P), respectively [11]. Given that SDG-P mice show evident glucose intolerance with mild obesity after HFD feeding as compared with SDG-R mice, these mice may serve as appropriate models for investigating hereditary predisposition to diet-induced diabetes. In this study, we examined pancreatic islet morphological features and functions in these novel mouse lines to investigate the predisposition to HFD-induced glucose intolerance in islets.

## Materials and Methods

### Animals

Male SDG-R and SDG-P mice ($15^{th}$–17th generations [11]) bred at the Institute for Animal Reproduction (Kasumigaura, Japan) were used. The mice were housed at 3–5 animals per cage and maintained in a temperature-controlled room on a 14-h light/10-h dark cycle, with free access to water and standard rodent chow (MF, Oriental Yeast, Tokyo, Japan), unless otherwise specified. SDG-R and SDG-P mice were fed with HFD (Quick Fat, CLEA Japan, Tokyo), providing 32% energy as fat, for 5 weeks (5–10 weeks of age). Before (4–5 weeks of age) and after HFD feeding (10–11 weeks of age), oral glucose tolerance test (OGTT), insulin tolerance test (ITT), and morphometric and functional analyses of pancreatic islets were performed. This study was conducted under approval from the institutional animal care and use committee of Nippon Medical School.

### Oral Glucose Tolerance Test

After overnight-fasted blood glucose levels were measured, a 20% glucose solution (40 and 60 mg glucose per mouse before and after HFD feeding, respectively) was orally administered, and blood glucose levels were measured at 30, 60, and 120 min after the administration with a glucose sensor (Glutest Neo Super, Sanwa Kagaku Kenkyusho, Nagoya, Japan) by tail bleeding. To evaluate early-phase insulin response, blood plasma was prepared from the tail vein blood before and at 15 and 30 min after the glucose challenge at 5 weeks of age. Plasma insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (Morinaga, Yokohama, Japan).

### Insulin Tolerance Test

ITT was performed in accordance with a recommendation from the Mouse Metabolic Phenotyping Center (MMPC) Consortium [12]. After 6-h-fasted blood glucose levels were measured, insulin (Humulin R, Eli Lilly Japan, Tokyo) was injected intraperitoneally at 0.5 U/kg of body weight. Blood glucose levels were measured at 15, 30, 60, and 90 min after the injection as described earlier.

### Morphometric Analysis of Islet Cells

The overnight-fasted mice were killed by blood withdrawal from the inferior vena cava under anesthesia. The pancreas was excised and fixed in neutral-buffered formalin and embedded in paraffin. After reviewing the sections (4-μm thickness) with hematoxylin-eosin staining, contiguous sections were doubly immunostained for glucagon and insulin to identify α and β cells, respectively [13,14]. Quantitative evaluations of the islet areas and volume densities of the α and β cells were performed using a computer-assisted point-counting method on Axio Image A1 microscope (Carl Zeiss, Oberkochen, Germany) and Nikon DS-Fi1-L2 digital camera system (Nikon, Tokyo, Japan) with the Image J software (version 1.46c, Wayne Rasband, National Institutes of Health) [13–15]. The morphometric analysis was performed by examiners who were unaware of the assignment of the specimens.

### Islet Isolation and Insulin Secretion *in Vitro*

Pancreatic islets were isolated by pancreatic duct injection of collagenase solution (1 mg/mL in Krebs-Ringer bicarbonate buffer [KRB] containing 0.2% bovine serum albumin [BSA]), followed by digestion at 37°C for 15 min with gentle shaking. Islets were then picked up manually under a stereomicroscope. A group of 10 islets of similar size was transferred into a cell culture filter insert (12-μm pores; Millicell, EMD Millipore, Billerica, MA, USA) in a 24-well plate containing Roswell Park Memorial Institute 1640 medium (containing 5.5 mmol/l glucose and 10% fetal bovine serum) and incubated overnight [16]. After the insert with islets was rinsed twice for 15 min with 1.4 mmol/l glucose in KRB containing 0.2% BSA, the islets were incubated in 2.8 mmol/l glucose for 60 min (low glucose concentration). The islets were then rinsed with 1.4 mmol/l glucose and treated with 16.7 mmol/l glucose for 60 min (high glucose concentration), after which the islets were rinsed again and treated with 34.8 mmol/l KCl for 60 min (high KCl concentration). The solutions (low glucose, high glucose, and high KCl concentrations) were collected for insulin assay. To analyze the intracellular insulin content, the islets were sonicated in distilled water and suspended in an acid-ethanol solution.

### RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from freshly isolated islets using the Isogen reagent (Nippon Gene, Tokyo, Japan), and cDNA was generated by PrimeScript RT reagents (Takara Bio, Otsu, Japan) according to the manufacturers' instructions. Gene expression was analyzed using the ABI 7500 Fast real-time polymerase chain reaction system with the use of commercial primers of TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The differences in gene expression were calculated by the comparative ΔΔCT method of relative quantification (normalized to *Gapdh*).

### Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). Mean values were compared using the Student *t* test, and $p<0.05$ was considered statistically significant. Statistical analyses were performed using the JMP 9.0.2 software (SAS Institute, Cary, NC, USA).

## Results

### HFD-induced Hyperglycemia in SDG-P Mice

In OGTT, SDG-P mice showed modestly higher postchallenge blood glucose levels before HFD feeding (Figure 1A). The glucose intolerance in SDG-P mice became more evident after the 5-week HFD feeding (Figure 1D). In ITT, although blood glucose concentrations of SDG-P mice were higher than those of SDG-R at 15 and 90 min before HFD feeding (Figure 1B), the percent changes form baseline were not significantly different between the 2 lines of mice (Figure 1C). After HFD feeding, SDG-P mice showed evidently higher blood glucose concentrations in ITT (Figure 1E), and the 15-min value was significantly higher than that of SDG-R mice even when the values were expressed as percent changes form baseline (Figure 1F).

No significant differences were observed in the post-glucose challenge insulin levels, whereas the fasting-state insulin levels in SDG-P mice were higher than those in SDG-R mice at 5 weeks of age (Figure 2B). Although no significant differences were observed in blood glucose and insulin levels at 15 min in OGTT (Figure 2A, B), the insulin response at 15 min (incremental insulin levels) in SDG-P mice was lower than that in SDG-R mice (Figure 2C).

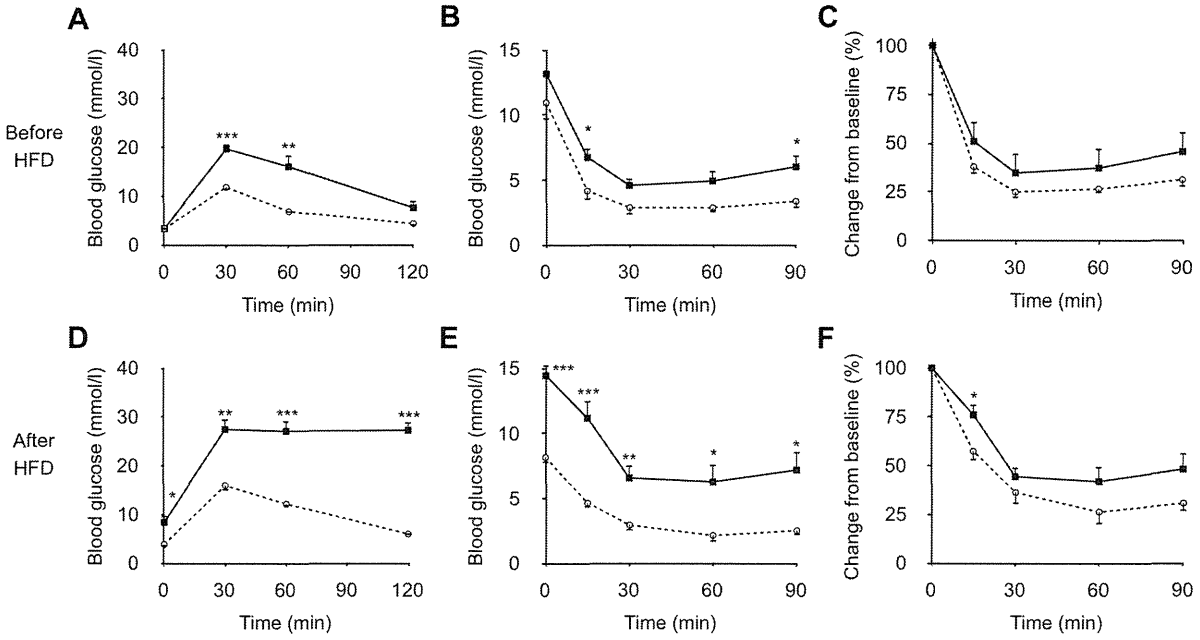


**Figure 1. Glucose tolerance and insulin sensitivity in SDG-R and SDG-P mice.** Blood glucose levels in OGTT before (**A**) and after HFD feeding (**D**). Blood glucose levels in ITT before (B) and after HFD feeding (E). Relative blood glucose levels from the baseline in ITT before (**C**) and after HFD feeding (**F**). Mean ± SEM (n = 5–6). SDG-R, open circle; SDG-P, closed square. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *versus* SDG-R mice.
doi:10.1371/journal.pone.0084725.g001

Consequently, the insulinogenic index ($\Delta[\text{insulin}]_{0-15\ \text{min}}/\Delta[\text{glucose}]_{0-15\ \text{min}}$) was significantly lower in SDG-P mice than in SDG-R mice (Figure 2D). The lower post-glucose challenge insulin response in SDG-P mice was also shown after HFD feeding (Figure 2E–H).

While no significant difference was observed in body weight between the 2 lines before HFD feeding (SDG-R mice, 15.2±0.9 g; SDG-P mice, 16.1±1.0 g: $p=0.53$), SDG-P mice gained more body weight than SDG-R mice during the 5-week

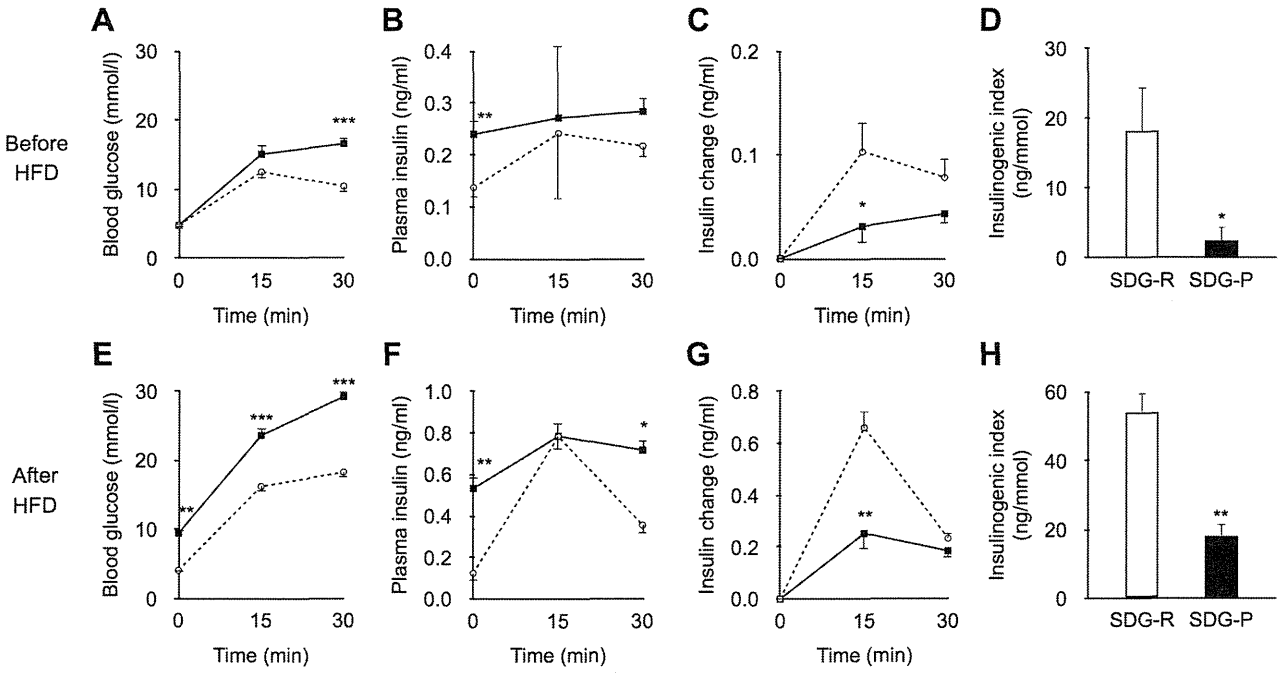


**Figure 2. Glucose induced insulin secretion *in vivo*.** Blood glucose and plasma insulin levels at 0, 15, and 30 min in OGTT before (**A–D**) and after HFD feeding (**E–H**). **A**, **E**; Blood glucose levels. **B**, **F**; Plasma insulin levels. **C**, **G**; Changes in plasma insulin levels from baseline. **D**, **H**; Insulinogenic index ($\Delta[\text{insulin}]_{0-15\ \text{min}}/\Delta[\text{glucose}]_{0-15\ \text{min}}$). Mean ± SEM (before HFD, n = 13–14; after HFD, n = 4–6). SDG-R, open circle/column; SDG-P, closed square/column. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *versus* SDG-R mice.
doi:10.1371/journal.pone.0084725.g002

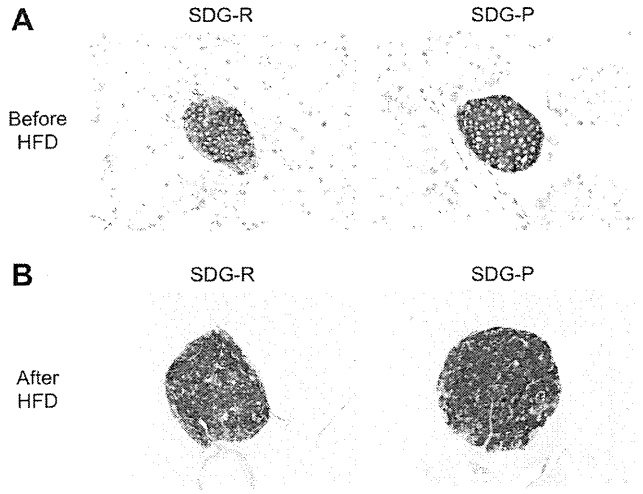


**Figure 3. Immunostaining images of pancreatic islet in SDG-R and SDG-P mice.** Immunostaining images of pancreatic islet before (**A**) and after 5-week HFD feeding (**B**). Double immunostaining for insulin (brown) and glucagon (red). Original magnification, ×200.
doi:10.1371/journal.pone.0084725.g003

HFD feeding period (body weight after HFD feeding: SDG-R mice, 25.6±1.4 g; SDG-P mice, 32.8±1.7 g: $p=0.008$).

## Morphological Analysis of Islets

Before HFD feeding, no significant differences in pancreatic weight, islet density, and composition of islet endocrine cells were observed in the morphometric analysis between the 2 mouse lines (Figure 3, Table 1). In contrast, HFD-fed SDG-P mice showed greater pancreatic weight and 2-fold larger β cell mass than SDG-R mice. Although the α cell density in HFD-fed SDG-P mice was lower than that in SDG-R mice, no significant differences in α cell mass were observed between the 2 lines before and after HFD feeding. The HFD-feeding induced pancreatic weight gain and β cell proliferation in both lines of mice, but it did not affect α cell mass.

In accordance with the immunohistochemical analysis, the appearance of isolated islets were closely similar between the 2 lines before HFD feeding (Figure 4A, D), whereas enlarged islets were observed in SDG-P mice after receiving HFD (Figure 4B, D). A greater number of islets were harvested from SDG-P mice before HFD feeding; however, no difference in the number of islets after receiving HFD was observed between the 2 lines (Figure 4C). Intracellular insulin content in SDG-P islets was higher than that in SDG-R islets before HFD feeding (Figure 4E). However, after receiving HFD, no difference in insulin content was observed between the 2 lines (Figure 4E). During the 5-week HFD feeding, the total number of islets was increased in SDG-R mice, whereas the average size of islets was increased but the cellular insulin content was decreased in SDG-P mice (Figure 4C–E).

## Insulin Secretion from Isolated Islets

In accordance with the difference in post-glucose challenge insulin response in vivo (Figure 2), the glucose-induced insulin secretion (GSIS) of the isolated islets from SDG-P mice were significantly lower than those from SDG-R mice regardless of whether it was before or after HFD feeding (Figure 5). A similar trend in insulin response was also observed in the KCl-induced insulin secretion (KSIS) (Figure 5).

## Gene Expression in Islets

The gene expression levels of a glucose transporter, *Glut2*, and a pancreas-specific transcriptional factor, *Pdx1*, were significantly lower in SDG-P islets than in SDG-R islets, regardless of whether it was before or after HFD feeding (Figure 6). The expression levels of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins *Snap25* and *Stx1a* in SDG-P islets were significantly lower than those in SDG-R before and after HFD feeding (Figure 6). SNARE proteins play a central role in insulin granule exocytosis in β cells [17]. Among lipid-handling and metabolism-related genes, a fatty acid transporter, *Cd36*, had a higher expression level in SDG-P islets, regardless of whether it was before or after HFD feeding, whereas a transcriptional factor, *Srebf1*, had a lower expression level after HFD feeding in SDG-P mice than in SDG-R mice (Figure 6).

# Discussion

Selective breeding has been applied to develop several animal models for diabetes research [2,18,19]. The polygenic background of selectively bred animal models is most likely to mimic the human pathophysiological features of type 2 diabetes. However, existing selectively bred diabetic models display marked hyperglycemia owing to spontaneous β cell loss even on normal chow

**Table 1.** Morphometric data of pancreatic islets in SDG-R and SDG-P mice.

| | Pancreas | Islet | β Cell | | α Cell | |
|---|---|---|---|---|---|---|
| | Weight (mg) | Density (%) | Density (%) | Mass (mg) | Density (%) | Mass (mg) |
| Before HFD[a] | | | | | | |
| SDG-R | 134±9 | 0.68±0.08 | 0.59±0.06 | 0.77±0.09 | 0.091±0.022 | 0.12±0.03 |
| SDG-P | 155±5 | 0.68±0.08 | 0.60±0.06 | 0.93±0.09 | 0.076±0.018 | 0.12±0.03 |
| After HFD[b] | | | | | | |
| SDG-R | 207±9$^{\dagger\dagger\dagger}$ | 0.92±0.08 | 0.85±0.08$^{\dagger}$ | 1.77±0.22$^{\dagger\dagger}$ | 0.072±0.011 | 0.15±0.02 |
| SDG-P | 302±28$^{*,\dagger\dagger\dagger}$ | 1.12±0.15$^{\dagger}$ | 1.08±0.15$^{\dagger}$ | 3.52±0.70$^{**,\dagger\dagger}$ | 0.039±0.004$^{**}$ | 0.13±0.02 |

Mean ± SEM (n = 5–6).
[a]Five weeks of age.
[b]Ten weeks of age.
*$p<0.05$, **$p<0.01$, *versus* SDG-R mice at the same age.
†$p<0.05$, ††$p<0.01$, †††$p<0.01$, *versus* before HFD feeding in the same line of mice.
doi:10.1371/journal.pone.0084725.t001

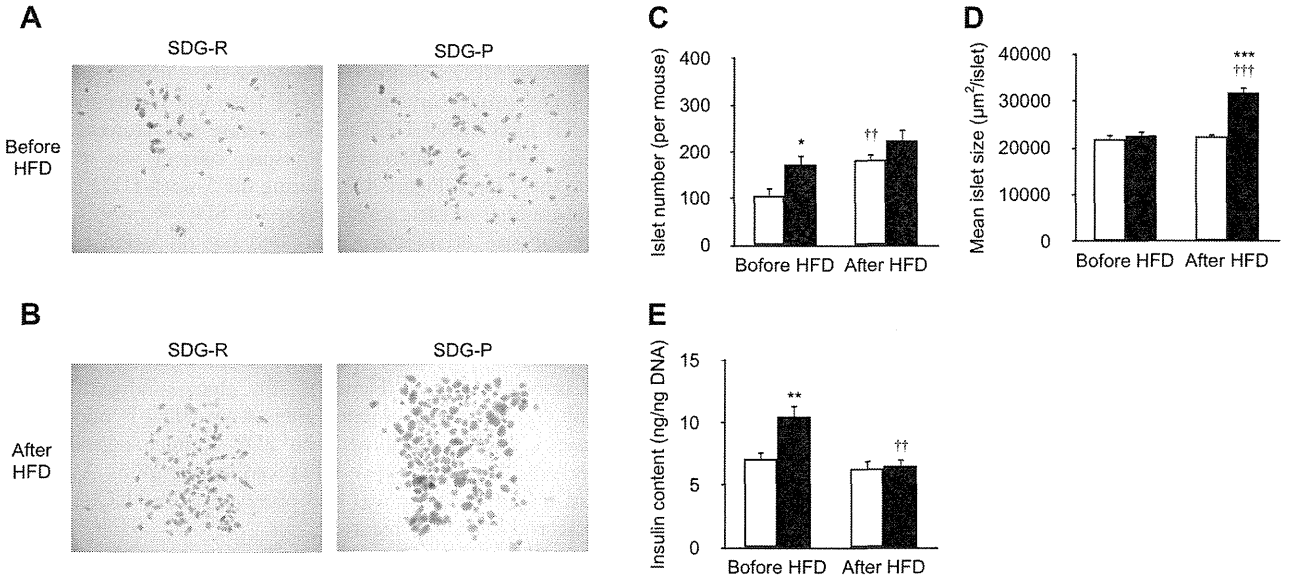


**Figure 4. Comparative analyses of isolated islets from SDG-R and SDG-P mice.** Stereomicroscopic images of the islets isolated before (**A**) and after 5-week HFD feeding (**B**). Islet numbers (**C**), apparent mean islet size (**D**), and insulin content (**E**) in the isolated islets. Mean ± SEM (n = 9–10). SDG-R, open column; SDG-P, closed column. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *versus* SDG-R mice. †$p<0.05$, ††$p<0.01$, †††$p<0.01$, *versus* before HFD feeding in the same line of mice.
doi:10.1371/journal.pone.0084725.g004

feeding [10]. Thus, hereditary predisposition to diet-induced impairment of glucose metabolism and consequent development of type 2 diabetes cannot be explained by the existing models. In the present study, SDG-P mice showed impaired glucose tolerance (moderate hyperglycemia in the postchallenge, not fasting, condition) before HFD feeding and developed overt diabetes after receiving HFD. Meanwhile, SDG-R mice maintained normal glucose tolerance even after receiving HFD. These novel mouse lines will therefore be appropriate to investigate the predisposition to diet-induced diabetes.

Before receiving HFD, SDG-P mice already displayed higher postchallenge blood glucose levels than SDG-R mice. In addition, the postchallenge insulin response was significantly lower in SDG-P mice than in SDG-R mice. These results suggest that SDG-P mice had innate defects in insulin secretion of pancreatic β cells. Several epidemiological studies also demonstrated that low early-phase insulin response in OGTT can predict future development of type 2 diabetes [20–22]. The preceding impairment of insulin secretion may predispose to exacerbated glucose tolerance under excessive dietary fat intake.

To elucidate the determinants of the difference in postchallenge insulin response between SDG-R and SDG-P mice, we included an analysis of the morphological features and functions of the pancreatic islets. Before HFD feeding, a greater number of islets with higher insulin content were harvested from SDG-P mice as compared with SDG-R mice, whereas no apparent differences were observed on immunohistological examination between the 2 lines, suggesting that functional defect (impaired insulin secretion) rather than β cell mass determined the impaired postchallenge insulin response in SDG-P mice in vivo. As expected, the isolated

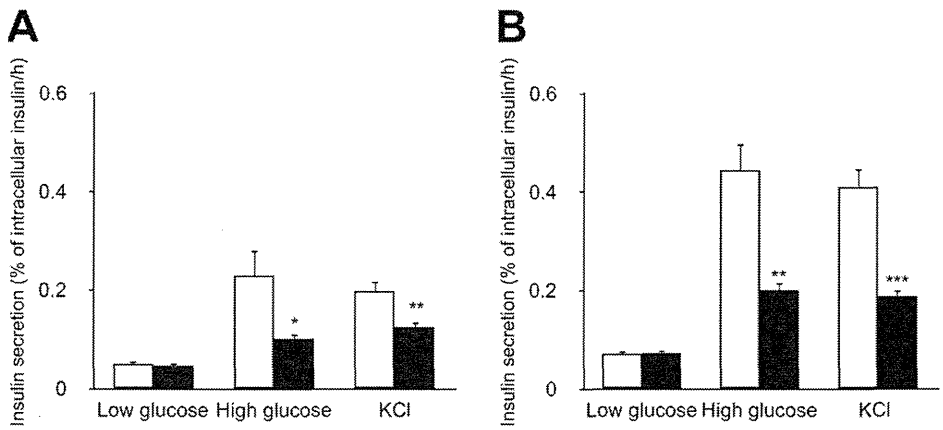


**Figure 5. Insulin secretion from isolated islets of SDG-R and SDG-P mice.** GSIS and KSIS from the islets isolated before (**A**) and after 5-week HFD feeding (**B**). Insulin secretion in 60 min was normalized to intracellular insulin content and expressed as percentages. Mean ± SEM (n = 9–10). SDG-R, open column; SDG-P, closed column. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *versus* SDG-R mice.
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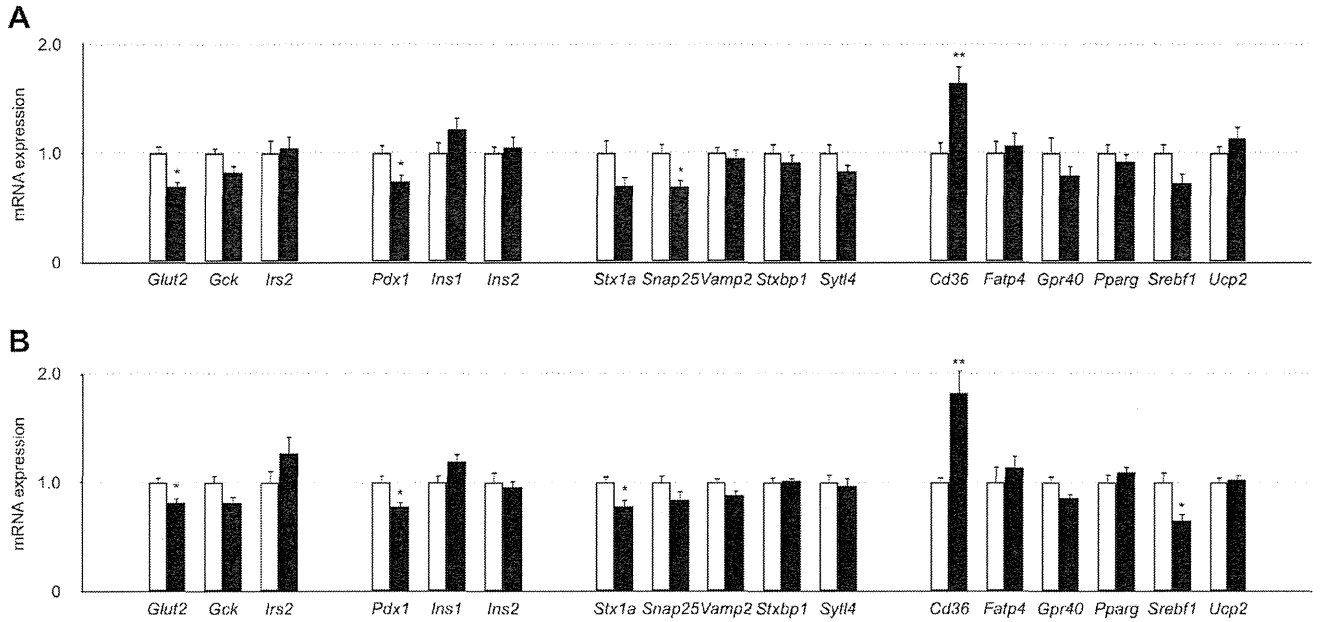


**Figure 6. Gene expression levels in isolated islets of SDG-R and SDG-P mice.** Relative gene expression levels in the islets isolated before (**A**) and after 5-week HFD feeding (**B**). Gene expression levels were normalized to *Gapdh*, and the normalized expression levels in SDG-P mice (closed column) were expressed as relative values to those in SDG-R mice (open column). Mean ± SEM (n = 7–9). *$p<0.05$, *versus* SDG-R mice.
doi:10.1371/journal.pone.0084725.g006

islets from SDG-P mice showed lower GSIS in vitro even before receiving HFD. The feature of impaired insulin secretion in the islets of prediabetic SDG-P mice resembled that of diabetic GK rats [23] and NSY mice [18].

Prevailing rodent models for type 2 diabetes (*e.g.*, GK rats and db/db mice) exhibit a progressive decline in β cell mass owing to the induction of apoptosis or dedifferentiation [24–27]. Several reports suggest that oxidative stress and endoplasmic reticulum stress are related to the β cell loss in these animals [14,26,28]. In contrast to the advanced diabetic animals, SDG-P mice showed increased β cell mass with preserved structural integrity of islets after HFD feeding. In addition, we could not observe increased apoptotic cell death in SDG-P islets even after HFD feeding (by TUNEL staining, data not shown). However, longer-term HFD feeding than the present study may eventually lead to apoptotic β cell death in SDG-P mice by excessive metabolic stress.

The gene expression patterns in SDG-P islets were notably almost unchanged (relative to SDG-R islets) after receiving HFD, implying that the β cell dysfunction (*i.e.*, impaired insulin secretion) is a hereditary character, not an acquired one. During HFD feeding, the islet size was increased in SDG-P mice, most likely due to the β cell proliferation as a compensatory response to hyperglycemia [29,30]. However, the β cell adaptation only in mass, but not in function, was insufficient to ameliorate the glucose intolerance in SDG-P mice. In accordance with the results of islet studies of GK rats and patients with type 2 diabetes [31], reduced gene expression levels of *Glut2* and SNARE proteins were observed in SDG-P islets, suggesting impairments in glucose uptake and exocytosis machinery of the insulin granules in β cells. Accordingly, GSIS and KSIS were impaired in SDG-P islets. A reduced gene expression level of *Pdx1*, a master regulator of β cell proliferation and function, may contribute to quantitative and qualitative defects in SDG-P islets.

In addition, higher *Cd36* expression level in SDG-P islets may participate in the acceleration of glucose intolerance under HFD feeding because CD36 protein (also known as fatty acid translocase) is postulated to facilitate fatty acid uptake, which leads to the attenuation of GSIS in β cells [32,33]. Chronic exposure to free fatty acids [34–36], as well as chronic hyperglycemia [37,38], is reported to reduce insulin content in pancreatic β cells. In this study, intracellular insulin content was decreased in SDG-P islets by HFD feeding. Thus, the possible involvement of CD36 in attenuated GSIS and decreased insulin content in islets is of further interest.

In conclusion, the present results indicate that the HFD-induced glucose intolerance-prone (SDG-P) mice had a hereditary defect in insulin secretion as compared with the glucose intolerance-resistant (SDG-R) mice. Lower gene expression levels involved in glucose uptake and insulin granule exocytosis may contribute to defects in SDG-P islets. The innate predisposition in pancreatic islets may determine the susceptibility to diet-induced acceleration of glucose intolerance. Recently, we have reported SDG-P mice showed 4-fold greater atherosclerotic lesion formation than SDG-R mice on an atherogenic diet [39], indicating that these mice may also serve as useful in vivo models for investigating the causal role of glucose intolerance in the pathogenesis of atherosclerosis. Further studies with these novel polygenic model mice are warranted to provide new strategies for the prevention and treatment of diet-induced type 2 diabetes and its complications.

## Author Contributions

Conceived and designed the experiments: MN AA HS SO. Performed the experiments: MN AA WI MK YS SK DS. Analyzed the data: MN AA WI MK SY. Wrote the paper: MN AA.

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# Significance of Imbalance in the Ratio of Serum n-3 to n-6 Polyunsaturated Fatty Acids in Patients With Acute Coronary Syndrome

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**This study aimed to assess the balance of serum n-3 to n-6 polyunsaturated fatty acids (PUFAs) in patients with acute coronary syndrome (ACS). We enrolled 1,119 patients who were treated and in whom serum PUFA level was evaluated in 5 divisions of cardiology in a metropolitan area in Japan. Serum levels of PUFAs, including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA), were compared between patients with and without ACS. We also evaluated the balance of serum n-3 to n-6 PUFAs, including EPA/AA and DHA/AA ratios. EPA/AA values were 0.46 ± 0.32 and 0.50 ± 0.32 in the ACS and non-ACS groups, respectively. DHA/AA values were 0.95 ± 0.37 and 0.96 ± 0.41 in the ACS and non-ACS groups, respectively. Next, we divided the patients into 3 groups based on the tertiles of EPA/AA or tertiles of DHA/AA to determine the independent risk factors for ACS. According to multivariate logistic regression analysis, the group with the lowest EPA/AA (≤0.33) had a greater probability of ACS (odds ratio 3.14, 95% confidence interval 1.16 to 8.49), but this was not true for DHA/AA. In conclusion, an imbalance in the ratio of serum EPA to AA, but not in the ratio of DHA to AA, was significantly associated with ACS.**  **(Am J Cardiol 2014;113:441–445)**

Lipid control with statins reduces the risk of acute coronary syndrome (ACS) through the regression or stabilization of coronary artery plaques.[1,2] However, patients treated with statins may still develop ACS. Sachdeva et al[3] analyzed a large cohort of patients hospitalized with coronary artery disease (CAD) and found that >1/2 were readmitted, although they had low-density lipoprotein cholesterol levels <100 mg/dl. Therefore, we need to focus on the residual risks in patients on statin therapy to further reduce cardiovascular events. Several observational studies reported that n-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), contributed to reduction in the risk of CAD in healthy subjects.[4,5] It is well known that PUFAs play important roles in the initiation and progression of CAD.[6] In the Japan EPA Lipid Intervention Study, which reported the beneficial effect of pure EPA administration for reducing coronary events, a high EPA/arachidonic acid (AA) ratio was associated with a low incidence of coronary events.[7] Based on these previous studies, n-3 PUFAs and the balance of n-3 to n-6 PUFAs may play important roles in residual cardiovascular risk reduction. However, it is still unclear which n-3 PUFA and the balance of which n-3 PUFA to n-6 PUFAs play an important role in the development of ACS. This study aimed to assess serum levels of PUFAs and the balance of n-3 to n-6 PUFAs, including EPA, DHA, AA, and dihomo-γ-linolenic acid in patients with ACS.

## Methods

This was a multicenter observational study performed at 5 centers (4 university hospitals and 1 community hospital) located in Tokyo. We enrolled 1,119 patients who were treated in the divisions of cardiology at these 5 centers from January 2004 to May 2011. All these patients had evaluation of serum PUFAs. This cohort consisted of 1,037 patients without ACS and 72 patients with ACS. Acute myocardial infarction was defined as a transient increase of the MB fraction of creatine kinase or troponin T level in patients with ischemic symptoms and/or typical electrocardiographic findings (ST elevation). Unstable angina was defined as angina at rest, accelerated exertional angina combined with typical electrocardiographic changes (ST depression), or an increase in the intensity of antiischemic therapy.[8] Patients were excluded if they were

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See page 444 for disclosure information.

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Table 1
Comparison of clinical data among eicosapentaenoic acid (EPA)/arachidonic acid (AA) ratio tertiles (n = 1,119)

| Variable | No. of Measurements | EPA/AA ≤0.33 (n = 366) | 0.33 < EPA/AA ≤ 0.55 (n = 386) | 0.55 < EPA/AA (n = 367) | p |
|---|---|---|---|---|---|
| Age (yrs) | 1,119 | 59.3 ± 13.6 | 65.4 ± 11.1 | 67.1 ± 8.9 | <0.001* |
| Men | 1,119 | 69.1 | 69.7 | 77.9 | 0.011* |
| Body mass index (kg/m$^2$) | 1,059 | 24.5 ± 4.3 | 24.3 ± 3.3 | 24.2 ± 3.1 | 0.514 |
| Hypertension | 1,119 | 52.5 | 53.1 | 58.3 | 0.217 |
| Diabetes mellitus | 1,119 | 29.0 | 35.0 | 37.9 | 0.034* |
| Dyslipidemia | 1,119 | 64.8 | 66.3 | 65.1 | 0.894 |
| Family history of ischemic heart disease | 1,119 | 16.1 | 16.1 | 13.6 | 0.56 |
| Smoking | 1,119 | 38.0 | 39.6 | 38.1 | 0.875 |
| Number of risk factors | 1,119 | 2.9 ± 1.4 | 3.2 ± 1.4 | 3.3 ± 1.4 | 0.001* |
| Total cholesterol (mg/dl) | 893 | 198.0 ± 36.6 | 196.5 ± 38.2 | 194.4 ± 33.2 | 0.47 |
| Triglyceride (mg/dl) | 913 | 153.7 ± 90.1 | 153.7 ± 130.0 | 137.2 ± 74.8 | 0.07 |
| Low-density lipoprotein cholesterol (mg/dl) | 924 | 115.6 ± 31.6 | 114.0 ± 31.4 | 112.0 ± 29.1 | 0.343 |
| High-density lipoprotein cholesterol (mg/dl) | 917 | 53.8 ± 17.6 | 53.5 ± 18.3 | 54.8 ± 17.9 | 0.607 |
| Low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio | 917 | 2.4 ± 1.0 | 2.4 ± 1.0 | 2.3 ± 0.9 | 0.218 |
| Fasting blood sugar (mg/dl) | 903 | 113.6 ± 50.0 | 112.9 ± 35.9 | 116.6 ± 33.4 | 0.495 |
| Hemoglobin A1c (%) | 898 | 6.1 ± 1.2 | 6.2 ± 1.0 | 6.2 ± 0.9 | 0.363 |
| Serum Cr (mg/dl) | 917 | 0.9 ± 0.3 | 0.8 ± 0.3 | 0.8 ± 0.3 | 0.736 |
| Estimated glomerular filtration rate (ml/min/1.73 m$^2$) | 917 | 64.8 ± 19.6 | 63.8 ± 16.8 | 63.3 ± 16.9 | 0.549 |
| EPA (μg/ml) | 1,119 | 38.8 ± 16.5 | 67.9 ± 19.7 | 122.7 ± 51.6 | <0.001* |
| AA (μg/ml) | 1,119 | 175.5 ± 75.3 | 158.3 ± 40.7 | 146.1 ± 34.3 | <0.001* |
| DHA (μg/ml) | 1,119 | 112.4 ± 38.2 | 147.6 ± 43.2 | 180.5 ± 59.1 | <0.001* |
| Dihomo-γ-linolenic acid (μg/ml) | 1,119 | 38.9 ± 13.4 | 33.7 ± 11.7 | 27.5 ± 9.2 | <0.001* |
| Statin | 1,119 | 40.2 | 41.5 | 46.9 | 0.149 |
| Antiplatelet agent | 1,119 | 39.1 | 48.4 | 53.1 | <0.001* |
| Renin-angiotensin system inhibitor | 1,119 | 7.9 | 11.1 | 6.8 | 0.089 |
| Calcium channel blocker | 1,119 | 43.2 | 42.2 | 56.1 | <0.001* |
| β Blocker | 1,119 | 31.4 | 33.9 | 32.2 | 0.749 |
| Hypoglycemic agents | 1,119 | 15.8 | 17.1 | 19.1 | 0.508 |
| ACS | 1,119 | 8.2 | 6.0 | 5.2 | 0.224 |
| Stable CAD | 1,119 | 37.4 | 47.2 | 45.2 | 0.182 |

Data are represented as mean ± SD or %.
Renin-angiotensin system inhibitor = angiotensin-converting enzyme inhibitor and angiotensin II receptor blocker.
* p <0.05. p Values are from analysis of variance for continuous data and from Mantel-Haenszel "analysis of variance" test of 2 degrees of freedom for presence-absence data.

receiving hemodialysis or taking pure EPA. Patients with ongoing congestive heart failure, severe liver dysfunction, or other systemic diseases, including malignancy and collagen disease, were also excluded. Patients with a medical history of percutaneous coronary intervention, coronary artery bypass grafting, and old myocardial infarction were also excluded. We also evaluated the use of the following medications: statins, antiplatelet agents, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, calcium channel blockers, β blockers, and hypoglycemic agents. This study was approved by the institutional ethics committee of each hospital, and all subjects gave informed consent.

Fasting blood samples were obtained in the morning, and serum levels of EPA, DHA, AA, and dihomo-γ-linolenic acid were measured at an external laboratory (SRL Inc., Tokyo, Japan). We also evaluated the following laboratory parameters: total cholesterol, fasting triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio, fasting plasma glucose, hemoglobin A1c, uric acid, serum creatinine (Cr), and estimated glomerular filtration rate. The estimated glomerular filtration rate was calculated based on the Japanese equation that uses serum Cr level, age, and gender as follows: estimated glomerular filtration rate (ml/min/1.73 m$^2$) = $194 \times Cr^{-1.094} \times age^{-0.287}$ (female × 0.739).[9] Blood samples from patients with ACS were obtained during admission and those from outpatients were obtained at the outpatient clinic.

Patients were stratified into 3 groups based on the tertiles of EPA/AA ratio (tertile cut-off values: 0.33 and 0.55) or DHA/AA ratio (tertile cut-off values: 0.78 and 1.06). The risk of ACS in patients in the different tertiles of EPA/AA and DHA/AA was compared using crude odds ratios (ORs) and their 95% confidence intervals. To adjust ORs for patients' clinical characteristics, we used a multivariate logistic regression model that included age, gender, body mass index, hypertension, diabetes mellitus, dyslipidemia, smoking, family history of CAD, serum Cr level, and the use of statins, antiplatelet agents, renin-angiotensin system inhibitors, calcium channel blockers, β blockers, or hypoglycemic agents. Patients with missing information for each variable were excluded in the multivariate analysis. The α level for

Table 2
Comparison of clinical data among docosahexaenoic acid (DHA)/arachidonic acid (AA) ratio tertiles (n = 1,119)

| Variable | No. of Measurements | DHA/AA ≤0.78 (n = 371) | 0.78 < DHA/AA ≤ 1.06 (n = 384) | 1.06 < DHA/AA (n = 364) | p |
|---|---|---|---|---|---|
| Age (yrs) | 1,119 | 58.7 ± 13.6 | 65.7 ± 10.4 | 67.3 ± 9.3 | <0.001* |
| Men | 1,119 | 68.5 | 72.1 | 76.1 | 0.069 |
| Body mass index (kg/m$^2$) | 1,059 | 24.6 ± 4.3 | 24.1 ± 3.3 | 24.4 ± 3.1 | 0.213 |
| Hypertension | 1,119 | 53.1 | 53.9 | 56.9 | 0.558 |
| Diabetes mellitus | 1,119 | 28.8 | 36.2 | 36.8 | 0.039* |
| Dyslipidemia | 1,119 | 63.6 | 66.1 | 66.5 | 0.668 |
| Family history of ischemic heart disease | 1,119 | 12.4 | 19.5 | 13.7 | 0.015* |
| Smoking | 1,119 | 33.7 | 39.8 | 42.3 | 0.047* |
| Number of risk factors | 1,119 | 2.8 ± 1.4 | 3.2 ± 1.4 | 3.3 ± 1.3 | <0.001* |
| Total cholesterol (mg/dl) | 893 | 199.2 ± 35.2 | 193.9 ± 39.5 | 195.7 ± 33.0 | 0.185 |
| Triglyceride (mg/dl) | 913 | 140.0 ± 84.1 | 140.3 ± 100.0 | 165.6 ± 118.1 | 0.002* |
| Low-density lipoprotein cholesterol (mg/dl) | 924 | 115.1 ± 30.8 | 113.7 ± 33.4 | 112.7 ± 27.7 | 0.635 |
| High-density lipoprotein cholesterol (mg/dl) | 917 | 57.3 ± 19.0 | 53.5 ± 17.8 | 51.1 ± 16.2 | <0.001* |
| Low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio | 917 | 2.2 ± 1.0 | 2.3 ± 1.0 | 2.4 ± 0.9 | 0.06 |
| Fasting blood sugar (mg/dl) | 903 | 114.6 ± 49.3 | 112.2 ± 31.2 | 116.3 ± 38.0 | 0.462 |
| Hemoglobin A1c (%) | 898 | 6.1 ± 1.1 | 6.2 ± 1.1 | 6.2 ± 0.9 | 0.207 |
| Serum Cr (mg/dl) | 917 | 0.8 ± 0.3 | 0.8 ± 0.3 | 0.9 ± 0.3 | 0.968 |
| Estimated glomerular filtration rate (ml/min/1.73 m$^2$) | 917 | 64.3 ± 18.7 | 64.3 ± 18.0 | 63.3 ± 16.5 | 0.69 |
| EPA (μg/ml) | 1,119 | 48.5 ± 26.3 | 72.8 ± 35.7 | 108.5 ± 56.1 | <0.001* |
| AA (μg/ml) | 1,119 | 182.8 ± 72.8 | 154.9 ± 37.8 | 141.9 ± 36.0 | <0.001* |
| DHA (μg/ml) | 1,119 | 109.9 ± 36.1 | 141.0 ± 33.4 | 190.9 ± 58.6 | <0.001* |
| Dihomo-γ-linolenic acid (μg/ml) | 1,119 | 37.8 ± 13.3 | 31.7 ± 10.8 | 30.6 ± 11.9 | <0.001* |
| Statin | 1,119 | 43.7 | 43.5 | 41.2 | 0.754 |
| Antiplatelet agent | 1,119 | 33.7 | 52.1 | 54.9 | <0.001* |
| Renin-angiotensin system inhibitor | 1,119 | 8.6 | 7.3 | 10.2 | 0.378 |
| Calcium channel blocker | 1,119 | 43.1 | 44.5 | 53.8 | 0.007* |
| β Blocker | 1,119 | 28.0 | 35.4 | 34.1 | 0.072 |
| Hypoglycemic agents | 1,119 | 14.6 | 17.7 | 19.8 | 0.169 |
| ACS | 1,119 | 5.7 | 7.8 | 5.8 | 0.397 |
| Stable CAD | 1,119 | 29.4 | 48.4 | 52.2 | <0.001* |

Data are represented as mean ± SD or %.
Renin-angiotensin system inhibitor = angiotensin-converting enzyme inhibitor and angiotensin II receptor blocker.
* p <0.05. p Values are from analysis of variance for continuous data and from Mantel-Haenszel "analysis of variance" test of 2 degrees of freedom for presence-absence data.

Table 3
Relation between eicosapentaenoic acid (EPA)/arachidonic acid (AA) ratio and acute coronary syndrome (ACS)

| EPA/AA Category | ACS | n | Prevalence (%) | Crude OR | Adjusted OR |
|---|---|---|---|---|---|
| EPA/AA ≤0.33 | 30 | 366 | 8.2 | 1.64 (0.90–2.96) | 3.14 (1.16–8.49) |
| 0.33 < EPA/AA ≤ 0.55 | 23 | 386 | 6.0 | 1.16 (0.62–2.17) | 0.80 (0.27–2.37) |
| 0.55 < EPA/AA | 19 | 367 | 5.2 | Reference | Reference |
| No. used in the analysis | | | | 1,119 | 861 |

Table 4
Relation between docosahexaenoic acid (DHA)/arachidonic acid (AA) ratio and acute coronary syndrome (ACS)

| DHA/AA Category | ACS | n | Prevalence (%) | Crude OR | Adjusted OR |
|---|---|---|---|---|---|
| DHA/AA ≤0.78 | 21 | 371 | 5.7 | 0.98 (0.53–1.83) | 2.55 (0.88–7.40) |
| 0.78 < DHA/AA ≤ 1.06 | 30 | 384 | 7.8 | 1.38 (0.78–2.47) | 1.49 (0.54–4.13) |
| 1.06 < DHA/AA | 21 | 364 | 5.8 | Reference | Reference |
| No. used in the analysis | | | | 1,119 | 861 |

all statistical tests was set at 0.05; thus, all confidence intervals were presented at the 95% level. All analyses were carried out with SAS, version 9.2 (SAS Institute, Cary, North Carolina).

## Results

In all 1,119 subjects, the mean ± SD serum levels of EPA, AA, DHA, and dihomo-γ-linolenic acid were 76.4 ± 47.8,

159.9 ± 54.3, 146.9 ± 55.0, and 33.4 ± 12.4 μg/ml, respectively. EPA/AA ratio was 0.498 ± 0.320 and DHA/AA ratio was 0.962 ± 0.408. EPA/AA ratio was 0.456 ± 0.321 and 0.501 ± 0.320 in the ACS and non-ACS groups, respectively. DHA/AA ratio was 0.951 ± 0.368 and 0.963 ± 0.411 in the ACS and non-ACS groups, respectively.

Tables 1 and 2 list a comparison of the clinical data among the patient groups stratified according to the tertiles of EPA/AA or tertiles of DHA/AA. There was an inverse trend between the magnitude of EPA/AA and the incidence of ACS, but there was no such trend for DHA/AA. A positive trend was observed between the magnitudes of both EPA/AA and DHA/AA and the average number of conventional coronary risk factors, including age (≥65 years), obesity (body mass index ≥25 kg/m$^2$), hypertension, diabetes mellitus, dyslipidemia, smoking history, and family history of CAD. Table 3 lists the risk of ACS in each EPA/AA group based on the OR determined by logistic regression analysis, and Table 4 lists the same analysis for DHA/AA group. According to the results of multivariate logistic regression analyses, the patients in the group with the lowest EPA/AA (≤0.33) were more likely to have ACS, but this was not true for DHA/AA. A multivariate logistic regression analysis demonstrated that apart from low EPA/AA, diabetes mellitus (OR 3.88, 95% confidence interval 1.54 to 9.73) showed significant and positive correlations with ACS risk.

## Discussion

The present study showed that a low EPA/AA ratio but not DHA/AA ratio had a significant relation with occurrence of ACS in a multicenter observational study.

It has been reported that n-3 PUFAs have multiple actions to prevent CAD, including an anti-inflammatory effect,[10] reduction of platelet aggregation,[11] stabilization of atherosclerotic plaques,[12] and an effect on red cell deformability.[13] Although EPA and DHA are both n-3 PUFAs, it is still unclear which fatty acid has a greater effect on cardiovascular risk reduction. Domei et al[14] measured the serum concentrations of various fatty acids and evaluated their relation with major adverse cardiac events that occurred in 284 patients who underwent elective percutaneous coronary intervention. They reported that the patients with a higher EPA/AA ratio (>0.40), but not DHA/AA, had significantly fewer major adverse cardiac events than subjects with a low EPA/AA ratio. The results of the present study are consistent with their results; however, the present study included a large control group without ACS and was conducted at multiple centers.

Nozue et al[15] recently reported the relation between the percent change in plaque volume and the changes in the EPA/AA ratio, DHA/AA ratio, and (EPA + DHA)/AA using virtual histology intravascular ultrasound in statin-treated patients with CAD. Negative correlations were observed between the percent change in plaque volume and change in not only EPA/AA but also DHA/AA and (EPA + DHA)/AA. A recent study reported the association between plaque progression and/or regression and coronary events.[16] Further large-scale prospective studies are needed to investigate the associations among the EPA-DHA-AA balance, plaque volume and/or morphology, and cardiovascular events.

We surmise that one of the reasons responsible for the difference between EPA and DHA is a difference in the uptake of EPA and DHA into plaques. The result of an intervention study in patients before carotid endarterectomy showed that the phospholipid EPA value in carotid plaques in patients who received oral EPA and DHA significantly increased to twice the value of the control patients, whereas there was no significant change in DHA level compared with that of the control group.[17] It was also reported that the relation between serum and erythrocyte membrane levels is stronger for EPA than DHA.[18] EPA and DHA may have differences in integration characteristics into cells or organs as those reports indicate, and this mechanism needs to be investigated in future studies.

There are several limitations of the present study. This was a multicenter study with >1,100 patients, but all the centers were located in the metropolitan Tokyo area. Therefore, the study results are not necessarily applicable to patients living in rural areas. Furthermore, we were unable to determine if a low EPA/AA value was the cause of ACS because of the cross-sectional design of our study. ACS prevalence was relatively low in the present study because patients with a history of percutaneous coronary intervention, coronary artery bypass grafting, and old myocardial infarction were excluded. Therefore, a large-scale, multicenter, prospective study is necessary to confirm the result of this study. In addition, no data were obtained on inflammatory markers such as high-sensitivity C-reactive protein that is assumed to have a strong association with ACS.[19]

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## Disclosures

Kazunori Shimada received lecture fees from Mochida Pharmaceutical Company. Katsumi Miyauchi received lecture fees from Mochida Pharmaceutical Company and Takeda Pharmaceutical Company. Michihiro Yoshimura received scholarship funds and lecture fees from Mochida Pharmaceutical Company and Takeda Pharmaceutical Company. Issei Komuro received lecture fees and Scholarship funds from Takeda Pharmaceutical Company. Ryozo Nagai received consultation and lecture fees from Mochida Pharmaceutical Company. Hiroyuki Daida received scholarship funds and lecture fees from Mochida Pharmaceutical Company and Takeda Pharmaceutical Company. The other authors report no conflicts.

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Committee Report 1

# Executive Summary of the Japan Atherosclerosis Society (JAS) Guidelines for the Diagnosis and Prevention of Atherosclerotic Cardiovascular Diseases in Japan—2012 Version

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Among the various atherosclerotic cardiovascular diseases (CVDs), these guidelines primarily deal with cerebrovascular disease, peripheral arterial disease (PAD) and coronary artery disease (CAD), which occur in association with atherosclerosis and is closely related to dyslipidemia.

## 1. Comprehensive Risk Management for the Prevention of Atherosclerotic CVD

To prevent CVD, it is important to manage dyslipidemia in addition to other risk factors. For this purpose, we propose comprehensive risk management for the prevention of CVD. Risk factors that should be considered include dyslipidemia, hypertension, diabetes mellitus, smoking, chronic kidney disease (CKD), a family history of premature CAD, a history of CAD, noncardiogenic cerebral infarction, PAD, age and sex. In this article, we describe the comprehensive management of CVD.

## 2. Diagnostic Criteria for Dyslipidemia

It has been shown in epidemiological studies conducted in Japan, as well as in Western countries, that the incidence of CAD increases in association with increases in the levels of LDL-cholesterol (LDL-C)[1] and triglycerides (TGs)[2, 3] and decreases in the level of HDL cholesterol (HDL-C)[4-7]. Currently in Japan, the incidence of CAD is much lower than that observed in Western countries[2, 3, 8, 9]; however, this incidence is anticipated to increase in the near future due to the recent Westernization of the Japanese lifestyle. Therefore, the current guidelines provide screening criteria for dyslipidemia to prevent CVD with a specific emphasis on the prevention of CAD, as shown in **Table 1**.

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Regarding the diagnosis of dyslipidemia, the total cholesterol (TC), TG and HDL-C levels should be measured after an overnight fast. The LDL-C level is then calculated using the Friedewald formula (LDL-C=TC−HDL-C−TG/5).

This formula cannot be used if blood is collected without fasting or if the TG is ≥400 mg/dL. In such cases, using the non HDL-C level is recommended, which is calculated by subtracting the HDL-C level from the TC level. Data obtained in Japan indicate that the non HDL-C level is approximately 30 mg/dL higher than the LDL-C level. This view is shared by the National Cholesterol Education Program (NCEP). When lipids are evaluated based on the non HDL-C level, the target value of non HDL-C is determined by adding 30 mg/dL to the value of LDL-C (**Table 2**).

The incidence and mortality of CAD increase continuously in association with increases in the LDL-C level. At present, the incidence of CAD is lower in Japanese individuals than in Westerners. To maintain this low rate, efforts directed toward early prevention are required. Therefore, from the perspective of the prevention and treatment of CAD, the current guidelines propose an LDL-C level of 140 mg/dL as the reference value when screening Japanese individuals for hyper-LDL cholesterolemia. This value was selected because it corresponds to a TC level of 220 mg/dL, at which point the relative risk is approximately 1.5-fold higher than that observed at a TC level of <180 mg/dL, according to the NIPPON DATA80[10]. Since the LDL-C goal may vary depending on concomitant risk factors, an LDL-C level between 120 and 139 mg/dL is defined as indicating borderline hyper-LDL cholesterolemia.

Hypo-HDL cholesterolemia has also been established to be a risk factor for CVD. The current guidelines define an HDL-C level of <40 mg/dL as indicating hypo-HDL cholesterolemia, as determined in

**Table 1.** Dyslipidemia: Diagnostic Criteria for Screening (Fasting*)

| | | |
|---|---|---|
| Low-density lipoprotein cholesterol (LDL-C) | ≥140 mg/dL | Hyper-LDL cholesterolemia |
| | 120-139 mg/dL | Borderline hyper-LDL cholesterolemia** |
| High-density lipoprotein cholesterol (HDL-C) | <40 mg/dL | Hypo-HDL cholesterolemia |
| Triglycerides (TG) | ≥150 mg/dL | Hypertriglyceridemia |

- The LDL-C level is calculated using the Friedewald formula (TC−HDL-C−TG/5) (for TG <400 mg/dL).
- If the TG level is ≥400 mg/dL or non-fasting blood is used, the non HDL-C (TC−HDL-C) level should be used with a cutoff value of LDL-C + 30 mg/dL.

*Fasting is defined as deprivation of food for at least 10 to 12 hours; however, the ingestion of noncaloric beverages, such as water and tea, is allowed.

**If a patient is found to have borderline hyper-LDL cholesterolemia during screening, he/she should be examined for any high-risk conditions and the need for treatment should be considered.

**Table 2.** Lipid Management Targets for Patients with Different Risk Levels

| Therapeutic principle | Management category | Lipid management target (mg/dL) | | | |
|---|---|---|---|---|---|
| | | LDL-C | HDL-C | TG | Non HDL-C |
| Primary prevention<br>Drug therapy should be considered after lifestyle modification | Category I | <160 | ≥40 | <150 | <190 |
| | Category II | <140 | | | <170 |
| | Category III | <120 | | | <150 |
| Secondary prevention<br>Drug therapy should be considered, together with lifestyle modification | History of CAD | <100 | | | <130 |

- For patients at low absolute risk, such as the young, the relative risk chart (Supplementary Table) should be used and changes in the absolute risk should be monitored carefully while encouraging the patient to modify their lifestyle.
- These values should be considered general, not mandatory, goals.
- A 20%-30% reduction in the level of LDL-C is considered to be a prime target for pharmacological intervention.
- The management target for the non HDL-C level is the secondary target to be used after a patient with hypertriglyceridemia has achieved the management target for the LDL-C level. The non HDL-C level should be used if blood is collected after meals or if the TG level is ≥400 mg/dL.
- For patients in any category, the management goals should generally be achieved via lifestyle modification.
- For patients in category I, drug therapy should be considered if the LDL-C level is ≥180 mg/dL.

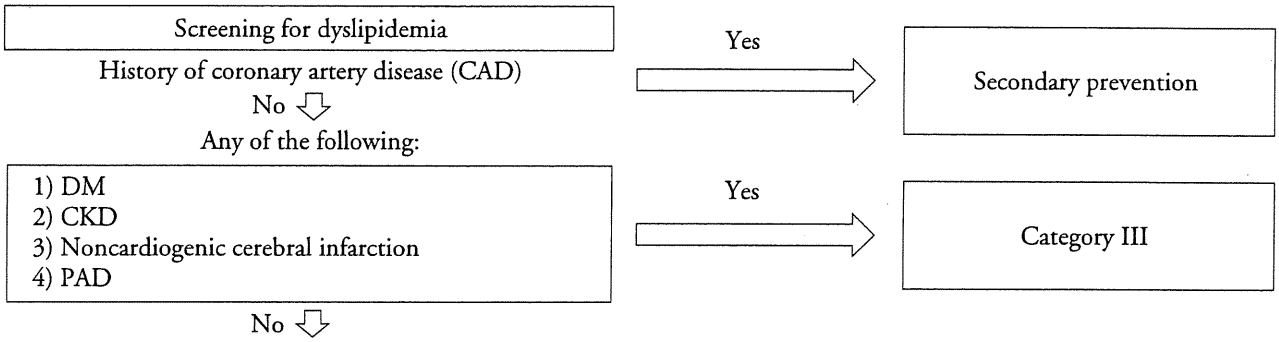
our previous guidelines. A number of studies have demonstrated sex differences in the HDL-C levels; however, it remains unclear whether these sex differences are reflected in the diagnosis of hypo-HDL cholesterolemia.

Hypertriglyceridemia has been found to occur in association with various conditions. Although some researchers insist that more intensive management is required in patients with certain diseases, such as diabetes mellitus, the current guidelines define a TG level of ≥150mg/dL as indicating hypertriglyceridemia, based on epidemiological data obtained during screenings of the general population.

### 3. Risk Stratification Based on Absolute Risk

The current guidelines stratify the risk of CVD for primary prevention according to the absolute risk calculated based on the results of the NIPPON DATA80[11]. This study identified age, sex, diabetes mellitus, current smoking, systolic blood pressure and the TC level as risk factors and determined the absolute risk of death from CAD depending on the degree or existence of these factors.

How absolute risk categories should be determined is based on clinical consensus and/or conventional wisdom. The U.S. NCEP Adult Treatment Panel III classifies a 10-year risk of death from CAD or the development of nonfatal myocardial infarction of ≥20% (based on the Framingham score) as high risk[12], whereas European guidelines classify a 10-year risk of death from CVD (including strokes and CAD) of ≥5% as high risk[13]. The current guidelines classify




Management categories based on absolute risk for the primary prevention of CAD

| 10-year probability (absolute risk) of CAD death derived from NIPPON DATA80 | Additional risk factors | |
|---|---|---|
| | No additional risk factors | One or more of the following:<br>(1) Hypo-HDL cholesterolemia (HDL-C <40 mg/dL)<br>(2) Family history of premature CAD in first-degree relatives (a man aged <55 years or a women aged <65 years)<br>(3) Impaired glucose tolerance |
| <0.5% | Category I | Category II |
| ≥0.5%–<2.0% | Category II | Category III |
| ≥2.0% | Category III | Category III |

This flow chart is not applicable to patients with FH.

**Fig. 1.** Flow chart for setting management targets for LDL cholesterol

patients with a 10-year risk of death from CAD of ≥ 2% as belonging to the high-risk group (category Ⅲ), those with a risk of ≥0.5% to <2% as belonging to the intermediate-risk group (category Ⅱ) and those with a risk of <0.5% as belonging to the low-risk group (category Ⅰ), considering that there is little evidence of an association between hypercholesterolemia and cerebrovascular diseases in Japanese individuals. Since diabetes mellitus, CKD and a history of noncardiogenic cerebral infarction or PAD are considered to be important risk factors, patients with any of these conditions are classified as belonging to the high-risk group (**Fig. 1**).

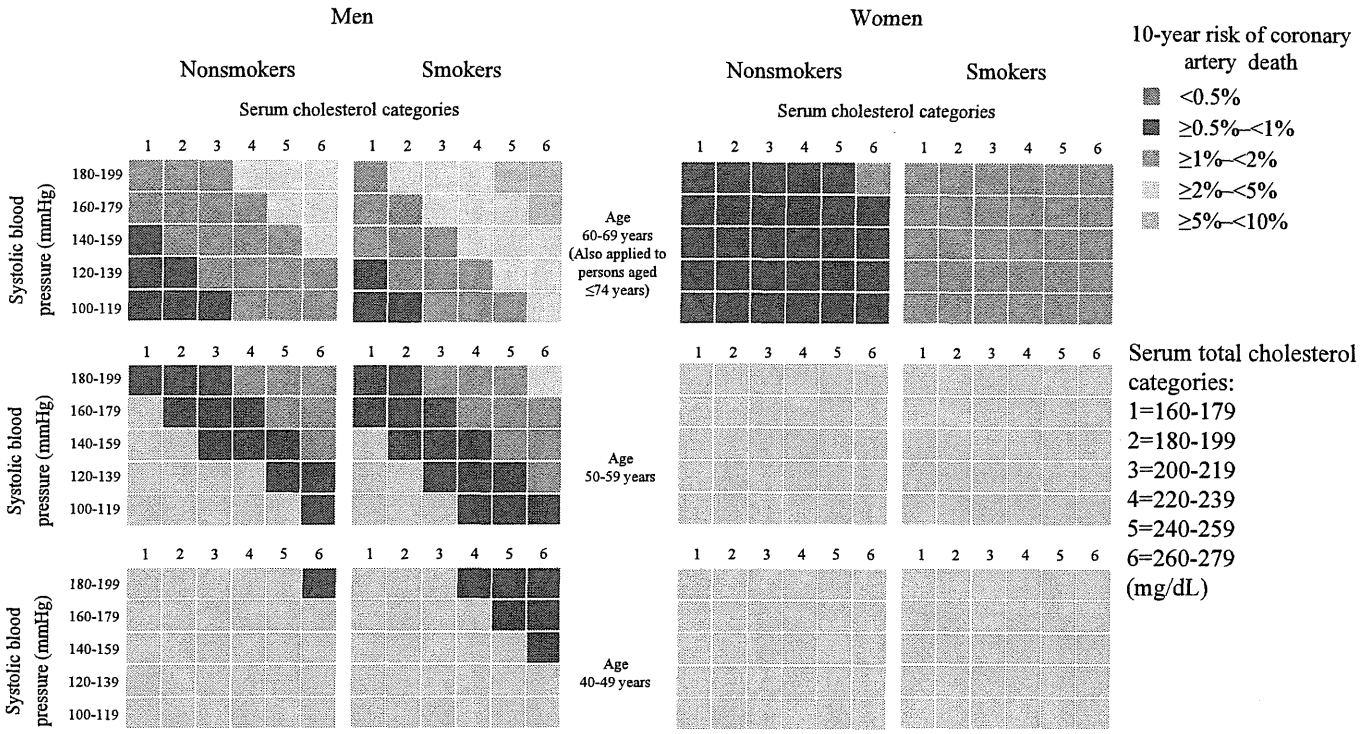
The 10-year absolute risk of CAD-related death should be determined based on the risk assessment chart provided in the NIPPON DATA80[11]. However, since this chart does not include hypo-HDL cholesterolemia, a family history of premature CAD or impaired glucose tolerance, the category should be raised if the patient meets one or more of these criteria (**Fig. 2**).

The chart obtained from the NIPPON DATA80 addresses the risk of CAD-related death in individuals between 40 and 79 years of age. While the current guidelines are intended for adults younger than 65 years of age, they can also be applied to persons between 65 and 74 years of age. To calculate the absolute risk for individuals ≥70 and <75 years of age, the table for individuals between 60 and 69 years of age should be used. For adults <40 years of age, the table for individuals between 40 and 49 years of age should be used.

When assessing the absolute risk, it should be noted that the absolute risk greatly depends on age. If a low absolute risk is obtained for a young individual with a risk factor, such as hypertension or smoking, the risk factors should be managed appropriately. When secondary prevention is required, each risk factor should be dealt with separately, as outlined in the previous guidelines.

### 4. Management Targets for Dyslipidemic Patients

The management targets for dyslipidemic patients are presented by category in **Table 2**. For primary prevention, drug therapy should be considered after lifestyle factors have been improved for a certain




The section of hyperglycemia from the NIPPON DATA80 risk assessment chart is omitted here. These charts cannot be applied to high-risk patients, such as those with DM or CKD.

**Fig. 2.** Absolute risk assessment charts for death from coronary artery disease (primary prevention).

Absolute risk should be reassessed at least once a year since it may be affected by either risk factors or aging.

Step 1: The applicable portion of the above figures should be assessed based on gender, age, the present smoking status, systolic blood pressure (mmHg) and the TC level (mg/dL).
Absolute risk ≥ 2% → Category Ⅲ
Absolute risk < 2% → To Step 2
Step 2: Any of the following conditions: hypo-HDL-cholesterolemia (< 40 mg/dL), a family history of CAD and/or impaired glucose tolerance
Absolute risk ≥ 0.5% < 2% + Yes → Category Ⅲ
Absolute risk ≥ 0.5% < 2% + No → Category Ⅱ
Absolute risk < 0.5% + Yes → Category Ⅱ
Absolute risk < 0.5% + No → Category Ⅰ
Supplementary notes
(1) The TC category 160-179 mg/dL should be used in patients with a TC level of < 160.
(2) The TC category 260-279 mg/dL should be used in patients with a TC level of ≥ 280 mg/dL.
(3) The systolic blood pressure category of 100-119 mmHg should be used in patients with a systolic blood pressure of < 100 mmHg, while the systolic blood pressure category of 180-199 mmHg should be used in patients with a systolic blood pressure of ≥ 200 mmHg.
(4) The guidelines cannot be applied to persons 75 years of age or older. "The Elderly." For patients < 40 years of age, the relative risk chart (Supplementary Table) should be used.
(5) Blood pressure should be managed according to the guidelines established by the Japanese Society of Hypertension, while diabetes mellitus should be managed according to the guidelines established by the Japan Diabetes Society.
(6) It is desirable to encourage smokers to stop smoking irrespective of the level of absolute risk.

period and the response has been evaluated. For individuals in category Ⅰ low absolute risk group), the management target for the LDL-C level is set at < 160 mg/dL. The target for individuals in category Ⅱ is set at < 140 mg/dL, while that for individuals in category Ⅲ (high absolute risk group) is set at < 120 mg/dL. It should be noted that achieving these targets is recommended but not obligatory. A meta-analysis of preventive clinical trials demonstrated that a 20%-30% reduction in the LDL-C level results in a decrease in the incidence of CAD of approximately 30%. Based on this finding, a 20%-30% decrease in

the LDL-C level can be considered a target. For secondary prevention, since the patient has already been diagnosed with CAD, the administration of drug therapy targeting an LDL-C level of <100 mg/dL is recommended in addition to lifestyle modification.

For the management of hypertriglyceridemia and hypo-HDL cholesterolemia, targeting a TG level of < 150 mg/dL and an HDL-C level of ≥40 mg/dL is recommended, as in the previous guidelines.

Some researchers have the opinion that stricter targets should be established for high-risk patients (such as those with diabetes mellitus or CKD) or those who require secondary prevention, depending on the patient's condition and severity of disease; however, there is insufficient evidence to support setting such goals. Nevertheless, the current guidelines also suggest that high-risk patients be stratified according to risk factors and that lower targets be established for such patients.

**5. Treatment**

Dyslipidemia should be treated with lifestyle modification, including smoking cessation and the administration of diet and/or exercise therapy. In primary prevention patients, drug therapy should only be considered when the lipid management targets are not achieved after sufficient effort has been made to improve lifestyle factors. In patients with a history of CAD, the use of drug therapy should be considered simultaneously with lifestyle modification.

When drug therapy is provided for patients with hyper-LDL cholesterolemia, statins are the first drug of choice. Resin, probucol and/or ezetimibe are used in combination with statins or selected when statins cannot be administered. The combination of statins and EPA is useful for treating high-risk patients with hyper-LDL cholesterolemia. For treating hypertriglyceridemia accompanied by hypo-HDL cholesterolemia, drugs such as fibrates and nicotinic acid derivatives should be considered.

**6. High-Risk Conditions for CVD**

The current guidelines include CKD in addition to a history of CAD (secondary prevention), diabetes mellitus, noncardiogenic cerebral infarction and PAD as high-risk conditions based on the findings of epidemiological studies, including evidence showing that the presence of CKD increases the incidence of CAD by at least two-fold. The previous guidelines classified a history of cerebral infarction as a high-risk condition, while the current guidelines classify a history of noncardiogenic cerebral infarction as a high-risk condition because cardiogenic cerebral infarctions are not caused by atherosclerotic disease.

**7. Familial Hypercholesterolemia**

Familial hypercholesterolemia occurs in approximately one in 500 individuals and is associated with a high risk of CAD. The current guidelines reference the diagnostic criteria for FH reported by the 2011 Primary Hyperlipidemia Research Group and set a target of an LDL-C level of <100 mg/dL or a decrease in the LDL-C level of at least 50%.

**8. Evaluation of CVD**

To prevent CVD, the presence or absence and severity of atherosclerosis must be evaluated before symptoms occur and risk factors must be managed or treated with the objective of preventing progression or possibly achieving regression. For this purpose, correctly staging CVD is important. At present, the degree of atherosclerosis is primarily evaluated using imaging techniques. Invasive techniques include angiography (to assess the severity of stenosis) as well as angioscopy and intravascular ultrasonography (to qualitatively assess the vessel walls). Noninvasive techniques include transcutaneous ultrasonography of the arteries, such as the carotid artery, to qualitatively and quantitatively evaluate the degree of atherosclerosis. Carotid artery ultrasonography is often used in general practice because the extent of carotid sclerosis has been shown to be correlated with the risk of cerebrovascular disease and/or CAD. The development of multidetector CT (MDCT) has allowed for easier detection of coronary artery lesions. At present, carotid artery ultrasonography and MDCT are less invasive and easier to perform than other imaging modalities. In the near future, developing guidelines for the assessment of atherosclerosis that can be employed before the onset of symptoms is necessary. At present, however, assessing the degree of atherosclerotic lesions using the above-mentioned imaging techniques is associated with some limitations. CVD should be diagnosed based on a clear understanding of these limitations.

## Footnotes

This is an English version of the guidelines of the Japan Atherosclerosis Society (Chapter 1) published in Japanese in June 2012.

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**Supplementary Table.** Relative Risk Charts for the Young, etc. with a Low Absolute Risk (based on the risk charts of the NIPPON DATA80)

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Nonsmokers | | | |
| Systolic blood pressure | | | | | | |
| Second-degree or higher hypertension (≥160 mmHg) | 2.2 | 2.8 | 3.6 | 4.6 | 5.8 | 7.4 |
| First-degree hypertension (140-159 mmHg) | 1.7 | 2.2 | 2.8 | 3.5 | 4.5 | 5.7 |
| Normal (≤140) | 1.0* | 1.3 | 1.6 | 2.1 | 2.6 | 3.4 |
| TC category (mg/dL) | 160-179 | 180-199 | 200-219 | 220-239 | 240-259 | 260+ |
| | | | Smokers | | | |
| Systolic blood pressure | | | | | | |
| Second-degree or higher hypertension (≥160 mmHg) | 3.2 | 4.1 | 5.2 | 6.6 | 8.4 | 10.7 |
| First-degree hypertension (140-159 mmHg) | 2.5 | 3.1 | 4.0 | 5.1 | 6.5 | 8.2 |
| Normal (≤140 mmHg) | 1.4 | 1.8 | 2.3 | 3.0 | 3.8 | 4.8 |
| TC category (mg/dL) | 160-179 | 180-199 | 200-219 | 220-239 | 240-259 | 260+ |

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# Increased lysophosphatidic acid levels in culprit coronary arteries of patients with acute coronary syndrome

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ABSTRACT

*Background:* Lysophosphatidic acid (LPA) is a platelet activator and highly thrombogenic lipid constituent of atherosclerotic plaque. However, whether or not LPA locally released from culprit lesions is associated with acute coronary syndrome (ACS) remains unclear.
*Methods:* We studied 52 patients with ACS who were treated by emergency percutaneous coronary intervention and thrombectomy. Levels of LPA and other established biomarkers were enzymatically assayed in samples of culprit coronary arterial and systemic peripheral arterial blood. Levels of LPA and lysophosphatidylcholine (LPC) were measured in plasma, and those of autotaxin, soluble CD40 ligand (sCD40L), hs-CRP and Lp-PLA2 were measured in serum.
*Results:* Median LPA levels were significantly higher in coronary (CB) than in peripheral (PB) arterial blood ($p = 0.009$). Levels of sCD40L were higher in CB than in PB, but the difference did not reach statistical significance ($p = 0.177$). In contrast, autotaxin and Lp-PLA2 levels were significantly higher in PB than in CB ($p = 0.005$ and $p = 0.038$, respectively). Levels of LPC and hs-CRP were also higher in PB than in CB ($p = 0.129$ and $p = 0.121$, respectively). Levels of LPA in both CB and PB were positively and significantly associated with those of LPC ($r = 0.632$, $p < 0.01$ and $r = 0.465$, $p < 0.001$).
*Conclusions:* Culprit coronary arteries of ACS contained significantly more LPA than the systemic arterial circulation. Higher LPA concentrations might be associated with the pathophysiology of ACS.


## 1. Introduction

Lysophosphatidic acid (LPA) has emerged as an important lipid mediator with various biological activities that are particularly important to vascular biology [1–3]. Accumulating evidence indicates that LPA plays several biological roles related to blood cells and cells of vessel walls (endothelial cells, smooth muscle cells), which are all key players in atherosclerotic and atherothrombotic processes [4,5]. Atherogenic oxidized low-density lipoprotein (LDL) contains lysophosphatidylcholine (LPC) that serves as a substrate for the production of LPA by autotaxin (lysophospholipase D, LysoPLD) [6]. The production of LPA is also associated with changes in atherosclerotic plaque formation. The increased deposition of potent platelet-activating and proinflammatory LPC species in advanced atherosclerotic lesions indicates that thin cap fibroatheromas can be characterized not only by cellular and morphological features, but also by their prothrombotic lipid profiles [7]. In addition, LPA is abundant in the lipid-rich core of human atherosclerotic plaque lesions [8]. After plaque rupture or erosion, exposure to LPA in the lipid-rich core might play a key role in triggering or potentiating platelet responses during acute thrombosis [9,10]. Our previous cross-sectional study found significantly higher concentrations of circulating plasma LPA in patients with acute coronary syndrome (ACS) than in those with stable angina or normal coronary arteries [11].

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However, clinical trials have not uncovered significant evidence of a mechanism of LPA production or the circulatory status of LPA in pathophysiological ACS. In particular, little is known about an association between LPA and plaque rupture during ACS. We therefore determined the clinical significance of LPA levels using coronary catheter intervention in patients with ACS and coronary plaque instability, and examined relationships between LPA and other established biomarkers.

## 2. Methods

### 2.1. Study participants

This prospective cross-sectional study comprised 52 consecutive patients with ACS who underwent emergency PCI and thrombectomy at Juntendo University Hospital between January and December 2009. The entry criteria were as follows: no history of coronary intervention or coronary artery bypass grafting and having evaluable coronary trees. Information about demographics, medical history and current medications was collected from all participants at the time of enrollment. The diagnosis of ACS was determined according to the 2007 American College of Cardiology/American Heart Association criteria [12,13]. The definition of ACS depended on the specific characteristics of individual elements of each clinical presentation, electrocardiographic findings and highly specific markers of cardiac damage.

The Ethics Review Committee at our institution approved the study, all participants signed informed consent forms and the study was registered in the UMIN protocol registration system (#UMIN000002103).

### 2.2. Study protocol

We collected all local blood samples in the operating room at the time of emergency coronary angiography. Patients received standard medication for ACS that comprised aspirin (162 mg) and clopidogrel (300 mg). Heparin (100 IU/Kg) was administered after identifying culprit lesions. A 0.014-inch guide wire was then advanced across the culprit lesion, and a thrombectomy catheter (Thrombuster, Kaneka Medics, Osaka, Japan) was inserted through the guiding catheter. A 20-cc syringe was used to withdraw both coronary blood (CB) from the culprit coronary artery through the thrombectomy catheter and peripheral blood (PB) from the arterial sheath (6-Fr or 7-Fr). Samples were then transferred using an 18-gauge needle into glass vacutainer tubes with or without EDTA to obtain plasma and serum, respectively, and immediately placed on ice. The anticoagulated samples were centrifuged at 2500× *g* for 30 min at 4 °C, and then the plasma supernatant was carefully decanted to avoid contamination with cell components. Samples of whole blood sample were left at room temperature for 15 min to allow blood clots to form, and then the serum was separated by centrifugation at 2500× *g* for 30 min at 4 °C. All samples were stored at −80 °C.

### 2.3. Laboratory measurements

We measure plasma levels of LPA and LPC, and serum levels of autotaxin (ATX), soluble CD40 ligand (sCD40L), hs-CRP, and Lp-PLA2.

Plasma LPA and LPC concentrations were enzymatically determined as described [14–16]. In brief, LPA was hydrolyzed with lysophospholipase to glycerol 3-phosphate, followed by enzymatic cycling using glycerol 3-phosphate oxidase and glycerol 3-phosphate dehydrogenase. The amplified concentrations of hydrogen peroxide, a product of enzymatic cycling, were then colorimetrically measured using an automatic analyzer (JCA-BM8040, JEOL, Tokyo, Japan). Lysophosphatidylcholine concentrations in human plasma were measured using our validated enzymatic assay in which LPC is converted by lysophospholipase into glycerophosphorylcholine, from which glycerophosphorylcholine phosphodiesterase generates choline. The amount of hydrogen peroxide produced from choline by choline oxidase was determined in the presence of peroxidase using an oxidative chromogenic reagent and 4-aminoantipyrine by measuring changes in absorbance. Serum ATX antigen concentrations were determined using a specific sandwich enzyme immunoassay that has proven useful for clinical laboratory testing [17]. Serum ATX antigen concentrations are associated with both plasma LPA concentration and serum lysoPLD activity, which is responsible for LPA production [18]. Serum hs-CRP was measured using a validated, highly sensitive immunoassay and particle-enhanced immunonephelometry (Dade Behring Holding GmbH, Liederbach, Germany). We quantified sCD40L using a human sCD40L ELISA kit (R&D Systems, Minneapolis, MN, USA). Serum Lp-PLA2 activity was determined spectrophotometrically as described [19]. Levels of serum cardiac troponin T were measured using a chemiluminescent enzyme immunoassay kit (Determiner CL TnT, Kyowa Medex, Tokyo, Japan). Other markers were routinely determined.

### 2.4. Statistical analysis

All data were statistically analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) and JMP version 9.0 (SAS Institute Inc., Cary, NC, USA). Results are presented as medians and inter-quartile ranges (IQR), means ± standard deviation or as ratios (%) and numbers for categorical data. Data from pairs were compared using the Wilcoxon rank-sum test. Because of known differences between coronary and systemic alterations in biomarker levels, the natural log transformation of the biomarker data achieved a normal distribution, and thus we used log-transformed marker values. Correlations were searched using Spearman's rank correlation. A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Clinical characteristics of study participants

Table 1 shows the characteristics of the 52 patients (male, 65%; mean age, 63.2 ± 13.6 years), 65% ($n = 34$) of whom had ST elevation myocardial infarction (STEMI). The left anterior descending artery was the culprit coronary artery in >50% of the patients. All patients received aspirin and clopidogrel, and most were also taking β-blockers at the time of blood collection, before undergoing emergency percutaneous coronary intervention and thrombectomy. Four patients had cardiogenic shock, so hemodynamic support was provided using intraaortic balloon pumping (IABP).

### 3.2. Comparison between coronary circulation and systemic circulation

Only median LPA levels were significantly higher in culprit CB than in PB (0.266 [IQR, 0.192–0.300] vs. 0.230 [IQR, 0.180–0.287] μM, $p = 0.009$). Median sCD40L levels were higher in CB than in PB, but the difference did not reach statistical significance (2055.0 [IQR, 1017.5–3375.0] vs. 1410.0 [IQR, 874.0–2485.0] pg/mL, $p = 0.177$) In contrast, autotaxin and Lp-PLA2 levels were significantly higher in PB than in CB (autotaxin: 0.671 [IQR, 0.589–0.837] vs. 0.632 [IQR, 0.496–0.738] mg/L, $p = 0.005$; Lp-PLA2: 442.0 [IQR, 347.0–556.5] vs. 436.5 [IQR, 318.8–537.3] IU/L, $p = 0.038$). In addition, LPC and hs-CRP levels were higher in PB than in CB (LPC: 163.0 [IQR, 133.5–187.5] vs. 158.0 [IQR, 119.8–191.8] μM, $p = 0.129$; hs-CRP: 1235 [IQR, 435–3250] vs. 993 [IQR, 395–2845] ng/mL, $p = 0.121$) (Fig. 1).