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Identification of novel serum markers for the progression of coronary atherosclerosis in WHHLMI rabbits, an animal model of familial hypercholesterolemia

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ABSTRACT

Background and aims: The development of serum markers specific for coronary lesions is important to prevent coronary events. However, analyses of serum markers in humans are affected by environmental factors and non-target diseases. Using an appropriate model animal can reduce these effects. To identify specific markers for coronary atherosclerosis, we comprehensively analyzed the serum of WHHLMI rabbits, which spontaneously develop coronary atherosclerosis.

Methods: Female WHHLMI rabbits were fed standard chow. Serum and plasma were collected under fasting at intervals of 4 months from 4 months old, and a total of 313 lipid molecules, 59 metabolites, lipoprotein lipid levels, and various plasma biochemical parameters were analyzed. The severity of coronary lesions was evaluated with cross-sectional narrowing (CSN) corrected with a frequency of 75% - 89% CSN and CSN> 90%.

Results: There was a large variation in the severity of coronary lesions in WHHLMI rabbits despite almost no differences in plasma biochemical parameters and aortic lesion area between rabbits with severe and mild coronary lesions. The metabolites and lipid molecules selected as serum markers for coronary atherosclerosis were lysophosphatidylcholine (LPC) 22:4 and diacylglycerol 18:0-18:0 at 4 months old, LPC 20:4 (sn-2), ceramide d18:1-18:2, citric acid plus isocitric acid, and pyroglutamic acid at 8 months old, and phosphatidylethanolamine plasminogen 16:1p-22:2 at 16 months old.

*Conclusion*s: These serum markers were coronary lesion-specific markers independent of cholesterol levels and aortic lesions and may be useful to detect patients who develop cardiovascular disease.

Key words

coronary atherosclerosis, lipidome analysis, metabolome analysis, serum marker, WHHLMI rabbit

1. Introduction

The discovery of serum markers specific for coronary lesions is important to prevent coronary events. In epidemiological studies, several marker candidates for coronary artery disease (CAD), such as the levels of serum cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, the ratio of LDL cholesterol to HDL cholesterol, lipoprotein (a), small dense LDL, and oxidized LDL, have been reported. These conventional factors are not always associated with the onset of CAD [1-3], and metabolome and lipidome analyses have revealed other candidates for serum markers of CAD or atherosclerosis in animal models [1,4-8] and humans [9-14]. Because serum levels of metabolites or lipid molecules are regulated by gene expression and environmental stresses, these metabolites or lipid molecules reflect the development and progression of the disease [15]. In clinical or epidemiological studies, several factors, such as dietary habits, environmental conditions, non-target diseases, social stress, and alcohol, affect analysis of markers related to CAD [16-19]. In studies using animals, these confounding factors can be kept constant, and age-related changes can be investigated in candidate markers. Although animals fed a cholesterol diet or a high fat diet [6-8] or raised in a smoke-filled environment [1,4] develop atherosclerotic lesions, these conditions directly or indirectly affect the serum concentration of metabolites or lipid molecules. Therefore, animal models that develop atherosclerosis spontaneously without the influence of environmental factors that regulate the concentration of serum parameters are better alternative animal models. In previous studies using these animals, candidate marker substances were analyzed for aortic atherosclerosis. These studies were performed under the assumption that there are no differences between aortic lesions and coronary lesions. Because the volume of aortic lesions is extremely large compared with that of coronary lesions, serum concentrations of marker substances for aortic lesions may mask those of coronary lesions. Therefore, markers that are specific for coronary lesions are unknown. Previous studies using WHHLMI rabbits, a spontaneous animal model for hypercholesterolemia from LDL receptor deficiency, coronary atherosclerosis, and myocardial lesions [20], suggest that factors associated with coronary

lesions differ from those of aortic lesions [21,22]. In this study, we identified serum markers specific for coronary lesions in WHHLMI rabbits using lipidome and metabolome analyses.

2. Materials and Methods

2.1. Animals

We examined serum markers for coronary lesions using 22 WHHLMI rabbits (12 females and 10 males), and 363 WHHLMI rabbits born between 2010 and 2016 were examined for age-dependent changes in the progression of coronary lesions. In addition, 6 male JW rabbits were used as normal controls. The details are described in the Supplementary Methods.

2.2. Fractionation of plasma lipoproteins and measurement of lipid concentration

Lipoproteins were fractionated with an ultracentrifuge to yield very low-density lipoproteins (VLDL, d<1.006 g/ml), LDL (1.006<d<1.063 g/ml), and HDL (d>1.063 g/ml). The details are described in the Supplementary Methods.

2.3. Metabolome analyses of serum

Metabolomic profiling using gas chromatography/mass spectrometry (GC/MS) was performed as reported previously [23]. Fifty-nine metabolites were recorded. The details are described in the Supplementary Methods.

2.4. Lipidome analyses of serum

Lipids were quantified using supercritical fluid chromatography triple quadrupole mass spectrometry (SFC/MS/MS) in the multiple reaction monitoring mode [24]. There were 313 lipid molecules detected in the serum. The relative concentration (RC) of lipid molecules was corrected with serum total cholesterol concentrations using the following equation:

[Corrected relative concentration (CRC)] = [relative concentration] / serum total cholesterol concentration x 1,000.

The details are described in the Supplementary Methods.

2.5. Evaluation of atherosclerotic lesions

Aortic lesions were evaluated as the ratio of the surface area of the lesions to the surface area of the entire lumen. The severity of coronary lesions was evaluated using a coronary severity score based on the following equation:

[Coronary severity score] = [average cross-sectional narrowing (CSN)] x (1 + [frequency of sections with 75%-89% CSN] x 10 + [frequency of sections with >90% CSN] x 20)

The details are described in the Supplementary Methods.

2.6. Age-dependent progression of coronary lesions

To examine age-dependent progression of coronary lesions in WHHLMI rabbits, we examined the maximum CSN from one month old to 30 months old using 363 dead rabbits or rabbits euthanized in other experiments between 2010 and 2016. Coronary lesions were examined in the manner described above. The details are described in the Supplementary Methods.

2.7. Other assays

At the start of the experiment (4 months old) and at the end of experiment (20 months old or at death), we assayed the plasma levels of glucose, insulin, NO_2, NO_3, and oxidized LDL, and superoxide dismutase (SOD) activity. The details are described in the Supplementary Methods.

2.8. Data and statistical analyses

We used data only from females because of sex differences in coronary lesions and serum levels of metabolites and lipid molecules. The serum marker candidates for coronary atherosclerosis were determined in two steps at 4 and 8 months old, and in 3 steps at over 8 months old, and at 20 months old or death. The details are shown in the supplementary

information.

3. Results

3.1. Atherosclerotic lesions of WHHLMI rabbits

Table 1 shows that the parameters of coronary lesions were significantly higher in the severe group than in the mild group, but there were no differences in aortic surface lesion area (96±2% vs 94±1%). These results suggest that the factors related to the progression of atherosclerotic lesions were different between aorta and coronary arteries.

3.2. Plasma biochemical parameters in WHHLMI rabbits

There were no significant differences in lipoprotein lipid levels and plasma biochemical parameters between the severe and mild groups (Supplementary Table 5 and 6). These results indicate that factors other than lipoprotein lipids and other plasma parameters were involved in the progression of coronary lesions.

3.3. Examination of serum metabolites as markers specific for coronary lesions

In analyses of serum metabolites, the most abundant metabolites were glycine, lactic acid, and alanine (data not shown). In volcano plot analyses of differences in serum metabolites between the severe and mild groups (Figure 1-A) for the first selection, 9 metabolites were selected at various ages. However, in the second selection, the Spearman's rank-correlation coefficient at 8 months old was 0.655 ($p = 0.029$) in pyroglutamic acid and 0.609 ($p = 0.047$) in citric acid plus isocitric acid (Table 2). The RC of these metabolites did not show a sex difference (data not shown). We selected these metabolites as markers for the rapid progression of coronary atherosclerosis at an early stage. In the sparse partial least square discriminant analysis (SPLS-DA) of each metabolite, the distribution of rabbits was distinctly separated between the two groups, except at 4 months old (Figure 1-B). Comparing normal and WHHLMI rabbits, the RC of 26 of 59 serum metabolites was significantly different (Supplementary Figure 4-A). The RCs of metabolites selected as markers of

coronary atherosclerosis (citric acid plus isocitric acid and pyroglutamic acid) were high in the WHHLMI rabbit severe group compared to those in normal rabbits, but the difference was not significant (Supplementary Figure 5-A).

3.4. Examination of serum lipid molecules as markers specific for coronary lesions

In analyses of serum lipid molecules, the most abundant lipids were cholesterol ester (CE), triacylglycerol (TAG), and phosphatidylcholine (PC) (data not shown). In volcano plot analyses of differences in the CRC of serum lipid molecules between the severe and mild groups for the first selection (Figure 2-A), 27 of 293 lipid molecules were selected (5 lipids at 4 months old, 4 lipids at 8 months old, 3 lipids at 12 months old, 12 lipids at 16 months old, and 3 lipids at 20 months old or death). In the second selection by the Spearman's rank-correlation coefficient (Table 3), 2 of 5 lipid molecules were selected at 4 months old [LPC 22:4 ($r = 0.700$, $p = 0.016$) and DAG 18:0-18:0 ($r = -0.636$, $p = 0.035$)] as markers for the generation of coronary lesions. At 8 months old, 2 of 4 lipid molecules were selected [LPC 20:4 (sn-2) ($r = 0.773$, $p = 0.005$) and Cer d18:1-18:2 ($r = 0.673$, $p = 0.023$)] as markers for the rapid progression of coronary lesions. In the third analyses for occluded coronary lesions or myocardial ischemia at over 8 months old (Figure 3-A), only PE 16:1p-22:2 was selected at 16 months old. In SPLS-DA of serum lipid molecules (Figure 2-B), the distribution of the rabbits was distinctly separated between the two groups. For sex differences, the CRC of Cer d18:1-18:2 was lower in females than males, but other selected lipid molecules were not different between males and females (data not shown). In comparison to normal JW rabbits, the relative concentrations of many serum lipid molecules in WHHLMI rabbits were extremely high (Supplementary Figure 4-B). In lipid molecules selected for markers of coronary atherosclerosis, the RCs of LPC 22:4, LPC 20:4 (sn-2) and Cer d18:1-18:2 were significantly high in WHHLMI rabbits compared to those in normal JW rabbits (Supplementary Figure 5-B), but others were below the detection limitation in normal JW rabbits.

4. Discussion

We identified serum markers specific for the generation, rapid progression, and occlusion of coronary lesions, which may be different from markers of aortic surface lesion area in female WHHLMI rabbits. Because many factors, such as the environment, lifestyle habits, feeding, social stress, non-target diseases, sex, cholesterol levels, and degree of aortic lesions, that influence serum marker analysis in humans [16-19] and other experimental animals [6-8] were controlled in our study, many of these previously described metabolites and lipid molecules were not selected. Therefore, the serum marker candidates found in the present study may reflect the development and progression of coronary lesions and myocardial ischemia independent of environmental factors.

At 4 months old, which corresponded to when coronary lesions were generated (Supplementary Figure 6), the lipids LPC22:4 and DAG18:0-18:0 were selected as markers. LPC is involved in monocyte recruitment, macrophage proliferation, increased expression of endothelial adhesion molecules, and endothelial dysfunction [25]. In patients with coronary artery disease, the serum levels of four LPCs containing unsaturated fatty acids were higher than in normal subjects, whereas the serum levels of two LPCs containing saturated fatty acids were lower than in normal subjects [12]. LPC22:4 is an LPC that contains adrenic acid, which enhances inflammation [26]. Therefore, differences in the side chain of LPC may be important in the generation of coronary lesions. DAG18:0-18:0 was negatively associated with coronary lesions. The odds ratio of stable CAD and normal subjects was high in unsaturated fatty acid-containing DAG but low in saturated fatty acid-containing DAG [27]. This observation indicates that the atherogenicity of DAG differs depending on side chain fatty acids and suggests that DAG18:0-18:0 is not atherogenic at an early stage.

At 8 months old, which corresponded to when coronary lesions rapidly progressed (Supplementary Figure 6), citric acid plus isocitric acid, pyroglutamic acid, LPC20:4 (sn-2), and Cer d18:1-18:2 were selected as markers for the rapid progression of coronary lesions at an early stage. For citric acid, Yamashita et al. [28] reported that in macrophage-rich lesions in the iliac artery of fat-fed rabbits, intermediates in the TCA cycle including citric acid was high compared to that of normal control arteries. Intermediates in the citric acid cycle are G-

protein-coupled receptor ligands and are involved in atherosclerosis [29]. Pyroglutamic acid is a cyclic derivative of glutamine and is independently associated with heart failure in humans [30]. In vitro studies demonstrated that pyroglutamate is associated with monocyte chemoattractant protein-1 activity and expression of HUVEC adhesion factor [31]. An increase in LPC20:4 (sn-2) is also observed in patients with angina pectoris and myocardial infarction [12] and carotid plaques [32]. We found that LPC22:4 increased when lesions were generated, and LPC20:4 (sn-2) increased when lesions rapidly progressed. These results suggest that there are differences in LPC molecules that are associated with coronary lesions during lesion development. Several studies also showed that several ceramide molecules were related to cardiovascular disease [12,33-36]. Ceramides have several functions related to atherosclerosis involving cell survival or death, the aggregation of lipoproteins, transcytosis of oxidized LDL across endothelial cells, regulation of monocyte adhesion to vascular walls, and promotion of LDL uptake [37]. Furthermore, knockout of sphingomyelin synthase 2, an enzyme for ceramide synthesis, decreases atherosclerosis in mice [38]. Therefore, reduction of ceramide by medication may be effective in suppressing arteriosclerosis. However, ceramide levels were lower in females (data not shown) than in males similar to those in mice [39]. Analysis of the relationship between ceramide and coronary artery lesions using males is needed in the future.

At 16 months old, which corresponds to when the average of maximum CSN was over 90% (Supplementary Figure 6) and the development of myocardial ischemia, the CRC of PE16:1p-22:2, a phosphatidylethanolamine plasminogen species (PE-pln), was selected as a marker for occluded coronary lesions or myocardial ischemia. PE-pln has anti-oxidative and anti-inflammatory functions [40]. However, it is unclear how PE16:1p-22:2 is associated with occluded coronary lesions or myocardial ischemia. The serum levels of several PE-pln species are significantly higher in patients with angina pectoris than in normal subjects [12]. Because the frequency of sections with more than 90% CSN was 45 ± 13% (82%, 32%, and 18% of sections in three rabbits), which suggests ischemic damage of the myocardium in the severe group, occlusion of coronary lesions may cause an increase in serum PE16:1p-22:2

similar to that of patients with angina pectoris.

There were several limitations in the present study. Many serum metabolites and lipid molecules showed sex differences, and variations in coronary lesion severity were large in females, although 7 of 9 males had severe coronary lesions. Therefore, we used only females in the present study. To compensate for the decrease in the number of rabbits by limiting our analysis to female rabbits, we used a rigorous selection criteria for marker candidates. However, we need to analyze serum markers using males in the future. It is unclear whether the serum markers we found can be used in non-FH patients. Because there is no difference in the serum total cholesterol level between the mild group and the severe group, it is considered that the selected markers were not affected by hypercholesterolemia. Therefore, these markers may be useful for patients with cardiovascular disease without hypercholesterolemia.

In conclusion, we found serum markers specific for the generation of coronary lesions, for the rapid progression of coronary lesions at an early stage, and for occluded coronary lesions. These serum markers may be useful to detect patients who develop cardiovascular disease.

Conflict of interests

The authors do not have anything to disclose or conflicts of interest with respect to this manuscript.

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Author contributions

The study was conceived and designed by MShio; the experiments were performed by

MShio, HT, YIr, NKi, SY, NKu, AK, YY, TK, and YIz; the data were analyzed by MShio, YIz, MShin, TB, and TI; the manuscript was prepared by MShio, MShin, TB, and TI.

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Figure legends

Fig. 1. Profiles of serum metabolites in female WHHLMI rabbits with severe and mild coronary atherosclerosis. (A) Upper panels indicate the results of the volcano plot. The cutoff value was set at a p-value of 0.05 and a 30% change in the relative concentration of plasma metabolites between the severe and mild groups. Dotted lines indicate the cutoff line. *P*-values were calculated by a Student's t-test or Welch's t-test. (B) Lower panels indicate the results of a direct comparison of the SPLS-DA score plots of 59 metabolites.

Fig. 2. Profiles of serum lipid molecules in female WHHLMI rabbits with severe and mild coronary atherosclerosis. (A) Upper panels indicate the results of the volcano plot. The cutoff value was set at a p-value of 0.05 and a 30% change in relative concentration of plasma metabolites between the severe and mild groups. Dotted lines indicate the cutoff line. *P*-values were calculated by a Student's t-test or Welch's t-test. (B) Lower panels indicate the results of a direct comparison of the SPLS-DA score plots of 310 lipid molecules.

Fig. 3. The corrected relative concentration (CRC) of serum PE16:1p-22:2. (A) Distribution of the CRC of PE16:1p-22:2 at 16 months old for 11 rabbits. (B) Changes in the CRC of PE16:1p-22:2 with aging. Data are mean ± SEM. FC in panel B indicates the fold changes between the CRC of PE16:1p-22:2 of the severe group at 16 months old and that of the mild group at the indicated age. Statistical analyses were performed by a Student's t-test in panel A and by a Dunnett test in panel B. A correlation coefficient was calculated by Spearman's rank-correlation coefficient. Diamonds indicate the severe group (n=3), circles indicate the mild group (n=6), and squares indicate the intermediate group (n=2).

Figure 1

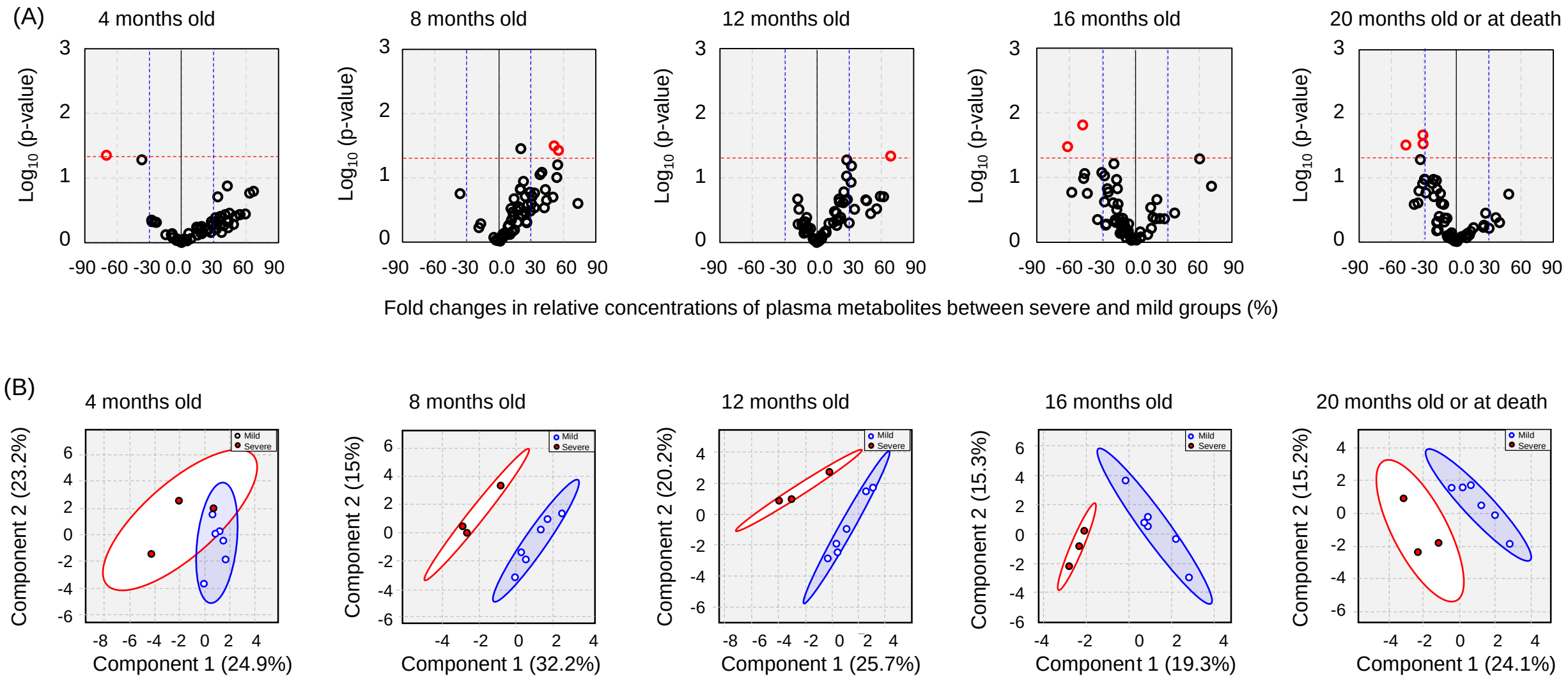
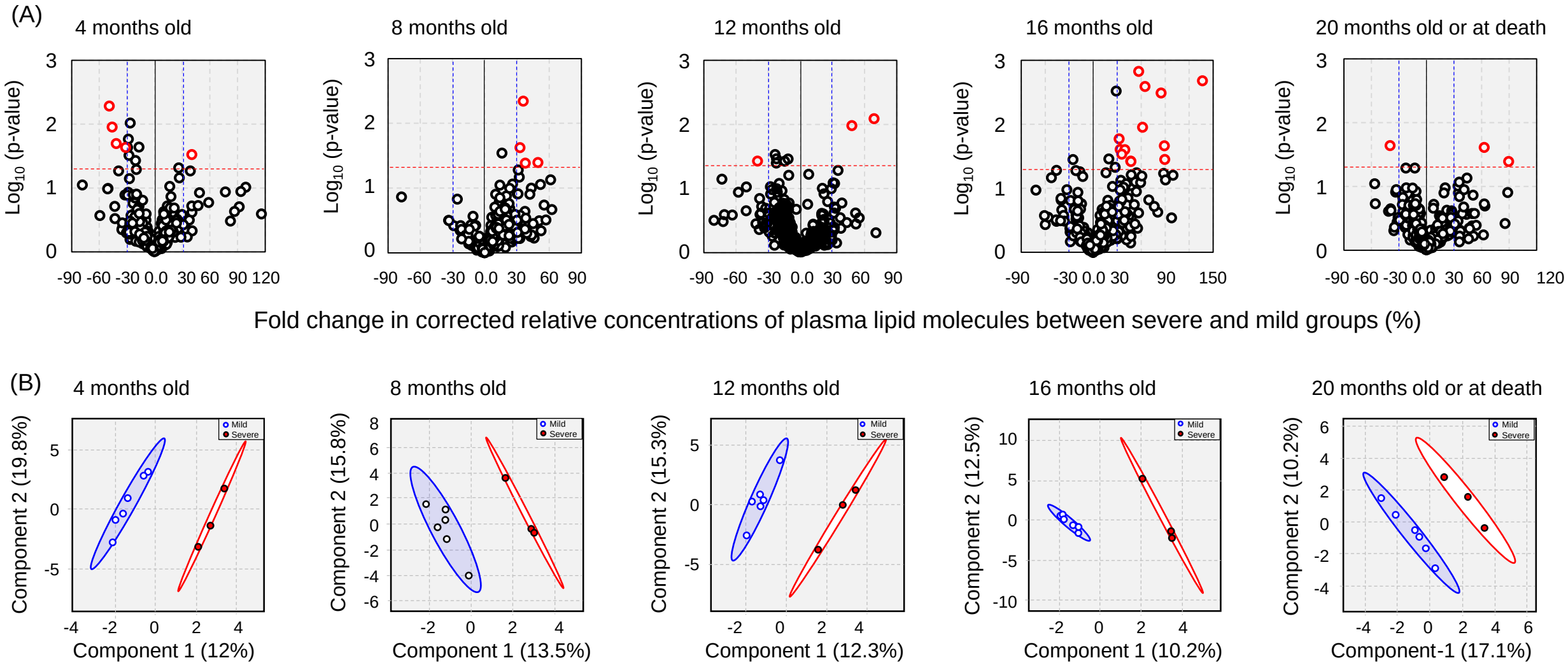

(A)
4 months old
8 months old
12 months old
16 months old
20 months old or at death
Log10 (p-value)
Fold changes in relative concentrations of plasma metabolites between severe and mild groups (%)
(B)
4 months old
8 months old
12 months old
16 months old
20 months old or at death
Mild
Severe
Component 2 (23.2%)
Component 1 (24.9%)
Component 2 (15%)
Component 1 (32.2%)
Component 2 (20.2%)
Component 1 (25.7%)
Component 2 (15.3%)
Component 1 (19.3%)
Component 2 (15.2%)
Component 1 (24.1%)
Sparse partial least squares discriminant analysis (SPLS-DA) of relative concentration of plasma metabolites in severe and mild groups

Figure 2



Sparse partial least squares discriminant analysis (SPLS-DA) of corrected relative concentration of plasma lipid molecules in severe and mild groups

Figure 3

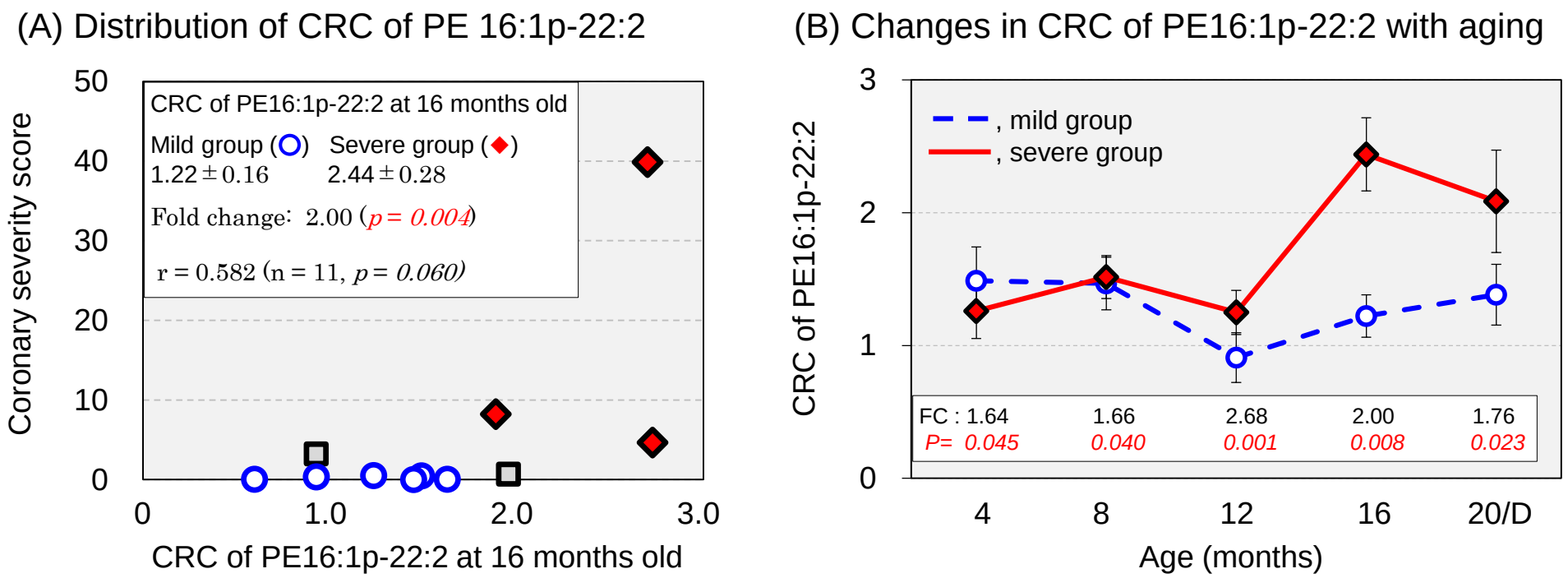

(A) Distribution of CRC of PE 16:1p-22:2
CRC of PE16:1p-22:2 at 16 months old
Mild group (○) Severe group (◆)
1.22 ± 0.16 2.44 ± 0.28
Fold change: 2.00 (p = 0.004)
r = 0.582 (n = 11, p = 0.060)
Coronary severity score
CRC of PE16:1p-22:2 at 16 months old
0
10
20
30
40
50
0
1.0
2.0
3.0
(B) Changes in CRC of PE16:1p-22:2 with aging
, mild group
, severe group
FC : 1.64 1.66 2.68 2.00 1.76
P= 0.045 0.040 0.001 0.008 0.023
CRC of PE16:1p-22:2
Age (months)
0
1
2
3
4
8
12
16
20/D

Table 1

Table 1. Development of atherosclerotic lesions in female WHHLMI rabbits

	Severe group	Mild group	P-value
Animals	3	6	
Coronary lesions			
Examined sections	22 ± 0	20 ± 1	0.013
Sections with lesions	17 ± 1	10 ± 3	0.010
Frequency of sections with lesions (%)	76 ± 15	49 ± 16	0.059
Maximum CSN (%)	98 ± 2	49 ± 12	<0.001
Average CSN (%)	62 ± 15	26 ± 15	0.005
Sections with >90% CSN	10 ± 4	0 ± 0	<0.001
Frequency of sections with >90% CSN (%)	45 ± 13	0 ± 0	<0.001
Sections with >75% CSN (%)	12 ± 4	0 ± 0	0.001
Frequency of sections with >75% CSN (%)	56 ± 20	0 ± 0	0.001
Coronary severity score	18 ± 11	0.2 ± 0.1	<0.001
Aortic lesions			
Percentage of surface lesion area (%)	96 ± 2	94 ± 1	0.157

Data are the mean ± SEM. Statistical analyses were performed with the Mann-Whitney U-test. CSN (cross-sectional narrowing) was calculated by dividing the lesion area by the area of the lumen and lesions. The percentage of the surface lesion area was calculated by dividing the surface area of the lesions by the surface area of the aortic lumen.

Table 2

Table 2. Selected serum metabolites as markers for progression of coronary lesions.

	Severe group	Mild group	Fold		Correlation coefficient	
	(n = 3)	(n = 6)	Change	p-value	r	p-value
8 months old						
Citric acid + Isocitric acid	1.03±0.16	0.67±0.07	1.552	0.037	0.609	0.047
Pyroglutamic acid	0.082±0.012	0.054±0.005	1.513	0.032	0.655	0.029

Data indicate the relative concentration of each metabolite and are mean ± SEM. A difference was calculated by dividing the relative concentration of plasma metabolites in the severe group by that in the mild group. Statistical analyses of the difference were performed with a Student t-test or Welch's t-test. Correlation coefficients were calculated with Spearman's rank-correlation coefficient using all 11 females in the intermediate group.

Table 3

Table 3. Selected serum lipid molecules at 4 and 8 months old as markers for the progression of coronary lesions.

	Severe group (n=3)	Mild group (n=6)	Fold Change	p-value	Correlation coefficient r	p-value
4 months old						
LPC22:4	0.47 ± 0.05	0.34 ± 0.03	1.404	*0.030*	0.700	0.016
DAG18:0-18:0	0.11 ± 0.01	0.21 ± 0.02	0.510	*0.005*	-0.636	0.035
8 months old						
LPC20:4 (sn-2)	0.81 ± 0.03	0.60 ± 0.03	1.359	*0.005*	0.773	0.005
Cer d18:1-18:2	0.08 ± 0.01	0.06 ± 0.00	1.377	*0.041*	0.673	0.023

Data indicate the corrected relative concentration (CRC) of each lipid molecule and are mean ± SEM. A difference was calculated by dividing the CRC of serum metabolites in the severe group by that in the mild group. Statistical analyses of the difference were performed with a Student t-test or Welch's t-test. Correlation coefficients were calculated with Spearman's rank-correlation coefficient using all 11 females in the intermediate group.

Supplementary Material for online publication only

Click here to download Supplementary Material for online publication only: Supplemental Data-2019_2_5.pdf

Supplementary information

Materials and Methods

2.1. Animals

We examined serum markers for coronary lesions using 22 WHHLMI rabbits (12 females and 10 males), and 363 WHHLMI rabbits born between 2010 and 2016 were examined for age-dependent changes in the progression of coronary lesions. WHHLMI rabbits were bred at the Institute for Experimental Animals, Kobe University Graduate School of Medicine (Kobe, Japan). In addition, 6 male JW rabbits (Kitayama Labes Co., Ltd., Ina, Japan) were used as a normal control. Rabbits were fed a standard rabbit chow (LPRC4, Oriental Yeast Co., Ltd., Tokyo, Japan). Rabbits were housed individually in metal cages (550 mm x 600 mm x 450 mm in width, depth, and height, respectively) with a flat metal floor. Animals were maintained under specific pathogen-free conditions with a constant temperature (22 ± 2 °C), relative humidity (50–60%), ventilation rate (15 cycles/h), air supply (through a HEPA filter), and lighting cycle (12 h light/dark). This study was approved by the Kobe University Animal Care and Use Committee (approval number: P110511 and P110511-R1), and all animal experiments were conducted in accordance with the Regulations for Animal Experimentation of Kobe University, the 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). Blood samples were collected at 4-month intervals from 4 months old to 20 months old. At the end of the experiments (20 months old), rabbits were euthanized with an intravenous injection of pentobarbital, and the heart and aorta were excised.

2.2. Fractionation of plasma lipoproteins and measurement of lipid concentration

After overnight fasting, samples were collected every 4 months from the marginal ear vein, and lipoproteins were fractionated with an ultracentrifuge to yield very low-density lipoproteins (VLDL, $d < 1.006$ g/mL), LDL ($1.006 < d < 1.063$ g/mL), and HDL ($d > 1.063$ g/mL). Lipoprotein lipid concentration was assayed using enzymatic methods.

2.3. Metabolome analyses of sera

Metabolomic profiling using gas chromatography/mass spectrometry (GC/MS) was

performed as reported previously [S-1]. Low molecular weight metabolites were extracted, and sinapinic acid (WAKO, Tokyo, Japan) was used as an internal standard to correct for the losses of analytes during sample preparation. Metabolites were analyzed with a GCMS-QP2010 Ultra (Shimadzu Co, Kyoto, Japan), which was equipped with a fused silica capillary column (CP-SIL 8 CB low bleed/MS; 30 mm × 0.25 mm inner diameter, 0.25-μm film thickness; Agilent Co., Palo Alto, CA). Fifty-nine metabolites (Supplementary Table 1) were recorded using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co, Kyoto, Japan) and were analyzed using MetAlign (Wageningen UR The Netherlands) and AI-output software (PMID 21641865, 21542920).

2.4. Lipidome analyses of sera

Lipids were extracted from sera using Bligh and Dyer's method [S-2] with minor modifications [S-3]. A dodecanoyl or heptadecanoyl-based synthetic internal standard mixture (Supplementary Table 2) was used as an internal standard. Lipid molecules were quantified using supercritical fluid chromatography triple quadrupole mass spectrometry (SFC/MS/MS) in the multiple reaction monitoring mode [S-3]. The SFC/MS/MS system was composed of an ACQUITY UltraPerformance Convergence

Chromatography (UPC²) system (Waters Co., Milford, MA, USA) and Xevo TQ–S micro tandem mass spectrometer with an electrospray ionization ion source (Waters Co.), which were controlled by MassLynx software version 4.1 (Waters Co.). An HPLC 515 pump (Waters Co.) was used as a make-up pump to enhance ionization efficiency. There were 313 lipid molecules detected in the serum (Supplementary Table 3). The relative concentrations (RC) of detected lipid molecules were corrected with serum total cholesterol concentrations using the following equation:

[Corrected relative concentration (CRC)] = [relative concentration] / [concentration of serum total cholesterol] x 1,000.

2.5. Evaluation of atherosclerotic lesions

We examined atherosclerotic lesions of WHHLMI rabbits that died from myocardial ischemia during the experiment in addition to rabbits euthanized at the end of the experiment (20 months old). Coronary sections were prepared as reported previously [S-4]. We examined the atherosclerotic lesions of the left circumflex arteries because this artery is the major coronary artery, and atherosclerotic lesions frequently develop there in rabbits [S-5]. Sections were stained with Victoria blue-HE staining. The degree of coronary atherosclerosis was evaluated as cross-sectional narrowing

(CSN). The severity of coronary lesions was evaluated using a coronary severity score based on the following equation:

[Coronary severity score] = [average CSN] x (1 + [frequency of sections with 75%-89% CSN] x 10 + [frequency of sections with >90% CSN] x 20)

Supplementary Figure 1 shows the development of atherosclerotic lesions in the aorta and coronary circumflex artery of WHHLMI rabbits. As a result of the evaluation of the coronary severity score, we classified WHHLMI rabbits into three groups (severe, intermediate, or mild). In the severe group, every rabbit had coronary sections with more than 90% CSN, and no rabbits in the mild group had coronary sections with more than 75% CSN. Aorta were excised from the aortic root to the bifurcation of the femoral artery. Aortic lesions were evaluated as the ratio of the surface area of the lesions to the surface area of the entire lumen. Yu et al. [S-6] demonstrated that in cholesterol-fed rabbits, the percentage of surface lesion area on aortic lumen surface correlated with the intimal lesion area of the aortic histopathological sections.

2.6. Age-dependent progression of coronary lesions

To examine age-dependent progression of coronary lesions in WHHLMI rabbits, we examined the maximum CSN from one month old to 30 months old using 363 dead

rabbits or rabbits euthanized in other experiments between 2010 and 2016. Coronary lesions were examined in the manner described above.

2.7. Other assays

At the start of the experiment (4 months old) and at the end of experiment (20 months old or at death), we assayed the plasma levels of glucose, insulin, NO_2, NO_3, and oxidized LDL, and superoxide dismutase (SOD) activity using the Autokit Glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), Lbis (R) Insulin-Rat-T (FUJIFILM Wako Pure Chemical Corporation), NO_2/NO_3 Assay Kit-CII (Dojindo Laboratories, Co., Ltd., Kumamoto, Japan), Rabbit Oxidized Low-density Lipoprotein (OxLDL) ELISA kit (MyBioSource Inc., San Diego, USA), and SOD Assay Kit-WST (Dojindo Laboratories, Inc.), respectively.

2.8. Data and statistical analyses

Three rabbits died during the experiment. Because the frequency of sections with >90% CSN was 86%, 82%, and 87% in each of these rabbits, the cause of death was likely related to occlusion of the coronary arteries from an atherosclerotic lesion. In analyses of serum marker candidates for coronary lesions, we also used data from these

three rabbits in addition to data from the 17 rabbits sacrificed at 20 months old because the purpose was to identify serum marker candidates for the progression of coronary atherosclerosis. Two rabbits that died for renal tumor or lymphoma were excluded from analysis. In analyses of sex differences, the severity of coronary lesions and the RC of serum metabolites and the CRC of serum lipid molecules were significantly different between males and females (Supplementary Table 4, Supplementary Figure 2), despite no differences in aortic surface lesion area and conventional plasma biochemical parameters. In addition, 7 of 9 males and 3 of 11 females were in the severe group. Consequently, we performed metabolomic and lipidomic analyses using only females. Serum marker candidates for coronary atherosclerosis were determined by the method of Supplementary Figure 3. In lipidome analyses, because 20 of 313 lipid molecules were below the detection limit in at least one rabbit, 293 lipid molecules were used to select serum marker candidates. In brief, for serum metabolites or lipid molecules at 4 and 8 months old, marker candidates were selected using two statistical analysis steps. The first step was a volcano plot analysis [S-7] using a cutoff p-value of 0.05 and 30% fold-changes in the CRC between the severe and mild groups. The second step was a Spearman's rank-correlation analysis using a cutoff correlation coefficient-value of 0.550. For serum metabolites and

lipid molecules at 12, 16, and 20 months old or death, marker candidates were selected after an additional Dunnett analyses between the CRC of selected candidate molecules of the severe group at the selected age and those of the mild group at each age. Metabolites or lipid molecules with significant >50% fold changes from the severe group at the selected age from that in the mild group at each age were selected as markers.

Differences in the profiles of serum metabolites or lipid molecules between the severe and mild groups were analyzed by sparse partial least square discriminant analysis (SPLS-DA) using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/) [S-8].

Data are the mean ± SEM. $P < 0.05$ was considered significant. Correlation analyses between the coronary severity score and the relative concentration of plasma metabolites or lipid molecules were performed with Spearman's rank correlation coefficient.

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Supplementary Table 1. Fifty nine metabolites examined in metabolome analyses

1,5-Anhydro-D-glucitol	1-Hexadecanol	1-Methyl Histidine	2-Aminoethanol	3-Hydroxy-Butyrate	Alanine	a-Phenylglycine
Arabitol	Asparagine	Aspartic acid	b-Alanine	Benzen-1,3-Dicarboxylic acid	Citric acid + Isocitric acid	Citrulline
Cystamine	Cystathionine	Cysteine+Cystine	Fumaric acid	Galactosamine_2	Glucuronate_1	Glutamic acid
Glutamine	Glyceraldehyde_2	Glycerol	Glycine	Glycolic acid	Histidine	Hypoxanthine
Inositol	Ketoisoleucine_1	Ketovaline_1	Lactic acid	Lauric acid	Lysine	Lyxose_2
Malic acid	Mannose_1	meso-erythritol	Methionine	N-Acetyl-D-Glucosamine_1	N-FormylGlycine	Nonanoic acid
O-Phosphoethanolamine	Ornithine	Phenylalanine	Phosphate	Prolinamide	Proline	Pyroglutamic acid
Pyruvate+Oxalacetic acid	Ribitol	Serine	Threo-b-HydroxyAspartic acid	Threonine	trans-4-Hydroxy-L-proline	Tryptophan
Tyrosine	Uracil	Valine				

Supplementary Table 2. Internal standard substances in lipidome analyses

Lipid class	ISD conc. (μM)	Final conc. (nM)	Injection (fmol)	Total extraction volume (pmol)	Serum concentration (nmol/ml)
FFA 17:0	500	2244	2244	4999	250
LPC 17:0	25	112	112	250	12. 5
LPE 17:1	500	2,243	2,244	4,999	250
PC 17:0-17:0	500	2,243	2,244	4,999	250
PE 17:0-17:0	200	1,794	1,795	3,999	200
PS 17:0-17:0	2,000	17,948	17,948	399,887	1,999
SM d18:1-17:0	5	22.4	22.4	50.0	2.50
Cer d18:1-17:0	5	22.4	22.4	50.0	2.50
CE 17:0	250	2244	2244	4999	250
MAG 17:0	50	224	224	500	25
DAG 12:0-12:0	50	224	224	500	25
TAG 51:0	50	224	224	500	25

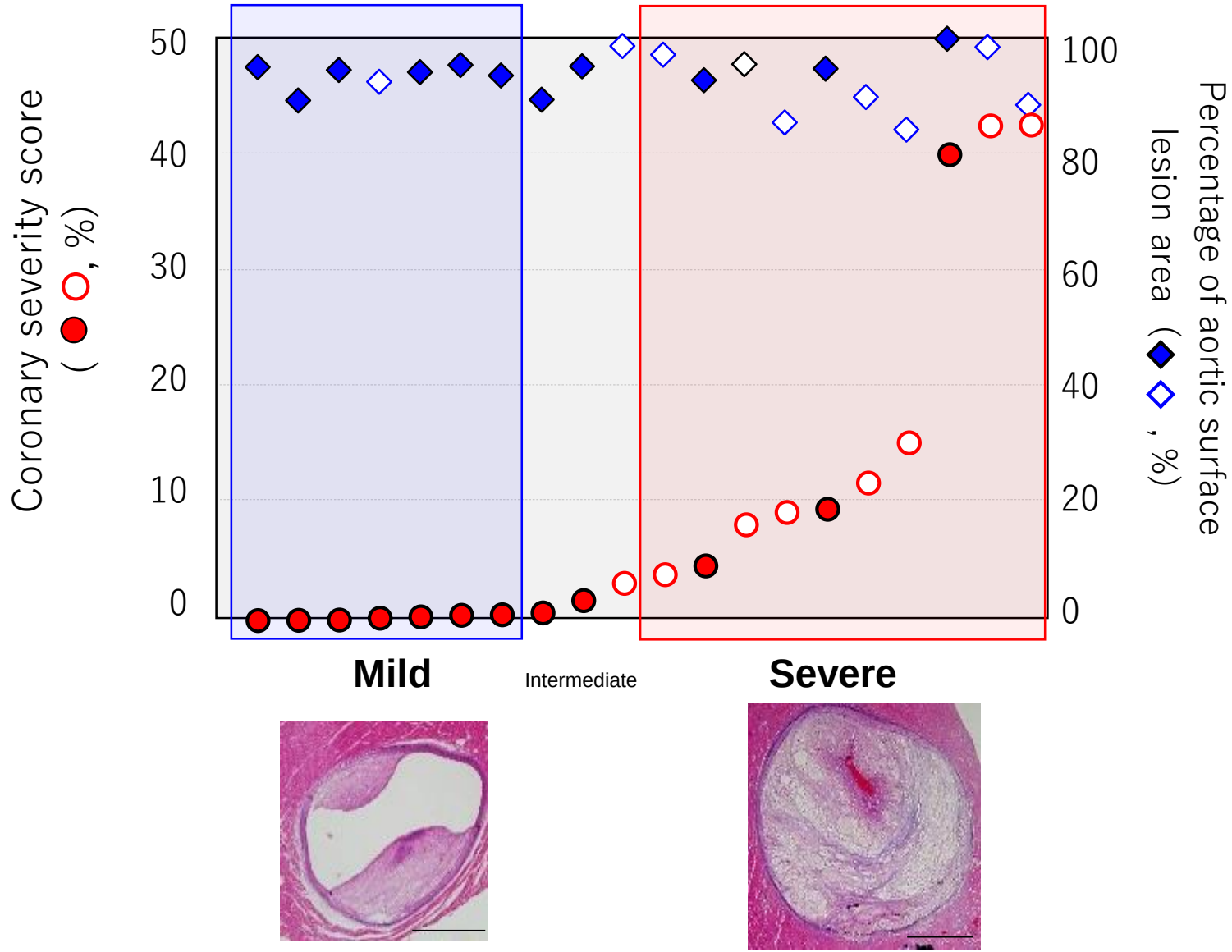
ISD, internal standard; FFA, free fatty acids; LPC, Lysophosphatidylcholine; LPE, Lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, Phosphatidylethanolamine; PS, phosphatidylserine; SM, Sphingomyelin; Cer, Ceramide; CE, cholesterol ester; MAG, monoacylglycerol: DAG. Diacylglycerol; TAG, triacylglycerol

Supplementary Table 3. 322 lipid molecules examined in lipidome analyses

Free fatty acids (FFA): 8 lipids										
FFA 14:0	FFA 16:0	FFA 16:1	FFA 18:0	FFA 18:1	FFA 18:2	FFA 18:3	FFA 20:4			
Lysophosphatidylcholine (LPC): 26 lipids										
LPC 14:0	LPC 16:0 (sn-1)	LPC 16:0 (sn-2)	LPC 16:1 (sn-1)	LPC 16:1 (sn-2)	LPC 18:0 (sn-1)	LPC 18:0 (sn-2)	LPC 18:1 (sn-1)	LPC 18:1 (sn-2)	LPC 18:2 (sn-1)	LPC 18:2 (sn-2)
LPC 18:3 (sn-1)	LPC 18:3 (sn-2)	LPC 20:1 (sn-1)	LPC 20:1 (sn-2)	LPC 20:2 (sn-1)	LPC 20:2 (sn-2)	LPC 20:3 (sn-1)	LPC 20:3 (sn-2)	LPC 20:4 (sn-1)	LPC 20:4 (sn-2)	LPC 20:5
LPC 22:0	LPC 22:4	LPC 22:5	LPC 22:6							
Lysophosphatidylethanolamine (LPE): 6 lipids										
LPE 16:0	LPE 18:0 (sn-1)	LPE 18:0 (sn-2)	LPE 18:1	LPE 18:2 (sn-1)	LPE 18:2 (sn-2)					
Phosphatidylcholine (PC): 33 lipids										
PC 16:0-16:0	PC 16:0-16:1	PC 16:0-18:0	PC 16:0-18:1	PC 16:0-18:2	PC 16:0-18:3	PC 16:0-20:0	PC 16:0-20:2	PC 16:0-20:3	PC 16:0-20:4	PC 16:0-22:4
PC 16:0-22:5	PC 16:1-18:1	PC 16:1-18:2	PC 18:0-18:0	PC 18:0-18:1	PC 18:0-18:2	PC 18:0-18:3	PC 18:0-20:2	PC 18:0-20:3	PC 18:0-20:4	PC 18:0-22:4
PC 18:0-22:5	PC 18:1-18:1	PC 18:1-18:2	PC 18:1-18:3	PC 18:1-20:4	PC 18:2-18:2	PC 18:2-18:3	PC 18:2-20:1	PC 18:2-20:2	PC 18:2-20:3	PC 18:2-20:4
Ether Phosphatidylcholine (EPC): 11 lipids										
PC 16:1e-16:0 / PC 16:0p-16:0	PC 16:1e-18:1 / PC 16:0p-18:1	PC 16:1e-18:2 / PC 16:0p-18:2	PC 16:1e-20:4 / PC 16:0p-20:4	PC 16:2e-20:5 / PC 16:1p-20:5						
PC 18:1e-18:1 / PC 18:0p-18:1	PC 18:1e-18:2 / PC 18:0p-18:2	PC 18:1e-20:4 / PC 18:0p-20:4	PC 18:2e-18:2 / PC 18:1p-18:2	PC 18:2e-20:4 / PC 18:1p-20:4	PC 18:2p-20:5					
Phosphatidylethanolamine (PE): 9 lipids										
PE 16:0-18:1	PE 16:0-18:2	PE 16:0-20:4	PE 18:0-18:1	PE 18:0-18:2	PE 18:0-18:3	PE 18:0-20:4	PE 18:1-18:2	PE 18:2-18:2		
Phosphatidylethanolamine plasmalogen (PEpln): 40 lipids										
PE 16:0p-16:0	PE 16:0p-18:1	PE 16:0p-18:2	PE 16:0p-18:3	PE 16:0p-20:3	PE 16:0p-20:4	PE 16:0p-20:5	PE 16:0p-22:4	PE 16:0p-22:5	PE 16:0p-22:6	PE 16:1p-20:2
PE 16:1p-22:2	PE 16:1p-22:3	PE 18:0p-16:0	PE 18:0p-16:1	PE 18:0p-18:0	PE 18:0p-18:1	PE 18:0p-18:2	PE 18:0p-18:3	PE 18:0p-20:3	PE 18:0p-20:4	PE 18:0p-20:5
PE 18:0p-22:4	PE 18:0p-22:5	PE 18:0p-22:6	PE 18:1p-16:0	PE 18:1p-18:1	PE 18:1p-18:2	PE 18:1p-18:3	PE 18:1p-20:2	PE 18:1p-20:3	PE 18:1p-20:4	PE 18:1p-22:4
PE 18:1p-22:5	PE 18:1p-22:6	PE 18:2p-18:1	PE 18:2p-18:2	PE 18:2p-20:2	PE 18:2p-20:4	PE 18:2p-22:2				
Phosphatidylinositol (PI): 10 lipids										
PI 16:0-16:0	PI 16:0-18:1	PI 16:0-18:2	PI 18:0-18:1	PI 18:0-18:2	PI 18:0-20:2	PI 18:0-20:3	PI 18:0-20:4	PI 18:1-18:1	PI 18:1-18:2	
Sphingomyelin (SM): 14 lipids										
SM d18:1-14:0	SM d18:1-14:1	SM d18:1-16:0	SM d18:1-16:1	SM d18:1-18:0	SM d18:1-18:1	SM d18:1-18:2	SM d18:1-20:0	SM d18:1-20:1	SM d18:1-20:2	SM d18:1-22:0
SM d18:1-22:1	SM d18:1-22:2	SM d18:1-22:3								
Ceramide (Cer): 14 lipids										
Cer d18:1-16:0	Cer d18:1-18:0	Cer d18:1-18:2	Cer d18:1-18:4	Cer d18:1-20:0	Cer d18:1-20:1	Cer d18:1-20:2	Cer d18:1-20:4	Cer d18:1-22:0	Cer d18:1-22:1	Cer d18:1-22:2
Cer d18:1-22:3	Cer d18:1-22:4	Cer d18:1-22:5								

(**Supplementary table 3—continued**)

Cholesterol ester (CE): 20 lipids									
CE 14:0	CE 14:1	CE 16:0	CE 16:1	CE 18:0	CE 18:1	CE 18:2	CE 18:3	CE 18:4	CE 20:1
CE 20:2	CE 20:3	CE 20:4	CE 20:5	CE 22:1	CE 22:2	CE 22:3	CE 22:4	CE 22:5	CE 22:6
Monoacylglycerol (MAG): 2 lipids									
MAG 16:0	MAG 18:0								
Diacylglycerol (DAG): 51 lipids									
DAG 14:0-16:0	DAG 14:0-16:1	DAG 14:0-18:0	DAG 14:0-18:1	DAG 14:0-18:2	DAG 14:0-18:3	DAG 14:0-20:0	DAG 14:1-18:1	DAG 14:1-18:2	DAG 14:1-22:2
DAG 16:0-16:0	DAG 16:0-16:1	DAG 16:0-18:0	DAG 16:0-18:1	DAG 16:0-18:2	DAG 16:0-18:3	DAG 16:0-20:2	DAG 16:0-20:3	DAG 16:0-20:4	DAG 16:0-22:4
DAG 16:0-22:5	DAG 16:1-16:1	DAG 16:1-18:0	DAG 16:1-18:1	DAG 16:1-18:2	DAG 16:1-18:3	DAG 16:1-20:2	DAG 16:1-20:3	DAG 16:1-20:4	DAG 18:0-18:0
DAG 18:0-18:1	DAG 18:0-18:2	DAG 18:0-18:3	DAG 18:0-20:4	DAG 18:1-18:1	DAG 18:1-18:2	DAG 18:1-18:3	DAG 18:1-18:4	DAG 18:1-20:2	DAG 18:1-20:3
DAG 18:1-20:4	DAG 18:1-22:4	DAG 18:1-22:5	DAG 18:2-18:2	DAG 18:2-18:3	DAG 18:2-20:2	DAG 18:2-20:3	DAG 18:2-20:4	DAG 18:2-22:4	DAG 18:3-18:3
DAG 18:3-20:4									
Triacylglycerol (TAG): 69 lipids									
TAG 44:0	TAG 44:1	TAG 44:2	TAG 44:4	TAG 44:5	TAG 46:0	TAG 46:1	TAG 46:2	TAG 46:3	TAG 48:0
TAG 48:1	TAG 48:2	TAG 48:3	TAG 48:4	TAG 48:6	TAG 48:7	TAG 48:8	TAG 50:0	TAG 50:1	TAG 50:2
TAG 50:3	TAG 50:4	TAG 50:5	TAG 50:6	TAG 50:7	TAG 50:8	TAG 50:9	TAG 52:0	TAG 52:1	TAG 52:2
TAG 52:3	TAG 52:4	TAG 52:5	TAG 52:6	TAG 52:8	TAG 52:9	TAG 54:0	TAG 54:1	TAG 54:2	TAG 54:3
TAG 54:4	TAG 54:5	TAG 54:6	TAG 54:7	TAG 54:8	TAG 54:9	TAG 54:10	TAG 54:11	TAG 54:12	TAG 56:1
TAG 56:2	TAG 56:3	TAG 56:4	TAG 56:5	TAG 56:6	TAG 56:7	TAG 56:8	TAG 56:9	TAG 56:10	TAG 56:11
TAG 56:12	TAG 56:13	TAG 58:12	TAG 58:13	TAG 60:12	TAG 60:13	TAG 60:15	TAG 62:15	TAG 62:16	

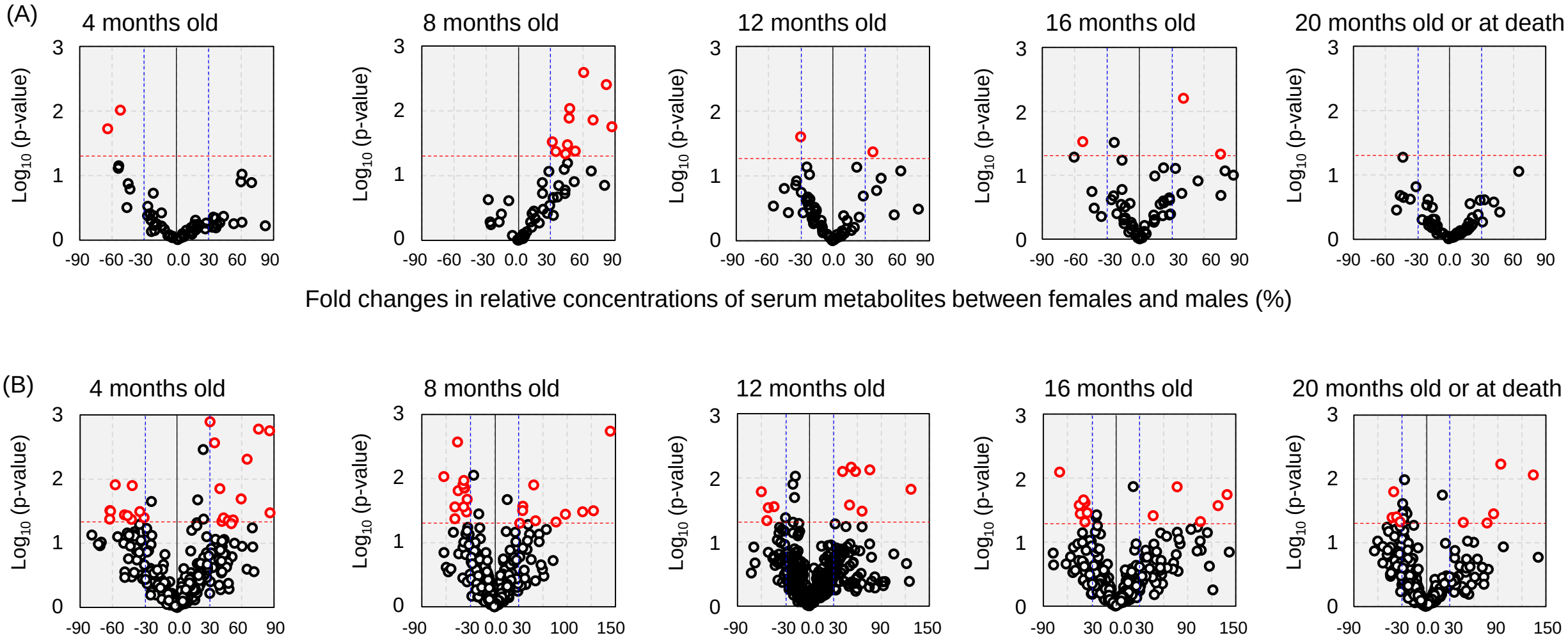


Supplementary Figure 1. Development of atherosclerotic lesions in aorta and coronary arteries of 20 WHHLMI rabbits analyzed in this study. Bars in photomicrographs indicate 500 micrometers.
Coronary severity score = [average CSN] x (1 + [frequency of sections with 75-89% CSN] x 10+ [frequency of sections with >90% CSN] x 20); CSN, cross-sectional narrowing; Solid symbols, female; Open symbols, males

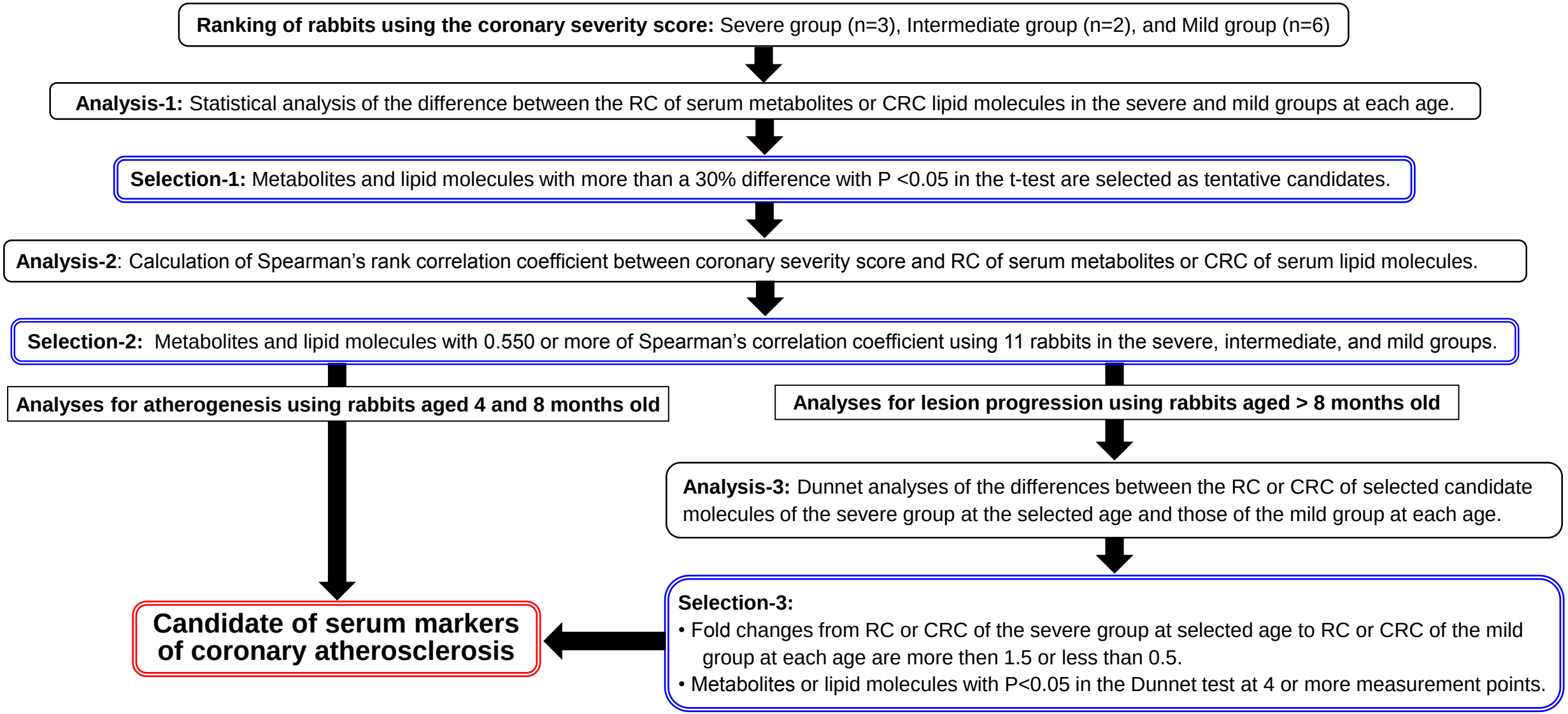
Supplementary Table 4. Sex differences in coronary severity score of WHHLMI rabbits

		Coronary severity score	Examined sections	Sections with lesions	Frequency of sections with lesions	Average CSN	Maximum CSN	Sections with >75% CSN	Frequency of sections with >75% CSN	Sections with >90% CSN	Frequency of sections with >90% CSN	Aortic lesion
Mean	**Female (n=11)**	**5.3**	**20.3**	**13.5**	**65%**	**39%**	**66%**	**5**	**21%**	**2.3**	**10%**	**94%**
	Male (n=9)	**15.2**	**19.4**	**15.4**	**82%**	**63%**	**94%**	**9**	**51%**	**5.7**	**33%**	**92%**
SEM	Female	5.4	0.6	2.3	11%	9%	10%	2.1	10%	1.6	7%	2%
	Male	3.5	1.1	1.3	8%	8%	3%	1.8	11%	1.6	11%	1%
	P-value	***0.020***	***0.726***	***0.909***	***0.136***	***0.044***	***0.030***	***0.052***	***0.036***	***0.035***	***0.021***	***0.271***

Statistical analyses were performed by Mann-Whitney U-test. CSN, cross-sectional narrowing; average CSN, mean of CSN in all coronary cross-sections; Maximum CSN, The largest CSN in all coronary cross-sections.



Supplementary Figure 2. Sex differences in plasma metabolites (A) and lipid molecules (B) . Data were analyzed by volcano plot analyses. Horizontal red broken lines indicate levels of *p=0.05* in Student's *t-test* or Welch's *t-test.*



Supplementary Figure 3. Method for selecting blood marker candidates relating to progression of coronary lesions.

RC, relative concentration; CRC, corrected relative concentration with plasma total cholesterol concentration

Supplementary Table 5. Plasma lipid levels of WHHLMI rabbits.

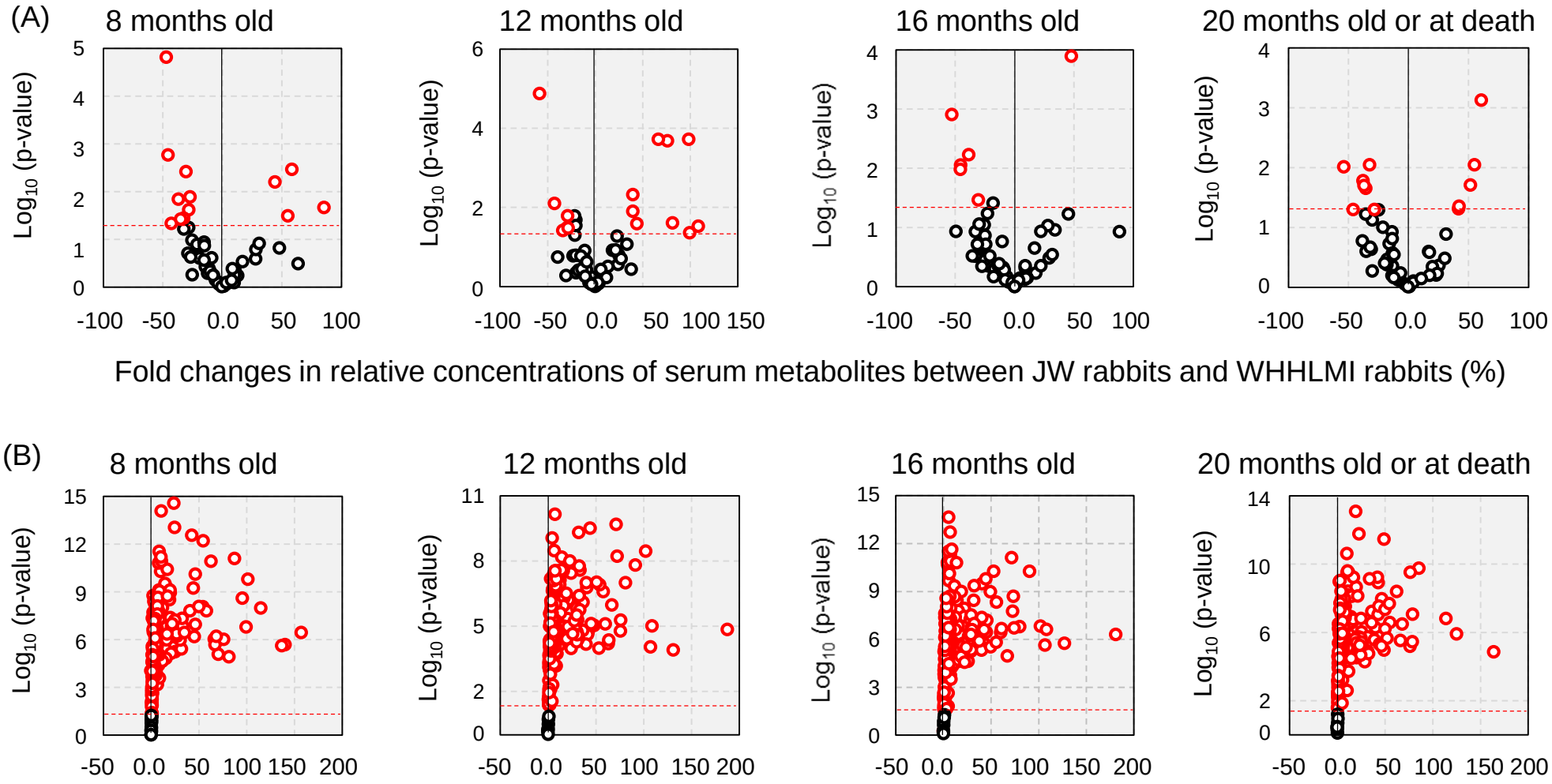
	Severe group	Mild group	*P-value*
Plasma cholesterol (mg/dl)			
At 4 months old	1346 ± 46	1394 ± 35	*0.446*
At 20 months old or at death	1367 ± 251	1019 ± 79	*0.146*
Plasma triglyceride (mg/dl)			
At 4 months old	290 ± 71	257 ± 19	*0.560*
At 20 months old or at death	417 ± 171	303 ± 35	*0.576*
VLDL cholesterol(mg/dl)			
At 4 months old	149 ± 17	133 ± 11	*0.428*
At 20 months old or at death	233 ± 110	96 ± 18	*0.340*
VLDL triglyceride (mg/dl)			
At 4 months old	50 ± 14	39 ± 4	*0.348*
At 20 months old or at death	132 ± 81	64 ± 10	*0.489*
LDL cholesterol (mg/dl)			
At 4 months old	1184 ± 38	1248 ± 35	*0.290*
At 20 months old or at death	1025 ± 204	910 ± 67	*0.197*
LDL triglyceride (mg/dl)			
At 4 months old	234 ± 56	208 ± 15	*0.566*
At 20 months old or at death	270 ± 85	216 ± 26	*0.454*
HDL cholesterol (mg/dl)			
At 4 months old	12.8 ± 2.4	12.6 ± 1.1	*0.937*
At 20 months old	15.8 ± 2.6	12.1 ± 1.0	*0.153*
LDL-cholesterol / HDL cholesterol			
At 4 months old	99 ± 17	102 ± 8	*0.842*
At 20 months old	72 ± 5	78 ± 9	*0.640*

Data are presented as the mean ± SEM. Statistical analyses were performed with Student's t-test or Welch's t-test.

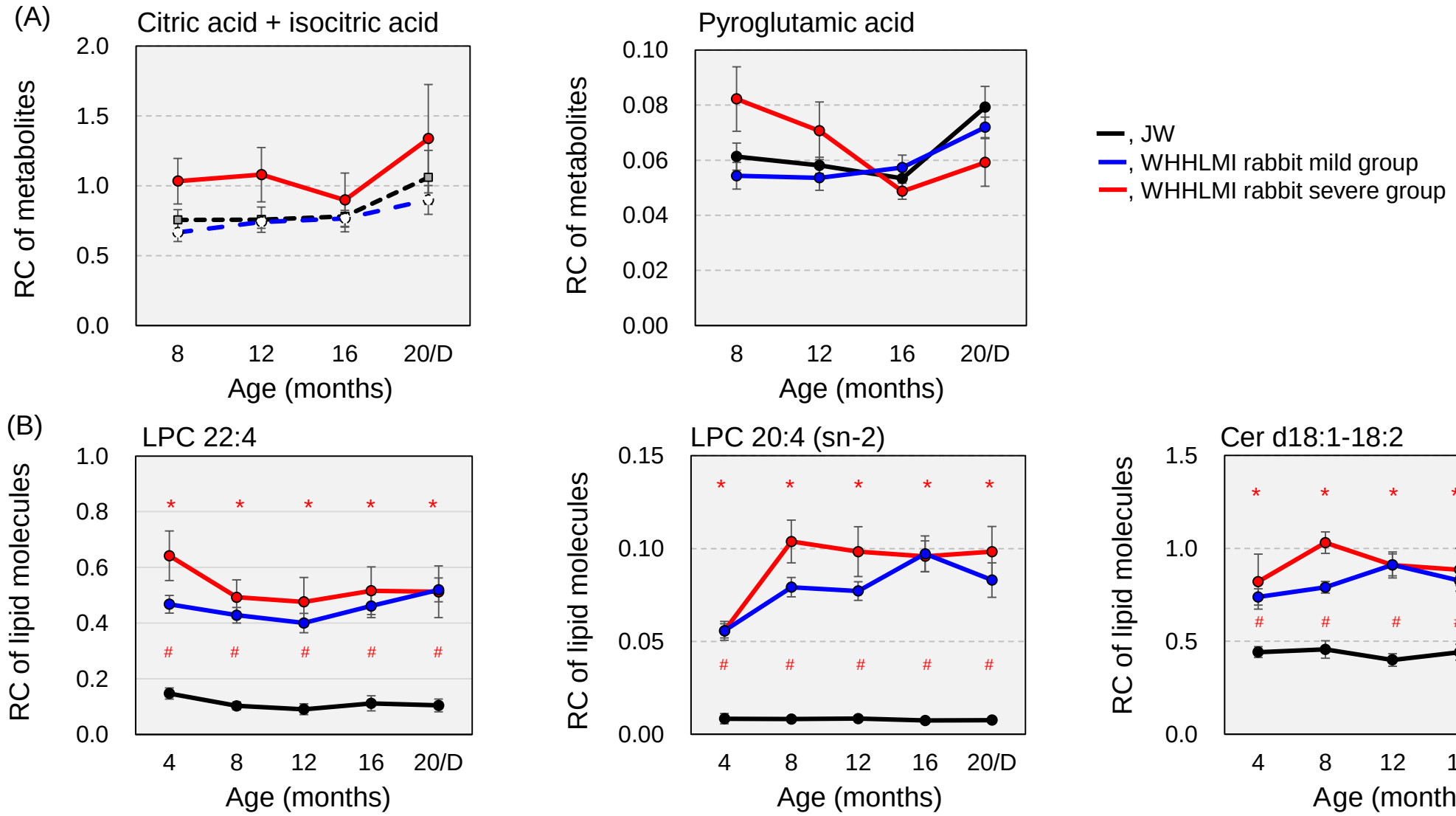
Supplementary Table 6. Plasma levels of glucose, insulin, oxidized LDL, SOD, NO_2, and NO_3 of WHHLMI rabbits.

	Severe group	Mild group	*P-value*
Plasma glucose (mg/dl)			
At 4 months old	137 ± 9	133 ± 5	*0.712*
At 20 months old or at death	115 ± 6	124 ± 4	*0.201*
Plasma insulin (ng/ml)			
At 4 months old	0.48 ± 0.06	0.54 ± 0.09	*0.640*
At 20 months old or at death	0.78 ± 0.30	0.74 ± 0.20	*0.913*
NO_2(μmol/l)			
At 4 months old	16.6 ± 2.0	16.2 ± 2.4	*0.913*
At 20 months old or at death	21.7 ± 7.4	15.0 ± 2.8	*0.329*
NO_3 (μmol/l)			
At 4 months old	31.2 ± 14.4	32.2 ± 6.5	*0.943*
At 20 months old or at death	21.0 ± 2.6	20.6 ± 4.0	*0.943*
Oxidized LDL (ng/ml)			
At 4 months old	0.55 ± 0.10	0.52 ± 0.06	*0.789*
At 20 months old or at death	0.90 ± 0.15	0.76 ± 0.20	*0.668*
Superoxide dismutase activity (U/ml)			
At 4 months old	1580 ± 754	1676 ± 359	*0.900*
At 20 months old or at death	1227 ± 399	1383 ± 353	*0.795*

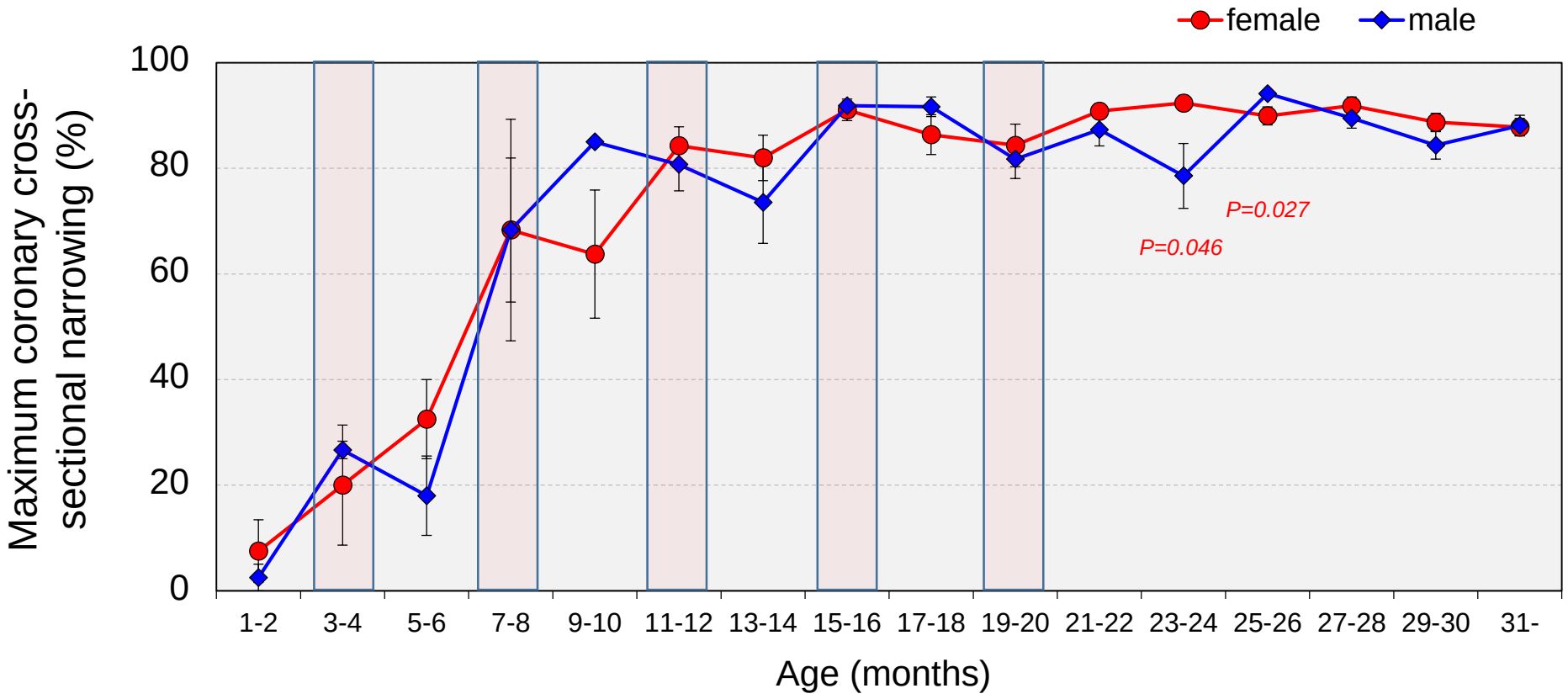
Data are presented as the mean ± SEM. Statistical analyses were performed with Student's t-test or Welch's t-test.



Supplementary Figure 4 Differences in plasma metabolites (A) and lipid molecules (B) between normal JW rabbits and WHHLMI rabbits. Data were analyzed volcano plot analyses. Horizontal red broken lines indicate levels of *p=0.05* in Student's *t-test* or Welch's *t-test*.



Supplementary Figure 5. Differences in selected serum metabolites (A) and lipid molecules (B) between JW rabbits and WHHLMI rabbits. Error bars indicate SEM. Statistical analyses were performed by the Dunnet method (*, *p=0.05* for JW vs WHHLMI rabbit severe group, #, *p=0.05* for JW rabbits vs WHHLMI rabbit mild group). Concentration of other selected lipid molecules were below the detection limit in JW rabbits. RC, relative concentration



Supplementary figure 6. Development and progression of coronary atherosclerosis in 363 WHHLMI rabbits that died between 2010 and 2016. Data are represented as the mean ± SEM. Sex differences were observed in the maximum coronary cross-sectional narrowing at 23-24 months old and 25-26 months old by Welch's t-test.