

Salmon consumption by pregnant women reduces ex vivo umbilical cord endothelial cell activation^{1–3}

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ABSTRACT

Background: In vitro exposure of endothelial cells (ECs) to n–3 (omega-3) long-chain PUFAs (LCPUFAs) reduces cell adhesion molecule (CAM) expression. However, to our knowledge, no previous human studies have examined the influence of an altered diet on CAM expression.

Objective: We assessed whether salmon (rich in n–3 LCPUFAs) consumption twice a week during pregnancy affected offspring umbilical vein EC CAM expression.

Design: Women were randomly assigned to maintain their habitual diets or to consume 2 portions of salmon per week during pregnancy months 4–9. ECs were isolated from umbilical cord veins collected at birth and cultured. The cell surface expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) was assessed by flow cytometry after the culture of ECs in the presence and absence of bacterial LPS for 24 h. Cytokine and growth factor concentrations in culture supernatant fluid were measured by using a multiplex assay.

Results: LPS increased the expression of VCAM-1 and the production of several cytokines and growth factors. The level of ICAM-1 expression per cell [ie, the median fluorescence intensity (MFI)] was increased by LPS stimulation in the control group (16.9 ± 2.4 compared with 135.3 ± 20.2 ; $P < 0.001$) and to a lesser extent in the salmon group (14.1 ± 3.8 compared with 65.8 ± 22.4 ; $P = 0.037$). The ICAM-1 MFI in the salmon group after LPS stimulation was lower than in the control group ($P = 0.006$).

Conclusion: Increased dietary salmon intake in pregnancy dampens offspring EC activation, which implicates a role for n–3 LCPUFAs in the suppression of inflammatory processes in humans. This trial was registered at clinicaltrials.gov as NCT00801502. *Am J Clin Nutr* 2011;94:1418–25.

INTRODUCTION

CVD⁴ has become the leading cause of morbidity and mortality in developed countries. The most important contributor to CVD is atherosclerosis, which is a complex, chronic inflammatory process accompanied by the accumulation of lipids and fibrous material within the walls of the large arteries (1). Vascular inflammation is often involved in the early stages of atherosclerosis and CVD. Vascular inflammation can lead to the upregulation of endothelial CAM expression and proinflammatory cytokine production (1, 2). The activated endothelium expresses selectins that are required for adhesion of

leukocytes to the vessel wall. Leukocyte integrins become activated by chemokines and bind to endothelial ICAM and VCAM, which permits firmer adhesion and diapedesis (3–5). Adhesion molecule expression on ECs, together with the secretion of proinflammatory cytokines such as TNF- α and IL-6, growth factors including G-CSF and transforming growth factor- β , and chemokines such as IL-8, forms an important event in inflammatory responses, vascular injury, and atherogenesis (6–11).

Epidemiologic and intervention studies have indicated that n–3 LCPUFAs such as EPA (20:5n–3) and DHA (22:6n–3) reduce risk of cardiovascular morbidity and mortality (12–18). Part of this protective effect may involve reduced inflammation (4). However, whether dietary n–3 LCPUFAs target EC responses has not been established. In vitro studies have shown that EPA and DHA can decrease the expression of CAMs on cultured ECs and decrease the production of inflammatory cy-

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⁴ Abbreviations used: CAM, cell adhesion molecule; CVD, cardiovascular disease; EC, endothelial cell; FITC, fluorescein isothiocyanate; G-CSF, granulocyte colony stimulating factor; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; LCPUFA, long-chain PUFA; MFI, median fluorescence intensity; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; SiPS, Salmon in Pregnancy Study; VCAM, vascular cell adhesion molecule.

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tokines by these cells (8, 19–21). To our knowledge, there are no human studies that investigated whether dietary n–3 LCPUFAs affect the inflammatory response of ECs, including CAM expression. The SiPS (22) provided an opportunity to study human ECs after a dietary intervention that increased both the intake of n–3 LCPUFAs and n–3 LCPUFA status in the mother and newborn infant. The aim of this work was to assess whether salmon consumption, by providing an enhanced intake of n–3 LCPUFAs, twice a week during the second half of pregnancy affects the response of HUVECs to an inflammatory stimulus *ex vivo*; CAM expression and inflammatory mediator release in response to LPS were measured as outcomes. It was hypothesized that CAM expression and inflammatory mediator release would be lower in the salmon group than in the control group.

SUBJECTS AND METHODS

Study design and population

Samples used in this study were from a small subgroup of participants involved in the SiPS, which was a dietary intervention study in pregnant women that investigated the impact of maternal oily fish intake on health outcomes in pregnancy and early infancy (22). At 12 wk of pregnancy, women at risk of giving birth to an atopic baby were identified at antenatal clinics in Princess Anne Hospital, Southampton, United Kingdom, and recruited into the study. This study was approved by the Southampton and South West Hampshire Research Ethics Committee (07/Q1704/43) and was registered at clinicaltrials.gov as NCT00801502.

Recruited women were randomly assigned to one of 2 groups. Women in the control group ($n = 61$) were asked to continue their habitual diet, and women in the salmon group ($n = 62$) were asked to incorporate 2 portions of farmed salmon (150 g/portion) into their diet per week from study entry (week 20) until they gave birth. Farmed salmon for use in the SiPS was raised by using dietary ingredients selected to contain low amounts of contaminants. Each 150-g salmon portion contained (on average) 30.5 g protein, 16.4 g fat, 0.57 g EPA, 0.35 g docosapentaenoic acid, 1.16 g DHA, 3.56 g total n–3 PUFAs, 4.1 mg α -tocopherol, 1.6 mg γ -tocopherol, 6 μ g vitamin A, 14 μ g vitamin D₃, and 43 μ g selenium. Thus, 2 portions of salmon per week would typically provide 3.45 g EPA + DHA, 28 μ g vitamin D₃, and 86 μ g selenium. Contaminants contributed <12.5% of the Food and Agricultural Organization/WHO provisional tolerable weekly intake for dioxin and dioxin-like polychlorinated biphenyls, <11.5% for arsenic, <0.00000008% for cadmium, 0.0000025% for mercury, and <0.00000002% for lead.

At birth, umbilical cords were collected from all pregnancies. Some umbilical cords were used for immunohistochemical staining ($n = 14$ in the control group and $n = 22$ in the salmon group), whereas other umbilical cords ($n = 5$ in the control group and $n = 4$ in the salmon group) were used for isolation of primary HUVECs.

Immunohistochemical staining of umbilical cord vasculature

After birth, a segment of the umbilical cord was stored at 4°C in sterile Hanks salt solution. The vein and artery were isolated, placed in ice-cooled acetone that contained protease inhibitors,

processed in glycolmethacrylate resin as previously outlined (23), and stored at –20°C until staining. Sequential sections (2 μ m) were cut and stained with toluidine blue to assess the morphology. The specimens were stained by using monoclonal antibodies against human CD31 (Monosan), human VCAM-1 (Abcam), and human ICAM-1 (Invitrogen) and visualized by using 3-amino-9-ethylcarbazole according to a previously described protocol (23). Staining was scored by using the quality and intensity method as previously described (24). A rating from 0 to 5 was given for the quantity of staining, where 0 = no staining, 1 = patchy staining, and 5 = continuous staining. The same was done for the intensity of staining where 0 = no staining, 1 = very light staining, and 5 = very dark staining. Values of quantity and intensity were added to yield a total score. All scoring was done blind by one investigator (MAvDM).

EC isolation and culture

Chemicals were purchased from Sigma-Aldrich Co Ltd unless otherwise stated. HUVECs were isolated and cultured by adapting the method of Jaffe et al (25). After birth, a segment of the umbilical cord was stored at 4°C in sterile Hanks salt solution. Cords were processed in ≤ 24 –48 h after delivery. HUVECs were obtained by collagenase perfusion of the large vein. Briefly, the umbilical vein was cannulated at both ends, rinsed with PBS, and infused with 5 mL filter-sterilized 1% (wt:vol) collagenase type II (284 kU/mL in PBS) (prepared from *Clostridium histolyticum*; Gibco; Invitrogen) to digest the interior. The cord was placed in a container with sterile PBS and incubated in a water bath at 37°C for 15–20 min, and the cord vein was rinsed with ~ 30 mL sterile PBS to obtain the collagenase solution that contained ECs. The solution was centrifuged at $220 \times g$ for 10 min at room temperature and washed with cell culture medium that consisted of medium 199 supplemented with 30 U/mL sodium heparin (from porcine intestinal mucosa), L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μ g/mL), 50 μ g EC growth factor/mL, and 20% heat-inactivated fetal calf serum (PAA Laboratories). The cell pellet was resuspended in fresh medium and cultured in a gelatin-coated [0.1% gelatin (from bovine skin, type B)] tissue culture flask (Greiner Bio One). Cells were grown to confluency in ~ 10 d under standard cell culture conditions (humidified atmosphere, 5% CO₂, 37°C) before experimental use. Cultured cells were identified as vascular ECs by their characteristic cobblestone morphology at confluence. In separate experiments, cells isolated and cultured under the same conditions were double-stained with FITC conjugated mouse anti-human CD31 (platelet EC adhesion molecule) and phycoerythrin-conjugated mouse anti-human CD144 (vascular endothelial cadherin) to confirm that they were HUVECs. Both antibodies were from R&D Systems. After antibody incubation, cells were washed with BD Cell Wash ($140 \times g$, 7 min) and fixed with BD Cell Fix (BD Biosciences), and 10,000 gated events per sample were acquired with a FACSCalibur flow cytometer (Becton Dickinson). Typically, >95% of cells stained positively for both these markers, which confirmed the cells as HUVECs.

Cytokine secretion and CAM expression

For experimental use, confluent HUVEC were harvested, plated, and grown to semiconfluence in 4 d before stimulation



with 1 $\mu\text{g/mL}$ LPS (from *Escherichia coli*) for 24 h. HUVECs used for these cultures were between passages 1 and 3 (passages 1, 1, 2, 2, and 3 in the control group and passages 1, 2, 3, and 3 in the salmon group; not significantly different). Twenty-four hours after stimulation with LPS, supernatant fluid was collected for assessment of inflammatory mediator concentrations by using a multiplex assay (Bio-Plex Pro, 27-plex M50-0KCAF0Y; Bio-Rad Laboratories). Mediators measured with their limits of detection (all pg/mL) were as follows: monocyte chemotactic protein 1 (1.81), basic fibroblast growth factor (1.21), eotaxin (2.00), G-CSF (1.31), granulocyte macrophage colony stimulating factor (0.74), interferon- γ (1.61), IL-1 β (2.67), IL-1 receptor antagonist (4.97), IL-2 (1.42), IL-4 (0.32), IL-5 (2.51), IL-6 (2.01), IL-7 (2.43), IL-8 (1.77), IL-9 (1.43), IL-10 (1.78), IL-12(p70) (2.55), IL-13 (2.68), IL-15 (1.82), IL-17 (1.87), interferon- γ induced protein 10 (2.38), macrophage inflammatory protein 1 α (1.42), macrophage inflammatory protein 1 β (1.41), PDGF-BB (2.04), regulated on activation, normal T cell expressed and secreted (1.59), TNF- α (6.42), and vascular endothelial growth factor (2.24). ECs were detached and stained for the EC marker CD31 to confirm their phenotype. The cell surface expression of ICAM-1 (CD54) and VCAM-1 (CD106) was assessed by flow cytometry. After washing with 10% fetal calf serum in PBS, cells (1×10^5 to 5×10^5) were treated at 4°C for 30 min with FITC-conjugated mouse anti-human CD31, FITC-conjugated mouse anti-human CD54, or FITC-conjugated mouse anti-human CD106. FITC-conjugated isotype-matched negative controls (IgG₁ and IgG_{2a}) were used. All antibodies were from R&D Systems. After antibody incubation, cells were washed with BD Cell Wash (140 \times g, 7 min) and fixed with BD Cell Fix (BD Biosciences), and 10,000 gated events per sample were acquired with a FACSCalibur flow cytometer (Becton Dickinson). Data on CAM expression were analyzed with CellQuest Pro software. HUVEC with a forward scatter >200 were included for ICAM-1 and VCAM-1 analysis. Both the percentage of positive cells and the MFI were obtained to analyze adhesion molecule expression. All positive staining was corrected for isotype background levels.

Statistical analysis

Graphs were made with GraphPad Prism software (GraphPad Prism for Windows, version 4; GraphPad Software Inc). Adhesion molecule staining on umbilical cord vasculature determined by immunohistochemistry was compared between groups by using the unpaired Student's *t* test. A 2-way mixed-model ANOVA with the subject as a random factor was used to assess the effects of diet (control and salmon) and LPS (in the absence and presence of LPS stimulation) on CAM expression, inflammatory mediator production, and growth-factor production by cultured HUVECs. The hypothesis that the effect of LPS would differ between the salmon and control groups was tested by including an interaction term between LPS and diet in the model. Data in the absence and presence of LPS stimulation within a dietary group were tested by paired comparisons, whereas comparisons between dietary groups (control compared with salmon) were tested unpaired. Statistical Analysis Systems statistical software package version 9.1.3 (SAS Institute) was used for these analyses. Pearson correlation coefficients were calculated with GraphPad Prism software (GraphPad Prism

for Windows, version 4; GraphPad Software Inc). In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Adhesion molecule expression on umbilical cord vasculature

There were no differences between groups in the quality and intensity score for either VCAM-1 (mean \pm SEM: 0.9 ± 0.5 in the control group compared with 1.7 ± 0.5 in the salmon group) or ICAM-1 (mean \pm SEM: 3.5 ± 0.8 in the control group compared with 3.2 ± 0.5 in the salmon group).

HUVEC culture and phenotype

HUVECs were isolated by using collagenase perfusion of the umbilical vein according to Jaffe et al (25) and cultured in medium 199. For experimental use, cells were grown to semi-confluence and stained for the EC marker CD31 by using flow cytometry. The morphology of cultured HUVECs is shown in **Figure 1A**, and the cell population as a forward-scatter plot compared with a side-scatter plot is shown in **Figure 1B**. The phenotype of every culture was confirmed by staining for CD31 and corrected for isotype background (**Figure 1, C and D**). The CD31 expression for HUVEC cultures varied between 84.1% and 96.7% of cells (mean \pm SEM: $89.6 \pm 1.9\%$).

Effect of salmon during pregnancy on ICAM-1 and VCAM-1 expression on cultured HUVECs

After growing to semiconfluence, HUVECs were stimulated for 24 h with the inflammatory stimulus LPS. Adhesion molecule expression on the cell surface in response to LPS stimulation was analyzed by flow cytometry. Representative histograms for ICAM-1 and VCAM-1 expression on HUVECs from control and salmon groups in the presence or absence of LPS stimulation are shown in **Figure 2A**.

The expression of ICAM-1 and VCAM-1 in cultured HUVECs is represented in **Figure 2, B–E**. In **Figure 2, B and C**, the percentages of cells positively stained for the respective adhesion molecules are depicted, whereas the level of expression (MFI) is shown in **Figure 2, D and E**.

There was a significant effect of LPS ($P < 0.001$), but not of diet ($P = 0.147$), on the percentage of HUVECs that expressed ICAM-1, and there was no diet \times LPS interaction ($P = 0.768$) (**Figure 2B**). About 10–20% of HUVECs expressed ICAM-1 in the basal state, with no differences between groups ($17.8 \pm 2.1\%$ in the control group and $11.6 \pm 2.0\%$ in the salmon group). The percentage of ICAM-1–positive cells was significantly increased by LPS stimulation ($73.6 \pm 2.6\%$ in the control group and $65.4 \pm 7.4\%$ in the salmon group) (**Figure 2B**). There was a significant effect of LPS ($P < 0.001$) and a trend toward a significant effect of diet ($P = 0.069$) on the level of expression of ICAM-1 on HUVECs, and there was a significant diet \times LPS interaction ($P = 0.043$) (**Figure 2D**). The level of ICAM-1 expression per cell (ie, MFI) was significantly increased by LPS stimulation in the control group (16.9 ± 2.4 compared with 135.3 ± 20.2 ; $P < 0.001$) and to a lesser extent in the salmon group (14.1 ± 3.8 compared with 65.8 ± 22.4 ; $P = 0.037$)



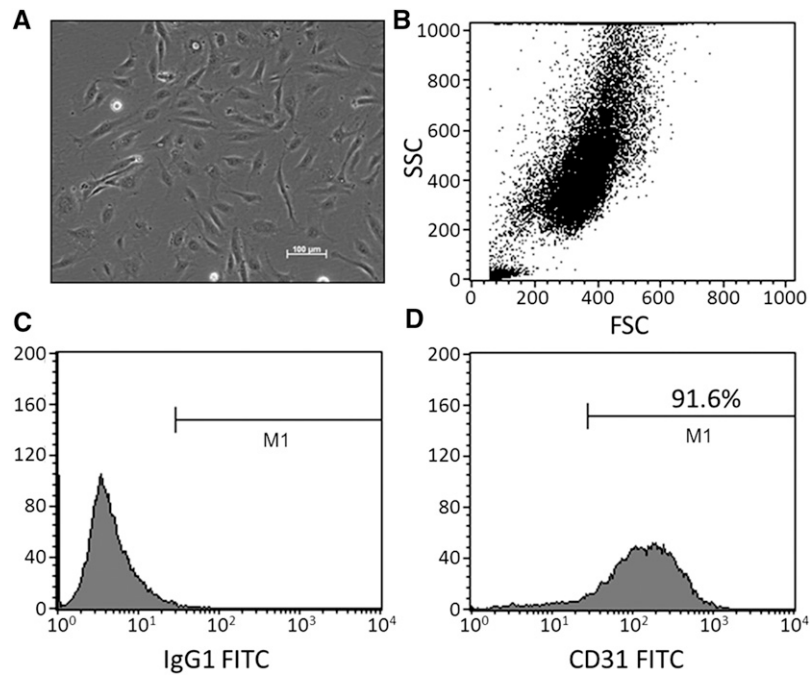


FIGURE 1. A. Morphology of semiconfluent HUVECs. B. FSC compared with SSC plot showing a distinct population of HUVECs present. C. Isotype control (marker M1 <math><1\%</math> of events)–staining of HUVECs. D. Cultured cells were positive for CD31 (in this case, 91.6% of cells were CD31⁺), which confirmed their phenotype. FITC, fluorescein isothiocyanate; FSC, forward scatter; HUVEC, human umbilical vein endothelial cell; SSC, side scatter.

(Figure 2D). The MFI in the salmon group in the LPS-stimulated state was significantly lower than in the control group ($P = 0.006$).

There was a significant effect of LPS ($P = 0.005$), but not of diet ($P = 0.138$), on the percentage of HUVECs that expressed VCAM-1, and there was no diet \times LPS interaction ($P = 0.139$) (Figure 2C). The percentage of HUVECs that expressed VCAM-1 was low under basal conditions ($1.2 \pm 0.2\%$ in the control group and $0.9 \pm 0.3\%$ in the salmon group), and this percentage was increased by LPS stimulation ($21.4 \pm 4.6\%$ in the control group and $9.3 \pm 5.7\%$ in the salmon group) (Figure 2C). There was a significant effect of LPS ($P = 0.006$), but not of diet ($P = 0.188$), on the level of expression of VCAM-1 on HUVECs, and there was a trend toward a significant diet \times LPS interaction ($P = 0.088$) (Figure 2E). The level of VCAM-1 expression (ie, MFI) was increased by LPS stimulation (Figure 2E).

Effect of salmon during pregnancy on inflammatory mediator secretion by HUVECs

In addition to CAM surface expression, the effect of dietary salmon intake on inflammatory mediator secretion by HUVECs was assessed. Data for the proinflammatory cytokines IL-6 and TNF- α are shown in Figure 3, A and B, respectively.

There was a significant effect of LPS ($P = 0.004$), but not of diet ($P = 0.229$), on IL-6 secretion, and there was no diet \times LPS interaction ($P = 0.300$) (Figure 3A). The IL-6 secretion by HUVECs increased on stimulation with LPS (724.5 ± 125.05 compared with 3969 ± 1044 in the control group; 436.8 ± 170.2 compared with 2299 ± 741.8 in the salmon group) (Figure 3A). There was a significant effect of LPS ($P < 0.001$), but not of diet ($P = 0.229$), on TNF- α secretion, and there was no diet \times LPS interaction ($P = 0.716$) (Figure 3B). TNF- α secretion by HUVECs increased on stimulation with LPS (166.4 ± 23.4 compared with 303.3 ± 25.8 in the control group; 108.1 ± 46.7

compared with 261.6 ± 44.4 in the salmon group) (Figure 3B). Concentrations of the other inflammatory mediators measured were not different between control and salmon groups although several were induced by LPS stimulation (data not shown).

Growth factors are known to exert protective effects on ECs. There was a significant effect of LPS ($P = 0.008$) and trends toward an effect of diet ($P = 0.066$) and toward a diet \times LPS interaction ($P = 0.066$) on G-CSF secretion (Figure 3C). G-CSF secretion by HUVECs increased on stimulation with LPS (4.2 ± 1.1 compared with 658.6 ± 121.7 in the control group; 87.8 ± 46.8 compared with 2680 ± 1044 in the salmon group) (Figure 3C). There was a significant effect of LPS ($P < 0.001$), but not of diet ($P = 0.200$), on PDGF-BB secretion (Figure 3D). There was no diet \times LPS interaction ($P = 0.309$). PDGF-BB secretion by HUVECs increased on stimulation with LPS (297.5 ± 49.3 compared with 489.9 ± 61.2 in the control group; 485.3 ± 165.4 compared with 772.2 ± 197.0 in the salmon group) (Figure 3D).

The expression (MFI) of ICAM-1 and VCAM-1 after LPS stimulation was positively correlated ($r = 0.9315$, $P < 0.001$; Figure 4A). Both ICAM-1 expression and VCAM-1 expression were positively correlated with IL-6 secretion from HUVECs (Figure 4, B and C; $P < 0.001$ and $P = 0.013$, respectively), whereas TNF- α was not correlated with CAM expression (data not shown). PDGF-BB showed a negative correlation with VCAM-1 after stimulation (Figure 4D; $P = 0.044$), whereas G-CSF showed a trend toward negative correlation with VCAM-1 ($P = 0.061$; data not shown). In addition, PDGF-BB tended to correlate negatively with ICAM-1 expression ($P = 0.065$; data not shown).

DISCUSSION

Study of the effects of n-3 LCPUFAs on EC responsiveness is important because such responses are involved in CVD and



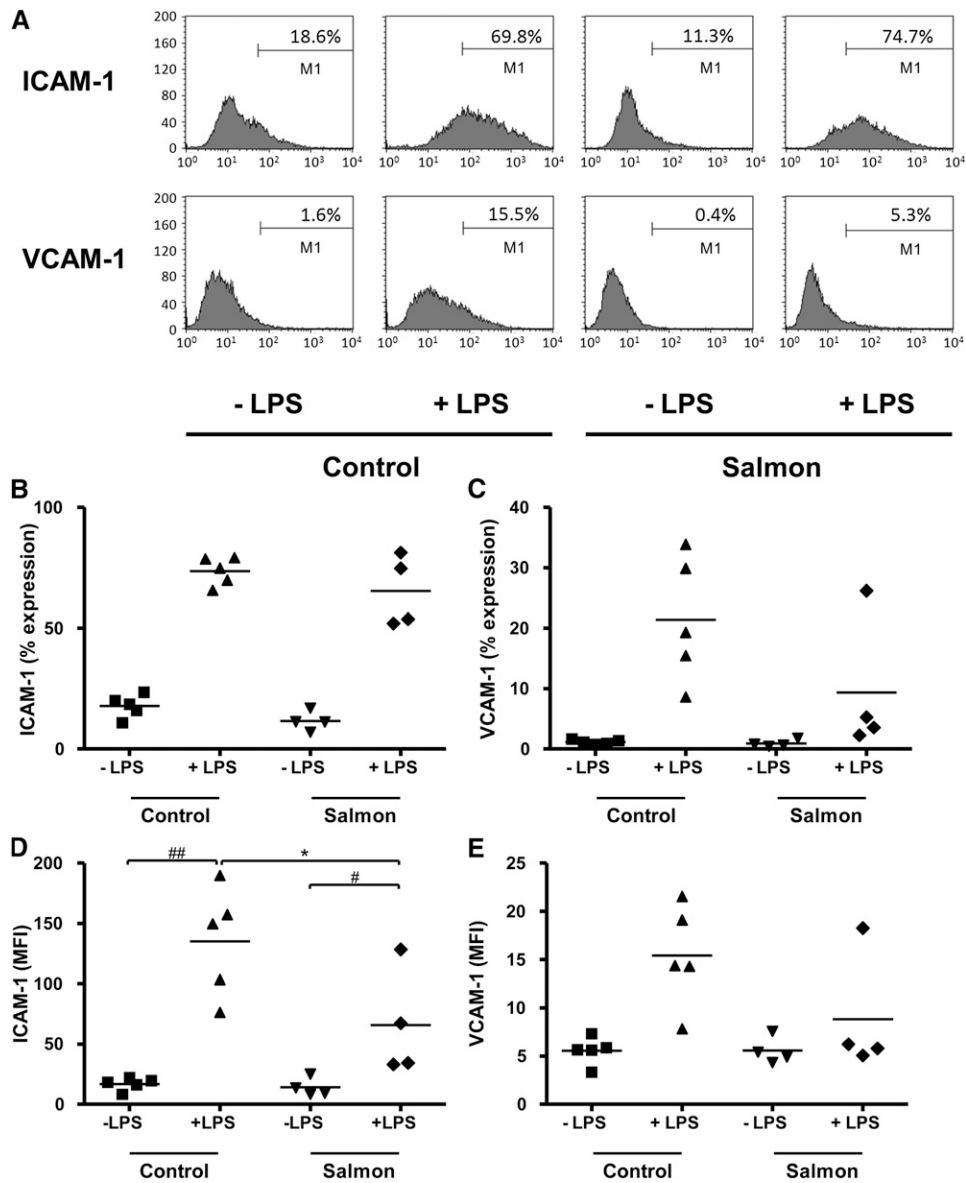


FIGURE 2. ICAM-1 and VCAM-1 for HUVECs isolated from women who consumed the control ($n = 5$) or salmon ($n = 4$) diet in the presence (+ LPS) or absence (- LPS) of 24 h of LPS stimulation. A. Histogram plots for an individual cell preparation showing the marker M1 set by using the relevant isotype control. Adhesion molecule expression as a percentage of positive cells (B and C) and MFI (D and E). B–E. Data for individual volunteers are shown. A 2-way mixed-model ANOVA with the subject as a random factor was used to assess the effects of diet (control and salmon) and LPS (+ LPS, - LPS). For the percentage of ICAM-1–positive cells, there was a significant effect of LPS ($P < 0.001$) but not diet ($P = 0.147$) and no diet \times LPS interaction ($P = 0.768$). For the percentage of VCAM-1–positive cells, there was a significant effect of LPS ($P = 0.005$) but not diet ($P = 0.138$) and no diet \times LPS interaction ($P = 0.139$). For ICAM-1 MFI, there was a significant effect of LPS ($P < 0.001$), a trend toward a significant effect of diet ($P = 0.069$), and a significant diet \times LPS interaction ($P = 0.043$). For VCAM-1 MFI, there was a significant effect of LPS ($P = 0.006$) but not diet ($P = 0.188$) and a trend toward a significant diet \times LPS interaction ($P = 0.088$). * $P < 0.01$ (control compared with salmon diets); # $P < 0.05$, ## $P < 0.001$ (- LPS compared with + LPS). HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; MFI, median fluorescence intensity; VCAM, vascular cell adhesion molecule.

other inflammatory conditions (1). Cell culture studies have identified that EPA and DHA can decrease the expression of ICAM-1 and VCAM-1 on ECs (8, 19, 21), adhesive interactions between ECs and leukocytes (8, 21), and the production of IL-6 by ECs (8, 20). Similarly, EPA and DHA decreased ICAM-1 expression by cultured monocytes (26). However, few studies have shown an effect of dietary $n-3$ LCPUFAs on adhesion molecule expression. Feeding fish oil to rodents decreased the expression of several adhesion molecules on lymphocytes (27), macrophages (28), and dendritic cells (29), whereas a fish-oil

supplementation study in humans showed decreased ICAM-1 expression on interferon- γ -stimulated monocytes (30). To our knowledge, no human studies have investigated the effect of dietary intervention on adhesion molecule expression or other inflammatory responses of ECs. The current, highly novel study used unique samples obtained from the SiPS to identify the effects of dietary $n-3$ LCPUFAs on adhesion molecule expression and mediator secretion by human ECs. It was hypothesized that EC responses would be lower in the group whose mothers received an increased dietary intake of $n-3$ LCPUFAs

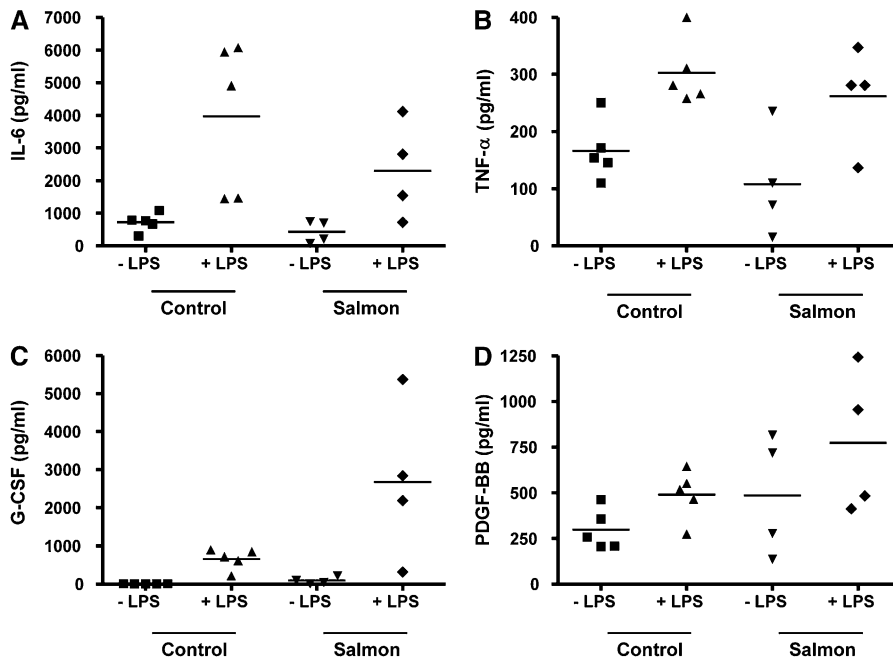


FIGURE 3. Secretion of cytokines IL-6 (A) and TNF- α (B) and growth factors G-CSF (C) and PDGF-BB (D) by HUVECs isolated from women who consumed the control ($n = 5$) or salmon ($n = 4$) diet in the presence (+ LPS) or absence (- LPS) of 24 h of LPS stimulation. Data for individual volunteers are shown. A 2-way mixed model ANOVA with the subject as a random factor was used to assess the effects of diet (control and salmon diets) and LPS (+ LPS, - LPS). For IL-6, there was a significant effect of LPS ($P = 0.004$) but not diet ($P = 0.229$) and no diet \times LPS interaction ($P = 0.300$). For TNF- α there was a significant effect of LPS ($P < 0.001$) but not diet ($P = 0.289$) and no diet \times LPS interaction ($P = 0.716$). For G-CSF, there was a significant effect of LPS ($P = 0.008$), a trend toward a significant effect of diet ($P = 0.066$), and a trend toward a significant diet \times LPS interaction ($P = 0.066$). For PDGF-BB, there was a significant effect of LPS ($P < 0.001$) but not diet ($P = 0.200$) and no diet \times LPS interaction ($P = 0.309$). G-CSF, granulocyte colony stimulating factor; HUVEC, human umbilical vein endothelial cell; PDGF, platelet-derived growth factor.

(ie, the salmon group). In accordance with this hypothesis, an increased dietary salmon intake was shown to result in the lower EC surface expression of ICAM-1 after ex vivo LPS stimulation;

the surface expression of VCAM-1 also tended to be lower in the salmon group after LPS exposure. In contrast, the production of G-CSF in response to LPS tended to be higher by ECs in the

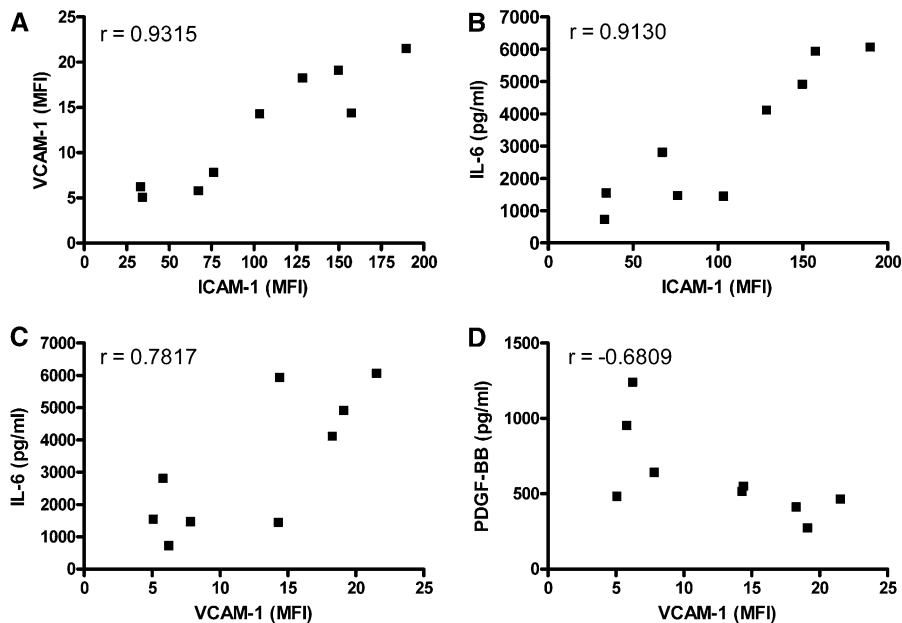


FIGURE 4. Correlations between CAM expression as MFI and mediator secretion after LPS stimulation. There were positive correlations between ICAM-1 expression and VCAM-1 expression (A; $r = 0.932$; $P = 0.0003$), ICAM-1 expression and IL-6 secretion (B; $r = 0.913$; $P = 0.0006$), and VCAM-1 expression and IL-6 secretion (C; $r = 0.782$; $P = 0.013$). There was a negative correlation between VCAM-1 expression and PDGF-BB secretion (D; $r = -0.681$; $P = 0.044$). CAM, cell adhesion molecule; ICAM, intercellular adhesion molecule; MFI, median fluorescence intensity; PDGF, platelet-derived growth factor; VCAM, vascular cell adhesion molecule.

salmon group. The production of a number of other mediators was not significantly different between the salmon and control groups. Thus, there was some selectivity or specificity to the antiinflammatory effects of dietary n-3 LCPUFAs, which perhaps suggested an effect on a single signaling pathway.

The increased intake of oily fish from week 20 of pregnancy did not affect the expression of ICAM-1 or VCAM-1 on cord tissue or on resting (ie, unstimulated) HUVECs but decreased the endothelial activation in response to LPS as indicated by a lower ICAM-1 expression. The lack of a group difference in CAM expression on cord tissue and on unstimulated HUVECs suggested that n-3 LCPUFAs did not affect basal expression levels of these molecules. This finding is in accordance with in vitro studies of n-3 LCPUFAs with ECs. For example, the incubation of unstimulated HUVECs with EPA or DHA had no effect on messenger RNA for ICAM-1 or VCAM-1 (19), whereas DHA had no effect on the basal ICAM-1 expression on HUVECs (21).

Stimulation with LPS for 24 h resulted in an increase in both the percentage of cells that expressed ICAM-1 and the level of expression (MFI); however, in the salmon group, the level of expression was ~50% lower than in the control group. The salmon diet was also associated with a tendency toward a lower VCAM-1 MFI compared with that in the control group. Both ICAM-1 and VCAM-1 are involved in the firm adhesion of leukocytes to the endothelium (9). Thus, lower CAM expression on ECs could result in decreased adherence and transmigration of leukocytes into vascular tissue, which is a key event in atherosclerosis. These data were supported by studies in which HUVECs were directly exposed to LCPUFAs during in vitro cultures and then stimulated with LPS or an inflammatory cytokine. De Caterina et al (8) showed that DHA, but not EPA, decreased the endothelial expression of ICAM-1 and VCAM-1 in a dose-dependent manner in human saphenous vein ECs after incubation with a proinflammatory stimulus. Weber et al (21) showed that DHA, but not EPA, dose-dependently decreased TNF- α -induced VCAM-1 expression on HUVECs and also lowered subsequent monocytic cell adhesion. Collie-Duguid et al (19) showed that messenger RNA amounts for both ICAM-1 and VCAM-1 in IL-1-activated HUVECs were lower in the presence of either EPA or DHA. These earlier studies were all conducted in vitro and involved the direct exposure of ECs to pure n-3 LCPUFAs in a cell culture setting. As far as we are aware, the current study is the first dietary intervention study that investigated the possible effects of salmon rich in n-3 LCPUFAs on EC activation in humans.

In the current study, dietary n-3 LCPUFAs have been shown to exert similar effects to those seen for individual n-3 LCPUFAs in in vitro experiments (8, 19–21). That effects of the salmon diet were seen, despite the HUVECs being cultured for 2 wk in a standard culture medium before activation, suggests a possible programming effect of the salmon diet with regard to endothelial responsiveness to an inflammatory stimulus. In utero exposure to n-3 LCPUFAs could modulate gene expression via epigenetic changes (eg, DNA methylation or histone modifications) and lead to an altered susceptibility to atherosclerosis (31). DNA hypermethylation of specific genes is important in atherosclerosis. n-3 LCPUFAs are PPAR activators, and PPAR can alter epigenetic processes (32, 33). Several PPAR activators have been shown to reduce the induced expression of CAM (34). Dietary n-3 LCPUFAs are associated with decreased plasma homocysteine concentrations (35, 36). Homocysteine upregu-

lates the expression of adhesion molecules such as ICAM-1 on ECs, and this expression is reduced by PPAR agonists (37, 38). In addition, a number of chromatin modifying enzymes have PPAR-response elements in their promoters and, thus, can be regulated via n-3 LCPUFAs; these enzymes contribute to the regulation of CAM expression (39–41). Furthermore, n-3 LCPUFAs could affect CAM expression via a reduced activation of nuclear transcription factor κ B (2, 21). Early exposure to salmon-derived n-3 LCPUFAs may contribute to a subtle but persistent effect on the development of CVD later in life.

The effect of salmon on the production of soluble mediators by ECs was also studied. The secretion of proinflammatory mediators by ECs is important in the inflammatory response and contributes, together with adhesion molecules, to the early phases of atherogenesis. n-3 LCPUFAs, in particular DHA, have been shown to decrease IL-6 expression in human EC after stimulation (8, 20). The salmon diet had little effect on the cytokine secretion by HUVECs in response to LPS stimulation.

Some growth factors are known to play an antiinflammatory role and exert protective effects on ECs (42, 43). The salmon diet tended to increase the production of G-CSF by LPS-stimulated ECs. Endogenous G-CSF is known to downregulate IL-1-induced ICAM-1 expression and granulocyte adhesion on human ECs (42). The concentration of G-CSF in HUVEC supernatant fluid of the salmon group on LPS stimulation was 4 times higher than in the corresponding control group, which may have been related to the antiinflammatory effects of the salmon diet. PDGF-BB has been shown to promote the structural integrity of the vessel wall and can stimulate the secretion of other growth factors. PDGF-BB has been shown to improve survival in severe sepsis via an effect on vessel wall homeostasis and a tissue healing capacity (43). LPS stimulation led to an increase in PDGF-BB production, but the salmon diet did not significantly affect this response. However, PDGF-BB was negatively associated with VCAM-1 expression and tended to be negatively correlated with ICAM-1 expression after stimulation, which suggested a possible contribution of PDGF-BB in the control of LPS-induced VCAM-1 and ICAM-1 expression.

In conclusion, the increased intake of salmon, which provided n-3 LCPUFAs, from week 20 of pregnancy was associated with a lower responsiveness of offspring umbilical vein ECs to LPS with regard to the upregulation of ICAM-1, and there was a trend toward a greater responsiveness with regard to the upregulation of G-CSF compared with in controls. These results suggested an antiinflammatory effect on human ECs through dietary n-3 LCPUFAs, which is an effect that, to our knowledge, has not been shown previously. If such an effect was a generalizable action of n-3 LCPUFAs on ECs at all sites of the vasculature, then this action would be suggestive of an important mechanism by which these fatty acids could lower cardiovascular risk.

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