# Role of Fat and Calcium in Cancer Causation by Food Mutagens, Heterocyclic Amines (43717)

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*Abstract.* **We investigated the modulation by dietary corn oil and calcium levels of carcinogenesis by heterocyclic amines (HCA), a new class of important carcinogens in the human nutritional environment, since they are formed during cooking. Two approaches involved (i) a chronic bioassay in male and female F344 rats, and (ii) an abbreviated test, the induction of foci of aberrant crypts in the colon in male F344 rats. One typical HCA, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was fed at 75 ppm for 12 months to male and female rats that were held three and six months longer, respectively, on control diets. Neoplasms were induced in the Zymbal gland, skin (predominantly in male rats), liver, mammary and preputial glands, colon, and lung. Diets with 23.5% corn oil increased carcinomas in the liver in males, and in the mammary gland in females, compared with a 5% corn oil diet. Males on the low-fat diet had more cancers in the lip, and females had more ear duct cancers, than did rats on the high-fat diet. Another HCA, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), fed at 400 ppm for nine weeks induced foci of aberrant crypts in the lower intestinal tract of male F344 rats. There were significantly more aberrant crypts on the high-fat than on the low-fat diet. On the low-fat diet, there were fewer aberrant crypts on the higher calcium level. Thus, dietary fat modulates the carcinogenic action of HCA food carcinogens in specific organs of male and female F344 rats. Also, both fat and calcium affected the induction of aberrant crypts in the distal intestinal tract of male F344 rats.**

[P.S.E.B.M. 1994, Vol 205]

In models of breast and colon cancer, induced by administration of standard synthetic chemical carcinogens like 7,12-dimethylbenz[a]anthracene (DMBA), N-nitrosomethylurea (NMU), azoxymethane (AOM), and 2,3-dimethyl-4-aminobiphenyl (DMAB), it has been established that a diet with 40% fat calories, corresponding to the traditional fat intake by Western people, potentiated the effect of these carcinogens, compared with providing 10% of fat calories, mimicking the diet of traditional Japanese (1–3). Until recently, the nature of the genotoxic carcinogens for human breast or colon cancer was unknown. We suggested in 1977, after Sugimura and colleagues discovered potent mutagens at the surface of fried meat (4), that these mutagens might be the relevant genotoxic carcinogens (5). They were identified as a new class of chemicals, heterocyclic amines (HCA). Since the discovery of their high mutagenic activity in the test in *Salmonella typhimurium* of Ames, particularly in the frame-shift sensitive mutants TA1538 or TA98, they have displayed uniform positive responses in tests providing evidence of DNA-reactivity and genotoxicity (6–8). As expected, they are potent carcinogens in animal models, including in nonhuman primates, as recently summarized (9). In particular, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) induced cancer in the colon, small intestine, Zymbal gland, liver, skin, oral cavity, and clitoral gland (10). In the sensitive female Sprague-Dawley rats, IQ-induced mammary gland cancer with a potency of the same order of magnitude as the positive control 4-aminobiphenyl, a human carcinogen (11, 12). The chemical PhIP, most

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Received October 12, 1993. [P.S.E.B.M. 1994, Vol 205]
Accepted December 21, 1993.

0037-9727/94/2054–0347$10.50/0

prevalent in fried or grilled meat (13), induced cancer of the colon and the mammary gland in male and female F344 rats (14).

The current paper will describe a role of fat dietary level in carcinogenesis by one HCA, IQ, fed at a low level to simulate, to some extent, human exposure conditions. Also, in view of the suggestion (15, 16) that calcium reduces carcinogenesis in the intestinal tract, this area was explored by determining the role of fat and of calcium in rats given another food HCA, PhIP, with the end point being the induction of foci of aberrant crypts, an indicator of early colon carcinogenesis (17, 18).

## Materials and Methods

**Chemicals.** IQ and PhIP were procured from Toronto Research Chemicals, Toronto, Canada and from Nard Institute, Amagasaki, Osaka, Japan. Standard high-purity chemicals were purchased from local supply houses. Diets were obtained from Dyets Inc., Bethlehem, PA. The composition of the diets is given in Table I. The fat was corn oil, and the dietary levels were 5% and 23.5% by weight, which are levels often used in research on the role of fat in cancer development (2).

**Treatment of Animals.** The animals were maintained in the Institute Research Animal Facility according to the institutional animal care guidelines, the Animal Welfare Act and the DHHS Guide for the Care and Use of Laboratory Animals. The specialized AAALAC-approved Facility is temperature and humidity controlled (24° ± 2°C; 50%), and has a 12-hr light cycle. Rats were housed two per plastic cage with free access to diets and water (the latter through an automatic tap water supply system). Cages were changed twice per week. The rats were weighed every two weeks for the first 16 weeks, monthly thereafter. All animals were inspected daily.

In the first experiment, exploring the effect of dietary fat level on cancer induced by IQ, weanling male and female F344 rats were purchased from Charles River, Kingston, NY. After maintenance in the quarantine unit for two weeks, all rats were weighed and the low- and high-weight animals culled. Those found suitable for the experiments were randomized into four groups of 34 or 35 males and females, and placed on the low- and high-fat diets, with 75 ppm IQ. In addition, there were four control groups of males and females on high- and low-fat diets, without IQ.

The animals were maintained on the IQ-containing diets for 53 weeks. Then the male rats were continued on the low- and high-fat control diets without IQ for 10 weeks longer, and the females for 25 weeks longer. When necessitated by the condition of the animals, or at the end of the planned experimental period, the rats were killed in a $CO_2$ atmosphere. Complete necropsies were performed, followed by the histopathologic evaluation of microscope sections stained with H&E or other appropriate stains.

In a second series of experiments dealing with colon carcinogenesis, male weanling F344 rats were acquired from Charles River, kept in quarantine and randomized into eight experimental and control groups of five rats each. Still on quarantine and beginning at five weeks of age, they were fed one of the four diets, high and low in fat, and high and low in calcium (Table I). At six weeks of age, rats in four groups received their respective diets with 400 ppm PhIP added. The other four groups continued on the diets without PhIP. The rats were weighed at the beginning of the test, every two weeks thereafter, and at the end. After nine weeks, the rats were killed by a $CO_2$ atmosphere.

**Table I.** Composition of Diets with Different Corn Oil and Calcium Levels

| Ingredient | Diet | | | |
|---|---|---|---|---|
| | Low fat/low Ca | High fat/low Ca | Low fat/high Ca | High fat/high Ca |
| Casein, vitamin free | 200.0 | 235.0 | 200.0 | 235.0 |
| Cornstarch | 516.9 | 325.9 | 495.3 | 304.3 |
| Dextrose | 130.0 | 83.0 | 130.0 | 83.0 |
| Cellulose | 50.0 | 59.0 | 50.0 | 59.0 |
| Corn oil | 50.0 | 235.2 | 50.0 | 235.2 |
| Calcium lactate, 5 $H_2O$ | 3.1 | 3.1 | 24.7 | 24.7 |
| Salt mix #200150 | 35.0 | 41.1 | 35.0 | 41.1 |
| Vitamin mix #300050 | 10.0 | 11.8 | 10.0 | 11.8 |
| Choline bitartrate | 2.0 | 2.4 | 2.0 | 2.4 |
| DL-Methionine | 3.0 | 3.5 | 3.0 | 3.5 |

The diets were AIN-76A-based, purchased from Dyets, Inc., Bethlehem, PA, shipped and stored at 4°C. The amounts listed are g ingredient/kg diet. The low- and high-fat, low-calcium diets were used in the first experiment, all four diets in the second experiment. The salt and vitamin mixes are standard mixtures prepared by Dyets.
In experimental Series 1, groups of male and female F344 rats received the low-calcium, low-fat, or the low-calcium, high-fat diets, with or without 75 ppm incorporated in each of the two diets, for a total of four groups of males and four groups of females. In experimental Series 2, groups of male F344 received all four diets above, with or without 400 ppm PhIP added, so that there were 8 groups of rats.

They were immediately necropsied; the distal colon and rectum were removed and cleansed of fecal material by flushing with a 0.2% Krebs-Ringer buffer solution, adjusted to pH 7.4. The descending intestinal tract was cut longitudinally and fixed between two filters papers, soaked in 10% buffered formalin. Segments 6 cm long of the distal colon and rectum were placed flat on microscope slides and stained with 0.2% methylene blue solution in the buffer for 30 min. The stained tissue was rinsed with buffer and immediately examined by light microscopy at a magnification of ×40 or ×200 for foci of aberrant crypts in three segments of 2 cm each. The total number of foci per 6-cm colon and the total number of aberrant crypts were recorded.

The statistical significance of the difference between the mean number of foci and aberrant crypts, by dietary fat and calcium levels, was evaluated using the Student's *t*-test with 2(n − 1) degrees of freedom. The differences in tumor incidence in high- versus low-fat groups were tested separately for each sex, through the critical ratio test for comparing two independent proportions (19).

## Results

**Effect of Dietary Level of Corn Oil in IQ Carcinogenesis.** At the beginning of this experiment, the mean body weight of males in the IQ-fed and the control group was 111 g, and that of females was 90 g. At the 26-week point, the IQ-fed males and females on the high-fat diet weighed 13.5%–15% ($P < 0.01$) more than those on the low-fat diet. In the male control group, the difference was 9.4% ($P < 0.01$) and in the female group it was 8.4% (not significant).

At the 54-week point, the fat-related difference ranged from 20% to 21% ($P < 0.01$). These differences between groups were maintained to termination at 63 weeks for males and 78 weeks for females. The control rats without IQ also had a 9%–12% higher weight gain on the higher fat level, that was statistically significant in males ($P < 0.01$) at all time points, but in females the difference at 26 weeks was not significant.

During the first year, 15 of 34 male rats in the low-fat group came to autopsy, with a total of 10 Zymbal gland (ear duct) neoplasms, five skin cancers, four in the colon, three in the liver, and one lung adenoma. Several of the rats had several types of cancer. In the males in the high-fat group, 10 rats had six Zymbal gland cancers, two skin, and two liver cancers. In the females in the low-fat group, one had a Zymbal gland cancer, and three died without evidence of neoplasms, but had pneumonia. In the female in the high-fat group, one rat had a Zymbal gland cancer, which was the earliest cancer seen, about seven months after the beginning of these experiments (Table II).

Overall, there were substantial numbers of rats with Zymbal gland/earduct neoplasms in all groups, with little influence of dietary fat in males (Table II). Female rats on the high-fat regimen had significantly fewer such tumors. There were numerous multiple malignant and benign sebaceous tumors of the skin at both dietary fat levels. They occurred mainly in males (36/69 rats), and there was only one in a female—an interesting difference. The group of males on the high-fat diet displayed more liver tumors than did the females. Mammary gland carcinomas and neoplasms in the preputial gland were induced to a greater extent on the high-fat diet than on the low-fat diet in the female rats. Pulmonary adenocarcinomas were more frequent in males, (9/69) than in females (3/68), and there was no association with dietary fat. There were only a few colon cancers. With the usual dose of 300 ppm IQ, a high incidence of colon cancer is usually found, in contrast to the small yield with 75 ppm IQ used here (10).

**Table II.** Effect of Dietary Corn Oil Level on Induction of Various Types of Neoplasms with 75 ppm IQ

| Group | Number of rats[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sebaceous tumors[b] | | | Liver | | Mammary gland tumors | Preputial gland tumors | Colon tumors | Lung tumors | Lung metastasis |
| | Zymbal gland | Lip | Skin | Carcinoma | Adenoma | | | | | |
| 5% fat, male, 75 ppm IQ | 14 | 5[S] | 16 | 2[S] | 0[S] | — | 3 | 2 | 3[c] | 3[g] |
| 23.5% fat, male, 75 ppm IQ | 14 | 0 | 20 | 17 | 5 | — | 2 | 3 | 6[d] | 5[h] |
| 5% fat, female, 75 ppm IQ | 12[S] | 2 | 1 | 6 | 3 | 10[S] | 9 | 0 | 2[e] | 1[i] |
| 23.5% fat, female, 75 ppm IQ | 4 | 3 | 0 | 6 | 2 | 17 | 13 | 1 | 1[f] | 0 |

[a] 34 rats/group, except 35 in males on 23.5% fat diet; the tumor incidence in controls on these diets without IQ was 0 or 1. Statistical comparisons in groups on high- and low-fat diets were made by sex. A significant difference ($P < 0.05$) as a function of fat is shown in each appropriate pair by the superscript *S*.
[b] In many cases these were multiple malignant and benign neoplasms of sebaceous origin.
[c] One adenoma, two adenocarcinomas.
[d] One adenoma, five adenocarcinomas.
[e] Two adenomas.
[f] One adenoma.
[g] One from skin and two from Zymbal gland squamous carcinoma.
[h] One from hepatocarcinoma, one from preputial gland carcinoma, two from Zymbal gland carcinoma, and one from skin squamous carcinoma.
[i] One from Zymbal gland carcinoma.

There were metastases to the lung, mainly from the squamous skin cancers, and one from a hepatocellular carcinoma.

**Effect of Dietary Level of Corn Oil and Calcium in Short-Term Colon Carcinogenesis, the Induction of Foci of Aberrant Crypts.** Administration of 400 ppm PhIP in the diet induced foci of aberrant crypts in the nine-week experimental period (Tables III and IV). Under the conditions of the experiment, 18/20 rats given PhIP displayed such foci. On a low- and a high-calcium diet, there were more aberrant crypts present with a 23.5% fat diet than with a 5% fat diet. Also, animals on the higher calcium level had fewer foci and fewer aberrant crypts than animals on the lower calcium level, on the low fat level. Control animals without carcinogen displayed few or no foci of aberrant crypts, in the 6-cm distal intestinal segment examined, and there was no association with diet.

## Discussion

The action of the food mutagens and carcinogens IQ and PhIP are susceptible to modulation by dietary factors. These heterocyclic amines, formed during cooking of meat or fish, are active in many of the rapid bioassay test for genotoxicity, including the tests of Ames and Williams (8, 9). Virtually all human carcinogens display this type of activity (20). Therefore, it seems most likely that this class of carcinogens, to which a large number of human populations in the world are exposed, represent human cancer risks. Careful analysis of diverse foods have indicated that the absolute amount of the dietary intake of these carcinogens per day, especially in populations eating grilled, fried or broiled meat/fish, is small (13). A number of bioassays were conducted, utilizing low levels or mixtures of low levels of these carcinogens and little effect was obtained (21). Populations, as, for example, the Japanese, that consume such carcinogens in cooked fish or meat, but are on a low-fat diet, have a relatively small risk of disease.

**Table III.** Effect of Dietary Corn Oil and Calcium Levels on Induction of Foci of Aberrant Crypts in Male F344 Rats After Nine Weeks on 400 ppm PhIP

| Group | No. rats with foci | Total foci | Total aberrant crypts |
|---|---|---|---|
| 1<br>5% Fat + 0.04% $Ca^{2+}$ + 400 ppm PhIP | 5/5 | 17 ± 0.6 | 23 ± 0.6 |
| 2<br>23.5% Fat + 0.04% $Ca^{2+}$ + 400 ppm PhIP | 4/5 | 30 ± 7.6 | 55 ± 7.6 |
| 3<br>5% Fat + 0.32% $Ca^{2+}$ + 400 ppm PhIP | 4/5 | 13 ± 1.6 | 16 ± 1.8 |
| 4<br>23.5% Fat + 0.32% $Ca^{2+}$ + 400 ppm PhIP | 5/5 | 20 ± 0.8 | 34 ± 1.8 |
| 5<br>5% Fat + 0.04% $Ca^{2+}$ | 0/5 | 0 | 0 |
| 6<br>23.5% Fat + 0.04% $Ca^{2+}$ | 1/5 | 1 ± 0.2 | 7 ± 1.4 |
| 7<br>5% Fat + 0.32% $Ca^{2+}$ | 1/5 | 3 ± 0.6 | 5 ± 1.0 |
| 8<br>23.5% Fat + 0.32% $Ca^{2+}$ | 0/5 | 0 | 0 |

Groups of five male six-week-old F344 rats were given diets containing 5% or 23.5% corn oil and 0.04 or 0.32% $Ca^{2+}$ as calcium lactate, 5 $H_2O$, in a modified AIN76A diet. The diets of groups 1–4 contained 400 ppm PhIP. Rats in Groups 5–8 fed the same diets without PhIP. After nine weeks, the rats were killed in a $CO_2$ atmosphere, their descending colon and rectum was removed, and a 6-cm segment from the anus was analyzed for foci of aberrant crypts. Data expressed as means ± SE. All data in Groups 1–4 of rats fed PhIP are significantly different, $P < 0.05$, from control Groups 5–8 not given PhIP. A statistical analysis of data in Groups 1–4 is given in Table IV.

However, in the Western world, people consume more fried or grilled meat, and importantly, they are on a high-fat diet, with about 40% of calories in fat. The experiments described were designed to mimic these contrasting epidemiologic situations. Thus, we found that when a relatively low level of IQ was fed in low- and high-fat diets, the carcinogenicity at several target organs was increased with the high-fat diet. For example, this experiment demonstrated that females on the high fat level had more cancers in the mammary glands, and that in male rats, there was an increased effect in the liver. We do not believe that the increased incidence was associated with the higher weight gain of male and female rats on the higher fat intake. We have observed increased incidence, especially of cancer in the mammary gland and colon without weight gain at the higher fat level (2). Also, with some oils, like monounsaturated olive oil, containing adequate amounts of essential fatty acids, carcinogenesis was not increased (2). Thus, the findings in the mammary gland may mimic the situation in humans. Classic epidemiological data show that in the Western world on a high-fat diet, the breast cancer rate is a major disease, but on the traditional low-fat Japanese diet, postmenopausal breast cancer was relatively infrequent. Currently, with an increasing intake of total fat, breast cancer and other fat-linked cancers such as distal colon cancer are rising in Japan (3, 22).

In a parallel study, on the induction of foci of aberrant crypts in the colon of animals fed PhIP, a higher number of aberrant crypts was found in rats on a high-fat diet, compared with rats on a low fat diet. Also, the low dietary level of fat, a higher calcium intake resulted in a lower number of aberrant crypts. Overall, the experiments described suggest that the effect of the heterocyclic amines can be modified by other dietary components. These findings strengthen the hypothesis that the genotoxic carcinogens for certain cancers prevalent in the Western world stem from the

**Table IV.** Statistical Comparison of Mean Foci and Aberrant Crypts, as a Function of Dietary Fat and Calcium Levels

| I. 5% vs 23.5% Fat | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1. 0.04% Calcium | | | | | 2. 0.32% Calcium | | | | |
| Groups | Total | Foci | Total aberrant crypts | | Groups | Total | Foci | Total aberrant crypts | |
| | *t* | *P* | *t* | *P*[a] | | *t* | *P* | *t* | *P* |
| 1 vs 2 | −1.6 | NS[b] | −3.9 | <0.01 | 3 vs 4 | −2.9 | <0.02 | −5.0 | <0.01 |
| II. 0.04% vs 0.32% Calcium | | | | | | | | | |
| 1. 5% Fat | | | | | 2. 23.5% Fat | | | | |
| Groups | Total | Foci | Total aberrant crypts | | Groups | Total | Foci | Total aberrant crypts | |
| | *t* | *P* | *t* | *P* | | *t* | *P* | *t* | *P* |
| 1 vs 3 | 0.5 | NS | 2.9 | <0.02 | 2 vs 4 | 1.2 | NS | 2.2 | NS |

[a] Based on a one-tailed Student's *t*-test.
[b] Statistically not significant ($P > 0.05$).

mode of cooking. Their effect is promoted by fat intake, and as other experiments suggest, inhibited by dietary fiber, vegetables and fruits, and active components in tea (9).

Pioneering findings from the laboratory of Tudek *et al.* (17) established the value of determining the induction of foci of aberrant crypts in the colon of animals given appropriate carcinogens, including those found in fried and grilled meats. An association between aberrant crypts and colon carcinogenesis with the colon carcinogen azoxymethane was observed (23, 24). A major food carcinogen, PhlP has been studied in the laboratories of Sugimura and of Ito, who also observed that virtually all treated animals displayed foci and found about the same number of aberrant crypts per focus in the colon as was noted in the present study (14, 18). As an important extension, we found that the number of foci and of aberrant crypts was subject to control directly by dietary fat and, to some extent, inversely by dietary calcium. These effects may stem from action on the rate of cell cycling (15, 25, 26).

Thus, our results show that with carcinogens present in fried foods, of the heterocyclic aromatic amine type widely prevalent in the human environment, there is an association between dietary conditions favoring or inhibiting carcinogenesis in an abbreviated nine-week test, in the determination of foci of aberrant crypts, and in a standard carcinogenicity bioassay extending over 15–18 months.

Research in Valhalla was supported by Grant CN-29 from the American Cancer Society, USPHS-NIH Grant CA-42381 from the National Cancer Institute, and, in Tokyo, by a grant-in Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control, Japan. We are grateful to J. Braley, C. Aliaga, J. Reinhardt, K. Stamatis, and B. McKinney for valuable assistance in phases of these experiments. We are indebted to W. R. Bruce and X.-M. Zhang, Ontario Cancer Institute, Toronto, Canada, for instruction on the procedures to determine foci of aberrant crypts.

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