1 **Title**

2 Lipidomic analysis of plasma lipoprotein fractions in myocardial infarction-prone
3 rabbits

4

5 **Running title**

6 Lipidomic analysis of lipoprotein fractions

7

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29 **Keywords**

30 Lipidomics, Lipoprotein, Supercritical fluid chromatography mass spectrometry,
31 Coronary artery disease, Myocardial infarction-prone Watanabe heritable
32 hyperlipidemic rabbit, Phosphatidylethanolamine plasmalogen

33

34 **Footnotes**

35 The study represents a portion of the dissertation submitted by Hiroaki Takeda to Osaka
36 University in partial fulfillment of the requirement for his PhD.

37

38 **Abstract**

39 Lipids play important roles in the body and are transported to various tissues via
40 lipoproteins. It is commonly assumed that alteration of lipid levels in lipoproteins leads
41 to dyslipidemia and serious diseases such as coronary artery disease (CAD). However,
42 lipid compositions in each lipoprotein fraction induced by lipoprotein metabolism are
43 poorly understood. Lipidomics, which involves the comprehensive and quantitative
44 analysis of lipids, is expected to provide valuable information regarding the pathogenic
45 mechanism of CAD. Here, we performed a lipidomic analysis of plasma and its
46 lipoprotein fractions in myocardial infarction-prone Watanabe heritable hyperlipidemic
47 (WHHLMI) rabbits. In total, 172 lipids in plasma obtained from normal and WHHLMI
48 rabbits were quantified with high throughput and accuracy using supercritical fluid
49 chromatography hybrid quadrupole-Orbitrap mass spectrometry (SFC/Q-Orbitrap-MS).
50 Plasma levels of each lipid class (i.e., phosphatidylcholine, phosphatidylethanolamine,
51 phosphatidylinositol, lysophosphatidylcholine, lysophosphatidylethanolamine,
52 sphingomyelin, ceramide, triacylglycerol, diacylglycerol, and cholesterol ester, except
53 for free fatty acids) in 21-month-old WHHLMI rabbits were significantly higher than
54 those in normal rabbits. High levels of functional lipids, such as
55 alkyl-phosphatidylcholines, phospholipids including ω -6 fatty acids, and plasmalogens,
56 were also observed in WHHLMI rabbit plasma. In addition, high-resolution lipidomic
57 analysis using very low density lipoprotein (VLDL) and low density lipoprotein (LDL)
58 provided information on the specific molecular species of lipids in each lipoprotein
59 fraction. In particular, higher levels of phosphatidylethanolamine plasmalogens were
60 detected in LDL than in VLDL. Our lipidomics approach for plasma lipoprotein
61 fractions will be useful for in-depth studies on the pathogenesis of CAD.

62

63 INTRODUCTION

64 Lipids, which have a wide variety of structures with a hydrophilic head group and
65 hydrophobic fatty acid (FA) tails, play important roles in energy storage, the cell
66 membrane, and cellular signal transduction (1). Lipids such as triacylglycerol (TAG) are
67 stored in subcutaneous, internal, and intramuscular adipose tissues, and these lipids are
68 transported throughout the body in lipoprotein particles. Lipoproteins consist of a core
69 of hydrophobic lipids, including cholesterol ester (CE) and TAG, covered by a layer of
70 apoproteins and phospholipids such as phosphatidylcholine (PC),
71 phosphatidylethanolamine (PE), and sphingomyelin (SM), which allows them to be
72 transported in the aqueous environment of blood (2). Based on their lipid composition
73 and physical structure, these lipoproteins are classified as chylomicron (CM), very low
74 density lipoprotein (VLDL), low density lipoprotein (LDL), and high density
75 lipoprotein (HDL). VLDL and LDL are involved in the same metabolic pathway and
76 transport lipids from the liver to peripheral tissues. In contrast, HDL returns excess
77 lipids to the liver. In this manner, lipids play important roles in the body after being
78 transported to various tissues via lipoproteins.

79 Coronary artery disease (CAD), including myocardial infarction and cardiac angina,
80 is the major cause of death in developed countries. The basic pathogenesis of CAD
81 involves abnormal lipid metabolism and a maladaptive immune response associated
82 with chronic inflammation of the arterial wall (3). CAD has very specific symptoms
83 (e.g., angina) in the advanced stages but does not have any symptoms in the early stages.
84 Although low levels of HDL-cholesterol (HDL-Cho) and high levels of
85 LDL-cholesterol (LDL-Cho) are considered risk factors for CAD, reliable diagnostic
86 markers for measuring the severity of CAD have not been found. Additionally, the
87 regulatory mechanism of lipid metabolism in CAD remains unclear.

88 Recently, lipidomics, which is the mass spectrometry-based comprehensive study of
89 lipid molecular species, has been developed. Lipidomic analysis has attracted attention
90 not only for elucidating the cellular processes involved in health and diseases such as
91 cancer and atherosclerosis but also for enabling personalized medicine (4). In our
92 previous studies, we developed a lipidomic analytical system using supercritical fluid
93 chromatography mass spectrometry (SFC/MS) (5–7). We can achieve high-speed
94 analysis while maintaining the resolution of chromatography, thus enabling profiling of
95 hundreds of different lipid molecular species including glycerolipids (e.g.,
96 monoacylglycerol, MAG; diacylglycerol, DAG; and TAG), glycerophospholipids (e.g.,
97 lysophosphatidylcholine, LPC; lysophosphatidylethanolamine, LPE;
98 phosphatidylinositol, PI; PC; and PE), sphingolipids (e.g., ceramide, Cer and SM), and
99 sterol lipids (e.g., CE) in mouse plasma within only 20 min (7). Although a plasma or
100 serum lipidomics approach will be useful for biomarker discovery for CAD, the details
101 of the lipid metabolism including the role of each lipoprotein are poorly understood.
102 Because plasma contains various lipoproteins (CM, VLDL, LDL, and HDL), lipid
103 profiling of plasma samples currently provides only general information. Therefore, the
104 analysis of lipid class composition and species pattern of each lipoprotein (i.e.,
105 high-resolution lipidomic analysis) is expected to shed light on the pathogenic
106 mechanism of CAD. Recently, only a few groups reported studies using lipidomic
107 techniques for human lipoproteins of hyperlipidemic patients (8) or CAD patients (9), as
108 alteration of lipid levels in lipoproteins leads to cardiovascular disease. However,
109 because the only target lipids were phospholipids, the available lipidomic information
110 for lipoproteins is limited.

111 Pathological animal models are important for preclinical analyses that elucidate
112 pathological features, toxicity, and drug effects in detail. Myocardial infarction-prone

113 Watanabe heritable hyperlipidemic (WHHLMI) rabbits, which are developed by
114 selective breeding of Watanabe heritable hyperlipidemic (WHHL) rabbits, are an animal
115 model of familial hypercholesterolemia in humans (10). In previous studies, WHHLMI
116 rabbits were used to investigate the progression of coronary atherosclerotic lesions (11)
117 to observe the process of LDL oxidation with a radioiodinated short peptide probe
118 developed (12), and to investigate the relationship between mesenteric fat accumulation
119 and insulin resistance, hyperlipidemia and atherosclerosis (13). These studies revealed
120 that WHHLMI rabbits are potentially useful for the study of myocardial infarction.
121 However, to date, the levels of various lipid molecular species associated with
122 myocardial infarction in WHHLMI rabbits remains unclear. Therefore, it is expected
123 that high-resolution lipidomic analyses using lipoprotein fractions will be able to
124 elucidate the lipid metabolism in myocardial infarction. In the present study, we
125 therefore aimed to demonstrate the utility of high-resolution lipidomic analysis using
126 plasma lipoprotein fractions in WHHLMI rabbits.

127

128 MATERIALS AND METHODS

129 Chemicals and reagents

130 JIS special grade sodium chloride and sodium bromide, sodium hydroxide solution (1
131 mol/L), and ethylenediaminetetraacetic acid disodium salt dihydrate were purchased
132 from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Ammonium acetate was
133 obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). MS grade methanol was
134 purchased from Kanto Chemical Co., Ltd (Tokyo, Japan), HPLC grade chloroform was
135 obtained from Kishida Chemical (Osaka, Japan), and HPLC grade distilled water was
136 purchased from Wako Pure Chemical Industries. An authentic sample of diacyl
137 12:0/12:0 phosphatidylcholine (PC 12:0/12:0) was purchased from Avanti Polar Lipids
138 (Alabaster, AL, USA). Carbon dioxide (99.99% grade, Nerikigasu, Osaka, Japan) was
139 used as the SFC mobile phase.

140

141 Animals

142 All animal procedures were approved by the Kobe University Animal Care
143 Committee. Five WHHLMI rabbits (21 months old) and three Japanese white (JW)
144 rabbits (23 months old, Kitayama Labes, Co. Ltd., Ina, Japan) were used. The rabbits
145 were housed individually in metal cages in a room with constant temperature ($22 \pm 2^\circ\text{C}$)
146 and a consistent lighting cycle (12 h light/dark), and they were fed standard rabbit chow
147 (120 g per day; LRC4, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum
148 (11).

149

150 Sample preparation

151 Plasma lipoproteins were fractionated by ultracentrifugation with a stepwise method
152 (VLDL, $d < 1.006 \text{ g mL}^{-1}$; LDL, $1.006 \text{ g mL}^{-1} < d < 1.063 \text{ g mL}^{-1}$; and HDL, $d > 1.063$

153 g mL⁻¹) (14,15).

154 Lipid extraction from rabbit plasma, LDL, and VLDL was performed using Bligh and
155 Dyer's method (16) with minor modifications. In short, 40 µL of plasma or each
156 lipoprotein fraction was mixed with 150 µL of a solvent mixture (methanol:chloroform
157 = 2:1, v/v) containing PC 12:0/12:0 dissolved in methanol as an internal standard (20 ng
158 mL⁻¹ for plasma in JW rabbits, 200 ng mL⁻¹ for plasma in WHHLMI rabbits, 20 ng mL⁻¹
159 for VLDL in WHHLMI rabbits, and 200 ng mL⁻¹ for LDL in WHHLMI rabbits), and
160 then the solution was vortexed at the maximum setting for 30 s. After 50 µL of
161 chloroform and 50 µL of distilled water were added, the solution was vortexed again.
162 Phase separation of aqueous and organic layers was performed by centrifugation at 800
163 ×g for 10 min at 4°C. Aliquots (100 µL) of the organic layer were transferred to clean
164 tubes. The plasma lipid extract (100 µL) for JW rabbits and WHHLMI rabbits was
165 diluted to a final volume of 200 µL and 2000 µL, respectively, with a solution of 2:1
166 (v/v) methanol:chloroform for SFC/MS analysis. VLDL and LDL lipid extracts (100
167 µL) from WHHLMI rabbits were diluted to final volumes of 200 µL and 2000 µL,
168 respectively, as described above.

169

170 **Biochemical analysis for total cholesterol and total TAG**

171 Total cholesterol (Cho) and TAG levels were measured using a Cholesterol E-Test
172 Assay Kit and Triglyceride E-Test Assay Kit (Wako Pure Chemical Industries, Ltd)
173 following the manufacturer's instructions.

174

175 **SFC/Q-Orbitrap-MS conditions**

176 Lipid molecular species were separated using an Analytical SFC Method Station
177 (Waters, Milford, MA, USA) controlled by SuperChrom software (Waters). The SFC

178 conditions were as follows: column, Inertsil ODS-4 column (4.6×250 mm, particle size
179 of $5 \mu\text{m}$, GL Sciences, Tokyo, Japan); mobile phase, carbon dioxide (A) and 0.1% (w/v)
180 ammonium acetate in methanol as modifier (B); flow rate, 3.0 mL min^{-1} ; gradient curve,
181 10% B at 0 min, 30% B at 15 min, 10% B at 20 min; injection volume, $5 \mu\text{L}$; column
182 temperature, 35°C ; and back pressure, 10 MPa.

183 Individual lipid molecular species were identified using hybrid quadrupole-Orbitrap
184 mass spectrometer, Q Exactive MS fitted with an electrospray ionization (ESI) ion
185 source (Thermo Scientific, Waltham, MA, USA) and controlled by Xcalibur software
186 version 4.1 (Thermo Scientific). The Q Exactive MS parameters for high-mass-accuracy
187 MS^1 and data-dependent MS^2 (dd- MS^2) analysis were described previously (7) and used
188 here with minor modifications. The modified ionization conditions were as follows:
189 capillary temperature, 380°C ; and heater temperature, 350°C . For lipidomic analysis of
190 plasma and lipoproteins, full MS/dd- MS^2 (top 3) was used. The experimental conditions
191 for the data-dependent product ion scanning were as follows. The isolation width,
192 normalized collision energy (NCE), and stepped NCE were set to m/z 2.0, 25 eV, and
193 20% in the positive ion mode, and m/z 2.0, 30 eV, and 20 % in the negative ion mode,
194 respectively.

195

196 **Data analysis**

197 Identification of lipid molecular species was performed using Lipid Search software
198 (Mitsui Knowledge Industry, Tokyo, Japan) (17), and the product search mode, which
199 identifies using accurate mass and MS^2 spectrum pattern, was used. The precursor-ion
200 and product-ion tolerances were set to 5 and 10 ppm mass windows, respectively. The
201 absolute intensity threshold for precursor ions and the relative intensity threshold for
202 product ions were set to 5000 and 10%, respectively.

203 The chromatographic peak area for each analyte (precursor ion) was calculated using
204 Xcalibur software Ver. 2.2. (Thermo Scientific) based on the results obtained from Lipid
205 Search. The quantitative values were calculated using the ratio of the chromatographic
206 peak area of each analyte to that of the internal standard, PC 12:0/12:0. Furthermore,
207 lipid data obtained from lipoprotein fractions were normalized to total cholesterol levels
208 in each lipoprotein sample.
209

210 **RESULTS**

211 **Characteristics of JW rabbits and WHHLMI rabbits**

212 In the present study, we performed a lipidomic analysis of plasma and its lipoprotein
213 fractions (LDL and VLDL) in WHHLMI rabbits at the age of 21 months. Table 1 shows
214 the age, sex, weight, total plasma Cho level, and total plasma TAG level in JW rabbits
215 and WHHLMI rabbits. No significant difference in weight was observed between the
216 two rabbit strains, whereas total plasma Cho and total plasma TAG levels in WHHLMI
217 rabbits were higher (approximately 70-fold and 6-fold, respectively) than those in JW
218 rabbits.

219

220 **Optimization of lipidomic analytical system for lipid profiling of plasma and its** 221 **lipoprotein fractions in WHHLMI rabbits**

222 Lipids include various isobaric and isomeric lipid molecular species in the blood. To
223 simultaneous and accurately analyze lipids, we used SFC/Q-Orbitrap-MS, which
224 enables high-speed, high-efficiency chromatographic separation in addition to a high
225 mass resolution. In this study, we optimized the SFC/Q-Orbitrap-MS analytical
226 conditions for the rabbit plasma, LDL, and VLDL analyses according to a previously
227 described method (7) with minor modifications. In SFC separation, supercritical carbon
228 dioxide, which has almost the same polarity as hexane (5) is frequently used as an SFC
229 mobile phase, and its polarity can be changed by adding a modifier such as methanol.
230 Furthermore, the addition of MS-favorable organic solvents to the modifier is especially
231 important for chromatographic peak shape and ionization efficiency in the ESI source.
232 We investigated the analytical sensitivity of various types of lipids including free fatty
233 acid (FFA) associated with different additive agents, i.e., ammonium formate or
234 ammonium acetate. Using 0.1 % (w/v) ammonium acetate in methanol as a modifier in

235 the SFC, the sensitivity for FFAs was approximately 100-fold higher than that when
236 0.1 % (w/v) ammonium formate in methanol was used. On the other hand, the
237 sensitivity for other lipids was almost same (see Electronic Supplementary Material
238 Table S1). Therefore, in this study, we selected ammonium acetate for comprehensive
239 lipid analysis.

240 Figure 1 shows the total ion current chromatogram of SFC/Q-Orbitrap-MS data
241 obtained from the plasma of WHHLMI rabbits. In the present study, various lipids were
242 identified according to the accurate information for the precursor ion (MS) and its
243 representative product ions (MS/MS) in the positive-ion mode and in the negative-ion
244 mode (Table 2). However, some isomers of DAGs or TAGs were co-eluted and could
245 not be identified precisely because the separation efficiency for these lipids was
246 insufficient in the present analytical system. Therefore, quantitative values for DAGs
247 and TAGs were calculated based on the each molecular subset such as TAG 48:0 (total
248 acyl carbons : total double bonds). Similarly, many PC molecular species could not be
249 identified completely. To identify the molecular species of PC in Q-Orbitrap-MS,
250 detection of specific fragment ions derived from both the head group (phosphocholine)
251 in positive-ion mode and FA moieties in the negative-ion mode is required. However,
252 the detection sensitivity for the product ions obtained from FA moieties in the
253 negative-ion mode was several hundred-fold lower than that for the phosphocholine
254 product ion (m/z 184.0729) in the positive-ion mode. Thus, it was difficult to identify
255 the PCs in the rabbit plasma. Therefore, the quantitative values for PC were calculated
256 according to each molecular subset (e.g., PC 32:0). In total, the values of 172 molecular
257 species of lipid, including FFA, phospholipids (LPC, PC, LPE, PE, and PI),
258 sphingolipids (Cer and SM), glycerolipids (DAG and TAG), and sterol lipids (CE), in
259 plasma obtained from JW rabbits and WHHLMI rabbits were quantified within 15 min

260 using the SFC/Q-Orbitrap-MS system developed in this study.

261

262 **Comparison of plasma lipid profiles between JW rabbits and WHHLMI rabbits**

263 First, the difference of plasma lipid levels between JW rabbits and WHHLMI rabbits
264 was evaluated based on the peak area for all detected lipids using scatter plot (Fig. 2).
265 Plasma levels of all lipid classes except for FFA showed higher tendency in WHHLMI
266 rabbits than in JW rabbits. In particular, SM and CE levels in WHHLMI rabbit plasma
267 were significantly higher than those in JW rabbit plasma, respectively. Similarly,
268 WHHLMI rabbit plasma contained higher amounts of phospholipids (PC, PE, and PI)
269 than JW rabbit plasma.

270 Second, to determine individual lipid levels, the plasma lipid profiles of JW rabbits
271 and WHHLMI rabbits were compared using a volcano plot (Fig. 3), which is a method
272 used to visualize the ratio of lipid levels (fold change) and the statistical significance
273 (p -value) of the differences between the two groups compared. The levels of more than
274 70% of lipids detected in the plasma of WHHLMI rabbits were statistically increased
275 compared to those in the plasma of JW rabbits ($p < 0.05$, Table S2). Therefore, in the
276 present study, we focused on the lipids whose contents were significantly different
277 between the two strains (fold change > 16 and $p < 0.01$). Notably, increased plasma
278 levels of functional lipids such as alkyl-phosphatidylcholines (e.g., PC 34:2e and PC
279 36:4e), phospholipids including ω -6 FA (PI 18:0/20:4), plasmalogens (e.g., PE
280 16:0p/18:2 and PE 18:0p/20:4), CEs (e.g., CE 18:1, CE 18:2, and CE 20:4), and SMs
281 (e.g., SM d18:1/14:0 and SM d18:1/16:0) were observed in WHHLMI rabbits.
282 Alkyl-phosphatidylcholine is a major membrane constituent of inflammatory cells and a
283 precursor for lyso-platelet-activating factor (lyso-PAF) (18). PI 18:0/20:4 is the primary
284 source of arachidonic acid via the action of phospholipase A₂ (PLA₂), which is required

for biosynthesis of prostaglandins (19). Plasmalogens are representative physiological antioxidants, as their vinyl ether functionality serves as a sacrificial trap for free radicals (19). Additionally, CEs are the main components of foam cells (20), and SMs abound in plaques (21). Thus, our findings show that a wide range of functional lipids related to inflammation was significantly increased in WHHLMi rabbits. However, many FFAs and TAGs did not show significant differences between the two rabbit strains.

High-resolution lipidomic analysis with plasma lipoprotein fractions in WHHLMi rabbits

To evaluate the utility of lipidomic analysis of plasma lipoprotein fractions, detailed lipid profiling was performed with VLDL and LDL lipoproteins obtained from JW rabbits and WHHLMi rabbits. However, the number of lipids quantified in each lipoprotein fraction (VLDL and LDL) derived from JW rabbits was dramatically limited, because plasma lipid levels in JW rabbits were significantly lowered compared to those in WHHLMi rabbits (Table 1 and Fig. 2). For this reason, we evaluated the alterations of lipid composition in VLDL and LDL fractions induced by lipoprotein metabolism in WHHLMi rabbits. Figure 4 shows the ratio of the lipid levels of the LDL fraction to those of the VLDL fraction in WHHLMi rabbits. The lipid levels for LPCs, SMs, and CEs in the LDL fractions were higher than those in the VLDL fractions, and significant differences were observed ($p < 0.01$, see Table S3). In contrast, the abundance of neutral lipids such as DAGs and TAGs in the LDL fractions was lower than that in the VLDL fractions (Fig. 4 and Table S3). Additionally, notable differences in lipid contents were observed: higher levels of PE plasmalogens including PE 16:0p/18:2, PE 16:0p/22:5, PE 18:0p/20:4, and PE 18:0p/22:6 were observed in the LDL fractions than in the VLDL fractions.

311 **DISCUSSION**

312 Atherosclerotic lesions develop spontaneously in WHHLMi rabbits because of
313 hypercholesterolemia, even in animals fed normal chow. A previous study confirmed
314 that the cumulative incidence of myocardial infarction increases with age (10).
315 Therefore, WHHLMi rabbits are widely used to elucidate pathological features such as
316 in myocardial infarction (11–13). Metabolomic approaches have been used to study the
317 metabolic profile induced by the administration of simvastatin (22). However, because
318 the only target metabolites were hydrophilic species, the available metabolic
319 information for myocardial infarction is limited. This is the first report regarding the
320 association of lipid molecules and lipoproteins in the plasma with abnormal lipid
321 metabolism in WHHLMi rabbits. Figure 2 shows that the levels of all lipid classes
322 except for FFAs were significantly higher in WHHLMi rabbit plasma than in JW rabbit
323 plasma. In these lipid classes, energy storage molecules such as DAGs and TAGs did
324 not show the clear changes observed for other lipid classes. Many reports have
325 described the association between lipid metabolites and CAD. For example, for
326 phospholipids, it has already been reported that PC metabolism in the gut flora is
327 associated with cardiovascular diseases (23). [SMs on the surface of lipoprotein particles](#)
328 [delay the elimination of the lipoprotein particles from the blood by reducing their](#)
329 [hepatocellular uptake. Therefore, SMs are a risk factor for dyslipidemia and are](#)
330 [involved in the progression of atherosclerosis](#) (24). In addition, production of Cer by
331 SMase promotes the uptake of lipoproteins by macrophages and is involved in the
332 formation of foam cells, thus Cer also promotes atherosclerosis (24,25). In sterol lipids,
333 accumulation of CE causes foam cell formation from macrophages, and imbalanced
334 sterol lipid metabolism is involved in the pathogenesis of diseases. It has been
335 suggested that imbalance in the metabolism of various lipids (glycerophospholipids,

336 sphingolipids, and sterol lipids) is involved in the pathogenesis of CAD, and changes in
337 lipid classes were also observed in the blood.

338 To gain insight into the imbalance of lipid metabolism, we focused on differences in
339 individual lipid levels between JW rabbit plasma and WHHLMI rabbit plasma. As
340 shown in Fig. 3, a wide range of functional lipids related to inflammation were
341 significantly increased in WHHLMI rabbit plasma. In particular, the levels of
342 alkyl-phosphatidylcholines (e.g., PC 34:2e and PC 36:4e) were significantly increased
343 compared to those in JW rabbit plasma. Alkyl-phosphatidylcholines including stearic
344 acid and palmitic acid (e.g., PC 16:0e/18:2 and PC 18:0e/20:4) function as precursors of
345 platelet-activating factor (PAF), which is produced in inflammatory cells and exerts
346 various effects including not only platelet aggregation but also leukocyte migration,
347 increased vascular permeability, and reduced blood pressure (26). In an acute
348 inflammation, lyso-PAF is produced from alkyl-phosphatidylcholine by PLA₂, and PAF
349 is produced by lyso-PAF acetyltransferase from lyso-PAF (18). In the present study, the
350 levels of alkyl-phosphatidylcholines were significantly increased in the plasma,
351 suggesting that the metabolic pathway was activated in WHHLMI rabbits.

352 In total, 11 PE plasmalogens (e.g., PE 16:0p/18:2 and PE 18:0p/22:6) were
353 significantly increased in WHHLMI rabbits. Alkenyl-phospholipids (PE plasmalogens,
354 PC plasmalogens, and plasmalogens) are susceptible to oxidation because they contain
355 vinyl ether bonds, which are highly radical-sensitive. Moreover, they are also more
356 susceptible to oxidation because of their high proportion of polyunsaturated FAs and
357 alkenyl-linked fatty acids. For this reason, alkenyl-phospholipids have antioxidant
358 effects (19). On the other hand, phospholipids including ω -6 FA (e.g., PI 18:0/20:4)
359 were also significantly increased in WHHLMI rabbits. These lipids, via the action of
360 PLA₂, are the primary source of arachidonic acid. In inflammation, arachidonic acid is

361 produced by PLA₂ from phospholipids including ω -6 FA, and prostanoids are produced
362 by cyclooxygenase (COX) from arachidonic acid. Therefore, phospholipids including
363 ω -6 FA are required for the biosynthesis of the prostaglandins and other eicosanoids.
364 Prostanoids are involved in the activation of monocytes and macrophages and are
365 associated with matrix metalloproteinase production, which is a hallmark of plaque
366 instability (19). Taking of our findings into account, the increase of
367 alkenyl-phospholipids and/or phospholipids including ω -6 FA in plasma is related to the
368 progression of inflammation at the coronary atherosclerotic lesions in WHHLMi rabbits
369 (11).

370 In the present study, the lipidomic analysis of plasma lipoprotein fractions (i.e.,
371 high-resolution lipidomics approach) was applied to WHHLMi rabbits to provide a
372 detailed analysis of lipid metabolism. CM transports dietary lipids from small intestine
373 to the liver. In the present study, [since the WHHLMi rabbits had fasted at least 16 hours](#)
374 [before blood sampling](#), the transport of dietary lipids by CM was complete and there
375 was no CM in the blood. [Additionally, since the function of the LDL receptor is almost](#)
376 [deficient in WHHLMi rabbits, plasma HDL levels are extremely low \(10\). Therefore,](#)
377 [almost all of the Cho in the plasma accumulates in the LDL fraction \(see Table S4\).](#)

378 In this study, high-resolution lipidomic analysis was applied to plasma lipoprotein
379 fractions (VLDL and LDL) obtained by ultracentrifugation. Figure 4 shows the specific
380 lipid molecular species corresponding to the VLDL or LDL fractions obtained from
381 WHHLMi rabbits. In lipoprotein metabolism, VLDL transports lipids from the liver to
382 peripheral tissues. In this process, TAGs in VLDL are hydrolyzed by lipoprotein lipase
383 (LPL), and subsequently, LDL is produced. The LDL transports CE to peripheral tissues
384 via the LDL receptor, and the lipid compositions of VLDL and LDL are thus changed
385 through this lipoprotein metabolism. Therefore, the lipid levels of CE molecular species

386 in the LDL were higher than those in the VLDL in the present study (Fig. 4 and **Table**
387 **S3**). For the same reason, the lipid levels of TAG molecular species in the VLDL
388 fractions were higher than those in the LDL fractions.

389 PCs in LDL are catalyzed by PLA₂, and subsequently, they are converted to LPCs.
390 The level of LPCs increases in atherosclerotic lesions, resulting in atherogenesis (27).
391 Because LPC levels reflect atherosclerotic conditions in the LDL fraction, LPC levels in
392 the LDL fraction are highly associated with CAD. In the present study, although the
393 abundance of PCs was almost same between the VLDL fractions and the LDL fractions
394 in WHHLMI rabbits, the abundance of LPCs in the LDL fractions obtained from
395 WHHLMI rabbits was higher than that in the VLDL fractions (Fig. 4 and **Table S3**). Our
396 results suggest that LPC synthesis was accelerated in the LDL fraction, and that the
397 LPCs then facilitated the formation of atherosclerotic lesions.

398 In our high-resolution lipidomic analysis using lipoproteins, the levels of PEs and PE
399 plasmalogens were different between the LDL and the VLDL fractions in WHHLMI
400 rabbits. PE molecular species were less abundant in the LDL fractions than in the VLDL
401 fractions. In contrast, the levels of PE plasmalogens in the LDL fractions were higher
402 than those in the VLDL fractions. Quantitative changes in PEs and PE plasmalogens
403 between the LDL and the VLDL fractions might contribute to the formation and
404 development of coronary atherosclerotic lesions in WHHLMI rabbits. Taking all of the
405 present results into account, a high-resolution lipidomic analysis using plasma
406 lipoprotein fractions can provide new insights into the complicated lipid composition.

407 Our high-resolution lipidomic analysis using lipoprotein fractions represents
408 advancement the association of abnormal lipid metabolism with myocardial infarction
409 in WHHLMI rabbits. In total, 172 lipid molecular species in plasma from JW and
410 WHHLMI rabbits were quantified within 15 min using the SFC/Q-Orbitrap-MS system.

411 The plasma levels of each lipid class except for FFA were significantly higher in
412 WHHLMI rabbits than in JW rabbits. In particular, high levels of inflammation-related
413 functional lipids were also observed in WHHLMI rabbit plasma. In addition, using the
414 lipidomic approach applied to lipoproteins in WHHLMI rabbits, increase of CEs and
415 LPCs and decrease of TAGs was observed in the LDL fractions relative to the VLDL
416 fractions. Additionally, higher levels of PE plasmalogens were observed in the LDL
417 than in the VLDL. Our high-resolution lipidomics approach using lipoprotein fractions
418 represents to be a potential tool to elucidate the pathogenesis of myocardial infarctions
419 and search for biomarkers for CAD because WHHLMI rabbits spontaneously develop
420 myocardial infarction.

421

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426

427

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512

513 **Figure legends**

514

515 **FIG. 1.** Total ion current chromatogram for SFC/Q-Orbitrap-MS data obtained from
516 plasma in WHHLMI rabbits using an ODS-4 column. **LPL**, lysophospholipid (LPC and
517 **LPE**); and **PL**, phospholipid (PC, PE, and PI).

518

519 **FIG. 2.** Scatter plot of fold-change of all detected lipids between JW rabbits ($n = 3$)
520 and WHHLMI rabbits ($n = 5$).

521

522 **FIG. 3.** Individual plasma lipid profiles of JW rabbits ($n = 3$) and WHHLMI rabbits (n
523 $= 5$) by volcano plot. The gray area shows that levels of lipid molecular species were
524 remarkably different between the two rabbit strains (fold-change > 16 and $p < 0.01$).

525

526 **FIG. 4.** Ratio of lipid levels in the LDL fraction to those in the VLDL fraction in
527 WHHLMI rabbits. Blue-yellow color indicates the levels of each lipid (blue: LDL $<$
528 VLDL and yellow: LDL $>$ VLDL). Data were $\log_2$ transformed and the mean value of
529 five biological replicates is presented.

Figure1

Figure2

Figure3

Figure4

TABLE 1. Characteristics of JW rabbits and WHHLMI rabbits

		JW rabbit (<i>n</i> = 3)	WHHLMI rabbit (<i>n</i> = 5)
Age	Month	23 ± 0	21 ± 0
Sex	% male	100	40
Weight	kg	3.5 ± 0.1	3.4 ± 0.4
Plasma total Cho	mg dL ⁻¹	13 ± 2	914 ± 220 ^{***}
Plasma total TAG	mg dL ⁻¹	59 ± 38	305 ± 171

Values are mean ± standard deviation.

Statistical significance was determined using Student's *t*-test (**p* < 0.05, ***p* < 0.01, and ****p* < 0.001).

TABLE 2. MS and representative MS/MS fragment information for identification of each lipid class

Lipid class	MS ¹ (<i>m/z</i>)		MS ² (<i>m/z</i>)	
	Positive	Negative	Positive	Negative
LPC	[M + H] ⁺	[M + CH ₃ COO] ⁻	184.0729	[Acyl FA – H] ⁻
PC	[M + H] ⁺	[M + CH ₃ COO] ⁻	184.0729	[Acyl FA – H] ⁻
LPE	[M + H] ⁺	[M – H] ⁻	[M + H] ⁺ – 141.0184	[Acyl FA – H] ⁻
PE	[M + H] ⁺	[M – H] ⁻	[M + H] ⁺ – 141.0184	[Acyl FA – H] ⁻
PE plasmalogen	[M + H] ⁺	-	[M – FA – (CH ₂ =COHCH ₂ OH)] ⁺	-
PI	-	[M – H] ⁻	-	241.0119, [Acyl FA – H] ⁻
SM	[M + H] ⁺	-	184.0729	-
Cer	[M + H] ⁺	-	[So dFA + H – H ₂ O] ⁺	-
			[So dFA + H – 2H ₂ O] ⁺	
CE	[M + NH ₄] ⁺	-	369.3516	-
DAG	[M + NH ₄] ⁺	-	[M + NH ₄ – NH ₃ – Acyl FA] ⁺	-
TAG	[M + NH ₄] ⁺	-	[M + NH ₄ – NH ₃ – Acyl FA] ⁺	-

So, Sphingoshine