Widely-targeted quantitative lipidomics method by supercritical fluid chromatography triple quadrupole mass spectrometry

Hiroaki Takeda,1,8 Yoshihiro Izumi,1,8 Masatomo Takahashi,8 Thanai Paxton,1 Shohei Tamura,8 Tomonori Koike,8 Ying Yu,8 Noriko Kato,1 Katsutoshi Nagase,1 Masashi Shiomi,8 and Takeshi Bamba2,9

Division of Metabolomics,9 Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan; Nihon Waters K.K.,† Shinagawa-ku, Tokyo 140-0001, Japan; and Institute of Experimental Animals,3 Kobe University Graduate School of Medicine, Chuo-ku, Kobe 650-0017, Japan

ORCID IDs: 0000-0003-4608-4652 (Y.I.); 0000-0002-7442-2738 (T.B.)

Abstract Lipidomics, the mass spectrometry-based comprehensive analysis of lipids, has attracted attention as an analytical approach to provide novel insight into lipid metabolism and to search for biomarkers. However, an ideal method for both comprehensive and quantitative analysis of lipids has not been fully developed. Here, we have proposed a practical methodology for widely targeted quantitative lipidomic analysis using supercritical fluid chromatography fast-scanning triple-quadrupole mass spectrometry (SFC/QqQMS) and theoretically calculated a comprehensive lipid multiple reaction monitoring (MRM) library. Lipid classes can be separated by SFC with a normal-phase diethylamine-bonded silica column with high resolution, high throughput, and good repeatability. Structural isomers of phospholipids can be monitored by mass spectrometric separation with fatty acyl-based MRM transitions. SFC/QqQMS analysis using an internal standard-dilution method offers quantitative information for both lipid class and individual lipid molecular species in the same lipid class. Additionally, data acquired using this method has advantages, including reduction of misidentification and acceleration of data analysis. Using the SFC/QqQMS system, alteration of plasma lipid levels in myocardial infarction-prone rabbits to the supplementation of EPA was first observed. Our developed SFC/QqQMS method represents a potentially useful tool for in-depth studies focused on complex lipid metabolism and biomarker discovery.—Takeda, H., Y. Izumi, M. Takahashi, T. Paxton, S. Tamura, T. Koike, Y. Yu, N. Kato, K. Nagase, M. Shiomi, and T. Bamba. Widely-targeted quantitative lipidomics method by supercritical fluid chromatography triple quadrupole mass spectrometry. J. Lipid Res. 2018, 59: 1283–1293.

Supplementary key words quantitative analysis • multiple reaction monitoring • phospholipids • sphingolipids • neutral lipids • omega-3 fatty acids

Imbalance of lipid metabolites in vivo is believed to affect the onset of serious diseases such as dyslipidemia and resultant atherosclerosis (1, 2). In recent years, cohort studies using metabolomics have also helped discover reliable biomarkers (3, 4). Accordingly, the development of a novel lipidomic analytical system that enables the acquisition of...
comprehensive and quantitative information of individual lipid molecular species is needed for identification and validation of potential biomarkers across multiple specimens and different equipment and/or facilities (5). Recently, chromatographic and/or tandem mass spectrometric analytical approaches for lipidomics have been proposed (6). Features of representative lipidomic methodologies are summarized in supplemental Table S1.

The shotgun lipidomics approach, using direct infusion tandem mass spectrometry (DI/MS/MS), is often used to rapidly quantitate lipid molecular species in a short time (7–9). Each lipid level can be quantitated using mass spectrometry because the ion-suppression and/or ion-enhancement effects of the biological matrix can be normalized by adding the appropriate internal standards of each lipid class (e.g., chemically synthesized lipid standards not detected in vivo or stable isotope-labeled lipid standards). However, it is difficult to identify some isomeric compounds due to coelution, and low abundant lipid molecular species are poorly detected because of strong ionization suppression. In-source fragmentation caused by DI/MS/MS analysis can also confound lipid identification and quantification.

LC/MS/MS techniques have been developed to identify and quantitate lipid molecular species. The advantages of LC/MS/MS methods over DI/MS/MS are as follows: i) improvement of detection sensitivity, since ESI mass spectrometry (ESI-MS) is a concentration-sensitive device (10); ii) decrease in the effects of the biological matrix by liquid chromatography (LC) separation; and iii) consequently, an increase in the number of lipid detected. Reverse-phase LC (RPLC) is a widely used separation technique of lipid molecular species based on the hydrophobic interaction between nonpolar side chains of C18 particles and hydrophobic fatty acyl chains of lipids. RPLC can enhance the chromatographic resolution of a wide range of lipids including some isomers (11, 12). However, biological matrix effects cannot be normalized in all detected peaks, because it is not possible to prepare appropriate internal standards corresponding to all the detected peaks. In contrast, normal-phase LC MS/MS (NPLC/MS/MS) can be used to separate each lipid class according to the polar head group (13–15). In principle, the NPLC/MS/MS approach can lead to reliable identification and quantification of each lipid class and some of individual lipid species by the addition of appropriate internal standards. However, the mobile phase of NPLC (e.g., hexane, isopropanol, and chloroform) causes a reduction in ionization efficiency. Thus, the sensitivity of lipid molecular species is decreased. Instead, hydrophilic interaction chromatography MS/MS (HILIC/MS/MS)-based lipidome analysis has been applied to overcome this limitation (16–21). Separation behavior of HILIC for lipidomics is similar to that of NPLC. The sensitivity of HILIC/MS/MS is higher than that of NPLC/MS/MS because HILIC/MS/MS uses ESI-compatible solvents (e.g., acetonitrile, methanol, and water) as the mobile phase. However, since HILIC methods have insufficient retention and separation for nonpolar lipids [e.g., cholesteryl ester (CE), diacylglycerol (DAG), and triacylglycerol (TAG)] (18), HILIC methods are mainly applied to polar lipids such as glycerophospholipids and sphingolipids (16, 17, 19–21). In order to carry out HILIC/MS/MS-based lipidome analysis with high repeatability of retention times (RTs) and peak areas, long equilibration times are also required to form a water-rich layer on the surface of the polar stationary phase (17–21).

A supercritical fluid (SCF), kept beyond its critical temperature and pressure, is defined as a high-density and noncondensable fluid. SCF chromatography (SFC) is a chromatographic separation technique that uses an SCF as a mobile phase. Supercritical carbon dioxide (SCCO2) is the most frequently used mobile phase for SFC, because carbon dioxide can easily be converted to its supercritical state (critical temperature, 31.1°C; critical pressure, 7.38 MPa) and exhibits favorable properties such as being nonflammable, chemically inert, relatively nontoxic, easy to handle, and inexpensive. Moreover, like n-hexane, SCCO2 has relatively low polarity, and the polarity of the mobile phase in SFC can be changed considerably by adding a polar organic solvent such as methanol as a modifier. In previous work, we have presented that SFC/MS/MS with a reverse-phase column can be used for the analysis of global lipids without ESI-incompatible solvents (22–24). Very recently, Lisa and Holčapek reported that SFC coupled with a quadrupole TOF mass spectrometer (SFC/QTOFMS) system with a normal-phase silica-based column has been applied to perform high-throughput lipidome analysis (25). Lisa and Holčapek also revealed that lipid class separation using SFC would be superior to conventional lipid class separation methods (i.e., NPLC and HILIC) in terms of short analysis time and chromatographic resolution for a wide variety of lipids. However, SFC separation efficiency of individual lipid classes, chromatographic and mass spectrometric separation of individual lipid species, including positional or structural isomers, and quantitative evaluation have not been fully investigated. It is necessary to further investigate chromatographic and mass spectrometric separation for quantification of a wide range of lipids.

Multiple reaction monitoring (MRM) using triple-quadrupole mass spectrometry (QqQMS) has highly sensitive and selective quantitative performance, allowing for reliable monitoring of low-abundance lipids. Furthermore, mass spectrometric separation of structural isomers [e.g., phosphatidylcholine (PC) 16:0–20:4 and PC 18:2–18:2] can be provided using fatty acyl-based MRM transitions. In lipidome analysis, fragmentation was induced regularly based on the lipid class and FA side chain. A rapid development of fast-scanning QqQMS has also contributed to conduct multitargeted MRM transition settings (i.e., large-scale MRM mode) for simultaneous analysis of several hundred lipids (namely, widely targeted lipidomics) (26, 27).

In the present study, we aimed to develop an advanced methodology for widely targeted quantitative analysis of individual lipid molecular species of 22 lipid classes, including lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), lysophosphaticid acid (LPA), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), PC, alkyl-acyl PC, alkenyl-acyl
PC, phosphatidylethanolamine (PE), alkanyl-acyl PE, phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), SM, ceramide (Cer), CE, monoacylglycerol (MAG), DAG, TAG, and FFA, in complex biological samples using SFC coupled with fast-scanning QqQMS (SFC/QqQMS) and theoretically calculated a comprehensive lipid MRM library (Fig. 1). Based on this analytical method, we investigated the effect of administration of EPA on the plasma lipid compositions using myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits (28, 29).

MATERIALS AND METHODS

Chemicals and reagents

Ammonium acetate was obtained from Sigma-Aldrich Co. (St. Louis, MO). Distilled water (LC/MS grade) and methanol (LC/MS grade) were purchased from Kanto Chemical Co., Ltd (Tokyo, Japan). HPLC-grade chloroform was obtained from Kishida Chemical (Osaka, Japan). All synthetic lipid standards were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) except for FA 17:0 (Sigma-Aldrich Co.). Carbon dioxide (99.5% grade, Yoshida Sanso Co., Ltd., Fukuoka, Japan) was used as the SFC mobile phase. Supplemental Table S2 lists the FA composition used to construct an in-house lipid MRM library.

Animals

All animal procedures were approved by the Kobe University Animal Care and Use Committee and were conducted in accordance with the Regulations for Animal Experimentation of Kobe University, Act on Welfare and Management of Animals (Law No. 105, 1973, revised in 2006), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88, 2006), and Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the Jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (Notice No. 71, 2006). Twelve WHHLMI rabbits (10.2 ± 0.4 months old) were used. The rabbits were housed individually in metal cages in a room with constant temperature (22 ± 2°C) and a constant lighting cycle (12 h light/dark). EPA was suspended using a 0.5% carboxymethyl cellulose suspension, and 300 mg per weight (kg) of EPA was administered to six WHHLMI rabbits orally every day. Another set of six WHHLMI rabbits (placebo group) was administered only a 0.5% carboxymethyl cellulose suspension. After administration, the rabbits were fed standard rabbit chow (120 g per day; CR-3, Clea, Tokyo, Japan) and water ad libitum.

Rabbit plasma sample preparation

Blood was collected from the auricular central artery of the 12 WHHLMI rabbits, who had undergone a period of fasting of more than 12 h. Lipid extraction from rabbit plasma was performed using Bligh and Dyer’s method with minor modifications.
(30). Before lipid extraction, a dodecanoyl- or heptadecanoyl-based synthetic internal standard mixture was added to samples for quantification of the endogenous lipid molecular species (31). The concentrations of internal standard mixture spiked into the plasma were as follows: 1.6 µM (SM d18:1–17:0 and Cer d18:1–17:0); 8 µM (LPC 17:0); 16 µM (MAG 17:0, DAG 12:0–12:0, and TAG 17:0–17:0–17:0); 130 µM (PE 17:0–17:0); 160 µM (LPE 17:1, PC 17:0–17:0, CE 17:0, and FFA 17:0); 320 µM (PG 17:0–17:0); 450 µM (PA 17:0–17:0); 1,200 µM (LPG 17:1); 1,300 µM (LPl 17:1 and PS 17:0–17:0); and 1,600 µM (LPA 17:0 and LPS 17:1). In short, 20 µl of plasma was mixed with 30 µl of internal standard mixture and 1,000 µl of methanol/chloroform/water (10:5:3, vol/vol/vol) and vortexed at the maximum setting for 1 min. The plasma sample was further extracted by ultrasonication for 5 min before centrifugation at 16,000 g for 5 min at 4°C. The supernatant (700 µl) was transferred to clean tubes. After adding 195 µl of chloroform and 195 µl of water, phase separation of aqueous and organic layers was performed by centrifugation at 16,000 g for 3 min at 4°C. Aliquots (100 µl) of the organic layer were transferred to clean tubes. The plasma lipid extract (100 µl) for WHHLMI rabbit was diluted to a final volume of 400 µl with a solution of 2:1 (vol/vol) methanol/chloroform for SFC/QqQMS analysis. A quality control (QC) sample (240 µl) was prepared by mixing equal amounts (10 µl each) of 24 plasma extracts.

Flow injection conditions

An SFC/QqQMS system was composed of an ACQUITY Ultra-Performance Convergence Chromatography (UPC2) system and a Xevo TQS micro triple-quadrupole mass spectrometer with an ESI ion source (Waters, Milford, MA). In order to enhance ionization efficiency, HPLC 515 pump (Waters) was used as a make-up pump. The SFC and QqQMS systems and data acquisition were controlled by MassLynx software version 4.1 (Waters). Standard solutions (1 µM) of each synthetic lipid were prepared in methanol/chloroform (2/1, vol/vol). MRM transition settings for each lipid class were performed using flow injection. The SFC flow injection conditions were as follows: injection volume, 2 µl; mobile phase (A), SCCO2; mobile phase (B) (modifier) and make-up pump solvent; methanol with 0.1% (wt/vol) ammonium acetate; flow rate of mobile phase, 1.0 ml min⁻¹; flow rate of make-up pump, 0.1 ml min⁻¹; modifier condition; 40% B (isocratic condition); temperature of column manager, 40°C; temperature of sample manager, 10°C; active back pressure regulator (ABPR), 1,500 pounds per square inch (psi); analytical time, 1 min. QqQMS analytical conditions were as follows: capillary voltage, 3.0 kV; desolvation temperature, 500°C; cone gas flow rate, 50 lh⁻¹; and desolvation gas flow rate, 1,000 lh⁻¹. The MRM parameters per one time period were as follows: limit on number of MRM transitions, 150; dwell time, 1 ms; MS inter-scan and interchannel delay, 2 ms; and polarity switch interscan, 15 ms.

Method validation

For the creation of calibration curves of each lipid, their standard solutions were prepared at the following concentrations: 0, 1, 5, 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000, and 100,000 nM, each with the internal standard of 22 nM (SM d18:1–17:0 and Cer d18:1–17:0); 110 nM (LPC 17:0); 220 nM (MAG 17:0, DAG 12:0–12:0, and TAG 17:0–17:0–17:0); 1,800 nM (PE 17:0–17:0); 2,200 nM (LPE 17:1, PC 17:0–17:0, CE 17:0, and FFA 17:0); 4,400 nM (PG 17:0–17:0); 6,200 nM (PA 17:0–17:0); 16,000 nM (LPG 17:1); 18,000 nM (LPI 17:1 and PS 17:0–17:0); and 22,000 nM (LPA 17:0 and LPS 17:1).

Spike-and-recovery test

A standard spike-and-recovery test was carried out in optimal analytical conditions by adding the same amount of synthetic lipid standard as the actual amount in the representative plasma lipid extract for the WHHLMI rabbit (placebo group).

Data analysis for quantitation

Identification and quantification of lipid molecular species were performed using MassLyix software version 4.1. The quantitative values were calculated using the ratio of the chromatographic peak area of each analyte to that of the internal standard of its representative lipid class.

Biochemical analysis for total cholesterol

Total cholesterol level was measured using a Cholesterol E-Test Assay Kit (Wako Pure Chemical Industries, Ltd.) following the manufacturer’s instruction.

Statistical analysis

Statistical significance between baseline and 5 weeks after administration of EPA was determined using paired t-test (*P < 0.05, **P < 0.01, and ***P < 0.001). The difference of lipid profiles was examined by volcano plot which determined the relationship between fold-change and statistical significance.

RESULTS AND DISCUSSION

Overview of SFC/QqQMS system

A schematic diagram of the Waters SFC/QqQMS system used in this study is shown in supplemental Fig. S1. Recently, a modern analytical SFC system, namely, the Waters ACQUITY UPC2®, has been developed. The new SFC system benefits from a novel back pressure regulator design (i.e., ABPR) and is largely based on ultra-high-performance LC technology, including higher upper pressure limits and reduced void volume. Improved quantitative performance (accuracy, repeatability, robustness, etc.) using columns packed with sub-2 µm particles have broadened the bioanalytical applications (32). A new fast-scanning QqQMS, namely, the Waters Xevo TQS micro, has also been developed,
allowing for increased scan speed [maximum scan speed: previous QqQMS, approximately (approx.) 10,000 u/s; and Xevo TQ-S micro, 20,000 u/s] and large-scale MRM mode (minimum dwell time: previous QqQMS, approx. 5–10 ms; and Xevo TQ-S micro, 1 ms). Therefore, it is possible to conduct multitriggered MRM transition settings for simultaneous analysis of several hundred compounds.

In SFC/MS, SCCO₂ vaporizes at the inlet of the ESI-MS interface, and a small amount of organic solvent used as the modifier and make-up solvent is introduced into the ESI-MS. From a theoretical point of view, ionization/desorption efficiency in SFC/MS should be better than that in RPLC/MS, which uses an aqueous organic solvent (e.g., water/methanol). In our previous investigation, we confirmed that SFC/QqQMS showed much higher sensitivity than LC/QqQMS when the analytical conditions were fully optimized for SFC/QqQMS (33).

**Optimization of MRM transition settings**

Identification of the precursor ion and elucidation of the fragmentation patterns of each of the 22 lipid classes (LPC, LPE, LPG, LPA, LPI, LPS, PC, alkyl-acyl PC, alkenyl-acyl PC, PE, alkenyl-acyl PE, PG, PA, PI, PS, SM, Cer, CE, MAG, DAG, TAG, and FFA) were carried out by SFC flow injection analysis in both positive and negative ion modes. Precursor ions of each lipid were clearly observed in the injection analysis in both positive and negative ion modes. 

**Effect of modifier and make-up solvent on SFC lipid class separation and QqQMS detection**

The effect of mobile phase composition in SFC on peak shapes of targeted lipids was first evaluated by SFC analysis with a silica-based BEH column as the reference column because of the lack of functional groups. In the case of using 100% methanol with 0.1% (wt/vol) ammonium acetate, peak shapes of the polar lipids such as LPA, LPS, PA, and PS were broadened. On the other hand, their peak shapes were improved by the addition of 5% water to the modifier solvent (supplemental Fig. S2). It is known that use of a mixture of 5% water in methanol as the polar modifier led to an improvement in peak shape for hydrophilic compounds (e.g., flavonoid glycosides) because of increased analyte solubility and enhanced solvating power of the mobile phase (34). Thus, it is suggested that similar improvement in peak shape was observed in the above-mentioned relative polar lipid species. Using this water-containing modifier solvent, we then optimized the flow rate of the make-up solvent. The coupling with QqQMS consists of a double T with polyetheretherketone tubing with fixed dimensions. The first T enables the addition of make-up solvent to the column effluent to enhance ionization. In the second T, the flow is split between the ABPR and MS source in a ratio of approx. 9:1 (supplemental Fig. S1). In the initial solvent condition in SFC with the BEH column, the mobile phase consists of SCCO₂, and weakly retaining lipids such as CE are eluted under this condition. In order to enhance the ionization efficiency for CE, we investigated the flow rate of the make-up pump (supplemental Fig. S3A). As a result, the flow rate of the make-up pump was set to 0.2 ml·min⁻¹.

**Column screening for high-resolution SFC separation of lipid classes and their positional isomers**

A previous study described that SFC/QTOFMS system using a normal-phase silica-based BEH column (100 × 3.0 mm i.d., particle size: sub-1.7 μm, Waters) identified a total of 436 molecular species in porcine brain extract (25). However, lysosphospholipid positional isomers [e.g., LPC 16:0 (sn-1) and LPC 16:0 (sn-2)] and diacyl phospholipid structural isomers (e.g., PC 16:0–22:6 and PC18:2–20:4) were not identified in the previous methodology. To investigate the separation efficiency of each lipid class and some lipid isomers, including positional isomers (i.e., MAG, DAG, and lysosphospholipids), we used six different normal-phase columns including BEH, 2-EP, 2-PIC, 1-AA, DIOL, and DEA, which were developed based on 1.7 μm BEH particle technologies. First, we set the optimal SFC condition under the maximum system pressure limit (6,000 psi) in this SFC system. The initial modifier concentration was set to 1% in the first 1 min. From this isocratic condition, the peak shape of weakly retaining lipids such as CE was improved in all SFC conditions using each of the six different columns (supplemental Fig. S3B). Afterward, other lipid classes were eluted with gradient conditions. In SFC conditions using the DEA column, LPA had the strongest retention. Therefore, the modifier percentage was increased from 1% to 65%. Next, we checked the effect of column oven temperature on lipid class separation and system pressure. By raising the column oven temperature, the retention of each lipid class became slightly stronger, and system pressure decreased in all SFC conditions using each of the six different columns (supplemental Fig. S4A); however, lipid class separation did not improve dramatically. In this study, the optimal column oven temperature was set to 50°C. Subsequently, we investigated for the optimal ABPR condition. Separation of each lipid class and its peak shape showed the same behavior under each ABPR (1,500; 1,750; and 2,000 psi). Thus, we decided to set it to 1,500 psi, the minimum condition of ABPR. Finally, we investigated the best flow rate conditions (supplemental Fig. S4B). In the case of 1.0 ml·min⁻¹ flow rate in the SFC using the DEA column, LPA was eluted within 20 min, and the system pressure was kept within the upper limit of the instrument (6,000 psi).
We examined the separation behavior of lipid classes using six different SFC columns under the same SFC condition (supplemental Table S4). Figure 2A shows the visualization of retention behavior of each lipid class. In the case of using the three types of columns (2-EP, 2-PIC, and 1-AA) with a benzene ring as a functional group, more hydrophobic lipids, such as CE and TAG, had a relatively stronger retention than the other columns (supplemental Table S4). In contrast, among screening columns, SFC separation with the DEA column had the best hydrophilic interaction with acidic polar lipids such as PI, PS, PA, LPI, LPS, and LPA. Furthermore, peak width for these acidic polar lipids was improved to a greater extent in SFC separation using the DEA column rather than using the BEH column (Fig. 2B). One of the most plausible reasons for this result is that the DEA stationary phase, which has strongly basic properties, contributed to the retention and separation of acidic polar lipids. By using the DEA column, baseline separation of positional isomers such as lysophospholipids (e.g., LPC, LPE, and LPG) and neutral lipids (e.g., MAG and DAG) were also obtained (Fig. 3). Lysophospholipids are known as bioactive molecules, precursors, and intermediates in the metabolism of glycerophospholipids and sphingolipids. However, the difference in function between 1-acyl-2-lysophospholipid and 2-acyl-1-lysophospholipid in vivo is not well known at the moment (35). Similarly, the differences in the roles of 1-acyl-2-MAG and 2-acyl-1-MAG or 1,2-acyl-DAG and 1,3-acyl-DAG have not been elucidated. Thus, our analytical method will be useful in future research for elucidation of the function and roles of these molecules. Based on these observations, we decided to use the DEA column for widely targeted lipidome analysis. SFC with the DEA column achieved good separation of internal standard mixture of 19 lipid classes within 20 min (Fig. 4A). In SFC separation using the DEA column, the minimum peak width for DAGs was less than 5 s (supplemental Fig. S5). In order to maintain 10 data points across a peak, MRM parameters, including dwell time, interscan and interchannel delay, and polarity switch interscan, were optimized (see Materials and Methods). High-performance chromatographic separation of lipid classes not only avoids ion-enhancement and/or ion-suppression effects among lipid classes, but also leads to improved compound identification and quantitative accuracy.

Discrimination of diacyl phospholipid structural isomers by fast-scanning QqQMS

Discrimination of diacyl phospholipid structural isomers with different FA side chains (e.g., PC 16:0–20:4 and PC 18:2–18:2) was subsequently investigated based on MS/MS separation. We validated the fragmentation patterns of each of the 22 lipid classes and determined the MRM transition(s) for identification and quantification of each lipid molecule (supplemental Table S3). The optimized MRM transitions for phospholipids including PC, PE, PG, PA, PI, and PS were set to the MS/MS fragments derived from their FA side chains in the negative ion mode. Based on the MRM transitions derived from FA side chains, PC 16:0–20:4 (840.6 > 255.3 and 840.6 > 303.2) and PC 18:2–18:2 (840.6 > 279.3) could be monitored individually. Identification of individual TAG molecular species could not be achieved by MRM transitions derived from neutral loss of FA side chains. Since TAG comprises three fatty acyl tails at the sn-1, -2, and -3 positions of the glycerol backbone, the coeluted TAG structural isomers cannot be monitored individually. For example, TAG 16:0–18:1–20:4 (898.8 >
577.5, 898.8 > 599.5, and 898.8 > 625.5) and TAG 16:0–18:2–20:3 (898.8 > 575.5, 898.8 > 601.5, and 898.8 > 625.5) have the same MRM transition as 898.8 > 625.5. Thus, individual TAG levels cannot be obtained by the lipid class-based separation method of SFC with the DEA column. In the present study, we quantified TAG molecular species by summing lipid levels of these structural isomers using the same precursor-to-product ion transition (i.e., TAG 54:5, 898.8 > 898.8) (supplemental Table S3). In order to obtain detailed molecular information of TAG with FA side chain information, reverse-phase-based separation in LC/MS/MS or SFC/MS/MS is recommended (36).

Widely targeted quantitative lipidomics platform using SFC/QqQMS with theoretically calculated comprehensive lipid MRM library

The SFC/QqQMS analytical system was optimized by the investigation of SFC column screening, SFC separation conditions, and MRM parameter settings. In addition, according to the MS/MS fragment patterns of each lipid class and major FA composition (supplemental Table S3), we conducted widely targeted MRM transition settings (supplemental Table S3). In general, MRM assays using QqQMS are selected for quantitative analysis of targeted compounds, including minor components. However, this approach has limitations, which are inherent to the targeted acquisition. In other words, MRM-based methods are a problem in terms of comprehensive and simultaneous lipid measurements. In the present study, an in-house lipid MRM library is utilized to perform comprehensive lipids measurements using fast-scanning QqQMS. We targeted 22 lipid classes with a variety of 23 FA side chains; thus, targeted lipid molecular species totaled approx. 2,500 compounds (supplemental Table S5). Based on the structurally representative MS/MS fragments derived from each lipid, we selected approx. 5,000 MRM transitions for the screening of lipids contained in biological samples used for research (supplemental Table S5). Our strategy of a widely targeted quantitative lipidomics methodology was as follows: i) preparation of a mixture consisting of small aliquots from each sample extract containing the lipid class-specific internal standards (QC sample); ii) selection of target lipids by complete screening of the QC sample using 16 different RT-based scheduled MRM analyses in which the QqQMS is programmed to monitor specific MRM transitions only during the expected RT window; and iii) targeted quantitative analysis of selected lipids in all samples by performing a selected RT-based scheduled MRM method (Fig. 1 and supplemental Fig. S6).

Method validation

We validated the method using lipids standard solutions. The calibration curves were made using the ratio of the chromatographic peak area of each lipid class to that of the corresponding internal standard. The result of the analytical validation is summarized in Table 1. From these results, each lipid class displayed good RT repeatability [relative SDs (RSDs) < 3.8%] and peak areas (RSDs < 13.0%). As shown in supplemental Table S6, our SFC/QqQMS method using the DEA column also gave excellent repeatability for RTs (RSDs < 5.4%) on five different days (interday variation). The SFC/QqQMS method showed linearity with correlation coefficients ($R^2$) over 0.9922. The limits of detections (LODs) for the 22 lipid classes were in the range of 5–1,000 fmol. By our SFC/QqQMS method, the LOD of PC and PE standards were found to be 50 and 10 fmol, respectively, and these sensitivities were 20- and 100-fold higher, respectively, than those in the SFC/QTOFMS full-scan method (supplemental Table S7). QTOFMS allows nontargeted lipidome analysis; however, the sensitivity
of each lipid class was lower than that in the QqQMS-based MRM assay. The use of QC samples and in-house lipid MRM library also allowed for efficient selection of target lipid molecules. Thus, our SFC/QqQMS-based approach could also achieve comprehensive and simultaneous analysis of lipids.

Previous studies demonstrated that the ionization efficiency of lipid molecular species was mainly dependent on their polar head groups and slightly dependent on their FA side chains (7, 37). Furthermore, there were no significant differences in the ionization efficiency of lipid molecular species differing in FA chain lengths and number of double bonds (31). Therefore, the lipid class-based separation improves the quantification of individual lipid species as the internal standard elutes together with the endogenous species. In order to investigate the quantitative accuracy, a standard spike-and-recovery test was carried out. We analyzed each lipid molecular species in WHHLMI rabbit plasma using the SFC/QqQMS system. Among commercially available chemically synthesized lipid standards, 14 standards of lipid molecular species contained in WHHLMI rabbit plasma were picked up for evaluation of the spike-and-recovery test (Table 1). Afterward, standard spike-and-recovery tests were carried out by adding the same amount of synthetic lipid standard as calculated for lipid levels in the plasma lipid extract. The calculated recoveries ranged from 64.9% to 103.5%, which were within the generally accepted range for quantification (Table 1). These results indicated that this lipidomics method using SFC/QqQMS could simultaneously achieve quantitative analysis with good repeatability and accuracy.

**Widely targeted and quantitative lipidome analysis of myocardial infarction-prone animal model by SFC/QqQMS system**

Dietary n-3 FAs are essential nutrients to prevent serious diseases, such as atherosclerosis. Many reports have described the association between n-3 FAs and prevention of atherosclerosis from the viewpoint of supplementation (38, 39). For example, administration of n-3 PUFAs such as EPA and docosahexaenoic acid decreased macrophage accumulation and expression of inflammatory molecules in apolipoprotein E KO mice. It was suggested that suppressive effects of n-3 PUFAs against plaque destabilization occurred (39). In the present study, we investigated the alteration of plasma lipid compositions by administration of EPA using the constructed analytical method. We used 12 WHHLMI rabbits (6 rabbits, placebo group; and 6 rabbits, EPA administration group). Supplemental Table S8 shows the age, sex, weight, and total plasma cholesterol levels in both placebo and EPA administration groups. No significant difference in body weight was observed, whereas total cholesterol levels significantly decreased (approx. 24%) by administration of EPA.

First, we performed lipid screening with the QC sample, which was derived from a mixture of equal amounts of the 24 plasma extracts. A total of 413 lipid molecular species were identified in plasma extracts of WHHLMI rabbits.
Based on the results of lipid screening, we selected MRM transitions and performed quantitative analysis in the 24 plasma extracts by the SFC/MS/MS with the selected RT-based scheduled MRM. Fig. 4B shows MRM chromatograms of the target lipid molecular species in WHHLMI rabbit plasma. From these results, in SFC separation using the DEA column, lipid species with the same FA composition but different head groups were fully resolved, but entire classes had some overlap (Table 1).

By using our novel analytical system, the alteration of each lipid class as well as individual lipid molecular species, including structural isomers of phospholipids by administration of EPA, could be quantitatively obtained. Figure 5A shows the plasma concentration of each lipid class in both the placebo and the EPA administration groups. The quantitative levels of plasma LPC, LPE, PC, alkenyl-acyl PC, PE, PI, DAG, and TAG were significantly decreased by administration of the EPA. To determine individual lipid levels, the PI, DAG, and TAG were significantly decreased by administration of the EPA. Therefore, a decrease in some lipid classes (i.e., LPC, LPE, PC, alkenyl-acyl PC, PE, PI, DAG, and TAG) was mainly due to a decrease in lipids with a linoleic acid side chain. This phenomenon may suggest that balance of the n-3 and -6 FAs changes with EPA supplementation.

CONCLUSIONS

In the present study, we have developed a practical methodology for widely targeted quantitative lipidome analysis using SFC/QqQMS and theoretically calculated a comprehensive lipid MRM library. Lipid classes can be separated by SFC using the DEA column with high resolution, high throughput, and good repeatability. The lipid class-based separation and multitetargeted MRM assay also improve the quantification of individual lipid species as the internal standard elutes together with the endogenous species. Our SFC/QqQMS-based analytical platform enabled us to quantitatively capture the changes of individual lipid classes as well as individual lipid molecular species, including positional and structural isomers in WHHLMI rabbit plasma. Quantitative analysis of comprehensive lipids in various biological samples (e.g., blood, cell, and organ) will enable comparison of measurements on different days, using different equipment, and/or facilities. Information of

| Lipid class | Ion mode | Analyte | Internal standard | RT for each analyte (min) | RSD (%) | RPA (%) | Linear range | Range of RTs | Spike-and-recovery test (%) |
|-------------|----------|---------|-------------------|--------------------------|---------|---------|--------------|-------------|--------------------------|
| LPC         | ESI+     | LPC 18:0| LPC 17:0          | 6.12                     | 0.09    | 2.7     | 50–50,000    | 0.9998      | 6.03–6.30 LPC 18:0        |
| LPE         | ESI+     | LPE 18:0| LPE 17:1          | 7.45                     | 0.07    | 1.73    | 50–100,000   | 0.9987      | 7.32–7.56 LPE 18:0        |
| LPG         | ESI+     | LPG 18:0| LPG 17:1          | 10.87                    | 0.08    | 8.18    | 50–100,000   | 0.9940      | 8.05–8.36 LPG 18:0        |
| LPA         | ESI−     | LPA 18:0| LPA 17:0          | 17.43                    | 0.57    | 8.97    | 1,000–100,000| 0.9942      | –                       |
| LPI         | ESI−     | LPI 18:0| LPI 17:1          | 14.02                    | 0.09    | 3.5     | 5–50,000     | 0.9966      | –                       |
| LPS         | ESI+     | LPS 18:0| LPS 17:1          | 15.36                    | 0.07    | 2.27    | 100–100,000  | 0.9999      | –                       |
| PC          | ESI−     | PC 18:0–18:2| PC 17:0–17:0 | 5.46                    | 0.08    | 4.35    | 50–50,000    | 0.9922      | 5.33–5.51 PC 16:0–20:4/77.7–3.5 |
| Alkenyl-acyl| PC (e)   | PC 16:0e–18:1| PC 16:0          | 5.40                    | 0.08    | 2.44    | 5–10,000     | 0.9993      | –                       |
| Alkenyl-acyl| PC (p)   | PC 18:0p–20:4| PC 17:0          | 5.45                    | 0.1    | 3.66    | 50–50,000    | 0.9777      | 5.33–5.45 PC 18:0p–20:4/78.0–10.4 |
| PE          | ESI−     | PE 18:0–20:4| PE 17:0          | 6.32                    | 0.11    | 3.09    | 10–50,000    | 0.9992      | 5.80–6.32 PE 18:0–20:4/101.9–17.0 |
| Alkenyl-acyl| PE (p)   | PE 18:0p–20:4| PE 17:0          | 6.22                    | 0.09    | 9.91    | 10–100,000   | 0.9976      | 6.05–6.33 PE 18:0p–20:4/95.8–17.3 |
| PG          | ESI−     | PG 16:0–18:1| PG 17:0–17:0    | 8.99                    | 0.08    | 1.72    | 5–50,000     | 0.9775      | –                       |
| PA          | ESI−     | PA 16:0–18:1| PA 17:0–17:0    | 12.43                   | 0.82    | 4.17    | 10–100,000   | 0.9856      | –                       |
| PI          | ESI−     | PI 18:0–20:4| PS 17:0–17:0    | 12.06                   | 0.21    | 2.66    | 100–50,000   | 0.994        | 11.84–11.98 PI 18:0–20:4/101.5–7.6 |
| PS          | ESI−     | PS 18:0–18:1| PS 17:0–17:0    | 12.23                   | 0.09    | 1.87    | 50–70,000    | 0.9959      | –                       |
| SM          | ESI+     | SM d18:1–18:0| SM d18:1–17:0   | 5.83                    | 0.09    | 2.56    | 10–10,000    | 0.9550      | 5.74–5.92 SM d18:1–18:0/64.9–3.0 |
| Cer         | ESI+     | Cer d18:1–18:0| Cer d18:1–17:0 | 4.48                    | 0.12    | 12.95   | 10–100,000   | 0.977    | 4.48–4.51 Cer d18:1–18:0/91.0–14.2 |
| CE          | ESI+     | CE 16:0   | CE 17:0          | 1.12                    | 1.81    | 11.32   | 50–50,000    | 0.9977      | 1.12–1.30 CE 16:0/67.2–5.7 |
| MAG         | ESI+     | MAG 16:0  | MAG 17:0         | 3.97                    | 0.18    | 3.49    | 500–100,000  | 0.9974      | 4.02–4.15 MAG 16:0/98.4–1.8 |
| DAG         | ESI+     | DAG 18:0–20:4| DAG 12:0–12:0   | 3.25                    | 0.14    | 8.56    | 5–10,000     | 0.9767      | 3.14–3.40 DAG 16:0–16:0/86.6–9.1 |
| TAG         | ESI+     | TAG 54:2  | TAG 51:0         | 1.61                    | 0.76    | 2.86    | 500–100,000  | 0.9992      | 0.97–1.76 TAG 54:2/72.8–5.0 |
| FFA         | ESI−     | FFA 18:0  | FFA 17:0         | 4.02                    | 0.11    | 1.18    | 500–100,000  | 0.9888      | 3.97–4.40 FFA 18:0/91.8–7.1 |

Supplementary Table S9. Based on the results of lipid screening, we selected MRM transitions and performed quantitative analysis in the 24 plasma extracts by the SFC/MS/MS with the selected RT-based scheduled MRM. Fig. 4B shows MRM chromatograms of the target lipid molecular species in WHHLMI rabbit plasma. From these results, in SFC separation using the DEA column, lipid species with the same FA composition but different head groups were fully resolved, but entire classes had some overlap (Table 1).

By using our novel analytical system, the alteration of each lipid class as well as individual lipid molecular species, including structural isomers of phospholipids by administration of EPA, could be quantitatively obtained. Figure 5A shows the plasma concentration of each lipid class in both the placebo and the EPA administration groups. The quantitative levels of plasma LPC, LPE, PC, alkenyl-acyl PC, PE, PI, DAG, and TAG were significantly decreased by administration of the EPA. To determine individual lipid levels, the PI, DAG, and TAG were significantly decreased by administration of the EPA. Therefore, a decrease in some lipid classes (i.e., LPC, LPE, PC, alkenyl-acyl PC, PE, PI, DAG, and TAG) was mainly due to a decrease in lipids with a linoleic acid side chain. This phenomenon may suggest that balance of the n-3 and -6 FAs changes with EPA supplementation.
endogenous amounts of lipids will also develop the biological function of lipid molecular species. Taking all of the present results into account, our developed analytical system is a promising new tool for in-depth studies on complex lipid metabolism, drug administration testing, and biomarker discovery.

REFERENCES

1. Ekoos, K., M. Jänis, K. Tarasov, R. Hurme, and R. Laaksonen. 2010. Lipidomics: a tool for studies of atherosclerosis. Curr. Atheroscler. Rep. 12: 273–281.

2. Takeda, H., T. Koike, Y. Isumi, T. Yamada, M. Yoshida, M. Shiomi, E. Fukusaki, and T. Bamba. 2015. Lipidomic analysis of plasma lipoprotein fractions in myocardial infarction-prone rabbits. J. Biosci. Bioeng. 120: 476–482.

3. Kettunen, J., T. Tukiainen, A. P. Sarin, A. Ortega-Alonso, E. Tikkanen, L. P. Lytikäinen, A. J. Kangas, P. Soininen, P. Wirtz, K. Silander, et al. 2012. Genome-wide association study identifies multiple loci influencing human serum metabolite levels. Nat. Genet. 44: 269–276.

4. Rhee, E. P., J. E. Ho, M. H. Chen, D. Shen, S. Cheng, M. G. Larson, A. Ghorbani, X. Shi, I. T. Helenius, C. J. O’Donnell, et al. 2013. A genome-wide association study of the human metabolism in a community-based cohort. Cell Metab. 18: 130–143.

5. Bowden, J. A., A. Heckert, C. Z. Ulmer, C. M. Jones, J. P. Koehmel, L. Abdullah, L. Ahonen, Y. Ahouazi, A. Armando, J. M. Asara, et al. 2017. Harmonizing lipidomics: NIST interlaboratory comparison exercise for lipidomics using standard reference material 1950 metabolites in frozen human plasma. J. Lipid Res. 58: 2275–2288.

6. Cäjka, T., and O. Fiehn. 2014. Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry. Trends Analit. Chem. 61: 192–206.

7. Han, X., and R. W. Gross. 2005. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipids directly from crude extracts of biological samples. Mass Spectrom. Rev. 24: 367–412.

8. Stahlin, M., C. S. Ejsing, K. Tarasov, J. Perman, J. Borén, and K. Ekoos. 2009. High-throughput shotgun lipidomics by quadrupole time-of-flight mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877: 2654–2672.

9. Heikans, L. A., M. Suonimi, H. X. Ta, K. Tarasov, and K. Ekroos. 2013. Long-term performance and stability of molecular shotgun lipidomic analysis of human plasma samples. Anal. Chem. 85: 8757–8763.

10. Hopfgartner, G., K. Bean, J. Henion, and B. Henry. 1993. Ion-spray mass spectrometric detection for liquid chromatography: a concentration- or a mass-flow-sensitive device? J. Chromatogr. A. 647: 51–61.

11. Yamada, T., T. Uchikata, S. Sakamoto, Y. Yokoi, E. Fukusaki, and T. Bamba. 2013. Development of a lipid profiling system using reverse-phase liquid chromatography coupled to high-resolution mass spectrometry with rapid polarity switching and an automated lipid identification software. J. Chromatogr. A. 1292: 211–218.

12. Tsugawa, H., T. Cäjka, T. Kind, Y. Ma, B. Higgins, K. Ikeda, M. Kanazawa, J. VanderGheynst, O. Fiehn, and M. Arita. 2015. MS-DIAL data-independent MS/MS deconvolution for comprehensive metabolome analysis. Nat. Methods. 12: 523–526.

13. Karlsson, A. Å., P. Michelsen, Å. Larsen, and G. Odham. 1996. Normal-phase liquid chromatography class separation and species determination of phospholipids utilizing electrospray mass spectrometry/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 10: 775–780.

14. Urán, S., Á. Larsen, P. B. Jacobsen, and T. Skotland. 2001. Analysis of phospholipid species in human blood using normal-phase liquid chromatography coupled with electrospray ionization ion-trap tandem mass spectrometry. J. Chromatogr. B Biomed. Sci. Appl. 758: 265–275.

15. Pang, L. Q., Q. L. Liang, Y. M. Wang, L. Ping, and G. A. Luo. 2008. Simultaneous determination and quantification of seven major phospholipid classes in human blood using normal-phase liquid chromatography coupled with electrospray mass spectrometry and the application in diabetes nephropathy. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 869: 118–125.

16. Schwabel-Herrmann, M., J. Willmann, and D. Leibfritz. 2010. Separation of phospholipid classes by hydrophilic interaction chromatography detected by electrospray ionization mass spectrometry. J. Chromatogr. A. 1217: 5179–5183.

17. Zhao, Y. Y., Y. Xiou, and J. M. Curtis. 2011. Measurement of phospholipids by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry: the determination of choline containing compounds in foods. J. Chromatogr. A. 1218: 5470–5479.

18. Chekkova, E., M. Holčapek, M. Liša, M. Ovčáčková, A. Lyčka, P. Lynen, and P. Sandera. 2012. Nontargeted quantitation of lipid classes using hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry with single internal standard and response factor approach. Anal. Chem. 84: 10064–10070.
Based on a diol column. J. Chromatogr. A. 2012: 26–34.

Okazaki, Y., Y. Kamide, M. Y. Hirai, and K. Saito. 2013. Plant lipidomics based on hydrophilic interaction chromatography coupled to ion trap time-of-flight mass spectrometry. Metabolomics. 9: 121–131.

Sonomura, K., S. Kudo, T. Sato, and F. Matsuda. 2015. Plasma lipid analysis by hydrophilic interaction liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J. Sep. Sci. 38: 2033–2037.

Bamba, T., N. Shimomish, A. Matsubara, K. Hirata, Y. Nakazawa, A. Kobayashi, and E. Fukusaki. 2008. High throughput and exhaustive analysis of diverse lipids by using supercritical fluid chromatography-mass spectrometry for metabolomics. J. Biochem. Biophys. 105: 460–469.

Uchikata, T., A. Matsubara, S. Nishiumi, M. Yoshida, E. Fukusaki, and T. Bamba. 2013. Supercritical fluid chromatography/Orbitrap mass spectrometry for metabolomics. J. Chromatogr. A. 1350: 257–242.

Liu, J., E. L. Regalado, I. Mergelsberg, and C. J. Welch. 2013. Extending the range of supercritical fluid chromatography by use of water-rich modifiers. Org. Biomol. Chem. 11: 4925–4929.

Koistinen, K. M., M. Suoniemi, H. Simolin, and K. Ekroos. 2015. Quantitative lysophospholipidomics in human plasma and skin by LC-MS/MS. Anal. Bioanal. Chem. 407: 5091–5099.

Lee, J. W., T. Nagai, N. Gotoh, E. Fukusaki, and T. Bamba. 2014. Profiling of regiosomeric triacylglycerols in edible oils by supercritical fluid chromatography/tandem mass spectrometry. J. Chromatogr. A. 1508: 138–147.

Han, X., and R. W. Gross. 1994. Electrospray ionization mass spectrometric analysis of human erythrocyte plasma membrane phospholipids. Proc. Natl. Acad. Sci. USA. 91: 10635–10639.

Connor, W. E. 2000. Importance of n-3 fatty acids in health and disease. Am. J. Clin. Nutr. 71: 1715–1755.

Takashima, A., D. Fukiya, K. Tanaka, Y. Higashikuni, Y. Hirata, S. Nishimoto, S. Yagi, H. Yamada, T. Soeki, T. Wakatsuki, et al. 2016. Combination of n-3 polyunsaturated fatty acids reduces atherosclerosis in apolipoprotein E-deficient mice by inhibiting macrophage activation. Atherosclerosis. 254: 142–150.

Shiomi, M., M. Ito, S. Yamada, S. Kawashima, and J. Fan. 2003. Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). Arterioscler. Thromb. Vasc. Biol. 23: 1239–1244.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917.

Jung, H. R., T. Sylvänne, K. M. Koistinen, K. Tarasov, D. Kauhanen, and K. Ekroos. 2011. High throughput quantitative molecular lipidomics. Biochim. Biophys. Acta. 1811: 925–934.

Nováková, L., A. G.-G. Perrenoud, I. Francois, C. West, E. Leselier, and D. Guillarme. 2014. Modern analytical supercritical fluid chromatography using columns packed with sub-2 µm particles: A tutorial. Anal. Chem. 824: 18–35.

Fujito, Y., Y. Hayakawa, Y. Izumi, and T. Bamba. 2017. Importance of optimizing chromatographic conditions and mass spectrometric parameters for supercritical fluid chromatography/mass spectrometry. J. Chromatogr. A. 1508: 138–147.

Okazaki, Y., Y. Kamide, M. Y. Hirai, and K. Saito. 2011. High throughput and comprehensive lipidomic analysis using ultra-high-performance supercritical fluid chromatography-mass spectrometry. Anal. Chem. 87: 7187–7195.

Tsugawa, H., M. Arita, M. Kanazawa, A. Ogwara, T. Bamba, and E. Fukusaki. 2013. MRMPROBS: a data assessment and metabolite identification tool for large-scale multiple reaction monitoring based widely targeted metabolomics. Anal. Chem. 85: 5191–5199.

Zhou, J., and Y. Yin. 2016. Strategies for large-scale targeted metabolomics quantification by liquid chromatography-mass spectrometry. Analyst. 141: 6362–6373.

Shiomi, M., and T. Ito. 2009. The Watanabe heritable hyperlipidemic (WHHL) rabbit: its characteristics and history of development: a tribute to the late Dr. Yoshio Watanabe. Atherosclerosis. 207: 1–7.