

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



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### HIGHLIGHTS

- Potential differences between serum markers of coronary lesions and aortic lesions.
- Stronger association with coronary lesion of factors other than conventional markers.
- Difference in the serum markers between generation of coronary lesions and advanced coronary lesions.

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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.

**Conclusions:** 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.

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## 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 Materials.

### 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 Materials.

### 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 Materials.

### 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:

$$[\text{Corrected relative concentration (CRC)}] = [\text{relative concentration}] / \text{serum total cholesterol concentration} \times 1000$$

The details are described in the [Supplementary Materials](#).

### 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:

$$[\text{Coronary severity score}] = [\text{average cross-sectional narrowing (CSN)}] \times (1 + [\text{frequency of sections with 75\%–89\% CSN}] \times 10 + [\text{frequency of sections with } > 90\% \text{ CSN}] \times 20)$$

The details are described in the [Supplementary Materials](#).

### 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 Materials](#).

### 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<sub>2</sub>, NO<sub>3</sub>, and oxidized LDL, and superoxide dismutase (SOD) activity. The details are described in the [Supplementary Materials](#).

### 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 Materials](#).

## 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 ( $96 \pm 2\%$  vs.  $94 \pm 1\%$ ). 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

**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.

(Supplementary Tables 5 and 6). These results indicate that factors other than lipoprotein lipids and other plasma parameters were involved in the progression of coronary lesions.

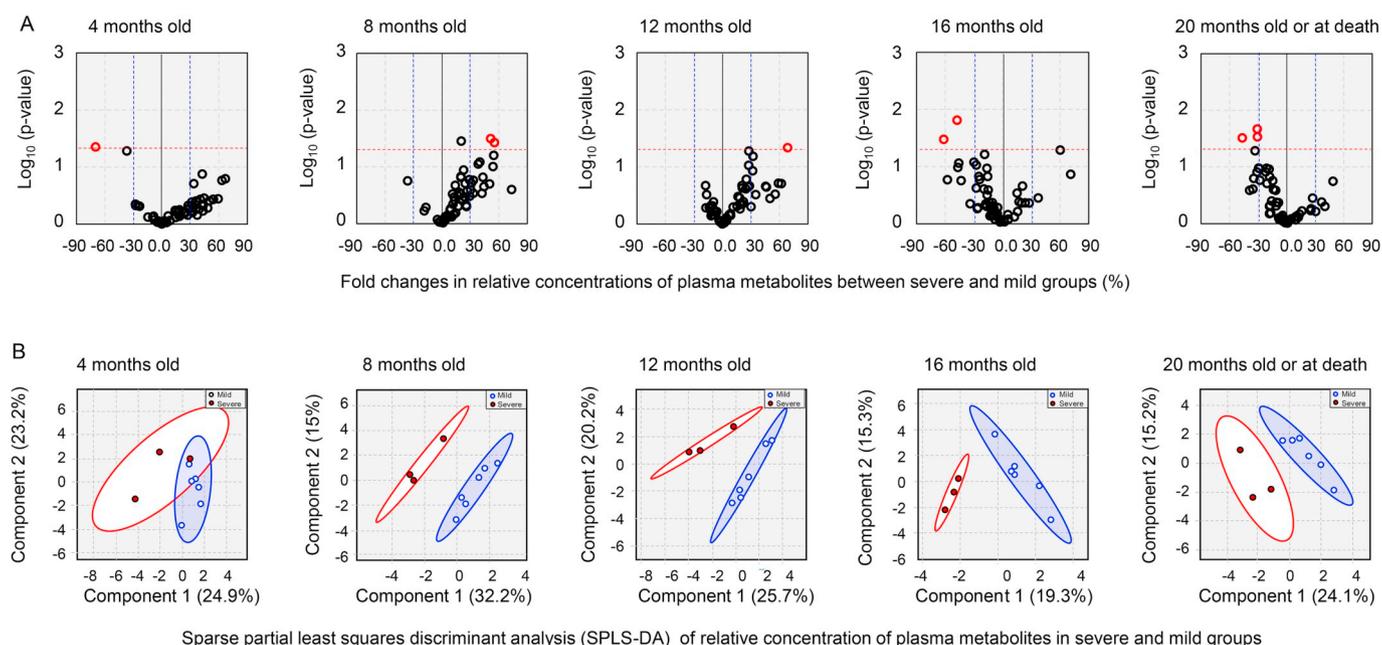
### 3.3. Examination of serum metabolites as markers specific for coronary lesions

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 (Fig. 1A) 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 (Fig. 1-B). Comparing normal and WHHLMI rabbits, the RC of 26 of 59 serum metabolites was significantly different (Supplementary Fig. 4A). 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 Fig. 5A).

### 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 (Fig. 2A), 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 (Fig. 3A), only PE 16:1p-22:2 was selected at 16 months old. In SPLS-DA of serum lipid molecules (Fig. 2B), 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



**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.

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

|                              | Severe group<br>(n = 3) | Mild group<br>(n = 6) | Fold<br>change | p-value | Correlation coefficient<br>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.

relative concentrations of many serum lipid molecules in WHHLM rabbits were extremely high (Supplementary Fig. 4B). 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 WHHLM rabbits compared to those in normal JW rabbits (Supplementary Fig. 5B), but others were below the detection limitation in normal JW rabbits.

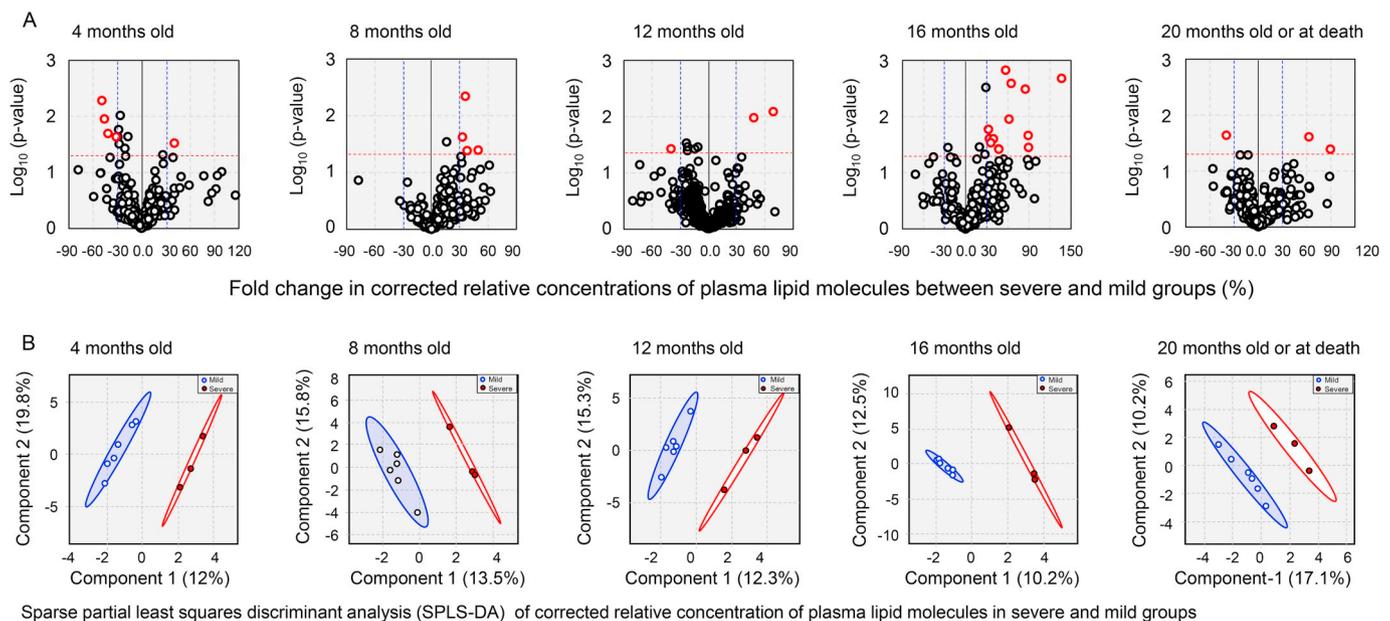
#### 4. Discussion

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 WHHLM 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 Fig. 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 Fig. 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



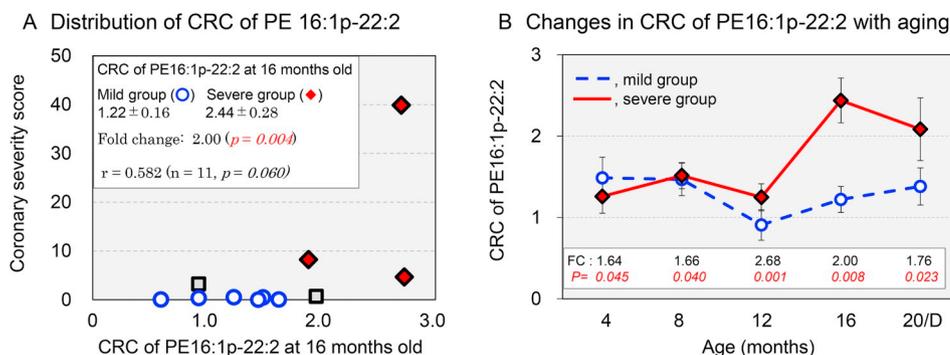
**Fig. 2.** Profiles of serum lipid molecules in female WHHLM rabbits with severe and mild coronary atherosclerosis.

(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.

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

|                | Severe group<br>(n = 3) | Mild group<br>(n = 6) | Fold<br>change | p-value | Correlation coefficient<br>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.



**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 (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 (A) and by a Dunnett test in (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).

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 Fig. 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.

#### Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

#### Author contributions

The study was conceived and designed by MShio; the experiments were performed by MShio, HT, YI, 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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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2019.02.020>.

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