[CANCER RESEARCH 43, 1910–1913, April 1983]
0008-5472/83/0043$02.00

Fecal Neutral Steroids in Two Japanese Populations with Different Colon Cancer Risks[1]

Abraham M. Y. Nomura,[2] Tracy D. Wilkins, Sigetosi Kamiyama, Lance K. Heilbrun, Akio Shimada, Grant N. Stemmermann, and Howard F. Mower

Japan-Hawaii Cancer Study, Kuakini Medical Center, Honolulu, Hawaii 96817 [A. M. Y. N., L. K. H., G. N. S.]; Department of Anaerobe Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061 [T. D. W.]; Department of Hygiene, Akita University School of Medicine, Akita, Japan [S. K., A. S.]; and Department of Biochemistry and Biophysics, University of Hawaii School of Medicine, Honolulu, Hawaii 96822 [H. F. M.]

ABSTRACT

Several workers have associated fecal neutral steroids with colon cancer frequency. They suggested that the risk for colon cancer increases with a rise in the level of total and certain neutral steroids. The Japanese in Hawaii, who are at high risk for this cancer, had a higher concentration of cholesterol and total animal steroids in their fecal specimens than did the people in Akita, Japan, who are at low risk. However, the rest of the findings on neutral steroids were unremarkable or inconsistent in comparison with those of other studies. These data are suggestive but not strongly supportive of a relationship between fecal neutral steroid patterns and colon cancer risk.

INTRODUCTION

Several studies have suggested that there is an association between fecal neutral steroids and colon cancer risk. Fecal concentrations of cholesterol, coprostanol, coprostanone, and total neutral steroids were higher among colon cancer patients than were those among controls (8, 15, 17). Patients who are at high risk for colon cancer because of ulcerative colitis or adenomatous polyps also had higher levels of fecal cholesterol, coprostanol, and coprostanone than did controls (14, 17). When subjects from ethnic or diet groups in different countries were tested, it was found that high-colon cancer-risk groups had higher levels of coprostanone, coprostanol, and total neutral steroids than did low-risk groups (7, 16, 19).

Other studies, however, have found conflicting results. Moskovitz *et al.* (12) noted no differences in the concentrations of fecal neutral steroids between colon cancer patients and controls. Patients with familial polyposis of the colon who are at very high risk for colon cancer did not have higher concentrations of fecal neutral steroids than did controls (2, 15, 22). Furthermore, there was no difference in total neutral steroids between the Danes in Copenhagen and the Finns in a rural province in Finland, 2 groups which differ in their colon cancer risks (9).

In the present study, we compared the fecal neutral steroid patterns in 2 ethnically similar but geographically separate groups with a marked difference in their colon cancer risks. Our study included 279 subjects, substantially more than the number of participants in any of the reports cited previously.

[1] Supported in part by Contracts N01 CP 61060 and N01 CB 55685 from the National Cancer Institute, USPHS.

[2] To whom requests for reprints should be addressed, at Japan-Hawaii Cancer Study, Kuakini Medical Center, 347 North Kuakini Street, Honolulu, Hawaii 96817.

Received November 23, 1981; accepted December 29, 1982.

MATERIALS AND METHODS

Participants of the Japan-Hawaii Cancer Study in Honolulu, Hawaii, and Akita Prefecture, Japan, formed the population base of this investigation (13). Japanese men and women, ages 30 to 82, were randomly selected to submit a stool specimen for neutral steroid analysis. If a person had a previous resection for a gastrointestinal lesion or was currently receiving antibiotic medication, he or she was disqualified from the study. Over 95% of the qualified selectees agreed to participate. In all, there were 163 participants from Hawaii and 116 participants from Akita.

The method of stool collection has been described elsewhere (4). The donor took home a dry ice container, a polyethylene bag, and a metal toilet bowl frame. After the fecal specimen was expelled into the bag, it was frozen at −20°. Later, it was lyophilized and shipped to Virginia Polytechnic Institute for analysis (23).

From each lyophilized sample, 0.3-g aliquots of finely ground powder were put into 25- x 100-mm screw-capped tubes, and 20 ml of saponification mixture, composed of ethanol and water (95:5) and 4% sodium hydroxide, were added to each tube. The tubes were mixed vigorously on a vortex mixer for 30 sec and then incubated for 90 min in an 80–90° water bath. After cooling, 10 ml of distilled water were added to the saponified fecal samples. This was followed by the addition of 10 ml of reagent grade petroleum ether (Fisher Scientific Co., Raleigh, N. C.) containing 0.4 mg cholestane per ml (Sigma Chemical Co., St. Louis, Mo.) as an internal standard for neutral steroid quantitation. The tubes were inverted 40 times, and approximately 1.5 ml of the petroleum ether layer were transferred to gas-liquid chromatography vials.

A Hewlett-Packard Model 5830A gas-liquid chromatograph (Hewlett-Packard Co., Richmond, Va.) with a flame ionization detector was used for quantitation of neutral steroids without further derivatization of the steroids. Identification of peaks was based on the work of Eneroth *et al.* (3), and major peaks were confirmed by mass spectroscopy. A 2-m glass column (inside diameter, 2 mm) packed with 3% QF-1 on Chromosorb Q, 100 to 120 mesh (Supelco, Inc., Bellefonte, Pa.) was used for the analysis. Operating conditions were: column temperature, 225°; injection temperature, 275°; and detector temperature, 275°. The carrier gas was N_2, used at a flow rate of 30 ml/min. Prior to sample analysis, the chromatograph was calibrated by running the following neutral steroid standards at concentrations of 0.15 to 1 mg/ml: coprostanol; coprostanone; campesterol; sitosterol; and cholesterol.

The efficiency of neutral steroid extraction was determined by adding 0.1 μCi of [^{14}C]cholesterol (New England Nuclear, Boston, Mass.) to each sample prior to saponification. After petroleum ether extraction of the saponified samples, 1 ml of the ether was transferred to scintillation vials. Ten ml of a scintillation mixture were added to each vial and were then counted on a Beckman Model LS-250 liquid scintillation counter (Beckman Instruments, Inc., Columbia, Md.). The percentage of cholesterol recovered was determined by multiplying a quench correction factor by the ratio of cpm in the sample per cpm in the standard. These values were used to adjust the concentrations of neutral steroids detected by the chromatograph.

A diet recall questionnaire was used to estimate each subject's weekly consumption of different food items found in a Western or Japanese diet.

The details of the dietary method were published previously (5). Briefly, it was designed to quantitate the recent intake of specified food items with the assistance of photographs of weighed small, medium, and large portions of each item.

The blood pressure was recorded on the left arm when the person was sitting. Diastolic blood pressure was based on the disappearance of the sound. The hematocrit was based on the microtechnique.

When comparing study subjects by clinical and dietary variables, adjustment for age and sex was made via the direct method (18), with the entire 279 subjects as the reference population. Age- and sex-adjusted neutral steroid measurability rates were computed by multiple logistic regression methods (11). Analysis of covariance models (20) were constructed for each of the different fecal neutral steroids in Table 4. Because of significant departures from normality in the distributions of measurable steroid values, log transformations (Naperian) were applied before the covariance analyses were carried out. To facilitate interpretation of our own results and for easier comparison with other studies, the log-transformed adjusted mean values were exponentiated to return them to the original scale of measurement. They are therefore presented as geometric means (1) in Table 4.

The statistical basis for excluding subjects with no measurable value from Table 4 analyses was to avoid severely violating the normality assumption of the analysis of covariance models. As shown in Table 3, the measurability rates vary a great deal across the different neutral steroid variables. They were essentially piecewise distributions with a "spike" at zero (nonmeasurable level) which sometimes comprised well over 50% of the subjects for a particular neutral steroid. The remainder of each distribution was continuous but was almost always heavily skewed. The best approach seemed to be a piecewise analysis of the inescapably piecewise, discontinuous distributions of neutral steroid values. This meant using logistic regression models for the nonmeasurable values and analysis of covariance models for the (log-transformed) measurable values. Finally, standard partial correlations were used to relate consumption levels of food items with the (log-transformed) measurable neutral steroids while controlling for geographic location (Japan or Hawaii).

RESULTS

The comparison of Japanese men and women in Hawaii and Akita by weight, height, blood pressure, and other parameters is presented in Table 1. The men in Hawaii were taller, were heavier, and had higher hematocrit values than did their counterparts in Akita, who had higher levels of systolic blood pressure. The Japanese women in Hawaii also were taller, were heavier, and had higher hematocrit values than did the women in Akita.

The food items in the questionnaire were separated into Western or Japanese foods on an *a priori* basis. The age- and sex-adjusted mean quantitative weekly intake levels of the different items were then computed and are shown in Table 2. Of the Western foods, the Japanese in Hawaii consumed much more beef, bacon, lettuce, and coffee but less tomatoes than did their counterparts from Akita. With respect to the Japanese foods, the people of Akita ate much more dried fish, tofu (soybean curd), rice, hakusaizuke (pickled Chinese cabbage), takuwan (pickled turnip), ume (pickled plum), tsukudani (seaweed paste), and genmai cha (tea) than did Japanese residents in Hawaii. The same pattern of dietary differences was present for both men and women.

We then compared the neutral steroid patterns between the 2 groups in Table 3, which gives the age- and sex-adjusted percentages of subjects having measurable amounts (≥0.1 mg/g of dry-weight feces) of the individual neutral steroids. Significantly more Hawaiian-Japanese subjects had measurable amounts of cholesterol and the plant steroids, sitosterol and campesterol. In contrast, significantly more subjects from Akita had detectable amounts of the degraded animal steroids, coprostanol and coprostanone, as well as other plant steroids. In the logistic models, age was significantly related ($p < 0.05$) to the measurability rates of 4 of the 6 neutral steroids, coprostanol, coprostanone, campesterol, and other plant steroids. Older subjects had significantly higher measurability rates of all 4 of these steroids except campesterol, for which they had a significantly lower measurability rate. The covariate sex was significantly related to choles-

Table 2
Age- and sex-adjusted mean weekly consumption of Western and Japanese food items by study subjects in Hawaii and Akita

Food item	Mean weekly consumption (g/wk)		
	Hawaii	Akita	p[a]
Western			
Beef	242.5	12.8	<0.001
Wieners	32.4	48.0	0.472
Bacon	9.3	1.2	<0.001
Sliced tomatoes	110.8	175.1	0.044
Lettuce	267.3	103.0	<0.001
Celery	23.1	12.2	0.224
Coffee	2623.6	25.5	<0.001
Milk	571.8	462.1	0.323
Japanese			
Sashimi (raw fish)	48.1	68.2	0.536
Dried fish	12.0	819.4	<0.001
Dried cuttlefish	2.5	2.6	0.984
Kamaboko (fish cake)	24.3	31.3	0.660
Tofu (soybean curd)	90.9	233.7	<0.001
Rice	1461.9	2134.9	<0.001
Hakusaizuke (pickled Chinese cabbage)	45.2	170.3	<0.001
Takuwan (pickled turnip)	11.8	82.9	<0.001
Ume (pickled plum)	4.1	16.5	<0.001
Tsukudani (seaweed paste)	0.5	2.2	0.002
Green tea	593.3	946.8	0.082
Genmai cha (tea)	468.7	1031.7	0.002

[a] Two-tailed *t* test.

Table 1
Age-adjusted comparison between study subjects in Hawaii and Akita

	No.		Av. age		Colon cancer incidence[a]		Height (cm)		Wt (kg)		Systolic blood pressure (mm Hg)		Diastolic blood pressure (mm Hg)		Hematocrit (%)	
	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women
Hawaii	100	63	56.4	52.0	22.4	18.8	165.3	153.0	66.9	54.4	130.6	127.1	84.8	81.3	44.6	40.0
Akita	64	52	56.9	52.9	5.6	5.4	161.0	147.6	56.4	50.3	137.2	129.7	83.5	79.1	43.2	38.4
p[b]							<0.001	0.002	<0.001	0.030	0.043	0.455	0.536	0.344	0.005	0.014

[a] Annual age-adjusted incidence per 100,000 (21). Rates in Miyagi Prefecture, which adjoins Akita, were used to represent the Akita rates.
[b] Two-tailed *t* test.

Table 3

Age- and sex-adjusted percentages (adjusted by multiple logistic regression methods) of study subjects from Hawaii and Akita with measurable amounts of selected fecal neutral steroids

Neutral steroid	% of subjects with measurable amounts: Hawaii[a]	Akita[b]	p[c]
Animal origin			
Cholesterol	90.8	75.0	0.001
Coprostanol	75.4	90.4	0.002
Coprostanone	45.4	57.7	0.029
Plant origin			
Sitosterol	88.3	71.5	0.001
Campesterol	35.0	4.3	<0.001
Other plant steroids	73.0	94.0	<0.001

[a] $n = 163$.
[b] $n = 116$.
[c] For testing whether the adjusted percentages are significantly different (2-sided test) in the multiple logistic model.

terol, coprostanone, and sitosterol. Females had significantly higher measurability rates of all 3 of these neutral steroids.

For only those persons having measurable amounts, we then compared age- and sex-adjusted geometric mean values of the various neutral steroids (and the degraded fraction of total animal steroids) of subjects in Hawaii and Japan, as shown in Table 4. The Hawaiian men and women had significantly higher mean levels of cholesterol, total animal steroids, sitosterol, campesterol, and other plant steroids. The only mean level that was higher (and significantly so) in the Akita subjects was the degraded fraction of total animal steroids. In the 8 analysis of covariance models of measurable steroid levels, age was a statistically significant ($p < 0.05$) covariate for cholesterol only. Older subjects had significantly lower mean levels of fecal cholesterol. Sex was significantly ($p < 0.10$) related only to the degraded fraction of total animal steroids, and female subjects had a significantly higher degraded fraction than did males.

The data in Table 4 were also analyzed using mg/g, wet weight, of feces because of the difference in the mean percentages of water in the fecal samples: 82.2% for Hawaiian specimens and 79.4% for Japanese specimens ($p = 0.009$). When this was done, it did not change the findings for animal neutral steroids in Table 4. For fecal neutral steroids of plant origin, the differences in the concentrations of campesterol and other plant steroids were no longer significant with the use of wet instead of dry weight.

The 8 different neutral steroid parameters listed in Table 4 were correlated with the 20 food items. After controlling for the geographic location of the subjects, none of 160 partial correlations had a (2-sided) p value less than 0.02. The highest partial correlations were −0.29 ($p = 0.02$) between beef and fecal campesterol and −0.15 ($p = 0.02$) between celery and fecal cholesterol.

DISCUSSION

It has been postulated that a Western diet, rich in fatty foods, affects the intestinal bacterial flora and increases the concentrations of neutral steroids in the colon where degradation takes place and, possibly, intermediate carcinogens can be formed (7, 16). To investigate this possibility, we studied the diet and fecal neutral steroid patterns of people of Japanese ancestry in Hawaii and in Akita Prefecture, Japan. Because of the wide variation in daily diets and in fecal neutral steroid concentrations, large numbers of subjects were included in the study. Marked dietary differences were noted between the 2 population groups. The Hawaiian-Japanese consumed much more American foods but much less Japanese foods than did their peers in Akita, Japan.

Differences of a lesser magnitude were also present between Japanese men and women in Hawaii and Akita with respect to weight, height, and hematocrit values. Similar findings among men have also been observed by others using larger population samples (10).

When comparisons were made in fecal neutral steroids of animal origin between the 2 Japanese groups, we noted that the Hawaiian-Japanese had significantly higher concentrations of cholesterol and total animal steroids. This observation among the Japanese in Hawaii who are at high risk for colon cancer corresponded to the findings of other studies (7, 16, 19), but there were no meaningful differences between the 2 Japanese groups in the concentrations of degraded coprostanol and coprostanone. Furthermore, the degraded fraction [(coprostanol plus coprostanone)/total animal steroids] was greater in Akita than in Hawaii. This finding did not agree with the observations of several past studies (7, 16, 19).

The reason for the dissimilar findings is not clear. Salyers *et al.* (19) collected a single specimen from each individual, as was done in the present study, while Reddy and Wynder (16) used 2-day fecal samples. We observed, as did others (6, 19), that fecal specimens are well mixed and homogeneous with respect to neutral steroid levels and cholesterol patterns. Subjects in our study were chosen in a random fashion from over 10,000 participants in a population-based study, while the subjects of other studies (7, 16, 19) usually came from selected small groups such as hospital staff members, university students, or members of a village. We were able to control for ethnicity in our study by limiting it to persons of Japanese ancestry in 2 separate areas. Other studies (7, 16, 19) looking at high- and low-risk groups for colon cancer usually included subjects of different ethnic backgrounds. Whether or not these differences contributed to the disparity in findings between the studies cannot be determined at this time.

Neutral steroids of plant origin accounted for approximately 30% of the total neutral steroids in the Japanese in both Hawaii

Table 4

Age- and sex-adjusted geometric mean values (adjusted by analysis of covariance) of neutral steroids among study subjects from Hawaii and Akita with measurable amounts

Neutral steroid	Geometric mean values (mg/g dry feces): Hawaii	Akita	p[a]
Animal origin			
Cholesterol	7.92 (1.1, 41.7)[b]	4.14 (1.0, 22.1)	<0.001
Coprostanol	7.85 (0.1, 50.0)	7.41 (0.1, 32.7)	0.720
Coprostanone	1.05 (0.1, 14.1)	1.05 (0.1, 5.0)	0.995
Total animal steroids	18.35 (5.8, 52.9)	11.88 (0.1, 32.7)	<0.001
Degraded fraction[c]	0.39 (0.01, 1.0)	0.64 (0.03, 1.0)	0.001
Plant origin			
Sitosterol	2.36 (0.2, 10.3)	1.65 (0.3, 4.8)	<0.001
Campesterol	1.14 (0.2, 3.6)	0.62 (0.4, 0.8)	0.027
Other plant steroids	3.99 (0.1, 32.9)	2.85 (0.2, 14.0)	0.008

[a] For testing whether the adjusted means are significantly different (2-sided test) in the analysis of covariance.
[b] Numbers in parentheses, minimum and maximum values.
[c] Degraded fraction of total animal steroids (TAS) = $\frac{\text{Coprostanol + coprostanone}}{\text{TAS}}$.

and Akita. Two past studies (14, 17) in subjects with colon cancer, adenomatous polyps, or ulcerative colitis showed no differences in sitosterol or campesterol concentrations in patients and controls. Our data, however, indicate that the Hawaiian-Japanese had higher concentrations of these plant steroids than did their counterparts in Akita. The sources of campesterol, sitosterol, and other plant steroids in the feces are not known. Because the Japanese in Hawaii are frequent consumers of certain plants and vegetables (namely, lettuce and celery), the observed differences may be real. Another possibility is that the differences may reflect a greater use of certain plant oils in cooking in Hawaii than in Akita.

The lack of strong correlations between the fecal concentrations of different neutral steroids and the intake of only 20 food items was not surprising. Many additional food items that the subjects consumed in a recent week were not included in the questionnaire. Furthermore, the countless interactions of different foods in varying amounts in the digestive tract would make it extremely difficult to find strong correlations of fecal neutral steroids with any particular food item.

Overall, our findings support the presence of differences in the fecal neutral steroid patterns between the high-risk Japanese in Hawaii and the low-risk Japanese in Akita. However, there were consistencies and inconsistencies compared with findings from other similar studies. Whether or not these differences are related to large-bowel cancer risk remains to be resolved, since it is apparent that the 2 Japanese groups also differ by many other factors besides fecal neutral steroid concentrations.

REFERENCES

1. Armitage, R. Statistical Methods in Medical Research, pp. 33–34. Oxford, England: Blackwell Scientific Publications, 1971.
2. Bone, E., Drasar, B. S., and Hill, M. J. Gut bacteria and their metabolic activities in familial polyposis. Lancet, *1:* 1117–1120, 1975.
3. Eneroth, P., Hellstrom, K., and Ryhage, R. Identification and quantification of neutral fecal steroids by gas-liquid chromatography and mass spectrometry: studies of human excretion during two dietary regimens. J. Lipid Res., *5:* 245–262, 1964.
4. Glober, G. A., Moore, J. O., Klein, K. L., and Abba, B. C. Bowel transit times in two populations experiencing similar colon-cancer risks. Lancet, *2:* 80–81, 1974.
5. Hankin, J. H., Rhoads, G. G., and Glober, G. A. A dietary method for an epidemiologic study of gastrointestinal cancer. Am. J. Clin. Nutr., *28:* 1055–1060, 1975.
6. Hill, M. J., and Aries, V. C. Faecal steroid composition and its relationship to cancer of the large bowel. J. Pathol., *104:* 129–139, 1971.
7. Hill, M. J., Crowther, J. S., Drasar, B. S., Hawksworth, G., Aries, V., and Williams, R. E. O. Bacteria and aetiology of cancer of large bowel. Lancet, *1:* 95–100, 1971.
8. Hill, M. J., Drasar, B. S., Williams, R. E. O., Meade, T. W., Cox, A. G., Simpson, J. E. P., and Morson, B. C. Faecal bile acids and clostridia in patients with cancer of the large bowel. Lancet, *1:* 535–539, 1975.
9. International Agency for Research on Cancer Intestinal Microecology Group. Dietary fibre, transit-time, faecal bacteria steroids, and colon cancer in two Scandinavian populations. Lancet, *2:* 207–211, 1977.
10. Kagan, A., Harris, B. R., Winkelstein, W. J., Johnson, K. G., Kato, H., Syme, S. L., Rhoads, G. G., Gay, M. L., Nichaman, M. Z., Hamilton, H. B., and Tillotson, J. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: demographic, physical, dietary and biochemical characteristics. J. Chronic Dis., *27:* 345–364, 1974.
11. Lee, J. Covariance adjustment of rates based on the multiple logistic regression model. J. Chronic Dis., *34:* 415–426, 1981.
12. Moskovitz, M., White, C., Barnett, R. N., Stevens, S., Russell, E., Vargo, D., and Floch, M. H. Diet, fecal bile acids, and neutral sterols in carcinoma of the colon. Dig. Dis. Sci., *24:* 746–751, 1979.
13. Nomura, A., Stemmermann, G. N., Rhoads, G. G., and Glober, G. A. The Japan-Hawaii Cancer Study: a progress report. Hawaii Med. J., *34:* 309–316, 1975.
14. Reddy, B. S., Martin, C. W., and Wynder, E. L. Fecal bile acids and cholesterol metabolites of patients with ulcerative colitis, a high-risk group for development of colon cancer. Cancer Res., *37:* 1697–1701, 1977.
15. Reddy, B. S., Mastromarino, A., and Wynder, E. Diet and metabolism: large-bowel cancer. Cancer (Phila.), *39:* 1815–1819, 1977.
16. Reddy, B. S., and Wynder, E. L. Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer. J. Natl. Cancer Inst., *50:* 1437–1442, 1973.
17. Reddy, B. S., and Wynder, E. L. Metabolic epidemiology of colon cancer. Cancer (Phila.), *39:* 2533–2539, 1977.
18. Remington, R., and Schork, M. A. Statistics with Applications to the Biological and Health Sciences, pp. 330–333. Englewood Cliffs, N. J.: Prentice-Hall, Inc., 1970.
19. Salyers, A. A., Sperry, J. F., Wilkins, T. D., Walker, A. R. P., and Richardson, N. J. Neutral steroid concentrations in the faeces of North American white and South African black populations at different risks for cancer of the colon. S. Afr. Med. J., *51:* 823–827, 1977.
20. Snedecor, G. W., and Cochran, W. G. Statistical methods, Ed. 6, pp. 419–446. Iowa State University Press, 1967.
21. Waterhouse, J., Muir, C., Correa, P., and Powell, J. Cancer incidence in five continents. III. I. A. R. C. Sci. Publ., *15:* 496, 498, 538, 539, 1976.
22. Watne, A. L., Lai, H. L., Mance, T., and Core, S. Fecal steroids and bacterial flora in patients with polyposis coli. Am. J. Surg., *131:* 42–46, 1976.
23. Wilkins, T. D., and Hackman, A. S. Two patterns of neutral steroid conversion in the feces of normal North Americans. Cancer Res., *34:* 2250–2254, 1974.