Effect of Different Degrees of Hydrogenated Fish Oil on Intestinal Carcinogenesis in *Min*/+ mice

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Abstract. *Intake of trans fatty acids from hydrogenated fish oils has been related to increased risk of coronary heart diseases. The possible effect on colorectal carcinogenesis is unclear. Materials and Methods: Multiple intestinal neoplasia (Min/+) mice were fed one of four experimental diets: either raw fish oil (FO), low (LHFO)-, high (HHFO)- or fully-hydrogenated fish oil (FFHO), from 0 to 9 weeks of age. The number and size of intestinal tumors were recorded. Results: There was no difference between the intervention groups in the numbers of developed intestinal tumors. The tumor size was statistically significantly lower in HHFO vs. the FO-group in male Min/+ mice. The HHFO and FHFO groups had lower weight gain than did the FO group (p=0.008 and p=0.04, respectively), but gender differences, due to effect of dietary intervention on weight gain, were found in Min/+ mice. Conclusion: When compared with raw fish oil, different degrees of hydrogenation of the fish oil had no effect on intestinal carcinogenesis in Min/+ mice.*

Partially hydrogenated oils rich in *trans* fatty acids are one of the suspected dietary risk factors for colorectal cancer (CRC). These are found mainly in Western dietary patterns, characterized by high total fat, meat and sugar intake, and low fiber intake. Such dietary patterns are associated with high CRC incidence (1). However, it is unclear whether a possible independent effect of *trans* fatty acids on CRC risk exists. In two studies in rats, dietary *trans* fatty acids did not enhance dimethylhydrazine-induced colorectal carcinogenesis (2, 3). Partially-hydrogenated fish oil (PHFO) was a major component in margarine and shortenings in bakery products in Norway until the late 1990s, and was one of the major sources of dietary *trans* fatty acids in Norway during the past decades. The composition of *trans* fatty acids differs between PHFO and partially-hydrogenated vegetable oil (PHVO). While PHVO mainly contains the monounsaturated elaidic acid (18:1*trans*), PHFO additionally contains several long-chain mono- and polyunsaturated *trans* fatty acids (18-22 carboatoms). Dietary raw fish oil has been shown to suppress intestinal polyp formation and growth in multiple intestinal neoplasia (*Min*/+) mice (4). The effect of dietary hydrogenated fish oils on the formation and growth of intestinal tumors has been poorly investigated. However, one recent human prospective study found no association between intake of PHFO and the risk of colonic or rectal cancer (5). Neither how dietary fully-hydrogenated fish oil (FHFO), in which all polyunsaturated fatty acids (PUFA) have been transformed into long-chained saturated fatty acids (SFA), might affect intestinal carcinogenesis, is known. However, it has been shown that dietary FHFO in rats has a low absorption rate, resulting in reduced weight gain (6). Multiple intestinal neoplasia (C57BL/6J *Min*/+) mice (*Min*/+ mice) are, similarly to humans with familial adenomatous polyposis (FAP) syndrome, heterozygous for a mutation in the tumor suppressor gene (Adenomatous polyposis coli) (*Apc*). This results in the development of numerous neoplastic lesions in the intestines. While human patients with FAP mainly develop the lesions in the colon, *Min*/+ mice mainly develop

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lesions in the small intestine (7). Since the initial molecular pathogenesis with loss of APC function is similar in sporadic colonic carcinogenesis in humans, the *Min*/+ mouse provides a model for colonic carcinogenesis (8).

The aim of this experiment was to investigate the effect of dietary fish oil hydrogenated to different degrees, on the number and size of intestinal tumors in *Min*/+ mice using raw fish oil as a reference.

Materials and Methods

C57BL/6J-*Min*/+ male mice and C57BL/6J-+/+ wild-type (genetic normal, wt) female mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed at the animal facility at the Norwegian Institute of Public Health for approximately 10 days before the study. The mice were kept in plastic cages with woodchip in the bottom, in a room with a 12-h light/dark cycle, controlled humidity (55±5%) and temperature (20-24˚C). During breeding, the mice were given SDS RM3 feed (Special Diets Services, Witham, Essex, UK) and water *ad libitum*.

Diets and experimental design. All experimental diets consisted of basic AIN 76 diet without oil (Research Diets, New Brunswick, NJ, USA; 50% sucrose, 20% casein, 15% corn starch, 5% fiber, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, 0.3% DL-methionine and 0.2% choline bitartrate), to which experimental fat and corn oil were added (AIN 76 diet/experimental fat/corn oil in 88/11/1 weight %, respectively). Corn oil was added to avoid lack of essential fatty acids (9). The four experimental fats were raw fish oil (FO), low-hydrogenated fish oil (LHFO), high-hydrogenated fish oil (HHFO) or fully-hydrogenated fish oil (FHFO). Because the aim was to examine whether an increased degree of hydrogenation of fish oil had any impact on intestinal carcinogenesis, we used the mice receiving FO as a reference/control group. Fatty acid compositions of the experimental fats are given in Table I. The total content of *trans* fatty acids (both monounsaturated fatty acids (MUFA) and PUFA) in the experimental fats were 0% in FO, 54% in LHFO, 38% in HHFO and <1% in FHFO. All experimental fats were produced from the same raw fish oil originating from South-American fish and subsequently routinely analyzed at Denofa AS, Fredrikstad, Norway. The fats were immediately wrapped in aluminum foil and kept frozen at –30˚C until the start of the experiment. During the experiment, the experimental fats were kept in boxes wrapped in aluminum foil at 4˚C, which upon opening were filled with nitrogen gas to avoid oxidation of fatty acids. The study design is given in Figure 1. When the pups were born, the four experimental treatments were randomized by cages. When three weeks old, the *Min*/+ and wt pups were identified by an allele-specific (PCR)-assay, as previously described (10). Wt female mice were terminated. The first two weeks after birth, the pups were nursed, and the experimental diets and water were given *ad libitum* in animal feed boxes from 3-5 days of age. At approximately two weeks of age, the animals started to eat the experimental diet. The mice were weighed weekly from 32 until 60 days old. At nine weeks of age, the animals were terminated by cervical dislocation. The small intestine and the colon were removed separately and rinsed in ice-cold phosphate-buffered saline (PBS) and the small intestine was divided into proximal, middle and distal parts. Before being longitudinally incised, the intestines were fixed flat between wet PBS filter papers for a minimum of 24 h in 10% neutral buffered formalin. The intestines were stained in 0.2% methylene blue (George T. Gurr Ltd., London, UK) and kept in 10% neutral buffered formalin for a minimum of 24 h before the number, diameter (mm) and location of lesions (cm distally from the ventricle) were examined and scored using an inversed light microscope (Nikon TMS-F, Melville, NY, USA). Tumor size (mm^2) was calculated considering the lesion as a circle, using the formula of circle area: Area=$\pi \times$ (diameter/2)2, where π=3.14. The small intestines were examined from the duodenum to the ileum, while the colon was

Table I. *Fatty acid composition of the four experimental fats*.

	Experimental fat			
	FO	LHFO	HHFO	FHFO
Fatty acid	(%)	(%)	(%)	(%)
14:0	8.3	8.7	8.8	7.7
15:0	0.6	0.6	0.6	0.5
16:0	21.7	21.4	25.6	36.4
17:0	0.5	0.7	0.5	0.6
18:0	4.2	4.8	9.5	22.7
19:0	0.3		0.1	0.2
20:0	0.2	0.5	3.4	17.7
22:0		0.3	1.8	11.7
24:0			0.2	0.8
Sum saturated fatty acids	35.8	37.0	50.5	98.3
14:1, n5	0.2	0.2	0.2	0.1
16:1, n7	10.5	11.8	6.8	0.1
17:1	1.5	0.2		
18:1, n7+n9	13.6	17.3	11.1	0.1
20:1, n7	0.2			0.2
20:1, n9	2.0			
22:1, n9	0.2			
22:1, n11	1.1			
24:1	0.3			
Sum monounsaturated fatty acids	29.6	29.5	18.1	0.5
C16:2, n6	1.3		0.1	
C18:2, n6	1.6	0.2	0.1	
C18:3, n3	0.8			
C16:4, n3	0.7		0.1	
C18:4, n1	0.2			
C18:4, n3	1.7			
C20 poly[a]		17.2	15.1	
C20:2	0.2			
C20:4, n3	1.0			
C20:5, n3	12.1			
C20:4, n6	1.1			
C22 poly[b]		9.9	11.5	
C22:4, n6	0.3			
C21:5, n3	0.6			
C22:5, n3	2.6			
C22:6, n3	8.4			
Sum polyunsaturated fatty acids	29.5	27.1	26.7	0
Sum *trans* fatty acids	0	54.0	38.0	0
Other	2.0	6.2	4.5	1.2
Total	100	100	100	100

FO: Fish oil; LHFO: low-hydrogenated fish oil; HHFO: high-hydrogenated fish oil and FHFO: fully-hydrogenated fish oil. [a,b]All fatty acids with this chain length are included.

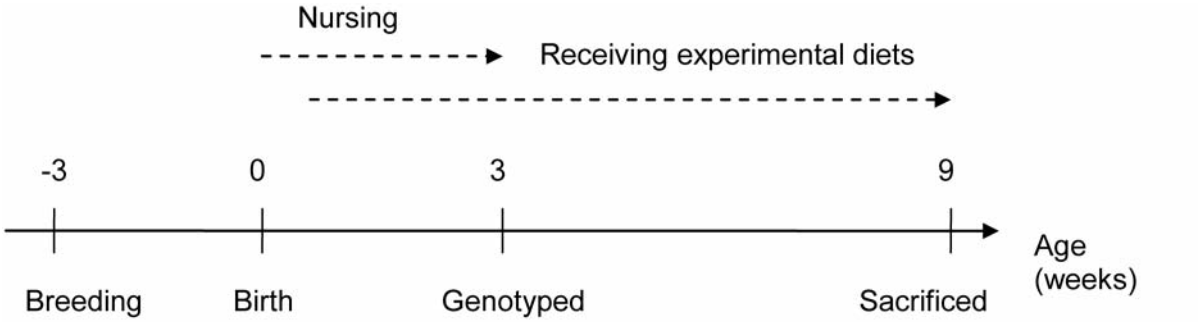

Figure 1. *Study design. Min/+ mice and their litter mates were treated in four different treatment groups: FO (raw fish oil), LHFO (low hydrogenated fish oil), HHFO (high hydrogenated fish oil) and FHFO (fully hydrogenated fish oil). The experimental diets were placed in the cages when the pups were 3-5 days old. The mother nursed the pups until they were genotyped at three weeks of age. From approximately two weeks of age, the pups started eating the experimental diet until sacrifice at nine weeks of age.*

examined from the proximal part to the anus. The animal experiment was approved by the Norwegian Animal Research Authority.

Statistical analysis. Data are presented as medians (25th, 75th quartiles). We analyzed differences in final body weight, number of tumors and tumor area among *Min*/+ mice in different intervention groups by independent samples median test, the FO group serving as the control group. We assessed body weight development in different types of mice and dietary intervention groups by fitting a growth model (the SAS MIXED procedure) with random intercepts for mice and fixed time, gender and intervention effects. *p*-Values <0.05 were considered as being statistically significant. We conducted the statistical analysis using the software SAS 9.2 (SAS Institute, Inc., Cary, NC, USA) and SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

A total of 86 mice were used in this experiment; 65 were *Min* mice (34 females and 31 males) and 21 wt mice (males) (Table II).

Effect of dietary intervention on the number of intestinal tumors. The total number of tumors in the small intestine in *Min*/+ mice was 3,220, with a median of 45 tumors per mouse. There was no difference in the median number of intestinal tumors between the dietary intervention groups (Table II). In comparison with the reference group (FO), the median tumor number did not differ in the three other dietary groups (data not shown). We did not observe any sex differences in the association between dietary intervention and tumor number. In the colon, a total number of 30 tumors were developed, with no significant differences between the dietary intervention groups (data not shown). The wt mice did not develop any intestinal tumors.

Effect of dietary intervention on intestinal tumor size and total tumor load. The mean tumor size was 0.39 mm^2. The median tumor load was 17.46 mm^2. There was no difference in the mean tumor size of each animal or tumor load between the dietary intervention groups (Table II). The mean tumor size was significantly lower in the HHFO group when compared with the control group (0.43 *vs*. 0.34 mm^2, respectively, $p=0.009$) (Figure 2). When stratified for gender, this difference was significant only in males. The tumor size and tumor load in the other three dietary groups did not differ from that of the control group (Figures 2 and 3). No gender differences were found in the association between other dietary intervention groups and tumor size or tumor load.

Final body weight. Overall, *Min*/+ mice males had a lower final body weight than did wt mice (median final weight 21.0 *vs*. 25.1 g, respectively, $p<0.001$). Within the group of *Min*/+ mice, males had a significantly higher final weight than did females (median final weight 23.9 *vs*. 19.3 g, respectively, $p<0.001$) (data not shown).

Effect of dietary intervention on body weight. There was no statistically significant difference in the final median weight between the dietary intervention groups in *Min*/+ mice (Table II). Among *Min*/+ males, there was a significant difference in final median body weight among the four dietary groups ($p=0.01$); the final median weight was lower in the FHFO group when compared to the control FO group (25.9 *vs*. 23 g, $p=0.041$). In *Min*/+ females, a significant difference in the final median body weight among the four dietary groups ($p=0.012$) was also found; the final weight was significantly higher in the LHFO group compared with the control group (21.3 *vs*. 18.9 g, respectively, $p=0.015$) (data not shown).

Weight gain was almost linear at the beginning of the study period and plateaued later (second-order time variable was significantly different from zero). Weight gain was significantly faster for male *Min*/+ mice as compared with female mice ($p<0.001$ for interaction between time of measurement and gender) (Figure 4, Table III). The *Min*/+ mice dietary intervention groups HHFO and FHFO had

Table II. *Min/+ mouse characteristics.*

	FO	LHFO	HHFO	FHFO	*p*-Value[a]
wt mice					
N	5	6	5	5	
Final body weight, day 60					
Median (25th, 75th)[b]	27.0 (24.9; 27.9)	23.6 (23.0; 25.5)	27.1 (25.6; 27.9)	24.4 (24.1; 24.8)	0.002
Min mice					
N	17	15	21	12	
Final body weight, day 60					
Median (25th, 75th)	23.9 (18.9; 25.9)	21.6 (20.9; 24.7)	20.4 (19.0; 22.1)	20.6 (18.0; 23.2)	0.113
Number of tumors					
Total	822	711	1073	614	
Median (25th, 75th)	49.0 (36.5; 52.5)	50.0 (27.0; 58.0)	42.0 (31.5; 66.0)	41.5 (30.5; 75.5)	0.451
Mean tumor size (mm^2)					
Median (25th, 75th)	0.43 (0.36; 0.51)	0.38 (0.26; 0.45)	0.34 (0.29; 0.41)	0.40 (0.29; 0.47)	0.068
Total tumor load (mm^2)[c]					
Median (25th, 75th)	21.9 (16.6; 24.9)	16.2 (11.4; 21.7)	14.5 (10.1; 24.4)	17.0 (10.9; 29.3)	0.174

FO: Fish oil; LHFO: low hydrogenated fish oil; HHFO: high hydrogenated fish oil and FHFO: fully hydrogenated fish oil; wt: wild-type. [a]independent-samples median test. [b]first and third quartiles, all such numbers in parentheses. [c]number of tumors × mean tumor area.

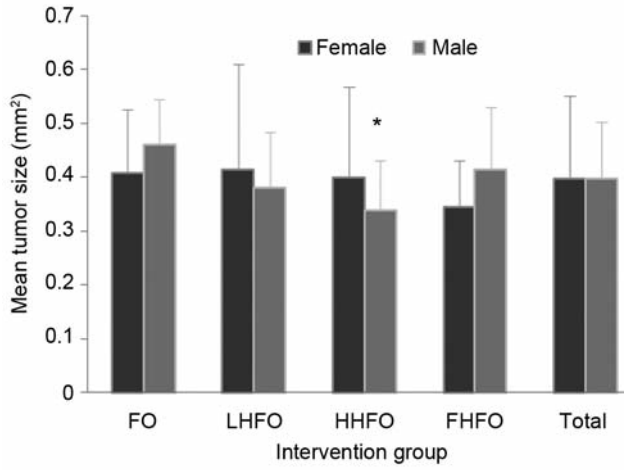

Figure 2. *Mean (±standard deviation) tumor size in the small intestine in male and female Min/+ mice in the four dietary intervention groups (FO; fish oil, LHFO; low-hydrogenated fish oil, HHFO; high-hydrogenated fish oil and FHFO; fully-hydrogenated fish oil). *Significantly different from the reference group for both sexes combined, p=0.009.*

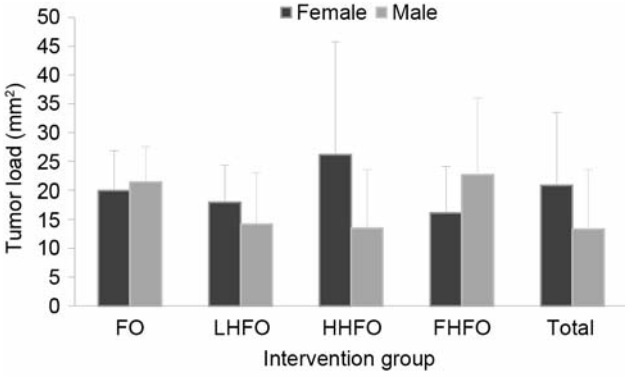

Figure 3. *Tumour load (mean tumor size × number of tumours) with standard deviation in small intestine in male and female Min/+ mice in the four dietary intervention groups (FO: fish oil; LHFO: low-hydrogenated fish oil; HHFO: high-hydrogenated fish oil; and FHFO: fully-hydrogenated fish oil).*

significantly lower weight gains than did the control group (p=0.008 and p=0.043, respectively), while no significant difference was observed between the FO and LHFO groups (Table III). There was a significant gender difference in the effect of dietary intervention on weight gain (a statistically significant interaction between genders in HHFO *vs*. FO, p=0.017, data not shown). The weight gain was significantly lower in *Min*/+ than wt males (p=0.001) (Table III). However, timing of the weight gain did not differ between wt and *Min* mice as no significant interaction between the type of mouse and time was found. When comparing *Min*/+ and wt males, we found no significant differences in the effect of dietary intervention on weight gain (data not shown).

Correlation between the final body weight, tumor number, size and load. There was no statistically significant correlation between final body weight and the number of tumors (Pearson's r=–0.156, p=0.214). However, there was a statistically significant positive correlation between final body weight and mean tumor size (Pearson's r=0.313, p=0.011). There was no difference in the number of tumors or the mean size between the control group and any of the intervention groups, when adjusted for starting or final body weight.

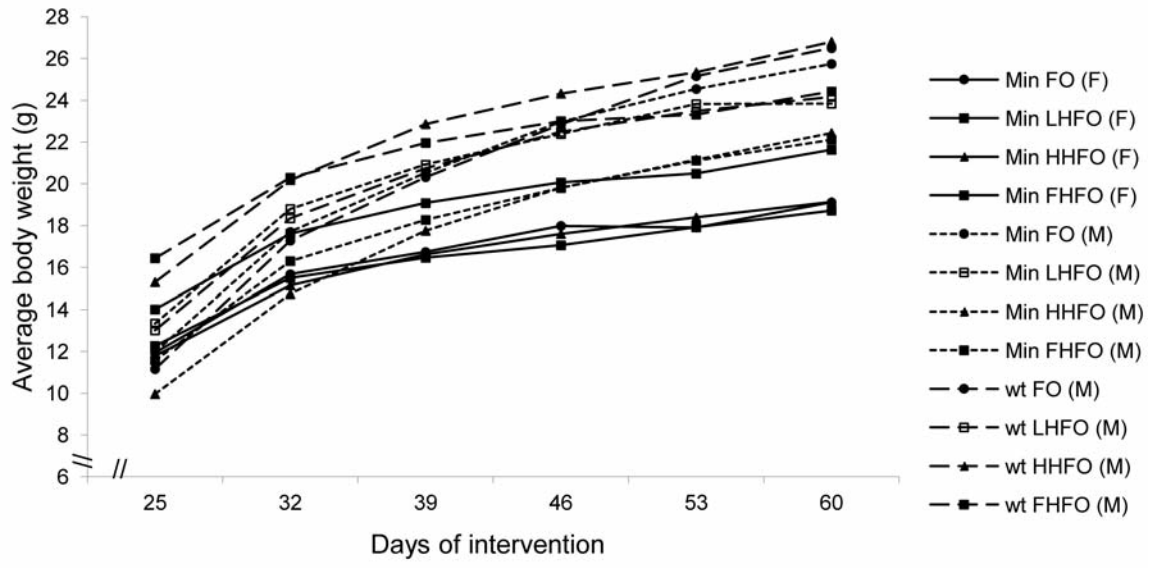

Figure 4. *Body weight of male (M) and female (F) Min/+ mice and male wild-type (wt) mice by dietary intervention group (FO: fish oil; LHFO: low-hydrogenated fish oil; HHFO: high-hydrogenated fish oil and FHFO: fully-hydrogenated fish oil).*

Table III. *Weight development in Min/+ mice and wild-type (wt) mice.*

	Min/+ mice		Male *Min*/+ and wt mice	
	Coefficient (95% CI)	*p*-Value	Coefficient (95% CI)	*p*-Value
Intercept[a]	12.90 (11.86; 13.94)	<0.001	14.12 (13.14; 15.10)	<0.001
Time, days	3.22 (2.97; 3.47)	<0.001	4.48 (4.21; 4.75)	<0.001
Time × Time	–0.38 (–0.42; –0.33)	<0.001	–0.45 (–0.50; –0.40)	<0.001
Gender[b]				
Male	–0.16 (–1.13; 0.81)	0.746	NA	
Mice[c]				
Min/+	NA		–2.12 (–3.34; –0.90)	0.001
Time × Gender	0.96 (0.82; 1.09)	<0.001		
Intervention				
FO – ref				
LHFO	1.13 (–0.15; 2.41)	0.085		
HHFO	–1.60 (–2.78; –0.43)	0.008		
FHFO	–1.40 (–2.76; –0.05)	0.043		

[a]Represents initial average weight of mice. [b]Reference, Female *Min*/+ mice. [c]Reference, wt mice. FO: Fish oil; LHFO: low-hydrogenated fish oil; HHFO: high-hydrogenated fish oil; FHFO: fully-hydrogenated fish oil.

Discussion

The incidence of colorectal cancer has, during past decades, increased more rapidly in Norway than in any other Nordic country (11), in parallel with the increase in margarine consumption (12), which was the main dietary source of PHFO and *trans* fats in Norway from the 1930s until 1998. Considering the high consumption of margarine during the past decades, PHFO is a dietary factor that makes the standard Norwegian diet different from that of other Nordic countries, and therefore it is of interest to investigate its effect on colorectal carcinogenesis. In this experimental study on *Min*/+ and wt mice, we showed that dietary hydrogenated fish oil did not affect spontaneous colon carcinogenesis differently from FO. However; hydrogenation of the fish oil had a negative effect on weight gain. The negative effect on weight gain in *Min*/+ mice was observed only for HHFO and FHFO, and not for the LHFO group. Some gender differences in the effect of different fat types on body weight was found in *Min*/+ mice, indicating that hydrogenated fish oil might have a negative effect on weight gain only in males. Increased body weight was associated with increased mean tumor size.

Our study is, to the best of our knowledge, the first of its kind to investigate the effect of hydrogenation of fish oil on intestinal carcinogenesis in a *Min*/+ mouse model. Only one earlier study has investigated the effect of hydrogenated fish oil on carcinogenesis in rats, concluding, in agreement with the present study, that hydrogenated fish oil had no effect on carcinogenesis (13). Results from rat experiments on the effect of dietary, partially-hydrogenated vegetable oils rich in *trans* fats on colorectal carcinogenesis are also in agreement with these results (2, 3, 14, 15). The reference (control) fat in the present study was raw fish oil, which has been found to reduce intestinal carcinogenesis in the *Min* mouse (4). Therefore, a considerably higher tumor load, as a result of hydrogenated fish oil treatment would have been expected if hydrogenated fish oil had a truly negative effect on colon carcinogenesis.

Epidemiological evidence is similar to our findings and does not confirm an association between dietary *trans* fat intake and CRC risk. A recent prospective study found no increased risk of cancer in the colon or rectum after intake of PHFO (5) and two large prospective studies in women found no association between dietary *trans* fat and CRC risk (16, 17). Retrospective studies have found increased risk (18-21), no effect (22, 23) or increased risk-only in those with low dietary calcium intake (22), or postmenopausal women not receiving postmenopausal hormone therapy (18).

Although there is a lack of convincing evidence of any direct association between dietary *trans* fat and colorectal cancer risk, several potential cancer-promoting mechanisms of *trans* fatty acids have been suggested. Such mechanisms are through incorporation of *trans* fatty acids into cell membrane phospholipids, resulting in oxidative stress and inflammation (24). Replacement of dietary SFA and cis-MUFA with *trans* fatty acids has been found to change the lipid profile in the serum and liver of rats (25). A *trans* fat-rich diet not only increased inflammatory markers in rats, but also in their offsprings (26). Human data suggest an association between *trans* fatty acid intake and markers of oxidative stress (27) and inflammation, at least in those who are overweight (28, 29). Whether this applies to the general population is, however, uncertain (30).

Restriction of energy intake reduces intestinal tumorigenesis in *Min*/+ mice (31). Hydrogenation of fish oil has been shown to reduce its absorption from the diet (6), and this is possibly the explanation for the lower weight gain in the HHFO and FHFO groups, as compared with the FO group. Thus, a possible negative effect on colonic carcinogenesis of HHFO or FHFO in the present study might have been compensated by the effect of the lower energy intake in these groups. The melting point of *trans* fatty acids is higher and digestibility lower, as compared with *cis*-unsaturated fatty acids of the same chain length. Therefore, comparison of their biological effects in a mouse model will in any case be confounded by differences in energy intake (32). Human studies support a protective effect of low energy balance; restricted energy intake in childhood has been associated with decreased risk of colorectal cancer in adulthood (33). On the other hand, a high energy intake is associated with increased cancer risk (34, 35). A weakness of this study is the short intervention time. A possible effect of hydrogenated fish oil on intestinal tumorigenesis in *Min*/+ mice cannot be excluded if it becomes apparent after a longer exposure time than 60 days, which was the age at termination in this study. We observed several very small tumors in female *Min*/+ mice in the HHFO group (data not shown). This might indicate a stronger effect of this fat type on intestinal carcinogenesis, which following a longer intervention time could have resulted in larger differences between the groups.

In conclusion, no effect of ingestion of fish oil-hydrogenated to different degrees, was found on the number or size of intestinal tumors in *Min*/+ mice, when using raw fish oil as a reference oil. However, intake of HHFO and FHFO resulted in reduced body weight gain when compared with the group receiving FO, possibly related to reduced bioavailability of the oil.

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