed 1,514 secondary prevention patients with coronary artery disease (CAD) for 5 years[41]. BARI study showed that non-HDL-C was a strong predictor of nonfatal myocardial infarction and angina pectoris but not related to mortality. In addition, LDL-C did not predict the cardiovascular events during follow-up. However, in 2018, Shiba *et al.* reported the discordance of non-HDL-C and LDL-C as predictors of CVD risk[42]. They selected 801 patients with successful coronary artery stenting with 18-month follow-up. These patients were classified into three groups in accordance with the baseline levels of non-HDL-C and LDL-C. Contrary to studies described above, non-HDL-C is less important than LDL-C as a predictor of CVD in patients with stable angina after stent implantation. Therefore, non-HDL-C will not necessarily outweigh LDL-C as a predictor of the CVD. However, we reported that the FHS risk score was positively correlated with non-HDL-C, IDL-C, and VLDL-C in patients who underwent medical check-ups ($p<0.0001$)[36], and the Suita score was also highly correlated with non-HDL-C and chylomicron-C in healthy men ($p<0.005$)[39]. These results suggest that non-HDL-C and TG-rich lipoproteins are considered second lipid targets next to LDL-C for the management of CVD risk presumably because of their significant associations with the FHS and Suita scores.

In 1981, Tatami et al. reported that the increased IDL-C was associated with the severity of CAD[43]. The severity of coronary atherosclerosis was estimated by the sum of coronary lesion scores based on stenosis rates and lesion numbers determined by coronary angiographic data. The coronary atherosclerosis severity was correlated with IDL-C ($P<0.01$). Liu *et al.* reported that non-HDL-C was a stronger predictor of CHD risk than LDL-C, and VLDL-C was an independent predictor of CHD risk after adjusting for LDL-C in the FHS[17]. We estimated lipoprotein profiles of CHD patients by AEX-HPLC[32]. IDL-C and VLDL-C of patients with CHD were higher than those of healthy subjects.

Nordestgaard showed that the elevated level of

TG-rich lipoprotein cholesterol estimated as "total cholesterol minus LDL-C and HDL-C" is causally associated with CVD and inflammation, and the calculated value is mainly VLDL-C and IDL-C[44, 45]. In the subendothelial space of artery, macrophages take up oxidized LDL via scavenger receptors. However, TG-rich lipoproteins (beta-VLDL, IDL, or chylomicron remnants) are mainly taken up by macrophages via LDL receptor and others to the lesser extent without modification[46-48]. These lipoproteins uptake by macrophages results in the formation of foam cells. Therefore, these lipoproteins are thought to be atherogenic. Two- to threefold increases of large VLDL in patients with insulin resistance cause the generation of small, dense LDL. A large amount of circulating oxidized LDL are present in small, dense LDL fraction[48]. Scheffer *et al.* reported an inverse relationship between LDL size and circulating oxidized LDL in patients with type 2 diabetes (T2DM)[49]. We also reported that LDL particles from T2DM patients are small-sized phenotypes and more susceptible to oxidation[50]. However, small, dense LDL cannot be separated by AEX-HPLC, but oxidized LDL can be separated[33]. As such, AEX-HPLC should be partially improved to separate small, dense LDL.

In a substudy of the JUPITER, VLDL-C and VLDL subfractions of patients on statin medication were evaluated by 400 MHz proton nuclear magnetic resonance spectroscopy, and this substudy investigated its relationships with CVD incidents[51]. The incrementally greater reduction of VLDL-C and small VLDL were associated with lower CVD risk, but reductions in TG and large VLDL were not associated[51]. Small VLDL is richer in cholesterol and protein than large VLDL[52]. Redgrave et al. estimated the changes of VLDL concentrations by density gradient preparative ultracentrifugation using plasma of normo- and hypertriglyceridemic subjects in the fasting state and after a fatty meal[53]. The increased changes of TG concentration of VLDL 6 hours after a fatty meal in these subjects were 141% and 126%. However, changes of VLDL-C concentrations in these subjects were 102% and 103%, respectively.

Therefore, cholesterol levels of IDL and VLDL measured by AEX-HPLC will be useful in clinical practice because they are risk makers for CVD, and postprandial changes of these lipoprotein cholesterol levels are relatively minor in contrast to TG levels.

Diabetes

The risk of CHD is markedly increased in patients with T2DM. Diabetes mellitus is included in the factors for estimated CHD risk scores[35, 38]. Patients with T2DM frequently present with dyslipidemia, which are characterized by increased TG, decreased HDL-C, and slightly increased LDL-C. In addition, large VLDL (VLDL1) and small, dense LDL are increased, and apolipoproteins are glycated[54]. Obesity and overweight are defined as an abnormal body fat accumulation and cause metabolic disorders, such as T2DM. Adiponectin is released by adipose tissue, and the plasma levels inversely correlate with body mass index (BMI)[55]. Adiponectin also exerts anti-inflammatory functions[56] and downregulates adhesion molecule expression in endothelial cells[57]. Therefore, adiponectin is thought to protect against atherosclerosis. Many studies on the relevance of plasma or serum adiponectin levels to CHD risk have been conducted worldwide[58-60]. However, Kanhai *et al.* indicated that plasma adiponectin was not related to CHD risk in a meta-analysis study[61]. We estimated correlations between lipoprotein profiles and serum adiponectin levels in patients with T2DM[62]. The adiponectin levels were inversely correlated with VLDL-C but were uncorrelated with HDL-C, LDL-C, and IDL-C. We also estimated the effects of aerobic exercise training (60 min/day, 2 or 3 times/week) on serum levels of lipids and adiponectin in moderate dyslipidemic patients without diabetes[63]. The results indicated that levels of LDL-C, IDL-C, and VLDL-C were significantly decreased after 8 and 16 weeks ($p<0.05$, $p<0.001$, and $p<0.001$, respectively). The adiponectin levels were not changed after 8 weeks but were significantly increased after 16 weeks ($p<0.001$).

RLPs, which increase in the impaired lipoprotein metabolism, are associated with the progression of atherosclerosis and CAD[64, 65]. A high RLP-C (>0.12 mmol/L) is a significant risk factor for CAD in Japanese patients with T2DM[66]. RLPs include chylomicron remnant and VLDL remnant (IDL)[64, 65]. RLP-C is significantly correlated with IDL-C and VLDL-C measured by AEX-HPLC, and VLDL ratio estimated by agarose gel electrophoresis (AGE) with lipid staining in patients with T2DM ($p<0.0001$)[67]. LDL and VLDL peaks in all sera could be separated by AEX-HPLC, but those in 8 out of 194 sera could not be separated by AGE. **Fig. 5** indicates AEX-HPLC chromatograms and AGE patterns of a healthy serum and a diabetic patient's serum. LDL and VLDL cannot be separated by AGE in a diabetic patient's serum. Another electrophoresis for the analysis of lipoprotein profile [polyacrylamide gel electrophoresis (PAGE) with lipid staining] shows that a mid-band appeared at a position between LDL and VLDL in a part of patients with familial dyslipidemia and dyslipidemic diabetes, and the mid-band lipoproteins promote atherosclerosis[68, 69]. In a previous study, an independent

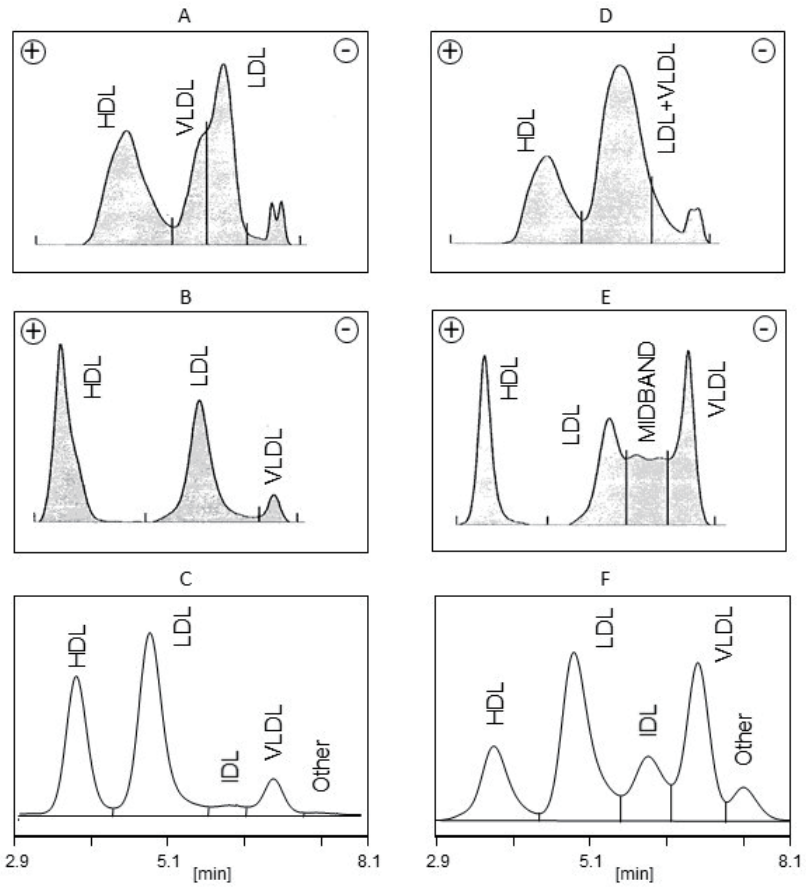

Fig. 5. Comparison between AEX-HPLC and electrophoresis

Panels A and D, B and E, and C and F show the patterns of agarose gel electrophoresis, polyacrylamide gel electrophoresis, and the chromatograms of AEX-HPLC, respectively.
A, B, and C are patterns and chromatogram of healthy serum. The data were as follows: TC=179 mg/dL, TG=4 mg/dL; AEX-HPLC method, HDL-C=61.7 mg/dL, LDL-C=95.8 mg/dL, IDL-C=5.1 mg/dL, VLDL-C=16.0 mg/dL, Other-C=0.9 mg/dL; agarose gel electrophoresis, HDL=43%, LDL=37%, VLDL=20%; polyacrylamide gel electrophoresis, HDL=49%, LDL=46%, VLDL=5%. D, E, F are of patients with T2DM and dyslipidemia. The data were as follows: TC=23 mg/dL, TG=336 mg/dL; AEX-HPLC method, HDL-C=35.5 mg/dL, LDL-C=81.6 mg/dL, IDL-C=31.2 mg/dL, VLDL-C=62.3 mg/dL, Other-C=12.9 mg/dL; agarose gel electrophoresis, HDL=31%, LDL+VLDL=69%; polyacrylamide gel electrophoresis, HDL=23%, LDL=26%, VLDL=28%, MID-BAND=23%.
This figures are referred in part to Reference #66.

VLDL2 peak was observed between peaks of VLDL1 and LDL on PAGE of hyperlipoproteinemic serum[70]. Another study indicated retardation factors (Rfs) of 0.2–0.45, 0.45–0.7, 0.7–0.85, and 0.85–1.0 for VLDL1 (Sf 60-400), VLDL2 (Sf 20-60), IDL, and LDL, respectively, on PAGE[71]. VLDL2 secretion from the liver, depending on cholesterol synthesis, cholesterol ester availability, and microsomal transfer protein activity, is enhanced in hypercholesterolemia, and the cholesterol content of VLDL2 is high[72]. Mid-band lipoproteins may include IDL and VLDL2. The levels of mid-band lipoproteins are significantly correlated with cholesterol levels of IDL and VLDL by AEX-HPLC in 34 patients with T2DM ($r=0.866$, $p<0.0001$ and $r=0.842$, $p<0.0005$, respectively). **Fig. 5** indicates AEX-HPLC chromatograms and PAGE patterns of a healthy serum and a diabetic patient's serum. Mid-band findings between LDL and VLDL appeared in the PAGE pattern of a diabetic patient's serum.

Lp(a) is one of the atherogenic lipoproteins and a target of therapy to lower CVD risk[73-75]. As indicated in many studies, an increased Lp(a) mass measured by enzyme-linked immunosorbent assay or latex agglutination assay is associated with CHD risk[76-79], and the risk of diabetes was increased by twofold[80]. However, Mora *et al.* showed that increased Lp(a) mass was inversely associated with incident T2DM risk in subjects without CVD[81]. A previous report indicated that high concentration of insulin suppressed apolipoprotein(a) synthesis in monkey hepatocytes[82]. An elevated Lp(a) level is known to be associated with the presence of CHD, and the Lp(a) contains small-molecular-weight apolipoprotein(a)[83]. However, low Lp(a) levels alone seem to not be causally associated with T2DM, but a causal association for large lipoprotein(a) isoform size cannot be excluded[84]. Niacin and PCSK9 inhibitors lower Lp(a), whereas niacin is associated with insulin resistance, but the relevance of therapy with PCSK9 inhibitors to increased incident T2DM has not been reported[85]. We reported that the insulin levels decreased in a 6-month period of dietary modification by calorie restriction, and the mass and cholesterol levels of Lp(a) increased in that period[86].

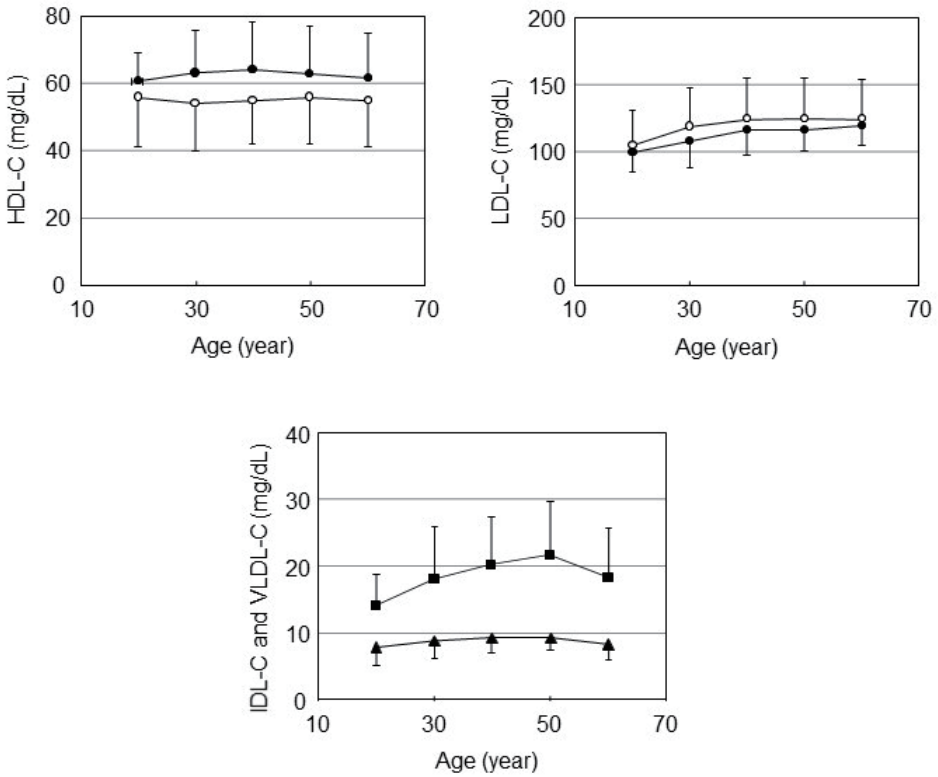

Fig. 6. Mean concentrations of cholesterol in HDL, LDL, IDL, and VLDL in relation to age

Close and open circles indicate mean concentrations of cholesterol measured by AEX-HPLC and of Japanese lipid survey, respectively. Square and triangle indicate mean concentrations of cholesterol in VLDL and IDL, respectively, measured by AEX-HPLC.
This figure is referred in part to Reference #39.

Kidney Disease

CVD is the most common cause of mortality in patients with CKD. Dyslipidemia is linked to an increased CVD risk in patients with CKD[87, 88]. In patients with CKD and proteinuria, a loss of apolipoprotein C-II, an activator of lipoprotein lipase (LPL), into urine impairs catabolism of VLDL[89]. In patients with CKD and reduced GFR, hepatic VLDL production is not elevated, and the catabolism of VLDL is impaired. Serum levels of apolipoprotein C-III, an inhibitor of LPL, is increased, and hepatic TG lipase activity is reduced[87, 88]. Therefore, serum IDL concentrations increase in the patients with CKD.

Shoji et al. reported that IDL-C and VLDL-C were elevated and HDL-C was reduced in patients with diabetic nephropathy or hemodialysis (HD)[89, 90]. They used an ultracentrifugation method for the separation analysis of lipoprotein classes. We examined the lipoprotein profiles measured by AEX-HPLC in patients undergoing HD or continuous ambulatory peritoneal dialysis (CAPD)[91, 92]. We also indicated decreased HDL-C and increased levels of IDL-C and VLDL-C in HD patients as compared with healthy subjects. Moreover, IDL-C only was consistently elevated regardless of CAPD duration. We also indicated that the earlier-eluting subfraction among two HDL subfractions was lower in HD patient's serum[93]. The earlier-eluting HDL subfraction contains HDL3[33]. The decreased HDL3 might be responsible for the HDL dysfunction of cholesterol efflux in HD patients.

Healthy Volunteer

To determine the recent serum lipid data in the general Japanese population, a survey was conducted in 36 districts of Japan in 2000[94]. The mean HDL-C was 59 mg/dL: 55 mg/dL in men and 65 mg/dL in women. HDL-C slightly decreased with increase of age. The mean LDL-C was 118 mg/dL: 121 mg/dL in men and 115 mg/dL in women. LDL-C slightly increased with advancing age. We studied lipoprotein profiles in 161 healthy men without mediations. The mean cholesterol levels of HDL and LDL in every age

were comparable to those of Japanese people in 2000 (**Fig. 6**)[39]. In addition, the lower eGFR was significantly correlated with higher levels of LDL-C ($p<0.005$), IDL-C ($p<0.001$), and VLDL-C ($p<0.0001$), and only VLDL-C was significantly correlated with eGFR independently of BMI ($p<0.0005$). Thus, the increased VLDL-C might be a good marker to predict renal dysfunction in healthy subjects.

Future Perspectives

The new method by using AEX-HPLC has a high capability to separate lipoprotein classes, which can be used in substitution for ultracentrifugation methods. We developed a new method for measurement of alpha-tocopherol (AT) in lipoprotein classes, which contains AEX-HPLC, for the separation of lipoprotein classes and reverse-phase chromatography to separate AT in each lipoprotein class[95]. The new automated method can measure AT concentrations of HDL, LDL, and VLDL in human plasma. AT is thought to be an antioxidant for lipoproteins[96, 97]. Oxidized LDL can promote the foam cell formation of macrophages, and the foam cell contributes to the development of atherosclerosis[98-100]. An index of the resistance to LDL oxidation is expressed as the lag time to copper ion-induced LDL oxidation[50, 101, 102]. The LDL lag time to oxidation is significantly correlated with the AT level in LDL[50, 95, 97]. The other antioxidant in lipoproteins is known to be beta carotene[102, 103]. We are going to develop another new method for the measurement of beta carotene in lipoprotein classes.

HDL is known to be an antiatherogenic lipoprotein, and low HDL-C is a risk factor of CVD. Some new assays for HDL function, e.g., cholesterol efflux, anti-inflammation, and antioxidative action, were recently developed[104]. Rohatgi *et al.* reported that HDL-mediated cholesterol efflux capacity has an inverse association with incident CVD, independently of HDL-C levels[105]. Some subfractions are found in HDL, and the functions of each HDL subfraction are different[106, 107]. We found two broad asymmetric HDL peaks in a chromatogram of AEX-HPLC with a linear gradient of chaotropic ion concentration (**Fig. 3A**). Now, we intend to study a separation method for HDL subfractions by AEX-HPLC.

Conclusions

We have developed the new method to separate lipoprotein classes by using AEX-HPLC. A column containing nonporous gel and a chaotropic ion-containing eluent were used to increase the performance of lipoprotein separation in AEX-HPLC. The clinical usefulness of AEX-HPLC was evaluated in samples of patient with CVD, diabetes, and CKD and of healthy volunteers. The diagnostic system with AEX-HPLC was approved for clinical use in the public health-care insurance in Japan in 2014. Furthermore, we applied AEX-HPLC to another new method for the measurement of AT in lipoprotein classes. The AEX-HPLC application for methods to measure various substances in lipoproteins will be expected in the future.

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Conflicts of Interest

Professor Hiroshi Yoshida received honoraria for speaking activities from Astellas, Amgen, Bayer, Denka Seiken, Kowa, Mochida, MSD, and Takeda, but these were not related to this study. Yuji Hirowatari PhD has no potential conflict of interest to disclose. The research funds from Tosoh Corporation to Drs. Hiroshi Yoshida and Yuji Hirowatari were less than the lower limit of the stipulated range that should be disclosed in accordance with Japan Atherosclerosis Society Guidelines for conflicts of interest.

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