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Breast cancer risk and erythrocyte compositions of n-3 highly unsaturated fatty acids in Japanese

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Dietary intake of fish rich in n-3 highly unsaturated fatty acids (HUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been proposed to decrease cancer risk. In contrast to results from laboratory studies, however, protective effects for breast cancer have proved equivocal in epidemiological studies. In the present case-control study, we examined associations between breast cancer risk and fatty acid compositions in erythrocyte membranes as biomarkers for those intakes. Dietary information and blood samples were collected from 103 incident breast cancer cases and 309 non-cancer controls (matched by age and season) and erythrocyte fatty acids were measured using accelerated solvent extraction and gas-liquid chromatography. Dietary intake of n-3 HUFAs demonstrated a negative association with risk (the highest to the lowest tertile, odds ratio (OR), 0.51; 95% confidence interval (CI), 0.27–0.98; $p_{trend} < 0.05$), but there was no association with those of saturated fatty acids (SFAs) and meat. Moreover, risk was inversely associated with erythrocyte compositions of EPA (OR, 0.27; 95% CI, 0.14–0.53; $p_{trend} < 0.0001$), DHA (OR, 0.06; 95% CI, 0.02–0.16; $p_{trend} < 0.0001$) and n-3 HUFAs (OR, 0.11; 95% CI, 0.05–0.24; $p_{trend} < 0.0001$), and positively with that of SFAs (OR, 12.29; 95% CI, 4.94–30.57; $p_{trend} < 0.0001$) and the ratio of SFAs/n-3 HUFAs (OR, 14.65; 95% CI, 5.67–37.82; $p_{trend} < 0.0001$). In conclusion, we showed that erythrocyte compositions of specific fatty acids derived from fish intake, as biomarkers, are associated with lower risk of breast cancer, but further studies are needed to investigate mechanisms linked to the etiology.

Key words: breast cancer; fish intake; n-3 polyunsaturated fatty acids; biomarkers; erythrocytes

Accumulated evidence from laboratory studies, such as animal experiments and cell culture studies, indicate that n-3 polyunsaturated fatty acids (PUFAs) inhibit the promotion and progression stages of tumor development.[1–3] n-3 PUFAs in our diets include both α-linolenic acid (18:3n-3) as a vegetable oil and n-3 highly unsaturated fatty acids (HUFAs), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), generally of marine origin. Especially, ecological studies have suggested that higher intake of fish rich in n-3 HUFAs is correlated with a lower cancer incidence in several sites.[4,5] Regarding suppression of carcinogenesis, n-3 HUFAs have been proposed to play critical roles in: (*i*) inhibition for biosynthesis of eicosanoids (*e.g.*, prostaglandin E_2) *via* the arachidonic acid (AA, 20:4n-6) cascade[1,6]; (*ii*) modulation of gene expression or the activities of signal transduction molecules involved in the control of cell growth, differentiation, apoptosis, angiogenesis and metastasis[7–9]; (*iii*) estrogen metabolism[1]; (*iv*) production of free radicals and reactive oxygen species;[7,8,10,11] (*v*) modulation of insulin sensitivity[1,12] and (*vi*) membrane fluidity.[1] For the AA cascade, such potential activities of EPA and DHA are estimated to be approximately 5 times higher than that of 18:3n-3.[6] EPA-derived eicosanoids also decrease the formation of AA-derived eicosanoids, which is linked to inflammation, tumorigenesis, angiogenesis, cell proliferation and apoptosis induction.[13] Anti-tumor effects have been suggested since EPA and DHA play key roles in inhibition of cell proliferation and induction of apoptosis, respectively.[1,2,9,10]

In contrast to the clearly positive findings of laboratory studies, influence of fish or n-3 HUFAs on breast cancer risk in many case-control studies has provided conflicting evidence, and no association was found in both prospective cohort studies and randomized controlled trials.[14–17] Several reasons for the discrepancy have been debated as follows: (*i*) higher n-3 HUFAs levels of exposure used in laboratory studies; (*ii*) measurement errors in epidemiological studies; (*iii*) other methodological issues; and (*iv*) the general difficulty in extrapolating results of laboratory to human studies.[18] In Japanese, however, dietary intake of fish or n-3 HUFAs has been demonstrated to have an inverse association with breast cancer risk in both large-scale case-control and prospective cohort studies.[19–21] Although dietary intake of fish in Japanese continues to be the highest in the world, the incidence rate of mammary cancer has markedly increased in recent years, linked to adoption of a Westernized diet with high consumption of meat, animal fat and/or saturated fatty acids (SFAs).[22,23]

PUFAs in biomaterials are useful as biomarkers for assessing dietary intakes of fat, fatty acids and fish, because they are not biosynthesized *in vivo*.[24,25] In 1 out of 2 cohort studies, breast cancer risk was inversely associated with compositions of n-3 HUFAs, especially DHA, in membrane phospholipids of erythrocytes (120 days half-life) in Italian women.[26] In the other study, no relation was found in Swedish postmenopausal women.[27] Estimated die-

Abbreviations: AA, arachidonic acid; ACCH, Aichi Cancer Center Hospital; CI, confidence interval; EPA, eicosapentaenoic acid; CV, coefficients of variation; DHA, docosahexaenoic acid; HUFAs, highly unsaturated fatty acids; MUFAs, monounsaturated fatty acids; OR, odds ratio; PUFAs, polyunsaturated fatty acids; SQFFQ, semi-quantitative food frequency questionnaire; SFAs, saturated fatty acids.

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tary intake of fish is high in Japanese; the percentage of energy from fish in a period of 1961–1994 was 6.21%, compared with 0.90% in Italians and 2.20% in Swedes,[22] so that Japanese are a particularly appropriate population to examine the role of n-3 HUFAs derived from fish intake. Correlation coefficients (r) between dietary intakes of EPA and DHA and the corresponding fatty acid compositions in erythrocyte membranes have been reported as 0.20 for EPA in Japan,[28] 0.48 for DHA in Italy,[26] and 0.23–0.27 for EPA and DHA in Swedes.[27] Recently, we have developed a new analytical method for measuring fatty acids in biomaterials using an automatic solvent extractor and gas-liquid chromatography, with confirmed high precision and accuracy, making it feasible to use small volume multi-samples, routinely, rapidly and cheaply.[29] To examine whether associations exist between breast cancer risk and erythrocyte fatty acids, therefore, we conducted the present case-control study in a Japanese population.

Material and methods

Within the framework of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC),[30] subjects (20–79 years) in the present study were recruited from December 2002 to May 2005. The study design has been elsewhere described,[30] and this study was executed in a series within the Aichi Fatty Acid (AiFat) Research project to clarify associations between a large number of blood parameters and cancers in several sites.[28,29] Briefly, all first-visit outpatients (n = 18,103), including all cancer cases (n = 3,972), are asked to fill out a self-administrated questionnaire regarding their lifestyle and to provide 7 mL blood samples. All subjects are provided with an explanatory document and requested to give their written informed consent for participation in the study, which was approved by the Ethics Committee of the Aichi Cancer Center. Dietary information from the questionnaire was systematically collected and checked by trained interviewers, and completed by 97.5% of 15,650 eligible subjects. Of 6,464 subjects (42.4%) provided blood samples. The participation rates for all cancer cases, breast cancer cases and controls were 43.6%, 42.7% and 41.9%, in that order. All subjects for the present study were Japanese, living in and around Aichi Prefecture, central Japan.

Case and control subjects

A total of 103 newly diagnosed (incident) cases (including 4 recurrent cases) completed the questionnaire and donated blood samples during the study period, and were histologically diagnosed as having breast cancers at Aichi Cancer Center Hospital (ACCH). Primary breast cancer of the 4 recurrent cases was diagnosed within 10 years. The cases were confirmed using hospital-based cancer registries. The control subjects were randomly selected from first-visit outpatients who visited ACCH at the same period, and were confirmed to have no cancer or any prior history of cancer according to a questionnaire. All cases and controls were also checked for current and/or previous history of cancer based on the cancer registry system in Aichi Prefecture. Subjects with or having a history of the following current or past diseases were excluded; hepatitis (3.2% of all subjects), liver cirrhosis (1.9%), chronic nephritis (2.3%), diabetes (5.4%), stroke (1.1%), ovary resection (3.7%) and uterus resection (4.9%). Finally, 309 controls were individually matched for age ($\pm$5 years) and season of sample collection to cases with a 1: 3 case-control ratio, considering seasonal differences in biomarkers for dietary intakes of fish, fat and fatty acids.[28,31] Approximately 57.9% of them had no current or past diseases according to the questionnaire, and others had the following current or past diseases; tuberculosis (1.6%), gastric ulcer (4.2%), duodenal ulcer (3.9), appendicitis (14.9%), hemorrhoids (10.4%), asthma (5.5%), hypertension (8.4%), angina (1.0%) and acute pneumonia (1.2%). Most of control subjects visited for their health check or cancer screening at ACCH. Our previous study demonstrated that it is feasible to use non-cancer outpatients at ACCH as controls in epidemiological studies because their general lifestyles are accordant with those of general population randomly selected from the electoral roll in Nagoya City, Aichi Prefecture.[32]

Questionnaire

The questionnaire covered height, weight, dietary habits, habitual exercise, drinking habit, smoking status and thorough medical information such as family history of cancer and current and prevalent history of diseases. A semi-quantitative food frequency questionnaire (SQFFQ) with 47 food items was developed,[33,34] and used for assessing the average daily intakes of various foods and nutrients. Compared with 3 days weighed diet records, an acceptable relative validity of the SQFFQ for their consumption has been demonstrated, and the Spearman's correlation coefficients for fatty acids ranged from 0.16 (PUFAs) to 0.34 (SFAs) for women, respectively.[35] Dietary intake of n-3 HUFAs based on the SQFFQ, moreover, has been demonstrated to have significant correlations with the corresponding fatty acid concentrations (mmol/L) in plasma, assessed as biomarkers,[36] with coefficients of 0.26 for n-3 HUFAs and 0.37 for the ratio of n-6 PUFAs/n-3 PUFAs in women. As with the general population, therefore, it appeared appropriate to use the SQFFQ for the study subjects.

The SQFFQ inquired about habitual dietary intake during the latest 1 year, and 8 category frequencies of 47 foods/food groups were divided as follows; never or seldom, 1–3 times/month, 1–2 times/week, 3–4 times/week, 5–6 times/week, once/day, twice/day and 3 times or more/day. For the control and case subjects, we asked for information about their lifestyle at the study enrolment and before the onset of disease, respectively. Dietary intakes of fat and fatty acids were computed by multiplying the standard portion size (in grams), frequency of consumption, and the content (per gram) of fat or each fatty acid in foods as listed in the Standard Tables of Food Consumption and the Follow-up version.[37,38] Dietary intakes (g/day) of individual fatty acids were summed up and categorized as follows; SFAs, monounsaturated fatty acids (MUFAs), n-6 PUFAs, n-3 PUFAs and n-3 HUFAs. Along with fat and energy intake, dietary intakes (g/day) of soy and the products, meat, total seafood, fish, other seafood, green-yellow vegetables and other vegetables were also calculated. Other seafood was defined as the sum of other fish (*i.e.*, bone-edible small fish rich in calcium such as smelt and dried whitebait), shellfish (*e.g.*, short-necked clam, corbicula and oyster), canned tuna, crustacean/mollusk (*e.g.*, cuttlefish, squid, octopus, shrimp and crab), fish past products. We summed fish and other seafood up as total seafood rich in n-3 HUFAs. Dietary intakes of fatty acids and foods were adjusted for total energy intake of each person and tertile cut-points (g/1,000 kcal) in control subjects were used to designate low, moderate, and high intakes. Likewise, non-energy adjusted dietary intakes (g/day) were also calculated. Lifestyle factors were also classified into 3 groups: *i.e.*, for habitual exercise other than work, less than once a week as "low," 1 to 2 times per week as "moderate," and 3 or more times per week as "high," for drinking habits, less than once a week as "low," 1 to 4 times per week as "moderate," and 5 or more times per week as "high"; and for smoking status, current, former and never-smokers. We defined former smokers as those who quit smoking more than 2 years before the questionnaire study.

Analysis of fatty acids in erythrocytes

Blood samples were collected using EDTA-2Na tubes and centrifuged at 2,000g for 15 min at 4°C and erythrocytes were stored at -80°C until analysis of individual fatty acids. Our approach uses an accelerated solvent extractor and a gas-liquid chromatography for measuring fatty acids in biomaterials.[28,29] In short, membranes (white ghosts) from 50 μL of erythrocytes were prepared with sodium phosphate buffer and an accelerated solvent extractor (ASE®) 200 (Nippon Dionex, Osaka, Japan) was applied for extracting lipids (first extraction) and fatty acid methyl esters (second extraction) with chloroform-methanol, 1:2, by volume and petroleum ether as

solvents, respectively. The two extraction processes were automatically achieved with computerized programs, and butylate hydroxytoluene applied as an antioxidant. All samples from the first extraction were treated with hydrochloride-methanol reagent for fatty acid conversion from lipids and subsequent methyl-transformation of fatty acids. The fatty acid methyl esters from the second extraction were analyzed by Shimadzu GC-2010 gas chromatography (Shimadzu, Kyoto, Japan) on a capillary column DB-225 (J&W Scientific, Folsom, CA), equipped with an auto-injector, auto-sampler, and flame ionization detector, under the conditions described elsewhere.[29] 17:0 (heptadecanoic acid) was used as an internal standard. Each fatty acid was identified with the use of commercial standards of known retention time, and integration of the peak areas was performed with the GC solution Ver.2 software (Shimadzu, Kyoto, Japan). The laboratory staffs for measuring fatty acids were completely blinded to case-control status, and links between the biomaterial and any other information on study subjects.

In line with our previous studies, we selected 13 fatty acids,[28,29] predominating in both dietary intakes and erythrocyte contents. Taking into consideration each molecular weight for individual fatty acids, their compositions of erythrocyte membranes were determined as mol percentage (mol %) of total fatty acid concentrations (mmol/L) because it was difficult to accurately determine the numbers of erythrocytes in 50 μL and evaluate the concentrations because erythrocytes vary in size and surface area. Intra-assay coefficients of variation (CVs) were based on analysis of a series of 10 samples measured within 1 day, and <4.0%, except a minor group of 14:0, 18:3n-6 and 18:3n-3 (≤0.5% of total fatty acids for each).[29] Inter-assay CVs were based on replicate analyses of pooled erythrocytes (a total 100 samples) over a period of 10 days, and also were <4.0%, except for a minor group of 14:0, 18:3n-6 and 18:3n-3.[29]

Selected fatty acids and grouping

Taking into account our previous findings,[28,29,39,40] we here selected the following thirteen fatty acids: 14:0 (myristic acid), 16:0 (palmitic acid), 16:1n-7 (palmitoleic acid), 18:0 (stearic acid), 18:1n-9 (oleic acid), 18:2n-6 (linoleic acid), 18:3n-6 (γ-linolenic acid), 18:3n-3 (α-linolenic acid), 20:3n-6 (dihomo-γ-linolenic acid), 20:4n-6 (AA), 20:5n-3 (EPA), 22:5n-3 (docosapentaenoic acid) and 22:6n-3 (DHA). The specification begins with 2 numbers separated by a colon; the first number refers to the number of carbons in the chain and the second is to that of double bonds. The next number, preceded by "n," indicates the distance of first double bond from the n-end of the chain. The geometric isomerism for all was *cis* type of the double bond nearest the n-end of the chain. To facilitate understanding the name of each fatty acid, systematic chemical names, without common names in parenthesis, were here used.[26,28]

The 7 groups of fatty acids in erythrocyte membranes were summarized as follows, with the use of total fatty acids as the denominator: SFAs (=14:0 + 16:0 + 18:0), MUFAs (=16:1n-7 + 18:1n-9), PUFAs (=n-6 PUFAs + n-3 PUFAs), n-6 PUFAs (=18:2n-6 + 18:3n-6 + 20:3n-6 + AA), n-3 PUFAs (=18:3n-3 + n-3 HUFAs) and n-3 HUFAs (=EPA + 22:5n-3 + DHA). We also defined the ratios of specific fatty acids as follows: 18:0/18:1n-9 (as a saturation $index_{n-9}$), 16:0/16:1n-7 (as a saturation $index_{n-7}$), SFAs/PUFAs, SFAs/n-6 PUFAs, SFAs/n-3 PUFAs, SFAs/n-3 HUFAs, n-6 PUFAs/n-3 PUFAs, n-6 PUFAs/n-3 HUFAs, AA/EPA and AA/DHA. The reciprocals of saturation indices are indicators of membrane fluidity and can be considered indices for activity of the rate-limiting enzyme delta 9 desaturases (stearoyl-CoA desaturase) that transform SFAs into the corresponding MUFAs.[26] The n-6 PUFAs/n-3 PUFAs ratio has been suggested to be particularly important for human health.[41,42]

Statistical methods

Body mass index (kg/m^2, BMI) was calculated from the self-reported height (m) and weight (kg). The differences in variables and proportions between cases and controls were tested using the Student's *t*-test and the χ^2 test, respectively. In control subjects, partial Spearman's correlation coefficients between fatty acids in diet (g/1,000 kcal) and those in erythrocyte membranes (mol %) were adjusted for age, BMI and season of sample collection. In erythrocyte phospholipids, SFAs is abundant at the α-position of glycerol-3-phosphate derivatives, whereas MUFAs and PUFAs (*e.g.*, 18:2n-6, AA, EPA and DHA) are predominantly composed at the β-position. To examine competitive incorporation of fatty acids into phospholipids in erythrocyte membranes, partial Pearson's correlation coefficients between the fatty acid contents in control subjects were adjusted for the same variables described above.

Cases were categorized according to the tertile levels of variables both for dietary intake and erythrocyte membranes among control subjects, and the odds ratios (ORs) for the middle and the highest to the lowest tertile were estimated. ORs and the 95% confidence intervals (CIs) were calculated, using conditional logistic regression models, after adjustment for BMI (continuous), habitual exercise, drinking and smoking habits, green-yellow vegetable intake (g/1,000 kcal), menopausal status (pre- or post-menopause), family history of breast cancer in parents and/or siblings (yes or no), age at menarche (≤12, 13–14 or ≥15 years), menopausal periods (continuous), parity (0, 1, 2 or ≥3) and female hormone users (yes or no). For non-energy adjusted variables of dietary intakes (g/day), green-yellow vegetable intake (g/day) was used instead of variables adjusted for energy intake (g/1,000 kcal). These possible confounding factors were considered with reference to previously published reports, including our recent results for breast cancer risk.[19–21] In Japanese, users of non-steroidal anti-inflammatory drugs are very few. A test for the trend with each variable was conducted by assigning the median values in control subjects. All statistical analyses were conducted with SAS version 9.1 (SAS Institute, Cary, NC), and $p < 0.05$ was considered statistically significant.

Results

Table I summarizes data for characteristics of the subjects and their dietary intakes of foods, fat and fatty acids. The percentage of energy from fat was ~26%, but the balance of fatty acid intakes significantly differed between the case and control groups. Dietary intakes (g/1,000 kcal) of total seafood, fish, other seafood and n-3 PUFAs had no associations with breast cancer risk (Table II). For n-3 HUFAs intake, however, the OR for the highest tertile to the lowest tertile was 0.51 (95% CI, 0.27–0.98; $p_{trend} < 0.05$). In contrast, risk was positively associated with n-6 PUFAs intake (OR, 2.19; 95% CI, 1.09–4.42; $p_{trend} = 0.07$) and dietary ratio of SFAs/PUFAs (OR, 0.41; 95% CI, 0.21–0.81; $p_{trend} < 0.05$). Regarding non-adjusted variables (g/day), risk was negatively associated with SFAs intake (OR, 0.51; 95% CI, 0.26–0.96; $p_{trend} < 0.05$) (data not shown), but no relation was found with total seafood, fish, other seafood, n-3 PUFAs and n-3 HUFAs. Dietary intakes of other foods and fatty acids had no associations with risk.

Table III shows mean percentage (mol %) fatty acid compositions of erythrocyte membranes. Excepting 14:0, 18:1n-9, 20:3n-6, 18:3n-3 and the saturation $index_{n-9}$, all variables significantly differed between the 2 groups. Erythrocyte compositions of SFAs and 16:0 were especially higher in cases than in control subjects, while those of both n-6 PUFAs and n-3 PUFAs series were lower. The ratios of SFAs/PUFAs and n-6 PUFAs/n-3 HUFAs were therefore higher in the cases. In the controls, erythrocyte levels of n-3 HUFAs, EPA and DHA had positive partial Spearman's correlation coefficients (*r*) with dietary intakes of total seafood, fish and n-3 HUFAs ($r = 0.14$ to 0.27, at least $p < 0.05$ for all) (Fig. 1), and those of n-6 PUFAs and 18:2n-6 had negative ones ($r = -0.12$ to -0.15, $p < 0.05$ for both). Moreover, n-6 PUFAs and the ratios of n-6 PUFAs/n-3 HUFAs in erythrocyte membranes and the diet had also positive correlation coefficients ($r = 0.14$ and 0.30, $p < 0.005$ for both), but dietary intakes of meat, SFAs and MUFAs had no relationship.

TABLE I – MEAN AND STANDARD DEVIATION (SD) OF SOME VARIABLES POSSIBLY RELATED TO BREAST CANCER IN CASE AND CONTROL SUBJECTS

	Case subjects (n = 103)		Control subjects (n = 309)		p value for χ^2 or t-test
	Mean	SD	Mean	SD	
Age (years)	52.1	11.0	52.0	10.7	NS
Body mass index (kg/m^2)	22.2	3.3	22.0	3.1	NS
Menopausal status					
Premenopause	46 (44.7%)		150 (48.5%)		NS
Postmenopause	57 (55.3%)		159 (51.5%)		
Family history of breast cancer in parents and/or siblings (n, %)	11 (10.7)		21 (6.8)		NS
Age at menarche (years)	13.4	1.5	13.3	1.5	NS
Menstrual periods (years)	33.1	6.4	33.8	6.3	NS
Number of parities (n)	1.8	1.1	1.8	1.1	NS
Hormone users[1] (n, %)	15 (14.7)		41 (13.6)		NS
Habitual exercise (n, %)					
High	36 (35.0)		102 (33.0)		NS
Moderate	26 (25.2)		113 (36.6)		
Low	41 (39.8)		94 (30.4)		
Drinking status (n, %)					
Drinkers	38 (36.9)		127 (41.1)		NS
Ex-drinkers	1 (1.0)		6 (1.9)		
Non-drinkers	64 (62.4)		176 (57.0)		
Smoking status (n, %)					
Smokers	12 (11.7)		38 (12.3)		NS
Ex-smokers	4 (3.9)		17 (5.5)		
Non-smokers	87 (84.5)		254 (82.2)		
Dietary intake					
Total energy (K cal)	1519	245	1536	248	NS
Soy and the products (g/1,000 kcal)	28.4	18.3	29.4	16.8	NS
Meat[2] (g/1,000 kcal)	20.8	11.2	22.4	13.6	NS
Total sea foods (g/1,000 kcal)	31.2	18.7	34.1	17.1	NS
Fish (g/1,000 kcal)	20.3	13.0	22.2	12.5	NS
Other seafood (g/1,000 kcal)	10.9	7.9	11.9	8.1	NS
Green-yellow vegetables (g/1,000 kcal)	46.0	32.1	48.6	28.8	NS
Other vegetables (g/1,000 kcal)	43.7	26.9	46.0	25.7	NS
Energy from fat (%)	25.6	5.6	25.9	6.1	NS
Total fat (g/1,000 kcal)	28.4	6.2	28.7	6.8	NS
SFAs[3] (g/1,000 kcal)	7.4	1.6	7.7	1.9	NS
MUFAs[3] (g/1,000 kcal)	10.6	2.5	10.4	2.5	NS
PUFAs[3] (g/1,000 kcal)	8.6	1.8	8.3	2.0	NS
n-6 PUFAs[3] (g/1,000 kcal)	7.3	1.6	7.0	1.8	NS
n-3 PUFAs[3] (g/1,000 kcal)	1.5	0.3	1.5	0.3	NS
n-3 HUFAs[3] (g/1,000 kcal)	0.45	0.19	0.48	0.19	NS
n-6 PUFAs/n-3 PUFAs	5.06	0.83	4.81	0.82	<0.01
n-6 PUFAs/n-3 HUFAs	18.77	8.1	16.6	7.26	<0.05
SFAs/PUFAs	0.88	0.23	0.96	0.29	<0.01

[1]Data from one case and eight control subjects was not collected.–[2]Meat included beef, pork and poultry.–[3]Dietary intakes of each fatty acid group were explained in "Material and methods" and mainly composed of the selected 13 fatty acids as follows; SFAs (saturated fatty acids) = 14:0 + 16:0 + 18:0; MUFAs (monounsaturated fatty acids) = 16:1n-7 + 18:1n-9; PUFAs (polyunsaturated fatty acids) = n-6 PUFAs + n-3 PUFAs; n-6 PUFAs = 18:2n-6 + 18:3n-6 + 20:3n-6 + 20:4n-6 (arachidonic acid, AA); n-3 PUFAs = 18:3n-3 + n-3 highly unsaturated fatty acids (HUFAs); and n-3 HUFAs = 20:5n-3 (eicosapentaenoic acid, EPA) + 22:5n-3 + 22:6n-3 (docosahexaenoic acid, DHA). NS, not significant.

The ORs for breast cancer according to fatty acid contents in erythrocyte membranes are shown in Table IV. The risk had strong positive associations with erythrocyte compositions of SFAs (the highest to the lowest tertile; OR, 12.29; 95% CI, 4.94–30.57; $p_{trend} < 0.0001$) and 16:0 (OR, 10.08; 95% CI, 4.02–25.23; $p_{trend} < 0.0001$). For those of MUFAs and 16:1n-7, the ORs were 3.58 (95% CI, 1.72–7.46; $p_{trend} < 0.001$) and 4.60 (95% CI, 2.07–10.19; $p_{trend} < 0.0005$), respectively, but that of 18:1n-9 exhibited no clear association. The analyses revealed a significant inverse association between the risk and PUFAs in erythrocyte membranes (OR, 0.09; 95% CI, 0.04–0.22; $p_{trend} < 0.0001$). Thus, the ORs were 0.11 (95% CI, 0.05–0.24; $p_{trend} < 0.0001$) for n-3 HUFAs, 0.27 (95% CI, 0.14–0.53; $p_{trend} < 0.0001$) for EPA and 0.06 (95% CI, 0.02–0.16; $p_{trend} < 0.0001$) for DHA, and, likewise, 0.23 (95% CI, 0.11–0.49; $p_{trend} < 0.005$) for n-6 PUFAs, 0.35 (95% CI, 0.18–0.68; $p_{trend} < 0.005$) for 18:2n-6 and 0.37 (95% CI, 0.19–0.72; $p_{trend} < 0.005$) for AA. In Figure 1, our findings regarding n-3 HUFAs series in dietary intakes and erythrocyte membranes are summarized.

The risk was also inversely related to the saturation index$_{n-7}$ (the highest to the lowest tertile, OR, 0.37; 95% CI, 0.18–0.78; $p_{trend} < 0.01$), but not associated with the saturation index$_{n-9}$ (Table IV). Strong positive links were noted between the risk and erythrocyte ratios for SFAs/PUFAs (OR, 12.56; 95% CI, 4.84–32.63; $p_{trend} < 0.0001$), especially SFAs/n-3 HUFAs (OR, 14.65; 95% CI, 5.67–37.82; $p_{trend} < 0.0001$), and n-6 PUFAs/n-3 HUFAs (OR, 7.20; 95% CI, 3.23–16.02; $p_{trend} < 0.0001$), AA/EPA (OR, 3.68; 95% CI, 1.84–7.34, $p_{trend} < 0.0001$) and AA/DHA (OR, 6.47; 95% CI, 2.96–14.17, $p_{trend} < 0.0001$). Although hormone use, one possible risk factor, was excluded from analyses due to missing data for 9 subjects, multivariate ORs and the trend tests did not change materially. Moreover, when 4 recurrent cases and the corresponding 12 controls were excluded, the risk was also not changed.

Discussion

We could here demonstrate that breast cancer risk exhibits a significant inverse association with dietary intake of n-3 HUFAs and high levels of n-3 HUFAs, EPA and DHA in erythrocyte membranes. Negative links with erythrocyte compositions of n-6 PUFAs, 18:2n-6 and AA, and positive correlations with erythro-

TABLE II – ODDS RATIOS (ORs) FOR BREAST CANCER AND THE 95% CONFIDENCE INTERVALS (CIs) ACCORDING TO TERTILE OF DIETARY INTAKES (g/1000 kcal) OF SEAFOOD AND FATTY ACIDS

Dietary intake	Odds ratios (95% CIs)[1] by tertiles			*p* for trend
	T1 (reference)	T2	T3	
Total energy (kcal)	<1437	1437–1516	> 1516	
	1.00	1.13 (0.65–1.97)	0.98 (0.53–1.80)	NS
Soy and the products (g/1000 kcal)	<20.34	20.34–32.78	> 32.78	
	1.00	0.95 (5.4–1.70)	0.70 (0.38–1.31)	NS
Meat[2] (g/1000 kcal)	<16.27	16.27–23.80	> 23.80	
	1.00	0.69 (0.38–1.26)	1.01 (0.57–1.78)	NS
Total sea foods (g/1000 kcal)	<23.91	23.91–38.76	> 38.76	
	1.00	0.70 (0.39–1.26)	0.61 (0.32–1.17)	NS
Fish (g/1000 kcal)	<12.77	12.77–26.10	> 26.10	
	1.00	0.69 (0.38–1.26)	0.59 (0.31–1.14)	NS
Other seafood (g/1000 kcal)	<7.87	7.87–12.32	> 12.32	
	1.00	0.66 (0.36–1.22)	0.68 (0.37–1.26)	NS
Energy from fat (%)	<23.36	23.36–27.57	> 27.57	
	1.00	0.83 (0.47–1.46)	0.74 (0.40–1.39)	NS
Total fat (g/1000 kcal)	<25.96	25.96–30.63	> 30.63	
	1.00	0.83 (0.47–1.46)	0.74 (0.40–1.39)	NS
SFAs[3] (g/1000 kcal)	<6.66	6.66–8.21	> 8.21	
	1.00	1.19 (0.69–2.04)	0.59 (0.31–1.12)	NS
MUFAs[3] (g/1000 kcal)	<9.30	9.30–11.27	> 11.27	
	1.00	1.06 (0.58–1.84)	1.04 (0.57–1.89)	NS
PUFAs[3] (g/1000 kcal)	<7.27	7.27–9.08	> 9.08	
	1.00	1.35 (0.72–2.52)	1.66 (0.89–3.08)	NS
n-6 PUFAs[3] (g/1000 kcal)	<5.90	5.90–7.73	> 7.73	
	1.00	3.15 (1.62–6.13)	2.19 (1.09–4.42)	0.07
n-3 PUFAs[3] (g/1000 kcal)	<1.30	1.30–1.57	> 1.57	
	1.00	0.99 (0.56–1.77)	0.87 (0.47–1.63)	NS
n-3 HUFAs[3] (g/1000 kcal)	<0.36	0.36–0.55	> 0.55	
	1.00	0.69 (0.39–1.22)	0.51 (0.27–0.98)	<0.05
n-6 PUFAs/n-3 PUFAs	<4.51	4.51– 5.17	> 5.17	
	1.00	1.36 (0.73–2.54)	1.51 (0.81–2.81)	NS
n-6 PUFAs/n-3 HUFAs	<12.46	12.46–18.50	> 18.50	
	1.00	1.12 (0.58–2.16)	1.66 (0.89–3.09)	NS
SFAs/PUFAs	<0.80	0.80–1.04	> 1.04	
	1.00	1.11 (0.65–1.91)	0.41 (0.21–0.81)	<0.05

[1]One case and eight control subjects were excluded from analyses (see Table 1). ORs and their 95% CIs were adjusted for BMI (continuous), habitual exercise, drinking and smoking status, green-yellow vegetable intake (g/1000 kcal), menopausal status (pre- or post-menopause), family history of breast cancer in parents and/or siblings (yes or no), age at menarche (≤12, 13–14 or ≥15 years), menopausal periods (continuous), parity (0, 1, 2 or ≥3) and hormone users (yes or no).–[2]Meat included beef, pork and poultry.–[3]Dietary intakes of each fatty acid group were explained in "Material and methods" and mainly composed of the selected 13 fatty acids as follows; SFAs (saturated fatty acids) = 14:0 + 16:0 + 18:0; MUFAs (monounsaturated fatty acids) = 16:1n-7 + 18:1n-9; PUFAs (polyunsaturated fatty acids) = n-6 PUFAs + n-3 PUFAs; n-6 PUFAs = 18:2n-6 + 18:3n-6 + 20:3n-6 + 20:4n-6 (arachidonic acid, AA); n-3 PUFAs = 18:3n-3 + n-3 highly unsaturated fatty acids (HUFAs); and n-3 HUFAs = 20:5n-3 (eicosapentaenoic acid, EPA) + 22:5n-3 + 22:6n-3 (docosahexaenoic acid, DHA). NS; Not significant.

cyte ratios of n-6 PUFAs/n-3 HUFAs, AA/EPA and AA/DHA were also observed. No association was found with dietary intakes of meat, total fat, SFAs and MUFAs, but erythrocyte compositions of SFAs, especially 16:0, demonstrated strong positive relationships. Increased risk, therefore, was strongly related to erythrocyte ratios of SFAs/PUFAs, especially SFAs/n-3 HUFAs, rather than SFAs/n-6 PUFAs.

In Japanese, Mediterranean and American diets, the amount of fat intake (energy intake, %) and the ratios of SFAs: MUFAs: PUFAs have been reported to be 40–50 g (20–25%) and 1: 1: 1, 70–80 g (30–35%) and 2: 5: 2, and 80–90 g (35–40%) and 2: 2: 1, in that order.[43] Since 1950, the traditional Japanese diet rich in fish has shifted toward a Westernized one rich in meat and fat,[23] and such dietary changes seem to be closely related to disease prevalence, including cancers in several sites.[43] Recent cancer registry data show that Japanese women are experiencing an increase in the incidence rate for breast cancer.[44] Evidence of an inverse association between breast cancer risk and dietary intakes of fish and n-3 HUFAs has been accumulated in Japanese,[19–21] who have the highest fish intake in the world. The risk, however, has hitherto not been assessed using appropriate biomarkers regarding dietary intakes of fish and n-3 HUFAs.

The relation between breast cancer and fish intake remains equivocal.[45] In Swedish postmenopausal women, no association was observed with any fatty acid compositions.[27] In Italian women, however, risk was found to be negatively associated with erythrocyte compositions of not only n-3 HUFAs and DHA, but also n-6 PUFAs and 18:2n-6.[26] In Russian pre- and post-menopausal women, risk was related to higher compositions of 18:2n-6 and AA, respectively.[46] Using erythrocyte membranes, serum phospholipids or adipose tissue (in the breast or subcutaneous) as biomaterials, risk has consistently been found to be inversely linked with n-3 PUFAs, EPA and DHA, but not 18:3n-3 in both cohort and case-control studies.[27,47,48] In postmenopausal women in cohort studies, decreased risk was associated with higher compositions of n-6 PUFAs and 18:2n-6, but not AA.[26,48] The AA cascade is suggested to play critical roles in tumor development from laboratory studies,[1–3,6] but an inverse association with breast cancer risk was unexpectedly here found for erythrocyte composition of AA. On the other hand, erythrocyte ratios of AA/EPA and AA/DHA had positive links with risk and the findings support the hypothesis regarding competitive inhibition for biosynthesis of AA-derived eicosanoids such as prostaglandin E_2.

Dietary intakes of total seafood, fish, n-3 HUFAs and n-3 PUFAs were positively correlated with erythrocyte compositions of n-3 HUFAs series and negatively with those of n-6 PUFAs series. Feeding studies with dietary supplementation of fish, fish oil, EPA and DHA have confirmed corresponding changes in biomate-

TABLE III – PERCENTAGE (MOL %) FATTY ACID COMPOSITIONS OF ERYTHROCYTE MEMBRANES IN CASE AND CONTROL SUBJECTS

Fatty acid	Common name	Case subjects (n = 103)		Control subjects (n = 309)		p value for χ^2 or t-test
		Mean	SD	Mean	SD	
SFAs[1]		55.7	2.5	53.8	3.1	<0.0001
14:0	Myristic acid	1.2	0.6	1.1	0.7	NS
16:0	Palmitic acid	32.6	2.0	31.3	2.4	<0.0001
18:0	Stearic acid	21.9	1.3	21.4	1.2	<0.001
MUFAs[2]		19.6	1.4	19.1	1.6	<0.005
16:1n-7	Palmitoleic acid	1.7	0.7	1.4	0.5	<0.0001
18:1n-9	Oleic acid	17.9	1.2	17.7	1.5	NS
PUFAs[3]		24.7	3.0	27.1	3.5	<0.0001
n-6 PUFAs[4]		19.6	2.1	20.5	2.1	<0.0005
18:2n-6	Linoleic acid	10.3	1.3	10.6	1.1	<0.05
18:3n-6	γ-Linolenic acid	0.04	0.02	0.06	0.03	<0.0001
20:3n-6	Dihomo-γ-linolenic acid	0.84	0.28	0.81	0.27	NS
20:4n-6	Arachidonic acid (AA)	8.4	1.5	9.0	1.6	<0.001
n-3 PUFAs[5]		5.1	1.6	6.7	2.1	<0.0001
18:3n-3	α-Linolenic acid	0.3	0.2	0.4	0.2	NS
n-3 HUFAs[6]		4.8	1.6	6.3	2.0	<0.0001
20:5n-3	Eicosapentaenoic acid (EPA)	0.9	0.6	1.2	0.6	<0.0001
22:5n-3	Docosapentaenoic acid	0.7	0.2	0.9	0.4	<0.0001
22:6n-3	Docosahexaenoic acid (DHA)	3.2	1.0	4.2	1.3	<0.0001
Saturation index$_{n-7}$	(16:0/16:1n-7)	22.65	10.19	26.49	12.47	<0.005
Saturation index$_{n-9}$	(18:0/18:1n-9)	1.23	0.12	1.22	0.13	NS
SFAs/PUFAs		2.30	0.38	2.03	0.38	<0.0001
SFAs/n-6 PUFAs		2.89	0.42	2.67	0.41	<0.0001
SFAs/n-3 PUFAs		12.26	4.92	9.08	3.56	<0.0001
SFAs/n-3 HUFAs		13.31	5.72	9.67	3.89	<0.0001
n-6 PUFAs/n-3PUFAs		4.21	1.40	3.36	1.05	<0.0001
n-6 PUFAs/n-3HUFAs		4.56	1.60	3.58	1.16	<0.0001
AA/EPA		13.11	9.68	9.66	6.60	<0.001
AA/DHA		2.80	0.77	2.31	0.60	<0.0001

[1]SFAs (saturated fatty acids) = 14:0 + 16:0 + 18:0.–[2]MUFAs (monounsaturated fatty acids) = 16:1n-7 + 18:1n-9.–[3]PUFAs (polyunsaturated fatty acids) = n-6 PUFAs + n-3 PUFAs.–[4]n-6 PUFAs = 18:2n-6 + 18:3n-6 + 20:3n-6 + 20:4n-6 (arachidonic acid, AA).–[5]n-3 PUFAs = 18:3n-3 + n-3 highly unsaturated fatty acids (HUFAs).–[6]n-3 HUFAs = 20:5n-3 (eicosapentaenoic acid, EPA) + 22:5n-3 + 22:6n-3 (docosahexaenoic acid, DHA). NS, not significant.

rials.[24,42,49–51] We here observed that erythrocyte compositions of DHA and n-3 HUFAs (not including EPA) had positive associations with those of n-6 PUFAs and AA (partial Pearson's correlation coefficients, $r = 0.47$ to 0.67, $p < 0.0001$ for all). We need to clarify competitive inhibition between AA and n-3 HUFAs series regarding (*i*) incorporation into membrane phospholipids; (*ii*) desaturation and elongation of 18:2n-6 and 18:3n-3 to biosynthesize AA and EPA; (*iii*) generation of corresponding AA- and EPA-derived eicosanoids by cyclooxygenases; and (*iv*) regulation of such eicosanoids biosynthesis and catabolism.

Regarding relationships between breast cancer risk and dietary intakes, high intakes of total fat, SFAs, animal fat and meat has been suggested as possible risk factors for breast cancer, whereas no association was found for those of MUFAs and PUFAs.[13,45,52] Higher erythrocyte compositions of SFAs and MUFAs series were associated with the increased risk, and our results were consistent with findings from a large-scale prospective cohort study.[26] We also observed strong positive associations between risk and erythrocyte ratios of SFAs/PUFAs, especially SFAs/n-3 HUFAs, whereas strong competitive incorporation of SFAs and PUFAs series in erythrocyte membranes was found [partial Pearson's correlation coefficients (r) = -0.73 to -0.90, $p < 0.0001$ for all]. In our present and previous studies, the erythrocyte saturation index$_{n-7}$ was negatively related to cancer risks on the breast and colorectum,[28] but the index$_{n-9}$ was negatively associated with breast cancer risk in Italian postmenopausal women.[26] No association was observed in Swedish women,[27] and therefore the inconsistent findings may be linked to the activity of delta 9 desaturase, depending on fat content, especially SFAs and n-3 HUFAs, in the diet.[26]

Potential limitations of the present study should be considered. The sample size was relatively small. Selection bias for study subjects might have arisen because the participation rate was less than 50%, but we requited the participants (including cases and controls) from all first-visit outpatients at ACCH. The control subjects were randomly selected and individually matched for age and season of sample collection with a 1: 3 case-control ratio, to increase the statistical power. We also could not assess the risk in pre- and post-menopausal women separately. This was a retrospective study for incident cases and the correspond controls. We could not clarify causal relationships between the development of breast cancer and erythrocyte compositions of fatty acids due to affects of the disease status for both nutrient intake and metabolism. With the following two ways to control for confounding, however, we showed that a series of erythrocyte n-3 HUFAs compositions are appropriate for use as biomarkers, not only for dietary intakes of fish and n-3 HUFAs, but also estimation of the suspected risk; (*i*) by designing the study controls, and (*ii*) by using statistical techniques removing the effects of confounding variables.

In a previous study of cancer patients receiving cytotoxic chemotherapy, significant positive correlations were found between dietary intake and plasma composition (wt %) of n-3 HUFAs by means of same method for cancer free subjects.[53] The findings support our results in the present study. Erythrocytes have a half life of 120 days and an intervention study showed that the EPA composition of erythrocyte phospholipids returns to the baseline level after 90 days from the endpoint of EPA supplementation.[24] Without recall bias regarding dietary intakes, n-3 HUFAs levels in adipose tissue and platelet phospholipids have also been suggested as discriminating biomarkers between long- or middle-term fish eaters and non-fish eaters, but these biomaterials are difficult to collect from the general population or are troublesome to separate from other contaminants in serum/plasma. Erythrocyte compositions of n-3 HUFAs series, therefore, provide good indices for evaluating dietary intakes of fish, fat and the corresponding fatty acids. With

TABLE IV – ODDS RATIOS (ORs) FOR BREAST CANCER AND THE 95% CONFIDENCE INTERVALS (CIs) ACCORDING TO TERTILE OF FATTY ACID COMPOSITIONS (MOL %) IN ERYTHROCYTE MEMBRANES

	Odds ratios (95% CIs)[1] by tertiles			*p* for trend
	T1 (reference)	T2	T3	
SFAs[2]	<52.11	52.11–55.34	>55.34	
	1.00	5.16 (1.96–13.54)	12.29 (4.94–30.57)	<0.0001
14:0	<0.74	0.74–1.22	>1.22	
	1.00	4.07 (1.97–8.44)	4.52 (2.02–10.08)	<0.01
16:0	<30.09	30.09–32.44	>32.44	
	1.00	8.16 (3.14–21.19)	10.08 (4.02–25.23)	<0.0001
18:0	<20.84	20.84–21.77	>21.77	
	1.00	1.44 (0.73–2.82)	2.65 (1.40–5.01)	<0.005
MUFAs[3]	<18.37	18.37–19.58	>19.58	
	1.00	2.15 (1.07–4.35)	3.58 (1.72–7.46)	<0.001
16:1n-7	<1.06	1.06–1.68	>1.68	
	1.00	2.15 (1.02–4.49)	4.60 (2.07–10.19)	<0.0005
18:1n-9	<17.00	17.00–18.07	>18.07	
	1.00	2.35 (1.23–4.51)	1.70 (0.87–3.36)	NS
PUFAs[4]	<25.02	25.02–28.86	>28.86	
	1.00	0.56 (0.31–1.01)	0.09 (0.04–0.22)	<0.0001
n-6 PUFAs[5]	<19.40	19.40–21.47	>21.47	
	1.00	0.96 (0.54–1.72)	0.23 (0.11–0.49)	<0.0005
18:2n-6	<10.15	10.15–11.12	>11.12	
	1.00	0.60 (0.34–1.07)	0.35 (0.18–0.68)	<0.005
18:3n-6	<0.04	0.04–0.07	>0.07	
	1.00	0.87 (0.49–1.55)	0.08 (0.03–0.22)	<0.0001
20:3n-6	<0.68	0.68–0.90	>0.90	
	1.00	0.97 (0.55–1.70)	1.11 (0.57–2.16)	NS
20:4n-6 (AA)	<8.17	8.17–9.68	>9.68	
	1.00	0.80 (0.45–1.44)	0.37 (0.19–0.72)	<0.005
n-3 PUFAs[6]	<5.57	5.57–7.57	>7.57	
	1.00	0.29 (0.15–0.54)	0.10 (0.04–0.23)	<0.0001
18:3n-3	<0.26	0.26–0.39	>0.39	
	1.00	0.72 (0.40–1.30)	0.69 (0.37–1.28)	NS
n-3 HUFAs[7]	<5.24	5.24–7.10	>7.10	
	1.00	0.28 (0.15–0.53)	0.11 (0.05–0.24)	0.0001
20:5n-3 (EPA)	<0.89	0.89–1.39	>1.39	
	1.00	0.27 (0.14–0.51)	0.27 (0.14–0.53)	<0.0001
22:5n-3	<0.73	0.73–1.06	>1.06	
	1.00	0.35 (0.19–0.64)	0.05 (0.02–0.13)	<0.0001
22:6n-3 (DHA)	<3.46	3.46–4.79	>4.79	
	1.00	0.46 (0.26–0.83)	0.06 (0.02–0.16)	<0.0001
Saturation index$_{n-7}$[8]	<18.47	18.47–29.75	>29.75	
	1.00	0.55 (0.29–1.06)	0.37 (0.18–0.78)	<0.01
Saturation index$_{n-9}$[9]	<1.17	1.17–1.28	>1.28	
	1.00	1.14 (0.63–2.05)	1.16 (0.62–2.17)	NS
SFAs/PUFAs	<1.81	1.81–2.19	>2.19	
	1.00	7.53 (2.77–20.49)	12.56 (4.84–32.63)	<0.0001
SFAs/n-6 PUFAs	<2.44	2.44–2.83	>2.83	
	1.00	4.99 (2.15–11.57)	6.15 (2.72–13.93)	<0.0001
SFAs/n-3 HUFAs	<7.24	7.24–10.39	>10.39	
	1.00	5.20 (1.89–14.32)	14.65 (5.67–37.82)	<0.0001
n-6 PUFAs/n-3 PUFAs	<2.82	2.82–3.65	>3.65	
	1.00	1.73 (0.78–3.82)	5.74 (2.69–12.27)	<0.0001
n-6 PUFAs/n-3 HUFAs	<2.97	2.97–3.87	>3.87	
	1.00	2.40 (1.05–5.50)	7.20 (3.23–16.02)	<0.0001
AA/EPA	<6.27	6.27–9.82	>9.82	
	1.00	1.56 (0.76–3.18)	3.68 (1.84–7.34)	<0.0001
AA/DHA	<2.02	2.02–2.51	>2.51	
	1.00	2.44 (1.08–5.47)	6.47 (2.96–14.17)	<0.0001

[1]One case and eight control subjects were excluded from analyses (see Table I). ORs and their 95% CIs were adjusted for BMI (continuous), habitual exercise, drinking and smoking status, green-yellow vegetable intake (g/1,000 kcal), menopausal status (pre- or post-menopause), family history of breast cancer in parents and/or siblings (yes or no), age at menarche (≤12, 13–14 or ≥15 years), menopausal periods (continuous), parity (0, 1, 2 or ≥3) and hormone users (yes or no).–[2]SFAs (saturated fatty acids) = 14:0 + 16:0 + 18:0.–[3]MUFAs (monounsaturated fatty acids) = 16:1n-7 + 18:1n-9.–[4]PUFAs (polyunsaturated fatty acids) = n-6 PUFAs + n-3 PUFAs.–[5]n-6 PUFAs = 18:2n-6 + 18:3n-6 + 20:3n-6 + 20:4n-6 (arachidonic acid, AA).–[6]n-3 PUFAs = 18:3n-3 + n-3 highly unsaturated fatty acids (HUFAs).–[7]n-3 HUFAs = 20:5n-3 (eicosapentaenoic acid, EPA) + 22:5n-3 + 22:6n-3 (docosahexaenoic acid, DHA).–[8]16:0/16:1n-7.–[9]18:0/18:1n-9.

the incident cancer cases of the present study, partial Spearman's correlation coefficients (*r*) with erythrocyte EPA composition were 0.28, 0.31 and 0.38 (at least $p < 0.01$) for dietary intakes of total seafood, fish and n-3 HUFAs, in that order. We here defined the selected thirteen fatty acids as total fatty acids, but noted that some of minor fatty acids (≤12 and ≥24 of the carbon number) might be important to evaluate the risk of breast cancer.

One other methodological issue is the selection of control subjects. Compared with the general population randomly selected from the electoral roll in the same area, the general lifestyle of non-cancer cases presenting at Aichi Cancer Center, including their dietary habits, was not found to demonstrate any differences.[32] Another potential source of bias is the medical background of control subjects, but we had already clarified that the majority did not

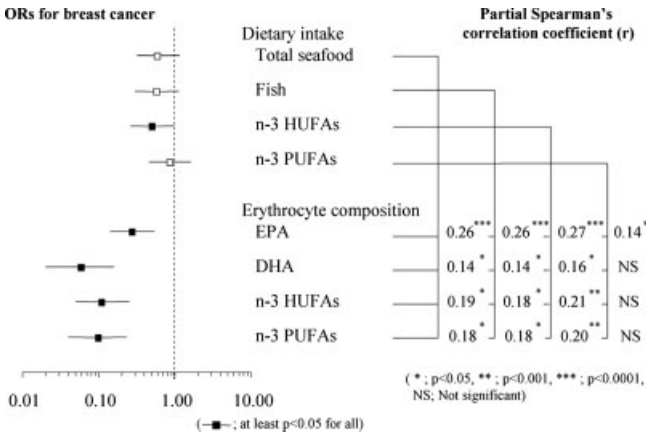

FIGURE 1 – Summary of odds ratios (ORs) for breast cancer and partial Spearman's correlation coefficients between dietary intakes of fish and n-3 HUFAs and their biomarkers in erythrocyte membranes. Total seafood was defined as the sum of fish and other seafood [other fish (e.g., bone-edible small fish), shellfish, canned tuna, crustacean/mollusk (cuttlefish, squid, octopus, shrimp and crab) and fish past products]. For both dietary intake and erythrocyte composition, n-3 polyunsaturated fatty acids (PUFAs) was summed up 18:3n-3 and n-3 highly unsaturated fatty acids (HUFAs), and n-3 HUFAs was also calculated by eicosapentaenoic acid (EPA), 22:5n-3 and docosahexaenoic acid (DHA). In control subjects ($n = 309$), partial Spearman's correlation coefficients between dietary intakes (g/1,000 kcal) of fish related variables and erythrocyte membranes (mol %) of n-3 HUFAs series were adjusted for age, body mass index (BMI) and season of sample collection. Cases ($n = 103$) were categorized according to the tertile levels of variables both in dietary intakes and erythrocyte membranes among control subjects, and ORs for the highest to the lowest tertile were estimated. ORs and the 95% confidence intervals (CIs) were calculated, using conditional logistic regression models, after adjustment for BMI (continuous), habitual exercise, drinking and smoking habits, green-yellow vegetable intake (g/1,000 kcal), menopausal status (pre- or post-menopause), family history of breast cancer in parents and/or siblings (yes or no), age at menarche ($\leq$12, 13–14 or $\geq$15 years), menopausal periods (continuous), parity (0, 1, 2 or $\geq$3) and female hormone users (yes or no) to control for the effects of potential confounding factors.

have any specific medical conditions.[28,54] In the present study, we excluded control subjects with any diseases related to fat and/or lipid metabolisms and history of resection in the ovary or uterus related to estrogen secretion. We therefore conclude that use of non-cancer outpatients as references for the present study was acceptable because it was reasonable to assume our case subjects arose within this population base. Blinded quality control for measuring erythrocyte compositions of fatty acids was not used during the period of laboratory work, but the measurement was completely blinded to links between biomaterials and any information on study subjects. Compared with other studies,[26,27,46] erythrocyte compositions of SFAs and n-3 HUFAs series were higher and lower, respectively, and a possible reason for the discrepancy in findings might be related to our method for measurement. However, within our series, there is no problem with comparisons.

In conclusion, using a newly established method for measuring fatty acids in biomaterials, we here demonstrate that erythrocyte compositions of n-3 HUFA series derived from fish intake, as biomarkers, are related to lower risk of breast cancer. Simultaneously, we also showed positive and negative associations between the cancer risk and higher erythrocyte compositions of SFAs and n-6 PUFAs series. Further research with large samples is now needed to clarify the mechanisms linked to SFAs, especially 16:0, for the cancer development and the discrepancy regarding the roles of n-6 PUFAs series, especially AA. Additional researches are also needed to investigate potential effects of anti-tumor drugs administrated during chemotherapy on the erythrocyte composition of DHA.[55]

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