# Retinol-induced Intestinal Tumorigenesis in *Min*/+ Mice and Importance of Vitamin D Status

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**Abstract.** *The effects of life-long dietary exposure, starting in utero, to high retinol, low vitamin D, or high retinol in combination with low vitamin D on intestinal tumorigenesis in Min/+ mice were investigated. In males, high retinol alone significantly increased the number (2.6-fold) and size (1.3-fold) of small intestinal tumours; in females no significant increase in tumour number or size was seen. In both genders, low vitamin D intake alone did not affect intestinal tumorigenesis. In males, intake of the combined high retinol/low vitamin D diet did not further increase the effects caused by high retinol alone. In females, however, the high retinol/low vitamin D-induced increase in tumour number (3.1-fold) and tumour size (1.5-fold) exceeded that of high retinol alone. In conclusion, a high dietary intake of retinol stimulated intestinal tumorigenesis in Min/+ mice. Furthermore, the results indicate a combined effect of high retinol and low vitamin D on tumorigenesis in females.*

The role of vitamin D produced by sun exposure in reduced risk of colon cancer rates was first suggested by Garland and Garland (1). Later studies focusing on vitamin D intake or serum concentration of 25-hydroxyvitamin D (25(OH)D) have substantially supported a protective effect of vitamin D for colon cancer (2-4). The association between vitamin D intake and risk of colorectal cancer may, however, be modified by other factors. A recent follow up of the Nurses' Health Study reported that retinol intake was related to increased risk of colorectal adenoma in women and suggested that high retinol intake may antagonize the protective effects of vitamin D (5). However, an inverse association between plasma 25(OH)D and colorectal cancer did not seem to differ by retinol intake in males within the Health Professionals' Follow-up Study (HPSF) (6).

Effects of retinol and vitamin D, or their active metabolites retinoic acid and $1\alpha,25(OH)\text{-}D_3$, on intestinal tumorigenesis have been studied in murine models of familial adenomatous polyposis (FAP). In the *Min*/+ mouse model, mutation in one of the *Apc* alleles leads to spontaneous polyp formation throughout the gastrointestinal tract. The *Apc* gene is also mutated in most (>80%) sporadic adenomas and carcinomas in human. This gene plays multiple roles in the intestinal and colorectal epithelia and inactivation of this tumour suppressor gene seems to be an early and important event in the development of colorectal cancer. We previously reported that increased dietary intake of retinoic acid stimulated spontaneous formation and growth of intestinal tumours in the *Min*/+ mouse (7). Regarding the effects of vitamin D on tumorigenesis in *Min*/+ mice, administration of the active metabolite $1\alpha,25(OH)_2\text{-}D_3$ resulted in a decreased tumour load (8). In contrast, a Western-style diet, which is low in vitamin D, led to increased incidence of carcinomas in heterozygote *Apc1638* mice (9).

In the present study, we wanted to investigate the effects of i) high retinol, ii) low vitamin D and iii) combined high retinol and low vitamin D on intestinal tumorigenesis in the same *Min*/+ mouse model as used previously. Since earlier studies have shown that for *Min*/+ mice, most of the intestinal tumours are initiated already at week 1 after birth (10), the different diets were given continuously to dams from four weeks before breeding and to the *Min*/+ offspring after weaning. The high retinol and low vitamin D was chosen at levels representative for human diets resulting in high retinol and low vitamin D consumption when compared to recommended daily intakes.

## Materials and Methods

*Animals. Min*/+ mice were bred at the Norwegian Institute of Public Health, Oslo, Norway, as described elsewhere (11). C57BL/6 J-*Min*/+ males and C57BL/6 J-+/+ (wild-type) females were mated and the *Min*/+ offspring were identified by an allele-specific PCR assay as described earlier (12). Water and feed were given *ad libitum*.

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*Diets*. The diets used in this study (Dyets Inc., Bethlehem, Pennsylvania, USA) were Standard AIN-76A and three iso-caloric variations in which the content of retinol, vitamin D or both was modified. With respect to all other components, the content in all diets was similar, and identical to Standard AIN-76A. Retinol and vitamin D content in the four diets was: Normal retinol/normal vitamin D (Standard AIN-76A, 4000 IU/kg vitamin A palmitate, 1000 IU/kg vitamin $D_3$), high retinol/normal vitamin D (40000 IU/kg vitamin A palmitate, 1000 IU/kg vitamin $D_3$), normal retinol/low vitamin D (4000 IU/kg vitamin A palmitate, 100 IU/kg vitamin $D_3$) and high retinol/low vitamin D (40000 IU/kg vitamin A palmitate, 100 IU/kg vitamin $D_3$); conversion factor for retinol, 1 IU=0.3 μg; for vitamin D, 1 IU=0.025 μg. The high level of retinol was 10 times that of the standard level and the low level of vitamin D was 1/10 of the standard level. In humans, a high consumption of retinol may give 7-8 times the recommended dose and a low vitamin D diet may give 1/5-1/10 of the recommended dose. During gestation and lactation, the mice were given supplementary ground pellets from a self feeding automat to compensate for a noticed hardness of the pellets.

*Experimental design*. Dams were fed the experimental diets from four weeks before breeding. After weaning, the four groups were separated by gender, and their dietary regimes were continued until the mice were killed with $CO_2$ at the age of 12 weeks. The total number of *Min*/+ offspring in the normal retinol/normal vitamin D group was 16 (7 females and 9 males), in the high retinol/normal vitamin D group 22 (14 females and 8 males), in the normal retinol/low vitamin D group 25 (10 females and 15 males), and in the high retinol/low vitamin D group 23 (9 females and 14 males). The wild-type litter mates in each group were injected subcutaneously with azoxymethane (AOM) (10 mg/kg bw/injection) at week 12 and 13 and fed the different diets until sacrifice at week 60.

*25-Hydroxyvitamin D and retinol in serum*. After termination, blood was collected by punctuating the heart. Serum was separated from blood cells by centrifugation (10 min at 3300 rpm) and stored at –70˚C. Serum levels of 25(OH)D were measured by a competitive radioimmunoassay (DiaSorin-RIA, Stillwater, MN, USA). This method measures the sum of $25(OH)D_3$ and $25(OH)D_2$ in nmol/l. Intra- and interassay CVs were 6% and 14-18% (depending on the serum level), respectively. The measurement range with this assay was 13-250 nmol/l. Total retinol levels in serum (μmol/l) were measured by HPLC with UV detection (Vitas, Oslo Research Park, N-0349 Oslo, Norway).

*Scoring of tumours*. The abdomen was cut open and intestines were removed, prepared and stained as described elsewhere (11). The intestines were examined by transillumination in an inverse microscope to determine tumour number, tumour area ($mm^2$) and tumour position along the small intestine. Tumour position was scored as the distance from the stomach in sections of 1 cm. Tumour load was defined as the total tumour area in a given part of the intestine.

*Statistical analysis*. Body weights, tumour number, tumour size and vitamin status in different dietary groups were compared using two-way ANOVA on ranks. The all pair-wise multiple comparison with Tukey test was used to isolate groups that were different (SigmaStat statistical software version 3.1, Jandel Scientific, Erkrath, Germany). Spearman's rank order correlation was used to disclose any associations between serum levels of 25(OH)D or retinol and tumour number. A value of $p<0.05$ was considered significant in all methods. Large intestinal tumour incidence was analysed by Fisher's exact test.

## Results

*Effects of high retinol in the diet*. In comparison with the standard diet (normal retinol/normal vitamin D, Standard AIN-76) the high retinol diet (high retinol/normal vitamin D) tended to increase the number and size of small intestinal tumours in both gender (Figure 1). In males, a statistically significant 2.5-fold increase in small intestinal tumour number and a 1.3-fold increase in small intestinal tumour size were observed (Figure 1A). In females, however, the high retinol diet led to a moderate and not statistically significant increase only in tumour number (Figure 1B). In males, a statistically significant higher incidence of tumours in the large intestine was seen when both groups fed high retinol were compared to the groups fed the standard level of retinol (Table I). The high retinol diet seemed to stimulate both formation and growth of large intestinal tumours, but the change was only statistically significant for the high retinol/low vitamin D group when groups were analyzed separately (Table I). Among all females, only 2 colonic tumours were observed (one in each of the low vitamin D diet groups) and no statistical analyses were performed. No corresponding tumour formation was observed in the small intestine or colon of the AOM-exposed wild-type litter mates (data not shown).

*Effects of low vitamin D in the diet*. In comparison with the standard diet, the low vitamin D diet (normal retinol/low vitamin D) tended to increase the number of small intestinal tumours in both genders (Figure 1A and 1B). However, none of these differences were statistically significant. In males, the number and size of colonic tumours did not differ between the low vitamin D group and the standard diet group (Table I).

*Effects of combined high retinol and low vitamin D in the diet*. In males fed the high retinol/low vitamin D diet, intestinal tumorigenesis was stimulated in comparison with males fed the standard diet. In the small intestine, there was a statistically significant 2.6-fold increase in tumour number and a 1.3-fold increase in tumour size (Figure 1 A). In the large intestine, the parallel increase in number and size was 9-fold and 2.9-fold, respectively (Table I). However, the stimulations seen with the high retinol/low vitamin D diet did not exceed the effect observed with the high retinol/normal vitamin D diet. Furthermore, independently of the vitamin D level, the number of tumours in each size class (Figure 2A) and tumour load distribution along the small intestine (Figure 3A) were similar in the two male groups fed high retinol diets.

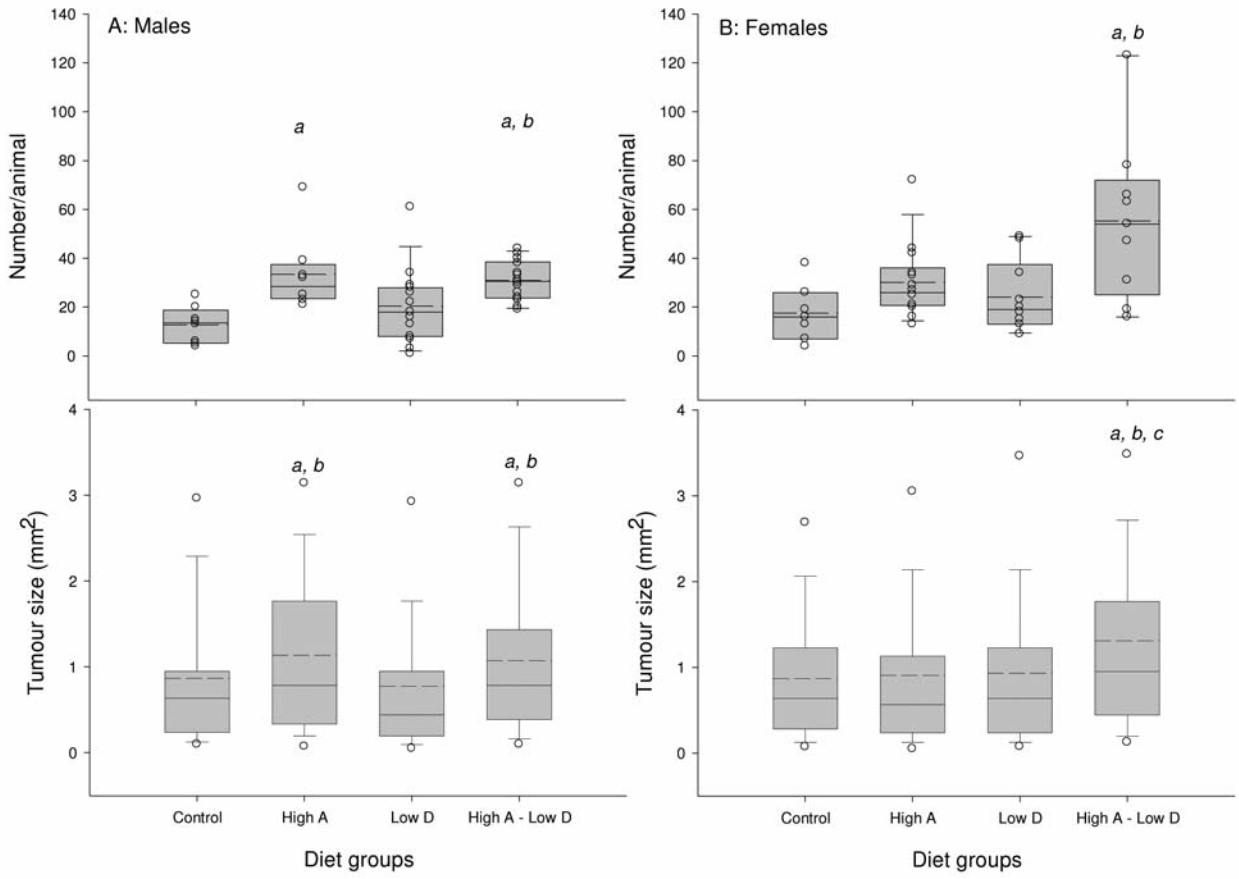


Figure 1. *Effects of dietary retinol and vitamin D on the number and size of tumours in the small intestine in A, male and B, female Min/+ mice. Boxes indicate the 25th and the 75th percentile; error bars indicate the 90th and 10th percentiles. Within the box, the continuous line marks the median and the dotted line the mean value. The circles represent individual values for each mouse. Two-way ANOVA on ranked values showed differences between diets regarding tumour number ($p<0.001$) and tumour size ($p<0.001$), and interaction between gender and diet on size of the tumours ($p<0.001$). All pair-wise multiple comparison procedures with Tukey test ($p<0.05$): a vs. normal retinol/normal vitamin D; b vs. normal retinol/low vitamin D; c vs. high retinol/normal vitamin D.*

Table I. *Effects of dietary retinol and vitamin D on the number and size of large intestinal tumours in male Min/+ mice. In females, there were too few tumours to perform statistical analysis.*

| Diet | Tumour incidence[c] | Mean tumour number/animal | Mean tumour size/animal ($mm^2$) |
|---|---|---|---|
| Normal retinol/normal vit. D | 1/8 | 0.1±0.1 | 1.57±1.5 |
| High retinol/normal vit. D | 3/8 | 0.8±0.4 | 4.30±2.22 |
| Normal retinol/low vit. D | 3/15 | 0.1±0.1 | 0.42±0.29 |
| High retinol/low vit. D | 9/14 | 0.9±0.3[a,b] | 4.58±1.44[a,b] |

Values are given as means±SEM. All pair-ways multiple comparison procedure with Tukey test ($p<0.05$): [a]*vs.* normal retinol/normal vitamin D, [b]*vs.* normal retinol/low vitamin D. Fisher's exact test ($p<0.05$): [c]higher incidence in both groups fed high retinol *vs.* both groups fed standard level.

In females fed the high retinol/low vitamin D diet, there was also a statistical significant increase in tumour number and tumour size in the small intestine in comparison with females fed the standard diet (3.1- and 1.5-fold increase, respectively). However, and contrary to the observations in males, in female mice the effect of the high retinol/low vitamin D diet exceeded that observed with high retinol alone, although only the tumour size data were statistically different (Figure 1 B). There was also a marked difference in the tumour size distribution between the two high retinol groups.

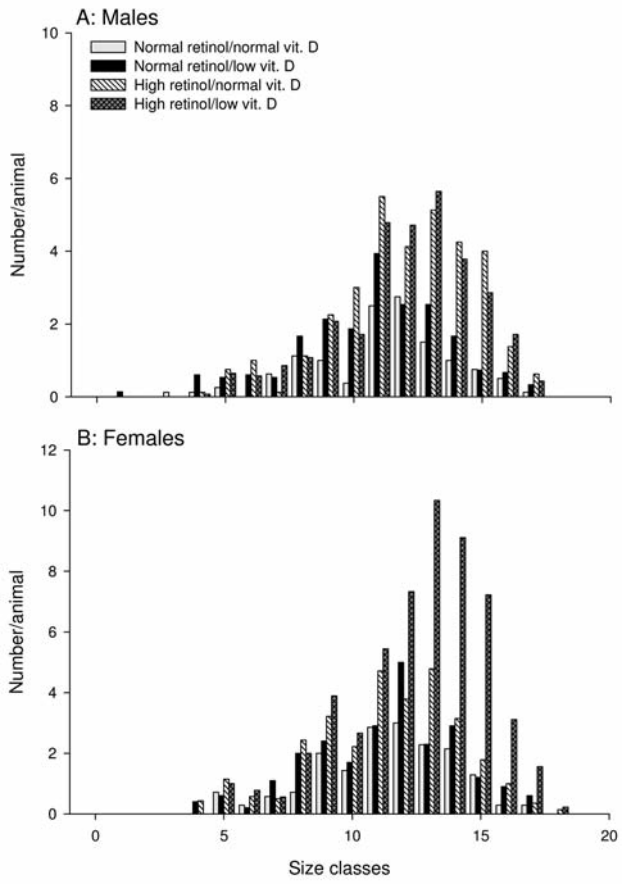


Figure 2. *Effects of dietary retinol and vitamin D on the size distribution of small intestinal tumours in A, male and B, female Min/+ mice, expressed as the mean tumour number/animal of increasing size classes. For each experimental group, the entire tumour population was divided into classes with subsequently increasing tumour sizes. Each size class represents a successive multiplication of the tumour size by 1.5: size class 1 ≤0.01 mm$^2$; size class 2 0.01 to ≤0.015 mm$^2$ etc. The mean tumour size/diet group is shown in Figure 1.*

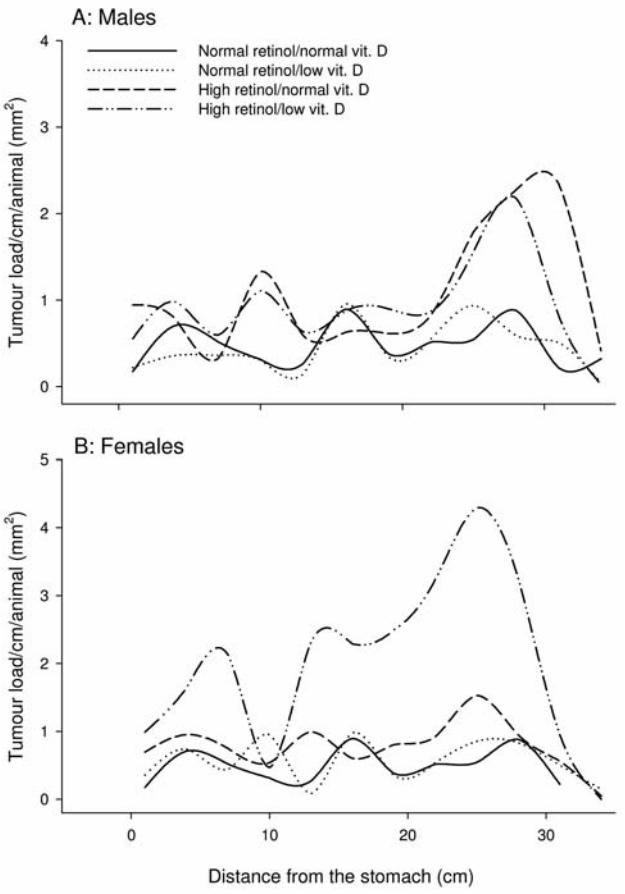


Figure 3. *Effects of dietary retinol and vitamin D on the distribution of tumour load (total tumour area) along the small intestine in A, male and B, female Min/+ mice. Distribution of tumour load is expressed as the mean tumour load/cm/animal (mm$^2$) in sections of 3 cm. Each tumour load score is given as the distance (cm) from the stomach.*

In the group fed the high retinol/low vitamin D diet, the larger size classes of tumours constituted the largest subset (Figure 2B). Furthermore, the combination of high retinol and low vitamin D induced a marked and statistically significant different profile of the tumour load distribution along the small intestine when compared with the tumour load distribution found after intake of high retinol combined with normal vitamin D (Figure 3B) ($p$<0.001, two-way ANOVA).

*Serum levels of 25(OH)D and correlation with tumour formation*. Vitamin D status (serum 25(OH)D concentration) depended significantly on vitamin D intake (Table II). Additionally, it was observed that in offspring on a low vitamin D diet, a high intake of retinol caused a further reduction of the vitamin D status. This possible effect of high retinol on vitamin D status reached statistical significance only in males. A similar effect was not seen in the dams. Among mice fed the two low vitamin D diets (low vitamin D with normal or high retinol content), there was a strong negative correlation between vitamin D status and tumour formation (Figure 4A) (r=–0.7, $p$<0.001, Spearman's rank correlation). In contrast, such correlation was not observed among mice fed the normal vitamin D diets (Figure 4B) (r=0.24, $p$=0.20). The strongest negative correlation between serum levels of 25(OH)D and the number of small intestinal tumours was observed among the females fed the high retinol/low vitamin D diet (r=–0.87, $p$<0.001).

*Serum levels of retinol and correlation with tumour formation*. Retinol in plasma of the mice was not dependent on retinol intake (Table II). Retinol in plasma is strictly regulated and may therefore not reflect the total availability

Table II. *Serum level of 25(OH)D and retinol, and body weights of male and female offspring and their dams at the time of sacrifice.*

| Diet | 25(OH)D (nmol/l) | | | Retinol (μmol/l) | | | Body weight (g) | | |
|---|---|---|---|---|---|---|---|---|---|
| | ♀ | ♂ | Dams | ♀ | ♂ | Dams | ♀ | ♂ | Dams |
| Normal retinol/normal vit. D | 65.3±14[a,c] (n=4) | 103±6.2[a,c] (n=5) | 102±7.8[a,c] (n=10) | 1.04±0.10 (n=4) | 1.26±0.06 (n=5) | 0.57±0.10 (n=10) | 21.5±0.5 (n=7) | 25.3±0.8 (n=9) | 31.7±1.1 (n=20) |
| High retinol/normal vit.D | 105±6.0[a,c] (n=12) | 121±9.8[a,c] (n=8) | 106±4.9[a,c] (n=9) | 0.92±0.03[c] (n=12) | 1.27±0.13 (n=8) | 0.79±0.10[c] (n=9) | 21.6±0.4 (n=14) | 26.1±1.2 (n=8) | 29.9±0.7 (n=10) |
| Normal retinol/low vit.D | 22.4±2.1 (n=8) | 26.6±1.7[c] (n=12) | 24.4±2.9 (n=11) | 1.10±0.07 (n=8) | 1.45±0.07 (n=12) | 0.84±0.08 (n=12) | 19.9±0.5 (n=10) | 24.6±0.6 (n=15) | 32.6±0.7 (n=11) |
| High retinol/low vit.D | 17.5±1.8 (n=8) | 17.5±1.5 (n=13) | 26.6±1.9 (n=11) | 1.17±0.06 (n=8) | 1.62±0.13 (n=13) | 0.97±0.06 (n=11) | 20.2±0.8 (n=9) | 24.8±0.9 (n=14) | 31.6±1.4 (n=9) |

Values are given as mean±SEM. Two-way ANOVA on ranked values: gender difference on 25(OH)D ($p$=0.039), retinol ($p$<0.001) and offspring body weight ($p$<0.001); differences between diets on retinol ($p$=0.022). All pair-wise multiple comparison procedures with Tukey test ($p$<0.05): [a]*vs*. normal retinol/low vitamin D; [b]*vs*. high retinol/normal vitamin D; [c]*vs*. high retinol/low vitamin D. Differences between number (n) of serum samples and animals within some groups are due to pooling of blood samples from litter mates of similar gender when serum volume from one animal was too low for analysis of 25(OH)D and retinol.

and storage of retinol in the body. However, higher serum levels of retinol were observed both in females ($p$=0.003, Mann-Whitney rank sum test) and males ($p$=0.031, Mann-Whitney rank sum test) fed the two low vitamin D diets in comparison with their respective female and male groups fed the two normal vitamin D diets. A corresponding observation was made in the dams ($p$=0.017, Mann-Whitney rank sum test). There were no correlations between serum levels of retinol and small intestinal tumours within any of the diet groups.

*Final body weight and general health observations*. The dietary treatments did not affect the final body weights of the offspring, but males were significantly larger than females (Table II) ($p$<0.001, two-way ANOVA). The litter size and body weight of the dams did not vary between the groups fed different diets (Table II). There were no clinical signs of rickets due to the reduced intake of vitamin D or other visible signs of toxicity among the dams or the offspring in any of the diet groups.

## Discussion

The main finding in our study was that high retinol intake increases tumorigenesis in the small intestine of Min/+ mice. In males, the observed increase seemed to be independent of the serum level of vitamin D, whereas in females the effect of retinol was enhanced in mice with a low vitamin D status. There was a strong negative correlation between vitamin D status and tumour formation among mice fed the low vitamin D diets. Serum 25(OH)D is widely accepted as indicator of vitamin D status, however the physiologically active form of vitamin D is 1,25$(OH)_2$D. Transformation of 25(OH)D to 1,25$(OH)_2$D by the enzyme 1α-hydroxylase takes place mainly

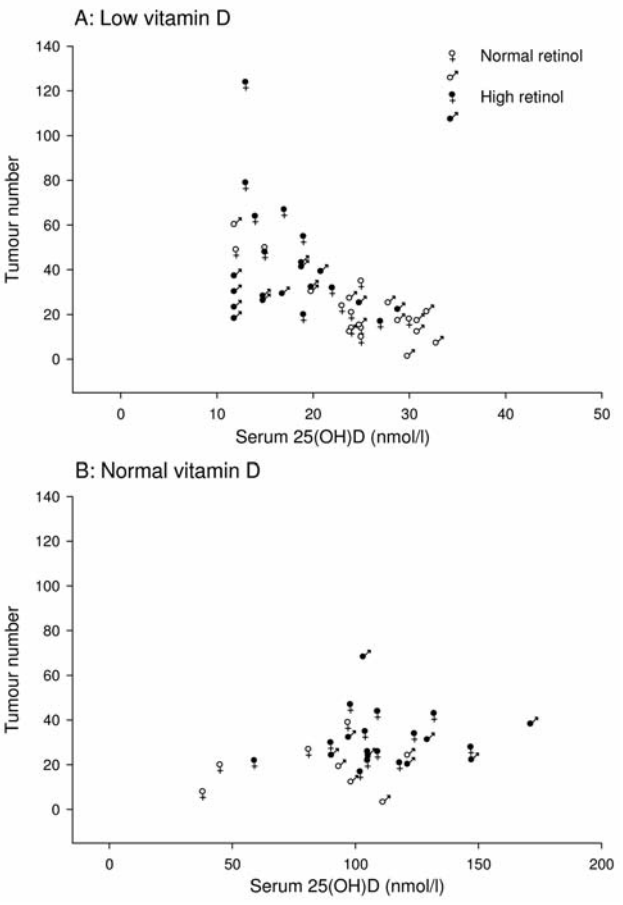


Figure 4. *Correlation between serum level of 25(OH)D and number of small intestinal tumours in Min/+ mice. A, Males and females fed low vitamin D and normal retinol (open symbols), or low vitamin D and high retinol (filled symbols). B, Males and females fed normal vitamin D and normal retinol (open symbols), or normal vitamin D and high retinol (filled symbols). The mean tumour number/animal/diet group is shown in Figure 1.*

in the kidneys, but the intestinal epithelium and other organs have also been shown to produce the active form. $1,25(OH)_2D$ is tightly regulated and is therefore not useful in measuring the total vitamin D status of an individual (13). However, potential differences in concentrations of the active form between the high and low vitamin D groups cannot be excluded.

The increased intestinal tumorigenesis in *Min*/+ mice by dietary retinol alone is in line with our previous study showing similar stimulatory effects with dietary retinoic acid in the same model (7). Neither retinoic acid (7) nor retinol (unpublished data) induced tumours in the unexposed (retinoic acid) or AOM-exposed (retinol) wild-type litter mates. The stimulatory effects of retinoic acid and retinol in *Min*/+ mice are in conflict with the general chemopreventive effect of retinoids reported in wild-type rodent models of chemically induced colorectal cancer (14, 15). More specifically, retinol has been shown to reduce β-catenin levels in retinoic acid-resistant colon cancer cell lines (16, 17), as well as in 1,2-dimethylhydrazine-exposed rats (18). Hence, our observations indicate that the described stimulatory effect of retinol and retinoic acid is linked to the *Apc* gene germline mutation and the loss of *Apc* function that initiate spontaneous intestinal tumorigenesis in *Min*/+ mice (19). The *Apc* gene plays multiple roles in the intestinal and colorectal epithelia, and inactivation of this tumour suppressor gene also seems to be an early and important event in sporadic adenomas and carcinomas in human. Results obtained from the *Min*/+ mouse model are therefore considered relevant for cancer development beyond those affected by FAP.

Recent studies show that *Apc* is involved in the regulation of retinol dehydrogenase L (20) and the retinoic catabolic enzyme CYP26A1 (21). It is not known whether lost *Apc* function and a subsequent dysregulation in intestinal retinol metabolism could be related to our findings. Another speculation for the stimulatory effect of retinol/retinoic acid may be linked to the increased level of peroxisome proliferator-activated receptor (PPAR)β/δ that we recently observed in intestinal lesions of *Min*/+ mice (22). PPARβ/δ has been proposed to be a downstream target of the *Apc*/β-catenin pathway (23), and it is reported that activation of PPARβ/δ by a specific synthetic ligand accelerated intestinal adenoma growth in *Min*/+ mice (24). Interestingly, retinoic acid is also a ligand for PPARβ/δ (25) and it has been demonstrated that shifted binding of retinoic acid from RAR to PPARβ/δ will enhance cell proliferation by the PPARβ/δ signalling pathway at the expense of the RAR signalling pathway of growth inhibition (26, 27).

The low vitamin D diet used in our study did not result in a significant increase in tumour formation. This is in contrast to the increased tumour formation in *Min*/+ mice following intake of the vitamin D-deficient New Western style diet (28). The New Western style diet is, however, also low in calcium. In a number of experimental models, the *Min*/+ mice model included, Yang *et al*. reported that supplementation of calcium and vitamin D antagonized the increased tumour formation caused by the New Western style diet (28). These observations could indicate that the low vitamin D diet used in our study, when combined with a normal calcium level, was not sufficient for a significant stimulation of intestinal tumorigenesis. Even if the tumorigenesis of mice fed low vitamin D as a group did not differ significantly from controls, there was a strong negative correlation between vitamin D status and tumour formation in these animals. In contrast, such correlation was not observed among mice fed the normal vitamin D diets. Dose–response gradients between serum 25(OH)D and risk of several types of cancer have been described in humans, and levels for increasing protection have been suggested (29). Our finding may support the idea of a critical level for serum 25(OH)D under which a concentration-dependent increase in colon cancer risk occurs.

When *Min*/+ mice were fed a diet high in retinol and low in vitamin D, a significant increase in tumour formation was induced in both genders. In males, however, the effect did not exceed that of the high retinol diet alone, indicating independence of vitamin D. The similar profiles of tumour size distribution and tumour load distribution along the small intestine observed for males fed high retinol with either low or normal vitamin D further illustrate this finding. In contrast, a combined effect of high retinol and low vitamin D seems to apply for females. A significant increase in tumour number per animal and mean tumour size was induced in the high retinol/low vitamin D group compared to the high retinol group. Furthermore, the increased number of larger tumours as well as the altered distribution of tumour load along the small intestine indicates a potential combined effect of high retinol and low vitamin D in females. Interestingly, the strongest negative correlation between vitamin D status and tumour number was observed among these females. A possible difference between females and males in response to the combined increased retinol and low vitamin D intake is supported by two recent studies. The follow up of the Nurses' Health Study showed that retinol intake was related to increased risk of colorectal adenoma in women, especially when combined with a low vitamin D intake (5). An inverse association between plasma 25(OH)D and colorectal cancer did not seem to differ by retinol intake in males within the Health Professionals' Follow-up Study (6).

An antagonistic effect of vitamin D and retinol was previously found on serum calcium after combined intake in humans (30). Antagonism of retinol on effects of vitamin D on bones and intestine was later demonstrated in female rats (31), and an antagonistic effect of retinol on calciferol and 25(OH)D3 was also shown in male rats (32). The past high retinol concentration in cod liver oil was also suggested as a possible explanation for the observed negative association

between childhood cod liver intake and bone mineral density in women (33). Several mechanisms may be involved in an antagonistic effect between retinol and vitamin D. Competition between the active metabolites of retinol and vitamin D, retinoic acid (RA) and 1,25-hydroxyvitamin $D_3$, for the retinoic X receptor (RXR), which forms heterodimers both with the vitamin D receptor (VDR) and the RA receptors (RARs), has been suggested (34). However, retinol could also interfere with the absorption, transport and conversion to the active form or the degradation of vitamin D. The observed difference in the tumorigenesis in males and females fed the high retinol/low vitamin D diet could be due to potential interactions of estrogen with vitamin D and/or calcium in the females. Such interactions were suggested for the different effect of calcium and vitamin D on colorectal cancer risk between estrogen users and non-users in a reanalysis of the Women's Health Initiative randomized trial (35). The observation that retinol lowers vitamin D status may also illustrate a potential antagonistic effect of retinol on vitamin D, and it accords well with previous findings in female rats (31).

In conclusion, a continued increased intake of retinol stimulated intestinal tumour formation in *Min*/+ mice. In males, the effect of high retinol intake seemed independent of the vitamin D intake. In females, however, the effect of high retinol was markedly increased when the intake of vitamin D was low. These results indicate a combined effect of high intake of retinol and low intake of vitamin D on intestinal tumour formation in females. Furthermore, a strong negative correlation between vitamin D status and tumour formation among mice fed the low vitamin D diets was demonstrated. Such correlation was not observed among mice fed the normal vitamin D diets.

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