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Modulation of natural killer cell activity by supplementation of fermented milk containing *Lactobacillus casei* in habitual smokers

Kanehisa Morimoto^{a,*}, Tatsuya Takeshita^{a,1}, Masanobu Nanno^b,
Shinkan Tokudome^c, Kunio Nakayama^a

^aDepartment of Social and Environmental Medicine, Osaka University Graduate School of Medicine, Osaka, Japan

^bYakult Central Institute for Microbiological Research, Tokyo, Japan

^cDepartment of Health Promotion and Preventive Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Abstract

Background. Habitual smoking significantly reduces natural killer (NK) cell activity. To clarify whether the intake of fermented milk containing lactic acid bacteria restores NK cell activity in habitual smokers, we conducted a placebo-controlled double-blind test.

Methods. Ninety-nine subjects with smoking habits were randomly divided into two groups and daily for 3 weeks were given fermented milk containing *Lactobacillus casei* or placebo. NK cell activity in the peripheral blood mononuclear cells (PBMNC) was determined before and after the intake. Average number of cigarettes smoked and number of cigarettes that the subjects smoked before giving blood after getting up on the inspection day were asked, and these data were used to adjust the influence of smoking on NK cell activity.

Results. NK cell activity in individuals was inversely correlated to numbers of cigarettes smoked. Averaged NK cell activity adjusted by the numbers of cigarettes was significantly higher in individuals drinking fermented milk containing *L. casei* than those drinking a placebo. However, the proportion of NK cells was not different between individuals drinking either fermented milk containing *L. casei* or the placebo.

Conclusions. Intake of fermented milk containing lactic acid bacteria was considered effective for restoring the NK cell activity of habitual smokers.

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Keywords: Smoking; NK cell activity; Lactic acid bacteria

Introduction

Cancer is now becoming the leading cause of death in Japan, and a change in lifestyle is closely associated with an increase in cancer [1]. We have analyzed the connection between lifestyle and immune functions and learned that low levels of natural killer (NK) cell activity and lymphokine-activated killer cell activity were closely linked with an unhealthy lifestyle [2,3]. We have also examined the

relationship between individual lifestyles and mutagen levels excreted in the urine and found that poor health practices may increase the urinary level of mutagens [4]. Good health practices are therefore indispensable for the maintenance of immune surveillance functions against cancer cells and the reduction of cancer.

Health practices can be assessed by asking the following eight questions and expressed as a health practice index (HPI): amount of smoking and drinking, eating breakfast, hours of sleep, hours of work, physical exercise, nutritional balance, and mental stress. These criteria were based on seven health practices demonstrated in an Alameda County (California) study [5] and modified by Morimoto to reflect Japanese situation more adequately [6]. Our findings revealed that a reduction of NK cell activity was strongly associated with smoking [3], suggesting that recovery of

* Corresponding author. Department of Social and Environmental Medicine, Osaka University School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 3929.

E-mail address: morimoto@envi.med.osaka-u.ac.jp (K. Morimoto).

¹ Present address: Wakayama Medical University, Kimiidera, Wakayama 641-8509, Japan.

NK cell activity in habitual smokers may be a valuable way for reducing the risk of cancer.

NK cells differentiate from their precursors in bone marrow and are activated by various cytokines. Some food components are known to increase NK cell activity by stimulating the differentiation and/or the activation of NK cells. Supplementation of vitamins in aged persons or in patients exposed to toxic chemicals enhances NK cell activity [7,8], and the administration of β -carotene is also able to augment NK cell activity in healthy elderly persons [9].

Probiotics are beneficial bacteria used to make dairy products such as yogurt or cheese. Gill et al. reported in a prepost intervention trial that supplementation of *Lactobacillus rhamnosus* and *Bifidobacterium lactis* enhances NK cell activity in humans [10–13]. These results demonstrate that supplementation of probiotics can restore NK cell activity that has been reduced. To more correctly evaluate the ability of probiotics to enhance NK cell activity, a double-blind placebo-controlled test is necessary.

Oral administration of *Lactobacillus casei* strain Shirota (LcS) reduces tumor incidence in mice given 3-methylcholanthrene subcutaneously, together with restoring NK cell activity in the spleen [14]. Intake of milk fermented with LcS enhances NK cell activity in healthy volunteers (20–40 years old) who have relatively low NK cell activity [15]. Moreover, an epidemiological study revealed that habitual intake of milk fermented with LcS significantly reduced bladder cancer [16]. These results strongly suggest that supplementation of fermented milk containing LcS may reduce the risk of cancer by augmenting NK cell activity. By analyzing the relationship between smoking and NK cell activity, we have already demonstrated that cigarette smoking significantly reduces NK cell activity [3]. In this study, we examined the effect of the intake of milk fermented with LcS on NK cell activity in habitual smokers to ascertain whether probiotics can restore NK cell activity in smokers.

Methods

Subjects

We chose male smokers of about 20–60 years old from workers employed at a corporation with a workforce of about 700. Thirty-eight males in experiment 1 and 62 males in experiment 2 were chosen. Informed consent was obtained from all the subjects. None of the subjects had any signs or symptoms of infectious disease. One subject was rejected in experiment 2 because he could not participate in the inspection after the intake duration, and so the data of 38 males in experiment 1 and 61 males in experiment 2 were used for analysis. These experiments were done in accordance with the guidelines of the Helsinki Declaration and the ethical committee for clinical experiments of Osaka University School of Medicine. The general characteristics of the subjects are shown in Table 1. Except

Table 1
Profile of subjects

Experiment		Placebo	LcS containing milk	P value
1	Number of subjects	19	19	
	Age	46.8 ± 7.8	47.4 ± 6.0	0.78
	Height (m)	1.69 ± 0.05	1.67 ± 0.07	0.44
	Weight (kg)	59.8 ± 6.0	64.3 ± 8.6	0.07
	Body mass index (kg/m ²)	21.1 ± 1.9	23.0 ± 2.3	0.01
	No. of cigarettes/day	20.7 ± 10.4	20.9 ± 9.8	0.96
	Health practice index	3.6 ± 1.5	3.7 ± 1.1	0.90
2	Number of subjects	30	31	
	Age	44.8 ± 9.2	44.3 ± 9.5	0.82
	Height (m)	1.70 ± 0.06	1.71 ± 0.07	0.80
	Weight (kg)	65.9 ± 10.9	68.5 ± 9.2	0.32
	Body mass index (kg/m ²)	22.7 ± 2.9	23.5 ± 2.5	0.25
	No. of cigarettes/day	22.8 ± 8.4	21.2 ± 8.0	0.44
	Health practice index	3.5 ± 1.3	3.4 ± 1.0	0.79

for body mass index in experiment 1, all the parameters were the same between groups drinking placebo or fermented milk containing LcS. The number of cigarettes smoked was also almost comparable between the two groups. The health practice indexes of both groups were either poor or moderate according to Morimoto [6].

Experimental design

The effect of the intake of *L. casei* strain Shirota (LcS) containing fermented milk was assessed by a placebo-controlled double-blind test. Subjects were randomly divided into two groups. One group drank fermented milk containing LcS (4×10^{10} live cells/80 ml) daily for 3 weeks, and the other drank a placebo whose components were the same as fermented milk except that LcS was not included. We collected the following data on the day before commencing the intake of samples and on the day just after ceasing the intake of samples. We gave then a questionnaire about body condition, average number of cigarettes they usually smoked, and the number of cigarettes that they smoked until giving blood after getting up on the inspection day. Peripheral blood was obtained, and NK cell activity and the proportion of NK cells were analyzed. Subjects did not know whether they drank fermented milk containing LcS or a placebo, neither did the technicians. After collecting all the data, the identity of the groups was disclosed.

Analysis of NK cell activity

Peripheral blood mononuclear cells (PBMNC) were isolated from heparinized venous blood by differential centrifugation through Ficoll-Hypaque. K562 cells were labeled by adding 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ and incubated at 37° for 1 h. PBMNC were suspended in 10% FCS/5 $\times 10^{-5}$ M β -

ME/RPMI1640 (medium) and put into a well of 96 U-well microtiter plate, and then [^{51}Cr]-labeled K562 cells (1×10^4 cells/well) were added. The effector–target cell ratios (E/T) ranged from 40:1 to 10:1. Plates were centrifuged at $150 \times g$ for 5 min and incubated in 5% CO_2 in air at 37° for 4 h. Spontaneous release was determined by incubating only target cells in medium, and maximal release was measured by standing target cells in medium containing 1% Triton X-100. The supernatants were collected to measure radioactivity released during the incubation, and the percentage of specific lysis was calculated according to the following formula: specific lysis = (experimental release – spontaneous release) / (maximal release – spontaneous release) \times 100. One lytic unit (L.U.) was defined as the cytotoxic activity giving 33.3% of maximal release.

Flow cytometry

PBMNC were incubated with FITC-conjugated anti-CD3 mAb (UCHT1; DakoCytomation Co. Ltd., Kyoto, Japan) and PE-conjugated anti-CD56 mAb (MY31; BD Biosciences, San Jose, CA) on ice for 20 min, and then the relative proportion of $\text{CD3}^+\text{CD56}^-$, $\text{CD3}^-\text{CD56}^+$, $\text{CD3}^-\text{CD56}^-$, and $\text{CD3}^+\text{CD56}^+$ cells was analyzed with a flow cytometer. The ratio of $\text{CD3}^-\text{CD56}^+$ cells was determined as the proportion of NK cells.

Statistical analysis

The significance of the mean between groups was evaluated by Student's *t* test. The difference of the

distribution of change in NK cell activity and the proportion of NK cells were assessed by χ^2 test.

Results

Relationship between cigarette smoking and NK cell activity

In this study, we analyzed in more detail the relationship between smoking and NK cell activity by using only smokers. We asked all the subjects about the average number of cigarettes that they usually smoked (X_1) and the number of cigarettes that they smoked until giving blood after getting up on inspection day (X_2). By investigating the relationship between the weighted combination of X_1 , X_2 , and NK cell activity, we found that $0.5X_1 + X_2$ was inversely correlated with NK cell activity (Fig. 1). These results support our previous report [2,3] and further demonstrate that NK cell activity is hampered more strongly when subjects smoke more cigarettes. Therefore, we adjusted NK cell activity measured before and after the supplementation of samples based on the regression line representing the relationship between $0.5X_1 + X_2$ and NK cell activity and then evaluated the effect of the intake of fermented milk containing LcS on NK cell activity.

Stably maintained NK cell activity in subjects drinking fermented milk containing LcS

In experiment 1, 19 subjects in each group participated in the trial. As shown in the top of Fig. 2, the distribution of

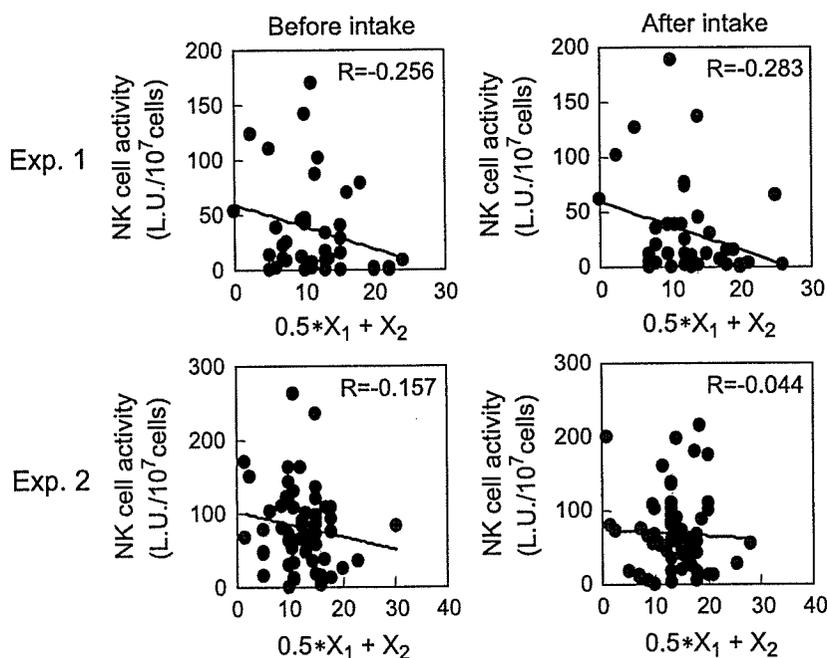


Fig. 1. Relationship between number of cigarettes smoked and NK cell activity in subjects. X_1 is the daily average number of cigarettes that subjects smoked, and X_2 is the number of cigarettes smoked until blood was taken after getting up on the inspection day. Values of $0.5X_1 + X_2$ and NK cell activity expressed as lytic unit/ 10^7 cells were plotted, and regression line was estimated. In both experiments, inverse relationship was detected.

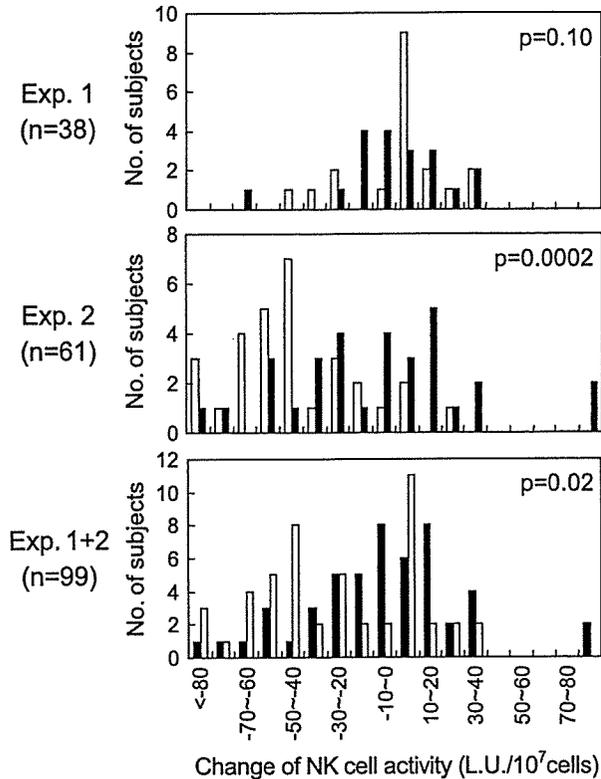


Fig. 2. Distribution of change in NK cell activity induced by drinking either placebo or fermented milk containing *L. casei* (LcS). Subjects were randomly divided into two groups. Daily for 3 weeks, one group drank placebo (□) and another drank fermented milk containing LcS (■). NK cell activity measured before and after the intake of samples was adjusted by regression line shown in Fig. 1, and distribution of change in adjusted NK cell activity in both groups was shown.

change in NK cell activity during the intake period was not different between both groups. However, since the NK cell activity (percentage of specific lysis) measured at E/T = 10 and E/T = 5 was significantly higher in subjects drinking fermented milk containing LcS, we decided to increase the numbers of subjects and repeat the same trial. In experiment 2, 30 subjects drank the placebo and 31 subjects drank fermented milk containing LcS. The results showed that the supplementation of fermented milk containing LcS maintained NK cell activity during the intake but supplementation of placebo did not, resulting in significant differences between the distribution of the change in NK cell activity in both groups ($P = 0.0002$; Fig. 2 center). Furthermore, even after the data from experiments 1 and 2 were combined, the distribution of the change in NK cell activity was significantly different whether subjects were drinking placebo or fermented milk containing LcS ($P = 0.02$; Fig. 2 below).

No change in the relative proportion of NK cells by drinking fermented milk containing LcS

To examine the effect of fermented milk containing LcS on the relative proportion of NK cells, we checked the change in the proportion of CD3⁻CD56⁺ cells in

PBMNC by drinking placebo or fermented milk containing LcS. We could not see any difference of distribution in the change of proportion of NK cells in either group in either experiment (Fig. 3).

Discussion

In this report, we reconfirmed that cigarette smoking is inversely associated with NK cell activity. We also found that not only the average number of cigarettes smoked every day (X_1), but also the number of cigarettes smoked just before the examination on the inspection day (X_2) were involved in the reduction of NK cell activity. Therefore, we adjusted NK cell activity based on the regression line of $0.5X_1 + X_2$ and NK cell activity and analyzed the effect of the intake of fermented milk containing *L. casei* strain Shirota (LcS) on NK cell activity. As a result, the intake of fermented milk containing LcS improved NK cell activity while the intake of the placebo did not. The daily intake of fermented milk containing LcS enhanced NK cell activity in healthy younger subjects with relatively lower NK cell activity [15]. The consumption of probiotic bacteria (*L. rhamnosus* and *B. lactis*) has been shown to enhance NK

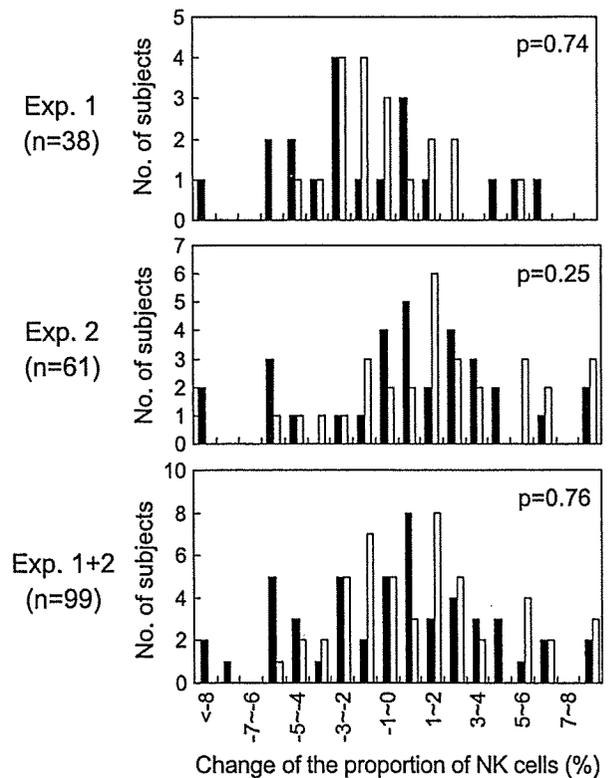


Fig. 3. Distribution of change in relative proportion of NK cells induced by drinking either placebo or fermented milk containing *L. casei* (LcS). Subjects were randomly divided into two groups. Daily for 3 weeks, one group drank placebo (□) and another drank fermented milk containing LcS (■). Distribution of change in relative proportion of NK cells adjusted by regression line shown in Fig. 1, and distribution of change in adjusted NK cell proportion in both groups was shown.

cell activity in healthy volunteers from a wider range of age groups living in Taiwan [10,11] and in elderly persons settling in New Zealand [12,13]. This study also clarified the effect of probiotics on healthy middle-aged subjects who were smoking.

It remains to be elucidated how the supplementation of fermented milk containing LcS can maintain NK cell activity that otherwise decreases due to habitual smoking. In a previous report, we showed that poor health practices are correlated with a decrease in NK cell activity per CD16⁺CD57⁻ cell and proportion of NK cell subset (CD16⁺CD57⁺ and CD16⁻CD57⁺) [3]. Flow cytometry analysis showed no difference in the relative proportion of NK cells in groups drinking either placebo or fermented milk containing LcS, suggesting that fermented milk containing LcS enhanced NK cell activity per cell but did not induce the expansion of NK cells. Taken together, we conclude that the supplementation of fermented milk containing LcS could slow down the reduction of NK cell activity without changing the relative proportion of NK cells.

When LcS was orally given to mice into which 3-methylcholanthrene had been injected subcutaneously, decline in NK cell numbers was improved and NK cell activity restored in the spleen [14]. Furthermore, the proportion of NK cells increased following the supplementation of *L. rhamnosus* and *B. lactis* in elderly persons [10,12]. Therefore, it is possible that whether NK cells increase in number after the supplementation of probiotics may be dependent on the species of lactic acid bacteria and/or the condition of the host (healthy subjects or cancer patients, younger or aged subjects, and so on).

NK cell activity has been found to be important for immune surveillance against cancer and pathogenic infection. An epidemiological cohort study revealed that cancer rates were significantly higher in populations with low NK cell activity than populations with intermediate or high NK cell activity [17]. Elderly persons with low NK cell activity exhibit higher mortality rates caused by infection than elderly persons with high NK cell activity [18]. These results argue that the maintenance of NK cell activity may be indispensable for reducing the risk of mortality due to cancer or infection. On the other hand, NK cell activity is vulnerable to environmental conditions, and smoking and insufficient physical exercise are the biggest culprits for reducing NK cell activity [2,3]. Moreover, NK cell activity in medical students declined on the day of examinations compared with 1 month before examinations, suggesting that mental stress decreases NK cell activity [19]. In contrast, viewing of humorous video reduces stress and improves NK cell activity [20]. These findings strongly suggest that psychological condition and NK cell activity are closely related.

In modern society, the causes of mental stress are increasing. Such circumstances may be one reason why smokers cannot stop. Adequate mental stress is necessary

for the maintenance of homeostasis, but excessive stress suppresses the immune system. Eating a balanced diet is an important factor to maintain NK cell activity [2,3], and we expect that the habitual intake of fermented milk containing LcS may help to reduce the risk of cancer and maintain health in smokers.

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'adiposity rebound' phenomenon, or 'thrifty phenotype'.^{3,4} While such epidemiological paradigms are fashionable, the narrow framework of Barker's hypothesis³ has also been extended to post-natal growth and developmental trajectories.⁴ However, it is difficult to disentangle the real culprit of nature from nurture: junk food, an elusive 'fat gene', gene-environment interactions, or the heterogeneity of biological pathways linking early life exposures to later outcomes.

To date, limited life course epidemiological evidence exists in developing countries. Such evidence reported in the developed world cannot be generalized. Therefore, historical birth cohorts and expertise are both necessary for the resource-poor countries where cost-effective public-health policies drawn on the conventional 'black box' (risk factor) epidemiology are also firmly established. So, how realistic can long-term untested policies based on interdisciplinary life course epidemiological approaches⁴ or utilizing sophisticated 'Mendelian randomization' techniques⁵ be in situations where 'joined-up thinking' is not even the norm! Should our policy makers and public-health leaders replace 'prevarication with imagination'⁶ as to empower our future generations with the ultimate health

prevention models that are built on life course approaches, and integrated into a macro-environment?

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Black tea and cardiovascular disease

From SHINKAN TOKUDOME,^{1*} IMAEDA NAHOMI,² CHIHO GOTO,³ YUKO TOKUDOME³ and MALCOLM A MOORE⁴

Dr Sesso *et al.*¹ could not detect a significant association between black tea consumption and risk of cardiovascular disease using well-established cohorts for the study on health and physical activity. Dr Poole *et al.*² have commented on the paper with regard to the concept of causation. We would like to add another view based on nutritional epidemiology and biological pathogenesis.

Potential protective effects of black tea against cardiovascular disease and cancer are attributed to polyphenol compounds and flavonoids/flavonols, including catechin/EGCG and theaflavin. The authors admit that they lacked a data-based approach³ for selecting foods/beverages contributory to certain nutrients in order to assess intake of flavonols/theaflavins from black tea.

We can assume from the literature⁴ that black tea is a major source of catechin/EGCG, but comparisons within several cups of black tea may not have enough power to detect any favourable effects of catechin/EGCG. In other words, a dose–response relationship could not be proven even after taking into account confounding coffee consumption. Although thus far inconsistent, some beneficial effects have been experienced with large intakes of black/green tea, such as

≥10 cups/day.^{5–8} We need a wide range of comparisons for cups of black tea to evaluate possible protective effects, if any, on cardiovascular disease.

The concentrations of catechin/EGCG in black tea are rather less than in green tea.⁴ In addition, antioxidant activity of black tea scored by oxygen radical absorbing capacity (ORAC) is lower than that for green tea. Furthermore, flavonoids are supplied to a greater extent by vegetables and fruit than several cups of black tea. Thus the authors should, at least, adjust for effects of consumption of vegetables and fruit.

Finally, it is known that folate is antiangiogenic because it is a cofactor in the metabolism of homocysteine to methionine. According to our recent study,⁹ folate is supplied by green tea along with vegetables and fruit; however, its content in black tea is far less than in green tea. Black tea thus seems generally less anticarcinogenic, antimutagenic, and antiangiogenic than green tea. Moreover, any fluids/beverages, including water, black/green tea, and coffee, may be important in terms of blood viscosity and excretion/dilution of mutagenic and carcinogenic substances.^{10,11}

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¹ Department of Health Promotion and Preventive Medicine, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya, Japan.

² Medical Welfare Center Kouseiin, Meito-ku, Nagoya, Japan.

³ Nagoya Bunri University, Inazawa, Japan.

⁴ Division of Experimental Pathology and Chemotherapy, National Cancer Center, Research Institute, Chuo-ku, Tokyo, Japan.

* Corresponding author. Department of Health Promotion and Preventive Medicine, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya 467-8601, Japan. E-mail: tokudome@med.nagoya-cu.ac.jp

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Authors' response

From CHARLES POOLE,¹ ULRIKE PETERS,² DORA IL'YASOVA³ and LENORE ARAB⁴

Dr Tokudome and colleagues¹ ask for more than any systematic review of the currently available epidemiological literature on tea and cardiovascular disease can deliver. If a sufficient number of investigators were to publish trend estimates for specific cardiovascular disease outcomes in relation to intake of specific compounds or classes of compounds in tea and other beverages and foods, those estimates would be able to be systematically reviewed. Until then, it is a hypothesis in search of a literature.

Very few populations contain sufficient numbers of people drinking ≥ 10 cups of tea per day to permit effects of intakes that high to be estimated with anything but the grossest imprecision. We evaluated trend estimates at an increment of three cups per day^{2,3} because that was the largest increment that fit well within the observed ranges of actual intakes in the published study populations.

¹ Department of Epidemiology, University of North Carolina, Chapel Hill, NC, USA.

² Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Bethesda, MD, USA.

³ Department of Epidemiology, Public Health Sciences, Wake Forest University School of Medicine, Winston-Salem, NC, USA.

⁴ Global Epidemiology, Amgen, Thousand Oaks, CA, USA.

Regarding potential interventions that might conceivably result someday from research on this topic, an increase of three cups per day in average tea intake would be a wildly unrealistic goal to set at the population level. Getting a population to increase its average tea intake by 10 cups per day would be pure fantasy. There is always the possibility that one or more preventive compounds might be identified and used for nutritional supplementation or dietary fortification, but the realization of such a possibility is a very long way off at best.

As a more general matter, Dr Tokudome and colleagues suggest that statistical significance constitutes proof. It does not.

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A hypothesis on the sexual behaviour of men who are destined to develop prostate cancer

From WILLIAM H JAMES

There is general agreement that prostatic cancer (PC) has genetic and environmental determinants. However, the putative susceptibility genes have so far largely evaded identification¹ and the environmental factors are also proving difficult to unravel. But for many years, attention has focussed on the possibility of

endocrine (particularly androgenic) determinants of one sort or another. The difficulty is that though it is accepted that androgens stimulate PC *in vitro* and *in vivo*, direct data on circulating concentrations of steroids are equivocal.^{2–4} However, men who are destined to develop the cancer reportedly have a statistically significant excess of sons,⁵ and there are good grounds for supposing that that indicates high androgen levels at the time of conception.^{6,7} Frequently these conceptions

The Galton Laboratory, University College London, Wolfson House, 4 Stephenson Way, London NW1 2HE, UK.

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Change in Food Intake Frequency at Five Years after Baseline in the JACC Study

Sadao Suzuki,¹ Miyuki Kawado,² Shuji Hashimoto,² Shinkan Tokudome,¹ Takesumi Yoshimura,³ Akiko Tamakoshi,⁴ for the JACC Study Group.

BACKGROUND: In a cohort study, information on an individual is taken at baseline, after which it usually remains fixed. There is some risk that this will lead to misclassification and cause weakened or biased results. To prevent such distortion, following up of exposure is important, although it is still scarce in practice.

METHODS: In the Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC Study) sponsored by Monbusho (Ministry of Education, Science, Sports and Culture of Japan), 37,838 (14,531 males and 23,307 females) subjects out of a cohort of 127,477 inhabitants answered an interim questionnaire on food intake frequency consisting of 33 items about five years after registration. The long-term reproducibility was assessed using Spearman's correlation coefficients and agreement. From data at two time points, longitudinal change, age effect, and secular trend were examined. Subjective changes in these items at the time of the interim survey were also compared to longitudinal changes.

RESULTS: Spearman's correlation coefficients varied from 0.27 (fruit juice in males) to 0.55 (beef in females and milk in males), and agreement from 29.9% (fruit juice in males) to 61.4% (liver in females). Correlation was relatively stronger in meat and dairy products and weaker in vegetables and fruits. In both males and females, most increased food item was edible wild plants followed by confectioneries (males) and yogurt (females).

CONCLUSION: Over five years, food intake was considerably changed. These interim data could be used for a long-term follow-up study to prevent the results becoming weakened or biased.

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Key words: epidemiologic method, food, questionnaire, cohort study, Japan

For a self-administered food frequency questionnaire, short-term reproducibility needs to be validated to prevent misclassification of true food intake.¹ However, long-term reproducibility is decreased not only by low reproducibility in the short term but also by real intake changes over time.² In a cohort study, information on an individual is taken at baseline and then usually remains fixed. However, if exposure changes over time, misclassification occurs which might cause weakened or biased results.^{3,4} To prevent such distortion, following up of food intake over the long term is important, although in practice this is still scarce.^{5,7} In the

Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC Study) sponsored by Monbusho (Ministry of Education, Science, Sports and Culture of Japan), an interim survey was designed to examine the changes in lifestyles. In this paper, the authors discuss long-term reproducibility and change in intake frequency of 33 food items over five years.

METHODS

JACC Study is a large-scale multi-center cohort study, which

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¹ Department of Health Promotion and Preventive Medicine, Nagoya City University Graduate School of Medical Sciences.

² Department of Hygiene, Fujita Health University School of Medicine.

³ Fukuoka Institute of Health and Environmental Sciences.

⁴ Department of Preventive Medicine/Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine.

Address for correspondence: Sadao Suzuki, Department of Health Promotion and Preventive Medicine, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. (ssuzuki@med.nagoya-cu.ac.jp)

aims to clarify the etiology of cancer mortality and incidence. Baseline information on physical status and lifestyle, as well as medical history, family history, education, and occupation, was gathered between 1988 and 1990 using a self-administered questionnaire. Baseline data are for 127,477 inhabitants (54,032 males and 73,445 females) enrolled from 45 study areas throughout Japan.⁹ About 5 years after the baseline survey, interim survey about lifestyle factors was conducted. Interim survey was asked to every participant in 18 areas. In contrast, it was asked to some of the cohort subjects in 13 areas, where for example, only examinees of basic health examinations approximately five years after the baseline survey, conducted under the Health and Medical Service Law for the Aged, were invited to the interim survey.¹⁰ In 14 areas interim survey was not conducted. The research was also done by using a self-administered questionnaire, including demographic information, past medical history, family cancer history in these 5 years, exercise/sports activities, frequency of food intake and change of intake compared with 5 years before, smoking and alcohol drinking status and so on. Out of 110,792 subjects between 40-79 years old at the time of registration, 46,680 (42.1%) individuals answered the interim questionnaire. Table 1 shows the number and response rate by how the interim survey was conducted. For 18 areas in which the interim questionnaire was asked to every participant, the response rate was 78.8%, while for the 13 area, in which it was not asked to all participants, the response rate was only 24.1%.

Among them, 37,838 (14,531 males and 23,307 females) were eligible subjects who answered an interim questionnaire on food intake frequency. In some areas, several items were not included in the questionnaire, and those areas were excluded from the analysis by food items.

In the baseline and interim surveys, the subjects were asked average intake frequency of the same 33 food items in a past year. They chose one appropriate frequency among five categories, i.e., (1) almost none, (2) 1-2 times per month, (3) 1-2 times per week, (4) 3-4 times per week, and (5) almost every day. Scores one to

five were used to evaluate the individual's food intake frequency, and the long-term reproducibility was assessed using Spearman's correlation coefficients and agreement (exact agreement and agreement allowing one category difference). Longitudinal change in intake frequency of food items was measured by the difference in scores on the two questionnaires.

In order to observe the difference in the change in food intake frequency by age, we divided the subjects into eight age specific sub-cohorts (40-44, 45-49, 50-54, 55-59, 60-64, 65-69, 70-74, and 75-79 years old). The variation of two scores for food intake frequency over five years consists of two parts: age effect and secular trend. We assumed the difference from a sub-cohort to one rank older at baseline as the age effect for five years, and that secular trend could be calculated as the longitudinal variation subtracted by the age effect. From this analysis, subjects aged 75-79 years old were excluded since there was no older age group at baseline. Each analysis was performed by sex.

After the long-term reproducibility and variation assessment of 33 food items using Spearman's correlation coefficients, agreement of the answer, longitudinal difference, age effect, and secular trend, we checked whether the results were consistent between males and females using Spearman's correlation coefficients of males and females for the indexes mentioned above.

In the interim questionnaire, subjective changes in intake were also asked for the same 33 items. The scores were 1 for 'increased', 0 for 'not changed', and -1 for 'decreased'. We examined the consistency of the food frequency variations taken from two different methods, i.e., the difference on two questionnaires and subjective changes at the time of the interim questionnaire using Spearman's correlation coefficients by sex. All analyses were performed using SAS[®] version 8.2 (SAS Institute).

Our entire study design, which comprised singular and collective use of epidemiologic data and biological materials (serum only), was approved in 2000 by the Ethical Board at Nagoya University School of Medicine, where the central secretariat of the JACC study is located.

Table 1. The number of the participants of the baseline and interim survey.

Target of interim survey	Baseline survey	Interim survey
All participants of the baseline survey (18 areas)	48,016	37,853 (78.8%)
Some participants of the baseline survey (13 areas)	36,460	8,797 (24.1%)
No participants (interim survey was not conducted) (14 areas)	26,316	0 (0.0%)
Total (45 areas)	110,792	46,650 (42.1%)

RESULTS

Table 2 shows the distribution of age and sex of the subjects. The mean age (standard deviation) of males and females was 58.1 (9.6) and 58.0 (9.5) years old, respectively. The mean (standard deviation) period was 4.71 (0.69) years and median was 4.83 years.

Table 3 shows the proportion of food intake frequency at the baseline and interim surveys. Missing values were common, more than 15%, for margarine, yogurt, butter, and cheese intake in both surveys. In contrast, missing values were fairly few, around 5%, for eggs, fresh fish, and tofu intake. Among the 33 items, the proportion of missing values was very consistent not only between males and females at baseline (Spearman's correlation coefficients: 0.98) and at the interim survey (0.98), but also between baseline and interim questionnaire in both males (0.89) and females (0.89). The occurrence of missing values strongly depended on the items regardless of sex or time.

We summarized in table 4 the results of long-term reproducibility and variation of the food intake frequency for five years. It contains Spearman's correlation coefficients, agreement of the categories (exact agreement and agreement allowing one category difference), mean scores of intake frequency, longitudinal difference, age effect, secular trend, and subjective change for 33 food items. Spearman's correlation coefficients ranged from 0.27 and 0.55, and the median was 0.38 in males and 0.39 in females. Correlation was highest for intake of beef (0.45 for males and 0.55 for females), milk (0.55, 0.54) and margarine (0.46, 0.54) both in males and females. The lowest Spearman's correlation coefficients were observed for fruit juice (0.27, 0.29) and Chinese cabbage (0.30, 0.30) in both males and females.

Exact agreement varied 29.5-61.4% (median 40.8 in males and 42.5% in females), and was the highest for liver (56.9%, 61.4%) and pickled vegetables (52.9%, 55.9%) in males and females. Agreement allowing one category difference varied 64.4-92.5% with a median of 82.9% in males and 83.8% in females. It was also the highest for liver (91.9%, 92.5%) followed by beef (90.5%, 91.3%). On the other hand, exact agreement was lowest for juice (67.5%, 64.4%), followed by confectioneries (traditional,

cakes, etc.) (73.6%, 73.5%).

Variations over five years which could not be assessed from Spearman's correlation coefficients or agreement were evaluated using the difference of two scores (Table 4). The most increased food items were edible wild plants (0.37, 0.44) and confectioneries (0.37, 0.34). Intake of yogurt was increased in females (0.40) but to a lesser extent in males (0.22). Conversely, intake frequency was decreased most for fruit juice (-0.53, -0.56) followed by seaweeds (-0.17, -0.215). Figure 1 shows intake changes over five years for yogurt, seaweeds, boiled beans, and confectioneries in every five-year age group by sex.

The age effect was defined as the difference between the food intake frequency score and the mean score of the subjects who belong to one-rank older sub-cohort in this study. The age effect was the largest for boiled beans (0.11 in males, 0.09 in females), indicating that aged people consume boiled beans more often than younger people. This was followed by confectioneries (0.09), oranges (0.07) in males, by oranges (0.04) and spinach (0.04) in females. In the opposite direction, age effect was the strongest for pork (-0.06 in males, -0.12 in females) followed by ham and sausage (-0.06 in males, -0.11 in females). Younger subjects consume these items more often than aged subjects.

Secular trend was also most increased for edible wild plants both in males and females (0.37 in males, 0.42 in females) followed by confectioneries (0.28, 0.34), and yogurt in females (0.40). Here again it was decreased most for fruit juice (-0.51, -0.52) followed by seaweeds (-0.21, -0.20) and oranges (-0.15, -0.25). The results are almost identical to those for longitudinal differences.

The changes over five years were consistent between males and females. Spearman's correlation coefficients of these indexes were high; Spearman's correlation coefficient of the intake frequency at the baseline and interim questionnaire (0.79), exact agreement (0.86), agreement allowing one rank difference (0.91), longitudinal difference (0.91), age effect (0.81), secular trend (0.89), and subjective variation over five years (0.96) of 33 food items.

In spite of the consistent results between males and females, the subjective difference and longitudinal difference from the scores

Table 2. Age and sex distribution of the subjects.

Age (year)	Males	Females	Total
40-44	1,569 (10.8%)	2,332 (10.0%)	3,901 (10.3%)
45-49	1,545 (10.6%)	2,690 (11.5%)	4,235 (11.2%)
50-54	1,854 (12.8%)	3,276 (14.1%)	5,130 (13.6%)
55-59	2,689 (18.5%)	4,183 (17.9%)	6,872 (18.2%)
60-64	3,164 (21.8%)	4,710 (20.2%)	7,874 (20.8%)
65-69	1,827 (12.6%)	3,324 (14.3%)	5,151 (13.6%)
70-74	1,248 (8.6%)	1,795 (7.7%)	3,043 (8.0%)
75-79	635 (4.4%)	997 (4.3%)	1,632 (4.3%)
Total	14,531	23,307	37,838

Table 3. Distribution of food intake frequency at baseline and interim survey.

	Males										Females																							
	Almost none		1-2/month		3-4/week		Almost every day		Subtotal		Missing value		Total		Almost none		1-2/month		3-4/week		Almost every day		Subtotal		Missing value		Total							
	(%)	No.	(%)	No.	(%)	No.	(%)	No.	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.							
Beef	Baseline	22.7	37.8	30.4	7.8	1.3	12,809	10.8	14,358	26.3	32.2	31.2	9.1	20,360	11.6	23,040	Baseline	26.3	32.2	31.2	9.1	20,360	11.6	23,040	Interim	14.9	33.3	6.2	8.3	14,358				
	Interim	14.9	33.3	6.2	8.3	1.3	13,166	8.3	14,358	20.2	38.6	34.1	6.4	20,897	9.3	23,040	Baseline	20.2	38.6	34.1	6.4	20,897	9.3	23,040	Interim	10.4	25.5	44.0	17.0	3.2	13,222	9.0	14,531	
Pork	Baseline	10.4	25.5	44.0	17.0	3.2	13,222	9.0	14,531	13.3	21.6	44.5	17.3	20,776	10.9	23,007	Baseline	13.3	21.6	44.5	17.3	20,776	10.9	23,007	Interim	7.6	31.5	46.5	13.0	1.5	13,096	10.2	14,531	
	Interim	7.6	31.5	46.5	13.0	1.5	13,096	10.2	14,531	26.1	26.7	47.4	14.3	20,690	11.2	23,307	Baseline	26.1	26.7	47.4	14.3	20,690	11.2	23,307	Interim	23.1	28.8	33.2	11.4	3.5	12,467	14.2	14,531	
Ham and sausages	Baseline	23.1	28.8	33.2	11.4	3.5	12,467	14.2	14,531	21.8	35.6	32.5	8.2	19,727	15.4	23,307	Baseline	21.8	35.6	32.5	8.2	19,727	15.4	23,307	Interim	19.6	36.8	32.3	9.2	2.1	12,576	13.5	14,531	
	Interim	19.6	36.8	32.3	9.2	2.1	12,576	13.5	14,531	9.1	24.2	47.4	17.0	20,862	10.5	23,307	Baseline	9.1	24.2	47.4	17.0	20,862	10.5	23,307	Interim	30.1	30.1	44.1	14.2	2.1	13,039	10.3	14,531	
Chicken	Baseline	9.4	36.8	30.1	44.1	14.2	13,039	10.3	14,531	48.6	38.8	8.7	14.1	18,787	14.9	22,084	Baseline	48.6	38.8	8.7	14.1	18,787	14.9	22,084	Interim	8.0	34.0	44.2	10.3	0.6	11,810	14.1	14,531	
	Interim	8.0	34.0	44.2	10.3	0.6	11,810	14.1	14,531	47.4	41.6	8.8	1.6	18,691	15.4	22,084	Baseline	47.4	41.6	8.8	1.6	18,691	15.4	22,084	Interim	39.5	48.1	9.8	1.9	3.9	13,913	4.3	14,531	
Liver	Baseline	39.5	48.1	9.8	1.9	3.9	13,913	4.3	14,531	2.5	5.0	24.7	26.5	22,233	4.6	22,443	Baseline	2.5	5.0	24.7	26.5	22,233	4.6	22,443	Interim	2.1	5.5	23.2	24.5	44.7	13,913	4.3	14,531	
	Interim	2.1	5.5	23.2	24.5	44.7	13,913	4.3	14,531	17.8	7.2	13.5	13.1	20,689	7.8	22,443	Baseline	17.8	7.2	13.5	13.1	20,689	7.8	22,443	Interim	5.7	5.7	23.7	30.8	38.2	13,716	5.6	14,531	
Eggs	Baseline	5.7	5.7	23.7	30.8	38.2	13,716	5.6	14,531	14.2	14.2	14.2	14.4	20,689	8.2	22,443	Baseline	14.2	14.2	14.2	14.4	20,689	8.2	22,443	Interim	20.4	9.6	14.7	13.0	42.3	12,813	8.6	14,024	
	Interim	20.4	9.6	14.7	13.0	42.3	12,813	8.6	14,024	54.0	19.8	14.2	6.1	18,471	17.7	22,443	Baseline	54.0	19.8	14.2	6.1	18,471	17.7	22,443	Interim	17.6	10.2	13.3	13.4	45.6	11,383	8.8	14,024	
Milk	Baseline	17.6	10.2	13.3	13.4	45.6	11,383	8.8	14,024	37.7	23.8	18.9	10.0	19,216	14.4	22,443	Baseline	37.7	23.8	18.9	10.0	19,216	14.4	22,443	Interim	59.0	19.8	10.8	10.8	5.9	11,847	15.5	14,024	
	Interim	59.0	19.8	10.8	10.8	5.9	11,847	15.5	14,024	57.1	24.3	11.9	4.2	18,570	16.5	22,351	Baseline	57.1	24.3	11.9	4.2	18,570	16.5	22,351	Interim	54.9	27.3	33.9	12.8	3.8	13,929	4.7	14,531	
Cheese	Baseline	46.7	33.9	12.8	3.8	2.8	11,785	15.4	13,929	53.8	30.6	14.3	5.4	18,360	16.0	22,351	Baseline	46.7	33.9	12.8	3.8	2.8	11,785	15.4	13,929	Interim	55.7	23.1	12.6	4.5	5.7	11,511	16.1	14,531
	Interim	55.7	23.1	12.6	4.5	5.7	11,511	16.1	14,531	36.3	23.4	18.9	9.2	18,840	15.7	22,351	Baseline	36.3	23.4	18.9	9.2	18,840	15.7	22,351	Interim	50.9	30.1	12.8	3.4	2.7	11,684	16.1	14,531	
Butter	Baseline	50.9	30.1	12.8	3.4	2.7	11,684	16.1	14,531	4.0	27.6	46.8	17.4	20,324	12.8	23,307	Baseline	4.0	27.6	46.8	17.4	20,324	12.8	23,307	Interim	47.6	20.9	15.6	6.7	6.7	11,665	16.3	13,929	
	Interim	47.6	20.9	15.6	6.7	6.7	11,665	16.3	13,929	3.2	27.8	49.1	25.2	21,423	8.1	23,307	Baseline	3.2	27.8	49.1	25.2	21,423	8.1	23,307	Interim	4.1	25.7	46.9	18.4	4.1	14,531	7.9	14,531	
Margarine	Baseline	4.1	25.7	46.9	18.4	4.1	14,531	7.9	14,531	16.9	41.5	30.9	9.0	20,696	7.4	22,351	Baseline	16.9	41.5	30.9	9.0	20,696	7.4	22,351	Interim	2.9	17.6	41.7	24.1	24.1	12,734	12.4	14,531	
	Interim	2.9	17.6	41.7	24.1	24.1	12,734	12.4	14,531	3.6	16.1	41.3	25.2	21,129	5.5	23,307	Baseline	3.6	16.1	41.3	25.2	21,129	5.5	23,307	Interim	2.3	15.3	33.8	30.3	1.4	13,539	6.8	14,531	
Deep fried foods	Baseline	2.3	15.3	33.8	30.3	1.4	13,539	6.8	14,531	1.7	7.1	30.8	39.3	21,930	5.9	23,307	Baseline	1.7	7.1	30.8	39.3	21,930	5.9	23,307	Interim	1.4	7.6	33.2	36.0	22.1	13,765	5.3	14,531	
	Interim	1.4	7.6	33.2	36.0	22.1	13,765	5.3	14,531	10.7	26.0	35.6	18.0	20,794	10.8	23,307	Baseline	10.7	26.0	35.6	18.0	20,794	10.8	23,307	Interim	8.3	25.3	37.9	18.5	9.9	13,236	8.9	14,531	
Fried vegetables	Baseline	8.3	25.3	37.9	18.5	9.9	13,236	8.9	14,531	6.2	24.4	39.3	21.7	21,360	8.4	23,307	Baseline	6.2	24.4	39.3	21.7	21,360	8.4	23,307	Interim	4.9	23.4	40.5	22.0	22.0	13,886	13.1	13,685	
	Interim	4.9	23.4	40.5	22.0	22.0	13,886	13.1	13,685	20.1	33.3	31.9	11.6	19,048	13.2	21,947	Baseline	20.1	33.3	31.9	11.6	19,048	13.2	21,947	Interim	23.4	34.0	29.0	10.3	3.2	12,119	11.4	13,685	
Boiled fish paste	Baseline	19.7	41.5	28.8	8.3	1.7	12,119	11.4	13,685	0.9	5.5	26.2	29.9	20,696	7.4	22,351	Baseline	0.9	5.5	26.2	29.9	20,696	7.4	22,351	Interim	1.4	8.5	29.9	28.1	32.1	13,929	7.1	14,531	
	Interim	1.4	8.5	29.9	28.1	32.1	13,929	7.1	14,531	0.6	5.5	26.2	36.7	21,129	5.5	23,307	Baseline	0.6	5.5	26.2	36.7	21,129	5.5	23,307	Interim	1.0	8.4	30.4	34.9	25.4	13,161	5.5	14,531	
Green-leafy vegetables	Baseline	1.0	8.4	30.4	34.9	25.4	13,161	5.5	14,531	1.4	11.4	34.8	32.0	20,4	7.2	23,307	Baseline	1.4	11.4	34.8	32.0	20,4	7.2	23,307	Interim	5.2	20.9	39.4	27.8	11.3	13,417	7.7	14,531	
	Interim	5.2	20.9	39.4	27.8	11.3	13,417	7.7	14,531	0.8	9.5	34.4	36.7	20,824	10.7	23,307	Baseline	0.8	9.5	34.4	36.7	20,824	10.7	23,307	Interim	2.4	19.2	39.4	30.3	18.3	13,095	9.9	14,531	
Carrots and squash	Baseline	2.4	19.2	39.4	30.3	18.3	13,095	9.9	14,531	11.5	21.3	30.0	20.2	17.0	10.9	23,307	Baseline	11.5	21.3	30.0	20.2	17.0	10.9	23,307	Interim	8.6	25.0	35.1	21.6	21.6	14,531	10.3	14,531	
	Interim	8.6	25.0	35.1	21.6	21.6	14,531	10.3	14,531	1.4	6.6	29.4	31.8	21,903	6.0	23,307	Baseline	1.4	6.6	29.4	31.8	21,903	6.0	23,307	Interim	1.9	10.3	34.4	30.5	15.9	13,584	6.5	14,531	
Tomatoes	Baseline	1.9	10.3	34.4	30.5	15.9	13,584	6.5	14,531	1.1	16.8	35.7	24.8	18,402	16.2	21,947	Baseline	1.1	16.8	35.7	24.8	18,402	16.2	21,947	Interim	1.4	9.8	36.1	35.7	16.9	13,584	6.8	14,531	
	Interim	1.4	9.8	36.1	35.7	16.9	13,584	6.8	14,531	4.4	11.7	36.6	23.7	19,551	10.9	23,307	Baseline	4.4	11.7	36.6	23.7	19,551	10.9	23,307	Interim	4.4	17.6	37.6	32.5	25.5	12,401	9.4	13,685	
Cabbage and lettuce	Baseline	4.4	17.6	37.6	32.5	25.5	12,401	9.4	13,685	5.6	42.5	35.7	12.9	18,402	16.2	21,947	Baseline	5.6	42.5	35.7	12.9	18,402	16.2	21,947	Interim	4.5	20.4	38.2	25.4	11.5	13,929	11.8	14,531	
	Interim	4.5	20.4	38.2	25.4	11.5	13,929	11.8	14,531	4.3	25.7	38.9	22.6	19,294	12.9	23,307	Baseline	4.3	25.7	38.9	22.6	19,294	12.9	23,307	Interim	37.2	40.0	14.1	6.4	3.8	12,301	11.7	14,531	
Chinese cabbage	Baseline	37.2	40.0	14.1	6.4	3.8	12,301	11.7	14,531	4.3	25.7	38.9	22.6	19,294																				

Table 4. Spearman's correlation coefficients, agreement of the categories, mean scores, longitudinal difference, age effect, secular trend, and subjective change for 33 food items.

	Males											Females																							
	Allowing one category					Secular Subjective					Allowing one category					Secular Subjective																			
	Agreement (%)	Mean score	Longitudinal	Difference	Age effect [†]	trend	change	Agreement (%)	Mean score	Longitudinal	Difference	Age effect [†]	trend	change	Agreement (%)	Mean score	Longitudinal	Difference	Age effect [†]	trend	change														
	SCC*	Exact	Baseline	Interim	Longitudinal	Age effect [†]	trend	change	SCC*	Exact	Baseline	Interim	Longitudinal	Age effect [†]	trend	change	Agreement (%)	Mean score	Longitudinal	Difference	Age effect [†]	trend	change												
Beef	0.45	47.8	90.5	2.27	2.33	0.05	0.01	-0.14	0.55	51.3	91.3	2.27	2.28	0.02	0.00	-0.20	0.41	45.7	89.0	2.77	2.69	-0.08	-0.06	-0.02	-0.16	0.48	48.0	89.6	2.76	2.70	-0.06	-0.12	0.06	-0.22	
Pork	0.41	40.8	84.4	2.43	2.38	-0.06	0.00	-0.15	0.42	42.5	83.9	2.38	2.33	-0.05	0.06	-0.24	0.41	40.8	84.4	2.43	2.38	-0.06	-0.06	0.00	-0.15	0.42	42.5	83.9	2.38	2.33	-0.05	-0.11	0.06	-0.24	
Ham and sausages	0.37	45.4	89.4	2.69	2.64	-0.06	0.00	-0.08	0.39	47.5	89.7	2.79	2.71	-0.08	-0.05	-0.11	0.40	46.9	91.9	2.77	2.76	-0.01	0.00	-0.02	-0.13	0.47	51.3	91.3	2.27	2.28	0.02	0.00	-0.02	-0.17	
Chicken	0.40	56.9	91.9	1.77	1.76	-0.01	0.00	-0.13	0.42	45.9	84.4	3.99	3.88	-0.11	-0.05	-0.03	0.43	47.0	84.4	4.04	3.98	-0.06	0.00	-0.06	0.03	0.42	45.9	84.4	3.99	3.88	-0.11	-0.05	-0.06	-0.03	
Eggs	0.55	50.7	74.7	3.47	3.60	0.13	0.05	0.08	0.54	54.3	77.2	3.67	3.87	0.20	0.01	0.16	0.38	57.9	81.5	1.57	1.79	0.22	0.05	0.17	-0.02	0.44	45.0	75.1	1.90	2.30	0.40	0.00	0.40	0.04	
Milk	0.44	53.1	87.3	1.72	1.82	0.10	-0.02	0.12	0.49	55.1	86.8	1.71	1.86	0.15	-0.05	0.20	0.44	53.1	87.3	1.72	1.82	0.10	-0.02	0.12	-0.09	0.49	55.1	86.8	1.71	1.86	0.15	-0.05	0.20	-0.11	
Yogurt	0.38	51.9	83.9	1.78	1.77	-0.01	0.00	-0.13	0.42	51.8	83.6	1.86	1.83	-0.03	-0.05	0.02	0.38	51.9	83.9	1.78	1.77	-0.01	0.00	-0.02	-0.13	0.42	51.8	83.6	1.86	1.83	-0.03	-0.05	0.02	-0.18	
Cheese	0.46	49.8	80.3	2.09	2.02	-0.07	0.01	-0.08	0.54	46.6	78.4	2.48	2.35	-0.13	-0.07	-0.13	0.46	49.8	80.3	2.09	2.02	-0.07	0.01	-0.08	-0.10	0.54	46.6	78.4	2.48	2.35	-0.13	-0.07	-0.05	-0.13	
Butter	0.35	45.5	88.9	2.94	2.95	0.00	-0.01	-0.08	0.36	46.1	89.7	2.90	2.88	-0.02	-0.04	0.02	0.35	45.5	88.9	2.94	2.95	0.00	-0.01	-0.08	-0.10	0.36	46.1	89.7	2.90	2.88	-0.02	-0.04	0.02	-0.20	
Margarine	0.37	41.4	85.0	3.26	3.32	0.07	0.03	0.02	0.41	42.3	86.2	3.30	3.37	0.07	0.00	-0.04	0.37	41.4	85.0	3.26	3.32	0.07	0.03	0.02	0.08	0.41	42.3	86.2	3.30	3.37	0.07	0.00	0.07	-0.04	
Deep fried foods	0.37	43.4	85.4	3.73	3.71	-0.03	0.01	-0.04	0.40	44.5	87.0	3.76	3.72	-0.04	-0.02	-0.03	0.38	43.4	85.4	3.73	3.71	-0.03	0.01	-0.04	0.08	0.40	44.5	87.0	3.76	3.72	-0.04	-0.02	-0.03	0.09	
Fried vegetables	0.38	37.9	82.5	2.96	3.07	0.11	0.00	0.11	0.39	37.8	81.8	2.90	3.02	0.12	-0.02	0.14	0.38	37.9	82.5	2.96	3.07	0.11	0.00	0.11	-0.05	0.39	37.8	81.8	2.90	3.02	0.12	-0.02	0.14	-0.08	
Flesh fish	0.39	41.3	85.2	2.36	2.32	-0.04	0.03	-0.07	0.40	42.5	85.6	2.44	2.38	-0.06	-0.01	-0.05	0.39	41.3	85.2	2.36	2.32	-0.04	0.03	-0.07	-0.10	0.40	42.5	85.6	2.44	2.38	-0.06	-0.01	-0.05	-0.16	
Dried/salted fish	0.32	38.8	81.5	3.81	3.77	-0.04	0.05	-0.09	0.34	42.6	84.4	3.98	3.93	-0.05	0.04	0.13	0.32	38.8	81.5	3.81	3.77	-0.04	0.05	-0.09	0.10	0.34	42.6	84.4	3.98	3.93	-0.05	0.04	-0.09	0.13	
Fish paste	0.35	37.8	82.3	3.17	3.26	0.09	0.03	0.06	0.36	40.8	85.8	3.59	3.63	0.04	0.00	0.12	0.35	37.8	82.3	3.17	3.26	0.09	0.03	0.06	0.06	0.36	40.8	85.8	3.59	3.63	0.04	0.00	0.04	0.12	
Green-leafy vegetables	0.40	36.0	77.8	2.90	3.03	0.13	0.03	0.09	0.39	35.8	76.4	3.10	3.28	0.18	0.00	0.06	0.40	36.0	77.8	2.90	3.03	0.13	0.03	0.09	0.04	0.39	35.8	76.4	3.10	3.28	0.18	-0.02	0.20	0.06	
Carrots and squash	0.33	39.4	83.7	3.62	3.57	-0.05	0.00	-0.05	0.36	41.6	85.3	3.84	3.76	-0.09	-0.04	0.08	0.33	39.4	83.7	3.62	3.57	-0.05	0.00	-0.05	0.08	0.36	41.6	85.3	3.84	3.76	-0.09	-0.04	-0.05	0.08	
Tomatoes	0.30	36.3	79.8	3.28	3.20	-0.08	0.04	-0.12	0.30	35.5	78.0	3.29	3.15	-0.14	0.03	-0.02	0.30	36.3	79.8	3.28	3.20	-0.08	0.04	-0.12	0.01	0.30	35.5	78.0	3.29	3.15	-0.14	0.03	-0.17	-0.02	
Cabbage and lettuce	0.33	38.4	80.4	1.97	2.34	0.37	0.00	0.37	0.31	37.2	78.5	1.91	2.35	0.44	0.02	0.42	0.33	38.4	80.4	1.97	2.34	0.37	0.00	0.37	-0.05	0.31	37.2	78.5	1.91	2.35	0.44	0.02	0.42	-0.07	
Chinese cabbage	0.30	39.3	83.5	2.78	2.79	0.00	0.04	-0.03	0.35	39.5	83.8	3.05	3.03	-0.02	0.00	0.06	0.30	39.3	83.5	2.78	2.79	0.00	0.04	-0.03	0.02	0.35	39.5	83.8	3.05	3.03	-0.02	0.00	-0.02	0.06	
Edible wild plants	0.39	39.4	84.1	3.08	3.15	0.07	0.05	0.02	0.38	42.3	86.2	3.46	3.43	-0.04	0.00	0.03	0.39	39.4	84.1	3.08	3.15	0.07	0.05	0.02	0.01	0.38	42.3	86.2	3.46	3.43	-0.04	0.00	-0.04	0.03	
Mushrooms	0.34	37.8	80.3	3.59	3.43	-0.17	0.05	-0.21	0.36	40.3	82.5	3.88	3.67	-0.21	-0.01	0.11	0.34	37.8	80.3	3.59	3.43	-0.17	0.05	-0.21	0.08	0.36	40.3	82.5	3.88	3.67	-0.21	-0.01	-0.20	0.11	
Potatoes	0.42	52.9	79.8	4.12	4.18	0.06	-0.01	-0.04	0.40	55.9	80.6	4.19	4.27	0.07	-0.02	-0.08	0.42	52.9	79.8	4.12	4.18	0.06	-0.01	-0.04	0.04	0.40	55.9	80.6	4.19	4.27	0.07	-0.02	0.09	-0.08	
Seaweeds	0.33	35.0	76.9	2.47	2.59	0.12	0.02	0.09	0.37	36.1	77.1	2.39	2.54	0.16	0.03	0.13	0.33	35.0	76.9	2.47	2.59	0.12	0.02	0.09	-0.10	0.37	36.1	77.1	2.39	2.54	0.16	0.03	0.13	-0.15	
Pickled vegetables	0.37	40.3	82.9	2.34	2.46	0.12	0.11	0.02	0.35	40.9	82.6	2.48	2.56	0.08	0.09	0.00	0.37	40.3	82.9	2.34	2.46	0.12	0.11	0.02	-0.06	0.35	40.9	82.6	2.48	2.56	0.08	0.09	0.00	-0.08	
Tsukudani (food boiled with soy)	0.42	44.5	87.4	3.76	3.84	0.07	0.03	0.04	0.43	47.3	88.6	3.91	3.95	0.04	-0.02	0.05	0.42	44.5	87.4	3.76	3.84	0.07	0.03	0.04	0.13	0.43	47.3	88.6	3.91	3.95	0.04	-0.02	0.05	0.17	
Boiled beans	0.40	34.7	75.9	3.33	3.26	-0.08	0.07	-0.15	0.37	38.3	76.5	3.88	3.67	-0.21	0.04	0.02	0.40	34.7	75.9	3.33	3.26	-0.08	0.07	-0.15	0.01	0.37	38.3	76.5	3.88	3.67	-0.21	0.04	-0.25	0.02	
Tofu (soybean curd)	0.37	36.8	77.3	3.48	3.42	-0.07	0.02	-0.09	0.36	41.5	79.0	3.96	3.85	-0.11	-0.03	0.08	0.37	36.8	77.3	3.48	3.42	-0.07	0.02	-0.09	0.05	0.36	41.5	79.0	3.96	3.85	-0.11	-0.03	-0.08	0.08	
Oranges	0.27	29.9	67.5	2.90	2.37	-0.53	-0.02	-0.51	0.29	29.5	64.4	2.97	2.42	-0.56	-0.04	-0.09	0.27	29.9	67.5	2.90	2.37	-0.53	-0.02	-0.51	-0.05	0.29	29.5	64.4	2.97	2.42	-0.56	-0.04	-0.52	-0.09	
Fruits other than oranges	0.46	34.4	73.6	2.89	3.26	0.37	0.09	0.28	0.39	34.7	73.5	3.26	3.61	0.34	0.01	0.34	0.46	34.4	73.6	2.89	3.26	0.37	0.09	0.28	-0.07	0.39	34.7	73.5	3.26	3.61	0.34	0.01	0.34	-0.14	
Fruit juice																																			
Confectioneries (traditional, cakes, etc.)																																			

* : Spearman's correlation coefficient.
 † : Age effect. Mean difference in five years at baseline.

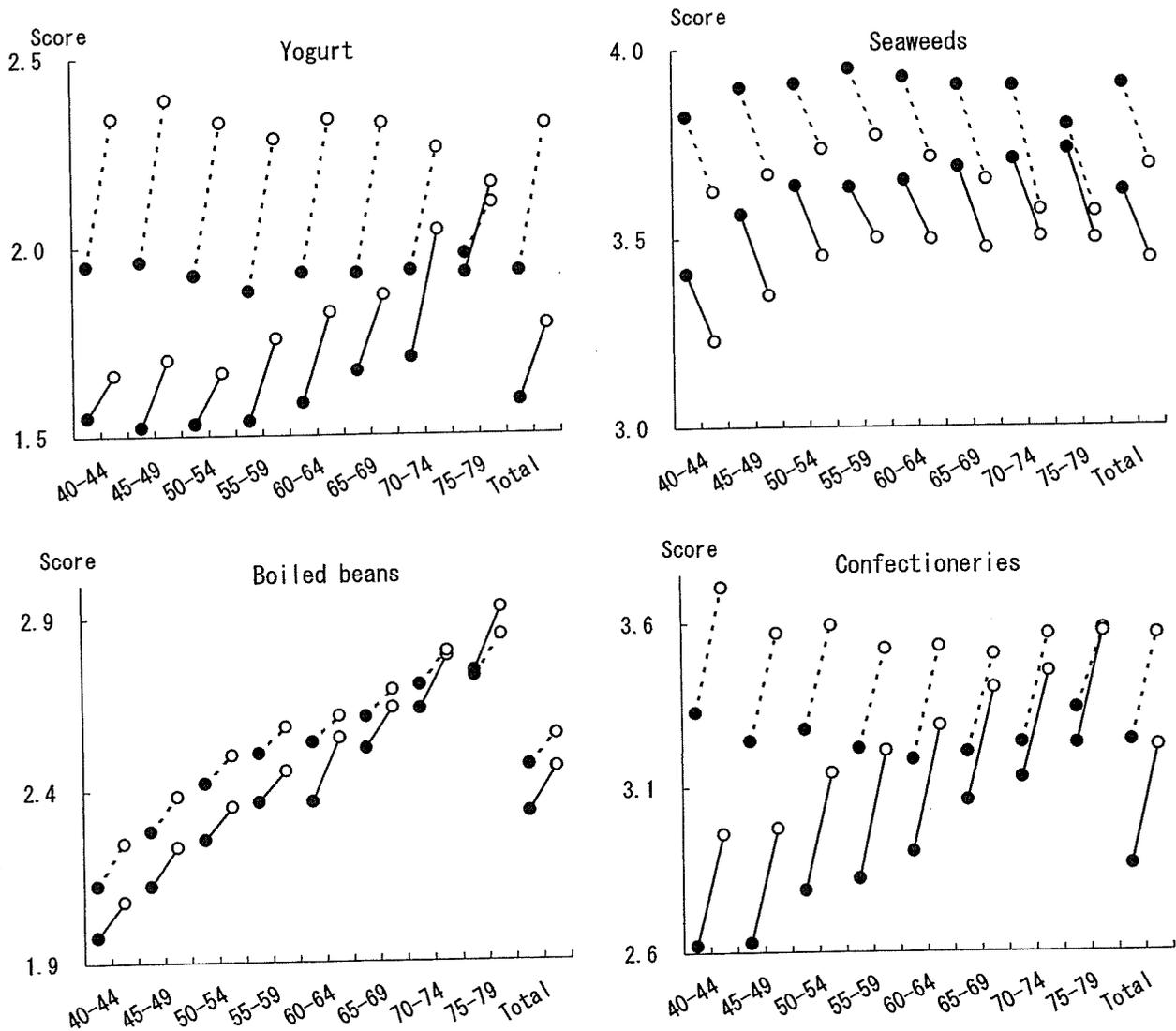


Figure 1. Intake changes over five years of yogurt, seaweeds, boiled beans and confectioneries in age groups. Closed circle and open circle stand for baseline and interim score, respectively. Solid line and dotted line stand for males and females, respectively.

were poorly related. Spearman's correlation coefficients were highest for milk (0.26 for males and 0.24 for females) and yogurt intake (0.20, 0.24), and lowest for pork (0.08, 0.09). Not only was there a poor correlation, but the direction of mean variation of longitudinal change and subjective change was inconsistent for 18 items for males and 17 for females among 33 items.

DISCUSSION

In the present study, long-term reproducibility of food intake frequency after five years was assessed using Spearman's correlation coefficients and agreement from basement and interim questionnaires. If both indexes are high, intake frequency is quite stable

over five years. If only agreement is high, it could be due to a cluster of distribution. In this study, liver and pickled vegetable are clustered in the lowest (almost none) and highest (almost every day) category, respectively.

Spearman's correlation coefficients in the study varied from 0.27 to 0.55, and agreement from 29.9% to 61.4%. Correlation was relatively higher for meat and dairy products and lower for vegetables and fruits in this study. The short-term reproducibility of the questionnaire in this study has been evaluated¹¹. Compared to the correlation coefficients 0.57-0.94, and agreement 55%-80% of questionnaires with an interval of one week, the correlations in this study were lower. In Finland, intraclass correlation coefficient of 32 foods over 4-7 years varied 0.10-0.54 (median: 0.36),

while that over 4-8 months varied 0.25-0.85.⁷ The median value is quite similar to our data. The decrease is considered to be due to a real change during the five years. Therefore, for valid evaluation for exposure, additional information on food intake would be needed over a long time course.

Overall increase or decrease which cannot be evaluated by correlation was assessed using a mean change in the score of intake frequency, and it was tested by the paired t-test. The longitudinal difference includes the effect of aging component, and we assumed the aging component could be substituted by the cross-sectional difference from one specific age sub-cohort to the one higher by one rank (five years). Thus the secular trend score was expressed by (longitudinal difference) - (age effect). For boiled beans, pork, ham and sausage intake, the age effect was larger than the secular trend. Especially, boiled bean intake was almost fully explained by the age effect. The trends were consistent between males and females.

The largest secular increase was observed for edible wild plants, yogurt and confectioneries in both males and females. Among them edible wild plants showed low correlation coefficients. Other than that, correlations were not so low, meaning that intake was increased as a whole while maintaining the relative order. On the other hand, fruit juice, orange and seaweed intake was considerably decreased. Decrease of orange intake frequency is consistent with the results from the national nutrition surveys^{12,13} in 1989 and 1994 (46.8g to 36.9g per day). However the intake of seaweeds is not greatly changed (5.9g to 5.8g). Intake of fruit juice is more inconsistent. It increased by 62% (6.6g to 10.7g) in the national surveys in this period. This discrepancy might be due to the difference in expression of the baseline and interim questionnaires. Only the baseline questionnaire included the comment of 'in summer' for fruit juice. It is consumed more in the hot season, and this comment caused the answer to be biased toward a larger score.¹⁵ Other than this item, seasonal effect did not distort the change in frequency, since they were asked average intakes in a past year, and the distributions of season of both surveys were not different so much (data not shown).

Subjective change of food intake frequency was poorly correlated to the longitudinal change of the same item. Subjectively increased items were tofu, spinach, milk, fresh fish, cabbage and lettuce, and seaweeds, while decreased items were pork, ham and sausage, beef, liver, and butter. These items are recommended to be consumed or avoided for healthy life, and responders' desire for health might have distorted the real intake status. It also could be due to unclear wording of questions about dietary change. The questions did not specify whether the change was in frequency or amount. Confusion between frequency and amount could weaken the relationship. Or responder paid little attention to the time frame of five years and answered changes in terms of a shorter time frame.¹⁴ Whether the reason, the poor correlation generates serious misclassification if the subjective change is used in the regression analysis and might lead to biased results. To use data on change of dietary habit, information should be obtained twice

and the difference evaluated.

In the interim survey, only 42.1% subjects of the baseline survey participated. However, the proportion from the 18 areas where all participants of the baseline survey were targeted to the interim survey was 78.8%, which can be interpreted as the response rate. Furthermore 81.1% subjects of the interim survey were from these areas. Thus, the problem of self-selection bias, which violates external validity, seems not to be serious in this study.

In conclusion, food intake was considerably changed over five years. Interim data should be considered for long-term follow-up study for a more valid evaluation of exposure. Subjective changes have a weak correlation to actual changes in food intake.

MEMBER LIST OF THE JACC STUDY GROUP

The present investigators involved, with the co-authorship of this paper, in the JACC Study and their affiliations are as follows: Dr. Akiko Tamakoshi (present chairman of the study group), Nagoya University Graduate School of Medicine; Dr. Mitsuru Mori, Sapporo Medical University School of Medicine; Dr. Yutaka Motohashi, Akita University School of Medicine; Dr. Ichiro Tsuji, Tohoku University Graduate School of Medicine; Dr. Yosikazu Nakamura, Jichi Medical School; Dr. Hiroyasu Iso, Institute of Community Medicine, University of Tsukuba; Dr. Haruo Mikami, Chiba Cancer Center; Dr. Yutaka Inaba, Juntendo University School of Medicine; Dr. Yoshiharu Hoshiyama, University of Human Arts and Sciences; Dr. Hiroshi Suzuki, Niigata University School of Medicine; Dr. Hiroyuki Shimizu, Gifu University School of Medicine; Dr. Hideaki Toyoshima, Nagoya University Graduate School of Medicine; Dr. Kenji Wakai, Aichi Cancer Center Research Institute; Dr. Shinkan Tokudome, Nagoya City University Graduate School of Medical Sciences; Dr. Yoshinori Ito, Fujita Health University School of Health Sciences; Dr. Shuji Hashimoto, Fujita Health University School of Medicine; Dr. Shogo Kikuchi, Aichi Medical University School of Medicine; Dr. Akio Koizumi, Graduate School of Medicine and Faculty of Medicine, Kyoto University; Dr. Takashi Kawamura, Kyoto University Center for Student Health; Dr. Yoshiyuki Watanabe, Kyoto Prefectural University of Medicine Graduate School of Medical Science; Dr. Tsuneharu Miki, Graduate School of Medical Science, Kyoto Prefectural University of Medicine; Dr. Chigusa Date, Faculty of Human Environmental Sciences, Mukogawa Women's University; Dr. Kiyomi Sakata, Wakayama Medical University; Dr. Takayuki Nose, Tottori University Faculty of Medicine; Dr. Norihiko Hayakawa, Research Institute for Radiation Biology and Medicine, Hiroshima University; Dr. Takesumi Yoshimura, Fukuoka Institute of Health and Environmental Sciences; Dr. Akira Shibata, Kurume University School of Medicine; Dr. Naoyuki Okamoto, Kanagawa Cancer Center; Dr. Hideo Shio, Moriyama Municipal Hospital; Dr. Yoshiyuki Ohno, Asahi Rosai Hospital; Dr. Tomoyuki Kitagawa, Cancer Institute of the

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Serum Carotenoids, Retinol, and Tocopherols, and Colorectal Cancer Risk in a Japanese Cohort: Effect Modification by Sex for Carotenoids

Kenji Wakai, Koji Suzuki, Yoshinori Ito, Masayo Kojima, Koji Tamakoshi,
 Yoshiyuki Watanabe, Hideaki Toyoshima, Norihiko Hayakawa, Shuji Hashimoto,
 Shinkan Tokudome, Sadao Suzuki, Miyuki Kawado, Kotaro Ozasa,
 and Akiko Tamakoshi for the Japan Collaborative Cohort Study Group

Abstract: To examine associations of serum carotenoids, retinol, and tocopherols with colorectal cancer risk, we conducted a case-control study nested within the Japan Collaborative Cohort Study. These micronutrients were measured in prediagnostic serum samples from 116 men and women who developed colorectal cancer during an 8-yr follow-up period and from 298 matched controls. In men, the higher level of serum total carotenoids was associated with a decreased risk: The multivariate-adjusted odds ratio (OR) for the highest vs. the lowest tertile was 0.34 (95% confidence interval [CI] = 0.11–1.00; trend *P* over tertiles = 0.040). In women, the higher levels of α - and β -carotenes and total carotenoids were instead related to an increased risk: The corresponding ORs were 4.72 (95% CI = 1.29–17.3), 2.00 (0.70–5.73), and 2.47 (0.73–8.34), respectively (trend *P* = 0.007, 0.040, and 0.064, respectively). We also found a somewhat decreasing risk with increased serum retinol in all subjects and α -tocopherol in men: The ORs (95% CI) for the highest tertiles were 0.29 (0.11–0.78; trend *P* over tertiles = 0.010) and 0.29 (0.07–1.17; trend *P* = 0.098), respectively. The effects of some carotenoids on colorectal cancer risk may be modified by sex or by factors associated with sex, including smoking and drinking habits.

Introduction

Consumption of vegetables and fruit has often been related to a decreased risk of colorectal cancer (1,2), suggesting the potential etiological importance of carotenoids and other phytochemicals contained in these foods.

Several studies have assessed dietary intake of carotenoids in relation to colorectal cancer risk but have reported inconsistent findings: Some suggested the protective effects of carotenoids (3–7) whereas others did not (8–11). In addition, only a few investigations (11–13) have examined possible associations of specific carotenoids other than β -carotene with the risk.

Studies using blood samples can assess the role of several carotenoids simultaneously (14) and allow for the bioavailability of compounds in individual subjects. Such studies may also provide possible explanations for the results of several recent cohort studies that have not demonstrated the presumed protective effects of vegetables and fruit (15,16). Nevertheless, there have been little data on the association of blood carotenoids with colorectal cancer risk.

We therefore examined the associations between serum carotenoids and the risk of colorectal cancer in a prospective study in Japan. Additionally, retinol and tocopherols, possi-

K. Wakai is affiliated with the Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya 464–8681, Japan and the Department of Preventive Medicine/Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, Nagoya 466–8550, Japan. K. Suzuki and Y. Ito are affiliated with the Department of Public Health, Fujita Health University School of Health Sciences, Toyoake 470–1192, Japan. M. Kojima, S. Tokudome, and S. Suzuki are affiliated with the Department of Health Promotion and Preventive Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya 467–8601, Japan. K. Tamakoshi and H. Toyoshima are affiliated with the Department of Public Health/Health Information Dynamics, Nagoya University Graduate School of Medicine, Nagoya 466–8550, Japan. Y. Watanabe and K. Ozasa are affiliated with the Department of Epidemiology for Community Health and Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto 602–8566, Japan. N. Hayakawa is affiliated with the Department of Epidemiology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, 734–8553, Japan. S. Hashimoto and M. Kawado are affiliated with the Department of Hygiene, Fujita Health University School of Medicine, Toyoake 470–1192, Japan. A. Tamakoshi is affiliated with the Department of Preventive Medicine/Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, Nagoya 466–8550, Japan.

bly important anticarcinogenic substances from foods other than carotenoids (14), were considered in relation to malignancy.

Materials and Methods

Study Population and Serum Samples

We carried out a nested case-control study as a part of the Japan Collaborative Cohort Study for Evaluation of Cancer Risk Sponsored by Monbusho (the JACC Study; the Monbusho is the Japanese name for the Ministry of Education, Culture, Sports, Science and Technology of Japan). The details of this study are described elsewhere (17,18). The study involved 110,792 residents, aged 40–79 yr at baseline, from 45 areas all over Japan. Potential subjects for the present study were restricted to 65,184 individuals who lived in 24 study areas, where cancer registries are available. An epidemiological survey on lifestyle factors was conducted using a self-administered questionnaire from 1988 to 1990. The questionnaire addressed demographic factors, personal and family medical histories, anthropometric factors, smoking and drinking habits, physical activity, dietary habits, use of vitamin supplements, and other lifestyles. We did not collect information on carotenoid supplements since they were uncommon in Japan at the time of the baseline survey.

In addition to completing the questionnaire, those survey participants who underwent health-screening checks sponsored by municipalities were asked to donate blood samples during the same period as the questionnaire survey. Eventually, 23,863 subjects (7,793 men and 16,070 women; 36.6% of the 65,184 respondents to the questionnaire survey in the 24 study areas) provided blood samples.

Those who donated blood samples were more likely to be women (67.3%) than those not providing samples (54.8%). The former were less likely to be highly educated (those attending school until the age of ≥ 19 ; 15.8% for men and 9.3% for women) than the latter (20.7% for men and 11.6% for women). In women, the mean age was lower in subjects with blood samples (56.8 [SD, 9.4] yr) than those without them (59.4 [10.5] yr), while it was similar between the two groups of men (58.4 [9.7] and 57.9 [10.6] yr, respectively). Among subjects providing blood samples, those with a previous history of cancer ($n = 409$) were omitted, leaving 23,454 (7,673 men and 15,781 women) for follow-up.

Sera were separated from the samples at laboratories in or near the surveyed municipalities as soon as possible after the blood draw. Serum of each participant was divided into three to five tubes (100 to 500 μ l per tube), and the tubes were stored in deep freezers at -80°C until analyzed in 2002.

Informed consent for participation was obtained individually from subjects, with the exception of those in some study areas in which informed consent was provided at the group level after the aim of the study and confidentiality of the data had been explained to community leaders. The Ethics Committee of Medical Care and Research of Fujita Health Uni-

versity approved the protocol of this investigation including the procedures to obtain informed consent.

Case Ascertainment and Control Selection

We used population registries in the municipalities to determine the vital and residential status of the subjects. Registration of death is required by the Family Registration Law in Japan and is followed across the country. For logistical reasons, we discontinued the follow-up of subjects who had moved out of the study areas.

The cases were defined as those of incident colorectal cancer (*International Statistical Classification of Diseases and Related Health Problems, Tenth Revision*, C18, C19, and C20). We ascertained the incidence of cancer by means of linkage with the records of population-based cancer registries and/or checking hospital-based registries or inpatient records of hospitals treating cancer patients (17), supplemented by systematic review of death certificates. The follow-up was conducted from the time of baseline survey through the end of 1997, except for three areas (to the end of 1994, 1995, and 1996, respectively). During the study period, only 2.1% ($n = 501$) of the 23,454 subjects were lost to follow-up due to moving. The mortality to incidence ratio for colorectal cancer was 0.28 in the cohort covered by cancer registries. This figure is comparable with those in representative population-based cancer registries in Japan (0.23 to 0.51; 19) and indicates the reasonably high quality of the case identification procedure.

During the mean follow-up of 7.9 (SD, 1.5) yr, 171 incident cases of colorectal cancer were documented among the subjects who had provided serum samples at baseline. Of the cases, we excluded 46 without sufficient samples for measurement. For each case, 2 or 3 controls were selected from the population at risk without incident cancer or previous history of cancer, matching for sex, age (as near as possible), and participating institution. We had to further exclude 9 cases because appropriate controls were not available. Eventually, 116 cases of colorectal cancer (including 84 cases of colon cancer) and 298 controls were involved in the analysis.

The baseline characteristics of cases were not materially altered by this exclusion. Those of all the 171 incident cases and of the 116 cases included in the analysis were as follows, respectively: female sex, 50.3% and 53.4%; mean age \pm SD, 61.4 ± 8.3 and 61.2 ± 8.8 yr in men and 62.7 ± 7.8 and 62.0 ± 7.6 yr in women; current smokers, 45.9% and 46.3% in men and 3.5% and 3.2% in women; current drinkers, 80.0% and 75.9% in men and 23.3% and 21.0% in women; multivitamin supplement users, 12.3% and 15.0% in men and 7.3% and 10.2% in women; and users of vitamin E supplement, 3.2% and 5.1% in men and 14.5% and 16.3% in women. In both sexes, the proportions of daily consumers of vegetables or fruit were comparable among all the cases and the cases involved in the analysis except for green leafy vegetables in women (38.7% of all cases and 52.8% of selected cases were daily consumers of green leafy vegetables).