REVIEW

Recent Advances in Analytical Methods on Lipoprotein Subclasses: Calculation of Particle Numbers from Lipid Levels by Gel Permeation HPLC Using “Spherical Particle Model”

Mitsuyo Okazaki^{1*} and Shizuya Yamashita^{2,3}

¹ Professor of Emeritus of Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8519, JAPAN

² Director, Rinku General Medical Center, 2-23 Ourai-kita, Rinku, Izumisano, Osaka 598-8577, JAPAN

³ Professor, Department of Community Medicine & Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, JAPAN

Abstract: Recently, we developed an analytical method for determining the lipid levels and particle numbers in lipoprotein subclasses covering a wide size range from chylomicrons to small high density lipoproteins, by using gel permeation high-performance liquid chromatography (GP-HPLC). The challenges in analytical methods on lipoprotein subclasses have been addressed from 1980 by Hara and Okazaki using commercial TSK gel permeation columns. Later, the improvements in the hardware, separation and detection of lipoproteins, and the data processing software, using a Gaussian distribution approximation to calculate lipid levels of lipoprotein subclasses, have been extensively utilized in these analytical methods for over thirty years. In this review, we describe on the recent advances in analytical methods on lipoprotein subclasses based on various techniques, and the calculation of particle numbers from lipid levels by GP-HPLC using the “spherical particle model”. Free/ester ratio of cholesterol in particular lipoprotein subclass was accurately estimated from triglyceride, total cholesterol (free and esterified) and the size of the particle based on this model originally proposed by Shen and Kezdy.

Key words: gel permeation chromatography, HPLC, lipoprotein subclasses, particle number, spherical particle model

1 Introduction

“Without cholesterol there can be no atherosclerosis”, this statement has been widely accepted since Anitschkow first demonstrated the role of cholesterol in the development of atherosclerosis by his classic experiments using rabbits in 1913¹⁾. The role of lipids contained in lipoprotein particles in atherosclerosis is now indisputable. Although the cholesterol levels of low density lipoproteins (LDL) and high density lipoproteins (HDL) are known to have the opposite effects on the development of cardiovascular diseases^{2,3)}, LDL and HDL are heterogeneous particles⁴⁻⁹⁾ in the metabolic origin and/or the function, and physicochemical properties such as density, size, charge, and protein and lipid compositions. Therefore, many analytical methods of lipoprotein subclasses have been developed using various

separation techniques or computer analyses such as self-organizing map analysis¹⁰⁾, multivariate and neural network analysis¹¹⁾. Because of no reference materials, the definition of lipoprotein subclasses used in various methods are different, and standardization is required to evaluate the risk of cardiovascular diseases in epidemiological studies based on lipoprotein subclass profiles. Our definition of lipoprotein subclasses based on particle size, has been determined by average profiles of healthy subjects and patients with lipoprotein metabolic disorder having a remarkable increase with respect to special lipoprotein subclasses^{12,13)}. For example, patients whose lipoprotein lipase (LPL) activity is absent or little have remarkable increases of chylomicrons (CM), large sized VLDL, small sized LDL, and small sized HDL particles, compared to

*Correspondence to: Mitsuyo Okazaki, Professor of Emeritus of Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8519, JAPAN

E-mail: okazaki.las@tmd.ac.jp

Accepted February 16, 2016 (received for review January 29, 2016)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjocs>

those of normal subjects¹⁴. Patients with cholesteryl ester transfer protein (CETP) deficiency have a remarkable increase of large HDL particles^{14, 15}. In type III hyperlipidemia with apolipoprotein (apo) E2/2, a remarkable increase of small sized VLDL particles has been observed due to the accumulation of remnant particles, which lead to the shift of LDL to a larger particle size than that of normal LDL^{14, 16}. These approaches for subclass definition incorporating the lipid disorders have been highly evaluated for reference particle sizes in nuclear magnetic resonance (NMR) analysis of lipoproteins by Ala-Korpela *et al.*^{17–26} (No. 8 in Table 1).

2 Background of development of gel permeation high-performance liquid chromatography (GP-HPLC) for lipoprotein subclass analysis

Serum lipoproteins can be separated according to the differences in particle sizes, densities, charges, immunochemical reactivity of protein moieties, among others. Measurements of the amounts of separated lipoprotein particles are successfully performed by the selective detection of lipoprotein components with high sensitivity and high accuracy. For this purpose, an enzymatic method is the most suitable, where colored substances are derived in proportion to the numbers of target molecule^{27, 28}.

Gel permeation chromatography has been evaluated as a lipoprotein analytical method. Its advantages as a tool of lipoprotein analysis are as follows: (1) All components in the loaded samples can be recovered from the columns, allowing repeated analysis; (2) Degradation or denaturation of samples during separation is the lowest among other separation techniques, especially ultracentrifugation^{29–31}; (3) Analysis of chromatogram is simple and easy, as the separation is only based on particle size. In 1966, Werner³² pre-

sented elution patterns of plasma lipoproteins using 2% agarose gel column, where lipid components of collected eluent fractions from the column were measured in dry weight, after extraction with organic solvents. For cholesterol (Cho), the determination was made by Lieberman Burchard reaction (heating, sulfuric acid, acetic acid and others). This required 5 mL of plasma and 4–12 h to fractionate one sample.

In 1970, Sata *et al.*³³ evaluated gel chromatography for plasma lipoprotein fractionation by comparing ultracentrifugation, paper electrophoresis, and thin-layer chromatography technique. They then revealed that plasma lipoproteins were eluted successively from the column in the four overlapping peaks of chylomicrons (CM), very low density lipoproteins (VLDL), LDL, and HDL, and showed continuous progressive changes in lipid composition as these fractions emerged, while LDL showed a relatively constant lipid composition. These widely accepted profiles for 4 major lipoprotein classes, as previously presented by analytical ultracentrifugation by Lindgren *et al.*^{34–36}, have been revealed using a traditional gel chromatography technique and chemical analysis.

In 1974, Allain *et al.*²⁷ developed an enzymatic method for the determination of Cho by using a single aqueous reagent, containing one of the key enzymes, cholesterol oxidase. In 1978, Kato *et al.*³⁷ developed gel permeation columns for using aqueous solution under high-speed and/or high pressure for the separation of macromolecules, and Hara *et al.*^{38–40} successfully applied them to HPLC for analysis of serum lipoproteins by combining the on-line detection of Cho using enzymatic reaction. In 1981, Okazaki *et al.*⁴¹ clarified the clinical relevance of the analysis using their GP-HPLC method, by the remarkable decrease of small-sized HDL subclasses (HDL3) Cho levels in patients with liver cirrhosis. In 1984, Matsuzawa *et al.*¹⁶ revealed

Table 1 Comparison of analytical methods for all lipoprotein subclasses from whole plasma or serum sample.

No.	Method/Principle	Subclasses	Cho	TG	PN**	Size
1	Gel permeation HPLC	2 CM, 5 VLDL, 6 LDL, 7 HDL	✓ *	✓ *	✓	✓
2	Ion exchange HPLC	CM, VLDL, IDL, 2 LDL, 2 HDL	✓ *	–	–	–
3	Analytical ultracentrifugation ^{a, f}	14 VLDL, 15 LDL, 15 HDL	–	–	✓	–
4	Density gradient ultracentrifugation	3 VLDL, 1 IDL, 5 LDL, 5 HDL	✓ *	–	–	–
5	Gradient gel electrophoresis (GGE) ^{b, f}	7 LDL, 5 HDL	–	–	–	✓
6	Ion Mobility Analysis ^c	3 VLDL, 2 IDL, 4 LDL, 2 HDL	–	–	✓	✓
7	NMR (Curve fitting method) ^{d, f}	3 CM&VLDL, IDL, 3LDL, 3HDL	–	–	✓	✓
8	NMR (Combination of different regression and statistical methods) ^e	6 VLDL, IDL, 3LDL, 4HDL	✓	✓	✓	✓

* lipid components are measured directly by on-line enzymatic reaction.

** particle number.

^a particle concentration is derived from schlieren pattern, as a whole lipoprotein mass.

^b LDL and HDL subclasses are measured separately by LDL gel and HDL gel.

^c Plasma protein previously removal from sample by ultracentrifugation.

^d group led by Otvos, NMR measurement at 47°C.

^e group led by Ala-Korpela, NMR measurement at 37°C.

^f Comparison of these methods with LipoSEARCH data are described in the text.

using this early GP- HPLC system, that patients with type III hyperlipidemia with apoE2/2 showed no lipoproteins eluted at the regular LDL position, and elution position of major component shifted to larger particle size than that of normal LDL, and absence of LDL-sized peak was also reported in the improved HPLC system¹⁴. In 1988, Yamashita *et al.*¹⁵ revealed, using the early GP-HPLC system that patients with CETP deficiency have abnormal lipoprotein profiles, especially, extremely larger HDL in size. These two abnormal profiles, derived from the lipid disorders were confirmed in an early GP-HPLC system, and they were used to determine the individual anchor peak positions out of the 20 component peaks^{12, 13} in the GP-HPLC system as shown in Fig. 2 and Table 2.

In 2002, a split in the column effluent (Fig. 1) made simultaneous detection of two lipid components possible by Usui *et al.*⁴². After that, the data processing of Gaussian curve fitting technique helped in the measurement of the 20 component peak areas^{12, 13}. The elimination of non-specific absorption of lipoproteins to the gel matrix in columns as well as reduction of particle size of the gel matrix have resulted in the increase of analytical performance in lipoprotein separation in a wide size range from CM to small HDL^{43–45}. By these improvements in hardware and software, that is, separation, detection, and data processing as described above, the GP-HPLC system for Cho and triglycerides (TG) measurements of lipoprotein subclasses, have been established. Skylight Biotech Inc. launched LipoSEARCH service by using this improved GP-HPLC system⁴⁶. In this article, we call this improved GP-HPLC system as “the LipoSEARCH system”. This system has been used by many researchers worldwide, and many papers over 300 using LipoSEARCH profiling data have been published⁴⁷.

More recently, an algorithm for the calculation of particle

number of lipoprotein subclasses has been developed⁴⁸ and applied for the LipoSEARCH system. Using the new system, Okada *et al.*⁴⁹ have revealed unique distribution of lipoprotein particle numbers in patients with CETP deficiency. Ai *et al.*⁵⁰ have also examined the changes in particle number and core lipid composition by pitavastatin therapy in patients with hypercholesterolemia.

3 Comparison of GP-HPLC with other methods for lipoprotein subclass analysis

The performances of the LipoSEARCH system were compared to those of other analytical methods for all lipoprotein subclasses from whole serum or plasma samples. In Table 1, comparison of analytical performances between 2 HPLC methods (No. 1 and 2), 2 ultracentrifugation methods (No. 3 and 4), gradient electrophoresis (No. 5), ion mobility analysis (No. 6) and 2 NMR methods (No. 7 and 8) are summarized.

In the LipoSEARCH system, amounts of Cho and TG, particle numbers and particle sizes can be determined for CM, 5 VLDL, 6 LDL and 7 HDL subclasses. Another method of HPLC (No. 2 in Table 1), combination of step-wise elution using ion-exchange column and on-line enzymatic reaction of Cho, allows measurement of the amounts of only Cho in seven lipoprotein subclasses⁵¹.

A spectrum of particles with differing flotation characteristics, which is a function of both size and hydrated density of lipoprotein particles, were obtained using analytical ultracentrifugation (AU). Krauss *et al.*⁵² reported the distribution of 14 VLDL, 15 LDL, and 15 HDL subclasses as a whole lipoprotein mass using by AU (No. 3 in Table 1), and we compared the particle number differences related to sex and age by GP-HPLC to those of particle

Fig. 1 Configuration of the LipoSEARCH system. Arrows indicate the direction of flow. Injection volume: 4 μ L; flow rate of the running buffer: 0.24 mL/min; flow rate of reaction reagents: 0.12 mL/min; column: SkylightPakLP1-AA gel permeation column (Skylight Biotech Inc., Japan, column size, 300 mm $\times$ 4.6 mm I.D. (internal diameter); particle diameter of gel matrix, 9 μ m.); column temperature: 25°C; reactor coil: PTFE., 25 m $\times$ 0.18 mm I.D.; reaction temperature: 37°C; UV-VIS detector λ : 550 nm.

Table 2 Definition of lipoprotein subclasses in the LipoSEARCH system.

component peak No.	1	2	3	4	5 ^a	6 ^a	7 ^a	8	9 ^a	10 ^a	11	12	13	14	15 ^a	16	17 ^a	18 ^a	19	20 ^b
subclass name	CM1	CM2	VLDL1	VLDL2	VLDL3	VLDL4	VLDL5	LDL1	LDL2	LDL3	LDL4	LDL5	LDL6	HDL1	HDL2	HDL3	HDL4	HDL5	HDL6	HDL7
major subclass name	CM	CM	large VLDL		medium VLDL		small VLDL	large LDL	medium LDL	small LDL	very small LDL		LDL		very large HDL	large HDL	medium HDL	small HDL	very small HDL	
major class name	CM	CM	VLDL		VLDL		VLDL		LDL		LDL		LDL		HDL		HDL		HDL	
particle diameter (nm)	>90	75	64	53.6	44.5	36.8	31.3	28.6	25.5	23	20.7	18.6	16.7	15	13.5	12.1	10.9	9.8	8.8	7.6
particle radius ^c (nm)	41.2		32	26.8	22.25	18.4	15.65	14.3	12.75	11.5	10.35	9.3	8.35	7.5	6.75	6.05	5.45	4.9	4.4	3.8

^a anchor peak determined experimentally as average peak positions of healthy subject and dyslipidemic patients.^b including color material in plasma, such as bilirubin.^c particle radius corresponding to a maximum hydration layer including hydrated water and sugar chains, by which lipoprotein particle separated (R_{hplc} in Fig. 3).

mass differences by AU for three HDL subclasses as described later (section 9).

Density gradient ultracentrifugation is used for whole lipoprotein profile (No. 4 in Table 1). The Vertical Auto Profile (VAP) II test measures the relative cholesterol distribution for different lipoprotein subclasses with on-line enzymatic detection⁵³⁾.

Gradient gel electrophoresis (No. 5 in Table 1) can fractionate LDL and HDL subclasses directly from the plasma according to their size and charge by pre-lipid staining, and the size and relative (%) distribution of subclasses can be determined⁵⁴⁾. We compared the LDL particle size calculated from the LDL peak time on the original chromatogram in the GP-HPLC system with the LDL scores by the Lipo-print LDL system⁵⁵⁾, where a higher value means small particle size. The moderate negative correlation ($r = -0.585$, $n = 87$) between the two methods was given⁵⁶⁾.

Ion mobility analysis depends on the differences in electrophoretic mobility of the gas-phased lipoprotein particles (No. 6 in Table 1). After an ultracentrifugation step to remove albumin, the size distributions are presented as particle number and mass (arbitrary unit) in eleven lipoprotein subclasses by Caulfield *et al.*⁵⁷⁾.

NMR spectroscopy is one of the most important methods in chemistry, particularly for molecular identification and absolute quantification of soluble compounds in liquid phase. Recently, NMR spectroscopy has been applied to analyze lipoproteins which are macro emulsion particles dispersed in an aqueous phase⁵⁸⁾.

Since the beginning of the 1990s, two groups made a significant progress in the analysis of ¹H-NMR for lipoprotein profiles. The first group, led by Otvos^{59–61)} (No. 7 in Table 1), created and perfected the LipoProfile test service, which was made commercially available in 1997 by LipoScience Inc. and huge amounts of clinical data derived by this system has been published^{62–67)}. The second group, led by Ala-Korpela^{68, 69)} (No. 8 in Table 1), has made in-depth studies on the possibilities of ¹H-NMR in the analysis of lipoproteins and the use of NMR spectra in the assessment of risk factors in population studies^{17–26)}. In the latter group, a recent systematic analytic approach verified that the significant modeling of 14 lipoprotein subclasses as shown in Fig. 2 of their article⁷⁰⁾, are completely consistent with our LipoSEARCH size definition (Table 2), and the latter group have stated in their papers^{17–26)} that the lipoprotein size of these subclasses was calibrated with the GP-HPLC method by Okazaki *et al.*¹²⁾.

Besides those two groups, many others also reported lipoprotein subclass analysis using NMR information by various approaches. They are referred to in the recent review by Mallol *et al.*⁵⁸⁾, where detailed information including principles of NMR application to lipoprotein analysis is presented.

All NMR applications for analyzing lipoprotein subclasses

share the common basic principle, as NMR signals depend on particle diffusion rates according to lipoprotein sizes. Unlike the GP-HPLC method, where Cho and TG levels are directly measured, each subclass analysis are performed using a whole ^1H -NMR signal intensity from methyl groups contained in the hydrocarbon chains; 2 methyl groups in free cholesterol (FC), 3 in TG, 3 in cholesterol ester (CE), and 2 in phospholipids, respectively. Therefore, the direct measurements for each lipid component can not be done from ^1H -NMR signal information.

There are many other methods focusing on only one subclass. For example, measurement of Cho levels in small dense LDL fraction has been developed by Ito *et al.*⁷¹⁾ by the combination of surface active surfactants and enzymatic determination of Cho. This method has been used in a large population clinical trial, the multi-ethnic study of atherosclerosis⁷²⁾, where the small LDL particle concentration was measured by the NMR technique by Otvos *et al.* (No. 7 in Table 1).

4 Principles and system configuration of the LipoSEARCH system, and its application in companion animal or cell culture

Figure 1 shows the configuration of the LipoSEARCH system. The system consists of two parts, (1) measurement system consisting three pumps, an auto-sampler, a degasser, a column oven, a reactor, two UV-VIS detectors, and a system controller, (2) analysis system (data processing: LipoSEARCH).

Briefly, lipoproteins in the whole plasma or serum (4 μL) were separated with tandemly connected SkylightPakLP1-AA gel permeation columns. The column effluent was then equally split into two lines by a micro splitter, and each effluent was allowed to react at 37°C with Cho and TG reagents customized for the LipoSEARCH system (Toyobo Inc., Japan), respectively. Absorbance at 550 nm was continuously monitored after each enzymatic reaction. Cho and TG levels of the major classes and subclasses of lipoproteins were calculated from the component peak areas by the Gaussian curve fitting technique¹³⁾ (Fig. 2). The detailed information about the validation of the analytical method such as reproducibility, linearity, limit of detection, limit of quantification, and effect of influence substances, are referred to in the review by Toshima *et al.*⁴⁶⁾.

The LipoSEARCH system was successfully applied to the evaluation of lipoprotein metabolism in companion animals, and it allowed the classification 4 types for dyslipidemia in dogs and cats. This classification has been very useful for diagnosis and disease treatment for companion animals^{46, 73)}. Skylight Biotech Inc. has also launched a LipoTEST service for companion animals, which has been applied for over 10,000 cases up to the present time.

Since the GP-HPLC method is very sensitive, analysis of lipoprotein profiles secreted in the medium from cultured cells can be successfully performed^{9, 74)}. (LipoCULTURE by Skylight Biotech Inc.) A novel screening system for anti-dyslipidemic agents using cultured cells has been reported by Takahashi *et al.*⁷⁵⁾.

A lot of papers on lipoprotein profile data by the two application system have been published⁴⁷⁾.

5 The definition of lipoprotein subclasses in the LipoSEARCH system

A significant numbers of subjects (over 1000), including normal and diseased, have been analyzed using the LipoSEARCH system, leading to the definition of the 20 lipoprotein subclasses as shown in Table 2.

The representative chromatograms for curve fitting analysis of normolipidemic and hyperlipidemic subjects are presented in Fig. 2. The 20 component peaks consists of 8 anchor peaks and 12 extra essential peaks and their elution positions are determined as follows: Positions of anchor peaks 6, 9, and 17 are determined as average elution positions for VLDL, LDL, and HDL peaks observed in healthy middle aged men, respectively. Positions of anchor peaks 5, 10, and 18 are determined as average elution positions of VLDL, LDL and HDL in patients with LPL deficiency, respectively. Position of anchor peaks 7 and 15 are determined as average elution position of VLDL in patients with apoE2/2 and that of HDL in patients with CETP deficiency, respectively. Positions of 12 extra essential peaks (thin broken line line in Fig. 2) other than 8 anchor peaks (bold solid in Fig. 2) among the 20 component peaks are mathematically determined in order to proceed with the curve fitting summation technique for the separation of 20 components peaks¹³⁾.

The conversion of peak position to particle diameter was performed using a column calibration curve, a plot of logarithm of the particle diameter of standard samples, latex beads (Magsphere Inc.) 25 and 37 nm in diameter, and a high molecular weight calibrator (Pharmacia Biotech) containing thyroglobulin (17 nm), ferritin (12.2 nm), catalase (9.2 nm), albumin (7.1 nm), and ovalbumin (6.1 nm) against their peak positions^{6, 12, 76)}.

We defined the subclass names corresponding to the 20 component peaks according to the major lipoprotein classes as follows: CM1 to CM2, peaks 1 to 2; VLDL1 to VLDL5, peaks 3 to 7; LDL1 to LDL6, peaks 8 to 13; HDL1 to HDL7, peaks 14 to 20; together with major subclass names, such as very small LDL etc., and the particle size of each peak are shown in Table 2. The particle size of peak 1 (CM1), which is separated as the excluded volume of the column, is defined over 90 nm. Therefore, in order to calculate the particle number from particle radius, it is inte-

grated with peak 2(CM2) to be a CM fraction equivalent and the particle radius is defined at 41.2 nm for the weight average size of TG levels in CM1 and CM2 of healthy subjects⁴⁸⁾.

6 Lipoprotein structures for apoB-containing lipoproteins and apoA-I-containing lipoproteins (HDL)

Almost all serum lipoproteins are either apoB-containing particles or apoA-I-containing particles, both of which are spherical particles with a lipid core consisting of TG and CE surrounded by a surface layer consisting of FC, phospholipids and apolipoproteins, like a micelle emulsion, originally reported by Shen *et al.*⁷⁷⁾, and then by others^{78–81)}. The conceptual structures of lipoproteins, “spherical particle model”, widely accepted are shown in Fig. 3. In apoB-containing lipoproteins, CM, VLDL, and LDL (peak 1 to 13 in the LipoSEARCH system), one molecule of apoB-48 or B-100 is contained per one lipoprotein particle^{80–82)} together with other apolipoproteins such as apoE, and apoCs. During the metabolism process in the blood, apoB is retained in the lipoprotein particles, unlike other apolipoproteins. On the other hand, HDL (peak 14 to 20 in the LipoSEARCH system) have 2 to 5 apoA-I molecules per one lipoprotein particle^{83, 84)} together with other apolipoproteins, such as apoA-II, apoE, and apoCs. There are non-spherical particles without core lipids in rare cases. For example, in patients with primary biliary cirrhosis (PBC), drug hepatopathy and lecithin cholesterol acyltransferase (LCAT) deficiency, liposome like particle, called Lp(X)^{85–87)} and discoidal HDL^{88, 89)}, which are eluted in the VLDL size and/or HDL size range, are present. In the subclass containing substantial amounts of these non-spherical particles, calculation of particle number using “spherical particle model” can not be applied as described in section 7. As expected, these cases deviated largely from a regression model curve as presented in VLDL4 and HDL4 for PBC and/or LCAT deficiency as shown in Fig. 5.

Among components in the serum lipoproteins, only FC is a pure compound with a molecular weight of 386.7, partial specific volume of 1.021 cm³/g, and molecular volume of 0.6223 nm³. As for the components containing fatty acids, such as CE, TG, and phospholipids, the oleic acid esters are used for the molecular data. As for apolipoproteins, especially apoB (apoB-48 in CM, apoB-100 in VLDL and LDL), the molecular weights can not be determined even though amino acid sequences are determined by cDNA analysis^{90–93)}, because unknown amounts of carbohydrate are contained. Moreover, some parts of apoB-48 or apoB-100 are estimated to insert into the core region as presented by Yang *et al.*⁹⁴⁾. We use the most popular molecular data on apoB-48 and apoB-100 of 264,000 and 550,000

Daltons, respectively. Considering these characteristics of the chemical properties on lipoprotein components, we have to use some estimated and/or modified molecular data from the literatures^{33, 48, 77, 95–97)}.

7 Algorithm for calculation of particle number of lipoprotein subclasses

Based on the spherical structure, so called “spherical particle model”, we developed an algorithm for calculation of particle numbers in lipoprotein subclasses defined as shown in Table 2. It can be assumed that the particle size of lipoproteins belonging to a certain subclass is almost constant. Thus, the particle numbers belonging to a certain subclass can be calculated by dividing the sum of the core volume of all lipoprotein particles (tVc) by the core volume occupied by TG and CE of one lipoprotein particle; hereafter, we call it “LipoSEARCH Vc”.

The tVc in each subclass is the sum of the total volume of TG (tVtg) and the total volume of CE (tVce). When using the molecular weight of 885.45 and 651.1, and the molecular volume of 1.607 nm³ and 1.1443 nm³, for TG and CE, respectively^{77, 95)}, tVtg and tVce are calculated as follows; tVtg = TG (mg/dL) / 88.545 × 1.607 (nm³), tVce = CE (mg/dL) / 65.11 × 1.1443 (nm³). Thus, for each subclass having the same radius, particle numbers of lipoproteins can be obtained from the experimental values of tVc and the LipoSEARCH Vc values specified by the LipoSEARCH system.

7.1 Determination of core volume occupied by CE and TG per one particle

Although the size of the lipoprotein subclass is defined by the diameter (nm), the radius (R_{hplc} , nm) will be used to determine the core volume occupied by CE and TG per one particle. The separation of lipoproteins by gel permeation depends on the differences in the size of the maximum hydration layer of the lipoprotein particle. The maximum size includes an amorphous hydration layer formed by sugar chains attached to apolipoproteins, such as apoB, apoE, and apoCs. Accordingly, the particle size (R_{hplc}) means the size of the maximum hydration layer of the lipoprotein particle as shown in Fig. 3. The LipoSEARCH Vc is constant in the lipoprotein particles having a constant size, and we determined it for each lipoprotein subclass in the following way⁴⁸⁾. The LipoSEARCH Vc is determined using the actual core radius (R_c), which is estimated from R_{hplc} by subtracting the surface layer thickness (t_s) and hydration layer thickness (t_{os}), that is, R_c (nm) = R_{hplc} (nm) - t_s (nm) - t_{os} (nm), and the LipoSEARCH Vc = $4/3\pi R_c^3$ (nm³) for subclasses without apoB (HDL1–7). In the case of apoB-containing lipoproteins (CM, VLDL1–5 and LDL1–6), some volume of apoB-48 or apoB-100 inserted into the core region is deducted from $4/3\pi R_c^3$.

Fig. 2 Representative patterns for separation of the 20 component peaks using the Gaussian curve fitting technique¹³⁾, for (A) a healthy woman and (B) a patient with LPL deficiency. Solid black curve is real pattern detected by online enzymatic reaction for Cho reagent. Solid colored curves are individual component peaks (brown, CM; blue, VLDL; red, LDL; green, HDL) and broken black curve is their sum of Gaussian curves. Vertical lines are the 20 component peak positions, bold lines are anchor peak positions determined by healthy male (blue, peaks 6, 9, 17), LPL deficiency (brown, peaks 5, 10, 18), type III with apoE2/2 (green, peak 7) and CETP deficiency (red, peak 15), and extra essential peak positions are shown by vertical thin broken lines.

Fig. 3 Lipoprotein structures, "spherical particle model" for (A) apoB-containing lipoproteins and (B) apoA-I-containing lipoproteins (HDL). PL, phospholipids; FC, free cholesterol; CE, cholesterol ester; TG, triglycerides; t_s , the surface layer thickness; t_{os} , hydration layer thickness; R , particle radius without amorphous hydration layer; R_{hplc} , particle radius corresponding to the maximum hydration layer of the lipoprotein particle including an amorphous hydration layer formed by sugar chains attached to apolipoproteins, such as apoB, apoE, and apoCs; R_c , actual core radius, which is estimated as $R_{hplc}(\text{nm}) - t_s(\text{nm}) - t_{os}(\text{nm})$; V_s , volume of surface layer; V_c , core volume occupied by CE and TG, called "LipoSEARCH V_c ", as presented yellow area in the figure.

7.2 Determination of ratio of FC to (CE+TG) for healthy subjects

In the “spherical particle model”, it is expected that the ratio of surface layer volume (V_s) to core volume (V_c) is constant in the particles having the same size as shown in Fig. 3. Moreover, the ratio of FC volume to core volume is estimated to be constant in the particles of the same size. Therefore, this assumption was examined experimentally for healthy control subjects.

The amounts of Cho and TG were measured by on-line enzymatic reaction in the LipoSEARCH system. In the enzymatic reagent for determination of Cho²⁷⁾, cholesterol oxidase and cholesterol esterase are contained as the key enzymes. Therefore, Cho values measured by the reagent are the total amounts of the FC, the original one and the FC derived from the CE. As CE is not the target molecule of cholesterol oxidase, CE can be measured after being hydrolyzed by cholesterol esterase to FC. Thus, the original amount of FC shall be measured in the same process without using cholesterol esterase. Based on the principle of enzymatic reaction, CE cannot be determined directly and is calculated from Cho and FC as follows: $CE = Cho - FC$ (in mol.) or $CE/651.1 = Cho/386.7 - FC/386.7$ (in mg/dL), that is, $CE = (Cho - FC) \times 1.684$ (in mg/dL).

Hereafter, the CE values determined by above equation are called “actual CE, aCE” in order to differentiate from the “estimated CE, eCE” values using the “spherical particle model” without FC measurement, as described later.

In order to examine the assumption of $FC/(CE + TG)$ is constant, the amounts of Cho, FC, and TG of 80 healthy subjects were measured by the on-line enzymatic reaction in the LipoSEARCH system, where FC was measured without cholesterol esterase, and aCE was calculated from FC and Cho. In Fig. 4, averages and 95% confidential intervals of $FC/(aCE + TG)$ ratio are presented for each subclass. The ratio is found to be almost constant except for HDL7. Moreover, the ratio is expected to increase continuously with the increase in the particle diameter, because of

a constant thickness of the surface layer (t_s in Fig. 3). However, the calculated ratios derived from the measured values have higher points in the range of the LDL subclass region than that of the HDL subclass region. Lund-Katz *et al.*^{78, 79)} reported a higher molar ratio of FC to phospholipids in the surface layer of LDL, compared to that in HDL, from the molecular packing data of the spherical particle model of human plasma LDL and HDL using proton-decoupled ¹³C-NMR spectrum study. Moreover, Tuzikov *et al.*⁹⁷⁾ proposed a mathematical model to describe the composition, structure, and dynamic equilibrium between different human serum lipoprotein particles using specially developed computer programs and data by the small-angle X-ray scattering method, and analyzing the data in the literatures. In their article, the percent distribution of FC volume to total particle volume has a maximum point at the LDL size particle, according to their mathematical model. Therefore, our data, $FC/(aCE + TG)$ ratio, obtained from healthy subjects was proved reliable enough to build a regression model to estimate CE values from TG and Cho values.

The ratio of HDL7 subclass is significantly higher than other HDL subclasses, which is explained by the elution of pre β_1 particle^{98, 99)}, consisting of phospholipids, apoA-I and FC, with no core components unlike the spherical particle, in the size range of HDL7. Therefore, the particle number calculated by the “spherical particle model” at the HDL7 should be distinguished from those of other subclasses.

7.3 Calculation of particle numbers from the amounts of Cho and TG without FC measurement

As described above, the lipoproteins particles of a certain subclass can be considered largely having uniform structure such as the same particle radius, and the same ratio of FC volume to core volume occupied by TG and CE. Based on this fact, we attempted to find abnormal lipoprotein particles deviated from this scheme with respect to particle structure by specific lipid disorder. Thus, $FC/(CE$

Fig. 4 Ratio of FC to (aCE + TG) for 80 healthy subjects in lipoprotein subclasses. $\blacktriangle$ and bar are the averages of the $FC/(aCE + TG)$ ratio (weight / weight) and 95 % confidential intervals. The amounts of FC was determined by the component peak areas detected by on-line detection using enzymatic Cho reagent without cholesterol esterase. The amounts aCE (actual CE) was calculated from Cho and FC values as described in the text.

+ TG) ratio is assumed to be constant (in volume/volume or weight/weight) for each subclass and $\text{Cho} = \text{FC} + \text{CE}$ (in mol), the ratio of aCE/Cho , y , was plotted against the ratio $\text{Cho}/(\text{Cho} + \text{TG})$, x , for each subclass VLDL4 (peak 6), LDL2 (peak 9), and HDL4 (peak 17) obtained from 80 healthy subjects as shown in Fig. 5. The arrows indicate the average values of 80 healthy subjects, which were used as the anchor points of the regression fitting. The best empirical fittings that pass the anchor points are shown in the following regression equation (1), for each subclass.

$$y = b_1 \times 1/x + b_0 \quad (0 < x \leq 1.0, y \geq 0) \quad (1)$$

The samples of type III with apoE2/2, LPL deficiency and CETP deficiency indicated as large circles in Fig. 5 were found within the range of the regression equation (1), indicating that lipoproteins in these lipid disorders mostly are spherical particles although the core lipid compositions were quite different from healthy subjects, where the values of $\text{Cho}/(\text{Cho} + \text{TG})$ in x axis of Fig. 5 are confirmed to reflect the core lipid composition, $\text{aCE}/(\text{aCE} + \text{TG})$ by empirical fitting analysis⁴⁸⁾. For example, TG enrichments in LDL2 and HDL4 were observed except for HDL4 in CETP deficiency. In the samples of LCAT deficiency and PBC, the data of LDL2, which is known to be TG-rich and spherical, so-called Lp(Y)⁸⁵⁾, were also within the range of the regression equation (1). However, the data of their VLDL4 and HDL4 fell far out of the range of the regression equation (1), perhaps because of the presence of substantial amounts of non-spherical particles, such as liposome-like particles, Lp(X)^{85–87)}, or discoidal particles^{88,89)}. Thus, this empirical analysis can detect unusual rare cases those have non-spherical lipoprotein particles.

In Fig. 6, eCE was plotted against aCE for the subclass of VLDL4 (peak 6), LDL2 (peak 9) and HDL4 (peak 17) for 80 healthy subjects, where eCE was calculated from the data of Cho and TG using the regression equation (1). Those values were in remarkably good agreement with a correlation coefficient $r > 0.987$. A high correlation was also obtained in all fractions with $r > 0.961$ except HDL7 (peak 20) containing pre β_1 HDL, for which spherical particle model cannot be applied. This is consistent with the results that the ratio of $\text{FC}/(\text{CE} + \text{TG})$ for HDL7 subclass is much higher than other HDL subclasses. Thus, we demonstrated that FC/CE ratio can be accurately estimated from the values of Cho, TG and particle size, without actual measurement of FC or CE. Furthermore, particle numbers in subclasses without substantial amounts of non-spherical particles can be confirmed to be calculated from the LipoSEARCH data (Cho and TG).

8 Stoichiometry in apoB-containing lipoproteins and apoA-I-containing lipoproteins (HDL): numbers of apolipoprotein molecule per one lipoprotein particle

As shown in Fig. 3, the stoichiometry that apoB-containing lipoproteins have one molecule of apoB per particle^{80–82)} has been widely accepted. On the other hand, in HDL, 2 to 5 apoA-I molecules are contained^{83,84)}. Therefore the relationship of serum apoB levels and apoA-I levels to the particle numbers in total apoB-containing lipoproteins and in total HDL, were examined, respectively, for individuals whose serum apoB and apoA-I levels were measured. In Fig. 7A and 7B, the molecular numbers (nM) of serum apoB and apoA-I (y axis) are plotted against particle numbers (nM) in total apoB-containing lipoproteins and total HDL (x axis), respectively. As expected, a very high correlation between apoB molecular number and particle number were obtained, $r = 0.964$, and apoB molecule of 1.041 ± 0.078 (95% CI 1.038 – 1.04), was confirmed to be contained per particle. In case of apoA-I in total HDL, very high correlation are also obtained, $r = 0.889$, however, an intercept of a regression line deviated from zero, unlike the apoB in Fig. 7A. In Japanese population, having a higher Cho level in total HDL^{100,101)}, the molecular number of 3.062 ± 0.242 (95% CI 3.051 – 3.072) of apoA-I in total HDL was within the reported stoichiometry of apoA-I in total HDL (2 – 5 molecules)^{83,84)}. Therefore, an algorithm for calculating the particle numbers in the lipoprotein subclasses described above was confirmed with respect to the stoichiometry of lipoprotein structure.

Regarding the two types of lipoprotein metabolic disorders, which were used to determine the anchor peak positions, patients with type III hyperlipidemia with apoE2/2 and patients with CETP deficiency, the stoichiometry was examined (see inserted plots in Fig. 7A and 7B). In three cases with CETP deficiency, as marked with large dotted open circle in the figures, data plots deviated from the regression lines in opposite directions; decreased number of apoB and increased number of apoA-I. As for these peculiar and rare cases among CETP deficiency, modifications of the algorithm for calculating particle numbers are needed. We estimated that a large amount of extremely very large HDL components was eluted between the LDL and HDL positions. Under these estimations, we examined the modification of algorithm to calculate the particle numbers for extremely very large HDL subclasses⁴⁸⁾. Here, the stoichiometry of apoB and apoA-I in our regression model were examined for patients with these two lipid disorders, excepting three cases in CETP deficiency marked in Fig. 7; the molecular number of apoB was 0.96 ± 0.16 (95% CI: 0.82 – 1.09) and 1.06 ± 0.10 (95% CI 0.98 – 1.13) in patients with apoE2/2 and CETP deficiency, respectively. An algorithm of the particle number was confirmed in patients with these lipid disorders as well as the general pop-

Fig. 5 Regression curves using “spherical particle model” for estimation of CE values without measuring FC using the regression equation (1) for representative subclasses of VLDL, LDL and HDL. (A), medium VLDL (VLDL4, peak 6); (B), medium LDL (LDL2, peak 9); (C), medium HDL (HDL4, peak 17). The regression equation (1) for three subclasses are presented in the figure. Cho / (Cho + TG) ratio in x axis is equivalent to aCE / (aCE + TG) ratio, see in the text. As for 80 healthy subjects (●), actual data of aCE / Cho ratio as y axis are plotted against the Cho / (Cho + TG) ratio as x axis and the averages are shown with arrows, which pass through the regression curve. The data for patients with lipid disorders are plotted as large circles, ●, Type III with apoE2/2; ●, LPL deficiency; ●, CETP deficiency; ●, LCAT deficiency, PBC.

Fig. 6 Plots of the estimated CE values (eCE) against the actual CE values (aCE) for 80 healthy subjects, for representative subclasses of VLDL, LDL and HDL. (A), medium VLDL (VLDL4, peak 6); (B), medium LDL (LDL2, peak 9); (C), medium HDL (HDL4, peak 17). The estimated CE values were obtained from experimental Cho and TG values using the regression equation (1), presented in Fig. 5, respectively.

Fig. 7 Stoichiometry of numbers of apoB molecule and apoA-I molecule per one lipoprotein particle for apoB-containing lipoproteins and apoA-I-containing lipoproteins, respectively. (A), Scatter plots of serum apoB molecular numbers (nM) against total particles numbers of apoB-containing lipoproteins (CM, total VLDL and total LDL); (B), Scatter plots of serum apoA-I molecular numbers (nM) against total particle numbers of apoA-I-containing lipoproteins (total HDL). ▲, general subjects (n=1992); ●, type III with apoE2/2 (n=8); ●, CETP deficiency (n=12), three cases having extremely large HDL subclass are marked with large dotted open circles.

ulation. On the other hand, the numbers of apoA-I in total HDL in the case of apoE2/2 ($n=8$), 2.65 ± 0.30 (95% CI 2.40 – 2.90) was significantly lower, and in those of CETP deficiency ($n=9$), 3.33 ± 0.20 (95% CI 3.18 – 3.48) significantly higher than those of the general population. These deviation of apoA-I molecular number from that of the general population might be explained by the difference of HDL particle size distribution, that is, smaller HDL size in the former case and larger HDL size in the latter case as previously reported^{14, 16}. Thus, the particle numbers in the lipoprotein subclasses are very useful to analyze the qualitative as well as quantitative differences based on lipid disorders.

9 Sex and age-related differences in lipoprotein particle numbers for Japanese healthy population

The normal ranges for Cho and TG levels in the serum lipoprotein subclasses derived by the LipoSEARCH system for healthy Japanese adults have been reported by Furusyo *et al.*¹⁰². The critical differences of TG levels as well as Cho levels were clearly observed among men, premenopausal women and postmenopausal women.

It is well known that age and sex influence the risk of cardiovascular diseases and lipoprotein profiles by various analytical methods^{103–108}. Therefore, the relationship of a new parameter in the LipoSEARCH system, the particle number, to sex and age were reviewed for the LipoSEARCH data (Cho and TG levels in the 20 subclasses) about 4731 subjects, which included healthy volunteers and participants in regular health checkups, from 2002 to 2012 (fresh sample or fresh frozen sample, storage time less than a few months).

Protocol for the operation and analysis was consistent with current Ethical Guidelines for Epidemiological Research by Ministry of Health, Labor and Welfare of Japan first installed in 2002 <<https://www.niph.go.jp/wadai/ekigakurinri/guidelines.pdf>>. The studies using a part of this population were published elsewhere^{14, 102, 109–113}.

From the 4731 subjects with the LipoSEARCH data, individuals with a history of cardiovascular diseases, critical liver and kidney diseases, and pregnancy were excluded, and the particle numbers were calculated for the remaining 4386 individuals; age 46 ± 15 (18 – 92) years, 1899 men, and 2487 women. Cho and TG levels in total lipoproteins were 201 mg/dL (in average) and 109 mg/dL, respectively. Averages and 95% confidential intervals in the lipoprotein particle numbers in apoB-containing lipoproteins and apoA-I-containing lipoproteins (HDL) are presented against age with 5-year intervals in Fig. 8 and Fig. 9, respectively.

The particle numbers in each subclass for apoB-containing lipoproteins (CM, VLDL1 – 5 and LDL1 – 6) obtained in the LipoSEARCH system can be converted to apoB con-

centrations (mg/dL) in each subclass, because a stoichiometry that apoB-containing lipoproteins have one molecule of apoB have been validated as shown in Fig. 7A (1.041 ± 0.078 apoB molecule per particle). Therefore, both particle numbers (nM, left axis) and apoB levels (mg/dL, right axis) are shown in the scales of y axis in Fig. 8. Thus, the particle numbers in total apoB lipoproteins (sum of CM, VLDL1 – 5 and LDL1 – 6) are equal to the serum apoB levels, and comparison of the serum apoB levels with many reported values in biochemical research, epidemiological studies, and clinical trials can be possible.

In this Japanese population, serum apoB levels in men and women are 97 ± 25 mg/dL and 85 ± 24 mg/dL, respectively, and men have a significantly higher value than women, as reported in many studies^{107, 114}. For example, very similar differences of serum apoB levels related to sex and age reported in Finnish population samples¹¹⁴, however the apoB levels was a little lower than the Japanese population (Fig. 8A).

It was reported by Nordestgaard *et al.*¹¹⁵ that the arterial wall permeability and efflux from intima of plasma lipoproteins depended on the particle size, and that the accumulation of atherogenic lipoproteins in the arterial intima was a key event in the development of atherosclerosis. Low permeability to the arterial wall of CM and large VLDL has been demonstrated. Thus, our method, providing particle numbers (apoB levels) in each subclass as well as in total lipoproteins, will greatly contribute to the prediction of cardiovascular disease risks.

As shown in Fig. 8, sex and age differences in large LDL (LDL1) are clearly distinct from those in total apoB-containing lipoproteins, total VLDL (VLDL1 – 5), and small and very small LDL (LDL3 – 6). The peculiarity of the large LDL subclass has been previously reported with respect to visceral fat accumulation¹². In 1980, unique correlation of the level of large LDL compared to other LDL subclasses by canonical analysis among lipoprotein subclasses were presented by Krauss *et al.*⁵² by using AU technique, where the lipoprotein levels were measured as whole particle mass transferred from schlieren curves (No. 3 in Table 1).

As the HDL particle contains 2 to 5 apoA-I molecules^{83, 84}, apoA-I levels cannot be calculated from particle number in total HDL unlike the case of apoB-containing lipoproteins. However, very high correlation was obtained between apoA-I molecular number and particle number in total HDL (as shown in Fig. 7B). Thus, we tried to calculate the serum apoA-I levels from the particle number under the assumption of three apoA-I molecules per HDL particle and the results of apoA-I levels were inserted in the right side of y axis (mg/dL) in Fig. 9A. Serum apoA-I levels in men (153 ± 26 mg/dL) was significantly lower than those in women (158 ± 25 mg/dL) as reported in many studies^{114, 116}. Unlike the case of apoB levels, clear increase of particle number or apoA-I level in total HDL with increasing age

Fig. 8 Relationship of particle numbers to sex and age for Japanese healthy population (n = 4386). (A), total apoB-containing lipoproteins (CM, total VLDL and total LDL); (B), total VLDL (VLDL1 – 5); (C), large LDL (LDL1); (D), small and very small LDL (LDL3 – 6) to age with 5-year intervals. ▲, women; ▲, men. Bar is 95% CI. In (A), data of serum apoB levels from literature for Finnish population¹¹⁴ are inserted against age with four decades, ●, women; ●, men.

Fig. 9 Relationship of particles numbers to sex and age for Japanese healthy population (n = 4386). (A), total apoA-I-containing lipoproteins (HDL1-7); (B), very large and large HDL (HDL1 – 3); (C), medium HDL (HDL4); (D), small and very small HDL (HDL5 – 7) to age with 5-year intervals. ▲, women; ▲, men. Bar is 95% CI. In (A) data of serum apoA-I levels from literature for Finnish population¹¹⁴ are inserted against age with four decades, ●, women; ●, men.

were not observed as reported in the Finnish population¹¹⁴). Regarding the particle numbers in HDL subclasses (Fig. 9B-D), the very large and large HDL(HDL1 – 3) and medium HDL(HDL4) numbers were significantly higher in women than those in men, but was the opposite in the case of small and very small HDL(HDL4 – 7). Anderson *et al.*¹⁰⁴) examined the HDL distribution for normal men and women subjects using AU(No. 3 in Table 1), where total mass of HDL was resolved into three components, HDL2b(d 1.063 – 1.100 g/mL), HDL2a(d 1.100 – 1.125 g/mL) and HDL3(d 1.125 – 1.200 g/mL). Mean serum levels of HDL2b and HDL2a were significantly higher in women than in men for each of the four age decades. On the other hand, mean HDL3 levels were significantly higher in men than in the women for the population as a whole. These data might suggest that HDL1 – 3, HDL4 and HDL5 – 7 by the LipoSEARCH system largely corresponded to HDL2b, HDL2a and HDL3 identified by AU, respectively.

Since sex and age related differences in particle numbers of lipoprotein subclasses measured by NMR(No. 7 in Table 1) on the Framingham Study were reported by Freeman *et al.*¹⁰⁶), we compared them to those of the Japanese population. Except for medium LDL and medium HDL, similar patterns related to sex and age were observed in the LDL and HDL subclasses. The particle numbers in total LDL were comparable to our LipoSEARCH data, but those in total HDL were two times higher.

Regardless of the differences in the time of sample collection, geographic position and race or ethnicity, the profile of size distribution in accordance with sex and age showed substantial similarities, though some differences in measured values perhaps partly because of the difference in the methods used, allowing us to evaluate new methods without conducting a new reference studies with subjects with matching backgrounds.

10 Conclusion

A significant number of subjects, including healthy individuals and patients with lipoprotein metabolic disorders, have been analyzed using GP-HPLC, for a long time. We developed our present method to calculate the lipoprotein particle numbers by using the amounts of lipids in the lipoprotein subclasses defined by the particle size and the “spherical particle model”. Nowadays, the following questions have often been discussed: which subclasses, which lipid levels, and which particle numbers in lipoprotein subclasses are good for the prediction of cardiovascular diseases? Our GP-HPLC method, “the LipoSEARCH system”, provides the required data and information to answer these questions, from small amounts of serum or plasma samples no more than 10 µL.

Particle numbers give the information on stoichiometry

of the lipoprotein particles, for example, the numbers of molecules of lipoprotein constituents such as apolipoproteins and lipid components in one lipoprotein particle. Data from the LipoSEARCH system can be compared with the accumulated evidence of previous experimental data about lipoprotein structure. The LipoSEARCH system will greatly contribute to both basic and clinical research on lipoproteins.

Acknowledgment

The authors are grateful acknowledge professor Kentaro Shimokado and professor Masumi Ai of Tokyo Medical and Dental University for their valuable discussion and supports.

References

- 1) Anitschkow, N.; Chaladow, S. Ueber experimentelle Cholesterinsteatose und ihre Bedeutung fuer die Entstehung einiger pathologischer Prozesse. *Zentrbl. Allg. Pathol. Pathol. Anat.* **24**, 1-9(1913). English translation version: On experimental cholesterol steatosis and its significance in the origin of some pathological processes by N. Anitschkow and S. Chaladow, translated by Mary Z. Pelias, 1913. *Arteriosclerosis* **3**, 178-182(1983).
- 2) Kannel, W. B.; Castelli, W. P.; Gordon, T.; McNamara, P. M. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. The Framingham study. *Ann. Intern. Med.* **74**, 1-8(1971).
- 3) Gordon, T.; Castelli, W. P.; Hjortland, M. C.; Kannel, W. B.; Dawber, T. R. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am. J. Med.* **62**, 707-714(1977).
- 4) Krauss, R. M.; Burke, D. J. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. Lipid Res.* **23**, 97-104(1982).
- 5) Berneis, K. K.; Krauss, R. M. Metabolic origins and clinical significance of LDL heterogeneity. *J. Lipid Res.* **43**, 1363-1379(2002).
- 6) Okazaki, M.; Hagiwara, N.; Hara, I. Heterogeneity of human serum high density lipoproteins on high performance liquid chromatography. *J. Biochem.* **92**, 517-524(1982).
- 7) Rosenson, R. S.; Brewer, H. B. Jr.; Chapman, M. J.; Fazio, S.; Hussain, M. M.; Kontush, A.; Krauss, R. M.; Otvos, J. D.; Remaley, A. T.; Schaefer, E. J. HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events. *Clin. Chem.* **57**, 392-410(2011).
- 8) Davidson, W. S.; Silva, R. A.; Chantepie, S.; Lagor, W.

- R.; Chapman, M. J.; Kontush, A. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler. Thromb. Vasc. Biol.* **29**, 870-876 (2009).
- 9) Hayashi, M.; Abe-Dohmae, S.; Okazaki, M.; Ueda, K.; Yokoyama, S. Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7. *J. Lipid Res.* **46**, 1703-1711 (2005).
- 10) Kaartinen, J.; Hiltunen, Y.; Kovanen, P. T.; Ala-Korpela, M. Application of self-organizing maps for the detection and classification of human blood plasma lipoprotein lipid profiles on the basis of ¹H NMR spectroscopy data. *NMR Biomed.* **11**, 168-176 (1998).
- 11) Ala-Korpela, M.; Hiltunen, Y.; Bell, J. D. Quantification of biomedical NMR data using artificial neural network analysis: lipoprotein lipid profiles from ¹H NMR data of human plasma. *NMR Biomed.* **8**, 235-244 (1995).
- 12) Okazaki, M.; Usui, S.; Ishigami, M.; Sakai, N.; Nakamura, T.; Matsuzawa, Y.; Yamashita, S. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in HPLC. *Arterioscler. Thromb. Vasc. Biol.* **25**, 578-584 (2005).
- 13) Okazaki, M. United States Patent, Patent No. US 8,268,626.
- 14) Okazaki, M.; Usui, S.; Nakamura, M.; Yamashita, S. Evaluation of an HPLC Method for LDL-cholesterol Determination in Patients with Various Lipoprotein Abnormalities in Comparison with Beta-quantification. *Clin. Chim. Acta* **395**, 62-67 (2008).
- 15) Yamashita, S.; Matsuzawa, Y.; Okazaki, M.; Kako, H.; Yasugi, T.; Akioka, H.; Hirano, K.; Tarui, S. Small polydisperse low density lipoproteins in familial hyperalphalipoproteinemia with complete deficiency of cholesteryl ester transfer activity. *Atherosclerosis* **70**, 7-12 (1988).
- 16) Matsuzawa, Y.; Sho, N.; Kameda, K.; Kubo, M.; Hirobe, K.; Tarui, S.; Yamamoto, A.; Okazaki, M.; Hara, I. Characterization of Lipoprotein abnormalities in type III hyperlipoproteinemia associated with apoE3 deficiency (E2/2 phenotype). *J. Jpn. Atheroscler. Soc.* **6**, 1243-1248 (1984).
- 17) Inouye, M.; Kettunen, J.; Soininen, P.; Silander, K.; Ripatti, S.; Kumpula, L. S.; Hamalainen, E.; Jousilahti, P.; Kangas, A. J.; Mannisto, S.; Savolainen, M. J.; Julia, A.; Leiviska, J.; Palotie, A.; Salomaa, V.; Perola, M.; Ala-Korpela, M.; Peltonen, L. Metabonomic, transcriptomic, and genomic variation of a population cohort. *Mol. Syst. Biol.* **6**, 441 (2010).
- 18) Makinen, V. P.; Tynkkynen, T.; Soininen, P.; Peltola, T.; Kangas, A. J.; Forsblom, C.; Thorn, L. M.; Kaski, K.; Laatikainen, R.; Ala-Korpela, M.; Groop, P. H. Metabolic Diversity of Progressive Kidney Disease in 325 Patients with Type 1 Diabetes (the FinnDiane Study). *J. Proteome Res.* **11**, 1782-1790 (2012).
- 19) Wurtz, P.; Soininen, P.; Kangas, A. J.; Makinen, V. P.; Groop, P. H.; Savolainen, M. J.; Juonala, M.; Viikari, J. S.; Kahonen, M.; Lehtimaki, T.; Raitakari, O. T.; Ala-Korpela, M. Characterization of systemic metabolic phenotypes associated with subclinical atherosclerosis. *Mol. Biosyst.* **7**, 385-393 (2011).
- 20) Stancakova, A.; Paananen, J.; Soininen, P.; Kangas, A. J.; Bonnycastle, L. L.; Morken, M. A.; Collins, F. S.; Jackson, A. U.; Boehnke, M. L.; Kuusisto, J.; Ala-Korpela, M.; Laakso, M. Effects of 34 risk loci for type 2 diabetes or hyperglycemia on lipoprotein subclasses and their composition in 6,580 nondiabetic Finnish men. *Diabetes* **60**, 1608-1616 (2011).
- 21) Surakka, I.; Isaacs, A.; Karssen, L. C. *et al.* ENGAGE Consortium. A genome-wide screen for interactions reveals a new locus on 4p15 modifying the effect of waist-to-hip ratio on total cholesterol. *PLoS Genet.* **7**, e1002333 (2011).
- 22) Wang, J.; Stancakova, A.; Soininen, P.; Kangas, A. J.; Paananen, J.; Kuusisto, J.; Ala-Korpela, M.; Laakso, M. Lipoprotein subclass profiles in individuals with varying degrees of glucose tolerance: a population-based study of 9399 Finnish men. *J. Intern. Med.* **272**, 562-572 (2012).
- 23) Vangipurapu, J.; Stancakova, A.; Kuulasmaa, T.; Soininen, P.; Kangas, A. J.; Ala-Korpela, M.; Kuusisto, J.; Laakso, M. Association between liver insulin resistance and cardiovascular risk factors. *J. Intern. Med.* **272**, 402-408 (2012).
- 24) Szymanska, E.; Bouwman, J.; Strassburg, K.; Vervoort, J.; Kangas, A. J.; Soininen, P.; Ala-Korpela, M.; Westerhuis, J.; van Duynhoven, J. P.; Mela, D. J.; Macdonald, I. A.; Vreeken, R. J.; Smilde, A. K.; Jacobs, D. M. Gender-dependent associations of metabolite profiles and body fat distribution in a healthy population with central obesity: towards metabolomics diagnostics. *OMICS* **16**, 652-667 (2012).
- 25) Tukiainen, T.; Kettunen, J.; Kangas, A. J.; Lyytikainen, L. P.; Soininen, P.; Sarin, A. P.; Tikkanen, E.; O'Reilly, P. F.; Savolainen, M. J.; Kaski, K.; Pouta, A.; Julia, A.; Lehtimaki, T.; Kahonen, M.; Viikari, J.; Taskinen, M. R.; Jauhiainen, M.; Eriksson, J. G.; Raitakari, O.; Salomaa, V.; Jarvelin, M. R.; Perola, M.; Palotie, A.; Ala-Korpela, M.; Ripatti, S. Detailed metabolic and genetic characterization reveals new associations for 30 known lipid loci. *Hum. Mol. Genet.* **21**, 1444-1455 (2012).
- 26) Kujala, U. M.; Makinen, V. P.; Heinonen, I.; Soininen, P.; Kangas, A. J.; Leskinen, T. H.; Rahkila, P.; Wurtz, P.; Kovanen, V.; Cheng, S.; Sipila, S.; Hirvensalo, M.;

- Telama, R.; Tammelin, T.; Savolainen, M. J.; Pouta, A.; O'Reilly, P. F.; Mantyselka, P.; Viikari, J.; Kahonen, M.; Lehtimäki, T.; Elliott, P.; Vanhala, M. J.; Raitakari, O. T.; Jarvelin, M. R.; Kaprio, J.; Kainulainen, H.; Ala-Korpela, M. Long-term Leisure-time Physical Activity and Serum Metabolome. *Circulation* **127**, 340-348 (2013).
- 27) Allain, C. C.; Poon, L. S.; Chan, C. S.; Richmond, W.; Fu, P. C. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20**, 470-475 (1974).
- 28) Fossati, P.; Prencipe, L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin. Chem.* **28**, 2077-2080 (1982).
- 29) Herbert, P. N.; Forte, T. M.; Shulman, R. S.; La Piana, M. J.; Gong, E. L.; Levy, R. I.; Fredrickson, D. S.; Nichols, V. Structural and compositional changes attending the ultracentrifugation of very low density lipoproteins. *Prep. Biochem.* **5**, 93-129 (1975).
- 30) Kunitake, S. T.; Kane, J. P. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. *J. Lipid Res.* **23**, 936-940 (1982).
- 31) Murdoch, S. J.; Breckenridge, W. C. Development of a density gradient ultracentrifugation technique for the resolution of plasma lipoproteins which avoids apoE dissociation. *Anal. Biochem.* **222**, 427-434 (1994).
- 32) Werner, M. Fractionation of lipoproteins from blood by gel filtration. *J. Chromatogr.* **25**, 63-70 (1966).
- 33) Sata, T.; Estrich, D. L.; Wood, P. D. S.; Kinsell, L. W. Evaluation of gel chromatography for plasma lipoprotein fractionation. *J. Lipid Res.* **11**, 331-340 (1970).
- 34) Lindgren, F. T.; Elliott, H. A.; Gofman, J. W. The ultracentrifugal characterization and isolation of human blood lipids and lipoproteins, with applications to the study of atherosclerosis. *J. Phys. Colloid Chem.* **55**, 80-93 (1951).
- 35) Gofman, J. W.; Lindgren, F. T.; Elliott, H. Ultracentrifugal studies of lipoproteins of human serum. *J. Biol. Chem.* **179**, 973-979 (1949).
- 36) Lindgren, FT.; Freeman, NK.; Ewing, AM.; Jensen, LC. Serum lipoprotein distribution, flotation rates and protein analysis. *J. Am. Oil Chem. Soc.* **43**, 281-285 (1966).
- 37) Kato, Y.; Sasaki, H.; Aiura, M.; Hashimoto, T. High-performance aqueous gel-permeation chromatography of oligomers. *J. Chromatogr.* **153**, 546-549 (1978).
- 38) Hara, I.; Okazaki, M. High performance liquid chromatography of serum lipoproteins. In *Methods in Enzymology* (Albers J.J., Segrest J.P. eds.) Vol. 129, Academic Press, San Diego, pp. 57-78 (1986).
- 39) Hara, I.; Okazaki, M.; Ohno, Y. Rapid analysis of cholesterol of high density lipoprotein and low density lipoproteins in human serum by high performance liquid chromatography. *J. Biochem. (Tokyo)* **87**, 1863-1865 (1980).
- 40) Okazaki, M.; Itakura, H.; Shiraishi, K.; Hara, I. Serum lipoprotein measurement—liquid chromatography and sequential floatation (ultra centrifugation) compared. *Clin. Chem.* **29**, 768-773 (1983).
- 41) Okazaki, M.; Hara, I.; Tanaka, A.; Kodama, T.; Yokoyama, S. Decreased serum HDL3 cholesterol levels in cirrhosis of the liver. *N. Engl. J. Med.* **304**, 1608 (1981).
- 42) Usui, S.; Hara, Y.; Hosaki, S.; Okazaki, M. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J. Lipid Res.* **43**, 805-814 (2002).
- 43) Kitamura, T.; Ito, S.; Moriyama, H.; Kato, Y.; Sasamoto, K.; Okazaki, M. Quantitative analysis of serum lipoproteins (CM, VLDL, LDL and HDL) by high-performance gel filtration chromatography. *Chromatography* **17**, 33-37 (1996).
- 44) Okazaki, M.; Sasamoto, K.; Muramatsu, T.; Hosaki, S. Evaluation of precipitation and direct methods for HDL-cholesterol assay by HPLC. *Clin. Chem.* **43**, 1885-1890 (1997).
- 45) Okazaki, M.; Usui, S.; Hosaki, S. Analysis of plasma lipoproteins by gel permeation chromatography. In *Handbook of lipoprotein testing*, 2nd ed. (Rifai, N.; Warnick, G. R.; Dominiczak, M. H. eds.) American Association of Clinical Chemistry Press, Washington, DC, pp. 647-669 (2000).
- 46) Toshima, G.; Iwama, Y.; Kimura, F.; Matsumoto, Y.; Miura, M.; Takahashi, J.; Yasuda, H.; Arai, N.; Mizutani, H.; Hata, K.; Usui, S.; Okazaki, M. LipoSEARCH®; Analytical GP-HPLC method for lipoprotein profiling and its applications. *J. Biol. Macromol.* **13**, 21-32 (2013).
- 47) Papers using LipoSEARCH profiles: URL; <http://www.lipo-search.com/eng/literature/>. This material is available free of charge via the Internet at <http://dx.doi.org/jos.65.10.5650/jos.ess.16020>.
- 48) Okazaki, M. PCT patent application, Application number: PCT/2015/060467, Publication number: WO/2015/152371.
- 49) Okada, T.; Ohama, T.; Okazaki, M.; Sairyo, M.; Zhu, Y.; Kawase, R.; Nakaoka, H.; Nakatani, K.; Masuda, D.; Koseki, M.; Nishida, M.; Sakata, Y.; Yamashita, S. Assessment of lipoprotein particle number by high-Performance gel permeation chromatography in patients with cholesteryl ester transfer protein deficiency, 17th International Symposium on Atherosclerosis May 23-26, 2015, Amsterdam, The Netherlands.
- 50) Ai, M.; Tanaka, A.; Yoshida, M.; Tomonaga, O.; Yamazaki, T.; Niwa, A.; Schaefer, E. J.; Morimoto, N.; Okazaki, M. Lipoprotein particle number and core lipid composition changes based on high-perfor-

- mance gel permeation chromatography with pitavastatin therapy in hypercholesterolemic patients with or without type 2 diabetes mellitus, 17th International Symposium on Atherosclerosis May 23-26, 2015, Amsterdam, The Netherlands.
- 51) Hirowatari, Y.; Tsunoda, Y.; Ogura, Y.; Homma, Y. Analyzing of high-density lipoprotein subfractions and low-density lipoprotein subfractions in human serum with anion-exchange chromatography. *Atherosclerosis* **204**, E52-E57 (2009).
- 52) Krauss, R. M.; Lindgren, F. T.; Ray, R. M. Interrelationships among subgroups of serum lipoproteins in normal human subjects. *Clin. Chim. Acta* **104**, 275-290 (1980).
- 53) Kulkarni, K. R.; Garber, D. W.; Marcovina, S. M.; Segrest, J. P. Quantification of cholesterol in all lipoprotein classes by the VAP-II method. *J. Lipid Res.* **35**, 159-168 (1994).
- 54) Warnick, G. R.; McNamara, J. R.; Boggess, C. N.; Clendenen, F.; Williams, P. T.; Landolt, C. C. Polyacrylamide gradient gel electrophoresis of lipoprotein subclasses. *Clin. Lab. Med.* **26**, 803-846 (2006).
- 55) Hoefner, D. M.; Hodel, S. D.; O'Brien, J. F.; Branum, E. L.; Sun, D.; Meissner, I.; McConnell, J. P. Development of a rapid, quantitative method for LDL subfraction with use of the Quantimetrix Lipoprint LDL System. *Clin. Chem.* **47**, 266-274 (2001).
- 56) Okazaki, M.; Usui, S.; Yamashita, S. Measurement of serum lipoprotein subclasses by an HPLC method. *The Fats of Life* **17**, 64-68 (2004).
- 57) Caulfield, M. P.; Li, S.; Lee, G.; Blanche, P. J.; Salameh, W. A.; Benner, W. H.; Reitz, R. E.; Krauss, R. M. Direct Determination of Lipoprotein Particle Sizes and Concentrations by Ion Mobility Analysis. *Clin. Chem.* **54**, 1307-1316 (2008).
- 58) Mallol, R.; Rodriguez, M. A.; Brezmes, J.; Masana, L.; Correig, X. Human serum/plasma lipoprotein analysis by NMR: Application to the study of diabetic dyslipidemia. *Progress NMR Spectrosc.* **70**, 1-24 (2013).
- 59) Otvos, J. D.; Jeyarajah, E. J.; Bennett, D. W. Quantification of plasma lipoproteins by proton nuclear magnetic resonance spectroscopy. *Clin. Chem.* **37**, 377-386 (1991).
- 60) Otvos, J. D. Measurement of lipoprotein subclass profiles by nuclear magnetic resonance spectroscopy. In *Handbook of lipoprotein testing*, 2nd ed. (Rifai, N.; Warnick, G. R.; Dominiczak, M.H. eds.) American Association of Clinical Chemistry Press, Washington, DC, pp. 609-623 (2000).
- 61) Jeyarajah, E. J.; Cromwell, W. C.; Otvos, J. D. Lipoprotein particle analysis by nuclear magnetic resonance spectroscopy. *Clin. Lab. Med.* **26**, 847-870 (2006).
- 62) Colhoun, H. M.; Otvos, J. D.; Rubens, M. B.; Taskinen, M. R.; Underwood, S. R.; Fuller, J. H. Lipoprotein subclasses and particle sizes and their relationship with coronary artery calcification in men and women with and without type 1 diabetes. *Diabetes* **51**, 1949-1956 (2002).
- 63) Festa, A.; Williams, K.; Hanley, A. J.; Otvos, J. D.; Goff, D. C.; Wagenknecht, L. E.; Haffner, S. M. Nuclear magnetic resonance lipoprotein abnormalities in prediabetic subjects in the Insulin Resistance Atherosclerosis Study. *Circulation* **111**, 3465-3471 (2005).
- 64) Mora, S.; Otvos, J. D.; Rifai, N.; Rosenson, R. S.; Buring, J. E.; Ridker, P. M. Lipoprotein particle profiles by nuclear magnetic resonance compared with standard lipids and apolipoproteins in predicting incident cardiovascular disease in women. *Circulation* **119**, 931-939 (2009).
- 65) Mora, S.; Otvos, J. D.; Rosenson, R. S.; Pradhan, A.; Buring, J. E.; Ridker, P. M. Lipoprotein particle size and concentration by nuclear magnetic resonance and incident type 2 diabetes in women. *Diabetes* **9**, 1153-1160 (2010).
- 66) Parish, S.; Offer, A.; Clarke, R.; Hopewell, J. C.; Hill, M. R.; Otvos, J. D.; Armitage, J.; Collins, R. Heart Protection Study Collaborative Group. Lipids and lipoproteins and risk of different vascular events in the MRC/BHF Heart Protection Study. *Circulation* **125**, 2469-2478 (2012).
- 67) Sanchez, O. A.; Duprez, D. A.; Daniels, L. B.; Maisel, A. S.; Otvos, J. D.; Peralta, C. A.; Lima, J. A.; Bahrami, H.; Jacobs, D. R. Jr. The association between N-terminal pro B-type natriuretic peptide and lipoprotein particle concentration plateaus at higher N-terminal pro B-type natriuretic peptide values: Multi-Ethnic Study on Atherosclerosis. *Metabolism* **64**, 857-861 (2015).
- 68) Ala-Korpela, M.; Korhonen, A.; Keisala, J.; Horkko, S.; Korpi, P.; Ingman, L. P.; Jokisaari, J.; Savolainen, M. J.; Kesaniemi, Y. A. ¹H NMR-based absolute quantitation of human lipoproteins and their lipid contents directly from plasma. *J. Lipid Res.* **35**, 2292-2304 (1994).
- 69) Ala-Korpela, M.; Lankinen, N.; Salminen, A.; Suna, T.; Soininen, P.; Laatikainen, R.; Ingman, P.; Jauhiainen, M.; Taskinen, M. R.; Héberger, K.; Kaski, K. The inherent accuracy of ¹H NMR spectroscopy to quantify plasma lipoproteins is subclass dependent. *Atherosclerosis* **190**, 352-358 (2007).
- 70) Soininen, P.; Kangas, A. J.; Wurtz, P.; Suna, T.; Ala-Korpela, M. Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. *Circ. Cardiovasc. Genet.* **8**, 192-205 (2015).
- 71) Ito, Y.; Fujimura, M.; Ohta, M.; Hirano, T. Development of a homogeneous assay for measurement of small dense LDL cholesterol. *Clin. Chem.* **57**, 57-65 (2011).

- 72) Tsai, M. Y.; Steffen, B. T.; Guan, W.; McClelland, R. L.; Warnick, R.; McConnell, J.; Hoefner, D. M.; Remaley, A. T. New automated assay of small dense low-density lipoprotein cholesterol identifies risk of coronary heart disease: the multi-ethnic study of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **34**, 196-201 (2014).
- 73) Mizutani, H.; Sako, T.; Arai, N.; Kuriyama, K.; Yoshimura, I.; Mori, A.; Iwase, K.; Hirose, H. Application of Gel Permeation HPLC for Lipoprotein Profiling in Dogs. *J. Vet. Med. Sci.* **72**, 813-817 (2010).
- 74) Itoh, M.; Abe, Y.; Iwama, Y.; Kimura, F.; Satoh, M.; Shoji, M.; Takahashi, J.; Toshima, G.; Sasaki, H.; Hiwatashi, K.; Hata, K. Lupeol reduces triglyceride and cholesterol synthesis in human hepatoma cells. *Bio-technol. Lett.* **31**, 953-957 (2009).
- 75) Takahashi, J.; Toshima, G.; Matsumoto, Y.; Kimura, F.; Kiuchi, T.; Hamada, K.; Hata, K. In vitro screening for antihyperlipidemic activities in foodstuffs by evaluating lipoprotein profiles secreted from human hepatoma cells. *J. Nat. Med.* **61**, 670-674 (2011).
- 76) Usui, S.; Nakamura, M.; Jitsukata, K.; Nara, M.; Hosaki, S.; Okazaki, M. Assessment of between-instruments variation in a HPLC method for serum lipoproteins and its traceability to reference methods for total cholesterol and HDL-cholesterol. *Clin. Chem.* **46**, 63-72 (2000).
- 77) Shen, B. W.; Scanu, A. M.; Kézdy, F. J. Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. USA* **74**, 837-842 (1977).
- 78) Lund-Katz, S.; Phillips, M. C. Packing of cholesterol molecules in human high-density lipoprotein. *Biochemistry* **23**, 1130-1138 (1984).
- 79) Lund-Katz, S.; Phillips, M. C. Packing of cholesterol molecules in human low-density lipoprotein. *Biochemistry* **25**, 1562-1568 (1986).
- 80) Baumstark, M. W.; Kreutz, W.; Berg, A.; Frey, I.; Keul, J. Structure of human low-density lipoprotein subfractions, determined by x-ray small-angle scattering. *Biochim. Biophys. Acta* **1037**, 48-57 (1990).
- 81) Laggner, P.; Kostner, G. M.; Rakusch, U.; Worcester, D. Neutron small angle scattering on selectively deuterated human plasma low density lipoproteins. The location of polar phospholipid headgroups. *J. Biol. Chem.* **256**, 11832-11839 (1981).
- 82) Wiklund, O.; Dyer, C. A.; Tsao, B. P.; Curtiss, L. K. Stoichiometric binding of apolipoprotein B-specific monoclonal antibodies to low density lipoproteins. *J. Biol. Chem.* **260**, 10956-10960 (1985).
- 83) Colvin, P. L.; Moriguchi, E.; Barrett, P. H.; Parks, J. S.; Rudel, L. L. Small HDL particles containing two apoA-I molecules are precursors in vivo to medium and large HDL particles containing three and four apoA-I molecules in nonhuman primates. *J. Lipid Res.* **40**, 1782-1792 (1999).
- 84) Segrest, J. P.; Cheung, M. C.; Jones, M. K. Volumetric determination of apolipoprotein stoichiometry of circulating HDL subspecies. *J. Lipid Res.* **54**, 2733-2744 (2013).
- 85) Kostner, G. M.; Laggner, P.; Prexl, H. J.; Holasek, A. Investigation of the abnormal low-density lipoproteins occurring in patients with obstructive jaundice. *Biochem. J.* **157**, 401-407 (1976).
- 86) Manzato, E.; Fellin, R.; Baggio, G.; Walch, S.; Neuback, W.; Seidel, D. Formation of lipoprotein-X. Its relationship to bile compounds. *J. Clin. Invest.* **57**, 1248-1260 (1976).
- 87) O, K.; Frohlich, J. Role of lecithin: cholesterol acyltransferase and apolipoprotein A-I in cholesterol esterification in lipoprotein-X in vitro. *J. Lipid Res.* **36**, 2344-2356 (1995).
- 88) Kodama, T.; Akanuma, Y.; Okazaki, M.; Aburatani, H.; Itakura, H.; Takahashi, K.; Sakuma, M.; Takaku, F.; Hara, I. Abnormalities in plasma lipoprotein in familial partial lecithin: cholesterol acyltransferase deficiency. *Biochim. Biophys. Acta* **752**, 407-415 (1983).
- 89) Caste, A.; Patterson, J. C.; Jones, M. K.; Jerome, W. G.; Bashtovyy, D.; Su, Z.; Gu, F.; Chen, J.; Aliste, M. P.; Harvey, S. C.; Li, L.; Weinstein, G.; Segrest, J. P. Novel changes in discoidal high density lipoprotein morphology: a molecular dynamics study. *Biophys. J.* **90**, 4345-4360 (2006).
- 90) Knott, T. J.; Pease, R. J.; Powell, L. M.; Wallis, S. C.; Rall, S. C. Jr.; Innerarity, T. L.; Blackhart, B.; Taylor, W. H.; Marcel, Y.; Milne, R. *et al.* Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature* **328**, 734-738 (1986).
- 91) Yang, C. Y.; Chen, S. H.; Gianturco, S. H.; Bradley, W. A.; Sparrow, J. T.; Tanimura, M.; Li, W. H.; Sparrow, D. A.; DeLoof, H.; Rosseneu, M. *et al.* Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature* **323**, 738-742 (1986).
- 92) Law, S. W.; Grant, S. M.; Higuchi, K.; Hospattankar, A.; Lackner, K.; Lee, N.; Brewer, H. B. Jr. Human liver apolipoprotein B-100 cDNA: complete nucleic acid and derived amino acid sequence. *Proc. Natl. Acad. Sci. USA* **83**, 8142-8146 (1986).
- 93) Chen, S. H.; Yang, C. Y.; Chen, P. F.; Setzer, D.; Tanimura, M.; Li, W. H.; Gotto, A. M. Jr.; Chan, L. The complete cDNA and amino acid sequence of human apolipoprotein B-100. *J. Biol. Chem.* **261**, 12918-12921 (1986).
- 94) Yang, C. Y.; Gu, Z. W.; Weng, S. A.; Kim, T. W.; Chen, S. H.; Pownall, H. J.; Sharp, P. M.; Liu, S. W.; Li, W. H.;

- Gotto, A. M. Jr.; Chan, L. Structure of apolipoprotein B-100 of human low density lipoproteins. *Arteriosclerosis* **9**, 96-108(1989).
- 95) Schumaker, V. N.; Phillips, M. L.; Chatterton, J. E. Apolipoprotein B and low-density lipoprotein structure: implications for biosynthesis of triglyceride-rich lipoproteins. In *Advances in Protein Chemistry* (Schumaker, V.N. ed) Vol 45, Academic Press, San Diego, pp. 205-248(1994).
- 96) McNamara, J. R.; Small, D. M.; Li, Z.; Schaefer, E. J. Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B. *J. Lipid Res.* **37**, 1924-1935(1996).
- 97) Tuzikov, F. V.; Tuzikova, N. A.; Galimov, R. V.; Panin, L. E.; Nevinsky, G. A. General model to describe the structure and dynamic balance between different human serum lipoproteins and its practical application. *Med. Sci. Monit.* **8**, MT79-MT88(2002).
- 98) Castro, G. R.; Fielding, C. J. Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein. *Biochemistry* **27**, 25-29(1988).
- 99) Lund-Katz, S.; Liu, L.; Thuahna, S. T.; Phillips, M. C. High density lipoprotein structure. *Front. Biosci.* **8**, d1044-d1054(2003).
- 100) Yokoyama, S.; Ueshima, H.; Miida, T.; Nakamura, M.; Takata, K.; Fukukawa, T.; Goto, T.; Harada-Shiba, M.; Sano, M.; Kato, K.; Matsuda, K. High-density lipoprotein levels have markedly increased over the past twenty years in Japan. *J. Atheroscler. Thromb.* **21**, 151-160(2014).
- 101) Yokoyama, S. Unique Features of High-Density Lipoproteins in the Japanese: In Population and in Genetic Factors. *Nutrients* **4**, 2359-2381(2015).
- 102) Furusyo, N.; Ai, M.; Okazaki, M.; Ikezaki, H.; Ihara, T.; Hayashi, T.; Hiramane, S.; Ura, K.; Kohzuma, T.; Schaefer, E. J.; Hayashi, J. Serum cholesterol and triglyceride reference ranges of twenty lipoprotein subclasses for healthy Japanese men and women. *Atherosclerosis* **231**, 238-245(2013).
- 103) Prospective Studies Collaboration; Lewington, S.; Whitlock, G.; Clarke, R.; Sherliker, P.; Emberson, J.; Halsey, J.; Qizilbash, N.; Peto, R.; Collins, R. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. *Lancet* **370**, 1829-1939(2007).
- 104) Anderson, D. W.; Nichols, A. V.; Pan, S. S. W.; Lindgren, F. T. High density lipoprotein distribution. Resolution and determination of three major components in normal population sample. *Atherosclerosis* **29**, 161-179(1978).
- 105) Levy, R. I. Cholesterol, lipoproteins, apoproteins, and heart disease: present status and future prospects. *Clin. Chem.* **27**, 653-662(1981).
- 106) Freedman, D. S.; Otvos, J. D.; Jeyarajah, E. J.; Shalurova, I.; Cupples, L. A.; Parise, H.; D'Agostino, R. B.; Wilson, P. W.; Schaefer, E. J. Sex and Age Differences in Lipoprotein Subclasses Measured by Nuclear Magnetic Resonance Spectroscopy: The Framingham Study. *Clin. Chem.* **50**, 1189-1200(2004).
- 107) Albers, J. J.; Cabana, V. G.; Hazzard, W. R. Immunoassay of human plasma apolipoprotein B. *Metabolism* **24**, 1339-1351(1975).
- 108) Ohta, T.; Hattori, S.; Murakami, M.; Nishiyama, S.; Matsuda, I. Age- and sex-related differences in lipoproteins containing apoprotein A-I. *Arteriosclerosis* **9**, 90-95(1989).
- 109) Kasezawa, N.; Tohyama, K.; Kiuchi, T.; Okazaki, M. Utility of lipoprotein profiling by high-performance liquid chromatography in metabolic syndrome-focused health check-up. *Ningen Dock* **24**, 673-680(2009).
- 110) Okazaki, M.; Miyake, S.; Yoshinaga, H.; Ai, M.; Toshima, G.; Kawamoto, R.; Shimokado, K. Relationship of cholesterol levels in LDL subclasses measured by gel permeation high-performance liquid chromatography and metabolic syndrome risk items in healthy population. *Ningen Dock* **25**, 644-651(2010).
- 111) Okazaki, M.; Usui, S.; Fukui, A.; Kubota, I.; Tomoike, H. Component Analysis of HPLC Profiles of Unique Lipoprotein Subclass Cholesterols for Detection of Coronary Artery Disease. *Clin. Chem.* **52**, 2049-2053(2006).
- 112) Usui, S.; Mizuno, T.; Okazaki, M.; Nakamura, M.; Sakurabayashi, I. Evaluation of a gel-permeation high-performance liquid chromatography for determining triglyceride levels in serum major lipoproteins, compared with the ultracentrifugation/precipitation method. *Clin. Biochem.* **42**, 114-117(2009).
- 113) Tsubakio-Yamamoto, K.; Sugimoto, T.; Nishida, M.; Okano, R.; Monden, Y.; Kitazume-Taneike, R.; Yamashita, T.; Nakaoka, H.; Kawase, R.; Yuasa-Kawase, M.; Inagaki, M.; Nakatani, K.; Masuda, D.; Ohama, T.; Matsuyama, A.; Nakagawa-Toyama, Y.; Ishigami, M.; Komuro, I.; Yamashita, S. Serum adiponectin level is correlated with the size of HDL and LDL particles determined by high performance liquid chromatography. *Metabolism* **61**, 1763-1770(2012).
- 114) Leino, A.; Impivaara, O.; Kaitsaari, M.; Jarvisalo, J. Serum concentrations of apolipoprotein A-I, apolipoprotein B, and lipoprotein(a) in a population sample. *Clin. Chem.* **41**, 1633-1636(1995).
- 115) Nordestgaard, B. G.; Nielsen, L. B. Atherosclerosis and arterial influx of lipoproteins. *Curr. Opin. Lipidol.* **5**, 252-257(1994).
- 116) Albers, J. J.; Wahl, P. W.; Cabana, V. G.; Hazzard, W. R.; Hoover, J. J. Quantitation of apolipoprotein A-I of human plasma high density lipoprotein. *Metabolism* **25**, 633-644(1976).