博士論文

Fish, n-3 and n-6 polyunsaturated fatty acids intake and risk of breast cancer by estrogen and progesterone receptor status: The Japan Public Health Center-based Prospective Study

（日本人における魚、n−3、n−6 脂肪酸摂取と乳がんリスクに関する研究：ホルモン受容体有無による比較（多目的コホート研究））

Grace Yurina Kiyabu

キヤブ グレイス ユリナ

Fish, n-3 and n-6 polyunsaturated fatty acids intake and risk of breast cancer by estrogen and progesterone receptor status: The Japan Public Health Center-based Prospective Study

（日本人における魚、n−3、n−6 脂肪酸摂取と乳がんリスクに関する研究：ホルモン受容体有無による比較（多目的コホート研究））

In partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

A thesis presented by

Grace Yurina Kiyabu

Supervisor: Professor Kenji Shibuya

to

Department of Global Health Policy
School of International Health
Graduate School of Medicine
The University of Tokyo

Tokyo, Japan
2016

論文の内容の要旨

Fish, n-3 and n-6 polyunsaturated fatty acids intake and risk of breast cancer by estrogen and progesterone receptor status:
The Japan Public Health Center-based Prospective Study

（日本人における魚、n-3、n-6 脂肪酸摂取と乳がんリスクに関する研究：ホルモン受容体有無による比較 （多目的コホート研究））

渋谷健司教授

東京大学大学院医学系研究科

平成２５年４月入（進）学

医学博士課程

国際保健専攻

キヤブグレイスユリナ

Grace Yurina Kiyabu

Abstract

Objective: Limited and inconsistent studies exist on the association between the intake of fish, n-3 polyunsaturated fatty acids (PUFA), and n-6 PUFA and breast cancer. Fish

and n-3 PUFA support various body functions and are thought to reduce the carcinogenesis risk while n-6 PUFA may have a positive association with cancer risk. The association between intake of fish, n-3 PUFA, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), alpha linolenic acid (ALA), n-6 PUFA, and n-6/n-3 ratio and breast cancer in Japan with further analyses on estrogen (ER) and progesterone receptor (PR) status were examined.

Methods: A total of 38,234 Japanese women aged 45-74 years from the Japan Public Health Center-based prospective Study (JPHC) were investigated, and during 14.1 years of follow-up time, 556 breast cancer cases were newly diagnosed. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were calculated by multivariable Cox proportional hazard models with age as the time scale.

Results: Breast cancer risk was not associated with the intake of total fish, n-3 PUFA, and n-6 PUFA when analyzed in totality through multivariable Cox proportional hazards regression models with age as the time-scale. Intake of total n-6 was positively associated with the development of ER+PR+ tumors [multivariable adjusted HR $_{Q4}$ vs $_{Q1}$=2.94; p-value=0.02 (95% CI: 1.26-6.89; ptrend =0.02)].

Conclusion: While the overall association between the intake of total fish, n-3 PUFA, and n-6 PUFA and breast cancer risk is null, for ER+PR+ tumors, a positive association was seen between n-6 intake and breast cancer.

Key words: breast cancer, fatty acids, diet, fish, receptor

Table of Contents

Acknowledgements

I am indebted to many people for even being able to sit at my desk in room S514 and write these acknowledgements. I would like to convey my sincere appreciation to these people.

I express my gratitude to Dr. Kenji Shibuya for his guidance and support throughout the past three years. Besides building research skills, through the Global Health Entrepreneurship (GHE) program, I gained practical skills to tackle imminent global health issues. I am grateful for the opportunity I had to go on a site visit to the Camotes Islands in the Philippines to gain on-hands project experience.

I am also very thankful for Dr. Manami Inoue's continuous support in guiding me to conduct efficient literature review, robust statistical testing and analyses, and concise reporting. I would also like to thank her for the valuable summer school experience in Italy that enabled me to not only gain more knowledge on biostatistics but also connect with professors and friends around the world.

Without the support and encouragement from my friends and professors at the Department of Global Health Policy, I would not have been able to go through this process smoothly. My sincere thanks go to Ms. Saki Narita; I will never forget the days we spent coding together and our journey to the Philippines and Venice. I am also thankful for the kindness and patience of Dr. Sarah K. Abe and Dr. Eiko Saito in helping me adjust to not only the datasets but also life at the University of Tokyo. The secretaries, Ms. Shino Yamazaki and Ms. Shiho Kato, have also been extremely generous in navigating me through my PhD life, and I am grateful for their unfailing assistance.

I would also like to thank the coauthors of my International Journal of Cancer manuscript at the National Cancer Center: Dr. Norie Sawada, Dr. Junko Ishihara, Dr. Motoki Iwasaki, Dr. Taiki Yamaji, Dr. Taichi Shimazu, Dr. Shizuka Sasazuki, and Dr. Shoichiro Tsugane. Their critical feedback and suggestions helped improve my manuscript especially in the results and discussion sections. I also express my gratitude to the researchers I met through the summer school program in Italy: Dr. Rino Bellocco (University of Milano-Bicocca and Karolinska Institutet), Dr. Paul Dickman (Karolinska Institutet), Ms. Daniela Mariosa (Karolinska Institutet), and Dr. David

Wypij (Harvard Medical School and Boston Children's Hospital) for their generous and invaluable support on improving my models.

Last but not least, I would like to thank my family including my parents, Yuki, Christine, and my grandparents for giving me the opportunity to pursue further education and encouraging me to always do my best.

While this may be my last degree, it does not mark the end to my pursuit for knowledge. I will continue my journey to improve health in this world with passionate curiosity.

List of Tables

List of Figures

List of Abbreviations

ALA	alpha linolenic acid
CI	confidence interval
DCO	death certificate only
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
ER	estrogen receptor
FFQ	Food Frequency Questionnaire
HR	hazard ratio
JPHC Study	The Japan Public Health Center-based prospective Study
PHC	public health center
PR	progesterone receptor
PUFA	polyunsaturated fatty acids

1. Introduction

1.1. Organization of thesis

The thesis begins with Chapter 1 on the background on breast cancer, fish intake, and PUFA intake followed by the rationale and objectives. In Chapter 2, the methods of the study are explained in detail. The results on breast cancer risk and fish and PUFA intake are presented in Chapter 3. In Chapter 4, the results are put in context of other studies, strengths and limitations are discussed, and the importance of this study is highlighted. Chapter 5 provides a summary of the thesis and implications of the results in research.

1.2. Background

Through comparison of breast cancer with other common cancers, section (1.2.1) provides background on the cancer trends in Japanese women over the past 30 years. This is followed by section (1.2.2) on the risk factors of breast cancer, (1.2.3) which highlights fish and polyunsaturated fatty acids intake, and (1.2.4) which explains the potential pathway of the association between fish and polyunsaturated fatty acids and breast cancer.

1.2.1. Breast cancer incidence trends

Breast cancer is the most common cancer in women living in the developed and developing world.[1] In 2012, there were approximately 1.67 million new cases and 522,000 deaths worldwide. Japan and China have been experiencing a steady increase in incidence while the incidence rates of countries such as the United States and Canada have been approximately stable..[1, 2] Figure 1 shows age standardized breast cancer incidence trends in selected countries among those with population-based registries from the International Agency for Research on Cancer. Data was provided by: the Australian Institute of Health and Welfare (Authoritative information and statistics to promote better health and wellbeing), China (Shanghai, Jianshan County, and Hong Kong registries), Government of Canada (Statistics Canada), Japan (Miyagi, Osaka, and Yamagata cancer registries), Norway (Cancer Registry of Norway), USA (Surveillance Epidemiology and End Results (SEER) program: Atlanta, Connecticut, Detroit, Hawaii, Iowa, New Mexico, San Francisco-Oakland, Seattle-Puget Sound, and Utah).

There is an overall increasing trend from 1975 while Japan and China have a lower rate compared to the Western population.[3] Within the Japanese population, a dramatic increase of approximately 70% can be seen since the 1980s.[3]

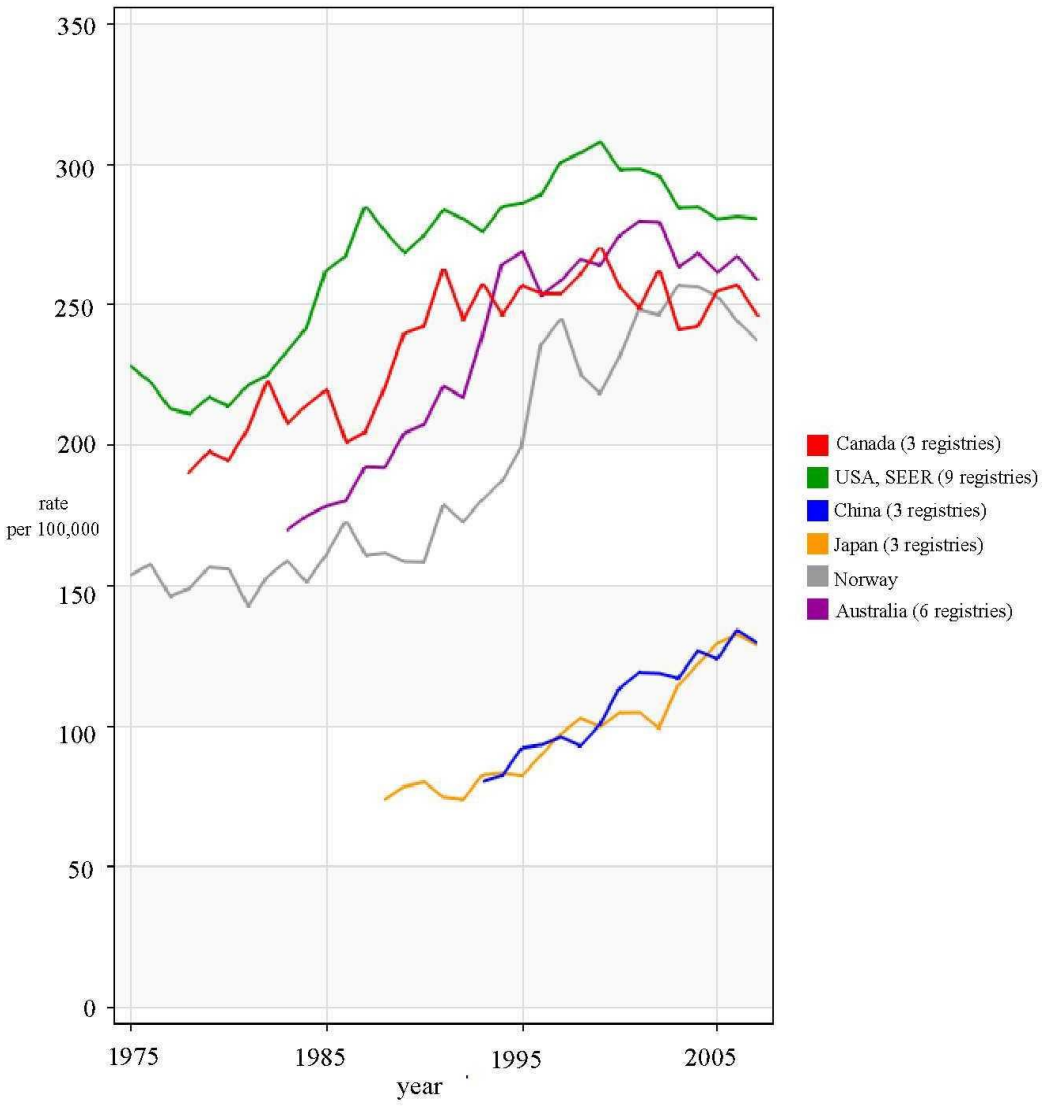


Figure 1: Breast cancer incidence trends in selected countries: age standardized rate per 100, 000 women

Source: International Agency for Research on Cancer[3]

Figure 2 shows the estimated age-standardized incidence and mortality in Japanese men and women in 2012.[3, 4] While stomach cancer has the highest incidence and lung cancer has the highest mortality among Japanese men, breast cancer has the highest incidence and mortality among Japanese women.[3, 4] The top five cancers with the highest

incidence trends in Japanese women are shown in Figure 3.[3] Breast cancer shows a sharp increase compared to colon, stomach, lung, and pancreatic cancers which have decreased or have not shown significant increases in incidence rates.

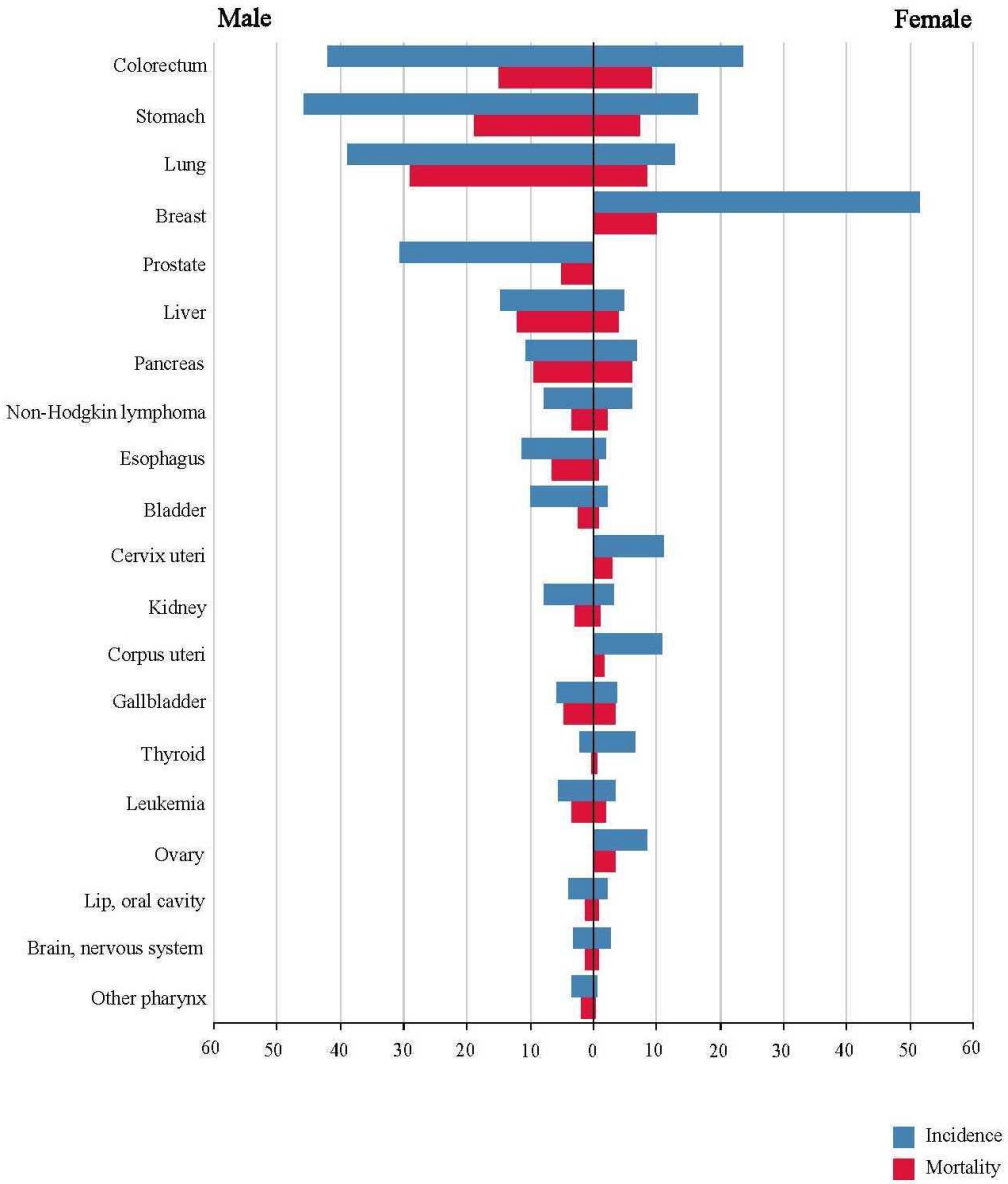


Figure 2: Estimated age-standardized incidence and mortality in Japanese men and women per 100,000 in 2012
Source: GLOBOCAN 2012[4] and International Agency for Research on Cancer[3]

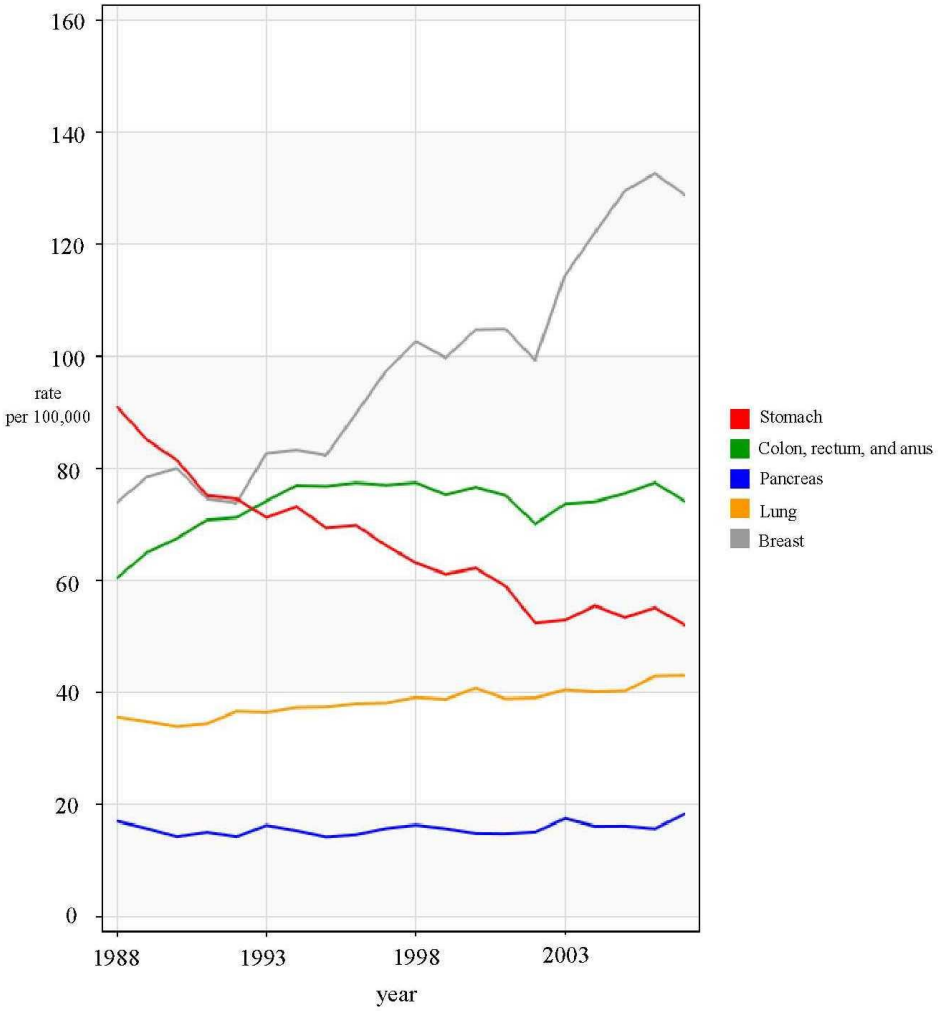


Figure 3: Cancer incidence trends in Japanese women for top 5 cancers: age-standardized rate per 100,000
Source: International Agency for Research on Cancer[3]

1.2.2. Breast cancer risk factors including receptor status

Reproductive factors including early menarche, late menopause, not bearing children and lifestyle factors such as lack of physical activity have generally been accepted to play etiological roles in breast cancer.[5, 6] In terms of diet, there is convincing evidence that alcohol consumption increases the risk of breast cancer.[7, 8]

As part of breast cancer categorization, testing for estrogen receptor (ER) status and progesterone receptor (PR) status is part of the standard care in making decisions for therapy because differences in receptor status can help determine how well the breast cancer case responds to hormone therapy.[9] Studies have also shown that risk factors for breast cancer differ depending on ER and PR status, which necessitates ER and PR analyses for accurate estimates of breast cancer risk.[10-12]

1.2.3. Fish and polyunsaturated fatty acids intake

The Japanese diet consists of relatively high intake of fish, a rich source of n-3 PUFA, and polyunsaturated fatty acids.[13] Figure 4 provides a global picture of fish consumption per capita supply on average from 2008 to 2010 in which Japan is in the highest-consumption category.[14] Figure 5 shows the food supply of fish and seafood (g/capita/day) of selected countries. Japan has the highest food supply of fish and seafood of 147 g/capita/day, followed by Norway with 146 g/capita/day.[14] Among the selected countries, the UK has the lowest food supply of fish and seafood of 52 g/capita/day.[14]

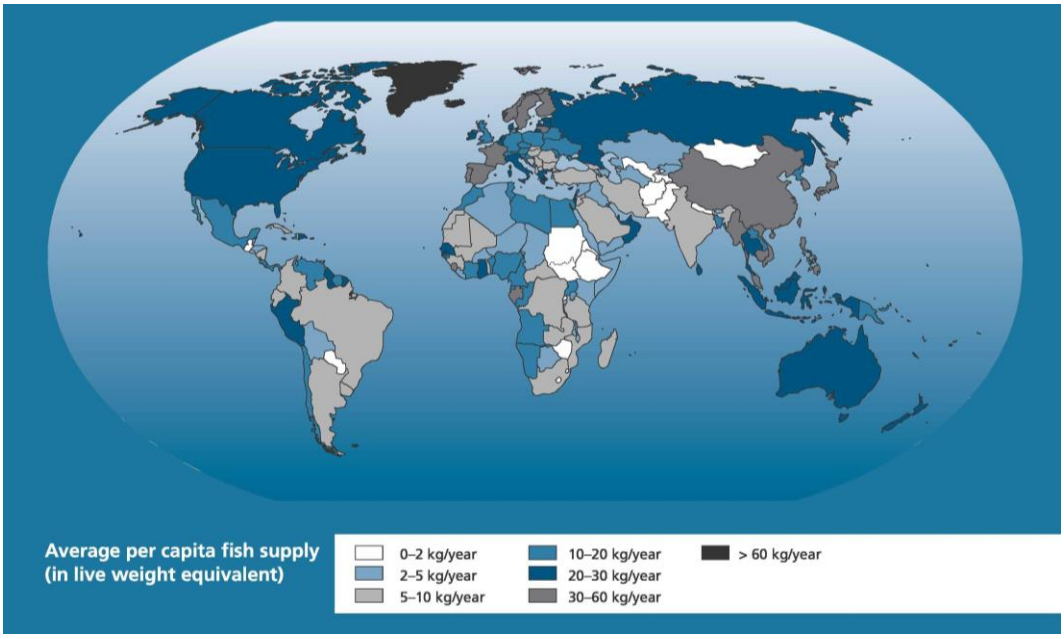


Figure 4: Global fish consumption per capita supply
Source: Food and Agriculture Organization of the United Nations[14]

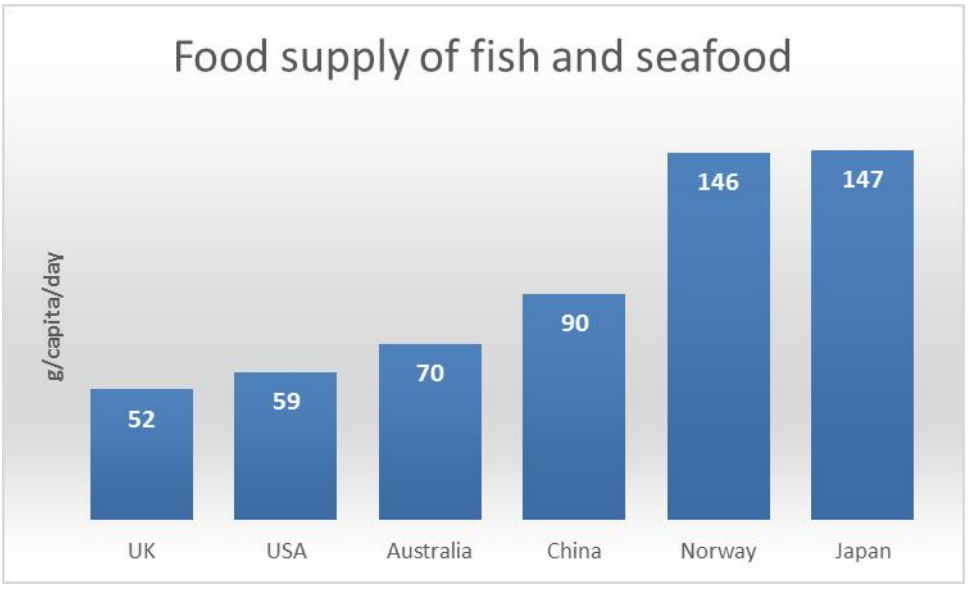


Figure 5: Food supply of fish and seafood (g/capita/day) of selected countries
Source: Food and Agriculture Organization of the United Nations[14]

Fish contains n-3 PUFA which can be further categorized into eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and alpha linolenic acid (ALA). Figure 6 shows the global seafood n-3 PUFA (mg/day) intake for adults from 20 years of age in 2010, and Figure 7 depicts global plant n-3 PUFA intake. Japan is in the highest group with an average intake of 718 mg/day of seafood based n-3 PUFA. For plant based n-3 PUFA intake, which includes vegetable oil, Japan lies in the mid-range of approximately 1,200 mg/day. Figure 8 shows the global n-6 PUFA intake (% energy) for adults from 20 years of age in 2010. Intake of n-6 PUFA in Japan is relatively low with approximately 4% of energy/day.

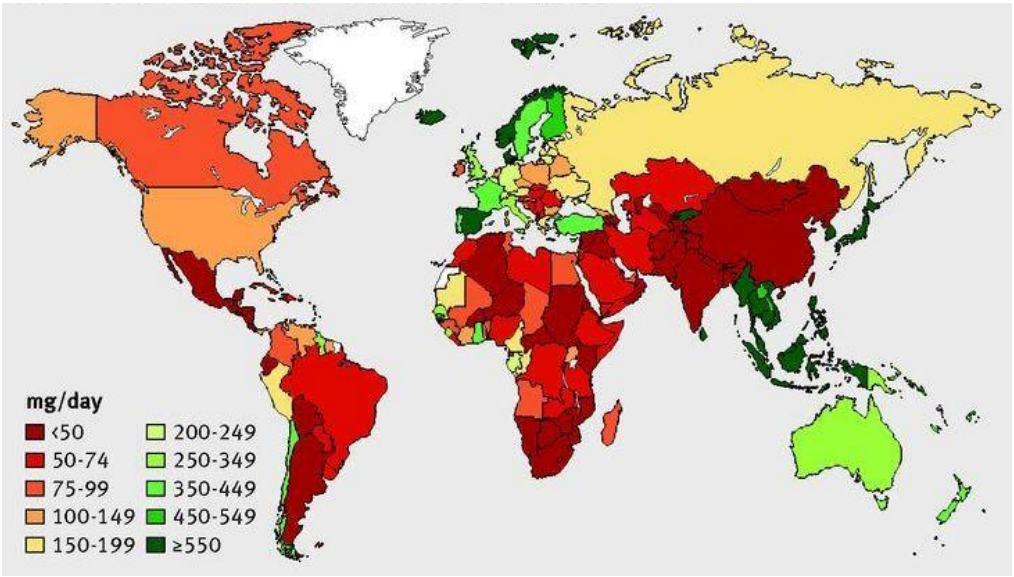


Figure 6: Global seafood n-3 PUFA (mg/day) intake in 2010
Source: Micha R. et al.[15]

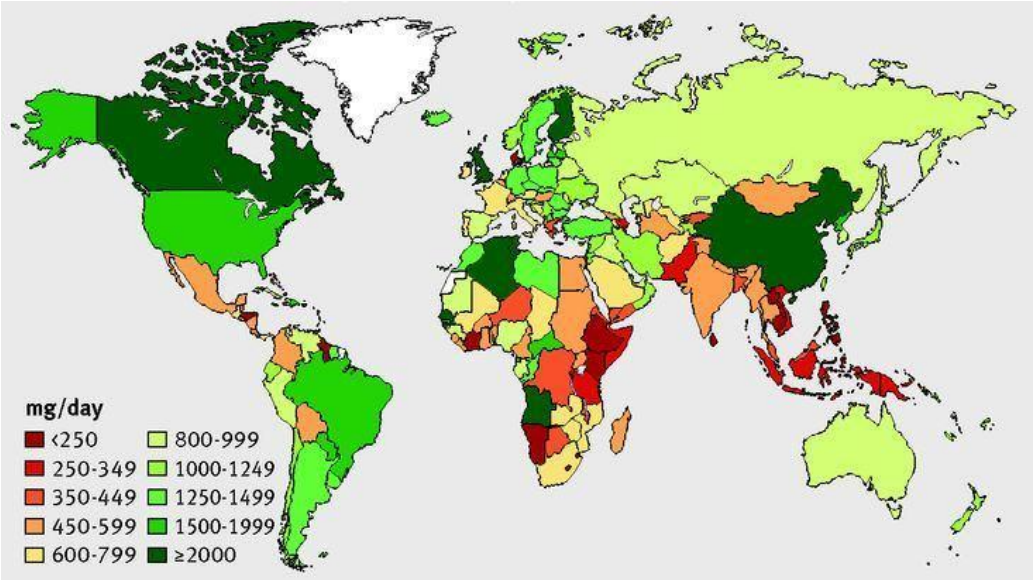


Figure 7: Global plant n-3 PUFA (mg/day) intake in 2010
Source: Micha R. et al.[15]

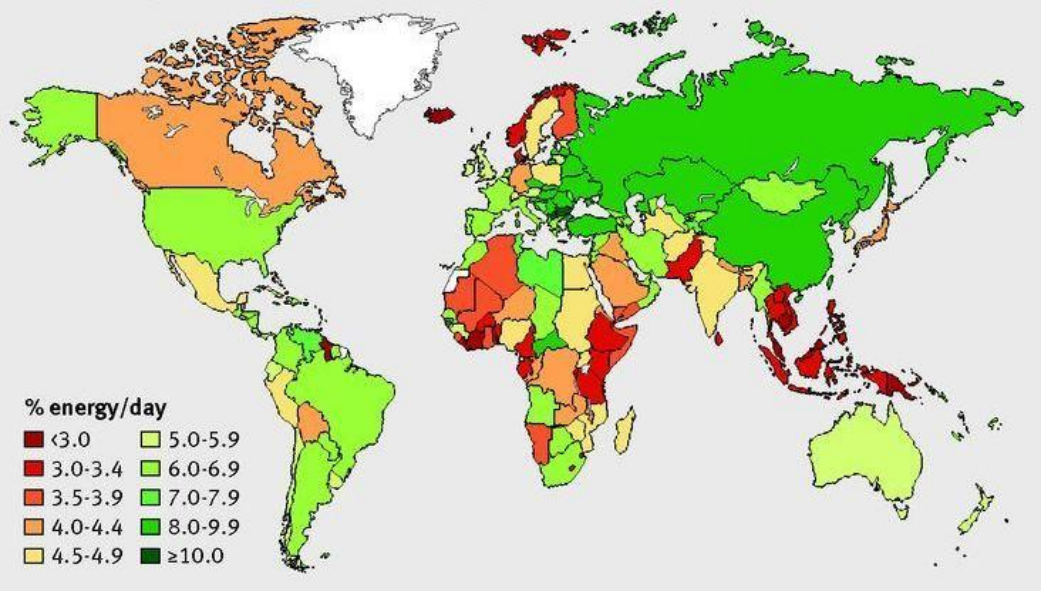


Figure 8: Global n-6 PUFA intake (% energy) in 2010
Source: Micha R. et al.[15]

Table 1 ranks the top five food categories for n-3 PUFA and n-6 PUFA intake from dietary records in Japan used in this study.[16] Vegetable oil contributes the most to both n-3 PUFA and n-6 PUFA dietary intake. For n-3 PUFA, fish including mackerel, pacific saury, and salmon contributes to n-3 PUFA intake, while n-6 PUFA consumption is mainly attributable to tofu and rice intake.

Table 1: Top 5 foods for n-3 PUFA and n-6 PUFA intake from dietary records in Japan (g/day)

n-3 PUFA		n-6 PUFA	
Food item	**g/day**	**Food item**	**g/day**
Vegetable oil	0.75	Vegetable oil	3.69
Mackerel	0.17	Tofu	0.61
Pacific saury	0.16	Rice	0.59
Mayonnaise	0.16	Mayonnaise	0.54
Miso	0.08	Miso	0.45
Tofu	0.08	Chicken egg	0.45
Salmon	0.07		

Source: Kobayashi M. et al.[16]

1.3. Rationale

The rationale behind studying the association between n-3 PUFA, and n-6 PUFA and breast cancer is explored in section (1.3.1). Based on this rationale, the main objectives are defined in section (1.3.2).

1.3.1. Fish, polyunsaturated fatty acids, and breast cancer

Epidemiological studies on the association between the intake of fish, n-3 PUFA, and n-6 PUFA and breast cancer have been limited and inconsistent (Table 2).[12, 17-19] When analyzing fish intake, previous epidemiological studies have yielded conflicting results on its association with breast cancer,[12, 20-22] while a recent meta-analysis of eleven independent cohort studies found no association between fish consumption and breast cancer risk but a protective effect of marine n-3 PUFA on breast cancer risk.[23]

A study of the Japanese population that analyzed dietary intake of fatty acids and breast cancer found protective effects of long-chain n-3 fatty acids, but did not analyze ER PR status.[24] In contrast, a positive association between n-6 PUFA and breast cancer was found in an epidemiological study on postmenopausal women in Sweden,[25] and possible tumor-enhancing effects have been biologically studied for the association between breast cancer and total n-6 PUFA which is considered to be the counterpart of total n-3 PUFA.[26-28] The n-6 PUFA to n-3 PUFA ratio has also been studied and prospective studies have generally found a null association between n-6/n-3 and breast cancer risk while a meta-analysis of 11 studies concluded a positive association between n-6/n-3 and breast cancer risk.[24, 25, 29, 30]

	Breast Cancer Risk		
Exposure	**Null**	**Positive**	**Inverse**
Fish	Vatten 1990 Holmes 1999 Engeset 2006 Zheng 2013	Stripp 2003	Hislop 1988 Vatten 1990 Key 1999
n-3	Park 2012	-	Wakai 2005 Gago-Dominguez 2003 Zheng 2013
n-6	Gago-Dominguez 2003 Park 2012	Wirfalt 2002	-
EPA	Park 2012	-	Zheng 2013
DHA	Park 2012	-	Zheng 2013
DPA	Zheng 2013	-	-
ALA	Park 2012 Zheng 2013	-	-
n-6/n-3	Wirfalt 2002 Wakai 2005 Park 2012	Yang 2014	

Figure 9: Summary of previous studies

Inconsistent results of previous studies may be due to the variations in breast tumor sub-type (ER PR status) given that previous laboratory[31] and epidemiological studies[29, 32, 33] have found significant associations between breast cancer according to sub-type and different exposures including body size and diet. While a multiethnic cohort study with hormone receptor status analysis on postmenopausal women showed no significant association of n-3 PUFA and n-6 PUFA intake with breast cancer risk,[29] other hormonal studies have shown that intake of different diets result in altered estrogen metabolism, which suggest dependence of breast cancer risk on ER PR status.[34, 35]

On the one hand, total n-3 PUFA contained in fish have been found to inhibit tumorigenesis and suppress transformation through enhanced apoptosis. [36-39] Total n-3 PUFA has been shown to not only decrease the proliferation and increase apoptosis of cancer cells, but also inhibit protein kinase B (Akt) phosphorylation (which promotes cell survival) and the DNA binding activity of NFkB, a prosurvival transcription factor that protects cells from apoptosis.[39] On the other hand, total n-6 PUFA may promote cancer tumorigenesis through prostaglandin synthesis.[40] These potentially opposing pathways motivated epidemiological analyses on a population with relatively high intake of PUFAs.

1.3.2. Main objectives

Many analyses of fish and PUFA have been conducted in breast cancer cases in Western populations; yet, compared to Western diets, the Japanese diet consists of relatively high intake of fish, a rich source of n-3 PUFA, which motivates this study on the Japanese population.[13] The objectives of this study are to:

1 Identify basic characteristics of fish, n-3 PUFA, and n-6 PUFA intake with breast cancer risk factors;

2 Assess breast cancer risk factors by fish, n-3 PUFA, and n-6 PUFA intake quartiles:

3 Analyze interaction between fish, n-3 PUFA, and n-6 PUFA and other risk factors;

and

4 Examine breast cancer risk factors according to ER and PR status.

2. Material and Methods

Background information on the study design of the Japan Public Health Center-based prospective Study (JPHC) is given in section (2.1). This is followed by section (2.2), which explains the exposure variables in detail by dividing the section further into (2.2.1) on the dietary assessment methods through the food frequency questionnaire and (2.2.2) on the other covariates of the study. Moreover, in section (2.3) detail on follow-up and identification of breast cancer cases is provided. Lastly, in section (2.4), methods of statistical analysis with a specific example on energy adjustment are described.

2.1 Study design and population

The JPHC Study consisted of participants who were Japanese residents from the catchment areas of 11 public health centers (PHCs) aged 40-59 years in Cohort I and

aged 40-69 years in Cohort II at time of baseline data collection.[41] Administration of follow-up surveys occurred at five and ten years. As shown in Figure 10, in 1990, Cohort I was enrolled from Akita-Yokote, Iwate-Nihone, Nagano-Saku, Tokyo-Katsushika, and Okinawa-Chubu, and in 1993, Cohort II was enrolled from Niigata-Nagaoka, Ibaraki-Mito, Osaka-Suita, Kochi- Chuohigashi, Nagasaki-Kamigoto, Okinawa-Miyako.[41]

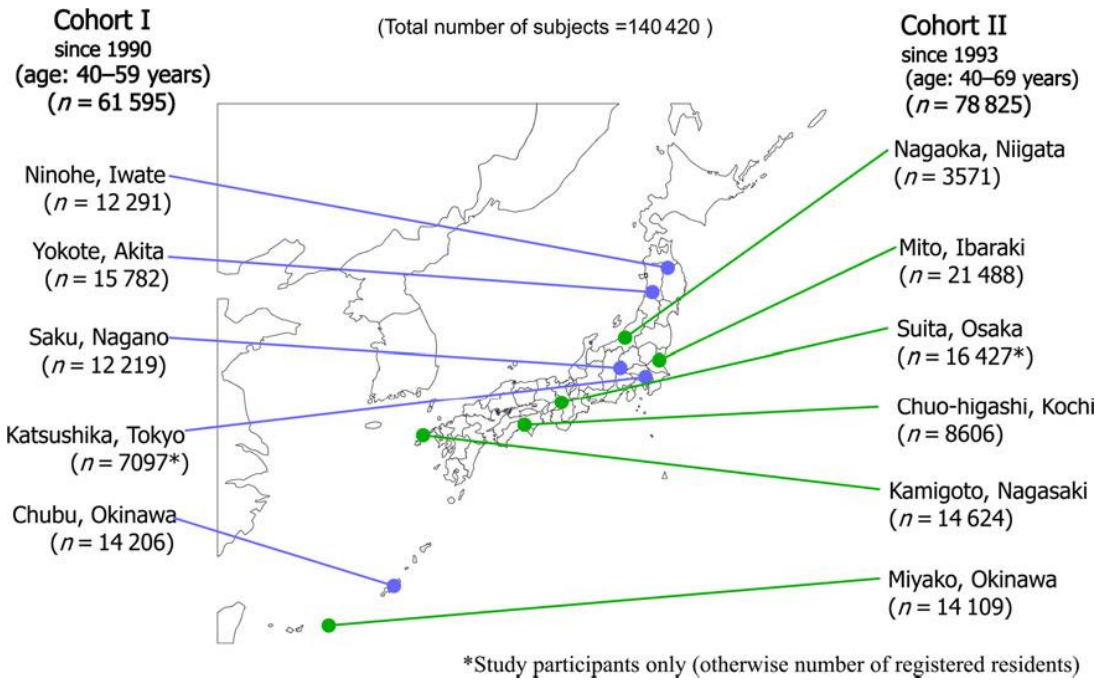


Figure 10: Map of the Japan Public Health Center-based prospective Study area
Source: Tsugane et al.[41]

The JPHC Study is an ongoing study scheduled to have a total of 30 years of follow-up. Further details regarding the JPHC study can be found in a previous publication.[41] The self-administered 5-year follow-up questionnaire (1995-1999) consisting of comprehensive information on dietary intake and lifestyle habits was used as the starting point of this study for subjects aged 45-74 years because the 5-year follow-up questionnaire was more detailed in the self-administered food frequency questionnaire (FFQ). Figure 11 outlines the study flow to obtain the final study sample.

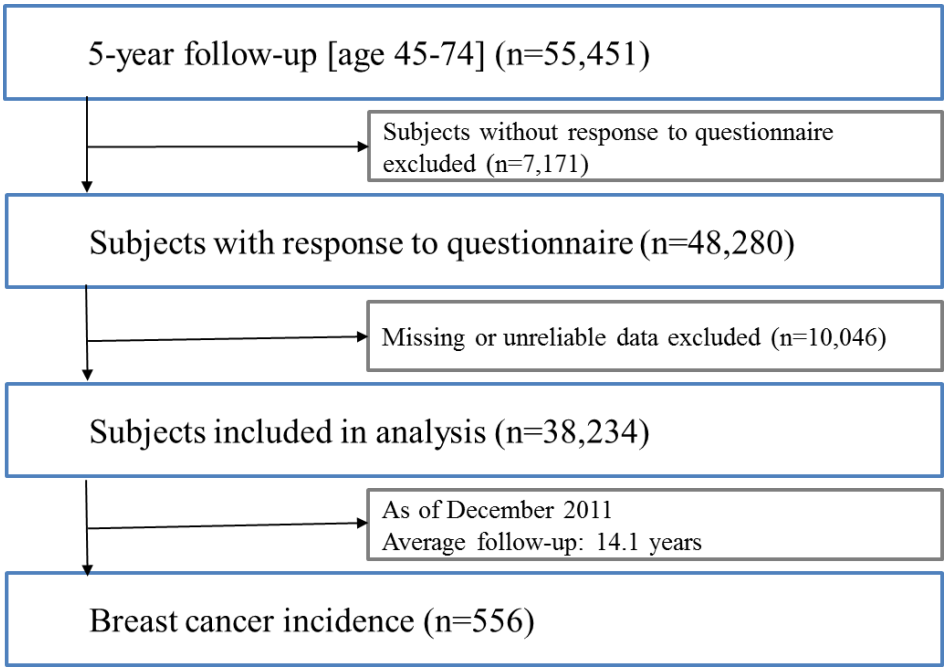


Figure 11: Study flow (*n*= number of subjects)

Subjects from the Tokyo-Katsushika (n=4,178) and the Osaka-Suita (n=8,337) PHC area were excluded due to the lack of access to data on cancer incidence or ER PR data. A total of 207 disqualified subjects, and 3,525 subjects who had died, moved prior to follow-up, or were lost to follow-up were excluded. Out of the 55,451 women eligible for participation, 48,280 responded to the questionnaire (response rate 87.1%). After excluding 10,046 women with incomplete or unreliable data on exposures and additional covariates, a total of 38,234 women were included in the multivariable adjusted analysis. The following provides an outline of the main criteria I used to check for unreliable data determined by the JPHC research group.

1. If total energy intake was extreme (upper or lower 2.5%), subjects were omitted (n=2,314).
2. BMI less than 14 or greater than 40 kg/m^2 (n=499).
3. When age at menarche was older than age at menopause, the ages were swapped because the participant most likely misread the question (n=1).
4. If menopausal age was greater than their current age, menopausal age was labeled as missing, and thus excluded because menopausal age cannot be older than their current age (n=5).
5. If menopause age was younger than current age, they did not have a history of

ovarian or uterine cyst, menopausal age was greater than or equal to 40, and age at questionnaire was greater than or equal to 55, menopausal status was checked to have been answered as “yes” because it is most likely that the question was overlooked and skipped (n=53).

6. If age at first birth was available, but parity was 0, parity was labeled as missing because it is most likely that the subject gave birth at least once (n=231).
7. If menopausal age was younger than age at first birth, menopausal age was labeled as missing because menopausal age cannot be younger than age at first birth (n=1,779).

Furthermore, ER PR data needed to be linked with the original dataset by merging by ID. Prior to merging the baseline data with updated ER PR data, through multiple visits at the National Cancer Center which stores data on ER PR status, I obtained, cleaned, and updated ER PR data. In the hospital records, ER PR data were mainly recorded as symbols (+) or (-), or as words (pos/neg, yes/no, or phrases in Japanese). For analyses, these were recoded as follows: 0 = negative, 1 = positive, and 2 = unknown.

This study was approved by the institutional review boards of the National Cancer

Center Japan (approval number: 13-021) and The University of Tokyo (approval number: 10508). The STROBE checklist was used as a guideline to check recommended items.[42]

2.2 Exposure variables

2.2.1 Dietary assessment

The JPHC Study questionnaire inquired about physical attributes including weight and height, lifestyle habits including alcohol consumption, smoking, and physical activity, and other categories such as occupation, working hours, and stress. Dietary intake was assessed through a validated self-administered food frequency questionnaire (FFQ) which was conducted at the 5-year follow up survey and inquired about 138 food and beverage items.[43, 44]

Within this questionnaire, 19 seafood item questions were included regarding the intake of salted fish, dried fish, canned tuna, salmon or trout, bonito or tuna, cod or flat fish, sea bream, horse mackerel or sardine, mackerel pike or mackerel, dried small fish, eel, salted roe, prawn, squid, octopus, short-necked clam or crab shell, freshwater snails,

kamaboko (fish paste product), and chikuwa (fish paste product). Of the 19 seafood items, 11 were considered fish (salted fish, dried fish, canned tuna, salmon or trout, bonito or tuna, cod or flat fish, sea bream, horse mackerel or sardine, mackerel pike or mackerel, dried small fish, and eel). If a participant answered any one of the questions on an item that is considered to be "fish" in this study, that participant had a value for fish consumption. For instance, if a participant reported eating salmon but did not respond to the question regarding tuna, then, for that participant, fish consumption was calculated from the reported intake of salmon. Based on the value of n-3 PUFA per 100g edible portion of fish from the Standard Tables of Food Composition in Japan (5^{th} revised and enlarged edition in 2005), five of the fish items were considered PUFA-rich fish (salmon or trout, sea bream, horse mackerel or sardine, mackerel pike or mackerel, and eel).[45] All fish and seafood PUFA content in the FFQ were calculated from wild-caught values indicated in the Standard Tables of Food Composition in Japan except for sea bream, which was calculated from the cultured value. Cultured data were only available for sea bream out of the fish items asked in the FFQ and relevant to this study. Moreover, the FFQ did not ask participants to differentiate their intake between wild-caught and cultured fish and seafood.

Average frequency/portion size from the previous year with nine frequency categories (never, 1-3 times/month, 1-2 times/week, 3-4 times/week, 5-6 times/week, once/day, 2-3 times/day, 4-6 times/day, and 7 or more times/day) was recorded. Three amount choices for standard portion/unit were designated with categories of small (50% smaller), medium (same as standard), and large (50% larger). The standard portion was specified for each food item. For example, the standard portion of salmon or trout was 1 slice of fish meat (about 70g). Frequency multiplied by the standard portion size for each food item gave food intake in grams/day.

To calculate the daily intake of n-3 PUFA, n-6 PUFA, EPA, DHA, DPA, ALA, and n-6/n-3 ratio, I used the fatty acid (FA) composition table developed by the substitute method[46] based on the supplemental FA composition table for Japanese foods.[45] The validity was assessed for subsamples from the 138-item FFQ for 113 women, and the Spearman rank correlation coefficients between the energy-adjusted intake estimated from dietary records and the PUFA intake estimated from the FFQ for women were the following: total n-3 (0.34), total n-6 (0.21), EPA (0.45), DPA (0.39), DHA (0.37), and ALA (0.25), and the validity of the FFQ was considered sufficient to use for analyses.[16] The 5-year follow-up survey also included information on other covariates including

smoking and drinking habits, menarche, and menopausal status.

2.2.2 Other covariates

The results were adjusted for the following covariates that have been previously found to have associations with breast cancer: body mass index (BMI),[47, 48] reproductive factors,[47, 49, 50] including (age at menarche, age at first birth, parity, menopausal age, and menopausal status), use of exogenous female hormones,[51] leisure-time physical activity,[52, 53] smoking status,[54-56] alcohol intake,[7, 8] and isoflavone consumption.[57, 58]

BMI was measured by dividing weight (kg) by height (m^2) from the 5-year follow-up survey. Menopausal status (premenopause, postmenopause) was recorded at baseline at the time of the 5-year follow-up survey. Use of exogenous female hormones included oral contraceptives and menopausal hormone treatment and was classified as either never or ever. Participants were asked about their leisure-time physical activity from the following choices: 3 days per month, 1 to 2 days per week, and 3 to 4 times per week, or almost every day. For smoking status, participants were asked whether they smoke (current smokers), quit (past smokers), or never smoked (never smokers), and current smokers were asked how many cigarettes they smoked on average per day.

Intake of alcohol was measured by categorizing consumption into the following: hardly ever drink, 1 to 3 days per month, 1 to 2 days per week, 3 to 4 days per week, 5 to 6 days per week, or drink every day. Drinkers were asked to choose their most usual combination of type of alcohol from the following: Japanese sake ("1 go" 180 ml, 23 grams of ethanol), shochu/awamori ("1 go" 180 ml, 36 grams of ethanol), beer (large bottle 633 ml, 23 grams of ethanol), whiskey (1 glass 30 ml, 13 grams of ethanol), wine (1 glass 100 ml, 6 grams of ethanol). For each alcohol category, the participants could choose from: do not drink, less than 0.5 "go"/bottle/glass, 1 "go"/bottle/glass, 2 "go"/bottles/glasses, 3 "go"/bottles/glasses, 4 "go"/ bottles/glasses, 5-6 "go"/ bottles/glasses, or 7 "go"/ bottles/glasses.

The FFQ had eight items on soy food including miso soup, soymilk, tofu for miso soup, tofu for other dishes, yushidofu (predrained tofu), koyadofu (freeze-dried tofu), aburaage (deep-fried tofu), and natto (fermented soybeans). Isoflavone consumption was calculated by multiplying genistein and daidzein content of each food and adding each food item to obtain the final amount of isoflavone consumption. The food composition table for isoflavones in the Japanese diet was used to measure the genistein and daidzein content of each food item.[59]

2.3 Follow-up and identification of breast cancer cases

Identification of breast cancer cases was based on active patient notification from major local hospitals and linked with data from population-based cancer registries. Death certificates were used as a supplementary information source. A total of 11 cases relied on death certificate notification (DCN= 2.0%), out of which diagnosis information was unavailable for 9 cases (DCO= 1.6%). Local governments responsible for the registries approved the use of the registries. Codes C500-C509 from the Third Edition of the International Classification of Diseases for Oncology classified breast cancer cases. For 97% of all cases, diagnosis was microscopically verified. Either the presence of any positive cells in a specimen in immunohistochemical assay or >=10 fmol/mg protein in enzyme-linked immunoassay defined ER PR status. Hormone-receptor positivity values were determined by assay (immunohistochemical assay or enzyme-linked immunoassay) or classified by clinical decision made for medical treatment. The number of cases identified by assays vs. those classified by clinical judgment cannot be determined because the main data source from each hospital in which each patient was diagnosed currently only provided information on whether the subject was diagnosed to have ER or PR positive or negative tumors without information on what method or

cutoff was used. This is the current reality of ER PR data collection in Japan. For women who had more than one incidence of breast cancer recorded, only the first incidence was used.

Follow-up commenced on the date of administration of the 5-year follow-up survey and accumulated follow-up time until the date of diagnosis of cancer, date of death, date of migration out of study area, or end of follow-up (December 31, 2011), whichever occurred first. Linkage with death registrations at the regional PHCs under the Ministry of Health, Labor, and Welfare verified dates of death.

2.4 Statistical analysis

Multivariable Cox proportional hazards regression models were used to estimate the hazard ratios (HRs) and 95% confidence intervals (CIs). Age was used as the time scale for the Cox proportional hazards models through nonparametric adjustment for age.[60] The rationale behind using age as the time scale was that age is considered a determinant of breast cancer and may also be correlated with dietary intake.[49, 50] Furthermore, cohorts differed by age and location at the time of recruitment. Moreover,

the hazard may change more as a function of age than a function of baseline time (when the subjects enter at baseline).[61] The process of using age as the time scale was divided into three major steps:

1 Clean up all date information including dates such as birthdate and date of diagnosis.

2 Set Cox proportional model to run with new option of setting age as the time scale

3 Run Cox proportional model without age as a covariate

An example can be given with the clean up process of birthdate.

Example:

1. Birthday must be ordered in day, month, year for recognition as the time scale.

   This can be done by generating a new label:

   generate new_bdate=1000000*bdate_d+10000*bdate_m+bdate_y

2. If the day of the birthday is only 1 digit, 0 must be added to the date to make all dates 8 digits for identification as a date.

   generate string_new_bdate_l=string(new_bdate_l, "%08.0f")

3. The date must be formatted to the DMY calendar to be used as the birthdate in the time scale model.

   generate string_new_bdate_l_date=date(string_new_bdate_l,"DMY")

format %td string_new_bdate_l_date

4. After cleaning up and formatting all of the data related to dates, the Cox proportional hazards model can be run by declaring data to be survival-time data with specifications on the **origin** (when a subject becomes at risk) and the **enter** date (when the subject first enters the study). By setting the birthdate as the origin, age becomes the time scale of the model.

   stset string_new_end1dt_date, failure(brca==1) id(id) ///

   origin(string_new_bdate_l_date) **enter**(string_new_q05_date) scale(365.25)

Women were subdivided by quartiles with respect to their energy-adjusted intake of fish, n-3 PUFA including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), alpha linolenic acid (ALA), n-6 PUFA, and n-6/n-3 ratio. The residual approach was used for energy- adjustment for all dietary intake data.[62-65]

Using the median dietary intake by quartiles, I energy-adjusted the intake of log-transformed nutrients. Specifically, I took the following steps:

1 Calculate the mean logarithmic energy

2 Log-transform nutrients

3 Perform regression analyses.

A specific example can be given with fish. The mean logarithmic energy was 7.53. When obtaining the logarithmic intake of fish, a constant of 0.01 was added before taking the natural log to eliminate zero and negative values. The coefficient of energy for fish was calculated by obtaining the slope when the logarithmic intake of fish was regressed on the logarithmic energy which was 1.68 for fish. In this same regression, the constant for fish was -8.69. From the log-transformed energy adjusted nutrients, I calculated the nutrient residuals. The following equations outline the mathematical steps taken.

Equations:

calories of intake = (mean log-transformed energy)*(slope: logarithmic intake of nutrient regressed on the logarithmic energy) + (intercept: constant)

final energy-adjusted log-transformed calories= nutrient residuals + calories of intake

Example:

calories of fish = (7.527237)*(1.682644) + (-8.689212) = 3.976448

final energy-adjusted log-transformed calories of fish = nutrient residuals + 3.976448

Covariates of the main multivariable Cox proportional hazards models (Model A) included area (9), BMI (<18.5, 18.5-23.9, >23.9 kg/m^2), age at menarche (<=13, 14, 15, >=16 years), age at first birth (<26, >=26 years), parity (nulliparous, 1-2, 3, >=4 children), menopausal age (<=44, 45-54, >=55 years), menopausal status at baseline (premenopause, postmenopause), use of exogenous female hormones which include oral contraceptives and menopausal hormone treatment (never, ever), leisure-time physical activity (<3 days/month, 1-2 days/week, >=3 days/week), smoking status (never, ever), alcohol intake (regular drinker >=150 g of ethanol per week, non-regular drinker <150 g of ethanol per week), and total energy-adjusted intake of isoflavones (mg/day). The cut-offs for age at first birth and age at menopause were chosen based on the distribution of the Japanese population in the study's generation. All covariates represent the status at the time of the 5-year follow-up. To calculate the p-value for trend, the continuous variable from the median value for each intake of fish and PUFAs

was included in the regression model. To observe the continuous effect of each exposure, the continuous variables of the exposures (per 10 grams increase for fish and PUFA-rich fish/ 1 gram increase for n-3, EPA, DHA, DPA, ALA, n-6, and n-6/n-3 ratio) were also regressed in the Cox proportional hazards models with the same covariates in the main model (Model A).

The linearity assumption in the Cox proportional hazards model was tested by plotting the predicted values against the Martingale residuals with smoothers (y = 0). Transformation was considered necessary if the smoothers were not approximately flat and horizontal.[60]

Stratification by smoking and baseline menopausal status (Model B), and calculation of the p-value for interaction (P_{int}) between the exposure variables and smoking and menopausal status were performed. Multiplicative interaction between n-3 PUFA and n-6 PUFA was also analyzed.

HRs by quartile subdivision were estimated for further analysis by ER PR status (Model C) with the same covariates as the general model except for ER-PR- cases (n=77), in

which smoking status was excluded from the covariates due to too few numbers of overall cases and smoker cases (n=4 for smokers vs n=73 non-smokers). However, the proportion of smokers compared to other ER PR categories did not differ significantly. All analyses were performed with Stata MP 13.[66]

3. Results

3.1 Basic characteristics of study participants

A total of 556 breast cancer cases were newly diagnosed among 38,234 women during an average follow-up of 14.1 years. ER PR status information was available for 272 cases and ER PR status information was unavailable for 284 cases. Total fish intake ranged from a mean of 25.9 g/day below the lowest quartile to 126 g/day above the highest quartile. Total n-3 PUFA intake ranged from a mean of 2.1 g/day in the lowest quarter to 4.6 g/day in the highest quarter, while total n-6 PUFA intake ranged from a mean of 6.9 g/day in the lowest quarter to 12.5 g/day in the highest quarter. Women who consumed more total n-3 were less likely to be current smokers and more likely to engage in leisure-time physical activity (**Table 2**). Participants who reported eating more fish generally reported eating less soy. To date, there have been no studies that

examined either the correlation between the intake of fish and of soy, or the reasoning behind a possible negative correlation. However, another JPHC study (on the consumption of n-3 PUFA and hepatocellular carcinoma) also found that people with the lowest fish consumption had the highest intake of soy (at baseline).[67] Participants who consume larger portions of main staple foods such as fish might consume smaller amounts of side dishes including soy, although no studies have tested this hypothesis.

Table 2: Study subjects' basic characteristics according to consumption of fish, n-3, and n-6 polyunsaturated fatty acids in the Japan Public Health Center-based prospective Study (Quarters)

Characteristic	**Total fish (g/day)**				**PUFA-rich fish (g/day)**			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Mean, g/day (SD)	25.9 (9.6)	49.5 (6.0)	72.9 (7.8)	126 (41.4)	10.3 (5.0)	23.1 (3.4)	36.8 (4.8)	72.1 (30.0)
Number of subjects (n)	9,559	9,558	9,559	9,558	9,559	9,558	9,559	9,558
Age at baseline, year, mean (SD)	57.3 (8.0)	57.1 (7.7)	57.8 (7.5)	58.9 (7.4)	57.3 (7.9)	57.1 (7.7)	57.7 (7.5)	58.8 (7.5)
Body mass index at 5-year follow-up, kg/m2, mean (SD)	23.6 (3.2)	23.5 (3.1)	23.5 (3.1)	23.6 (3.2)	23.6 (3.2)	23.5 (3.1)	23.5 (3.1)	23.6 (3.2)
Age at menarche, year, mean (SD)	14.9 (2.0)	14.6 (1.9)	14.6 (1.8)	14.8 (1.8)	14.9 (2.0)	14.6 (1.9)	14.7 (1.8)	14.7 (1.8)
Age at first birth, year, mean (SD)	24.9 (3.6)	24.9 (3.4)	24.8 (3.3)	24.7 (3.3)	24.8 (3.6)	24.9 (3.4)	24.8 (3.3)	24.7 (3.3)
Number of deliveries, n, mean (SD)	3.4 (1.8)	3.2 (1.7)	3.1 (1.6)	3.0 (1.6)	3.4 (1.8)	3.2 (1.6)	3.1 (1.6)	3.1 (1.7)
Age at menopause, year, mean (SD)	48.4 (4.5)	48.3 (4.6)	48.5 (4.5)	48.5 (4.6)	48.2 (4.6)	48.4 (4.5)	48.5 (4.5)	48.6 (4.6)
Use of exogenous female hormones (ever), %	3.1	2.6	2.7	2.8	3.0	2.9	2.6	2.7
Current smoker, %	5.9	5.1	4.7	5.3	6.2	5.1	4.8	4.9
Regular drinker (>150g/week), %	6.1	5.4	5.4	4.9	6.3	5.7	4.9	4.8
Leisure-time physical activity (>=3 days/week), %	10.1	10.0	11.0	10.6	9.9	9.9	10.9	11.0
Dietary intake								
Total energy intake (kcal/d)	1,948 (612)	1,906 (563)	1,890 (541)	1,769 (515)	2,006 (612)	1,920 (559)	1,864 (540)	1,724 (496)
Daidzein and genistein (mg/day)	42.6 (42.7)	43.6 (34.9)	44.1 (30.4)	41.5 (28.1)	44.6 (43.5)	43.8 (33.9)	43.5 (30.9)	39.9 (27.3)
Soyfood items (g/day)	75.0 (118)	68.4 (77.2)	65.9 (62.8)	61.2 (56.7)	76.7 (117)	67.8 (72.9)	65.4 (67.3)	60.5 (57.8)

Characteristic	Total n-3 PUFA (g/day)				Total n-6 PUFA (g/day)			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Mean, g/day (SD)	2.1 (0.4)	2.9 (0.2)	3.4 (0.2)	4.6 (0.8)	6.9 (0.9)	8.6 (0.4)	9.9 (0.4)	12.5 (1.9)
Number of subjects (n)	9,559	9,558	9,559	9,558	9,559	9,558	9,559	9,558
Age at baseline, year, mean (SD)	57.4 (8.0)	57.3 (7.6)	57.6 (7.6)	58.7 (7.3)	58.1 (7.8)	57.4 (7.6)	57.5 (7.5)	58.0 (7.7)
Body mass index at 5-year follow-up, kg/m2, mean (SD)	23.5 (3.2)	23.5 (3.1)	23.6 (3.1)	23.7 (3.2)	23.4 (3.2)	23.4 (3.1)	23.6 (3.1)	23.9 (3.3)
Age at menarche, year, mean (SD)	14.8 (2.0)	14.7 (1.9)	14.6 (1.8)	14.8 (1.8)	14.6 (1.8)	14.5 (1.8)	14.7 (1.9)	15.1 (2.0)
Age at first birth, year, mean (SD)	24.9 (3.6)	24.9 (3.5)	24.8 (3.3)	24.7 (3.3)	24.7 (3.5)	24.9 (3.3)	24.9 (3.4)	24.9 (3.5)
Number of deliveries, n, mean (SD)	3.3 (1.8)	3.2 (1.7)	3.2 (1.6)	3.1 (1.6)	3.1 (1.7)	3.1 (1.6)	3.2 (1.6)	3.4 (1.8)
Age at menopause, year, mean (SD)	48.4 (4.5)	48.4 (4.5)	48.5 (4.6)	48.5 (4.6)	48.4 (4.6)	48.4 (4.7)	48.4 (4.4)	48.5 (4.5)
Use of exogenous female hormones (ever), %	2.9	2.9	2.8	2.6	2.6	2.9	2.6	3.1
Current smoker, %	6.7	5.2	4.7	4.5	7.0	5.0	4.5	4.6
Regular drinker (>150g/week), %	7.6	5.5	4.5	4.2	8.9	5.2	4.1	3.6
Leisure-time physical activity (>=3 days/week), %	8.8	9.7	10.9	12.2	8.4	9.2	10.7	13.4
Dietary intake								
Total energy intake (kcal/d)	1,890 (611)	1,881 (544)	1,899 (542)	1,842 (548)	1,873 (592)	1,897 (549)	1,912 (545)	1,831 (560)
Daidzein and genistein (mg/day)	32.2 (24.0)	40.6 (29.1)	47.3 (35.9)	51.7 (42.8)	27.4 (17.8)	38.0 (21.8)	45.7 (25.9)	60.8 (52.0)
Soyfood items (g/day)	50.0 (54.4)	64.9 (70.5)	74.7 (93.6)	80.9 (99.1)	36.6 (31.3)	53.5 (42.1)	68.3 (52.0)	112 (136)

3.2 Breast cancer incidence by quarters of fish and polyunsaturated fatty acids intake, with analyses of interaction by major confounders

The multivariable adjusted model did not yield any significant association between fish, n-3 PUFA (total and sub-groups: EPA, DHA, DPA, and ALA), and n-6 PUFA intake and breast cancer risk [multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ =0.99 (95% CI: 0.77-1.28; p_{trend}=0.79) for fish, multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.99 (95% CI: 0.76-1.28; p_{trend}= 0.57) for total n-3 PUFA, and multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.96 (95% CI: 0.73-1.28; p_{trend}=0.51) for n-6 PUFA] (**Table 3**).

Table 3: Hazard ratios (HRs) and 95% confidence intervals (CIs) for the association between fish, n-3, and n-6 polyunsaturated fatty acids and breast cancer risk in the Japan Public Health Center-based prospective Study (Model A)

Food group	**All (*N*=38,234 subjects/ 556 total cases)**							
	Q1. Ref	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**	**Mean (SD) g/day**	**P_{trend}**	**Smoking P_{int}**	**10 g continuous HR (95% CI)**
Total fish								
Median (g/day)[a]	22.6 g/day	44.5 g/day	72.4 g/day	124 g/day	68.5 (42.9)			
Number of cases[a]	135	136	135	150				
Multivariable adjusted[a]	1.00 (ref)	0.93 (0.73-1.18)	1.10 (0.86-1.39)	0.99 (0.77-1.28)		0.79	0.29	1.00 (0.99-1.02)
PUFA-rich fish								
Median (g/day)[a]	10.3 g/day	21.1 g/day	37.5 g/day	66.7 g/day	35.6 (27.8)			
Number of cases[a]	151	129	134	142				
Multivariable adjusted[a]	1.00 (ref)	1.18 (0.93-1.49)	1.13 (0.89-1.45)	1.14 (0.88-1.48)		0.50	0.49	1.01 (0.98-1.04)
	Q1. Ref	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**		**P_{trend}**	**Smoking P_{int}**	**1g continuous HR (95% CI)**
Total n-3								
Median (g/day)[a]	1.7 g/day	2.6 g/day	3.5 g/day	5.2 g/day	3.3 (1.0)			
Number of cases[a]	141	135	123	157				
Multivariable adjusted[a]	1.00 (ref)	1.19 (0.94-1.50)	1.00 (0.78-1.28)	0.99 (0.76-1.28)		0.57	0.79	1.01 (0.94-1.10)
Total n-6								
Median (g/day)[a]	5.4 g/day	7.8 g/day	10.2 g/day	14.3 g/day	9.5 (2.3)			
Number of cases[a]	130	139	138	149				
Multivariable adjusted[a]	1.00 (ref)	1.10 (0.87-1.41)	0.89 (0.68-1.16)	0.96 (0.73-1.28)		0.51	0.17	1.01 (0.97-1.05)
EPA								
Median (g/day)[a]	0.1 g/day	0.2 g/day	0.4 g/day	0.7 g/day	0.4 (0.2)			
Number of cases[a]	134	144	129	149				
Multivariable adjusted[a]	1.00 (ref)	1.03 (0.80-1.32)	1.04 (0.80-1.34)	1.06 (0.81-1.39)		0.67	0.28	1.06 (0.78-1.45)

DHA								
Median (g/day)[a]	0.2 g/day	0.4 g/day	0.7 g/day	1.1 g/day	0.6 (0.4)			
Number of cases[a]	131	140	136	149				
Multivariable adjusted[a]	1.00 (ref)	1.02 (0.80-1.30)	0.98 (0.76-1.27)	1.13 (0.87-1.46)		0.35	0.52	1.03 (0.84-1.26)
DPA								
Median (g/day)[a]	0.03 g/day	0.06 g/day	0.1 g/day	0.2 g/day	0.1 (0.06)			
Number of cases[a]	138	138	134	146				
Multivariable adjusted[a]	1.00 (ref)	1.10 (0.87-1.41)	1.02 (0.79-1.32)	1.17 (0.90-1.51)		0.32	0.39	1.21 (0.34-4.34)
ALA								
Median (g/day)[a]	1.1 g/day	1.7 g/day	2.2 g/day	3.2 g/day	2.0 (0.6)			
Number of cases[a]	133	145	120	158				
Multivariable adjusted[a]	1.00 (ref)	0.98 (0.77-1.24)	0.89 (0.70-1.15)	0.96 (0.74-1.24)		0.68	0.21	1.00 (0.87-1.16)
n-6/n-3								
Median (g/day)[a]	2.2 g/day	2.8 g/day	3.3 g/day	4.0 g/day	3.1 (0.9)			
Number of cases[a]	142	120	146	148				
Multivariable adjusted[a]	1.00 (ref)	0.78 (0.61-1.01)	0.98 (0.77-1.24)	0.95 (0.73-1.23)		0.07	0.12	0.98 (0.88-1.09)

[a] Multivariable Cox proportional hazards models were adjusted for area (9), BMI (<18.5, 18.5-23.9, >23.9 kg/m2), age at menarche (<=13, 14, 15, >=16 years), age at first birth (<26, >=26 years), parity (nulliparous, 1-2, 3, >=4 children), menopausal age (<=44, 45-54, >=55 years), menopausal status at baseline (premenopause, postmenopause), use of exogenous female hormones which include oral contraceptives and menopausal hormone treatment (never, ever), leisure-time physical activity (<3 days/month, 1-2 days/week, >=3 days/week), smoking status (never, ever), alcohol intake (regular drinker >=150 g of ethanol per week, non-regular drinker <150 g of ethanol per week), and total energy-adjusted intake of isoflavones (mg/day)

There were no significant changes in the results after exclusion of former and current smokers [multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.96 (95% CI: 0.74-1.25; p_{trend} = 0.95 for fish, multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.96 (95% CI: 0.74-1.25; p_{trend} = 0.40) for total n-3 PUFA, and multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.99 (95% CI: 0.74-1.33; p_{trend} = 0.59) for n-6 PUFA).

The results after stratifying for menopausal status also did not provide any significant association with breast cancer risk [multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 1.08 (95% CI: 0.65-1.80; p_{trend} = 0.43) (premenopause) and multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.94 (95% CI: 0.70-1.27; p_{trend} = 0.79) (postmenopause) for fish, multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 1.10 (95% CI: 0.67-1.80; p_{trend} = 0.98) (premenopause) and multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.95 (95% CI: 0.70-1.29; p_{trend} = 0.47) (postmenopause) for total n-3 PUFA, and multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 0.71 (95% CI: 0.43-1.18; p_{trend} = 0.11) and multivariable adjusted HR $_{Q4}$ vs $_{Q1}$ = 1.12 (95% CI: 0.80-1.58; p_{trend} = 0.82) (postmenopause) for n-6 PUFA] (**Table 4**).

Table 4: Hazard ratios (HRs)[a] and 95% confidence intervals (CIs) for the association between fish, n-3, and n-6 polyunsaturated fatty acids and breast cancer risk stratified by menopausal status in the Japan Public Health Center-based prospective Study (Model B)

Food group	**Premenopausal** (*n*=8,928 155 cases)					**Postmenopausal** (*n*=29,306 401 cases)					P_{int}
	Q1. Ref	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**	P_{trend}	**Q1. Ref**	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**	P_{trend}	
Total fish											
Median (g/day)	23.5 g/day	44.0 g/day	72.0 g/day	120 g/day		22.2 g/day	44.7 g/day	72.6 g/day	125 g/day		
Number of cases	36	47	35	37		99	89	100	113		
Multivariable adjusted	1.00 (ref)	0.90 (0.57-1.41)	1.39 (0.90-2.16)	1.08 (0.65-1.80)	0.43	1.00 (ref)	0.94 (0.71-1.25)	1.00 (0.75-1.33)	0.94 (0.70-1.27)	0.79	0.35
PUFA-rich fish											
Median (g/day)	10.7 g/day	21.0 g/day	37.5 g/day	64.1 g/day		10.0 g/day	21.4 g/day	37.5 g/day	67.6 g/day		
Number of cases	45	39	32	39		106	90	102	103		
Multivariable adjusted	1.00 (ref)	1.26 (0.81-1.96)	1.19 (0.74-1.91)	1.50 (0.92-2.46)	0.15	1.00 (ref)	1.14 (0.86-1.52)	1.11 (0.83-1.48)	1.02 (0.75-1.37)	0.85	0.23
Total n-3											
Median (g/day)	1.7 g/day	2.6 g/day	3.5 g/day	5.1 g/day		1.7 g/day	2.6 g/day	3.5 g/day	5.2 g/day		
Number of cases	44	41	35	35		97	94	88	122		
Multivariable adjusted	1.00 (ref)	1.22 (0.80-1.86)	0.93 (0.58-1.50)	1.10 (0.67-1.80)	0.98	1.00 (ref)	1.18 (0.89-1.56)	1.03 (0.77-1.38)	0.95 (0.70-1.29)	0.47	0.98
Total n-6											
Median (g/day)	5.5 g/day	7.8 g/day	10.2 g/day	14.3 g/day		5.4 g/day	7.8 g/day	10.2 g/day	14.3 g/day		
Number of cases	38	45	40	32		92	94	98	117		
Multivariable adjusted	1.00 (ref)	0.79 (0.51-1.21)	0.47 (0.28-0.77)	0.71 (0.43-1.18)	0.11	1.00 (ref)	1.30 (0.96-1.75)	1.15 (0.84-1.58)	1.12 (0.80-1.58)	0.82	0.08
EPA											
Median (g/day)	0.1 g/day	0.2 g/day	0.4 g/day	0.7 g/day		0.1 g/day	0.2 g/day	0.4 g/day	0.7 g/day		
Number of cases	39	40	38	38		95	104	91	111		
Multivariable adjusted	1.00 (ref)	1.13 (0.71-1.80)	1.17 (0.72-1.93)	1.36 (0.81-2.29)	0.26	1.00 (ref)	0.99 (0.74-1.33)	0.98 (0.72-1.33)	0.96 (0.70-1.31)	0.77	0.46

DHA											
Median (g/day)	0.2 g/day	0.4 g/day	0.7 g/day	1.1 g/day		0.2 g/day	0.4 g/day	0.7 g/day	1.2 g/day		
Number of cases	39	41	39	36		92	99	97	113		
Multivariable adjusted	1.00 (ref)	1.11 (0.71-1.75)	1.10 (0.68-1.79)	1.38 (0.83-2.28)	0.22	1.00 (ref)	0.98 (0.73-1.31)	0.94 (0.69-1.27)	1.03 (0.76-1.39)	0.81	0.52
DPA											
Median (g/day)	0.03 g/day	0.06 g/day	0.1 g/day	0.2 g/day		0.03 g/day	0.06 g/day	0.1 g/day	0.2 g/day		
Number of cases	42	39	36	38		96	99	98	108		
Multivariable adjusted	1.00 (ref)	1.11 (0.71-1.74)	1.10 (0.68-1.78)	1.41 (0.86-2.31)	0.18	1.00 (ref)	1.11 (0.83-1.47)	0.99 (0.73-1.35)	1.08 (0.80-1.46)	0.79	0.52
ALA											
Median (g/day)	1.1 g/day	1.7 g/day	2.2 g/day	3.1 g/day		1.1 g/day	1.7 g/day	2.2 g/day	3.2 g/day		
Number of cases	41	43	36	35		92	102	84	123		
Multivariable adjusted	1.00 (ref)	0.76 (0.49-1.17)	0.83 (0.53-1.29)	0.70 (0.42-1.16)	0.21	1.00 (ref)	1.09 (0.82-1.46)	0.93 (0.69-1.26)	1.08 (0.80-1.47)	0.78	0.23
n-6/n-3											
Median (g/day)	2.3 g/day	2.8 g/day	3.3 g/day	4.0 g/day		2.2 g/day	2.8 g/day	3.3 g/day	4.1 g/day		
Number of cases	44	23	51	37		98	97	95	111		
Multivariable adjusted	1.00 (ref)	0.58 (0.36-0.95)	0.81 (0.52-1.25)	0.68 (0.42-1.12)	0.32	1.00 (ref)	0.86 (0.65-1.15)	1.05 (0.79-1.39)	1.08 (0.80-1.45)	0.78	0.31

[a] Multivariable Cox proportional hazards models were adjusted for area (9), BMI (<18.5, 18.5-23.9, >23.9 kg/m2), age at menarche (<=13, 14, 15, >=16 years), age at first birth (<26, >=26 years), parity (nulliparous, 1-2, 3, >=4 children), menopausal age (<=44, 45-54, >=55 years), menopausal status at baseline (premenopause, postmenopause), use of exogenous female hormones which include oral contraceptives and menopausal hormone treatment (never, ever), leisure-time physical activity (<3 days/month, 1-2 days/week, >=3 days/week), smoking status (never, ever), alcohol intake (regular drinker >=150 g of ethanol per week, non-regular drinker <150 g of ethanol per week), and total energy-adjusted intake of isoflavones (mg/day)

Hence, data did not provide any evidence of multiplicative interactions between the exposure variables and smoking and menopausal status. Furthermore, the interaction between n-3 PUFA and n-6 PUFA was not significant; HR for interactions were close to null, and the HR for the main effects were not significantly different from those in the model without interaction (**Table 5**).

Table 5: Interaction between n-3 PUFA and n-6 PUFA

Model specification	**n-3 PUFA * n-6 PUFA**
Model A	HR= 1.00 (95% CI:1.00-1.01), P_{int}=0.14
Model B (pre-menopause)	HR= 1.01 (95% CI:1.00-1.02), P_{int}=0.05
Model B (postmenopause)	HR= 1.00 (95% CI:0.99-1.01), P_{int}=0.55
Model C (ER+ PR+)	HR= 0.99 (95% CI:0.96-1.02), P_{int}=0.55
Model C (ER- PR-)	HR= 1.01 (95% CI:0.97-1.07), P_{int}=0.44

The linearity assumption of the Cox proportional hazards model was tested by plotting the predicted values against the Martingale residuals with smoothers. The smoothers (horizontal line y = 0) were approximately flat, which indicated no systematic pattern of deviation of the smoothed line from the horizontal. This finding supports the assumption of linearity, and thus transformation was not considered necessary (Appendix 3).[60]

3.3 Further analysis by estrogen and progesterone receptor status

Further analysis was performed by analyzing data on estrogen and progesterone receptor status. Intake of total n-6 PUFA was positively associated with the development of ER+PR+ tumors HR $_{Q4}$ vs $_{Q1}$=2.94 (95% CI: 1.26-6.89; ptrend =0.02)] (**Table 6**). Figure 12 summarizes the hazard ratios and 95% confidence intervals for ER+ PR+ cases. When analyzing by ERPR known vs. unknown, significant results were not obtained.

Table 6: Hazard ratios (HRs)[a] and 95% confidence intervals (CIs) for the association between fish, n-3, and n-6 polyunsaturated fatty acids and breast cancer risk stratified by ER and PR status in the Japan Public Health Center-based prospective Study by quarters (Model C)

	ER+PR+ (131 cases)				10 g/1g[b] continuous	P_{trend}
	Q1. Ref	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**	**HR (95% CI)**	
Food Group						
Total fish	1.00 (ref)	0.67 (0.33-1.39)	0.84 (0.43-1.68)	1.08 (0.49-2.40)	0.99 (0.92-1.06)	0.62
PUFA-rich fish	1.00 (ref)	0.97 (0.55-1.70)	0.61 (0.33-1.14)	0.83 (0.35-1.95)	0.95 (0.85-1.07)	0.44
Total n-3	1.00 (ref)	1.36 (0.70-2.64)	0.96 (0.50-1.84)	1.03 (0.44-2.41)	0.99 (0.72-1.36)	0.76
Total n-6	1.00 (ref)	1.95 (0.99-3.84)	2.13 (1.03-4.42)	2.94 (1.26-6.89)	1.05 (0.96-1.14)	0.02
EPA	1.00 (ref)	0.47 (0.25-0.89)	0.60 (0.31-1.17)	0.63 (0.27-1.46)	0.52 (0.13-2.07)	0.47
DHA	1.00 (ref)	0.64 (0.34-1.21)	0.77 (0.39-1.50)	0.83 (0.39-1.77)	0.68 (0.28-1.66)	0.75
DPA	1.00 (ref)	1.02 (0.56-1.87)	0.74 (0.37-1.46)	0.77 (0.36-1.63)	0.15 (0.0007-30.6)	0.45
ALA	1.00 (ref)	1.24 (0.64-2.39)	1.62 (0.83-3.18)	1.76 (0.80-3.86)	1.24 (0.77-1.99)	0.10
n-6/n-3	1.00 (ref)	0.87 (0.45-1.70)	1.17 (0.60-2.28)	1.69 (0.76-3.74)	1.19 (0.87-1.64)	0.17
	ER-PR- (77 cases)[c]				**10 g/1g[b] continuous**	**P_{trend}**
	Q1. Ref	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**	**HR (95% CI)**	
Food Group						
Total fish	1.00 (ref)	0.62 (0.22-1.74)	0.89 (0.31-2.56)	0.70 (0.24-1.99)	1.02 (0.95-1.09)	0.69
PUFA-rich fish	1.00 (ref)	1.24 (0.44-3.48)	1.85 (0.68-5.05)	0.85 (0.23-3.19)	1.03 (0.91-1.16)	0.96
Total n-3	1.00 (ref)	2.59 (0.73-9.17)	2.34 (0.68-8.03)	1.18 (0.39-3.60)	1.07 (0.79-1.46)	0.78
Total n-6	1.00 (ref)	0.46 (0.13-1.64)	0.89 (0.23-3.42)	0.72 (0.16-3.23)	0.93 (0.81-1.07)	0.69
EPA	1.00 (ref)	1.08 (0.29-4.11)	1.34 (0.49-3.65)	1.04 (0.30-3.58)	1.39 (0.38-5.03)	0.83
DHA	1.00 (ref)	0.92 (0.26-3.20)	1.15 (0.38-3.42)	0.93 (0.28-3.13)	1.15 (0.50-2.65)	0.98
DPA	1.00 (ref)	1.05 (0.35-3.11)	1.17 (0.45-3.01)	1.26 (0.41-3.88)	2.72 (0.01-562)	0.63
ALA	1.00 (ref)	0.67 (0.21-2.10)	0.92 (0.28-2.99)	1.44 (0.43-4.83)	1.10 (0.54-2.25)	0.28
n-6/n-3	1.00 (ref)	0.99 (0.31-3.11)	0.84 (0.21-3.33)	0.76 (0.23-2.55)	0.74 (0.46-1.18)	0.59

	ERPR Known (272 cases)				10 g/ 1g[c] continuous	P_{trend}
	Q1. Ref	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**	**HR (95% CI)**	
Food Group						
Total fish	1.00 (ref)	0.91 (0.65-1.29)	1.24 (0.89-1.73)	0.81 (0.55-1.20)	1.00 (0.97-1.03)	0.51
PUFA-rich fish	1.00 (ref)	1.19 (0.85-1.67)	1.25 (0.89-1.77)	0.97 (0.66-1.44)	1.00 (0.96-1.05)	0.75
Total n-3	1.00 (ref)	1.27 (0.92-1.75)	0.97 (0.68-1.38)	0.85 (0.58-1.25)	0.97 (0.86-1.09)	0.19
Total n-6	1.00 (ref)	1.06 (0.75-1.50)	0.88 (0.61-1.27)	0.85 (0.57-1.27)	0.99 (0.93-1.05)	0.29
EPA	1.00 (ref)	0.91 (0.65-1.29)	0.99 (0.69-1.41)	0.80 (0.54-1.19)	0.92 (0.56-1.53)	0.33
DHA	1.00 (ref)	0.90 (0.64-1.27)	0.95 (0.67-1.36)	0.86 (0.59-1.26)	0.94 (0.68-1.31)	0.50
DPA	1.00 (ref)	0.98 (0.70-1.37)	0.97 (0.68-1.38)	0.93 (0.64-1.36)	0.60 (0.08-4.72)	0.72
ALA	1.00 (ref)	1.15 (0.82-1.62)	1.06 (0.75-1.52)	0.89 (0.60-1.31)	0.92 (0.74-1.15)	0.43
n-6/n-3	1.00 (ref)	0.88 (0.62-1.26)	1.10 (0.78-1.55)	1.01 (0.69-1.47)	1.03 (0.88-1.20)	0.74

	ERPR Unknown (284 cases)				10 g/ 1g[c] continuous	P_{trend}
	Q1. Ref	**Q2. HR (95% CI)**	**Q3. HR (95% CI)**	**Q4. HR (95% CI)**	**HR (95% CI)**	
Food Group						
Total fish	1.00 (ref)	0.94 (0.67-1.32)	0.96 (0.68-1.35)	1.15 (0.82-1.62)	1.01 (0.99-1.03)	0.32
PUFA-rich fish	1.00 (ref)	1.16 (0.83-1.61)	1.02 (0.72-1.45)	1.28 (0.90-1.81)	1.02 (0.98-1.05)	0.22
Total n-3	1.00 (ref)	1.11 (0.79-1.55)	1.03 (0.73-1.46)	1.11 (0.79-1.58)	1.05 (0.95-1.15)	0.64
Total n-6	1.00 (ref)	1.13 (0.80-1.59)	0.89 (0.61-1.30)	1.08 (0.73-1.61)	1.02 (0.98-1.07)	0.90
EPA	1.00 (ref)	1.16 (0.81-1.65)	1.09 (0.74-1.59)	1.35 (0.93-1.96)	1.14 (0.78-1.69)	0.13
DHA	1.00 (ref)	1.14 (0.81-1.62)	1.01 (0.69-1.46)	1.43 (1.00-2.04)	1.08 (0.84-1.40)	0.06
DPA	1.00 (ref)	1.24 (0.88-1.75)	1.09 (0.75-1.58)	1.42 (0.99-2.03)	1.86 (0.38-9.03)	0.09
ALA	1.00 (ref)	0.83 (0.59-1.17)	0.75 (0.53-1.07)	1.02 (0.72-1.44)	1.07 (0.89-1.30)	0.84
n-6/n-3	1.00 (ref)	0.70 (0.50-1.00)	0.89 (0.64-1.24)	0.90 (0.63-1.29)	0.93 (0.79-1.09)	0.82

[a] Multivariable Cox proportional hazards models were adjusted for area (9), BMI (<18.5, 18.5-23.9, >23.9 kg/m2), age at menarche (<=13, 14, 15, >=16 years), age at first birth (<26, >=26 years), parity (nulliparous, 1-2, 3, >=4 children), menopausal age (<=44, 45-54, >=55 years), menopausal status at baseline (premenopause, postmenopause), use of exogenous female hormones which include oral contraceptives and menopausal hormone treatment (never, ever), leisure-time physical activity (<3 days/month, 1-2 days/week, >=3 days/week), smoking status (never, ever), alcohol intake (regular drinker >=150 g of ethanol per week, non-regular drinker <150 g of ethanol per week), and total energy-adjusted intake of isoflavones (mg/day)

[b] Unit (per 10g increase for fish and PUFA-rich fish, and per 1g increase for n-3, n-6, EPA, DHA, DPA, and ALA)

[c] Model for ER-PR- cases excluded smoking status as a covariate

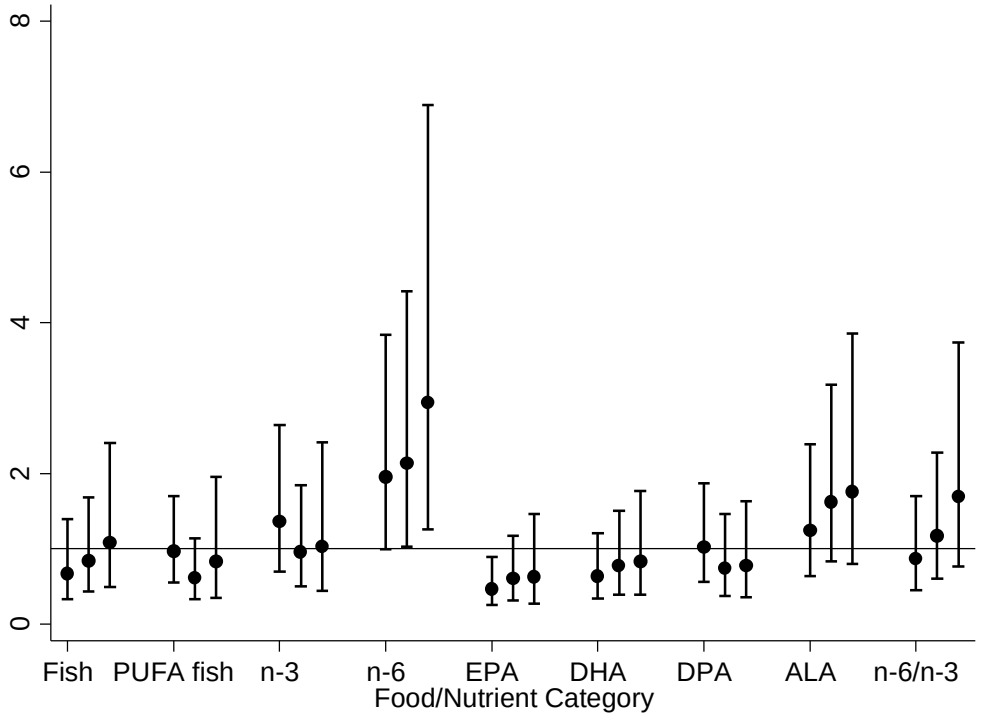


Figure 12: Overview of hazard ratios and 95% confidence intervals for ER+ PR+ cases

Furthermore, heterogeneity tests across ER+PR+ and ER-PR- subgroups were performed and there is no evidence to suggest any heterogeneity between the subgroups (**Table 7**).

Table 7: Heterogeneity tests across ER PR subgroups

Food Group	**p-value**
Total fish	0.20
PUFA-rich fish	0.17
Total n-3	0.13
Total n-6	0.28
EPA	0.22
DHA	0.20
DPA	0.17
ALA	0.37

4. Discussion

4.1 Summary of findings

This is the first study in Japan to find that when breast cancer cases are analyzed by ER PR status subtype, intake of total n-6 PUFA was positively associated with ER+PR+ tumors. The overall results of this study suggested that intake of fish, n-3 PUFA, and n-6 PUFA is not significantly associated with general breast cancer risk in this large prospective cohort study of Japanese women. Furthermore, consideration of smoking and menopausal status, potential effect modifiers for breast cancer, did not substantially alter the findings.[25, 68, 69]

Despite the fact that the 95% confidence intervals include 1, the ALA and n-6/n-3 hazard ratios point-estimates for ER+PR+ tumors show a dose-response relationship that fulfills Hill's biological-gradient criterion.[70] A few experimental studies have looked at this association and found growth-inhibitory and proapoptotic effects of ALA on breast cancer cells which contradicts the positive association found in this study.[71, 72] Moreover, a meta-analysis of 12 articles found no significant association between ALA and breast cancer risk.[23] To the best of the author's knowledge, there are no studies that have found a significant positive association between the intake of ALA and ER+ PR+ tumors that can support and suggest a mechanism for the positive dose-response relationship found between ALA and ER+PR+ in this study.

For the dose-response relationship in the n-6/n-3 hazard ratio point-estimates found for ER+ PR+ tumors, the results may be in line with the positive association found in a meta-analysis of 11 prospective studies.[30] Similar to the conclusion of this study, several of the prospective studies included in this meta-analysis concluded null associations between n-6/n-3 and breast cancer risk despite positive associations between n-6/n-3 and breast cancer risk because their 95% confidence intervals spanned 1.[24, 25, 29] In terms of the mechanism, studies have found that it is biologically plausible

that the ratio between n-6 and n-3 intake plays a role in breast cancer risk through competition over the same metabolic pathway to regulate tumor growth.[73, 74]

4.2 Comparison with other studies

Compared to previous studies, intake of fish ranged from 23.0 g/day in Q1 to 134.2 g/day in Q5 in quintiles in this study, while in the EPIC study the intake ranged from 1.9 g/day in Q1 to 89.5 g/day in Q5 in the UK and 4.7 g/day in Q1 to 115.5 g/day in Q5 in Norway (Table 8).[18] The Singapore Chinese Health study reported intake of fish which ranged from 21.3 g/day in Q1 to 75.0 g/day in Q4, while the intake of fish divided by quartiles in this study ranged from 25.9 g/day in Q1 to 126.0 g/day in Q4.[17] In the Malmo Diet and Cancer study in Sweden, intake of total n-3 PUFA ranged from 1.5 g/day in Q1 to 3.2 g/day in Q5 and intake of total n-6 PUFA ranged from 7.2 g/day in Q1 to 16.0 g/day in Q5 while in this study total n-3 PUFA ranged from 2.0 g/day in Q1 to 4.7 g/day in Q5 and total n-6 PUFA intake ranged from 6.7 g/day in Q1 to 12.9 g/day in Q5.[25] For fish and n-3 PUFA, the participants in this study had the highest dietary intake compared to the EPIC study, Singapore Chinese Health study, and Malmo Diet and Cancer study while n-6 PUFA intake was lower than the subjects in the Swedish study.

Table 8: Study subjects' basic characteristics according to consumption of fish, n-3, and n-6 polyunsaturated fatty acids for comparison with other studies in quintiles

Characteristic	**Total fish (g/day)**				
	Q1	Q2	Q3	Q4	Q5
Mean, g/day (SD)	23.0 (8.7)	43.3 (4.7)	60.3 (5.3)	81.6 (7.3)	134 (42.1)
Number of subjects (n)	7,647	7,647	7,647	7,647	7,646
	Total n-3 PUFA (g/day)				
	Q1	Q2	Q3	Q4	Q5
Mean, g/day (SD)	2.0 (0.3)	2.7 (0.1)	3.1 (0.1)	3.6 (0.2)	4.7 (0.8)
Number of subjects (n)	7,647	7,647	7,647	7,647	7,646
	Total n-6 PUFA (g/day)				
	Q1	Q2	Q3	Q4	Q5
Mean, g/day (SD)	6.7 (0.9)	8.3 (0.3)	9.3 (0.3)	10.4 (0.4)	12.9 (2.0)
Number of subjects (n)	7,647	7,647	7,647	7,647	7,646

In line with the results from this study, a recent meta-analysis reported no significant association between fish intake and breast cancer risk and an inverse association of EPA intake and breast cancer risk.[23] The European Prospective Investigation into Cancer and Nutrition (EPIC) study, one of the largest prospective cohort studies, also found no significant associations between dietary intake of fish and breast cancer risk.[18] Another systematic review reported insufficient consistent results to suggest a strong association between n-3 PUFA and breast cancer.[75] However, it should be also noted that there have been studies that have found an inverse association between fish and breast cancer, which contradicts the null association found in this study.[11, 20, 21] Furthermore, other studies have also found an inverse association between n-3 PUFA and breast cancer.[17, 24]

Limited studies exist on the association between n-6 PUFA and breast cancer risk; however, the Malmo Diet and Cancer study found a positive association between n-6 PUFA intake and breast cancer risk in postmenopausal women, which is consistent with the results from this study on the analyses of the positive association between ER+PR+ tumors and intake of total n-6 PUFA.[25] Other studies have found a null association between n-6 PUFA and breast cancer risk.[17, 29]Very few epidemiological studies have been conducted on the association between fatty acids and specific ER PR subtype breast cancer risk. One study found a significant increase in incidence rate of ER+ cases and high total fish intake but no significant changes for ER- cases.[12] Another study found an inverse association between fish consumption and ER- tumors.[11] Lack of studies involving ER PR analyses of breast cancer risk and fish and fatty acids motivate further experimental and epidemiological research.

Due to the relatively small number of cases for ER PR analysis, the results should be interpreted with caution. ER+ PR- and ER- PR+ cases could not be analyzed due to the small number of cases. Tests on heterogeneity between ER PR subgroups did not yield significant results.

4.3 Limitations and strengths

This study has several limitations. Due to missing values of covariates and exposure variables, a large number of participants were excluded, which may have led to selection bias from complete case analysis if the exclusion was associated with breast cancer outcome. If the exclusion occurred predominantly among the exposed participants in whom breast cancer developed, then the observed measure of disease frequency in those participants would have been lower than the truth; in that case, the hazard ratio would have been biased towards the null. If the exclusion occurred predominantly among the unexposed participants in whom breast cancer developed, then the observed measure of disease frequency in those participants would have been lower than the truth; in that case, the hazard ratio would have been biased upward. Yet, in this study, it is unlikely that the loss of breast cancer cases was associated with exposure. With that lack of an association, the loss would have been non-differential and any bias would have been towards the null. Further analysis of missing data showed that there were no significant differences at baseline between the participants whose data were not used due to missing covariates and the participants for whom data on covariates were available.

Moreover, the lack of ER PR data for approximately half of the cases lead to limited statistical power. However, having limited ER PR data was unavoidable because ER PR status was not substantially considered for clinical treatment during the early periods of follow-up. Since the JPHC study only has data on whether ER PR is positive or negative, lack of further information on what method was used to determine positivity for each participant may be perceived as a limitation.

Moreover, the self-reported nature of the questionnaires inevitably leads to measurement error, yet the FFQ was validated and has been established to reflect Japanese dietary intake.[16] The correlation coefficient for the validity of total n-6 PUFA was not as significant as other PUFA because cooking oil largely contributed to the intake of n-6 PUFA; however, cooking oil was not part of the inquiry of dietary records. Attenuation of the correlation coefficient could have also resulted due to the contribution of n-6 PUFA from lean foods including rice and tofu, which were consumed daily leading to minimal inter-person variability of study subjects.[16] However, it should be noted that FFQ validity and reproducibility still heavily depend on the type of food or nutrient as also seen in the range of correlation coefficients in this study. Having a low validity for a specific nutrient can indicate potential larger measurement

errors.

Misclassification of dietary habit changes throughout the study period could have also led to systematic bias because the FFQ assumes that there were no significant changes in dietary intake throughout the follow-up time. However, there is no reason to believe that there are unequal differences in changes in exposure between the breast cancer cases and non-cases. This would lead to non-differential misclassification and bias towards the null.

Furthermore, since the FFQ on fish and seafood intake did not ask subjects to differentiate their intake between wild-caught and cultured, the results may be also affected by misclassification. PUFA content can differ between wild-caught and cultured fish and other seafood.[76] Moreover, cultured fish may show more constant rates of EPA and DHA content because the feed is controlled and balanced throughout the farming period.[77] However, the trend in catches of wild-caught versus cultured marine fisheries in Japan from 1995 to 2013 shows a relatively constant proportion of cultured marine fisheries (Table 9).[78] Therefore, a significant change in the proportion of intake of wild-caught and cultured fish and seafood is unlikely. Yet, if bias were to exist, the

bias will be towards the null because of non-differential misclassification; it is most likely that cases and non-cases both had misclassification of PUFA content due to the inability to differentiate between the intake of wild-caught and cultured fish.

Table 9: Catches by sector of fisheries

Year		Wild marine fisheries (1,000t)	% of wild marine fisheries out of total	Cultured marine fisheries (1,000t)	% of cultured marine fisheries out of total	Total (1,000t)
1995		6,007	82%	1,315	18%	7,322
2000		5,022	80%	1,231	20%	6,253
2005		4,457	79%	1,212	21%	5,669
2010		4,122	79%	1,111	21%	5,233
2011	a)b)	3,824	81%	869	19%	4,693
2012	b)	3,759	78%	1,040	22%	4,799
2013	b)	3,734	79%	997	21%	4,731

a) Figures for "Marine fisheries and cultured" exclude some parts of Iwate, Miyagi and Fukushima Prefectures for which precise data are not available because of the Great East Japan Earthquake.

b) Excluding those items belonging to "Marine fisheries and culture" for which shipping was restricted due to the Great East Japan Earthquake.

Source: Adapted from data from the Statistics Department, Minister's Secretariat, Ministry of Agriculture, Forestry and Fisheries

The possibility of finding statistically significant associations by chance due to multiple comparisons also cannot be neglected. The significant positive association between total n-6 PUFA intake and ER+ PR+ tumors found in this study may be subject to the issue of multiple comparisons. While the mechanism between total n-6 PUFA intake and

breast cancer specific to ER+ PR+ tumors has not been clinically studied, cancer tumorigenesis found through prostaglandin synthesis of n-6 PUFA makes it biologically plausible for n-6 PUFA to be positively associated with ER+ PR+ tumors of breast cancer.

Another limitation of this study is that the true relationship between the exposures of interest and breast cancer risk may be non-linear. However, when the linearity assumption in the Cox proportional hazards model was tested by plotting the predicted values against the Martingale residuals with smoothers, the assumption of linearity was satisfied. Moreover, in line with the results for n-3 PUFA, the test for non-linearity in the meta-analysis for marine n-3 PUFA and the risk of breast cancer was also not significant.[23]

Moreover, while information on breast cancer screening would have been beneficial for analysis, the questionnaire only inquired whether the participant was screened in the previous year; information regarding the screening status throughout the follow-up period was not available. The questionnaire also did not inquire about breast feeding and its duration, factors which have been shown to lower breast cancer risk in some

studies.[79, 80] Furthermore, since menopausal status was recorded at baseline, premenopausal status at baseline could have changed to postmenopausal status at the time of diagnosis; this change could not be differentiated. Men were excluded in the study; therefore, the results cannot be generalized to breast cancer cases in men. The FFQ did not include questions to capture the intake of fish-oil supplements. However, the market growth for fish-oil supplements has been stagnant in Japan until 2003 and has approximately doubled in 2011 (Figure 13).[81] This is considered a slow growth in market size compared to the 10-fold increase in market size seen in the US market for fish-oil supplementation during the same time period. Yet, the lack of data on fish-oil supplements still could have affected the results. On the one hand, if the consumption of fish-oil supplements was high in both cases and non-cases, the results would be biased towards the null due to non-differential misclassification. On the other hand, differential misclassification is plausible if misclassification of the intake of n-3 occurred only among the non-cases (if n-3 intake has an inverse association with breast cancer) which could have led to an overestimate of the true hazard ratio.

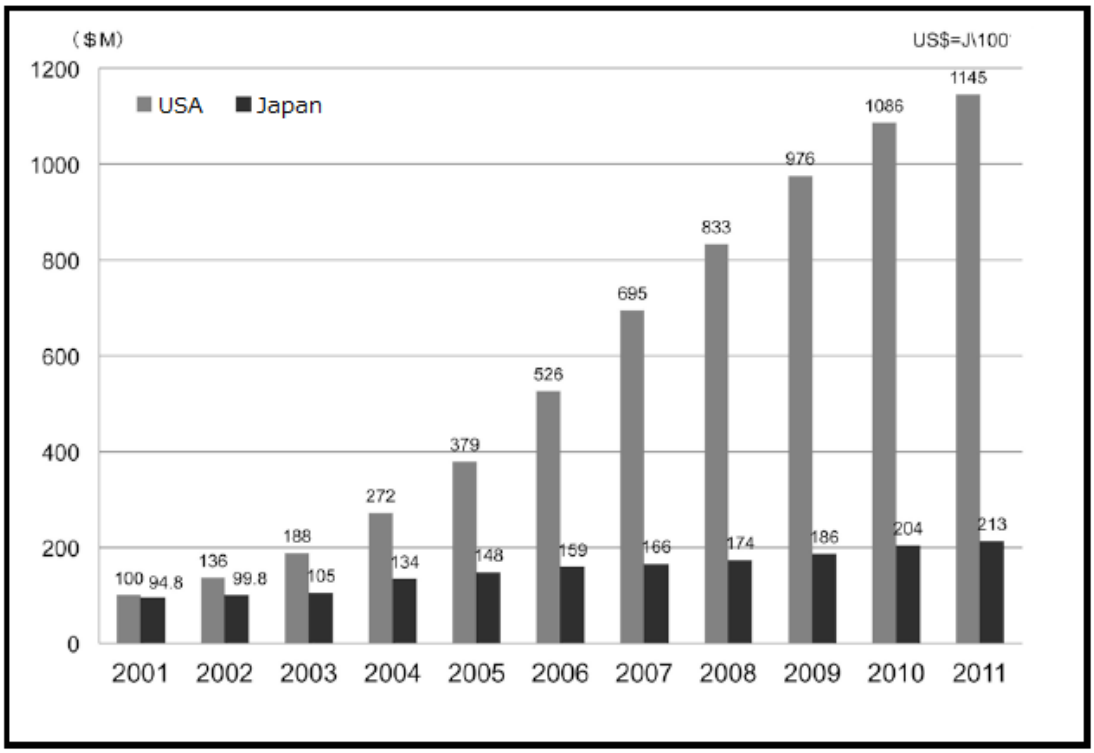


Figure 13: Market trend on DHA/EPA supplements in the United States and Japan (translated)
Source: UBM Japan Group[81]

While several studies have been already conducted on this association, they were mostly from Western populations[18, 20, 22, 25] where incidence of breast cancer is high and fish intake is low compared with that of Japanese, or China where the fish intake is not as high as Japan and the cooking method is different.[17] Only one study has been published from Japanese cohorts, but the study could not specify ER PR status.[24] As a whole, the association has been inconsistent. This is the first study to look at the association between the dietary intake of fish and PUFA and breast cancer by ER PR status on the

Japanese population which has relatively high intakes of fish and PUFA. From an epidemiological point of view, this study adds value because multiple epidemiological studies are required to confirm the association between dietary intake and cancer.

Moreover, the main strength of this study lies in the prospective population-based cohort design with a large overall sample size and high response rate. Recall bias was also unlikely because of the prospective nature of the study in which diagnosis occurred after collection of exposure data. Microscopic diagnosis of cases and limited reliance on death certificate notification made misclassification of breast cancer cases improbable.

4.4 Recommendations to improve the JPHC study in the future

Given the strengths and limitations of this study, the following are two recommendations on ways to improve the JPHC study in the future:

1. As mentioned in the limitations section, FFQ validity and reproducibility depend on the type of food or nutrient. Therefore, applying better methods to improve the validity of the FFQ dietary assessment can improve the JPHC study. Possible methods would be to computerize the FFQ and use a 24-hr dietary recall method which uses a structured interview method to obtain

detailed data about food and beverages consumed in the past 24 hours, which was used in the European Prospective Investigation into Cancer and Nutrition (EPIC) study.[82]

2. The assumption that exposure has not changed significantly throughout the study has also been raised as a limitation. Therefore, distributing the FFQ on a more frequent basis such as every three years would allow the study to have more information on the possible changes of exposure. An example is the Nurses' Health Study in which cohort members answered follow-up questionnaires every two years since 1978.[83]

3. Collecting DNA samples of participants may allow better understanding of the association between dietary intake and cancer. Only ER PR could be currently analyzed; if DNA samples can be obtained, other information on genetic susceptibility such as BRCA genes can be analyzed to confirm the role of genetics.

5. Conclusion

The overall intake of fish, n-3 PUFA, and n-6 PUFA were not significantly associated with breast cancer risk. However, significant positive associations were found between the intake of total n-6 PUFA and ER+ PR+ tumors.

The tumor subtype dependency of this study highlights that studies that have analyzed the association between dietary intake and breast cancer without consideration of different receptor statuses should include tumor analyses to better understand the relationship between dietary intake and breast cancer. Moreover, further laboratory studies performed in parallel to epidemiologic studies can help in understanding the mechanism of tumor growth in relation to fish, n-3 PUFA, and n-6 PUFA intake. With breast cancer incidence on the rise in Japan, studying the association and understanding the mechanisms to curb the growth through dietary intake is not only beneficial but also necessary.

6. References

1. Ferlay J SI, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0 Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11. 2013- [cited 2013 September 10]. Available from: http://globocan.iarc.fr.

2. Matsuda A, Matsuda T, Shibata A, Katanoda K, Sobue T, Nishimoto H. Cancer Incidence and Incidence Rates in Japan in 2008: A Study of 25 Population-based Cancer Registries for the Monitoring of Cancer Incidence in Japan (MCIJ) Project. *Jpn J Clin Oncol* 2014;**44**:388-96.

3. Ferlay J. Bray F. Steliarova-Foucher E. Forman D. Cancer Incidence in Five Continents, CI5plus. IARC CancerBase No. 9. Lyon: International Agency for Research on Cancer. 2014- [cited 24 June 2015]. Available from: http://ci5.iarc.fr.

4. Ferlay J. Soerjomataram I. Ervik M. Dikshit R. Eser S. Mathers C. Rebelo M. Parkin DM. Forman D. Bray F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. Lyon, France: International Agency for Research on Cancer. 2013- [cited 24 June 2015]. Available from: http://globocan.iarc.fr.

5. Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *Lancet* 2005;**366**:1784-93.

6. Vainio H, Bianchini F, eds. *IARC Handbook of cancer prevention*. Lyon: International Agency for Research on Cancer, 2002.

7. Hamajima N, Hirose K, Tajima K, Rohan T, Calle EE, Heath CW, Jr., Coates RJ, Liff JM, Talamini R, Chantarakul N, Koetsawang S, Rachawat D, et al. Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. *Br J Cancer* 2002;**87**:1234-45.

8. Smith-Warner SA, Spiegelman D, Yaun SS, van den Brandt PA, Folsom AR, Goldbohm RA, Graham S, Holmberg L, Howe GR, Marshall JR, Miller AB, Potter JD, et al. Alcohol and breast cancer in women: a pooled analysis of cohort studies. *Jama* 1998;**279**:535-40.

9. Hammond MEH, Hayes DF, Wolff AC, Mangu PB, Temin S. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer. *Journal of Oncology Practice* 2010;**6**:195-7.

10. Colditz GA, Rosner BA, Chen WY, Holmes MD, Hankinson SE. Risk Factors for Breast Cancer According to Estrogen and Progesterone Receptor Status. *Journal of the National Cancer Institute* 2004;**96**:218-28.

11. Hislop TG, Kan L, Coldman AJ, Band PR, Brauer G. Influence of estrogen receptor status on dietary risk factors for breast cancer. *CMAJ* 1988;**138**:424-30.

12. Stripp C, Overvad K, Christensen J, Thomsen BL, Olsen A, Møller S, Tjønneland A. Fish Intake Is Positively Associated with Breast Cancer Incidence Rate. *J Nutr* 2003;**133**:3664-9.

13. OECD Agricultural Outlook. 2014- [cited 2014 December 15]. Available from: http://dx.doi.org/10.1787/data-00285-en.

14. Food and Agriculture Organization of the United Nations The State of World Fisheries and Agriculture. 2014- [cited 2014 December 15]. Available from: http://www.fao.org/fishery/sofia/en.

15. Micha R, Khatibzadeh S, Shi P, Fahimi S, Lim S, Andrews KG, Engell RE, Powles J, Ezzati M, Mozaffarian D. Global, regional, and national consumption levels of dietary fats and oils in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys. *BMJ* 2014;**348**:g2272.

16. Kobayashi M, Sasaki S, Kawabata T, Hasegawa K, Tsugane S. Validity of a self-administered food frequency questionnaire used in the 5-year follow-up survey of the JPHC Study Cohort I to assess fatty acid intake: comparison with dietary records

and serum phospholipid level. *Journal of epidemiology / Japan Epidemiological Association* 2003;**13**:S64-81.

17. Gago-Dominguez M, Yuan JM, Sun CL, Lee HP, Yu MC. Opposing effects of dietary n-3 and n-6 fatty acids on mammary carcinogenesis: The Singapore Chinese Health Study. *Br J Cancer* 2003;**89**:1686-92.

18. Engeset D, Alsaker E, Lund E, Welch A, Khaw K-T, Clavel-Chapelon F, Thiébaut A, Chajès V, Key TJ, Allen NE, Amiano P, Dorronsoro M, et al. Fish consumption and breast cancer risk. The European Prospective Investigation into Cancer and Nutrition (EPIC). *International Journal of Cancer* 2006;**119**:175-82.

19. Patterson RE, Flatt SW, Newman VA, Natarajan L, Rock CL, Thomson CA, Caan BJ, Parker BA, Pierce JP. Marine fatty acid intake is associated with breast cancer prognosis. *J Nutr* 2011;**141**:201-6.

20. Vatten LJ, Solvoll K, Loken EB. Frequency of meat and fish intake and risk of breast cancer in a prospective study of 14,500 Norwegian women. *International journal of cancer Journal international du cancer* 1990;**46**:12-5.

21. Key TJ, Sharp GB, Appleby PN, Beral V, Goodman MT, Soda M, Mabuchi K. Soya foods and breast cancer risk: a prospective study in Hiroshima and Nagasaki, Japan. *Br J Cancer* 1999;**81**:1248-56.

22. Holmes MD, Colditz GA, Hunter DJ, Hankinson SE, Rosner B, Speizer FE, Willett WC. Meat, fish and egg intake and risk of breast cancer. *International journal of cancer Journal international du cancer* 2003;**104**:221-7.

23. Zheng JS, Hu XJ, Zhao YM, Yang J, Li D. Intake of fish and marine n-3 polyunsaturated fatty acids and risk of breast cancer: meta-analysis of data from 21 independent prospective cohort studies. *BMJ* 2013;**346**:f3706.

24. Wakai K, Tamakoshi K, Date C, Fukui M, Suzuki S, Lin Y, Niwa Y, Nishio K, Yatsuya H, Kondo T, Tokudome S, Yamamoto A, et al. Dietary intakes of fat and fatty acids and risk of breast cancer: A prospective study in Japan. *Cancer Sci* 2005;**96**:590-9.

25. Wirfält E, Mattisson I, Gullberg B, Johansson U, Olsson H, Berglund G. Postmenopausal breast cancer is associated with high intakes of ω6 fatty acids (Sweden). *Cancer Causes Control* 2002;**13**:883-93.

26. Fay MP, Freedman LS, Clifford CK, Midthune DN. Effect of different types and amounts of fat on the development of mammary tumors in rodents: a review. *Cancer Res* 1997;**57**:3979-88.

27. Hilakivi-Clarke L, Onojafe I, Raygada M, Cho E, Clarke R, Lippman ME. Breast Cancer Risk in Rats Fed a Diet High in n-6 Polyunsaturated Fatty Acids During Pregnancy. *J Natl Cancer Inst* 1996;**88**:1821-7.

28. Nair J, Vaca CE, Velic I, Mutanen M, Valsta LM, Bartsch H. High dietary omega-6 polyunsaturated fatty acids drastically increase the formation of etheno-DNA base adducts in white blood cells of female subjects. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 1997;**6**:597-601.

29. Park SY, Kolonel LN, Henderson BE, Wilkens LR. Dietary fat and breast cancer in postmenopausal women according to ethnicity and hormone receptor status: the Multiethnic Cohort Study. *Cancer Prev Res* 2012;**5**:216-28.

30. Yang B, Ren X-L, Fu Y-Q, Gao J-L, Li D. Ratio of n-3/n-6 PUFAs and risk of breast cancer: a meta-analysis of 274135 adult females from 11 independent prospective studies. *BMC Cancer* 2014;**14**:105-.

31. Brown K. Impact of Obesity on Mammary Gland Inflammation and Local Estrogen Production. *J Mammary Gland Biol Neoplasia* 2014;**19**:183-9.

32. Zhang CX, Ho SC, Cheng SZ, Chen YM, Fu JH, Lin FY. Effect of dietary fiber intake on breast cancer risk according to estrogen and progesterone receptor status. *Eur J Clin Nutr* 2011;**65**:929-36.

33. Zhang M, Yang H, Holman CD. Dietary intake of isoflavones and breast cancer risk by estrogen and progesterone receptor status. *Breast Cancer Res Treat* 2009;**118**:553-63.

34. Ingram DM, Bennett FC, Willcox D, de Klerk N. Effect of low-fat diet on female sex hormone levels. *J Natl Cancer Inst* 1987;**79**:1225-9.

35. Armstrong BK, Brown JB, Clarke HT, Crooke DK, Hähnel R, Masarei JR, Ratajczak T. Diet and Reproductive Hormones: A Study of Vegetarian and Nonvegetarian Postmenopausal Women. *J Natl Cancer Inst* 1981;**67**:761-7.

36. Rose DP, Connolly JM. Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol Ther* 1999;**83**:217-44.

37. Bougnoux P, Maillard V, Ferrari P, Jourdan ML, Chajes V. n-3 fatty acids and breast cancer. *IARC Sci Publ* 2002;**156**:337-41.

38. Terry PD, Rohan TE, Wolk A. Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. *The American journal of clinical nutrition* 2003;**77**:532-43.

39. Spencer L, Mann C, Metcalfe M, Webb M, Pollard C, Spencer D, Berry D, Steward W, Dennison A. The effect of omega-3 FAs on tumour angiogenesis and their therapeutic potential. *Eur J Cancer* 2009;**45**:2077-86.

40. Hubbard NE, Erickson KL. Enhancement of metastasis from a transplantable mouse mammary tumor by dietary linoleic acid. *Cancer Res* 1987;**47**:6171-5.

41. Tsugane S, Sawada N. The JPHC Study: Design and Some Findings on the Typical Japanese Diet. *Japanese Journal of Clinical Oncology* 2014;**44**:777-82.

42. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP, for the SI. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: Guidelines for Reporting Observational Studies. *PLoS Med* 2007;**4**:e296.

43. Tsubono Y, Takamori S, Kobayashi M, Takahashi T, Iwase Y, Iitoi Y, Akabane M, Yamaguchi M, Tsugane S. A data-based approach for designing a

semiquantitative food frequency questionnaire for a population-based prospective study in Japan. *Journal of epidemiology / Japan Epidemiological Association* 1996;**6**:45-53.

44. Tsugane S, Kobayashi M, Sasaki S. Validity of the self-administered food frequency questionnaire used in the 5-year follow-up survey of the JPHC Study Cohort I: comparison with dietary records for main nutrients. *Journal of epidemiology / Japan Epidemiological Association* 2003;**13**:S51-6.

45. Science and Technology Agency eds. *Standard tables of food composition in Japan, 5th revised and enlarged edn. (in Japanese)*. Tokyo: Printing Bureau: Ministry of Finance, 2005.

46. Sasaki S, Kobayashi M, Tsugane S. Development of Substituted Fatty Acid Food Composition Table for the Use in Nutritional Epidemiologic Studies for Japanese Populations: Its Methodological Backgrounds and the Evaluation. *Journal of epidemiology / Japan Epidemiological Association* 1999;**9**:190-207.

47. Van den Brandt PA, Spiegelman D, Yaun S-S, Adami H-O, Beeson L, Folsom AR, Fraser G, Goldbohm RA, Graham S, Kushi L, Marshall JR, Miller AB, et al. Pooled Analysis of Prospective Cohort Studies on Height, Weight, and Breast Cancer Risk. *American Journal of Epidemiology* 2000;**152**:514-27.

48. Endogenous Hormones Breast Cancer Collaborative Group. Body Mass Index, Serum Sex Hormones, and Breast Cancer Risk in Postmenopausal Women. *Journal of the National Cancer Institute* 2003;**95**:1218-26.

49. McPherson K, Steel CM, Dixon JM. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ* 2000;**321**:624-8.

50. MacMahon B. General Motors Cancer Research Prizewinners Laureates Lectures. Charles S. Mott Prize. Reproduction and cancer of the breast. *Cancer* 1993;**71**:3185-8.

51. IARC. Post-menopausal oestrogen-progestogen therapy. *IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer* 1999;**72**:531-628.

52. Sprague BL, Trentham-Dietz A, Newcomb PA, Titus-Ernstoff L, Hampton JM, Egan KM. Lifetime Recreational and Occupational Physical Activity and Risk of In situ and Invasive Breast Cancer. *Cancer Epidemiology Biomarkers & Prevention* 2007;**16**:236-43.

53. Schmidt ME, Chang-Claude J, Slanger T, Obi N, Flesch-Janys D, Steindorf K. Physical Activity and Postmenopausal Breast Cancer. Effect Modification by Other Breast Cancer Risk Factors. *Methods of Information in Medicine* 2009;**48**:444-50.

54. Catsburg C, Miller AB, Rohan TE. Active cigarette smoking and risk of breast cancer. *International Journal of Cancer* 2015;**136**:2204-9.

55. Johnson KC, Miller AB, Collishaw NE, Palmer JR, Hammond SK, Salmon AG, Cantor KP, Miller MD, Boyd NF, Millar J, Turcotte F. Active smoking and secondhand smoke increase breast cancer risk: the report of the Canadian Expert Panel on Tobacco Smoke and Breast Cancer Risk (2009). *Tobacco Control* 2011;**20**:e2.

56. Xue F, Willett WC, Rosner BA, Hankinson SE, Michels KB. CIgarette smoking and the incidence of breast cancer. *Archives of Internal Medicine* 2011;**171**:125-33.

57. Chen M, Rao Y, Zheng Y, Wei S, Li Y, Guo T, Yin P. Association between Soy Isoflavone Intake and Breast Cancer Risk for Pre- and Post-Menopausal Women: A Meta-Analysis of Epidemiological Studies. *PLoS ONE* 2014;**9**:e89288.

58. Nagata C, Mizoue T, Tanaka K, Tsuji I, Tamakoshi A, Matsuo K, Wakai K, Inoue M, Tsugane S, Sasazuki S, Development ftRGft, Japan EoCPSi. Soy Intake and Breast Cancer Risk: An Evaluation Based on a Systematic Review of Epidemiologic Evidence Among the Japanese Population. *Japanese Journal of Clinical Oncology* 2014;**44**:282-95.

59. Kimira M, Arai Y, Shimoi K, Watanabe S. Japanese intake of flavonoids and isoflavonoids from foods. *Journal of epidemiology / Japan Epidemiological Association* 1998;**8**:168-75.

60. Royston P, Lambert PC. *Flexible parametric survival analysis using Stata :*

beyond the Cox model. College Station, TX: Stata Press, 2011. xxvi, 347 p.

61. Kom EL, Graubard BI, Midthune D. Time-to-Event Analysis of Longitudinal Follow-up of a Survey: Choice of the Time-scale. *American Journal of Epidemiology* 1997;**145**:72-80.

62. Willett WC. *Nutritional epidemiology*. New York: Oxford University Press, 1990.

63. Brown CC, Kipnis V, Freedman LS, Hartman AM, Schatzkin A, Wacholder S. Energy adjustment methods for nutritional epidemiology: the effect of categorization. *Am J Epidemiol* 1994;**139**:323-38.

64. Kipnis V, Freedman LS, Brown CC, Hartman A, Schatzkin A, Wacholder S. Interpretation of energy adjustment models for nutritional epidemiology. *Am J Epidemiol* 1993;**137**:1376-80.

65. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. *The American journal of clinical nutrition* 1997;**65**:1220S-8S; discussion 9S-31S.

66. StataCorp. Stata Statistical Software: Release 13. TX: StataCorp LP, 2013.

67. Sawada N, Inoue M, Iwasaki M, Sasazuki S, Shimazu T, Yamaji T, Takachi R, Tanaka Y, Mizokami M, Tsugane S. Consumption of n-3 fatty acids and fish reduces risk of hepatocellular carcinoma. *Gastroenterology* 2012;**142**:1468-75.

68. Terry PD, Rohan TE. Cigarette Smoking and the Risk of Breast Cancer in Women: A Review of the Literature. *Cancer Epidemiology Biomarkers & Prevention* 2002;**11**:953-71.

69. Barnes BE, Chang-Claude J, Flesch-Janys D, Kinscherf R, Schmidt M, Slanger T, Bonaterra G, Steindorf K. Cancer risk factors associated with insulin-like growth factor (IGF)-I and IGF-binding protein-3 levels in healthy women: effect modification by menopausal status. *Cancer Causes Control* 2009;**20**:1985-96.

70. Aschengrau A, Seage GR. *Essentials of epidemiology in public health*. Sudbury, Mass.: Jones and Bartlett Publishers, 2008.

71. Vara-Messler M, Pasqualini M, Comba A, Silva R, Buccellati C, Trenti A, Trevisi L, Eynard A, Sala A, Bolego C, Valentich M. Increased dietary levels of α-linoleic acid inhibit mammary tumor growth and metastasis. *Eur J Nutr* 2015:1-11.

72. Kim J-Y, Park HD, Park E, Chon J-W, Park YK. Growth-Inhibitory and Proapoptotic Effects of Alpha-Linolenic Acid on Estrogen-Positive Breast Cancer Cells. *Annals of the New York Academy of Sciences* 2009;**1171**:190-5.

73. Karmali RA. Eicosanoids in neoplasia. *Preventive Medicine* 1987;**16**:493-502.

74. Wen ZH, Su YC, Lai PL, Zhang Y, Xu YF, Zhao A, Yao GY, Jia CH, Lin J, Xu S, Wang L, Wang XK, et al. Critical role of arachidonic acid-activated mTOR signaling in breast carcinogenesis and angiogenesis. *Oncogene* 2013;**32**:160-70.

75. MacLean CH, Newberry SJ, Mojica WA, et al. Effects of omega-3 fatty acids on cancer risk: A systematic review. *Jama* 2006;**295**:403-15.

76. Ackman RG. Nutritional composition of fats in seafoods. *Progress in food & nutrition science* 1989;**13**:161-289.

77. Cahu C, Salen P, de Lorgeril M. Farmed and wild fish in the prevention of cardiovascular diseases: assessing possible differences in lipid nutritional values. *Nutrition, metabolism, and cardiovascular diseases : NMCD* 2004;**14**:34-41.

78. Statistics Department Ministry of Agriculture Forestry and Fisheries. Chapter 8 Agriculture, Forestry and Fisheries. 2015- [cited Available from: www.stat.go.jp/english/data/nenkan/1431-08.htm.

79. Collaborative Group on Hormonal Factors in Breast Cancer. Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50 302 women with breast cancer and 96 973 women without the disease. *The Lancet*;**360**:187-95.

80. Ma H, Bernstein L, Pike MC, Ursin G. Reproductive factors and breast cancer risk according to joint estrogen and progesterone receptor status: a meta-analysis of epidemiological studies. *Breast Cancer Research* 2006;**8**:R43-R.

81. UBM Japan Group. Trends in the market for w3 *Food processing and ingredients* 2013;**48**:37.

82. Riboli E, Hunt K, Slimani N, Ferrari P, Norat T, Fahey M, Charrondière U, Hémon B, Casagrande C, Vignat J, Overvad K, Tjønneland A, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. *Public Health Nutrition* 2002;**5**:1113-24.

83. Belanger CF, Hennekens CH, Rosner B, Speizer FE. The nurses' health study. *The American journal of nursing* 1978;**78**:1039-40.

7. Appendix

7.1 Appendix 1: Ethical Approval

（様式６－1）

2015年06月19日

研究倫理審査委員会結果通知書

国立研究開発法人国立がん研究センター理事長　殿

国立研究開発法人国立がん研究センター研究倫理審査委員会委員長

研究計画に関する申請あるいは報告について、当センターの手順書に基づき審査を行い、以下のとおり判定した。

研究課題番号	2001-021		
研究課題名	多目的コホート研究(JPHC Study)		
研究責任者	がん予防・検診研究センター　がん予防・検診研究センター　津金　昌一郎		
適用となる倫理指針	□人を対象とする医学系研究に関する倫理指針 □臨床研究に関する倫理指針　■疫学研究に関する倫理指針 □ヒトゲノム・遺伝子解析研究に関する倫理指針 □その他		
研究計画書等に関する情報	研究計画書の作成日：2015年06月16日　第2015/06/16版（バージョン） 説明同意文書の作成日：－　第－版（バージョン）		
申請/報告の種別	□研究計画の新規申請　■研究計画の変更申請 □実施状況報告　□安全性情報に関する報告 □研究に関する不適切事案に関する報告 □その他		
審査方法・判断方法	□通常（合議）審査（委員会開催日：　年　月　日） ■迅速審査（適用条件：委員会手順書第12条第1項第1号　軽微な変更） □研究倫理審査委員会委員長決裁 □あらかじめ指名する者による審査不要の判断※ □		
委員会判定日（上記※の場合を除く）	2015年06月19日	判定	■承認　□条件付き承認　□却下 □保留（継続審査）　□差し戻し □非該当　□その他
付帯条件・勧告			
判定が承認以外の場合の理由、その他の意見			
備考	変更内容：　研究協力者追加 審査対象文書：研究実施計画書		

（様式６-2）

2015年06月25日

研究許可申請に関する指示・決定通知書

津金　昌一郎　殿

国立研究開発法人国立がん研究センター理事長

（押印省略）

貴殿から申請のあった上記の研究について、以下のとおり決定したので、通知する。

判定	■許可　□不許可　□差し戻し　□非該当 □その他
当センターにおける研究期間	自：1990年04月01日 至：2024年03月31日

7.2 Appendix 2: Example of Food Frequency Questionnaire (selected pages)

Health Promotion Questionnaire

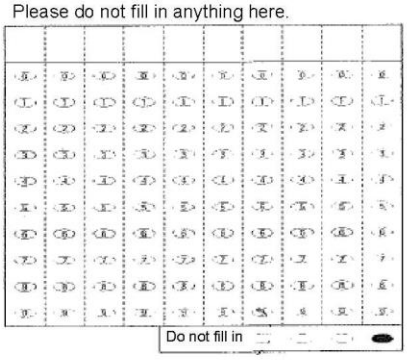
If you found an error in your name or address, please correct it with a red pen.

The Ministry of Health and Welfare: The Japan Public Health Center-based prospective Study on Cancer and Cardiovascular Disease Research Group
National Cancer Center
National Cardiovascular Center

We may call to ask you some questions regarding the contents of what you have filled in. Please provide your phone number below if you do not mind.

☎ ____ – ____

Please do not fill in anything here.



Before starting the questionnaire

The Epidemiology Research Group of the Ministry of Health, Labor and Welfare is working on research on "How do I prevent adult illnesses such as cancer, stroke, myocardial infarction, etc.?" In this context, we carried out the "Health Promotion Questionnaire" from 1990 to 1991 targeting people who were born from 1930 through 1949 who live in the cities, towns and villages of five health center districts throughout the nation.

Five years have passed since the survey, and we are carrying out a second questionnaire to find out whether there have been any changes in lifestyle or health status of people during this interval, and also details about the state of your dietary life. We would like to ask your cooperation now that you understand the background of this survey.

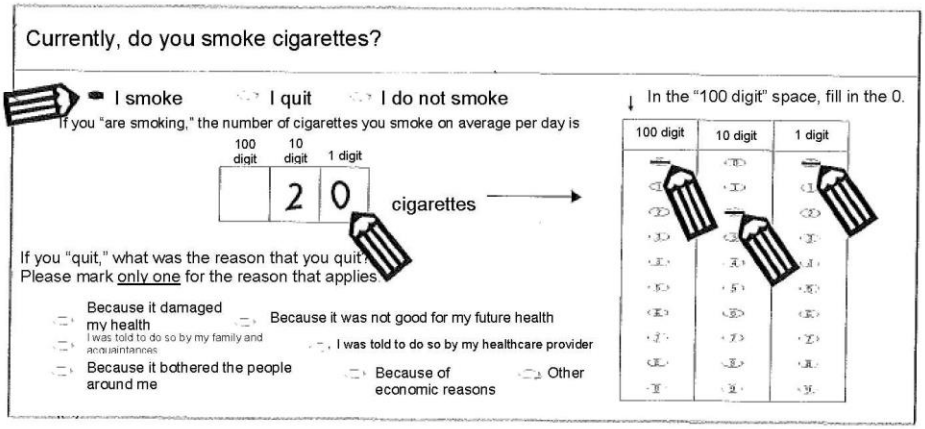
If you are willing to participate this time, please read the "Instructions on How to Filli in the Questionnaire" below and answer the questions beginning on the following page.

Instructions on How to Fill in the Questionnaire

1. As much as possible, please fill in this questionnaire by yourself.
2. Please fill in the given space (the oval circles) that apply with a black pencil, or enter a number of letter in the box. If you choose "Other" in the multiple choice selections, please fill in specific details in the parentheses.
3. Please use an HB, B or H graphite pencil.
4. Please do not use a fountain pen or ballpoint pen.
5. If you have any corrections, please erase them entirely with an eraser.
6. Please do not fill in anything in the blank spaces.

(example for filling in the mark)

For example, please fill it in as shown below if you currently smoke cigarettes, and you smoke 20 cigarettes on average per day.



What month is it today?

○ January ○ February ○ March ○ April ○ May ○ June
○ July ○ August ○ September ○ October ○ November ○ December

What is your gender? ○ Male ○ Female

About how tall are you currently?

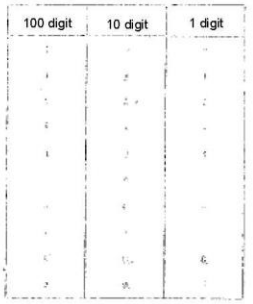
100 digit 10 digit 1 digit

cm (round off the fractions)

About how much do you weigh currently?

100 digit 10 digit 1 digit

kg (round off the fractions)



We are going to ask you about "rice (cooked rice)."

About what size rice bowl do you eat with?
○ Small rice bowl ○ Normal rice bowl ○ Donburi large rice bowl

About how many bowls do you eat in 1 day, combining breakfast, lunch and dinner?
○ Less than 1 bowl ○ 1 bowl ○ 2 bowls ○ 3 bowls 4 bowls
○ 5 bowls ○ 6 bowls ○ 7 - 9 bowls ○ 10 bowls or more

Do you eat vitamin-enriched rice?
○ No ○ Yes

Do you mix in wheat or millet or Japanese hie?
○ I do notmix it in ○ I sometimes mix it in ○ I always mix it in

We are going to ask you about "miso soup."

About how frequently do you eat it?
○ I hardly ever eat it ○ 1 - 3 days a month ○ 1 - 2 days a week ○ 3 - 4 days a week
○ 5 - 6 days a week ○ I eat it every day

About how many cups do you eat in 1 day, combining breakfast, lunch and dinner?
○ Less than 1 cup ○ 1 cup ○ 2 cups ○ 3 cups ○ 4 cups
○ 5 cups ○ 6 cups ○ 7 - 9 cups ○ 10 cups or more

How do you season it?
○ Fairly diluted ○ Normal ○ Fairly thick

Do not fill in ● ●

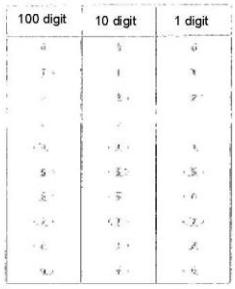
Currently, do you smoke cigarettes?

I smoke　　I quit　　I do not smoke

If you "are smoking," the number of cigarettes you smoke on average per day is

100 digit　10 digit　1 digit

cigarettes



If you "quit," what was the reason that you quit?
Please mark only one for the reason that applies.

- Because it damaged my health
- Because it was not good for my future health
- I was told to do so by my family and acquaintances
- I was told to do so by my healthcare provider
- Because it bothered the people around me
- Because of economic reasons
- Other

How frequently do you drink?

- I hardly ever drink
- 1 - 3 days a month
- 1 - 2 days a week
- 3 - 4 days a week
- 5 - 6 days a week
- I drink every day

Please choose the most usual combination that you drink in one day.

(Example) If normally after drinking one large bottle of beer you drink 2 go of Japanese sake, in the "Beer" area fill in "1 bottle" and in the "Japanese Sake" area fill in "2 go," and in the "Shochu or Awamori," "Whiskey," and "Wine" areas, fill in "I do not drink."

Japanese Sake 1 go (180ml)	I do not drink	less than 0.5 go	1 go	2 go	3 go	4 go	5 - 6 go	7 go or more
Shochu or Awamori 1 go (180ml)	I do not drink	less than 0.5 go	1 go	2 go	3 go	4 go	5 - 6 go	7 go or more
*Beer Large bottle (633ml)	I do not drink	less than 0.5 bottle	1 bottle	2 bottles	3 bottles	4 bottles	5 - 6 bottles	7 bottles or more
Whiskey Single (30ml)	I do not drink	less than 0.5 glass	1 glass	2 glasses	3 glasses	4 glasses	5 - 6 glasses	7 bottles or more
Wine Glass (100ml)	I do not drink	less than 0.5 glass	1 glass	2 glasses	3 glasses	4 glasses	5 - 6 glasses	7 bottles or more

*Please make the conversion at medium bottle or 500ml can, 0.8 of a bottle; small bottle or 350ml can, 0.6 of a bottle

Currently, is there a medicine that is prescribed by your healthcare provider and that you take periodically?

- Yes
- No

If "Yes," please mark all that apply.

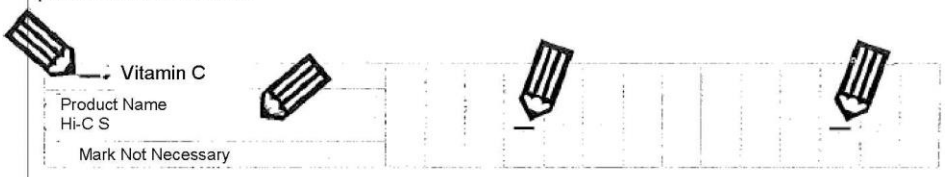
- High Blood Pressure Medicine
- Medicine to Lower Cholesterol
- Diabetes Medicine
- Gout Medicine
- Other

Mark Not Necessary

Are there any vitamins that you take once or more per week?

Yes No

If answered "yes" above, please fill in the product name, and mark the type of vitamin, frequency that you take it, and time period.
(Example) If you have been taking 1 tablet of the vitamin C agent called "Hi-C S" every day for 8 years, please fill it in as follows.



	Type and Product Name	Frequency								Time Period					
		1 - 2 tablets a week	3 - 4 tablets a week	5 - 6 tablets a week	1 tablet daily	2 - 3 tablets daily	4 - 6 tablets daily	7 tablets or more daily		Less than 1 year	1 - 2 years	3 - 4 years	5 - 9 years	10 - 19 years	20 years or more
	Multivitamin														
Product Name:															
	No Mark Necessary														
	Beta Carotene														
Product Name:															
	No Mark Necessary														
	Vitamin C														
Product Name:															
	No Mark Necessary														
	Vitamin E														
Product Name:															
	No Mark Necessary														
	Other														
Product Name:															
	No Mark Necessary														

In the past 5 years (from January 1, 1990 to present), have you been told by your healthcare provider that you have the following illness(es), and you had the following surgery(ies)? Please mark all that apply.

Disease

Stroke	Myocardial Infarction	Angina Pectoris	Diabetes	
Gout	Cataracts	Gall Stones	Urethral Stones or Kidney Stones	
Stomach Ulcers	Duodenal Ulcers	Stomach Polyps	Colon Polyps	Chronic Hepatitis or Cirrhosis of the Liver
Stomach Cancer	Colon Cancer	Liver Cancer	Lung Cancer	
Breast Cancer	Uterine Cancer	Other Cancer → Site		

Mark Not Necessary

Surgery

Stomach	Colon	Gall Stones	
Ovaries	Lung	Mammary glands	other → Site

Mark Not Necessary

Do not fill in

Questions about Your Dietary Life

Now some questions about your diet will follow.

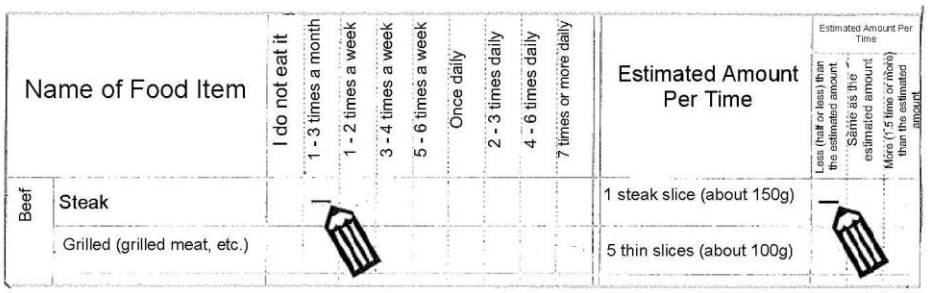
Recalling your diet over the past one year, please answer with average frequencies and amounts.

If you answer all the items, a detailed nutritional calculation of your normal dietary life can be made, so we will be able to report to each of you individually at a later date whether you have a nutritional balance, or whether your vitamins are enough, etc.

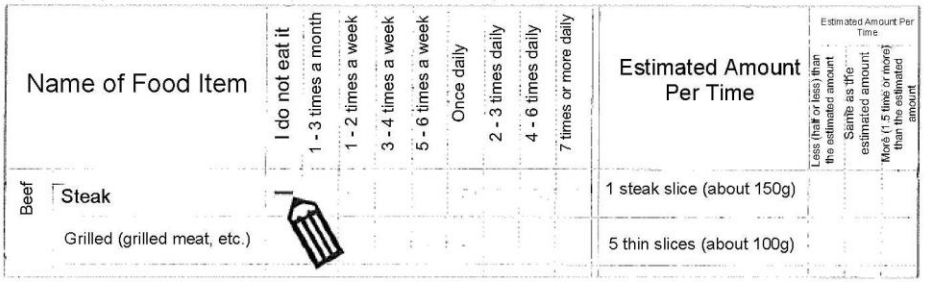
There are a lot of questions, and it this may be difficult for you, but we ask you to please complete it to the end.

Example

If you eat beef steak about 2 times a month, and the amount you eat per time is about half a steak, then fill it in as follows.



If you hardly ever eat beef steak (less than once a month), fill it in as follows.

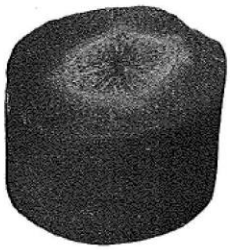


Do not fill in anything in the estimated amount. ↑

Estimated Amount of Vegetables (full size)

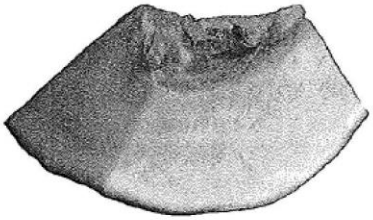
If the amount you eat per time is about the same as in the photograph, please fill in "Same." If it is more than what is in the photograph (1.5 times or more), please fill in "More," and if less (less than half), please fill in "Less."

(a) Carrot, 1/4 carrot
(about 50g)



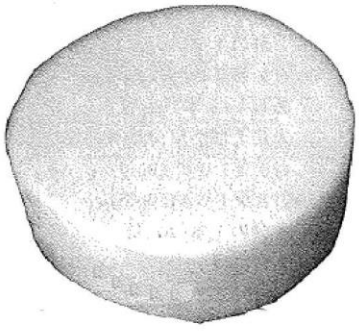
(b) Spinach, 2 bunches
(about 50g)

(c) Pumpkin, one 4 - 5cm cube
(about 40g)



(d) Cabbage, 1/2 medium-sized leaf
(about 30g)

(e) Radish, one 2-cm-thick round slice
(about 80g)



Recalling your diet over the past one year, please fill in average frequencies and amounts.

Name of Food Item	I do not eat it	1 – 3 times a month	1 – 2 times a week	3 – 4 times a week	5 – 6 times a week	Once daily	2 – 3 times daily	4 – 6 times daily	Estimated Amount Per Time	Estimated Amount Per Time		
										Less (half or less) than the estimated amount	Same as the estimated amount	More (1.5 time or more) than the estimated amount
Canned tuna (sea chicken flakes)									1/4 can (about 20g)			
Salmon or trout									1 slice of fish meat (about 70g)			
Bonito or tuna									4 raw slices (about 60g)			
Cod or flounder									1/2 slice (about 40g)			
Bream (Red Sea bream, Okinawan name: gurkun, Okinawan name: machi, etc.)									1 slice (about 70g)			
Horse mackerel or sardines									1 fish (about 80g)			
Pike or mackerel									1 fish (about 80g)			
Dried whitebait									2 tablespoonfuls (about 10g)			
Cod roe or salmon roe									1/4 sac (about 20g)			
Eel									1/2 fish (about 50g)			
Squid									3 raw slices (about 50g)			
Octopus									1/3 tentacle (about 50g)			
Shrimp									2 Taisho shrimp (about 40g)			
Clams or freshwater clams									10 shucked pieces of meat (about 20g)			
Snails									10 shucked pieces of meat (about 20g)			
Fish cake									1/6 tube (about 20g)			
Fish paste									2 slices (about 20g)			

For the following vegetables, please refer to the photographs on the page on the left, and fill in the frequency or amount you eat in the season when they appear on the market.

Carrot									Refer to photograph (a)			
Spinach									Refer to photograph (b)			
Pumpkin									Refer to photograph (c)			
Cabbage									Refer to photograph (d)			
Radish									Refer to photograph (e)			

Please mark only one as the preparation method you use most often.

	Raw	Boiled	Grilled	Deep-Fat Fried	Stir-Fried	Other
For meats?						
For fish?						
For vegetables?						

How do you most often eat steaks and grilled meats?

Close to raw (rare)	Medium (medium rare – rare)	Well grilled (well done)

When you eat grilled fish, do you eat the burned parts?

○ I hardly ever eat them ○ I eat about 1/3 of them ○ I eat about half of them ○ I eat about 2/3 of them ○ I eat almost all of them

Currently, whom do you live with? Please mark all of the people that you live with.

○ Spouse ○ Child ○ Parents ○ Other ○ I live alone

Has your work changed in the last 5 years?

○ It has not changed ○ I changed jobs ○ I retired and currently do not work

What is your current work? If it changes because you work more than one job or seasonally, please mark all that apply.

○ Agricultural industry ○ Forestry industry ○ Fishing industry ○ Office work ○ Self-employed
○ Specialty work ○ Housewife ○ Unemployed ○ Other

About how many hours do you work per day?

Less than 5 yours 5 hours or more and less than 9 hours 9 hours or more

Usually per day, about how many hours do you move your body including work?

In physical labor and extreme sports?	None	Less than 1 hour	1 hour or more
Time sitting?	3 hours or less	3 - 8 hours	8 hours or more
Time walking or standing?	Less than 1 hour	1 - 3 hours	3 hours or more

About how often do you have the opportunity to play sports or exercise outside of work?

Hardly ever 1 - 3 times a month 1 - 2 times a week 3 - 4 times a week Almost every day

Normally about how much sleep are you trying to get?

○ 5 hours or less ○ 6 hours ○ 7 hours ○ 8 hours ○ 9 hours ○ 10 hours or more

Are you regular in your everyday life? ○ I am regular ○ I am not regular

In your daily life do you have the feeling you are overworked?

I am not overworked I have the feeling I am a bit overworked I am always overworked

Daily, do you think that you have a lot of stress?

A bit Normal A lot

Do you think you are enjoying your life?

No Normal Yes

When it is cold, do you use an electric blanket? I do not I do

Do you use an electric carpet for heating? I do not I do

We are asking these only of women.

Currently, do you take female hormone medications? ○ I do not take them ○ I do take them

Currently, do you have menses (menstruation)?

○ I do ○ I have had menopause naturally ○ I have had menopause surgically, etc.

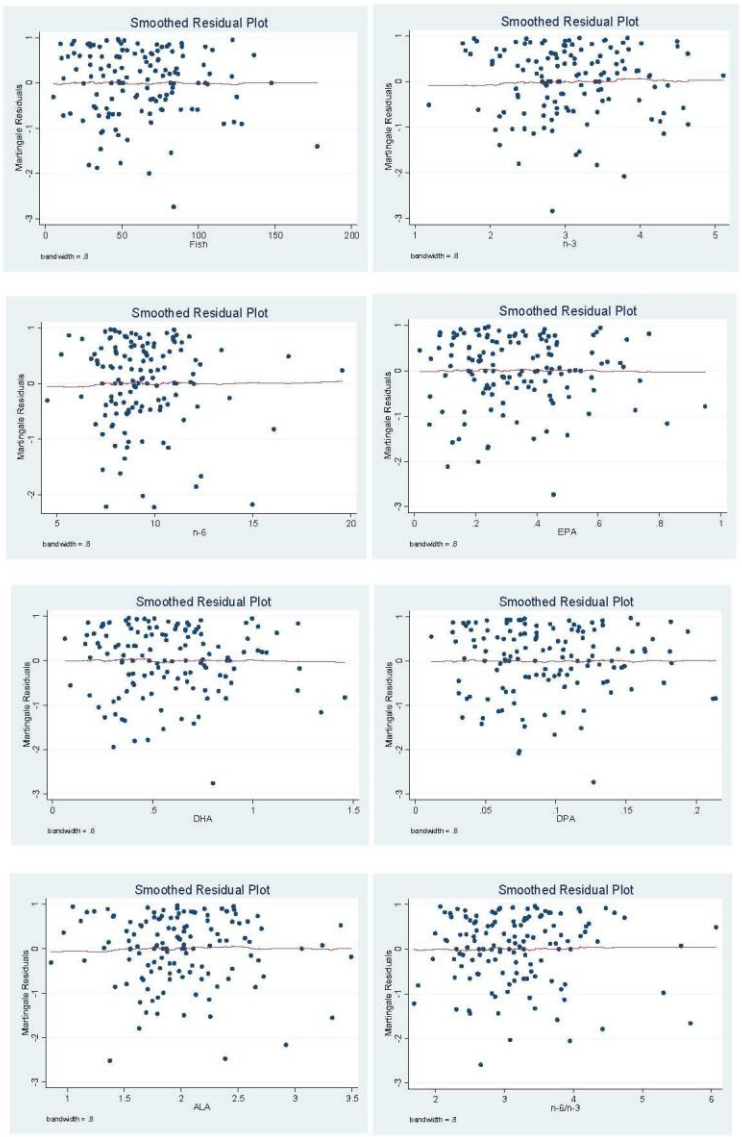
For persons who have had menopause, at what age did you have menopause?

○ Age 39 or under ○ Age 40 - 44 ○ Age 45 - 49 ○ Age 50 - 54 ○ Age 55 - 59 ○ Age 60 or over

Who filled this in? ○ Self ○ Representative

This is the end. We would appreciate it if you would check once more that you have not omitted anything. Thank you very much for your cooperation spending a long time on this.

7.3 Appendix 3: Example of smoothed residual plots of exposure of fish, n-3 PUFA, EPA, DHA, DPA, ALA, n-6 PUFA, and n-6/n-3 (g/day) against Martingale residuals



7.4 Appendix 4: Further acknowledgements

We are indebted to the Aomori, Iwate, Ibaraki, Niigata, Kochi, Nagasaki, and Okinawa Cancer Registries for providing their incidence data.

Members of the Japan Public Health Center-based Prospective Study (JPHC Study, principal investigator: S. Tsugane) Group are: S. Tsugane, N. Sawada, M. Iwasaki, S. Sasazuki, T. Yamaji, T. Shimazu and T. Hanaoka, National Cancer Center, Tokyo; J. Ogata, S. Baba, T. Manami, A. Okayama, and Y. Kokubo, National Cerebral and Cardiovascular Center, Osaka; K. Miyakawa, F. Saito, A. Koizumi, Y. Sano, I. Hashimoto, T. Ikuta, Y. Tanaba, H. Sato, Y. Roppongi, and T. Takashima, Iwate Prefectural Ninohe Public Health Center, Iwate; Y. Miyajima, N. Suzuki, S. Nagasawa, Y. Furusugi, N. Nagai, Y. Ito, S. Komatsu and T. Minamizono, Akita Prefectural Yokote Public Health Center, Akita; H. Sanada, Y. Hatayama, F. Kobayashi, H. Uchino, Y. Shirai, T. Kondo, R. Sasaki, Y. Watanabe, Y. Miyagawa, Y. Kobayashi, M. Machida, K. Kobayashi and M. Tsukada, Nagano Prefectural Saku Public Health Center, Nagano; Y. Kishimoto, E. Takara, T. Fukuyama, M. Kinjo, M. Irei, and H. Sakiyama, Okinawa Prefectural Chubu Public Health Center, Okinawa; K. Imoto, H. Yazawa, T. Seo, A. Seiko, F. Ito, F. Shoji and R. Saito, Katsushika Public Health Center, Tokyo; A. Murata,

K. Minato, K. Motegi, T. Fujieda and S. Yamato, Ibaraki Prefectural Mito Public Health Center, Ibaraki; K. Matsui, T. Abe, M. Katagiri, M. Suzuki, K. and Matsui, Niigata Prefectural Kashiwazaki and Nagaoka Public Health Center, Niigata; M. Doi, A. Terao, Y. Ishikawa, and T. Tagami, Kochi Prefectural Chuo-higashi Public Health Center, Kochi; H. Sueta, H. Doi, M. Urata, N. Okamoto, and F. Ide and H. Goto, Nagasaki Prefectural Kamigoto Public Health Center, Nagasaki; H. Sakiyama, N. Onga, H. Takaesu, M. Uehara, T. Nakasone and M. Yamakawa, Okinawa Prefectural Miyako Public Health Center, Okinawa; F. Horii, I. Asano, H. Yamaguchi, K. Aoki, S. Maruyama, M. Ichii, and M. Takano, Osaka Prefectural Suita Public Health Center, Osaka; Y. Tsubono, Tohoku University, Miyagi; K. Suzuki, Research Institute for Brain and Blood Vessels Akita, Akita; Y. Honda, K. Yamagishi, S. Sakurai and N. Tsuchiya, University of Tsukuba, Ibaraki; M. Kabuto, National Institute for Environmental Studies, Ibaraki; M. Yamaguchi, Y. Matsumura, S. Sasaki, and S. Watanabe, National Institute of Health and Nutrition, Tokyo; M. Akabane, Tokyo University of Agriculture, Tokyo; T. Kadowaki and M. Inoue, The University of Tokyo, Tokyo; M. Noda and T. Mizoue, National Center for Global Health and Medicine, Tokyo; Y. Kawaguchi, Tokyo Medical and Dental University, Tokyo; Y. Takashima and Y. Yoshida, Kyorin University, Tokyo; K. Nakamura and R. Takachi, Niigata University, Niigata; J.

Ishihara, Sagami Women's University, Kanagawa; S. Matsushima and S. Natsukawa, Saku General Hospital, Nagano; H. Shimizu, Sakihae Institute, Gifu; H. Sugimura, Hamamatsu University School of Medicine, Shizuoka; S. Tominaga, Aichi Cancer Center, Aichi; N. Hamajima, Nagoya University, Aichi; H. Iso and T. Sobue, Osaka University, Osaka; M. Iida, W. Ajiki, and A. Ioka, Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka; S. Sato, Chiba Prefectural Institute of Public Health, Chiba; E. Maruyama, Kobe University, Hyogo; M. Konishi, K. Okada, and I. Saito, Ehime University, Ehime; N. Yasuda, Kochi University, Kochi; S. Kono, Kyushu University, Fukuoka; S. Akiba, Kagoshima University, Kagoshima.