measured the same samples for inter-assay control every experiment and re-assayed if the inter-assay control value exceed more than 20 percent of the first control value. The sensitivity and specificity of the ELISA for each assay were described in the manufacturer's instruction. The assay's sensitivity is 3.5 pg/ml, 7.0 pg/ml and 9.0 pg/ml for sFlt-1, PlGF and VEGF respectively. If the measured values were below the limit of detection, they were excluded from statistical analysis. Actually, 15 samples of PlGF and 4 samples of VEGF were excluded, but none of the sFlt-1 samples were excluded from statistical analysis. The assay's specificity is described in the manufacturer's instruction that sFlt-1 assay recognizes only recombinant and natural human sFlt-1 and no significant cross-reactivity is observed with PlGF, VEGF, Flk-1 and so on. As for PlGF, the assay recognizes only recombinant and natural human PlGF and does not have cross-reactivity with VEGF or sFlt-1. As for VEGF, the assay recognizes recombinant and natural human VEGF and does not have cross-reactivity with PlGF or sFlt-1. Although the assay's specificity is described as above, we re-confirmed that the assay for sFlt-1 did not detect recombinant human PlGF and that the assay for PlGF did not detect recombinant human sFlt-1 by ourselves.

We used a specific primer that detected sFlt-1 mRNA. Before the experiment, we confirmed the primer's specificity by using mRNA extracted from human umbilical endothelial cell (HUVEC) as a positive control. As shown in Supplementary Figure 3A, a single band of

PCR product was observed in a dose dependent manner and a single band was also detected in patient's sample. The PCR for human sFlt-1 did not cross-react with full length human Flt-1 as shown in Supplementary Figure 3B. These data indicate that our PCR system works as specific PCR for sFlt-1. The specificity of the primer for mouse sFlt-1 was confirmed in a similar way.

***Experimental study***

*Preparation of recombinant human soluble Flt-1 (rhsFlt-1).* A DNA fragment encoding amino acids 1-338 of human Flt1 (sFlt-1 (D1-3)), which contains three Ig-like domains in its N-terminal region, was amplified by PCR using the specific primers 5'-CATCCATGGATCCTGAACTGAGTTTAAAAG-3' (forward) and 5'-CATGGATCCTCAATGTTTCACAGTGATGAATGC-3' (reverse), and human placental cDNA (Clontech Laboratories, Mountain View, CA, USA) serving as the template. The PCR product was subcloned into the pCRII-TOPO vector (Invitrogen), and the sequence was verified by sequencing. The appropriate plasmid was digested with NcoI and BamHI, and the resulting fragment was subcloned into the NcoI/BamHI sites of the pET-30a bacterial expression vector (Novagen, EMD Chemicals). This vector was then used to transform BL21 star (CE3) competent cells (Invitrogen). The transformants were cultured in LB medium containing 50 μg/ml kanamycin in a shaking flask at 37°C until the optical density at 600 nm reached 0.6,

after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was continued for an additional 4 h. The cells were then collected by centrifugation and washed, and the pellet was stored at -80°C until further purification of the protein. At that time, the cells were suspended in lysis buffer (50 mM Tris (pH 8.0) containing 100 mM NaCl and 5 mM EDTA) and lysed by incubation with 2 mg/ml lysozyme and sonication. The sFlt-1 (D1-3) protein was present predominantly in inclusion bodies, which were collected by centrifuging the lysate for 20 min at 10,000 rpm. The purified inclusion bodies were solubilized in 20 mM PBS containing 0.5 M NaCl, 6 M urea, 1 mM DTT, and 20 mM imidazole, after which the solution was run over a His-Trap HP (GE Healthcare) column. The eluate was dialyzed against graded concentrations of urea in 20 mM Tris (pH 8.0) buffer containing 0.5 M NaCl. Following the final dialysis against 20 mM Tris (pH 8.0) containing 0.5 M NaCl without urea, the sample was harvested.

*The Efficacy of rhsFlt-1 in Vitro and in Vivo.* Before the experimental study we confirmed that rhsFlt-1 binds to PlGF *in vitro* and *in vivo*. rhsFlt-1, which contains a histidine-tag, was initially diluted in buffer containing 20 mM imidazole, 0.5 M NaCl, 20 mM Tris, and 0.1 mg/ml BSA to five concentrations: 1 ng/20 μl, 10 ng/20 μl, 100 ng/20 μl, 1000 ng/20 μl, and 10,000 ng/20 μl. The rhsFlt-1 solutions were then mixed with Ni-agarose gel (Qiagen), which binds histidine-tagged sFlt-1. After centrifugation, the supernatant was

removed and recombinant human PlGF (rhPlGF) solution (10 ng/100 μl in 100 mM NaCl, 50 mM Tris, 20m M imidazole, and 0.1 mg/ml BSA) was added to the precipitate, which included the Ni-agarose-rhsFlt-1 complex, and incubated overnight at 4°C. After centrifugation to remove the Ni-agarose-rhsFlt-1 complex bound to rhPlGF, the uncombined rhPlGF in the supernatant was measured by ELISA, which confirmed that rhsFlt-1 bound rhPlGF in a dose-dependent manner (Supplementary Figure 3C). We next used ELISA to measure serum hsFlt-1 levels and free mouse PlGF-2 30 min after intraperitoneal administration of rhsFlt-1 to wild-type C57BL/6 mice (15 ng/g BW) to confirm that the injected rhsFlt-1 binds to endogenous mouse PlGF-2. After injection of rhsFlt-1, serum rhsFlt-1 increased to 3957 ± 916 pg/ml (Supplementary Figure 3D), and levels of endogenous mouse PlGF-2 were significantly reduced, as compared to control (PBS) (8.1 ± 1.4 vs. 13.7 ± 0.8 pg/ml; n=9, 8; $P$ <0.01, Supplementary Figure 3E). The serum concentration of endogenous VEGF tended to be lower in mice treated with rhsFlt-1 than in mice treated with PBS, but the difference was not statistically significant (58.1 ± 1.8 vs. 66.6 ± 8.4 pg/ml; n=9, 8; $P$ =0.308).

*Physical Examination.* Blood pressures and heart rates were measured once every 2 weeks using a tail-cuff system (BP-98A; Softron) that utilizes a photoelectric sensor to detect blood flow in the tail (2). The mice were familiarized to the procedure before they were 12 weeks old (Supplementary Figure 4).

**Supplementary Figure 1**

Plasma sFlt-1 levels and the relationship between plasma sFlt-1 and PlGF levels and coronary atherosclerosis. (**A**) Plasma levels of sFlt-1 from the renal vein were the highest among those from aorta, coronary sinus (CS), hepatic vein, and renal vein. *n=14*. **P*<0.05. (**B**) Plasma levels of sFlt-1 from aorta were positively correlated with plasma levels of sFlt-1 from renal vein. *n=126*. r=0.70. *P*<0.001. (**C, D**) When dividing patients into 5 groups according to renal function as described in supplemental method, the extent of coronary atherosclerosis in terms of both the number of coronary arteries showing >75% stenosis (**C**) and Gensini's score (**D**) in CKD patients was severer in patients with more severe renal dysfunction. *n=329*. **P*<0.05 vs. Group1 in **C** and **D.** sFlt-1 (**E**) and PlGF (**F**) in patients without coronary stenosis and in those with 1-, 2-, and 3-vessel disease. sFlt-1 tended to be lower and PlGF tended to be higher in accordance with coronary atherosclerosis severity, but neither difference reached the level of statistical significance. Data are means ± SEM.

**Supplementary Figure 2**

(**A-B**) Plasma levels of PlGF (**A**) and serum levels of VEGF (**B**) in samples collected from the aorta. Both values did not have significant correlation with eGFR. *n=314*. r=0.00. *P*=0.991.(**A**) *n=325*. r=0.10. *P*=0.079.(**B**) (**C-D**) Plasma PlGF levels (**C**) or serum VEGF levels (**D**) in

samples from aorta, CS, hepatic vein, and renal vein. PlGF levels did not significantly differ among these vessels. Serum levels of VEGF from hepatic vein were higher than those from renal vein, but not those from aorta or CS. $n = 14$. $^*P < 0.05$. (**E-F**) VEGF/sFlt-1 ratios plotted against renal function (**E**) and extent of atherosclerosis (**F**). VEGF/sFlt-1 ratios showed no significant relationship with eGFR (r= -0.10. $P$=0.073) and no tendency to differ according to the number of diseased coronary vessels. Data are means ± SEM.

**Supplementary Figure 3**

Validities of the specific primers for sFlt-1 and recombinant human sFlt-1 (rhsFlt-1). (**A**) Extracted mRNA from renal biopsy specimens was tested to confirm the specificity of the primer for human sFlt-1. Extracted mRNA from human umbilical endothelial cell (HUVEC) was used as a positive control at 4 graded concentrations. A single PCR product was observed in a dose dependent manner and a single band was also detected in patient's sample (Pt). (**B**) The PCR for human sFlt-1 did not cross-react with full length human Flt-1. In lanes 1 and 3, PCR products were amplified using full length Flt-1 cDNA as a template, in lanes 2 and 4, PCR products were amplified using sFlt-1 cDNA as a template. Lanes 1 and 2 were amplified using specific primers for full length Flt-1, lanes 3 and 4 were amplified using specific primer for sFlt-1. M stands for marker. (**C-E**) The efficacy of recombinant human sFlt-1 (rhsFlt-1) was

confirmed by demonstrating that it bound PlGF *in vitro* and *in vivo*. (**C**) When recombinant human PlGF (0.32 pM) was incubated with six different concentrations of rhsFlt-1 (0 to 100 pM), rhsFlt-1 bound to rhPlGF in a dose dependent manner. (**D-E**) Serum levels of human sFlt-1 and free mouse PlGF-2 were measured 30 min after intraperitoneal administration of rhsFlt-1 (15 ng/g BW) or PBS to wild-type C57/BL6 mice. (**D**) Human sFlt-1 was not detected in control mice. (**E**) Serum levels of mouse PlGF-2 were significantly reduced in rhsFlt-1-injected mice. $^{**}P < 0.01$. Data are means ± SEM.

**Supplementary Figure 4**

Body weight (**A**) and blood pressure (**B**) of Apo-E KO mice in the experimental study. Temporal differences in body weight and blood pressure existed between the 4 groups, but the differences were diminished at the end of the experiments. Cont. PBS stands for control mice administered with PBS, cont. sFlt-1 control mice administered with sFlt-1, 5/6NR PBS 5/6 nephrectomized mice administered with PBS, 5/6 NR sFlt-1 5/6 nephrectomized mice administered with sFlt-1, respectively. $^{*}P < 0.05$ between the 4 groups.

**Supplemental References**

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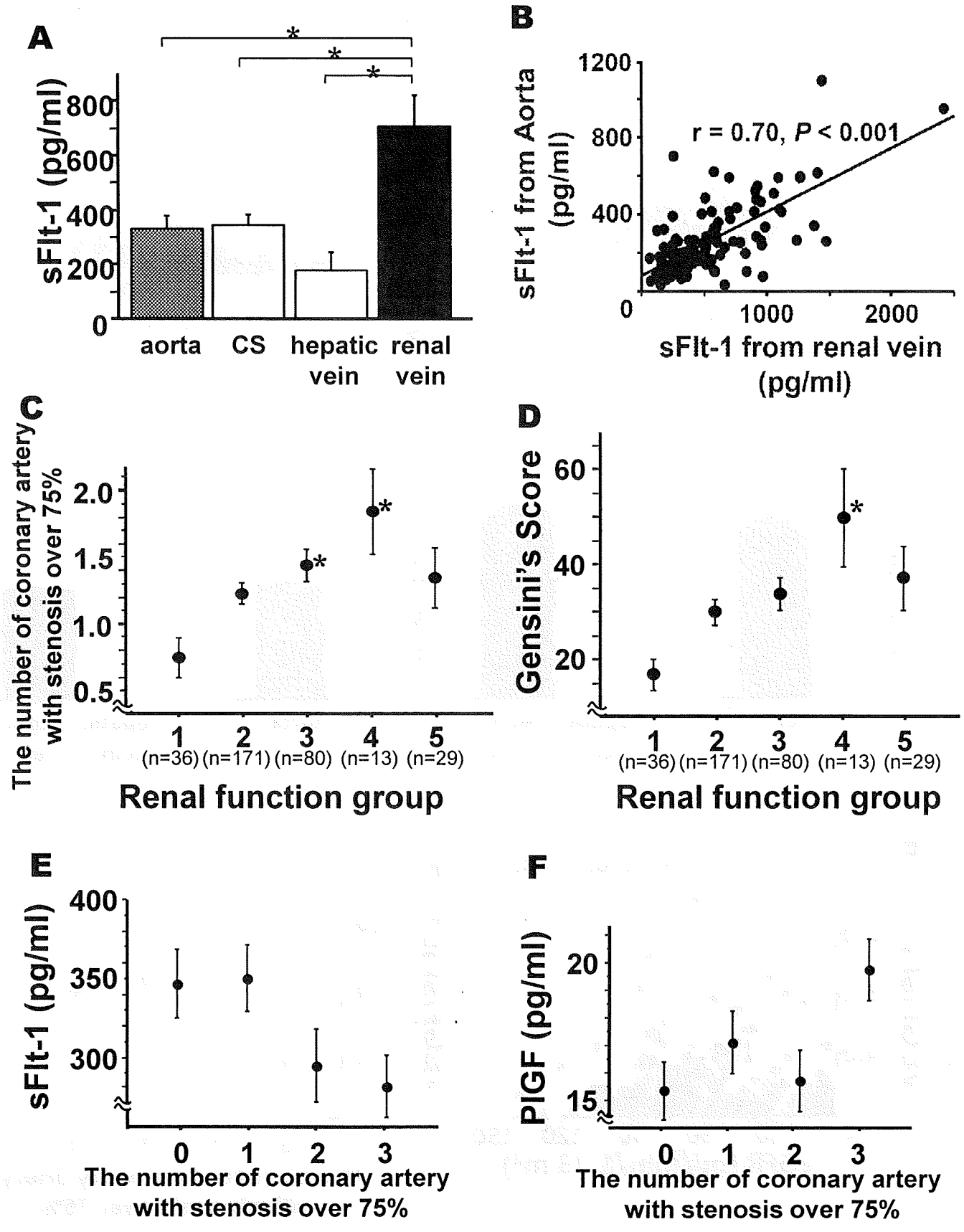
2. Krege, J.H., Hodgin, J.B., Hagaman, J.R., and Smithies, O. 1995. A noninvasive computerized tail-cuff system for measuring blood pressure in mice. *Hypertension.* **25**:1111-1115.

Supplementary Figure 1



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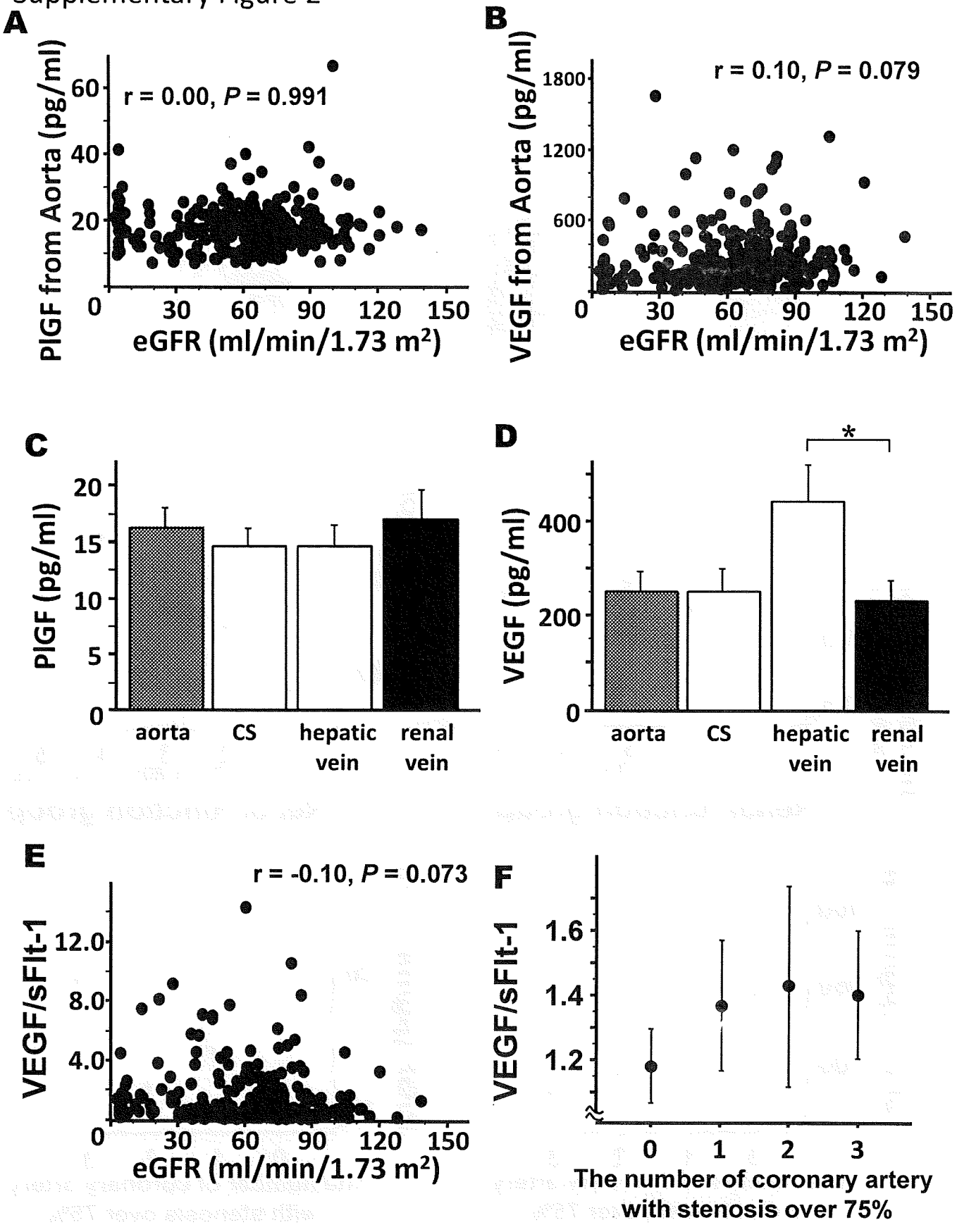
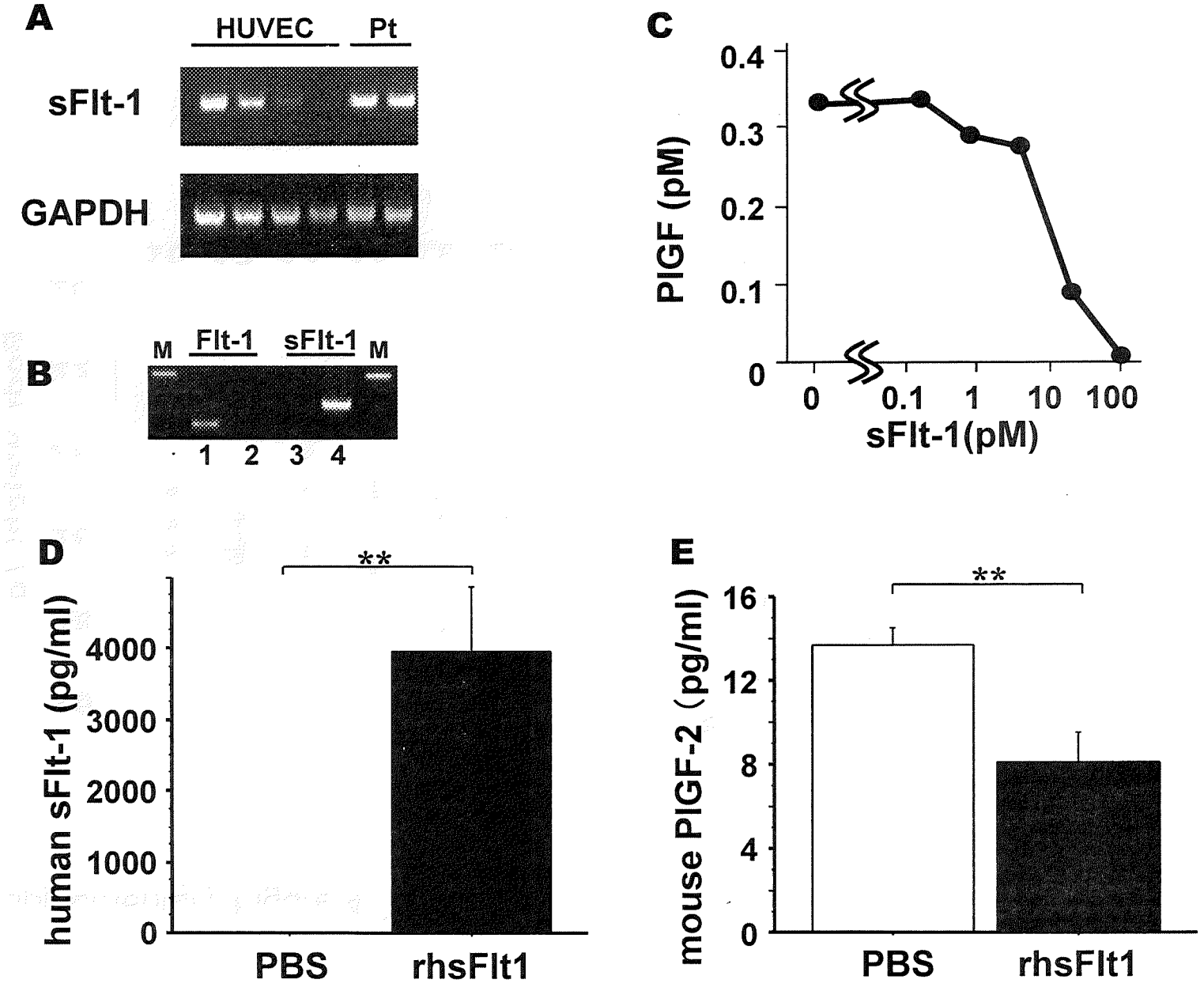
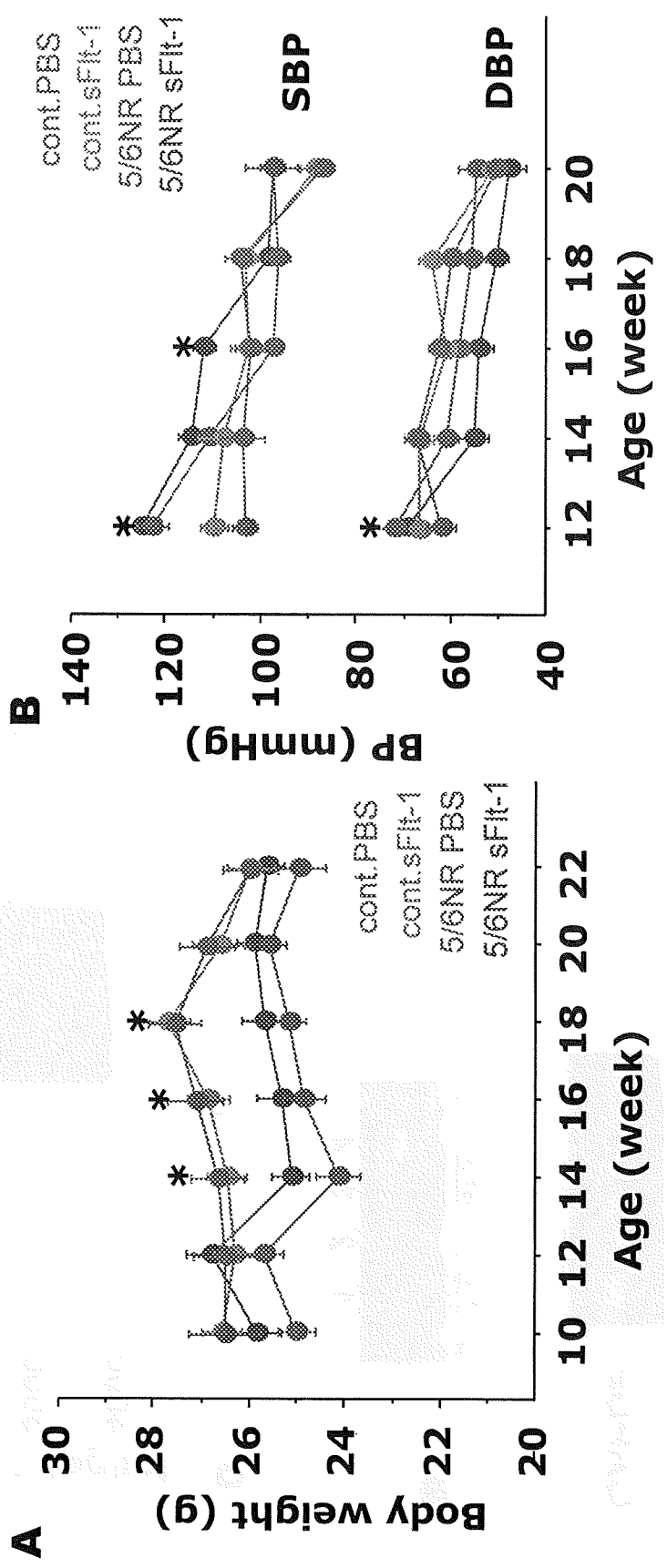


Supplementary Figure 3



Supplementary Figure 4



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# Incremental Effects of Eicosapentaenoic Acid on Cardiovascular Events in Statin-Treated Patients With Coronary Artery Disease

## — Secondary Prevention Analysis From JELIS —

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***Background:*** Results from JELIS (Japan EPA Lipid Intervention Study) demonstrated the efficacy of pure eicosapentaenoic acid (EPA) in preventing coronary artery disease (CAD) in hypercholesterolemic patients under statin treatment. The present study examined in detail whether EPA is effective for the secondary prevention of CAD.

***Methods and Results:*** Patients with established CAD and a total cholesterol level ≥250 mg/dl were observed with a mean follow-up of 4.6 years. They were randomly assigned to receive either 1,800 mg of EPA+statin (EPA group) or statin alone (control group). The incidence of major coronary events (MCE) were compared in the 2 groups. The incidence of MCE was significantly lower in the EPA group (8.7% vs 10.7%, adjusted hazard ratio=0.77, 95% confidence interval (CI) 0.63–0.96, P=0.017, number needed to treat (NNT)=49). Among 1,050 patients with prior myocardial infarction (MI), the incidence of MCE in the EPA group (15.0%) was significantly lower than that in the control group (20.1%, adjusted hazard ratio=0.73, 95%CI 0.54–0.98, P=0.033, NNT=19).

***Conclusions:*** EPA is effective for secondary prevention of CAD, especially in individuals with prior MI, and should be added to conventional treatment. (*Circ J* 2009; **73:** 1283–1290)

**Key Words:** Acute coronary syndrome; Fatty acids; Lipids; Secondary prevention

Several interventional studies have reported the clinical benefits of fish oil administration or fish consumption in patients with coronary artery disease (CAD), suggesting that n-3 polyunsaturated fatty acids (PUFAs) can reduce the risk of coronary events![1–7] Two large-scale secondary prevention trials, the Diet and Reinfarction Trial (DART)[7] and the Gruppo Italiano per lo Studio della Sopravivenza nell' Infarto Miocardico-Prevenzione Trial (GISSI)[8] reported that increased consumption of fish or fish-oil supplements reduced coronary death in postinfarction patients.

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However, those trials were performed in either United States or Europe, and different results might be obtained in the Japanese population, in which fish consumption is greater. In addition, assessment of individual fatty acids was not performed in the conventional trials because the intervention was performed with fish oil, which included various fatty acids, or with meals. Eicosapentaenoic acid (EPA) ethyl ester, which is purified from n-3 PUFAs present in fish oil, is approved by the Ministry of Health, Labour and Welfare of Japan as a treatment for hyperlipidemia and peripheral artery disease. The Japan EPA Lipid Intervention Study (JELIS)[9] was a prospective, randomized, open-label, blinded

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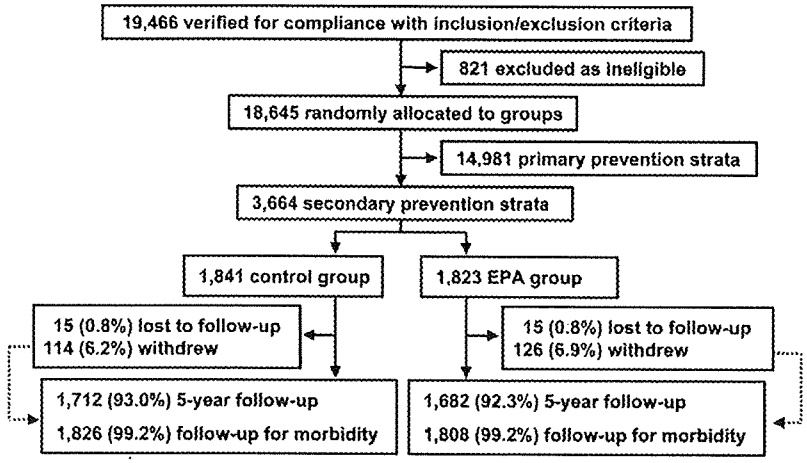



**Figure 1.** Trial profile. EPA, eicosapentaenoic acid.

endpoint trial that examined prevention of CAD by EPA (20:5 n-3) treatment in Japanese hypercholesterolemic patients. Secondary prevention strata of CAD were included in the trial population. To ensure that EPA was the only fatty acid tested, researchers administered a pure EPA capsule to patients in the active treatment group.

The biological effects of EPA include anti-arrhythmic effects[10,11] anti-inflammatory effects[12–14] decreased platelet aggregation[15] vasodilatory activity[16,17] and lipid-lowering effects[18,19] We began JELIS with the expectation that these effects of EPA, other than improvement of dyslipidemia, would reduce the risk of CAD.

The major findings of JELIS demonstrated 19% reduction by EPA treatment of major coronary events (MCE), including sudden cardiac death, fatal and non-fatal myocardial infarction (MI), and other non-fatal events, including unstable angina pectoris (AP), angioplasty, stenting, and coronary artery bypass grafting[20] In the present study, we performed an additional analysis of the JELIS data to determine whether EPA was effective for secondary prevention of CAD. We also examined a change in the ratio of plasma EPA to arachidonic acid (20:4 n-6) concentration and its relationship to the incidence of MCE.

## Methods

### Patient Population

A total of 18,645 patients with a total cholesterol (TC) level ≥250 mg/dl, which corresponds to a low-density lipoprotein-cholesterol (LDL-C) level ≥170 mg/dl at baseline, were examined in the JELIS trial. The design and inclusion and exclusion criteria are described in detail elsewhere[9] We used data from JELIS for 3,664 patients with established CAD defined as previous MI, coronary intervention, or confirmed AP for this analysis.

The exclusion criteria were acute MI within the past 6 months, unstable AP, a history of or complication by serious heart diseases (severe arrhythmia, heart failure, primary or secondary cardiomyopathy, valvular heart diseases, congenital heart diseases, and other related conditions). We also excluded cardiovascular reconstruction within the past 6 months, cerebrovascular disorder within the past 6 months, serious hepatic or renal disease, malignant tumor, uncontrollable diabetes mellitus, hyperlipidemia associated with effects of drugs such as steroid hormones, hemorrhage (hemophilia, capillary fragility, gastrointestinal ulcer, urinary tract hemorrhage, hemoptysis, vitreous hemorrhage), hemorrhagic diathesis, hypersensitivity to the study drug formulation, planned surgery, or other condition judged inappropriate for inclusion in the study by the physician-in-charge.

### Procedures

All patients received 10 mg of pravastatin or 5 mg of simvastatin once daily as the first-line treatment and were counseled to follow the National Cholesterol Education Program step I diet[21] The study population was randomly assigned to receive EPA with statin (EPA group) or statin alone (control group), after a 4- to 8-week washout from antihyperlipidemic drugs. In the EPA group, we administered a daily dose of 1,800 mg of EPA as 6 capsules each containing 300 mg of pure (>98%) EPA ethyl ester. Statin administration was continued until trial termination in 1,311/1,652 (79%) cases in the control group and 1,282/1,620 (79%) in the EPA group. EPA administration was continued until trial termination in 1,234 (76%) cases. The planned duration of follow-up of the patients was 5 years. Local physicians monitored compliance with dietary instructions and medication use at each clinic visit. Reported clinical endpoints were reviewed by expert cardiologists belonging to the Event Evaluation Committee without knowledge of treatment allocation. The primary endpoint was the cumulative incidence of MCE, which included sudden cardiac death, fatal and nonfatal MI, and other non-fatal events including unstable AP, angioplasty, stenting, and coronary artery bypass grafting (CABG). We sampled blood to measure serum lipids at 6 and 12 months, and then every year until the final follow-up visit. Plasma total fatty acid concentrations were measured annually by a central laboratory (BML Inc, Tokyo, Japan) for all patients who gave informed consent for blood sampling[22]

### Statistical Analysis

All analyses were based on the intention-to-treat principle. The Wilcoxon 2-sample test was used for comparisons involving continuous variables, and the chi-square test for those involving categorical variables. Time-to-event data were analyzed using the Kaplan-Meier method. The hazard ratio (HR) and its 95% confidence interval (CI) were computed with the Cox proportional hazard model adjusted for age, sex, smoking, prior MI, diabetes, and hypertension. Number needed to treat (NNT) was simply computed as the inverse of the absolute difference between 2 groups in terms

**Table 1. Baseline Characteristics of the Study Cohort**

| | Control group (n=1,841) | EPA group (n=1,823) | P value |
|---|---|---|---|
| General characteristics | | | |
| Age, years | | | |
| ≤49 | 128 | 116 | |
| 50–59 | 437 | 416 | |
| 60–69 | 781 | 809 | |
| ≥70 | 495 | 482 | |
| Mean (SD) | 63 (8) | 63 (8) | 0.638 |
| Male | 822 (45) | 844 (46) | 0.317 |
| Body mass index, mean (SD) | 24 (3) | 24 (3) | 0.735 |
| Systolic BP, mean (SD), mmHg | 137 (18) | 137 (18) | 0.887 |
| Diastolic BP, mean (SD), mmHg | 79 (11) | 79 (11) | 0.282 |
| Clinical history | | | |
| OMI | 502 (27) | 548 (30) | 0.062 |
| Stable angina | 1,484 (81) | 1,419 (78) | 0.039 |
| PTCA or CABG | 433 (24) | 462 (25) | 0.199 |
| Diabetes | 420 (23) | 405 (22) | 0.665 |
| Hypertension | 816 (44) | 799 (44) | 0.763 |
| Smoker | 442 (24) | 492 (27) | 0.039 |
| Laboratory data | | | |
| Total cholesterol, mean (SD), mg/dl | 270 (27) | 270 (24) | 0.817 |
| LDL-cholesterol, mean (SD), mg/dl | 178 (28) | 178 (28) | 0.789 |
| HDL-cholesterol, mean (SD), mg/dl | 55 (18) | 55 (16) | 0.615 |
| Triglyceride, median (IQR), mg/dl | 163 (120–230) | 160 (116–226) | 0.213 |
| Concomitant drugs | | | |
| Antiplatelet agent | 800 (43) | 755 (41) | 0.218 |
| Anticoagulant agent | 177 (10) | 192 (11) | 0.356 |
| Calcium antagonist | 930 (51) | 899 (49) | 0.467 |
| β-blocker | 341 (19) | 306 (17) | 0.168 |
| Other antihypertensive agent | 656 (36) | 638 (35) | 0.687 |
| Nitrate | 710 (39) | 685 (38) | 0.537 |
| Hypoglycemic agent | 294 (16) | 274 (15) | 0.432 |

Values are n (%) unless otherwise noted.
EPA, eicosapentaenoic acid; BP, blood pressure; OMI, old myocardial infarction; PTCA, percutaneous transluminal coronary angiography; CABG, coronary artery bypass grafting; LDL, low-density lipoprotein; HDL, high-density lipoprotein; IQR, interquartile range.

of event rate over the years of follow-up. In additional analyses, profiles of plasma fatty acid values were compared using repeated-measures ANOVA. Changes in serum lipid values were compared using the Wilcoxon 2-sample test. Probability values of 5% (2-sided) were considered significant. Analyses were performed using SAS statistical software (version 8.12, SAS Institute, Inc, Cary, NC, USA).

## Results

### Baseline Characteristics

**Figure 1** shows the trial profile. Data from all 3,664 patients in the secondary prevention strata were used for the analysis. Patients were followed for a mean of 4.6 years, and the 5-year follow-up rate was >92% in both groups.

Baseline characteristics of the study population are shown in **Table 1**. Of the 3,664 (1,823 in the EPA group and 1,841 in the control group) individuals with documented CAD, 1,050 had a history of MI, 2,903 with AP, and 895 of percutaneous transluminal coronary angioplasty (PTCA) or CABG. The rate of prior MI was higher, though not significantly so, in the EPA group; in contrast, AP was significantly less frequent in this group. There were more tobacco smokers in the EPA group. Except for these variables, the 2 treatment groups were well-matched at baseline, and the pattern of use of concomitant medications was similar between them (**Table 1**).

### Primary Endpoint

In the population for analysis comprising all 3,664 patients, 355 patients (158 in the EPA group, 197 in the control group) reached the composite primary endpoint. Kaplan-Meier curves for the primary endpoint showed that the 5-year cumulative total MCE rate was 8.7% in the EPA group and 10.7% in the control group (**Figure 2a**), with a significant relative risk reduction of 23% in the EPA group (P=0.017; NNT=49). As shown in **Figure 3**, EPA therapy was associated with a significant reduction of 30% in the incidence of unstable AP. The incidence of coronary death or MI was 30% lower in the EPA group than in the control group; this difference was not significant. In addition, the incidence of fatal or nonfatal MI was 30% lower in the EPA group; this difference, as well, was not significant. However, the incidence of nonfatal coronary events (nonfatal MI, unstable AP, and angioplasty/stenting or CABG) was 21%, and significantly lower in the EPA group than in the control group.

### Ancillary Analysis

Among the 895 patients with prior coronary intervention (PTCA or CABG), the incidence of MCE in the EPA group (14.7%) was significantly lower than that in the control group (22.0%, adjusted HR 0.65, P=0.007, NNT=13, **Figure 2b**). Among the 1,050 patients with prior MI, the incidence of MCE in the EPA group (15.0%) was significantly lower than that in the control group (20.1%, adjusted HR 0.73, P=0.033, NNT=19, **Figure 2c**). Among the 537 patients with

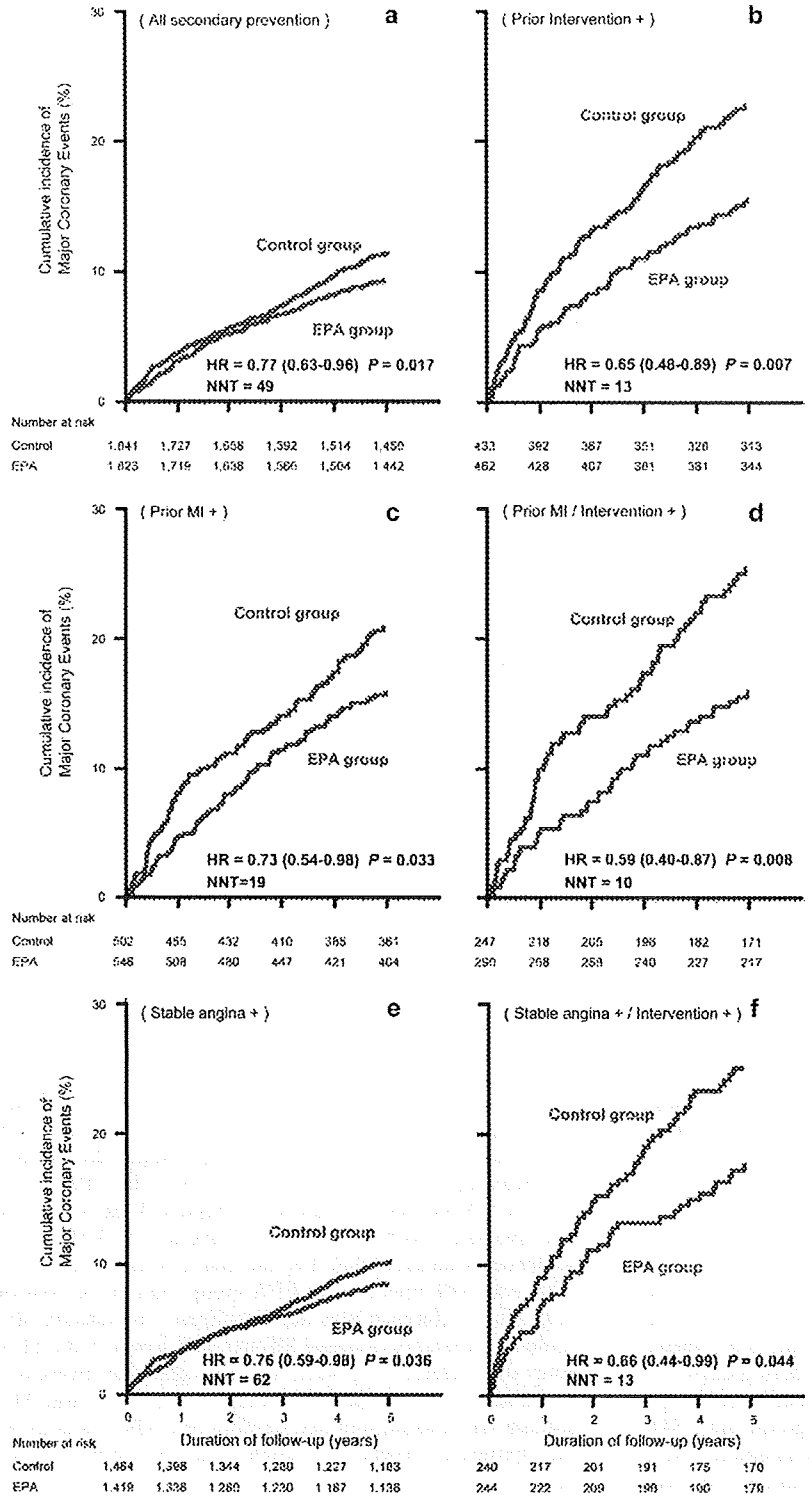



**Figure 2.** Kaplan-Meier estimates of the incidence of the primary endpoint of coronary events occurring in the group of all patients (**a**), those with an intervention (percutaneous transluminal coronary angioplasty or coronary artery bypass grafting (CABG)) (**b**), those with prior myocardial infarction (MI) (**c**), those with prior MI and an intervention (**d**), those with stable angina pectoris (AP) (**e**) and those with stable AP and an intervention (**f**). Major coronary events were considered to be sudden cardiac death, fatal and nonfatal MI, unstable AP, and angioplasty/stenting or CABG. EPA, eicosapentaenoic acid; HR, hazard ratio; CI, confidence interval; NNT, number needed to treat.

prior MI and coronary intervention, the incidence of MCE in the EPA group (15.2%) was also significantly lower than that in the control group (24.7%, adjusted HR 0.59, P=0.008, NNT=10, **Figure 2d**). Among the 2,903 patients with stable AP, the incidence of MCE in the EPA group (7.8%) was significantly lower than that in the control group (9.4%, adjusted HR 0.76, P=0.036, NNT=62, **Figure 2e**). Furthermore, among the 484 patients with stable AP and a coronary intervention, the incidence of MCE in the EPA group (16.8%) was also significantly lower than that in the control group (24.6%, adjusted HR 0.66, P=0.044, NNT=13, **Figure 2f**).

### Changes in Serum Lipid Values

**Figure 4** summarizes the post-treatment lipid profiles of

| Event | No. of events (%) Control ($n$ = 1,841) | EPA ($n$ = 1,823) | HR (95%CI) | $P$ value |
|---|---|---|---|---|
| Major coronary events | 197(10.7) | 158(8.7) | 0.77 (0.63 – 0.96) | 0.017 |
| Items of account | | | | |
| Sudden cardiac death | 13 (0.7) | 13 (0.7) | 0.93 (0.43 – 2.02) | 0.861 |
| Fatal MI | 8 (0.4) | 5 (0.3) | 0.57 (0.19 – 1.74) | 0.324 |
| Nonfatal MI | 38 (2.1) | 26 (1.4) | 0.66 (0.40 – 1.08) | 0.099 |
| Unstable angina | 123 (6.7) | 88 (4.8) | 0.70 (0.54 – 0.93) | 0.012 |
| CABG or PTCA | 148 (8.0) | 127 (7.0) | 0.83 (0.65 – 1.05) | 0.123 |
| Combined Endpoints | | | | |
| Coronary death | 21 (1.1) | 18 (1.0) | 0.79 (0.42 – 1.49) | 0.467 |
| Coronary death or MI | 58 (3.2) | 43 (2.4) | 0.70 (0.47 – 1.04) | 0.076 |
| Fatal or nonfatal MI | 42 (2.3) | 31 (1.7) | 0.70 (0.44 – 1.12) | 0.139 |
| Nonfatal coronary events | 178 (9.7) | 145 (8.0) | 0.79 (0.63 – 0.98) | 0.036 |

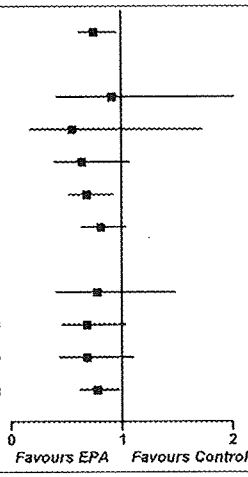
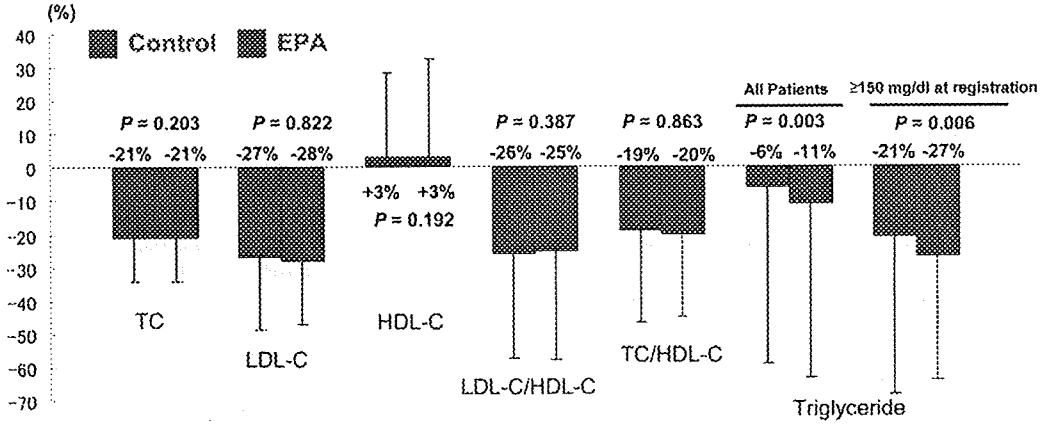
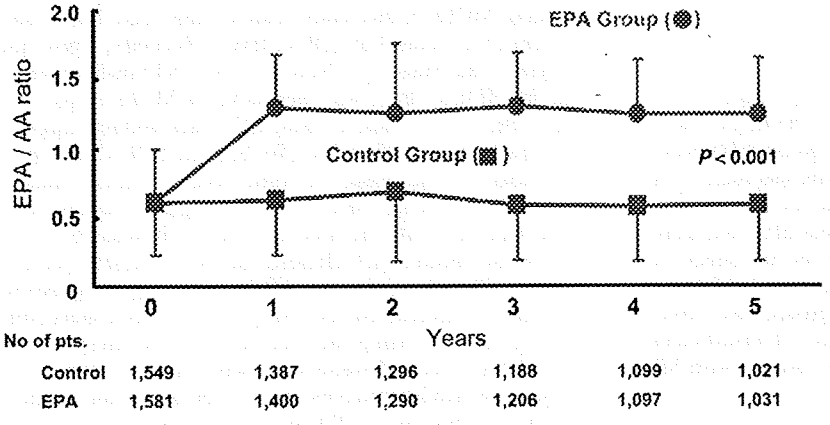



**Figure 3.** Incidence of primary endpoints and hazard ratios (HRs) with 95% confidence intervals (CIs). EPA, eicosapentaenoic acid; CABG, coronary artery bypass grafting; PTCA, percutaneous transluminal coronary angioplasty; MI, myocardial infarction.




| | TC mg/dl | LDL-C mg/dl | HDL-C mg/dl | LDL-C/HDL-C ratio | TC/HDL-C ratio | Triglyceride All patients | Triglyceride ≥150mg/dl at registration |
|---|---|---|---|---|---|---|---|
| Control | 218 (29) | 130 (27) | 56 (15) | 2.5 (0.9) | 4.1 (1.1) | 167 (96) | 202 (108) |
| No of pts. | 1,741 | 1,695 | 1,702 | 1,695 | 1,702 | 1,741 | 992 |
| EPA | 215 (30) | 130 (29) | 55 (14) | 2.5 (0.9) | 4.1 (1.1) | 154 (87) | 185 (101) |
| No of pts. | 1,709 | 1,669 | 1,677 | 1,669 | 1,677 | 1,707 | 921 |

**Figure 4.** Percentage change and on-treatment average levels (SD) in serum lipid profile in the eicosapentaenoic acid (EPA) group and Control group. TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol.




| No of pts. | | | | | | |
|---|---|---|---|---|---|---|
| Control | 1,549 | 1,387 | 1,296 | 1,188 | 1,099 | 1,021 |
| EPA | 1,581 | 1,400 | 1,290 | 1,206 | 1,097 | 1,031 |

**Figure 5.** Trends in mean eicosapentaenoic acid (EPA)/arachidonic acid (AA) ratio during observation. Points represent mean ± SD.

**Table 2. Relationships Between On-Treatment EPA, AA, and EPA/AA Ratio, and Adjusted Risk of Coronary Events**

| Endpoint | Major coronary events | | | Sudden cardiac death or fatal/nonfatal MI | | | Unstable angina | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low | Intermediate | High | Low | Intermediate | High | Low | Intermediate | High |
| EPA (mol%) | 0–2.59 | 2.60–4.79 | ≥4.80 | 0–2.59 | 2.60–4.79 | ≥4.80 | 0–2.59 | 2.60–4.79 | ≥4.80 |
| No. of patients | 1,047 | 1,125 | 1,089 | 1,047 | 1,125 | 1,089 | 1,047 | 1,125 | 1,089 |
| HR (95%CI) | 1.00 | 1.02 (0.78–1.35) | 0.82 (0.62–1.09) | 1.00 | 0.74 (0.45–1.24) | 0.65 (0.38–1.10) | 1.00 | 1.13 (0.80–1.59) | 0.84 (0.58–1.20) |
| P value (vs Low) | | 0.854 | 0.172 | | 0.258 | 0.107 | | 0.504 | 0.330 |
| AA (mol%) | 0–4.24 | 4.25–4.99 | ≥5.00 | 0–4.24 | 4.25–4.99 | ≥5.00 | 0–4.24 | 4.25–4.99 | ≥5.00 |
| No. of patients | 1,050 | 1,032 | 1,179 | 1,050 | 1,032 | 1,179 | 1,050 | 1,032 | 1,179 |
| HR (95%CI) | 1.00 | 0.99 (0.75–1.31) | 1.18 (0.91–1.55) | 1.00 | 1.43 (0.82–2.51) | 1.78 (1.04–3.02) | 1.00 | 0.83 (0.57–1.19) | 1.12 (0.80–1.56) |
| P value (vs Low) | | 0.927 | 0.212 | | 0.209 | 0.035 | | 0.308 | 0.519 |
| EPA/AA ratio | 0–0.55 | 0.56–1.05 | ≥1.06 | 0–0.55 | 0.56–1.05 | ≥1.06 | 0–0.55 | 0.56–1.05 | ≥1.06 |
| No. of patients | 1,064 | 1,108 | 1,089 | 1,064 | 1,108 | 1,089 | 1,064 | 1,108 | 1,089 |
| HR (95%CI) | 1.00 | 0.96 (0.73–1.26) | 0.80 (0.61–1.06) | 1.00 | 0.62 (0.37–1.04) | 0.58 (0.34–0.97) | 1.00 | 1.01 (0.71–1.43) | 0.82 (0.58–1.17) |
| P value (vs Low) | | 0.759 | 0.113 | | 0.069 | 0.038 | | 0.961 | 0.284 |

HRs with 95%CIs were compared and P values determined by comparison with the Low group. Cox proportional hazard model was used to compute them. AA, arachidonic acid; MI, myocardial infarction; HR, hazard ratio; CI, confidence interval. Other abbreviation see in Table 1.

the 2 treatment groups. TC and LDL-C levels decreased by 21% and 27% from baseline, respectively, in both groups. There were minimal changes in high-density lipoprotein-cholesterol (HDL-C) levels above the baseline in both treatment groups. These changes did not differ significantly between the 2 groups. Triglyceride levels decreased 11% from baseline in the EPA group and 6% in the control group. Triglyceride levels exhibited greater decrease in the group with levels above 150mg/dl at registration, at 27% from baseline in the EPA group and 21% in the control group. A significant decrease was observed in the EPA group.

### Relationships Among EPA, AA, and EPA/AA Ratio, and the Incidence of CAD

The mean value of the ratio of plasma EPA to arachidonic acid (AA, 20:4 n-6) at baseline was 0.6 in both treatment groups. The ratio did not change in the control group, but increased to 1.3 at 1 year in the EPA group and remained at that level through the follow-up period (P<0.001 in **Figure 5**). We divided the patients into 3 groups with approximately equal numbers of cases according to their mean on-treatment EPA and AA levels and EPA/AA ratio (**Table 2**). The incidence of MCE was lower, but not significantly so, in the group with highest EPA/AA ratio (≥1.06) (HR 0.80) compared with that in the group with lowest ratio (≤0.55) group, and the incidence of cardiac death or MI was significantly lower (adjusted HR 0.58, P=0.038) in the patient group with the highest EPA/AA ratio than in that with the lowest ratio.

## Discussion

The beneficial effects of EPA were remarkable in the secondary prevention subgroup in JELIS. The inclusion of larger numbers of patients in the EPA group who were smokers or had a history of MI was the principal reason for the reduction in the HR after adjustment for risk factors. Analysis of combined endpoints showed that EPA treatment led to a significant reduction of 21% in the incidence of nonfatal coronary events. Although the incidence of fatal or nonfatal MI was 30% lower in the EPA group, this difference was not significant, possibly because of insufficient statistical power (only a small number of patients with MI were included).

GISSI-Prevenzione is another large-scale clinical trial that demonstrated prevention of cardiovascular events by n-3 PUFAs[8] However, the n-3 PUFAs used in GISSI reduced mortality because of coronary events, though the same finding was not clearly obtained in JELIS. In the control group, the cardiac death rate per 1,000 person-years was 2.5 in JELIS compared with 17 in GISSI-Prevenzione. We suspect that a difference in dietary fish consumption was responsible for this difference in results. Our findings suggest that the number of all coronary events can be reduced by administration of EPA, even in Japanese who consume an abundance of fish. Furthermore, GISSI-Prevenzione evaluated the recurrence of acute coronary events in patients within 3 months after acute coronary syndrome, whereas we evaluated the recurrence of acute coronary events in stable patients more than 6 months after acute coronary syndrome. In addition, the difference in the inclusion criteria of the trials might have influenced the results.

Although previous epidemiological studies reported that the incidence of CAD in the Japanese population is lower than that in Western countries[23,24] a high LDL-C level is a risk factor for CAD in Japanese, as in the United States and Europe. Results from a clinical trial involving administration of simvastatin to Japanese patients with hypercholesterolemia have shown that lowering LDL-C can reduce the incidence of CAD[25,26] Although it is known that treatment with EPA has a LDL-C lowering effect[18,19] in JELIS only the triglyceride level, and not the LDL-C level, differed between the treatment groups. EPA had a weak LDL-C lowering effect beyond that of statins alone. The efficacy of EPA in reducing MCEs in this study design might have been independent of its control of LDL-C levels. Recently, a post hoc analysis of the Treating to New Targets (TNT) study reported that the HDL-C level was predictive of MCEs in patients with LDL-C level below 70mg/dl[27] That finding suggests that strategies providing benefits beyond LDL-C lowering may also be important. In some recent clinical studies designed to increase HDL-C levels, negative results have been reported with acyl-coenzyme A:cholesterol acyltransferase inhibitors[28] cholesterol ester transfer protein inhibitors[29] and succinobucol[30] in reversing progression of coronary atherosclerosis. Using EPA is thus a successful strategy for decreasing the risk of coronary artery events beyond the effect of statin treatment. In fact, changes in triglyceride-rich lipoproteins may also be important. In addition, administration of EPA leads to changes in membrane

fatty acid composition and function that could also improve cardiovascular function, independent of any changes in lipoprotein levels.

As noted, the benefits of n-3 PUFAs on cardiovascular events include its antiarrhythmic effect[10,11] anti-inflammatory effect[12–14] decreased platelet aggregation[15] vasodilatory activities[16,17] triglyceride-lowering effect[18,19] and increase in adiponectin[31,32] A randomized controlled trial that measured the concentrations of EPA, DHA, and linoleic acid in carotid artery plaque lipid fractions reported that fish oil increased plaque stability. Thies et al[33] reported that higher proportions of EPA and DHA and decreased numbers of macrophages in carotid plaque were observed in the fish oil treatment group than in the group treated with a blend of palm and soybean oil and the group treated with sunflower oil. Furthermore, fewer atheromatous plaques and thin fibrous cap atheromas were observed in the fish oil-treated group than in the other groups. A clinical observation study that used multidetector spiral computed tomography reported that there was an inverse correlation between serum n-3 PUFA (EPA and DHA) levels and the extent of coronary soft plaques and calcification in acute MI patients[34] Those findings suggest that EPA and DHA may have anti-arteriosclerotic effects.

In conclusion, the findings for the secondary prevention strata of JELIS were that EPA administration yielded a 23% reduction in the incidence of MCE. Notably, patients with prior MI exhibited a 27% reduction, and those with prior MI and an intervention (PTCA or CABG) exhibited a 41% reduction. These findings strongly suggest that the addition of EPA to conventional treatment should be considered for secondary prevention of CAD, and also suggest that n-3 PUFAs should be considered to reduce secondary prevention of CAD, which according to the results of this trial, might also include EPA alone.

## Study Limitation

The efficacy of preventing restenosis and acute coronary syndrome for acute-phase treatment of CAD could not be tested, because patients were excluded from this trial if the onset of CAD had been within 6 months.

When we devised the protocol for JELIS approximately 10 years ago, no target level of LDL-C had been clearly defined. The mean LDL-C value during the observation period of this study was 130 mg/dl, and high levels were controlled rather than meeting a current treatment target value. Though the control of LDL-C level was not satisfactory in this study, we considered the reduction of the risk of CAD with use of EPA to be clinically important. It appears possible that the prevention by EPA of CAD is independent of its control of the LDL-C level. We planned to emulate an evaluation in the real world of medical care, so we did not use a placebo in the control group, and for ethical reason we adopted the additional design parameter of treatment of hypercholesterolemia for all patients by statin administration.

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