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Review

LipoSEARCH®; Analytical GP-HPLC method for lipoprotein profiling and its applications

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We developed a novel evaluation system to conveniently classify and quantify lipoproteins with high sensitivity using gel-permeation high-performance liquid chromatography (GP-HPLC, LipoSEARCH®), which is an alternative method to ultracentrifugation. In LipoSEARCH®, cholesterol and triglycerides (TG) levels of the major classes and subclasses of lipoproteins were determined by their component peak analyses on the basis of lipoprotein particle sizes with the Gaussian curve fitting technique, and the particle sizes of each lipoprotein were calculated by their retention times on a chromatogram. LipoSEARCH® exhibited good reproducibility and linearity on determinations of cholesterol and TG in lipoproteins, and strong relationships with other analytical methods such as ultracentrifugation.

We recently used LipoSEARCH® to analyze lipoprotein profiles from companion animal serum (LipoTEST®) and cell culture media (LipoCULTURE). Furthermore, we developed a unique service to assess the progress risk of metabolic syndrome and/or atherosclerosis by determining LDL particle size and small dense LDL-cholesterol (MetaboCHART®).

Key words: Gel-permeation HPLC, Lipoprotein profiling, Small dense LDL, Lipoprotein particle size, Cholesterol, Triglycerides

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Introduction

Serum-insoluble lipids circulate in the bloodstream as lipoproteins, which are macromolecular complexes of free cholesterol, cholesterol esters, triglycerides (TG), phospholipids, and apolipoproteins. The hydrophobic components, TG and cholesterol esters, at the lipoprotein core are packed by components with hydrophobic and hydrophilic regions such as free cholesterol, phospholipids, and apolipoproteins (Fig. 1). Lipoproteins are mainly produced in intestinal epithelial tissues and the liver, and have been separated into 4 major classes based on their particle size and density. Chyromicron (CM, >80 nm) is produced in the intestine, and consists of lipids, apoA-1, and apoB-48. When TG in CM is digested by lipoprotein lipase into free fatty acids and glycerol, CM is converted to CM remnants, which are rich in cholesterol and are transported to the liver through the lymphatic vessels. Very low density lipoprotein (VLDL, 30-80 nm) and low density lipoprotein (LDL, 16-30 nm) are mainly synthesized in the liver, and consist of lipids and apoB-100. These lipoproteins transport serum lipids to each tissue; therefore, high levels of these lipoproteins have been commonly found in the serum of dyslipidemic patients (including hypercholesterolemia, hypo-HDL cholesterolemia, and hypertriglyceridemia). High density lipoprotein with apoA-1 (HDL, 8-16 nm,) is an important lipoprotein for the collection and transport of excess serum cholesterol. High blood cholesterol or LDL-cholesterol, and low levels of HDL-cholesterol have associated with an increased risk of atherosclerotic disease [1-3]. Furthermore, small dense LDL, a subclass of LDL, was shown to be of clinical significance [4, 5]. These findings have demonstrated that both quantitative and qualitative evaluations on lipoproteins are more important in detecting the early stage of diseases and controlling human health.

Ultracentrifugation is most commonly used for the separation and fractionation of lipoproteins into their major classes from plasma or serum samples according to their density [6]; however, long-time separation and a large volume are necessary for ultracentrifugation, and the separated lipoproteins should be carefully collected from centrifugal tubes for accurate examination.

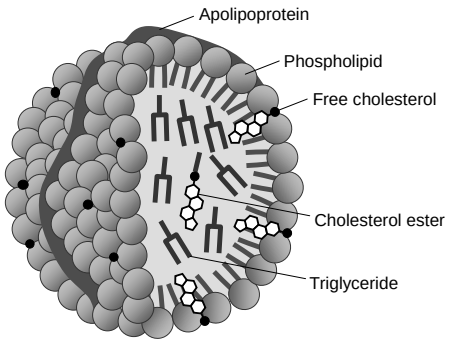


Fig. 1. General Schematic of the Structure of a Lipoprotein.

We previously developed a novel evaluation system for classifying and quantifying lipoproteins using gel-permeation high-performance liquid chromatography (GP-HPLC) as an alternative method to ultracentrifugation [7]. In this system, cholesterol and TG levels in lipoproteins could be simultaneously measured by splitting the column effluent equally into two lines following separation by gel permeation columns [8] (see ***Principles and system configuration of LipoSEARCH®***). We previously reported the good traceability of GP-HPLC to the reference methods, Abell-Kendall for total cholesterol, the ultracentrifugation (UC)/precipitation (heparin-$MnCl_2$) method for HDL-cholesterol [9], and the beta-quantification method for LDL-cholesterol levels [10]. Furthermore, LDL-TG levels measured by our GP-HPLC were comparable to those obtained from the UC/precipitation method [11]. Our developed method has been useful in the analysis of very small amounts and/or very low concentrations of lipoproteins from immunoaffinity-separated lipoprotein fractions [12], cell

culture media [13, 14], and plasma or serum from experimental small animals [15-18] and human subjects [19-21].

Herein, we report a novel analytical GP-HPLC system for lipoprotein profiling (LipoSEARCH®) and its applications.

Principles and system configuration of Lipo-SEARCH®

Figure 2 shows the configuration system of LipoSEARCH®. The system consists of a Shimadzu Prominence HPLC System (Shimadzu Inc., Japan); three pumps, an auto-sampler, a degasser, a column oven, a reactor, two UV-VIS detectors, and a system controller. The separation of lipoproteins and detection of cholesterol and TG were performed according to our previous method [8] with minor modifications.

Briefly, lipoproteins in whole plasma or serum (4 µl) were separated with tandemly connected SkylightPakLP1-AA gel permeation columns (Skylight Biotech Inc., Japan, 300 mm × 4.6 mm I.D.). 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 cholesterol reagent and TG reagent customized for LipoSEARCH® (Toyobo Inc., Japan), respectively. Absorbance at 550 nm was continuously monitored after each enzymatic reaction in two reactor coils (PTFE; 25 m × 0.18 mm I.D.). The particle sizes of each lipoprotein were determined by their retention times of the peaks observed on a chromatogram using a linear calibration curve [9]. Cholesterol and TG levels of the major classes and subclasses of lipoproteins were defined by component peak analyses on the basis of lipoprotein particle sizes with the Gaussian curve fitting technique (Fig. 3) [22, 23].

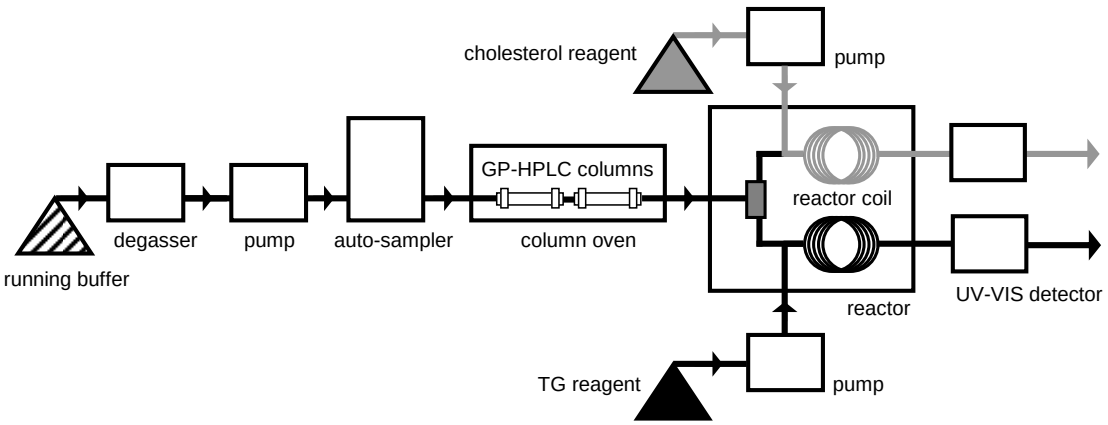


Fig. 2. Configuration System of LipoSEARCH®.

Arrows indicate the direction of flow. Injection volume: 4 µl; 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, 300 mm × 4.6 mm I.D.); column temperature: 25°C; reactor coil: PTFE., 25 m × 0.18 mm I.D.; reaction temperature: 37°C; UV-VIS detector λ: 550 nm.

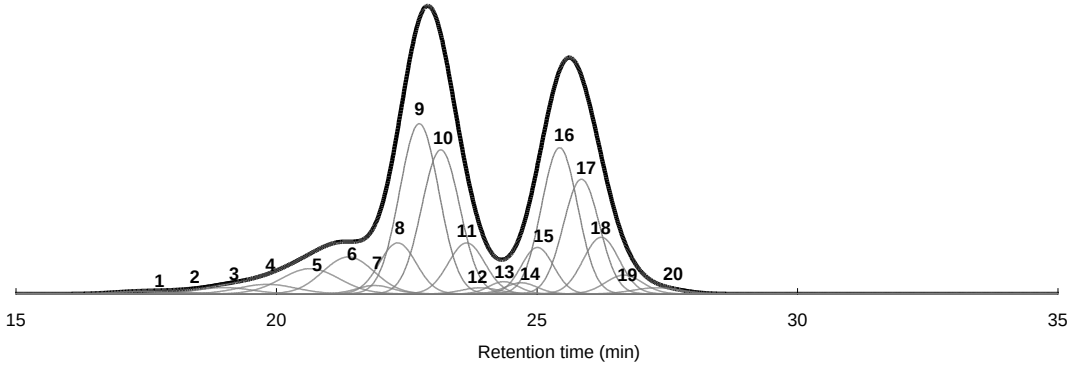


Fig. 3. Analysis Example by the Gaussian Curve Fitting Technique.

The black line shows the chromatogram detected by the enzymatic reaction for cholesterol. Gray lines are individual subclasses and their sum of Gaussian curves, which was determined by curve fitting using the Gaussian summation method. The number in the chromatogram is the component peak number of Gaussian curves.

Table 1. Definition of the Major Classes and Subclasses of Lipoproteins by LipoSEARCH®.

Component Peak No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Particle Diameter (nm)	>90	75	64	53.6	44.5	36.8	31.3	28.6	25.5	23.0	20.7	18.6	16.7	15.0	13.5	12.1	10.9	9.8	8.8	7.6
Subclass Name			L			M	S	L	M	S	VS			VL		L	M	S	VS	
Major Class	CM (>80 nm)		VLDL (30 - 80 nm)					LDL (16 - 30 nm)						HDL (8 - 16 nm)						

VL = very large; L = large; M = medium; S = small; VS = very small

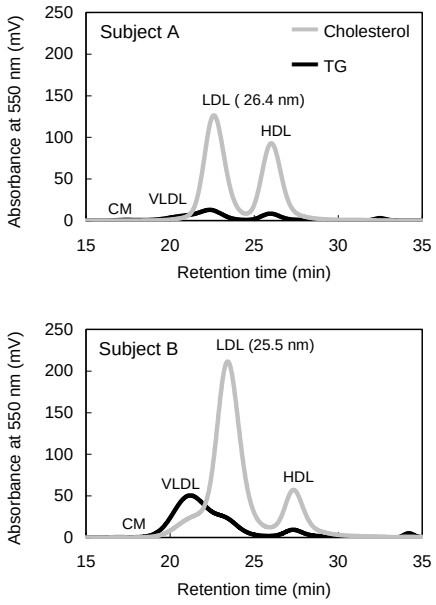


Fig. 4. Typical Chromatograms on LipoSEARCH® from a Normal Subject (A) and Dyslipidemic Patient (B).

Table 2. Typical Lipoprotein Profiles on LipoSEARCH® from a Normal Subject (A) and Dyslipidemic Patient (B).

		Subject A		Subject B	
Major Class	Subclass	Cholesterol (mg/dl)	TG (mg/dl)	Cholesterol (mg/dl)	TG (mg/dl)
CM		0.1	1.3	0.1	0.6
VLDL	Large	1.2	7.9	14.7	97.9
	Medium	1.1	5.6	10.5	46.6
	Small	6.6	5.1	10.3	16.6
LDL	Large	18.6	5.9	38.1	15.3
	Medium	32.5	7.5	57.2	15.2
	Small	19.4	3.7	37.3	9.1
	Very Small	8.4	1.6	27.5	8.1
HDL	Very Large	5.8	1.3	4.7	2.0
	Large	19.2	4.7	2.0	0.9
	Medium	22.5	5.0	11.3	5.4
	Small	9.5	1.8	16.4	7.2
	Very Small	5.1	1.5	10.7	4.9
Total		150.0	52.9	240.7	229.7

Plasma or serum lipoproteins were classified into 4 major classes and 12 subclasses (3 VLDL subclasses, 4 LDL subclasses, and 5 HDL subclasses) by their particle sizes on LipoSEARCH® (Table 1). Typical chromatograms on LipoSEARCH® from a normal subject (subject A) and dyslipidemic patient (subject B) and data on their major and subclasses are shown in Figure 4 and Table 2. VLDL-TG and LDL-cholesterol were significantly higher and HDL-cholesterol was lower in the serum of subject B than in the serum of subject A. Furthermore, increased levels of small dense LDL-cholesterol (the sum of small LDL-cholesterol and very small LDL-cholesterol) and smaller LDL particle sizes were obtained in the serum of subject B.

Validation of the analytical method

Reproducibility

We examined the reproducibility of LipoSEARCH® in four replicates/day for 5 days using serum-based standard material (Toyobo Inc., Japan). The results of reproducibility on lipoprotein analysis were summarized in Tables 3 and 4. Within-day imprecision CVs for lipoproteins were less than 4.1% for cholesterol and less than 1.7% for TG measurements, respectively. Within-day imprecision CVs for the particle sizes of LDL and HDL were less than 0.5%. Between-day imprecision CVs for lipoproteins were less than 4.5% for cholesterol and less than 4.3% for TG measurements, respectively. Between-day imprecision CVs for the particle sizes of LDL and

Table 3. Reproducibility of Cholesterol and TG Concentrations of each Major Lipoprotein and Cholesterol of Small Dense LDL.

	Concentration (mg/dl)					
	Within-Day			Between-Day		
	Mean	SD	CV(%)	Mean	SD	CV(%)
Cholesterol						
Total	213.5	0.6	0.3	213.3	1.9	0.9
CM	4.5	0.1	2.0	4.5	0.2	4.0
VLDL	48.3	0.9	1.8	48.0	1.1	2.2
LDL	102.2	0.3	0.3	101.8	0.9	0.9
Small dense LDL	33.4	1.4	4.1	33.7	1.5	4.5
HDL	58.5	0.2	0.3	59.0	1.1	1.9
TG						
Total	140.4	0.6	0.4	140.5	1.4	1.0
CM	6.7	0.1	1.7	6.8	0.3	4.3
VLDL	62.3	0.4	0.6	61.9	0.8	1.4
LDL	44.5	0.5	1.2	44.7	0.7	1.7
HDL	26.9	0.1	0.4	27.1	0.3	1.2

CV, coefficient of variation; SD, standard deviation

Table 4. Reproducibility of the Particle Sizes of LDL and HDL.

	Size (nm)					
	Within-Day			Between-Day		
	Mean	SD	CV(%)	Mean	SD	CV(%)
LDL particle size	26.2	0.1	0.5	26.2	0.2	0.6
HDL particle size	12.2	0.1	0.5	12.2	0.1	0.7

CV, coefficient of variation; SD, standard deviation

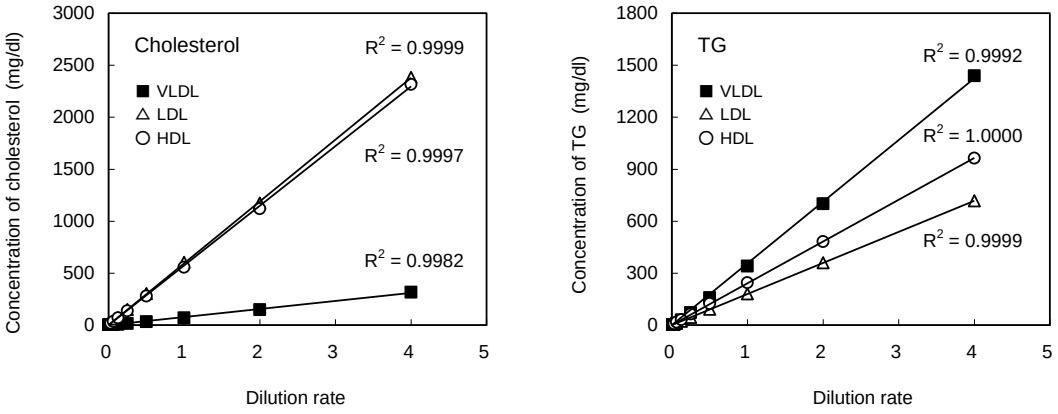


Fig. 5. Linearity of VLDL, LDL, and HDL Standard Materials.

HDL were less than 0.7%. These results indicated that LipoSEARCH® had high reliability for measurements of cholesterol, TG, and particle sizes in lipoproteins.

Linearity, limit of detection (LOD), and limit of quantification (LOQ)

The linearity of LipoSEARCH® was tested with 3 major lipoproteins, VLDL, LDL, and HDL standard material (prepared by ultracentrifugation, Calbiochem Inc., Germany). The high linearity between lipid concentrations in each major lipoprotein and dilution rate was confirmed (Fig. 5). Furthermore, limit of detection (LOD) and limit of quantification (LOQ) were calculated as below.

LOD = 3.3 * SD/S

LOQ = 10 * SD/S

where SD = standard deviation of the response calculated using the ASTM method of HPLC workstation software LCsolution (Shimadzu Inc., Japan), S = slope of the standard calibration curve.

LOD and LOQ values for both cholesterol and TG in each major lipoprotein were found to be less than 0.2 mg/dl and less than 0.5 mg/dl, respectively (data not shown).

Effect of interfering substances

The interfering effects of 5 substances, ascorbic acid (up to 50.0 mg/dl), free bilirubin (up to 19.1 mg/dl), conjugated bilirubin (up to 21.6 mg/dl), hemolytic hemoglobin (up to 494.0 mg/dl), or chyle (up to 1590 formazin turbidity unit) for LipoSEARCH® were investigated. The results showed that these substances did not interfere with the lipoprotein profiles in serum-based standard material (Kyowa Kirin Inc., Japan) (data not shown).

Applications

LipoTEST®, an evaluation of lipoprotein metabolism in companion animals

LipoSEARCH® requires a small amount of serum and is able to analyze serum lipoprotein profiles from many species of animals. Furthermore, we confirmed the strong relationships between LipoSEARCH® and other fractionation methods of lipoproteins such as ultracentrifugation, and used these advantages in clinical tests in the field of veterinary medicine, especially companion animals (termed LipoTEST®) [24].

The results obtained from over 5000 cases showed that dyslipidemia in dogs and cats could be classified into 4 types (Fig. 6). Type A is

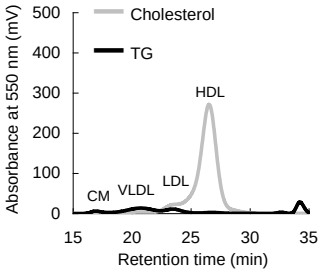
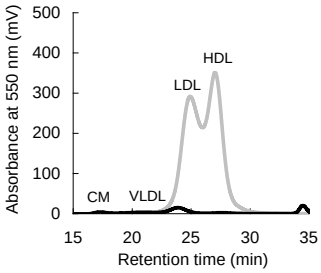
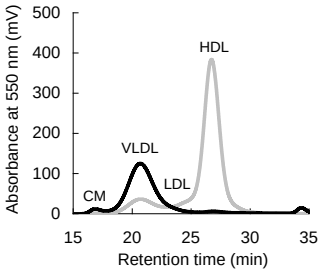
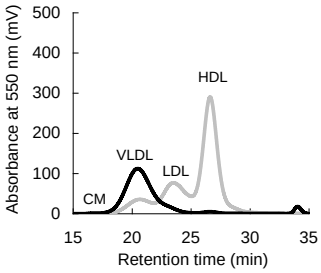
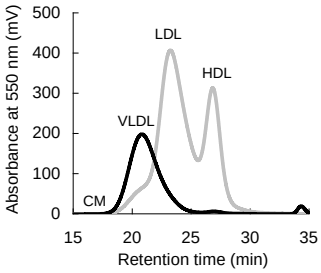
	Representative chromatogram	Lipoprotein metabolism abnormality / Related metabolic disease
Normal		
Type A		LDL-cholesterol ⬆ hypothyroidism * biliary sludge disease
Type B		VLDL-TG ⬆ diabetes * pancreatitis * fatty liver
Type C		LDL-cholesterol ⬆, VLDL-TG ⬆ hypercorticosteroidism diabetes * pancreatitis * biliary sludge disease fatty liver
Type D		LDL-cholesterol ⬆, VLDL-TG ⬆, LDL > HDL hypercorticosteroidism hypothyroidism diabetes * pancreatitis * biliary sludge disease * fatty liver

Fig. 6. Characteristic Patterns of LipoTEST®.

* Possible diseases.

typical hyper-LDL cholesterolemia. LDL-cholesterol in the serum of normal dogs and cats was shown to be low, whereas high levels of LDL-cholesterol were also found in this type. Type B is caused by insulin-resistance. The idiosyncrasy of type B is the high level of VLDL-TG, which is characteristic of insulin-resistance patients. Type C is a complication of type A and type B. Type D is hyper-LDL cholesterolemia, with the ratio of LDL-cholesterol and HDL-cholesterol being reversed by the progression of type C.

Serum lipoprotein analysis in LipoTEST® can be used to support treatments of various diseases, not only obesity and diabetes, but also endocrine diseases (hypothyroidism and hypercorticosteroidism), biliary sludge disease, fatty liver, and some dermatological diseases. In addition to conventional therapeutic guidance against every disease, better treatment management is enabled by performed therapy (for the purpose of improving lipid metabolism) based on the results of LipoTEST®. LipoTEST® is currently being used for the prevention, early checkup, and therapeutic management of diseases, and as an inspection item in companion animals.

LipoCULTURE, a novel screening system for antidyslipidemic agents using cultured cells

In developed countries, obesity, dyslipidemia, and a progressive increase in visceral adiposity due to excessive food intake has become a serious health problem. Experimental animals are commonly used in to discover drugs that can be used to treat dyslipidemia and obesity; however, studies using experimental animals are very expensive, and it is difficult to evaluate many test samples at one time. HepG2 human hepatoma cells can synthesize cholesterol and TG, pack them into lipoproteins, and release them into the culture medium [25]. We developed a novel *in vitro* assay system to screen antidyslipidemic agents by analyzing lipoprotein profiles from HepG2 cells (referred to as LipoCULTURE) [26].

We investigated the effects of two antidyslipidemic agents, simvastatin and fenofibrate, on lipoprotein release from HepG2 cells (Fig. 7). Simvastatin, which belongs to the statin group, is known to inhibit HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver, and was shown to reduce TG levels and raise HDL-cholesterol levels less than fibrates or niacin. This agent selectively sup-

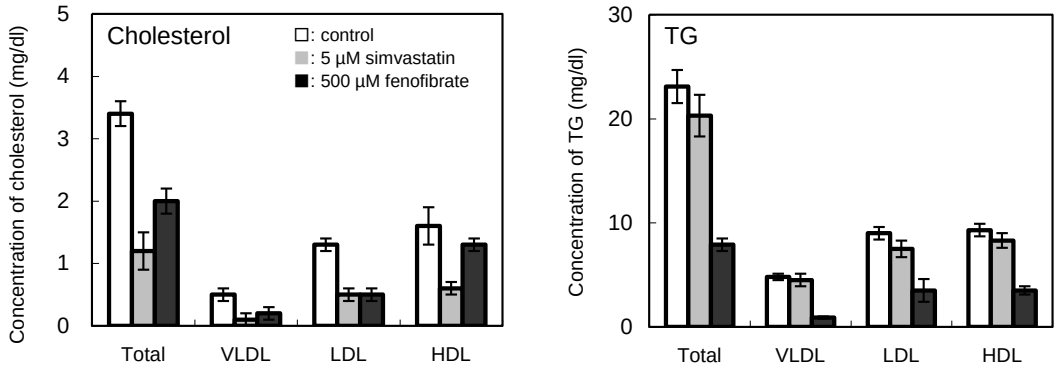


Fig. 7. Effects of Antidyslipidemic Agents on Cholesterol and TG Levels Released from HepG2 Cells.

Following HepG2 cell preculture in DMEM containing 10% (v/v) FCS for 2 days, cells were cultured in serum-free DMEM containing 0.1% BSA without (white bar) or with 5 μM simvastatin (gray bar) or 500 μM fenofibrate (black bar) for 4 days, and cholesterol and TG levels were determined in the culture medium. Data represent the mean ± SD (n = 4).

pressed cholesterol levels in VLDL and LDL from HepG2 cells without affecting the TG profile. Fibrates are PPAR activators and improve serum cholesterol and TG levels; therefore, these agents are typically used in combination with statins against dyslipidemic patients. In LipoCULTURE, fenofibrate reduced both cholesterol and TG levels from HepG2 cells in a clinical study.

Using LipoCULTURE, we screened the antidyslipidemic activities of foodstuffs in Akita [27]. The results showed that the ethanol extract of *Brasenia schreberi* (BSET) markedly suppressed lipid release from HepG2 cells and the expression of lipogenic enzymes at the mRNA level. Furthermore, the antidyslipidemic effects of BSET were confirmed in studies using dyslipidemic mice and clinical tests. These findings suggest that antidyslipidemic activities *in vitro* on LipoCULTURE were consistent with the *in vivo* test, and this system is very useful to screen for the antidyslipidemic activities of crude drugs and food materials.

MetaboCHART®, application to the physical checkup

Our recent study demonstrated that visceral fat accumulation (VFA) was closely linked to an increase in small dense LDL-cholesterol (the sum

Table 5. Comparison of Clinical Data in 2 Subjects with Similar Serum Cholesterol Levels and Different VFA.

	Subject C	Subject D
BMI (kg/m^2)	29.1	30.8
VFA (cm^2)	83.2	209.1
Total cholesterol (mg/dl)	225.5	224.7
LDL-cholesterol (mg/dl)	129.4	117.8
Small dense LDL-cholesterol (mg/dl)	22.0	48.4
LDL particle size (nm)	26.6	24.7

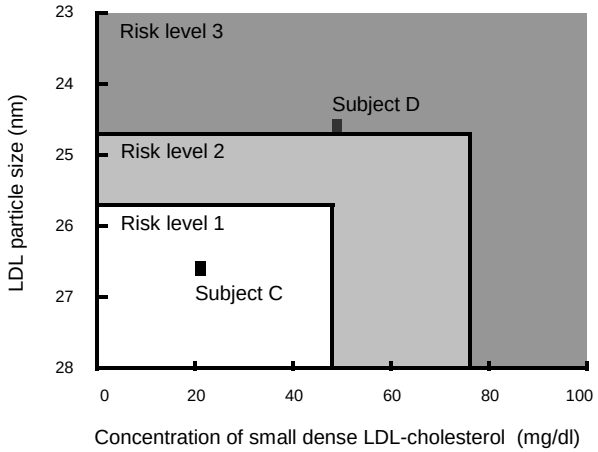


Fig. 8. Evaluation of the Progress Risk of Metabolic Syndrome and/or Atherosclerosis by MetaboCHART®.

of small LDL-cholesterol and very small LDL-cholesterol) [22]. Therefore, we examined the lipoprotein profiles of over 1,000 adults in a clinical survey, and found that both small dense LDL-cholesterol levels and LDL particle sizes were strongly associated with the progression of metabolic syndrome and/or atherosclerosis. On the basis of the findings of these clinical tests, we initiated novel clinical service (termed Metabo-CHART®), which evaluates the progress risk of metabolic syndrome and/or atherosclerosis by analyzing serum small dense LDL-cholesterol levels and LDL particle sizes. Figure 8 shows the evaluation standard on MetaboCHART®. Risk level 1 indicates a normal condition, in which the risk of metabolic syndrome is low. Risk level 2 indicates metabolic syndrome that requires monitoring for an atherosclerosis-related disease. Subjects in risk level 3 may be patients with progressed metabolic syndrome that are at high risk of atherosclerosis-related disease. Table 5 shows test examples of MetaboCHART®. BMI, total cholesterol, and LDL-cholesterol were similar in subjects C and D; however, subject C and D were judged to be in risk levels 1 and 3, respectively, because markedly higher small dense LDL-cholesterol levels and smaller LDL particle sizes were confirmed in subject D with a 2.5-fold higher VFA than in subject C (Fig. 8).

MetaboCHART® detects the early stage of metabolic syndrome in low risk subjects by analyzing blood samples only, and close inspection on the progress degree of diseases is enabled by a combination of MetaboCHART® and existing clinical tests such as the measurement of visceral fat with abdominal CT or examination of atherosclerosis by carotid artery echo.

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