(0.40; $P < .090$), because there were only 14 false-negative subjects. Second, the reference standard 75-g OGTT is not a reliable gold standard test. Nonetheless, 75-g OGTT is a valid standard test, and the unreliability was not considered to distort the results greatly because misclassification is expected to occur evenly in both tests.

In conclusion, we found that a cutoff HbA1c of ≥6.5% is as accurate as a FBG of ≥7.0 mmol/L in the diagnosis of PPHG. Further investigations on the relation between the reduced cutoff points and accuracy of HbA1c are needed.

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Influence of the C161T but not Pro12Ala polymorphism in the peroxisome proliferator-activated receptor-gamma on colorectal cancer in an Indian population

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The aim of the present study was to investigate associations between Pro12Ala and C161T polymorphisms in the peroxisome proliferator-activated receptor-gamma (*PPAR-γ*) gene and colorectal cancer (CRC) risk. We recruited 301 newly diagnosed CRC patients and 291 healthy control subjects at the Madras Cancer Institute in Chennai, India, from 1999 to 2001. Genotypes of the Pro12Ala and C161T polymorphisms were determined using the PCR-RFLP method. After adjustment for age, sex, smoking habit, family history and family income, an increased risk of CRC was observed for the C/T + T/T genotype compared to the C/C genotype of the C161T polymorphism (odds ratio = 1.61, 95% confidence interval: 1.10–2.36), whereas no significant association was found for Pro12Ala (odds ratio = 1.06, 95% confidence interval: 0.70–1.61). Analysis with estimated haplotypes showed a significant difference in haplotype frequencies between cases and controls (χ^2 = 11.62, P = 0.009, d.f. = 3). The relationship between the two polymorphisms and CRC risk was not significantly modified by dietary intake of fish. Although the biological mechanisms of the observed association remain to be elucidated, our findings suggest that the C161T polymorphism of the *PPAR-γ* gene is related to risk of CRC. Further research is needed to investigate functional implications of polymorphisms of the *PPAR-γ* gene in CRC development. (*Cancer Sci* 2005; 96: 507–512)

The peroxisome proliferator-activated receptor-gamma (*PPAR-γ*), a member of the nuclear hormone receptor super family, plays an important role in differentiation of adipocytes, lipid metabolism, insulin sensitivity, atherogenesis and immune regulation.[1–4] Recently, *PPAR-γ* has been implicated in the pathogenesis of colorectal cancer (CRC) in animal models and clinical studies. Colon cancer cells have been shown to express *PPAR-γ* at high levels, and somatic loss-of-function mutations have been identified.[5,6] *In vitro* studies have shown that ligand activation of *PPAR-γ* could inhibit the nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) inflammation pathways and cell growth, induce apoptosis and promote differentiation in colon, breast and prostate cell lines.[7–10] Furthermore, a *PPAR-γ* ligand was found to inhibit tumor growth in a xenograft model of colon cancer, and decrease premalignant intestinal lesions in mice treated with the chemical carcinogen azoxymethane.[11,12] In addition, increased susceptibility of *PPAR-γ* heterozygote knockout mice to colorectal carcinogenesis has been reported.[13] These observations raise the exciting hypothesis that *PPAR-γ* is a tumor suppressor gene in colorectal carcinogenesis. However, one study failed to find any *PPAR-γ* mutations in colon cancer samples, and another showed that administration of *PPAR-γ* ligands to Min mice resulted in development of more advanced colon cancers.[14,15]

The human *PPAR-γ* gene exists in three isoforms due to alternative promoters and differential splicing. PPAR-γ1 and PPAR-γ3 proteins are almost identical and are encoded by exons 1–6, whereas PPAR-γ2 has 28 additional amino acids at its N-terminus, encoded by the *PPAR-γ2*-specific exon B. The PPAR-γ1 and PPAR-γ3 isoforms are expressed in large intestinal, kidney and adipose tissues, while PPAR-γ2 exists exclusively in adipose tissue.[16,17] Common structural polymorphisms that have been detected in the *PPAR-γ* gene include a proline to alanine substitution (34C > G), located at codon 12 (Pro12Ala) of *PPAR-γ2*-specific exon B,[18] which reduces the promoter affinity by approximately 50%, and both ligand-independent and ligand-dependent *PPAR-γ* transactivation.[2] Another common polymorphism in exon 6 at nucleotide 161 results in a silent substitution from C to T (C161T).[3]

Recently, Landi *et al.* showed the Pro12Ala polymorphism to be related to a reduced CRC risk in a Spanish population.[19] Gong *et al.* also reported a decreased risk of colorectal adenomas associated with the 12Ala allele in *PPAR-γ*, with marginal significance.[20] Siezen *et al.* demonstrated a protective effect of the C/T genotype of the C161T polymorphism with reference to colorectal adenomas.[21] On the one hand, recent studies found

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no association between the Pro12Ala polymorphism and the risk of colorectal adenomas, prostate cancers or breast cancers, and there is some evidence that the C161T polymorphism may in fact be related to an increased risk of endometrial and prostate cancers, as well as glioblastoma multiforme.[21–25] Thus, the two common polymorphisms in the *PPAR-γ* gene may play a role in the etiology of cancer, but the results have been equivocal.

We therefore conducted the present study in an Indian population. Moreover, previous studies have shown that n-3 polyunsaturated fatty acids from fish may induce apoptosis in colon cells, and *PPAR-γ* mRNA expression levels were found to be elevated in fish oil-fed animals.[26,27] However, it has remained unclear whether fish consumption mediates effects on CRC development through interactions with *PPAR-γ*. We also investigated potential interactions between the two polymorphisms of the *PPAR-γ* gene and fish consumption with regard to CRC risk.

Methods

Subject selection and data collection

The case-control study was conducted with 301 colorectal cancer patients and 291 controls. All subjects were residents of Chennai and the surrounding area in south-eastern India. Cases were recruited between 1999 and 2001 at the Madras Cancer Institute in Chennai, India, with all patients with a first diagnosis of histologically confirmed colorectal cancer being enrolled. Control subjects were cancer-free individuals, selected among visitors who were attending with patients admitted for having cancers other than CRC during the time period of case collection. They were frequency matched to case patients by sex and age (within 5 years). Informed consent was obtained from all study subjects. Trained interviewers collected information on the socioeconomic status, medical histories, alcohol drinking habit, and smoking and tobacco-chewing habits using a standard questionnaire. A 114 food and beverage item food-frequency questionnaire (FFQ) specific to this population was used to measure long-term intake of foods and food groups. Interviewers asked the subjects about the average frequency of consumption of food items per week over the past 1-year period (for cancer cases, this was 1 year before the diagnosis of CRC). Foods and food groups were categorized as follows: cereals and breads ($n = 11$ food items), beans ($n = 6$), vegetables ($n = 22$), meats ($n = 4$, including mutton, beef, pork and chicken), fish ($n = 7$, including river fish, sea fish and shellfish), fruit ($n = 13$), dairy products and eggs ($n = 10$), beverages ($n = 6$), snacks and desserts ($n = 18$), spices ($n = 7$) and oil ($n = 10$). After the interview, 7 mL blood from each fasting subject was collected and stored at −80°C. The internal review board of the Madras Cancer Institute in Chennai approved the study.

Genotyping

DNA samples of subjects were extracted from peripheral blood leukocytes. To assess *PPAR-γ* genotypes, we used polymerase chain reaction to amplify the regions of the *PPAR-γ* gene that contain the Pro12Ala substitution and the C161T transition.[3,18] A 270-bp fragment including Pro12Ala was amplified using forward primers (5′-GCCAATTCAAGCCCAGTC-3′) and reverse primers (5′-GATATGTTTGCAGACAGTGTATCAGT-GAAGGAATCGCTTTCCG-3′), the Pro12Ala change creating a restriction site for the BstU-I enzyme. The expected products after digestion with BstU-I were 270 bp for Pro/Pro, 227 and 43 bp for Ala/Ala, and 270, 227 and 43 bp for Pro/Ala. A 200-bp fragment of C161T was amplified using forward and reverse primers (5′-CAAGACAACCTGCTACAAGC-3′ and 5′-TCCTTGTAGATCTCCTGCAG-3′, respectively), then digested with the PmlI restriction endonuclease. This resulted in two fragments (120 bp and 80 bp) for the wild type and one fragment (200 bp) when the restriction site was eliminated by the C161T transition. For quality control purposes, negative and positive controls were processed with each batch of samples. In addition, 10% of the subjects had their samples rerun to ensure agreement with the initial results.

Statistical analysis

We investigated the relationship between *PPAR-γ* genotypes and risk of CRC with the STATA statistical package (version 8.0; Stata Corporation, College Station, TX, USA). Differences of characteristics between cases and controls were assessed using the χ^2-test, as well as disparities of genotype and allele frequencies between the two groups. The Hardy–Weinberg equilibrium was checked using the χ^2-test. Unconditional logistic regression analysis was employed to estimate the odds ratios (OR) and confidence intervals (95% CI) for the association between genotypes and risk of CRC. Adjustments were made for matching variables (age, sex) and for possible confounders. Covariates were identified as potential confounders by examining their distribution by case-control status. As body mass index (BMI) in some cases were affected by the cancer, BMI was excluded from covariates to avoid information bias. The covariates were included in the model if they changed the OR by more than 20% or significantly changed the likelihood ratio statistic ($P < 0.05$) on univariate analysis. For all associations of genotypes with CRC, those subjects who were homozygous for the wild-type allele served as a reference. To increase statistical power, rare homozygotes were combined with heterozygotes assuming a dominant effect as their risk estimates were similar. To estimate linkage disequilibrium between *PPAR-γ* variants, pairwise linkage disequilibrium coefficients (D′) were calculated with the LINKAGE program.[28] The 'hapipf' command within STATA, which uses the expectation-maximization algorithm to resolve phase combined with a log-linear model, was used to estimate haplotype frequencies.[29] The χ^2-test was used to compare the distribution of haplotypes between cases and controls. The likelihood ratio test was used to examine the interaction among variables with respect to the risk of CRC. All statistical tests were two-sided and differences were considered to be statistically significant at $P < 0.05$.

Results

Demographic and lifestyle characteristics for the 301 colorectal cancer and 291 control subjects are shown in Table 1. In general, the CRC cases had a smaller BMI, a lower family income, and a higher prevalence of family history of CRC, and smoked more tobacco than the controls. In our population, after adjustment for sex, age, smoking habit, family history and family income, consumption of vegetables and fruit yielded a significant reduction of CRC risk ($P_{trend} = 0.001$ for vegetable intake, and $P_{trend} = 0.01$ for fruit intake). Fish intake

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Table 1. Characteristics of the colorectal cancer (CRC) patients and control subjects

	Cases (%) (n = 301)	Controls (%) (n = 291)	$P^{\dagger}$
Male	196 (65.1)	183 (62.9)	NS
Age (years)			
20–44	107 (35.6)	111 (38.1)	NS
45–59	109 (36.2)	121 (41.6)	
60–75	85 (28.2)	59 (20.3)	
BMI (kg/m^2)			
< 20.0	153 (50.8)	109 (37.5)	< 0.01
20.0–24.9	110 (36.6)	111 (38.1)	
≥ 25.0	38 (12.6)	71 (24.4)	
Education (years)			
< 5	104 (34.5)	88 (30.2)	NS
5–11	155 (51.5)	163 (56.0)	
> 11	42 (14.0)	40 (13.8)	
Religion			
Hindu	265 (88.0)	256 (88.0)	NS
Muslim	23 (7.7)	27 (9.3)	
Christian	13 (4.3)	8 (2.7)	
Family income (rupees/week)			
< 500	143 (47.5)	97 (33.3)	< 0.05
501–1300	69 (22.9)	101 (34.7)	
> 1300	89 (29.6)	93 (32.0)	
Smoking habit (pack-years)			
0	240 (79.7)	227 (78.0)	< 0.01
≤ 10	41 (13.6)	58 (19.9)	
> 10	20 (6.7)	6 (2.1)	
Drinking habit	56 (18.6)	56 (19.2)	NS
Tobacco chewing habit	39 (13.0)	28 (9.6)	NS
Family history of CRC	4 (1.3)	0	< 0.05
Vegetable intake (servings/day)			
< 2	117 (38.9)	65 (22.3)	< 0.01
2–3	109 (36.2)	111 (38.2)	
> 3	75 (24.9)	115 (39.5)	
Fruit intake (servings/week)			
< 4	132 (43.8)	102 (35.1)	< 0.05
4–8	126 (41.9)	129 (44.3)	
> 8	43 (14.3)	60 (20.6)	
Meat intake (servings/week)			
< 2	236 (78.4)	237 (81.4)	NS
≥ 2	65 (21.6)	54 (18.6)	
Fish intake (servings/week)			
< 2	251 (83.4)	219 (75.3)	< 0.05
≥ 2	50 (16.6)	72 (24.7)	

†Examined using the χ^2-test. BMI, body mass index; NS, not significant.

was related to a decreased risk of 0.63 (95% CI: 0.42–0.95), when comparing subjects who consumed two servings per week with those consuming less than two servings per week. In contrast, high meat intake (two servings per week) relative to low meat intake (less than two servings per week) conferred an increased risk (OR = 1.45, 95% CI: 0.92–2.35).

The genotype frequencies and association between the two polymorphisms and risk of CRC are shown in Table 2. The distribution of the observed genotypes did not deviate from the Hardy–Weinberg equilibrium for either the Pro12Ala (P = 0.77 in cases, and P = 0.83 in controls) or C161T (P = 0.33 in cases, and P = 0.71 in controls) polymorphisms. For the Pro12Ala polymorphism, the Pro/Pro, Pro/Ala and Ala/Ala genotype frequencies were 79.7%, 18.9%, and 1.3%, respectively, in the cancer cases compared with 79.0%, 19.6%, and 1.4%, respectively, for the controls. For the C161T polymorphism, the C/C, C/T and T/T genotype frequencies were 69.8%, 26.6% and 3.6%, respectively, in the cancer cases compared with 76.0%, 22.7% and 1.3%, respectively, for the controls. No significant differences in the genotype distribution of the Pro12Ala and C161T polymorphisms were observed between the cases and controls (P = 0.98 and P = 0.09). The T allele frequency for the C161T polymorphism was greater among cancer patients than controls (0.169 vs 0.127, P = 0.04), but no difference in the Ala allele frequency with the Pro12Ala polymorphism was found (0.108 vs 0.112).

After adjustment for sex, age, smoking habit, family history and family income, the OR was 1.52 (95% CI: 1.02–2.25) for the C/T genotype, and 2.71 (95% CI: 0.82–8.99) for the T/T genotype compared to the C/C genotype with the C161T polymorphism. When the C/T genotype and T/T genotypes were grouped, the OR was 1.61 (95% CI: 1.10–2.36). This association was essentially the same when colon and rectal cancers were analyzed separately. Compared to the Pro/Pro genotype, the OR was 1.07 (95% CI: 0.70–1.63) for the Pro/Ala genotype, and 1.02 (95% CI: 0.25–4.28) for the Ala/Ala genotype. When the Pro/Ala and Ala/Ala genotypes were grouped, the OR was 1.06 (95% CI: 0.70–1.61). Calculations based on the prevalence of the two polymorphisms and the size of our study population suggested an 80% power to detect an association at the 5% significance level (two-sided test) if the Pro12Ala and C161T polymorphisms conferred at least a two-fold increased risk (carriers of at least one variant allele vs no variant allele).

The haplotype frequency was computed from genotype data and the results are presented in Table 3. Linkage disequilibrium between Pro12Ala and C161T polymorphisms was observed ($D' = 0.69$, $\chi^2 = 234$ and $P < 0.001$ in controls; $D' = 0.88$, $\chi^2 = 282$ and $P < 0.001$ in cancer cases). A significant difference in haplotype frequencies between cancer cases and controls was found ($\chi^2 = 11.62$, $P = 0.009$, d.f. = 3). The frequency of the Pro-T haplotype (Pro allele for Pro12Ala and T allele for C161T) was higher in cancer cases than in controls (7.6 vs 3.9%). In contrast, the frequency of the Ala-C haplotype was lower (1.1% vs 2.7%).

Table 4 presents data for associations between the two polymorphisms in the *PPAR-γ* gene and CRC risk stratified for fish intake. A significant association between the C/T + T/T genotype in the C161T polymorphism and CRC risk limited to the subgroup of those who had a low fish intake was found. For the C/T + T/T genotype, high fish intake decreased the risk from 1.85 (95% CI: 1.20–2.89) to 0.69 (95% CI: 0.32–1.50). The P-value for the interaction was 0.10. There were no significant interactions between fish intake and the Pro12Ala polymorphism with regard to CRC risk.

Discussion

The present investigation, conducted to explore associations between the Pro12Ala and C161T polymorphisms in the *PPAR-γ* gene and CRC in an Indian population, showed the C/T + T/T genotype to be associated with a significant 1.61-fold increase in the OR compared with the C/C genotype with the C161T polymorphism. Analysis of the Pro-T haplotype

Table 2. Odds ratios (OR) for colorectal cancer (CRC) with reference to the *PPAR-γ* genetic polymorphisms

Variable	All cases		Colon cancer		Rectal cancer		Controls
	n	OR† (95% CI)	*n*	OR† (95% CI)	*n*	OR† (95% CI)	*n*
Pro12Ala							
Pro/Pro	240	1.00 (reference)	46	1.00 (reference)	194	1.00 (reference)	230
Pro/Ala	57	1.07 (0.70–1.63)	13	1.36 (0.66–2.78)	44	0.99 (0.63–1.57)	57
Ala/Ala	4	1.02 (0.25–4.28)	0	NA	4	1.27 (0.30–5.36)	4
Pro12Ala (grouped)							
Pro/Pro	240	1.00 (reference)	46	1.00 (reference)	194	1.00 (reference)	230
Pro/Ala + Ala/Ala	61	1.06 (0.70–1.61)	13	1.20 (0.59–2.43)	48	1.01 (0.65–1.58)	61
C161T							
C/C	210	1.00 (reference)	37	1.00 (reference)	173	1.00 (reference)	221
C/T	80	1.52 (1.02–2.25)	19	1.95 (0.99–3.81)	61	1.42 (0.96–2.18)	66
T/T	11	2.71 (0.82–8.99)	3	3.09 (0.53–18.06)	8	2.61 (0.75–9.07)	4
C161T (grouped)							
C/C	210	1.00 (reference)	37	1.00 (reference)	173	1.00 (reference)	221
C/T + T/T	91	1.61 (1.10–2.36)	22	2.00 (1.05–3.81)	69	1.50 (1.01–2.26)	70

†Adjusted for sex, age, smoking habit, family history and family income. CI, confidence interval; NA, not available.

Table 3. Haplotype frequencies for the *PPAR-γ* gene in the colorectal cancer patients and control subjects

	Frequency among cases	Frequency among controls	*P*§
PPAR-γ haplotype†			
Pro-C	0.816	0.849	0.009
Pro-T	0.076	0.039	
Ala-C	0.011	0.027	
Ala-T	0.097	0.085	
Disequilibrium			
D'‡	0.881	0.686	
χ^2	282	234	
P	0.0001	0.0001	

†The order of single-nucleotide polymorphisms in the haplotypes is Pro12Ala-C161T. ‡Pair-wise linkage disequilibrium coefficients. §The χ^2-test was used to compare the distribution of *PPAR-γ* haplotypes between cases and controls.

strengthened the relationship in our study population, and this proved consistent for both the colon and rectum.

However, evidence concerning the relationship between the C61T polymorphism and cancer is still limited and controversial. A protective effect on colorectal adenomas was earlier found for the C/T genotype of the C161T polymorphisms in *PPAR-γ*,[21] but other studies have shown an increased risk.[24,25] Clearly, functional aspects require further assessment.

Three hypotheses may be proposed for how CRC might be affected by the polymorphisms examined here. First, a new cryptic splice donor, acceptor or enhancer may be created by this C/T substitution, with decreased expression of the variant bearing the T allele, thus leading to a low level of functional activity. Alternatively, the substitution may influence the stability of the mRNA species. Second, it is reported that the T allele of the C161T polymorphism is associated with elevated plasma levels of leptin,[30] a 16 kDa adipokine that regulates proinflammatory immune responses,[31] and may be a growth factor for colonic epithelial cells.[32] Case-control studies have in fact suggested that leptin is a risk factor for colorectal cancer.[33,34] Third, C161T polymorphism may be in linkage disequilibrium with functional mutations in other *PPAR-γ* gene exons, or other unidentified genes near the *PPAR-γ* gene. Controversial results have been obtained for the associations between the C161T polymorphism and risk of colorectal adenomas.[21] Reasons for disagreements may be due in part to differences in study populations.

Analysis with estimated haplotypes showed the Pro-T haplotype to be more prevalent in cancer cases than in controls (7.6% vs 3.9%). Although relatively uncommon because

Table 4. Odds ratios for interactions between *PPAR-γ* genotypes and colorectal cancer stratified by fish intake

	Low fish intake†		*P*	High fish intake†	
	Cases/controls (*n*)	OR‡ (95% CI)		Cases/controls (*n*)	OR‡ (95% CI)
Pro12Ala					
Pro/Pro	197/176	1.00 (reference)		43/54	0.74 (0.46–1.18)
Pro/Ala + Ala/Ala	54/43	1.14 (0.72–1.81)		7/18	0.51 (0.20–1.27)
Interaction			0.36		
C161T					
C/C	171/173	1.00 (reference)		39/48	0.83 (0.51–1.36)
C/T + T/T	80/46	1.85 (1.20–2.89)		11/24	0.69 (0.32–1.50)
Interaction			0.10		

†Low fish intake, less than two servings per week; high fish intake, greater than two servings per week. ‡Adjusted for sex, age, smoking habit, family history, family income and consumption of meat, vegetables and fruit. CI, confidence interval; OR, odds ratio.

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of linkage disequilibrium between the two polymorphisms, its importance was also reported in two other studies regarding bodyweight and colorectal adenomas.[21,35] The validity of haplotype inference varied, depending on a number of factors, including sampling error, sample size, number of loci studied, allele frequencies, locus-specific allelic departures from the Hardy–Weinberg equilibrium and the linkage-disequilibrium structure of the region.[36] In the present study, to prevent genotyping bias, negative and positive controls were processed with each batch of samples, and 10% of the subjects had their samples rerun to ensure agreement with the initial results. Haplotype block predictions were based on all the complete genotype data available for the cases and controls with use of the expectation-maximization algorithm. In addition, the state of sample size (cases = 301, controls = 291), number of loci studied (two loci), allele frequencies (minor allele of the two polymorphisms < 0.17), Hardy–Weinberg equilibrium ($P = 0.83$ for Pro12Ala and $P = 0.71$ for C161T in controls; $P = 0.77$ for Pro12Ala and $P = 0.33$ for C161T in cancer cases), and linkage disequilibrium ($D' = 0.69$ in controls, and $D' = 0.88$ in cancer cases) in our study also supported the validity of haplotype estimation.[36] Certainly, more detailed investigations will be necessary to allow accurate haplotype inferences in future.

The Pro12Ala polymorphism in the *PPAR-γ* gene has been investigated in breast, prostate, lung and endometrial cancers and colorectal adenomas, but no significant associations were detected.[21–23,37,38] Landi *et al.* have reported the Ala allele to be related to a reduced risk of CRC in a hospital-based case-control study.[19] We could not confirm this finding in our study population. Tomita *et al.* have reported that the Pro12Ala polymorphism might be implicated in development of CRC in which the K-*ras* gene is not mutated.[39] Variation in lifestyle patterns or genetic background among the Indian and Spanish populations may explain to some extent the observed differences in risk. Our study used population-based controls, whereas Landi *et al.* adopted hospital controls, among which the Ala allele frequency was greater than in the Spanish general populace (0.11 vs 0.09).[40] In present study, the frequency of the Ala allele was similar to that in the general population in a diabetes study of Singapore Indians (0.112 vs 0.119).[41]

Epidemiological studies have shown that fish consumption is protective against CRC,[42] and the risk-reducing effects were also found in the present study. However, there were no significant interactions between the two polymorphisms and fish consumption in CRC development. Because only 20% of the study subjects consumed more than two servings of fish per week, this combined with the low allele frequency of the two polymorphisms may account for the non-significant results. In addition, as exposure information was collected after the diagnosis of CRC, differential dietary recall between cases and controls could yield biased results. Further larger studies in future are clearly warranted. While it is known that two polymorphisms in the *PPAR-γ* gene can affect the susceptibility to diabetes,[2,18] we failed to collect data on this disease in the present study.

In conclusion, our investigation here indicated that the *PPAR-γ* gene C161T substitution might be associated with an increased CRC risk, but no significant link was observed for the Pro12Ala polymorphism. As little is known about the underlying physiology, further genetic and epidemiological studies of the *PPAR-γ* gene loci and other associated genes should be conducted with an emphasis on functional aspects.

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Dietary intakes of fat and fatty acids and risk of breast cancer: A prospective study in Japan

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To examine the possible association of dietary fat and fatty acids with breast cancer risk in a population with a low total fat intake and a high consumption of fish, we analyzed data from the Japan Collaborative Cohort (JACC) Study. From 1988 to 1990, 26 291 women aged 40–79 years completed a questionnaire on dietary and other factors. Intakes of fat or fatty acids were estimated by using a food frequency questionnaire. Rate ratios (RR) were computed by fitting proportional hazards models. During the mean follow-up of 7.6 years, 129 breast cancer cases were documented. We found no clear association of total fat intake with breast cancer risk; the multivariate-adjusted RR across quartiles were 1.00, 1.29, 0.95, and 0.80 (95% confidence interval [CI] 0.46–1.38). A significant decrease in the risk was detected for the highest quartile of intake compared with the lowest for fish fat and long-chain n-3 fatty acids; the RR were 0.56 (95% CI 0.33–0.94) and 0.50 (0.30–0.85), respectively. A decreasing trend in risk was also suggested with an increasing intake of saturated fatty acids (trend $P = 0.066$). Among postmenopausal women at baseline, the highest quartile of vegetable fat intake was associated with a 2.08-fold increase in risk (95% CI 1.05–4.13). This prospective study did not support any increase in the risk of breast cancer associated with total or saturated fat intake, but it suggested the protective effects of the long-chain n-3 fatty acids that are abundant in fish. (*Cancer Sci* 2005; *96*: 590–599)

High intakes of total dietary fat have been postulated to increase breast cancer risk based on both animal experiments[1–3] and international ecological studies.[1,4] Dietary fat has been shown to be a promoter of mammary carcinogenesis,[1–3] and a strong positive correlation (0.7 or higher) has been reported between per capita fat consumption and the national incidence and mortality of breast cancer.[1,4] Many case-control and cohort studies have been conducted to address this hypothesis, but they have yielded contradictory results, so the role of dietary fat in the etiology of human breast cancer remains controversial.[5] Almost all large prospective studies have been undertaken in Western countries where total fat intake is rather high.[6] As some authors have suggested,[1,7] clear associations may not have been observed in Western populations because fat intake is so high that most study subjects may have had fat levels over the threshold for breast cancer risk.

Another possible explanation for the inconsistent results is that the intake of specific types of fat or fatty acids rather than the intake of total fat may influence breast cancer risk. N-3 fatty acids abundant in fish fat, particularly eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), have recently attracted attention.[8] They have been consistently shown to inhibit the proliferation of breast cancer cell lines *in vitro* and to suppress the progression of the tumors in animal experiments in part by inhibiting eicosanoid biosynthesis from arachidonic acid or by activating peroxisome-proliferator activated receptor-γ.[8] Furthermore, a cross-national ecological study demonstrated an inverse association between fish fat intake and the mortality rate of breast cancer.[4] Case-control or cohort studies, however, have reported conflicting findings on the association between the intake of fish and/or long-chain n-3 fatty acids and breast cancer risk.[8] In most of these studies conducted in countries with low fish consumption, fish fat intake among subjects may have been too low to detect the expected protective effects. Terry *et al.* pointed out that the null studies were often undertaken in areas with low consumption of n-3 fatty acids.[8] Japanese people may consume enough fish to test the hypothesis, because their intakes of fish fat[4] or n-3 fatty acids[8] are 2–40 times higher than those reported from Western countries.

However, diets high in n-6 fatty acids, particularly linoleic acid, increase chemically induced mammary gland carcinogenesis

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in rats.[3,9] The cyclooxygenase and lipoxygenase products of n-6 fatty acid metabolism may correlate with the growth of breast cancer cells.[9] N-6 fatty acids can also enhance mammary tumorigenesis by inhibiting the cellular gap junctions.[10] Nevertheless, the findings in laboratory animals have not necessarily been supported by case-control or cohort studies using a food frequency questionnaire[11,12] or biomarkers.[10]

To further examine the association of dietary fat and fatty acids with the risk of breast cancer in a population with a low total fat intake and a high consumption of fish, we analyzed the data from the Japan Collaborative Cohort Study (JACC) for Evaluation of Cancer Risk Sponsored by Monbusho (the Ministry of Education, Culture, Sports, Science and Technology of Japan).

Materials and Methods

The JACC Study

The JACC Study started in 1988–1990, during which period 110 792 male and female subjects aged 40–79 years completed a baseline questionnaire. The details of this study are described elsewhere.[13,14] In brief, participants were enrolled from 45 study areas throughout Japan, from general populations or participants in municipal health check-ups. In the JACC Study, researchers interested in initiating a multicenter cohort who could recruit subjects voluntarily participated in the study. The enrollment of subjects fell to each investigator, and study areas were arbitrarily defined. Thus, the background characteristics of areas (e.g. sea areas or agricultural areas) could not be considered in selecting study areas.

Informed consent for participation was obtained individually from each participant, except in a few study areas where informed consent was provided at the group level after the aim of the study and confidentiality of the data had been explained to community leaders. The Ethical Board of the Nagoya University School of Medicine approved the protocol of this investigation, including the procedures used to obtain informed consent.

Potential participants for the present analysis were restricted to 36 035 women who lived in 22 study areas where information on cancer incidence is available, and for whom a food frequency questionnaire (FFQ) to estimate food and nutrient intake was included in the baseline questionnaire.

Diet and other exposure data

The baseline questionnaire covered lifestyle factors including dietary habits, smoking and drinking, and physical activity, as well as medical history, education, family history of cancer, height and weight, and female reproductive factors.

The dietary component of the questionnaire included 40 food items.[15] For 33 foods or dishes, we asked about the average intake frequency without specifying portion size information. For rice, miso (fermented soybean paste) soup, and four non-alcoholic beverages, the number of bowls or cups consumed per day was inquired about. The frequency of alcohol consumption was asked about with respect to the usual amount consumed on any one occasion. Nutrient intakes were computed using the Japanese food composition table, assuming standard portion sizes. The portion sizes were not modified according to age and sex.

Energy-adjusted intakes of nutrients, including total fat and several types of fat or fatty acids, were calculated by using the residual method.[16] Natural logarithms of energy and nutrient intakes were used to improve the normality of their distribution, except for the ratio of n-6 fatty acid intake to that of n-3 fatty acids.

The FFQ was validated by referring to four 3-day weighed dietary records over a 1-year period as a standard.[15] Due to the limited number of food items, the FFQ underestimated intakes of total energy by 33%, but it was still able to appropriately rank respondents according to intakes of several nutrients. We reanalyzed data from the validation study to consider skewed distributions of nutrient intakes and within-person variation in intakes.[17] The de-attenuated correlation coefficients for energy-adjusted intakes between the FFQ and dietary records were 0.54 for total fat, 0.73 for animal fat, 0.43 for vegetable fat, 0.45 for fish fat, 0.57 for saturated fatty acids (SFA), 0.48 for monounsaturated fatty acids (MUFA), 0.29 for polyunsaturated fatty acids (PUFA), 0.36 for n-3 PUFA, 0.33 for n-6 PUFA, 0.37 for the n-6/n-3 ratio, and 0.48 for long-chain n-3 fatty acids (sum of EPA, docosapentaenoic acid [n-3], and DHA). Although the validity estimate for total energy was not high (crude correlation coefficient 0.24), the energy-adjusted nutrient intakes derived from the FFQ were scarcely correlated with energy intake from dietary records in the validation study: the correlation coefficients ranged from −0.08 to 0.01 for nutrients examined in the present study. This indicates that the adjustment for energy intake can be conducted using the energy intake estimated by the FFQ.

Of the 36 035 potential participants, we excluded 277 women with a history of breast cancer, 9377 without sufficient responses to the FFQ to estimate nutrient intake (judged by predefined criteria), and 90 with an implausibly high or low intake of total energy (< 500 or > 3500 kcal/day), leaving 26 291 women (73.0% of potential participants) eligible for the analysis. Women included in the analysis were younger (mean age ± SD: 56.6 ± 9.9 years), more likely to be more highly educated (proportion educated beyond high school: 11.4%), and had an earlier age at menarche (mean ± SD: 14.8 ± 1.8 years) and lower parity (mean ± SD: 2.6 ± 1.3) than those excluded due to inadequate responses to the FFQ or implausible energy intake (62.7 ± 9.4 years, 9.0%, 15.3 ± 1.8 years, and 2.8 ± 1.5, respectively). Other baseline characteristics, including family history of breast cancer, age at menopause and first birth, use of exogenous female hormones, drinking habits, consumption of green leafy vegetables, walking time, height, and body mass index (BMI), were comparable between the two groups.

Follow-up

We used population registries in the municipalities to determine the vital and residential status of the participants. Registration of death is required by the Family Registration Law in Japan, and is adhered to nationwide. For logistical reasons, we discontinued the follow-up of those who had moved out of their given study areas.

We ascertained the incidence of cancer by means of a linkage with the records of population-based cancer registries, supplemented by a systematic review of death certificates. In some study areas, medical records in major local hospitals were also reviewed. In three areas out of 22, population-based

cancer registries were not available. Therefore, hospital-based cancer registries or inpatient records of hospitals treating cancer patients were used to collect information on cancer incidence in such areas. The follow-up was conducted from the time of the baseline survey through to the end of 1997, except for one area (which was followed up to the end of 1994). During the study period, only 2.7% ($n = 717$) of the subjects were lost to follow-up because they moved away. In the analytic cohort, the proportion of death certificate only (DCO) registrations was 3.9% (five of 129 cases) for breast cancer. The mortality-to-incidence ratio was 0.13, which is lower than that available from representative population-based cancer registries in Japan (0.20–0.30).[18]

Statistical analysis

Baseline BMI was calculated from reported height and weight: BMI = (weight in kg)/(height in m)^2. The difference between two proportions was statistically tested by using the χ^2 test. We counted the person-time of follow-up for each participant from the date of filling out the baseline questionnaire to the date of diagnosis of breast cancer, the date of death from any cause, the date of emigration outside the study area, or the end of the follow-up period, whichever came first. For cases identified only with a death certificate, the date of death was assumed to be that of diagnosis. Those who died from causes other than breast cancer or who moved out of their study areas were treated as censored cases.

The rate ratios (RR) with 95% confidence intervals (CI) for breast cancer over quartiles of energy-adjusted intakes of fat or fatty acids (the RR for the second, third, and highest quartiles versus the lowest) were estimated using proportional hazards models[19] adjusted for age and other potential confounders. The RR were adjusted for age (using 10-year age groups), area (Hokkaido and Tohoku, Kanto, Chubu, Kinki, Chugoku, or Kyushu), educational level (attended school until the age of ≤ 15, 16–18, or ≥ 19 years), family history of breast cancer in mother or sisters (yes or no), age at menarche (≤ 13, 14–15, 16–17, or ≥18 years), age at menopause (premenopausal at baseline, ≤ 44, 45–49, or ≥ 50 years), age at first birth (≤ 24, 25–29, or ≥ 30 years), parity (0, 1, 2, 3, or ≥ 4), use of exogenous female hormones (yes or no), alcohol consumption (never drink, ex-drinker, or current drinker who consumes < 15 or ≥ 15 g of ethanol alcohol daily), smoking habits (never smoked, ex-smoker, or current smoker), consumption of green leafy vegetables (≤ 2 times/week, 3–4 times/week, or almost every day), daily walking habits (seldom or never, or approximately 30, 30–59, or ≥ 60 min/day), height (< 150.0, 150.0–159.9, or ≥ 160.0 cm), BMI (< 20.0, 20.0–24.9, 25.0–29.9, or ≥ 30.0 kg/m^2), and total energy intake (as a continuous variable). We considered walking time because that was the major physical activity undertaken by the study population.[20] The RR and 95% CI for breast cancer by intake frequency of fresh fish or green leafy vegetables were also computed using proportional hazards models with adjustment for the abovementioned variables.

Of the potential confounding variables mentioned here, area, educational level, family history of breast cancer, age at menarche, age at first birth, parity, alcohol drinking, consumption of green leafy vegetables, daily walking time, and BMI were significantly or marginally significantly ($P < 0.10$) correlated with the risk for all women and/or women who were postmenopausal at baseline. In addition to these variables, we also included age at baseline, age at menopause, use of exogenous female hormones, smoking, and height in the multivariate analyses, because these factors have been reported as risk factors for breast cancer.[21–23] We further considered total energy intake when estimating the multivariate-adjusted RR to elucidate the association between fat composition of the diet and breast cancer risk independently of total energy intake.[24]

Missing values for each covariate were treated as an additional category in the variable and were included in the proportional hazards model. As a basis for the trend tests, median values of each quartile of fat or fatty acid intake were included in the model. In the analysis for the level of intake frequency of food, ordinal scores (0, 1, or 2) were used for the tests.

The RR were computed for all women or for those who were postmenopausal at baseline. The cases of breast cancer in women who were premenopausal at baseline were too few to estimate the RR, but the RR for long-chain n-3 fatty acids and vegetable fat were calculated as an exception to compare the figures with those in women who were postmenopausal at baseline. We repeated all the analyses after excluding the first 2 years of follow-up, in which 27 cases of breast cancer were diagnosed. All P-values were two-sided, and all the analyses were performed using the Statistical Analysis System.[25]

Results

Mean intake of total fat was 75% higher in the highest quartile than in the lowest (Table 1). The proportions of highly educated women and daily consumers of green leafy vegetables increased markedly with increasing total fat intake, whereas that of women who were menopausal at baseline decreased with increasing intake. For these three variables, the differences in proportions between the lowest and the highest quartiles of fat intake were highly significant ($P < 0.001$). Although they were not striking, we found decreasing trends in age at baseline, age at menarche, parity, current drinkers, and BMI, and increasing trends in those with a family history of breast cancer, age at menopause, age at first birth, users of exogenous female hormones, and height with increasing intakes of total fat.

Mean fish fat intake differed four-fold between the highest and lowest quartiles of energy-adjusted intakes (Table 2). The percentage of daily consumers of green leafy vegetables increased with increasing intake of fish fat, whereas that of current drinkers slightly declined with an increasing consumption, with significant differences between the lowest and the highest quartiles of fish fat intake ($P < 0.001$ for daily consumers of green leafy vegetables and $P = 0.007$ for current drinkers). Women in the highest intake category were somewhat likely to be menopausal at baseline.

Within the 199 123 person-years of follow-up (mean per person ± SD: 7.6 ± 1.8 years), 129 cases of incident breast cancer were documented. We found no clear association of total fat intake with breast cancer risk (Table 3); the multivariate-adjusted RR across quartiles were 1.00, 1.29, 0.95, and 0.80 (95% CI 0.46–1.38).

A 40% significant decrease in the risk of breast cancer was detected in the highest quartile of fish fat intake compared

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Table 1. Baseline characteristics by quartile of energy-adjusted total fat intake among 26 291 women in the Japan Collaborative Cohort Study, 1988–1997

	Quartile of energy-adjusted total fat intake (% of energy)			
	1 (< 18.44) (n = 6572)	2 (18.44–21.53) (n = 6573)	3 (21.54–24.54) (n = 6573)	4 (≥ 24.55) (n = 6573)
Age (years)	57.2 ± 9.9	56.9 ± 9.9	56.4 ± 9.8	56.0 ± 10.1
Education beyond high school	8.3%	10.1%	12.4%	14.8%
Family history of breast cancer in mother and/or sisters	1.4%	1.6%	1.6%	1.7%
Age at menarche (years)	14.9 ± 1.8	14.8 ± 1.8	14.7 ± 1.8	14.7 ± 1.8
Menopause	71.2%	71.0%	69.3%	65.7%
Age at menopause (years)	48.5 ± 4.8	48.7 ± 4.5	48.8 ± 4.5	48.8 ± 4.6
Age at first birth (years)	24.9 ± 3.4	25.0 ± 3.2	25.2 ± 3.2	25.2 ± 3.1
Parity	2.7 ± 1.3	2.6 ± 1.3	2.6 ± 1.2	2.5 ± 1.2
Ever used exogenous female hormones	4.4%	5.0%	5.7%	5.5%
Alcohol consumption				
Current drinkers	25.7%	25.1%	23.2%	23.0%
Former drinkers	1.7%	1.6%	1.4%	2.0%
Smoking				
Current smokers	6.5%	5.1%	3.8%	4.5%
Former smokers	1.7%	1.5%	1.5%	1.5%
Daily consumer of green leafy vegetables	23.8%	32.1%	38.3%	42.3%
Walking time > 30 min/day	71.9%	72.6%	72.7%	70.3%
Height (cm)	151.0 ± 5.9	151.4 ± 5.8	151.7 ± 5.7	152.0 ± 5.6
Body mass index (kg/m^2)	23.0 ± 3.2	22.9 ± 3.1	22.8 ± 3.0	22.7 ± 3.6
Energy intake (kcal/day)	1294 ± 354	1309 ± 287	1339 ± 281	1293 ± 359
Total fat intake (g/day)	22.4 ± 7.1	29.2 ± 6.6	34.2 ± 7.4	39.4 ± 10.9

Plus-minus values are mean ± SD.

Table 2. Baseline characteristics by quartile of energy-adjusted fish fat intake among 26 291 women in the Japan Collaborative Cohort Study, 1988–1997

	Quartile of energy-adjusted fish fat intake (% of energy)			
	1 (< 1.41) (n = 6572)	2 (1.41–2.19) (n = 6573)	3 (2.20–3.26) (n = 6573)	4 (≥ 3.27) (n = 6573)
Age (years)	56.8 ± 10.2	56.0 ± 10.0	56.4 ± 9.9	57.3 ± 9.6
Education beyond high school	10.8%	11.5%	11.2%	12.1%
Family history of breast cancer in mother and/or sisters	1.5%	1.6%	1.8%	1.2%
Age at menarche (years)	14.8 ± 1.8	14.7 ± 1.8	14.8 ± 1.8	14.9 ± 1.8
Menopause	68.8%	66.2%	68.9%	73.3%
Age at menopause (years)	48.6 ± 4.6	48.6 ± 4.6	48.7 ± 4.6	48.8 ± 4.6
Age at first birth (years)	25.1 ± 3.3	25.1 ± 3.3	25.1 ± 3.2	25.1 ± 3.2
Parity	2.6 ± 1.3	2.6 ± 1.2	2.6 ± 1.2	2.6 ± 1.3
Ever used exogenous female hormones	4.5%	5.3%	5.5%	5.3%
Alcohol consumption				
Current drinkers	25.5%	24.6%	23.5%	23.4%
Former drinkers	1.9%	1.6%	1.5%	1.7%
Smoking				
Current smokers	5.8%	5.0%	4.4%	4.8%
Former smokers	1.7%	1.5%	1.3%	1.7%
Daily consumer of green leafy vegetables	26.8%	29.4%	34.7%	45.6%
Walking time > 30 min/day	71.7%	72.7%	71.3%	71.7%
Height (cm)	151.4 ± 5.8	151.6 ± 5.7	151.5 ± 5.8	151.5 ± 5.7
Body mass index (kg/m^2)	22.8 ± 3.1	22.8 ± 3.0	22.8 ± 3.6	22.9 ± 3.1
Energy intake (kcal/day)	1296 ± 309	1322 ± 334	1319 ± 342	1298 ± 304
Fish fat intake (g/day)	1.4 ± 0.6	2.7 ± 0.8	3.9 ± 1.2	6.1 ± 1.4

Plus-minus values are mean ± SD.

with the lowest quartile. The highest intake of long-chain n-3 fatty acids was associated with a halved risk. A decreasing trend in risk was also suggested, with an increasing SFA intake after adjustments for age and other potential confounders (trend $P = 0.066$). A further adjustment for SFA intake did not materially alter the reduced risk associated with fish fat or long-chain n-3 fatty acids; the RR (95% CI) across the quartiles of intake were: 1.00, 0.72 (0.45–1.16), 0.82 (0.51–1.31), and 0.59 (0.35–0.99) for fish fat (trend $P = 0.067$); and 1.00, 0.70 (0.43–1.13), 0.85 (0.54–1.35), and 0.53 (0.31–0.91) for long-chain n-3 fatty acids (trend $P = 0.044$). The findings for age-adjusted RR did not appreciably differ from those for the multivariate RR, except for a weaker association between SFA and breast cancer risk.

Furthermore, the intake frequency of fresh fish tended to be negatively associated with the risk (data not shown in the table); the multivariate-adjusted RR (adjusted for the same covariates as those in Table 3) were 0.81 (95% CI 0.54–1.22) for 3–4 times per week, and 0.63 (0.38–1.03) for almost every day, compared with two times per week or less (trend $P = 0.061$). A decreasing trend in risk was also observed with increasing frequency of consumption of green leafy vegetables; the RR, relative to two times per week or less, were 0.76 (95% CI 0.50–1.15) for 3–4 times per week, and 0.67 (0.43–1.04) for almost every day (trend $P = 0.067$).

Also in the analysis limited to participants who were postmenopausal at baseline (Table 4), total fat intake was not correlated with breast cancer risk. A 40–50% decrease in risk, although not statistically significant, was found for the highest quartile of intake of fish fat and long-chain n-3 fatty acids. An inverse association between SFA intake and the risk was also suggested by this analysis. In addition, women in the highest quartile of vegetable fat intake had twice the risk of those in the lowest group, and a significant, upward trend in risk was found with increasing intake. A similar elevating trend in risk was suggested for PUFA (trend $P = 0.071$).

For women who were premenopausal at baseline, the RR for higher quartiles of long-chain n-3 fatty acids were smaller than unity, but far from significance: the multivariate-adjusted RR (adjusted for the same covariates as those in Table 4 except for age at menopause) across quartiles were 1.00, 0.79 (95% CI 0.35–1.76), 0.77 (0.34–1.72), and 0.75 (0.33–1.68) (trend $P = 0.48$). An elevated risk associated with higher consumption of vegetable fat was not observed in this group of subjects; the RR over quartiles of intake were 1.00, 0.75 (95% CI 0.34–1.69), 0.95 (0.43–2.10), and 0.71 (0.30–1.73) (trend $P = 0.56$). All the findings in Tables 3 and 4 remained essentially the same when we excluded the first 2 years of follow-up from the analyses.

Discussion

In this prospective cohort study in Japan, we found no clear association of total fat intake with breast cancer risk. Intakes of fish fat and long-chain n-3 fatty acids were associated with a lower risk of breast cancer. A decreasing trend in risk was suggested with increasing SFA intake. In women who were postmenopausal at baseline, those with the highest intake of vegetable fat had an increased risk of breast cancer.

In general, the risk of breast cancer increases with age in Western countries.[18] When the crude incidence rate of breast cancer by menopausal status at baseline in our study was compared with the rate in another cohort study in Japan, the Japan Public Health Center-based Prospective (JPHC) Study,[26] the rates (per 100 000 person-years) were lower in our study than in the JPHC Study for both pre- and postmenopausal women: 76.4 in premenopausal women and 58.6 in postmenopausal women in the JACC Study, and 95.1 and 77.9, respectively, in the JPHC Study. The rate among premenopausal women, however, was higher than that among postmenopausal women also in the JPHC Study. Furthermore, the incidence rate of breast cancer peaks at age 45–49 in Japan.[27] The premenopausal dominance therefore is rather common in this country.

Our findings are in line with those from a pooled analysis of large cohort studies that reported no positive association of total fat or SFA with breast cancer risk.[11] However, we cannot exclude the possibility of a modest increase in risk associated with the highest level of total fat intake, for example 13%, as shown in the updated meta-analysis of 45 case-control or cohort studies,[6] considering the upper limit of 95% CI (1.38) for the highest quartile of fat intake in the present study.

It is unlikely, however, that total or saturated fat is more markedly linked with breast cancer risk in the low-intake range. When the percentage of energy from fat measured by dietary record was regressed on that measured by the FFQ using data from the validation study, the percentage was found to be underestimated by the FFQ. From the regression model, the median percentage of energy from fat in the present cohort would actually be 26.4% rather than 21.5%. Although this is comparable with the value in middle-aged Japanese women,[28] it is far lower than that in most Western populations, where the means or medians are often greater than 30%.[4,11] In a pooled analysis of large cohort studies in Western countries, fewer than 10% of women had fat intakes of 25% or less of total energy.[11]

Several case-control studies have suggested the protective effects of fish intake against breast cancer, particularly among postmenopausal women,[29–32] although only among premenopausal women in one study.[33] For example, Hirose *et al.* found a decreased risk in relation to frequent consumption of fish, particularly in postmenopausal women.[31] Maillard *et al.*[34] and Bagga *et al.*[35] confirmed the beneficial effect of long-chain n-3 fatty acids using breast adipose tissue as a biomarker. Our prospective data support these findings. In a Norwegian cohort, frequent consumption of poached fish was associated with a decreased risk of breast cancer.[36] High levels of dietary n-3 fatty acids from fish or shellfish were associated with a reduced risk in a prospective study of Chinese Singaporean women.[37] A meta-analysis of three cohort studies using the fatty acid composition of serum phospholipids or erythrocyte membrane related a 34% lower risk of postmenopausal breast cancer to the higher level of DHA.[10]

Other cohort studies, however, have found either no clear association[38–40] or actually an increased risk.[41,42] Wirfält *et al.*[39] found no association between breast cancer risk and fatty acids of erythrocyte membranes in postmenopausal women in a Swedish cohort not involved in the abovementioned meta-analysis.[10] Holmes and coworkers reported a 9% increase in risk for a 0.1% increase in energy from n-3 fat from fish in their Nurses' Health Study.[41]

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Table 3. Rate ratios (RR) with 95% confidence intervals (CI) for breast cancer by quartile of fat and fatty acid intake among all women in the Japan Collaborative Cohort Study, 1988–1997 (n = 26 291)

Quartile of intake (% of energy)‡	No. cases	Age-adjusted			Multivariate-adjusted†		
		RR	95% CI	*P* for trend	RR	95% CI	*P* for trend
Total fat				0.35			0.32
1 < 18.44	31	1.00			1.00		
2 18.44–21.53	41	1.31	0.82–2.08		1.29	0.80–2.08	
3 21.54–24.54	31	0.98	0.59–1.61		0.95	0.57–1.59	
4 ≥ 24.55	26	0.82	0.49–1.38		0.80	0.46–1.38	
Animal fat				0.34			0.13
1 < 7.41	34	1.00			1.00		
2 7.41–9.57	34	1.00	0.62–1.60		0.90	0.56–1.46	
3 9.58–11.78	37	1.09	0.68–1.73		0.96	0.60–1.56	
4 ≥ 11.79	24	0.71	0.42–1.20		0.61	0.36–1.06	
Vegetable fat				0.83			0.49
1 < 7.83	31	1.00			1.00		
2 7.83–9.40	32	1.00	0.61–1.63		1.06	0.64–1.76	
3 9.41–10.91	32	0.99	0.60–1.63		1.08	0.65–1.81	
4 ≥ 10.92	34	1.06	0.65–1.74		1.21	0.72–2.02	
Fish fat				0.034			0.042
1 < 1.41	41	1.00			1.00		
2 1.41–2.19	30	0.71	0.44–1.14		0.71	0.44–1.14	
3 2.20–3.26	34	0.80	0.51–1.26		0.80	0.50–1.27	
4 ≥ 3.27	24	0.55	0.33–0.92		0.56	0.33–0.94	
Saturated fatty acids				0.21			0.066
1 < 5.25	34	1.00			1.00		
2 5.25–6.36	42	1.23	0.78–1.93		1.13	0.72–1.79	
3 6.37–7.44	26	0.76	0.46–1.27		0.68	0.40–1.14	
4 ≥ 7.45	27	0.80	0.48–1.33		0.68	0.40–1.15	
Monounsaturated fatty acids				0.17			0.19
1 < 5.50	34	1.00			1.00		
2 5.50–6.48	34	0.99	0.61–1.59		0.96	0.59–1.55	
3 6.49–7.54	39	1.11	0.70–1.77		1.10	0.68–1.77	
4 ≥ 7.55	22	0.62	0.36–1.06		0.62	0.36–1.09	
Polyunsaturated fatty acids				0.44			0.83
1 < 4.39	32	1.00			1.00		
2 4.39–5.21	35	1.03	0.63–1.66		1.13	0.69–1.86	
3 5.22–6.02	31	0.88	0.53–1.44		1.01	0.60–1.71	
4 ≥ 6.03	31	0.85	0.52–1.40		1.10	0.63–1.90	
n-3 fatty acids				0.10			0.26
1 < 0.86	37	1.00			1.00		
2 0.86–1.06	31	0.80	0.50–1.29		0.84	0.51–1.36	
3 1.07–1.31	36	0.91	0.57–1.44		0.95	0.59–1.53	
4 ≥ 1.32	25	0.62	0.37–1.03		0.69	0.40–1.18	
n-6 fatty acids				0.35			0.96
1 < 3.46	34	1.00			1.00		
2 3.46–4.11	32	0.88	0.54–1.43		0.95	0.58–1.57	
3 4.12–4.77	32	0.85	0.52–1.37		0.96	0.58–1.61	
4 ≥ 4.78	31	0.80	0.49–1.30		1.02	0.59–1.74	
n-6/n-3 ratio				0.23			0.13
1 < 3.25	28	1.00			1.00		
2 3.25–3.90	26	0.91	0.54–1.56		0.95	0.55–1.62	
3 3.91–4.60	41	1.45	0.90–2.35		1.57	0.97–2.56	
4 ≥ 4.61	34	1.20	0.73–1.99		1.31	0.78–2.19	
Long-chain n-3 fatty acids				0.017			0.024
1 < 0.29	42	1.00			1.00		
2 0.29–0.42	29	0.67	0.42–1.08		0.68	0.42–1.10	
3 0.43–0.60	36	0.82	0.53–1.28		0.83	0.52–1.30	
4 ≥ 0.61	22	0.50	0.30–0.83		0.50	0.30–0.85	

†Adjusted for age, study area, educational level, family history of breast cancer, age at menarche, age at menopause, age at first birth, parity, use of exogenous female hormones, alcohol consumption, smoking, consumption of green leafy vegetables, daily walking, height, body mass index, and total energy intake.
‡Fat and fatty acid intake were adjusted to mean energy intake of 1309 kcal/day (5476 kJ/day).

Table 4. Rate ratios (RR) with 95% confidence intervals (CI) for breast cancer by quartile of fat and fatty acid intake among women postmenopausal at baseline in the Japan Collaborative Cohort Study, 1988–1997 (n = 17 538)

Quartile of intake (% of energy)[‡]	No. cases	Age-adjusted			Multivariate-adjusted[†]		
		RR	95% CI	*P* for trend	RR	95% CI	*P* for trend
Total fat							
1 < 18.38	19	1.00		0.93	1.00		0.90
2 18.38–21.40	18	0.94	0.49–1.78		1.01	0.52–1.94	
3 21.41–24.35	21	1.07	0.58–1.99		1.19	0.63–2.27	
4 ≥ 24.36	18	0.93	0.49–1.77		0.99	0.50–1.95	
Animal fat							
1 < 7.26	19	1.00		0.91	1.00		0.74
2 7.26–9.40	18	0.95	0.50–1.80		0.91	0.47–1.73	
3 9.41–11.54	21	1.12	0.60–2.08		1.02	0.54–1.92	
4 ≥ 11.55	18	0.99	0.52–1.89		0.85	0.44–1.67	
Vegetable fat							
1 < 7.86	15	1.00		0.25	1.00		0.043
2 7.86–9.44	19	1.20	0.61–2.36		1.47	0.74–2.95	
3 9.45–10.94	18	1.12	0.56–2.23		1.47	0.73–2.98	
4 ≥ 10.95	24	1.52	0.79–2.90		2.08	1.05–4.13	
Fish fat							
1 < 1.42	21	1.00		0.14	1.00		0.21
2 1.42–2.23	21	0.97	0.53–1.77		1.03	0.56–1.90	
3 2.24–3.34	21	0.95	0.52–1.73		0.99	0.54–1.84	
4 ≥ 3.35	13	0.57	0.29–1.14		0.60	0.30–1.23	
Saturated fatty acids							
1 < 5.20	25	1.00		0.20	1.00		0.090
2 5.20–6.30	19	0.76	0.42–1.38		0.74	0.40–1.35	
3 6.31–7.33	14	0.56	0.29–1.08		0.51	0.26–1.00	
4 ≥ 7.34	18	0.75	0.41–1.38		0.64	0.34–1.22	
Monounsaturated fatty acids							
1 < 5.47	15	1.00		0.96	1.00		0.82
2 5.47–6.44	22	1.45	0.75–2.79		1.50	0.77–2.93	
3 6.45–7.47	25	1.62	0.85–3.06		1.78	0.92–3.44	
4 ≥ 7.48	14	0.89	0.43–1.84		0.96	0.45–2.05	
Polyunsaturated fatty acids							
1 < 4.41	15	1.00		0.84	1.00		0.071
2 4.41–5.23	21	1.28	0.66–2.48		1.75	0.88–3.47	
3 5.24–6.05	20	1.16	0.60–2.28		1.81	0.89–3.68	
4 ≥ 6.06	20	1.11	0.56–2.18		1.98	0.94–4.18	
n-3 fatty acids							
1 < 0.87	19	1.00		0.32	1.00		0.87
2 0.87–1.07	22	1.08	0.59–2.00		1.23	0.65–2.30	
3 1.08–1.33	19	0.91	0.48–1.72		1.14	0.59–2.21	
4 ≥ 1.34	16	0.73	0.38–1.43		0.94	0.46–1.91	
n-6 fatty acids							
1 < 3.46	19	1.00		0.89	1.00		0.15
2 3.46–4.12	17	0.82	0.43–1.58		1.10	0.56–2.16	
3 4.13–4.79	18	0.82	0.43–1.56		1.22	0.61–2.43	
4 ≥ 4.80	22	0.96	0.52–1.79		1.68	0.85–3.35	
n-6/n-3 ratio							
1 < 3.21	16	1.00		0.44	1.00		0.28
2 3.21–3.86	17	1.05	0.53–2.07		1.09	0.55–2.18	
3 3.87–4.58	24	1.49	0.79–2.81		1.71	0.90–3.25	
4 ≥ 4.59	19	1.19	0.61–2.32		1.30	0.66–2.58	
Long-chain n-3 fatty acids							
1 < 0.29	24	1.00		0.079	1.00		0.12
2 0.29–0.42	17	0.68	0.37–1.27		0.72	0.38–1.35	
3 0.43–0.62	22	0.86	0.48–1.54		0.90	0.50–1.63	
4 ≥ 0.63	13	0.50	0.25–0.98		0.52	0.26–1.05	

[†]Adjusted for age, study area, educational level, family history of breast cancer, age at menarche, age at menopause, age at first birth, parity, use of exogenous female hormones, alcohol consumption, smoking, consumption of green leafy vegetables, daily walking, height, body mass index, and total energy intake.
[‡]Fat and fatty acid intake were adjusted to mean energy intake of 1307 kcal/day (5469 kJ/day).

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One possible reason for these inconsistencies is that halogenated hydrocarbons, including polychlorinated biphenyls (PCB) and dichlorodiphenyltrichloroethane (DDT), or heavy metals are concentrated in fish and may exert estrogenic effects that could predispose women to breast cancer.[42,43] In addition, genetic backgrounds such as polymorphisms of glutathione *S*-transferase may modify the effect of marine n-3 fatty acids.[44] Further investigations considering dietary intake of halogenated hydrocarbons or heavy metals and genetic factors would be valuable in clarifying the role of fish fat in the prevention of breast cancer.

A decreasing trend in risk was suggested with increasing SFA intake. This may partially be ascribable to the possible confounding by intake of long-chain n-3 fatty acids. The adjustment for intake of long-chain n-3 fatty acids attenuated the inverse correlation of SFA intake with breast cancer risk: the multivariate-adjusted RR (95% CI) over quartiles of SFA intake were 1.00, 1.18 (0.75–1.88), 0.73 (0.43–1.23), and 0.74 (0.44–1.27) (trend $P = 0.14$).

In the present study, a significantly high RR for breast cancer was found in relation to the highest intake of vegetable fat among who were women postmenopausal at baseline. This may be consistent with the results from a prospective study in Swedish women, in which postmenopausal breast cancer was positively associated with dietary n-6 fatty acids.[45] Although women with high intakes of n-6 fatty acids were not at a significantly elevated risk in our study, the relatively low validity of FFQ for n-6 PUFA (lower than that for vegetable fat) may have weakened the association.

The higher risk of breast cancer associated with high levels of consumption of vegetable fat may be biologically plausible and in line with findings from studies in laboratory animals.[3,9] However, this association has not always been supported by epidemiological studies.[11,12] De Stefani *et al.* reported that dietary linoleic acid was somewhat associated with a reduced risk.[46] Decreased risks were also found for the highest levels of total n-6 PUFA in serum or erythrocyte membrane.[10,47] To confirm our findings, including that for vegetable fat with a biomarker, we are planning a case-control study nested in the JACC Study using sera donated at baseline.[13] The n-6 to n-3 ratio in dietary fatty acids is much lower in Japan than in Western countries: approximately 4[48] and more than 10,[49] respectively. The role of n-6 PUFA in the development of breast cancer therefore could be different in Japanese and Western populations.

The strengths of the present study derive mainly from its prospective design, which avoids both the recall and selection bias inherent in case-control studies. Intakes of fat and fatty acids were assessed with a validated FFQ. Furthermore, our study in a population with a low total fat intake but a high consumption of fish provides a unique opportunity to examine the effects of dietary fat on breast cancer risk.

Some methodological issues, however, warrant further consideration. First, as has been the case in most cohort studies, we assessed dietary intake only at baseline and did not take into account changes in diet over time. Data collection during follow-up[41] may provide a more accurate assessment of long-term diets. Second, possible residual confounding cannot be ruled out, because, due to the data limitation, we could only roughly adjust for dietary factors other than fat, and for physical activity. Although the RR for fish fat and long-chain n-3 fatty acids remained almost the same after adjusting for potential confounding factors in the present analyses, insufficiently measured confounding variables may have failed to alter the RR estimates.

Third, we applied the best available method to ascertain incident cases of breast cancer in our study, and the indices for quality of registration were within the acceptable range. Some cases, however, may have been missed, particularly in study areas where well-established population-based cancer registries were not available. If the failure to record breast cancer cases was associated with dietary intakes of fat, it might have biased the estimates of RR. Studies with a more complete surveillance system may give more accurate information.

Finally, we adopted a simple 40-item FFQ to estimate dietary intakes of fat. This questionnaire with a small number of food items may not be suitable for estimating absolute levels of nutrient intakes, but it can be used to rank subjects according to intakes of selected nutrients as shown in the validation study. Thus, we consider that the RR by quartile of dietary intakes of fat could be estimated using the FFQ and that this investigation provided meaningful findings. The questionnaire, however, included only three items for fish and their products (fresh fish, dried fish, and boiled fish paste ["kamaboko" in Japanese]), and did not ask for details about the kind of fish. This prevented us from elucidating the effect of individual components of fat for each kind or type of fish. Investigations eliciting consumption habits according to the kind of fish would be required to further clarify the role of fish intake in the prevention of breast cancer, because the fat components differ widely among fish.

The portion size information was not specified in the FFQ in the JACC Study. Obtaining portion size data separately from intake frequencies for selected foods, however, may enhance the validity of assessment of nutrient intakes.[50,51] The inclusion of questions on portion sizes and the use of food models in FFQ therefore should be considered in future studies. Noethlings *et al.*[52] reported that the omission of individual portion size information would probably result in a notable reduction of interindividual variance of food consumption, but the assignment of a constant portion size seems to be adequate in large epidemiological studies. In addition, we assumed the standard portion sizes, irrespective of age and sex due to the limited data from dietary records. Because portion sizes differ greatly by age and sex, the validity of the FFQ could be improved by using age- and/or sex-specific portion sizes.[53] In another study,[52] however, the interindividual variance of dietary intake captured by the FFQ was not markedly increased when sex-, age-, and BMI-specific portion sizes were applied, which supported the assignment of a constant portion size for all study subjects.

In conclusion, this prospective study did not support any increase in the risk of breast cancer in relation to total or saturated fat intake, even in a population with a relatively low fat intake. However, the study suggested the protective effects of n-3 fatty acids abundant in fish.

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Original Article

Relative Validity of a Short Food Frequency Questionnaire for Assessing Nutrient Intake versus Three-day Weighed Diet Records in Middle-aged Japanese

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BACKGROUND: Validation studies on brief food frequency questionnaires (FFQs) for measuring consumption of macro- and micro-nutrients for the general populace are not fully executed in Japan.
METHODS: Two hundred and two middle-aged Japanese (73 males and 129 females) in Aichi Prefecture, Japan completed an FFQ and 3day-weighed diet records (3d-WDRs) in February 2004. We compared intakes of energy and 26 nutrients computed with the FFQ against those with the 3d-WDRs as a reference.
RESULTS: Mean daily intakes of selected nutrients determined with the FFQ were generally less than those with 3d-WDRs. The ratios assessed with the FFQ vs. 3d-WDRs (minimum - median - maximum) were distributed from 0.57 - 0.79 - 1.09 for males, and 0.61 - 0.86 - 1.04 for females. De-attenuated, log-transformed and energy-adjusted Pearson's correlation coefficients between intakes of selected nutrients quantified with both devices were distributed from 0.12 - 0.45 - 0.86 and energy-adjusted Spearman's rank correlation coefficients were from 0.13 - 0.35 - 0.76, for males. The respective values for females were 0.10 - 0.38 - 0.66, and 0.11 - 0.34 - 0.47. Median percentages for exact agreement, agreement within adjacent categories, and disagreement according to quartile classification of the energy-adjusted nutrient intakes measured with both methods were 33, 74, and 5 for males, and 35, 76, and 7 for females, respectively.
CONCLUSION: Satisfactorily high relative validity indices of most nutrient intakes computed with the FFQ were attained against those with the 3d-WDRs. The questionnaire therefore appears applicable for categorizing individuals according to consumption of energy and selected nutrients in dietary studies of middle-aged Japanese.
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Key words: Energy Intake, Questionnaire, Micronutrients, Diet Records, Validity.

It is well known that the leading causes of death are now chronic diseases such as cancer, cerebrovascular problems and heart disease in developed countries, including Japan.[1] They are related to daily lifestyle, including dietary habit, alcohol drinking, smoking, physical exercise, and factors for stress. Because dietary habit, in particular, appears to play a major role in their pathogenesis, batteries of tests to assess intake of foods/nutrients, including fats/fatty acids, antioxidants and dietary fibers, are needed for epidemiologic studies.

There are several tools available, including diet records (DRs)/weighed diet records (WDRs), 24-hour recall, food frequency questionnaires (FFQs), and duplicate methods.[2-4] Among

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these, the FFQ is most often employed to evaluate associations between long-term food intake and health/disease. We earlier developed a data-based semi-quantitative food frequency questionnaire (SQFFQ) using multiple regression analysis (MRA) as well as contribution analysis on the basis of WDRs,[5] and conducted a calibration/validation and reproducibility study, as detailed elsewhere.[6,7] However, the SQFFQ was primarily designed for the JADE (Japanese Dietitians' Epidemiologic) Study. We recently evolved a self-administered brief FFQ according to MRA as described elsewhere[8] for epidemiologic studies on lifestyle-related diseases of the middle-aged Japanese general populace.

In the present investigation, we carried out a relative validity study of intake of energy and 26 macro- and micro-nutrients measured with our FFQ versus reference values with three-day WDRs (3d-WDRs).

METHODS

Subjects

We recruited 222 (83 males and 139 females) middle-aged volunteers (30 - 70 years of age) who were attending physical exercise classes in communities, or were parents of college students in Aichi prefecture, central Japan. Twenty individuals were excluded from this study because eight persons were under 30 or over 70 years old, eight had not complied with the research regimen and four whose responses for energy lay beyond 3 standard deviations (SDs) from the mean measured with the FFQ. Finally, 202 participants (73 males and 129 females) were thus included in the present analysis.

FFQ and 3d-WDRs

In February 2004, we first administered the FFQ to the subjects by mail. The questionnaire inquired about habitual dietary intake during the previous one year for 47 foods/recipes and frequency in eight categories: never or seldom, 1-3 times/month, 1-2 times/week, 3-4 times/week, 5-6 times/week, once/day, twice/day and more than three times/day. For staple foods, including rice, noodle and bread, the portion size/serving size was requested. Approximately one week later, we administered the 3d-WDRs (two week-days and one weekend) and a disposable camera to photograph foods when eating out or take-out. Diet records not completed were checked and verified by research dietitians.

Nutrients selected

We earlier developed a brief FFQ for energy and 26 macro- and micro-nutrients, including protein, fat [saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), poly-unsaturated fatty acids (PUFAs), n-6 PUFAs, n-3 PUFAs, n-3 HUFAs (highly-unsaturated fatty acids) and cholesterol], carbohydrate, total dietary fiber (TDF) (soluble and insoluble), minerals (calcium and iron) and vitamins (carotene, and vitamins A, C, D and E),[8] and added 6 nutrients of interest, including carbohydrate energy%, protein energy %, fat energy %, vitamin B_1, vitamin B_2 and folate.

Calculation of intake of nutrients

We computed the average daily consumption of energy and selected nutrients using information from the FFQ and lifestyle questionnaire, including consumption of alcohol. According to the regression analysis, selected nutrients were adopted as dependent parameters and foods/food groups consumed, intake frequency, portion size (in grams) from our database,[5,8] or typical/standard values from the literature, nutrient contents per 100 grams of foods/food groups listed in the respective composition tables or of the model recipes were assumed to be independent variables.[9-13] With the WDRs, we calculated mean daily intakes of selected nutrients by multiplying the consumption of foods/food groups (in grams) and nutrient contents per 100 grams of foods as listed in the composition tables or model recipes.

Validation

First, we compared mean daily intakes of energy and 26 selected nutrients gauged with the FFQ against those with the 3d-WDRs. Differences in means and ratios were computed with the FFQ vs. 3d-WDRs values, and examined by t-test using Excel® and the SPSS®-10.0 software package.

Second, we calculated crude Pearson's correlation coefficients (CCs), log-transformed Pearson's CCs, log-transformed and energy-adjusted Pearson's CCs, and de-attenuated, log-transformed and energy-adjusted Pearson's CCs between intakes of selected nutrients based on the FFQ and 3d-WDRs. Energy adjustment was executed using regression models.[14] De-attenuated Pearson's CCs were computed by partitioning within- and inter-individual variations by one way of analysis of variance according to the formula described elsewhere.[3,15-17] Crude Spearman's rank CCs and energy-adjusted Spearman's rank CCs were also calculated.[18,19] Statistical significance was verified with the 95% confidence interval.

Third, after categorizing daily intakes of nutrients quantified with the FFQ and 3d-WDRs into quartiles, we computed percentages of exact agreement, agreement within adjacent categories, and disagreement.

Ethical issues

Our study protocol was reviewed and approved by the Internal Review Board at Nagoya City University Graduate School of Medical Sciences. Written informed consent was obtained from each participant.

RESULTS

Profile of study subjects

The mean ages ± standard deviations (SDs) (minimum - maximum) were 51.7 years ± 8.9 (30 - 68) for males, and 49.6 years ± 8.8 (30 - 68) for females. The values for height, weight and body mass index (kg/m^2) were 169.6 cm ± 6.6 (145.3 - 187.0), 65.5 kg ± 8.7 (53.0 - 93.0), and 22.7 ± 2.3 (18.0 - 28.1) for males, and 155.8 cm ± 5.2 (142.1 - 171.0), 53.1 kg ± 6.6 (38.5