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Effects of dietary fat and a vegetable–fruit mixture on the development of intestinal neoplasia in the *Apc^{Min}* mouse

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The variation in colorectal cancer (CRC) incidence worldwide strongly suggests a role for dietary influences. Based on epidemiological data, protective effects of vegetables and fruit intake on CRC are widely claimed, while other data indicate a possible increased CRC risk from (higher) dietary fat intake. Therefore, we have investigated single and interactive effects of dietary fat and a vegetable–fruit mixture (VFM) in the *Apc^{Min}* mouse, a mouse model for multiple intestinal neoplasia. In this study, four different diets (A–D) were compared, which were either low in fat (20% energy diets A/B) or high in fat (40% energy diets C/D). In addition, 19.5% (wt/wt) of the carbohydrates in diets B and D were replaced by a freeze-dried VFM. The diets were balanced so that they only differed among each other in fat/carbohydrate content and the presence of specific plant-constituents. Because the initiation of intestinal tumors in *Apc^{Min}* mice occurs relatively early in life, exposure to the diets was started *in utero*. Without the addition of VFM, mice maintained at a high-fat diet did not develop significantly higher numbers of small or large intestinal adenomas than mice maintained at a low-fat diet. VFM added to a low-fat diet significantly lowered multiplicity of small intestinal polyps (from 16.2 to 10.2/mouse, 15 animals/group), but not of colon tumors in male *Apc^{Min}* mice only. Strikingly, addition of VFM to female mice maintained on a low-fat diet and to both sexes maintained on a high-fat diet significantly enhanced intestinal polyp multiplicity (from 16.5 to 26.7 polyps/mouse). In conclusion, our results indicate that neither a lower fat intake nor consumption of VFM included in a high-fat diet decreases the development of polyps in mice genetically predisposed to intestinal tumor development.

Introduction

Colorectal cancer (CRC) is one of the leading cancers in the Western world with significant differences in incidence worldwide (1,2). Several epidemiological studies have associated these differences mainly with lifestyle, in particular dietary habits (3). In general, increased risk is associated with high dietary intake of animal and saturated fat and/or (red) meat and alcohol, whereas a decreased risk is associated with a high consumption of vegetables and, to a lesser extent, fruits (4,5). However, whether diets low in fat and rich in vegetables and fruit are causally involved in reducing the risk for developing colorectal cancer is still a matter of debate, because both epidemiological and animal studies have revealed inconsistent results (6,7). These conflicting results are partly due to confounders in epidemiological studies, such as recall-bias and uncertainties about the quality of the food consumed, or to low resolution because of the genetic heterogeneity within the populations studied (8). Animal studies performed under controlled conditions do not suffer from these limitations. However, in most of the animal studies dietary compositions are used that differ markedly from human diets in their source, preparation and content. Furthermore, single dietary components are usually studied in animal models in amounts that far exceed daily human intake. Such high amounts may limit the predictive value concerning colorectal cancer risk in humans. In addition, since diet is complex and dietary factors probably interact, it is likely that these interactions among the different dietary components, rather than individual components, are important risk factors in developing colorectal cancer (9,10). The inconsistent findings among animal studies may also be explained by the different study designs used, such as the use of different chemical colorectal cancer inducers and differences in genetic susceptibility for intestinal carcinogenesis among the rodent strains used (11). These dietary and methodological shortcomings in animal studies warrant the need to perform colorectal carcinogenesis experiments that use diets that resemble as much as possible realistic regular human diets, and that also take interactive effects into account. Applying such an approach, several studies on interactive effects between dietary fat and a vegetable–fruit mixture (VFM) in colorectal carcinogenesis in rats have been performed by Alink *et al.* (12–15). The results indicate that the cancer protective properties of the VFM depended mainly on the chemical carcinogen used to induce colorectal cancer, which underlines the importance of taking methodological limitations into account (11,16). To further dissect the complex dietary and genetic interactions into their primary causes, model systems are required where each variable is introduced into a homogeneous genetic background and a controlled environment. At present, this is best provided by inbred mouse models, and more specifically for intestinal cancer, by mice with an (genetically) altered *Apc* gene.

The adenomatous polyposis coli (*Apc*) gene on chromosome 5q is considered to be a gatekeeper of intestinal tumorigenesis. Its inactivation is involved in the onset of CRC, i.e. the formation of benign adenomas (polyps) (17,18). In the majority of colorectal neoplasia, both inherited and somatic, the *Apc* tumor suppressor gene is either mutationally inactivated by the introduction of premature stop codons and/or deleted by loss of heterozygosity (LOH) (19,20). In recent years, several

Abbreviations: *APC*, adenomatous polyposis coli; CRC, colorectal cancer; DMH, 1,2-dimethylhydrazine; KRA, ketamine-rompun-atropine; MIN, multiple intestinal neoplasia; MNNG, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; VFM, vegetables and fruit mixture.

mouse lineages that are heterozygous for specific mutations at the endogenous *Apc* gene have been developed and characterized with respect to their intestinal tumor multiplicity (reviewed in refs 21,22). Two of these strains of mutant *Apc* mice, Apc^{Min} (23) and $Apc^{\Delta 716}$ (24), are characterized by a relatively high tumor multiplicity in an identical inbred C57BL6 genetic background (~20–90 and 200–500 small intestinal tumors per mice in *Min* and $Apc^{\Delta 716}$ respectively).

The present study was performed to study the interactive effects between dietary fat (20 and 40% energy) and VFM (19.5% wt/wt), with exactly the same dietary composition as the previously mentioned chemically induced studies (14,15) on intestinal neoplasia in Apc^{Min} mice. Because the onset of intestinal carcinogenesis in *Min* mice is assumed to occur relatively early in life (25,26) exposure to the four experimental diets was already started during mating, and was continued through weaning and lasted until they were killed.

Materials and methods

Animals, housing and clinical observations

Min (C57BL/6J-$Apc^{Min/+Apc}$) heterozygote mice were obtained from a colony at Leiden University (R.Fodde, MGC-Department of Human Genetics) that were established with *Min* mice obtained from the original colony at the McArdle Laboratory (W.F.Dove, McArdle Laboratories, University of Wisconsin, Madison, WI). Breeding was with C57BL/6JIco (+/+) females purchased from Broekman Institute B.V. in The Netherlands. Using DNA isolated from mouse tails, progeny was genotyped before weaning by an allele-specific PCR for the nonsense mutation at codon 850 (27) as described by Jacoby *et al*. (28). The total number of 124 *Min* mice (63 males and 61 females) for this experiment were obtained from 56 breeding cages, each containing one male C57BL/6J-$Apc^{Min/Apc+}$ mouse and two female C57BL/6J$^{Apc+/Ap\ c+}$ mice. During the mating period, the breeding couples were randomly divided into the four dietary groups, and the different diets (A, B, C, D) were allocated *ad libitum*. After weaning, the newborn mice were housed in groups of 2–6 animals of the same sex per cage under controlled environmental conditions (temperature 21 ± 1°C, relative humidity 53% ± 2, 12/12 light/dark cycle, air changed eight times/h) and free access to feed and tap water. Body weight and food intake were recorded weekly. All mice were observed at least once per day for abnormalities in clinical appearance until being killed.

Diets

A detailed description of the diets tested is described elsewhere (14). In brief, the following four diets were used: a diet low in fat (20% energy; diet A), a diet low in fat (20% energy) to which a VFM was added (19.5% wt/wt; diet B), a diet high in fat (40% energy; diet C) and a diet high in fat (40% energy) to which the same VFM was added (19.5% wt/wt; diet D) as in diet B. The semi-synthetic Muracon-SSP/tox animal diet (Hope Farms, Woerden, The Netherlands) served as the basal diet for the four different diets.

The fat in the diets were a mixture of lard and sunflower oil, which resulted in a fatty acid composition similar to an average human diet in The Netherlands. The extra 20% fat added to diets C and D was at the expense of carbohydrates. In diets B and D, VFM was supplied to the Muracon-SSP/tox diet. The choice of the vegetables and fruit used approached the mean vegetables and fruit consumption in The Netherlands (29). The following products were separately cooked; potatoes, cauliflower, spinach, leek, red and white cabbage, sauerkraut, carrots, Brussels sprouts and beet. After freezing (–40°C) the mixture was freeze-dried, ground and homogenized. Before the VFM was mixed with the basal Muracon-SSP/tox and pelleted, it was analyzed for nutrient, vitamin and mineral content (data not shown). The final composition of the diets is shown in Table I. As a result of adjusting the diets for protein, mineral and vitamin contents, the diets differed only in the fat/carbohydrate content and in the presence of specific constituents (type of fibre, non-nutrients) in the VFM. Finally, the pelleted diets were stored at –40°C in air-closed plastic bags before use in the experiment. No antioxidants or other preservatives were added.

Tissue sampling and scoring tumors

Mice were killed with ketamine–rompun–atropine (KRA) mixture around day 90 (range 85–93 days) after birth. This point in time was chosen to minimize the risk of intercurrent mortality caused by severe progressive anemia, rectal prolaps or intestinal obstruction, which was typically for *Min* mice around day 120 (23). At post mortem, the complete gastrointestinal tract (except the distal rectum) was isolated, spread onto filter paper, dissected longitudinally with fine scissors, and the mucus and faeces were removed. The small intestine was separated from the colon and divided into three approximately equal sections (length ± 11 cm). These sections were macroscopically examined by a biotechnician who was unaware of the animals' diet, to record polyp number, size and location. After this macroscopic examination, Swiss rolls were made of the three small intestinal sections and the colon. The samples were fixed overnight at 4°C in 4% cold neutral buffered formaldehyde, washed twice with 70% ethanol and embedded in paraffin. Hematoxylin and eosin-stained 5 μm sections were histopathologically examined (the degree of dysplasia was scored as slight, moderate or severe). A selection of colon and small intestinal tumors was collected separately, snap frozen in liquid nitrogen and stored for further molecular analysis.

Table I. Final composition of the four experimental diets[a]

Ingredients	Diet A[b] low fat	Diet B low fat + VFM	Diet C high fat	Diet D high fat + VFM	VFM[h]
Fat[c]	8.50	8.50	20.00	20.00	0.40
Carbohydrate[d]	48.34	30.39	30.78	10.14	63.00
Protein[e]	23.19	23.19	26.50	26.50	8.80
Fibre (cellulose)	10.67	9.80	12.20	11.20	14.20
Vitamins[f]	0.35	0.31	0.40	0.35	0.80
Minerals[g]	8.95	8.31	10.12	9.51	1.30
VFM	–	19.50	–	22.30	
Water					11.50
kcal/kg	3603	3608	4126	4132	3548

[a]Values are expressed as % (wt/wt), and are calculated from analyses and represent approximate composition.
[b]Diet A is the basal Muracon-SSP/tox diet. In diets C and D, the carbohydrate, protein, fibre, vitamin and mineral contents of the Muracon-SSP/tox were adjusted to allow for decreased food consumption in animals that consume diets of high caloric density. In diets B and D, the amounts of carbohydrate, fibre, vitamins and minerals of the Muracon-SSP/tox were adjusted for the presence of these constituents in the VFM.
[c]84% lard and 16% sunflower oil.
[d]Diet A: 23.33 g cellulose/100 g food and 25.10 g corn starch/100g food.
[e]Diet A: 18.55 g acid casein/100 g food and 4.64 g soy protein/100 g of food.
[f]Diet A: cholin-Cl (50%) 0.303 g/100g; vitamin A 0.34 mg/kg; vitamin A/D3 1.50 mg/kg; vitamin E (50%) 6.54 mg/kg; vitamin K3 0.360 mg/kg; thiamin 0.650 mg/kg; riboflavin 0.920 mg/kg; pyridoxine HCl 0.270 mg/kg; niacin/nicotin 4.60 mg/kg; calcium pantothenate 2.86 mg/kg; vitamin B12 2.02 mg/kg; folic acid 0.300 mg/kg; biotin 2% 1.375 mg/kg; inositol 13.79 mg/kg; β-carotene 10% 2.86 mg/kg; vitamin C 12.39 mg/kg.
[g]Diet A: $CaHPO_4.2H_2O$ 3.34 g/100g; KCl 0.93 g/100g; NaCl 0.37 g/100g; Na_2CO_3 0.46g/100g; methionine synthetase 0.09 g/100g; PABA 18.18 mg/kg; MgO 90.93 mg/kg; $FeSO_4.H_2O$ 13.24 mg/kg; $ZnSO_4.5H_2O$ 7.00 mg/kg; $CoSO_4$ 0.225 mg/kg; ammonium heptamolybdate 0.01 mg/kg; NaF 0.01 mg/kg; Ca-iodate 0.05 mg/kg; $AlK(SO_4)_2.12H_2O$ 6.59 mg/kg; $CrCl_3.6H_2O$ 0.02 mg/kg; SiO_2 2.79 g/100g.
[h]Composition (% wet wt): potatoes (35.1), bananas (3.0), oranges (9.0), apples (19.10), lettuce (3.75), green pepper (1.25), tomatoes (3.75), cucumbers (3.75), cauliflowers (3.75), spinach (2.50), leek (2.50), red (2.5) and white (2.5) cabbage, sauerkraut (2.5), carrots (1.25), Brussels sprouts (1.25) and beet (2.50). Total glucosinolate content near detection level (i.e. glucobrassicin), flavonoid content 640 mg/100 g (i.e. quercetin, catechin, naringenin, hesperetin).

Statistical analysis

Differences in body weight were compared by analysis of variance and differences in tumor multiplicity by linear regression with log-link and Poisson distribution. For all tests, a confidence interval (CI) of 95% was chosen ($P \leqslant 0.05$).

Results

General observations

Although the body weights of mice consuming VFM in their diets (B and D) were in general somewhat higher (most pronounced for female mice; see Figure 1), no statistical

significant differences in body weights were observed between the four diet groups. This is in line with the data on daily food-intake (not shown), which showed a tendency for increased food-intake on addition of VFM. In particular, the 40% energy fat + VFM diet was consumed on average 15% (wt/wt) more than the other diets. Although the diets A and B are isocaloric, and this also holds for diets C and D, the differences in food-intake of the diets that contained VFM could have resulted in a slightly increased caloric intake for mice on diets B and D compared with diets A and C.

Intestinal tumor incidence and multiplicity

All of the histopathologically classified tumors in the small intestine, as well as in the colon, were adenomas (adenomatous polyps) with no evidence of local invasion of the lamina propria. On average, mice developed between 10 and 30 tumors in the small intestine, whereas in the colon it ranged between 0 and 3 tumors/mouse (Table II). As expected, almost all mice (71–100%) developed multiple small intestinal tumors. Therefore, no significant effects of fat or VFM on tumor incidence values for the small intestine were observed. For the colon, the tumor incidences were lower in general (40–80%). A marked decrease in incidence was observed only for male mice on a low-fat diet to which VFM was added, whereas on the high-fat diet, addition of VFM seems to increase the incidence of colon tumors in female mice (Table II). Mice that consume a high-fat diet without further additions did not develop significantly different numbers of small or large intestinal tumors compared with mice that consumed a low-fat diet. VFM added to a low-fat diet significantly lowered the multiplicity of small intestinal tumors, but not of colon tumors, in male *Apc*^{Min} mice only. In contrast, for both sexes maintained on a high-fat diet, addition of VFM significantly enhanced the small intestinal polyp multiplicity (Figure 2). In the colon, this observation was mainly restricted to male mice fed VFM in a high-fat diet. However, in the other groups, VFM also tended to enhance colon tumor development.

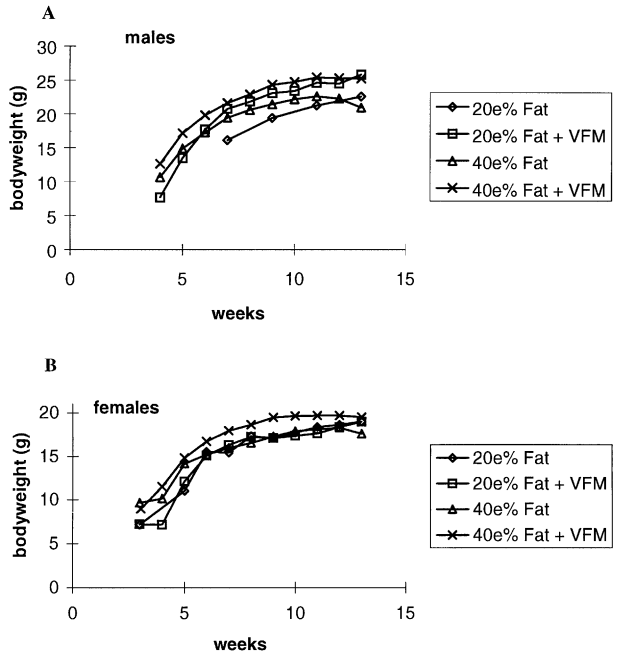

Fig. 1. Body weight of *Apc*^{Min} mice consuming the four diets as indicated. (**A**) male mice; (**B**) female mice.

Intestinal tumor size, localization and dysplasia

The mean size of the polyps in the small intestine averaged 2.0 ± 0.5 mm in both males and females, whereas in the colon it averaged 2.6 ± 0.4 mm in males and 3.2 ± 0.9 mm in females. The mean size of small and large intestinal polyps did not vary significantly among the dietary groups, or between males and females. Although not significant, small intestinal polyps tended to be more localized in the duodenum in females in groups B, C and D, and in males in group B, whereas in males in group A they tended to be more localized in the ileum (Table II). While there were some differences between the groups in degree of dysplasia of the small intestinal adenomas, any distinct or consistent trend could not be discerned. In the colon, an increase in the number of moderate dysplastic polyps was observed on addition of VFM to the 40% fat diet, which is in line with the previously mentioned increase in incidence of colon tumors on this diet (data not shown).

Discussion

In the present study, dietary fat showed no effect on the development of intestinal neoplasia in *Apc*^{Min} mice. The amount and fatty acid composition of the high fat diet is believed to

Table II. Intestinal polyp incidences, multiplicity and localization of intestinal polyps in *Min* mice

Diet group	Sex	No. of animals	Incidence (%)		Multiplicity[a,c]		Localization polyps[b] small intestine		
			Small intestines	Colon	Small intestines	Colon	Section 1	Section 2	Section 3
A 20e% fat	Male	15	100	80	16.4 ± 3.9	1.6 ± 0.2 (1.2)	5.1 ± 0.7	3.9 ± 1.4	7.5 ± 2.4
B 20e% fat + VFM		15	93	53	10.2 ± 3.2* (9.5)	1.9 ± 0.4 (1.0)	5.0 ± 1.4	1.9 ± 0.6	3.4 ± 1.7
C 40e% fat		16	88	63	17.1 ± 2.8 (14.9)	1.7 ± 0.2 (1.1)	5.4 ± 0.9	5.3 ± 1.0	6.4 ± 1.3
D 40e% fat + VFM		17	100	76	27.7 ± 5.3**	2.8 ± 0.6 (2.1)	9.5 ± 1.4	8.3 ± 1.9	9.9 ± 2.4
A 20e% fat	Female	14	71	71	16.6 ± 6.0 (11.9)	1.3 ± 0.2 (0.9)	5.7 ± 1.2	5.7 ± 2.6	5.3 ± 3.1
B 20e% fat + VFM		16	94	63	16.9 ± 2.5 (15.8)	2.4 ± 0.3 (1.4)	8.2 ± 1.0	3.4 ± 0.7	5.3 ± 1.5
C 40e% fat		15	80	40	16.0 ± 2.9 (12.8)	1.3 ± 0.2 (0.5)	6.8 ± 1.0	5.0 ± 1.2	4.2 ± 1.4
D 40e% fat + VFM		16	100	75	25.8 ± 3.9**	1.8 ± 0.3 (1.3)	10.9 ± 1.0	7.5 ± 1.4	7.4 ± 1.9

e, energy.
[a]Multiplicity is defined as the average number of tumors per tumor-bearing mouse, mean values ± standard error of the mean are shown; between brackets the mean of tumors per mouse is indicated if the incidence was <100%.
[b]Section 1: duodenum + first part jejunum (length 11 cm); section 2: jejunum (length 11 cm); section 3: last part jejunum + ileum (length 11 cm).
[c]Statistical significance is as follows: $^{*}P < 0.01$ between group A and B, $^{**}P < 0.01$ between group A/C and D.

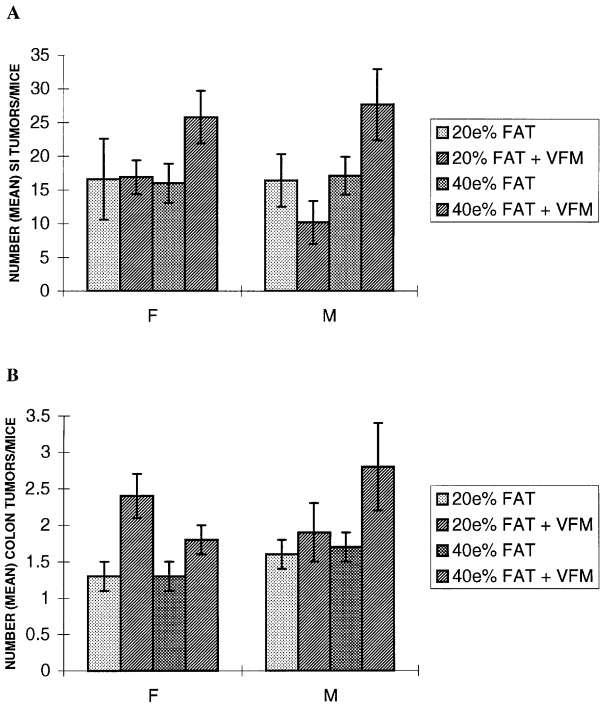

Fig. 2. Number of intestinal polyps in *Apc*^{Min} mice consuming the four diets as indicated. (**A**) Number of small intestinal polyps; (**B**) number of polyps in the colon.

be representative of the mean consumption and composition values in The Netherlands. This is in line with similar observations of 3 versus 15% wt/wt dietary fat (Dr P.Hambly, personal communication) and 10 and 20% wt/wt fat in *Apc*1638N mice (van Kranen, in preparation). However, a recent study by Wasan *et al.* (30) reported a significant increase in the number (~50%) and size of polyps in the small as well as in the large bowel of mice fed a diet that contained 15% wt/wt fat compared with a 3% wt/wt fat-containing standard diet. Studies with chemically-induced intestinal cancer in rodents provide evidence that not only the amount but also the types of fat (differing in fatty acid composition) are important factors in determining the enhancing effect of this nutrient in intestinal tumor development (31,32). This has also been demonstrated in *Apc*^{Min} mice by Paulsen *et al.*, who demonstrated a protective effect of a fish oil derived concentrate enriched in ω-3 polyunsaturated acids (54% eicosapentanoic acid–30% docosohexanoic acid) on intestinal neoplasia (33), whereas in *Apc*$^{\Delta 716}$ mice, a similar effect on female mice only, was observed after addition of docosohexanoic acid (34). To further complicate the dietary fat story, studies with so-called Western-style diets or high risk diets (HRD) in *Apc*-deficient mice have been reported. In general, these diets are not only relatively high in fat content but also high in phosphate and low in fibre, calcium and vitamin D. An increase in colonic polyploid hyperplasia has been reported after feeding a Western-style diet to *Apc*1638N mice (35). In the *Apc*$^{\Delta 716}$ mice, feeding a similar high risk diet for only 7 weeks resulted in a significant increase in intestinal polyp numbers but not in polyp size distribution (36). Overall, no firm conclusions on the role of dietary fat on polyp development in *Apc* deficient mice can be drawn at present.

Remarkably, the present study shows that a reasonable amount of specific plant constituents present in the VFM hardly protected against intestinal tumor development in *Apc*^{Min} mice. To the contrary, it enhanced tumor development in mice maintained on high-fat diets. This observation is consistent with some other animal experiments (13,37), but not supported by most epidemiological studies in which an inverse association is suggested between vegetables and fruit intake and intestinal cancer risk. Furthermore, protective effects on intestinal neoplasia in *Apc* deficient mice have been reported for specific plant constituents, such as for the soybean-derived Bowman–Birk inhibitor (38) and less pronounced for high fibre administered as short-chain fructo-oligosaccharides (39). The absence of a clear protection by the VFM against intestinal carcinogenesis as observed in the present study, may be ascribed to the use of whole vegetables and fruit, whereas in the aforementioned studies, isolated constituents were used at relatively high concentrations. In the present study, vegetables and fruit were used in regular amounts. Furthermore, the observed enhancement of intestinal carcinogenesis in *Apc*^{Min} mice by VFM cannot be excluded to result from an increased caloric intake in mice fed a high fat diet supplemented with VFM. However, such an explanation cannot account for the low-fat diet results. Therefore it is suggested that VFM may have neoplasia-stimulating activities in high-fat diets, whereas it may not stimulate or even prevent intestinal tumor formation at low amounts of fat in *Apc*^{Min} mice. This may point to some kind of yet unknown interaction between dietary fat and VFM. However, it should also be kept in mind that although most studies have been carried out with a C57BL/6 background, a wide range in the number of observed spontaneous polyps has been reported. The numbers of intestinal polyps reported in this study are on the lower bound of this range, with no apparent explanation. Three different possibilities frequently discussed are the methodological differences in estimating polyp numbers, intrastrain variations and the composition of the gut microflora. With regard to the latter, Dove *et al.* (40) report that the microbial status does not strongly alter the adenoma phenotype, whereas Wasan *et al.* (30) suggest that under specified pathogen free conditions polyp numbers increase.

The diets used here have revealed different outcomes when given to rats in which colorectal cancer was induced by the chemical carcinogens, 1,2-dimethylhydrazine and MNNG, as reported previously (12,14,15). In both animal studies, fat significantly enhanced colorectal carcinogenesis, whereas VFM gave a pronounced protection against colorectal carcinogenesis only in the MNNG study. Apart from the use of chemical carcinogens, differences in species and the duration of the experiments, the different outcomes between these two studies and the presently used *Apc*-deficient mice may be ascribed to differences in genetic susceptibility. In humans, mutations at the *APC* gene are found in patients having familial adenomatous polyposis (FAP) and in most patients who have sporadic colorectal cancer. Various studies have demonstrated that *Apc*-deficient mice are an appropriate animal model system for intestinal neoplasia, and in particular for FAP (20,41). Therefore, it cannot be excluded that consumption of moderate amounts of vegetables and fruit in diets high in fat may enhance tumor progression in people who are genetically predisposed. However, additional research is clearly needed to further elucidate the relationship between genetic defects in the *Apc* gene and dietary habits.

In conclusion, the results of the present study have shown the absence of a modifying effect on intestinal neoplasia by dietary fat in *Apc*^{Min} mice. Notably, VFM added to low-fat

diets did not consistently protect against intestinal neoplasia, whereas the same mixture added to high-fat diets even resulted in an enhancement of intestinal tumors.

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