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Gender-specific modulation of tumorigenesis by folic acid supply in the *Apc* $^{+/Min}$ mouse during early neonatal life

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Epidemiological studies suggest an inverse association between folic acid intake and colorectal cancer risk. Conversely, conventional treatment of existing tumours includes the use of folate antagonists. This suggests that the level of exposure to folate and its timing in relation to stage of tumorigenesis may be critical in determining outcomes. We hypothesised that folic acid depletion *in utero* and during early neonatal life may affect tumorigenesis in offspring. To investigate this hypothesis, female C57Bl6/J mice were randomised to a folic acid adequate (2 mg folic acid/kg diet) or folic acid depleted diet (0·4 mg folic acid/kg) from mating with *Apc* $^{+/Min}$ sires and throughout pregnancy and lactation. At weaning the *Apc* $^{+/Min}$ offspring were randomised to a folic acid adequate (2 mg folic acid/kg diet) or depleted (0·26 mg folic acid/kg diet) diet, creating four *in utero*/post-weaning dietary regimens. At 10 weeks post-weaning, mice were killed and the intestinal tumour number and size were recorded. Folic acid depletion during pregnancy and post-weaning reduced erythrocyte folate concentrations in offspring significantly. Folic acid depletion during pregnancy and lactation did not affect tumour multiplicity or size. However, female mice fed normal folic acid diets post-weaning had more, and larger, tumours when compared with depleted females and both depleted and adequate folic acid fed males. These data suggest that folate depletion post-weaning was protective against neoplasia in female *Apc* $^{+/Min}$ mice and highlights the need for further investigation of the optimal timing and dose of folic acid supplementation with regard to colorectal cancer risk.

Folate: Intestinal tumours: Gender: *In utero*

Variation in diet and nutritional factors contribute about 50 % of variability in risk of developing colorectal cancer (CRC) in Westernised countries[1]. Efforts have been focused on understanding the dietary factors that influence CRC development. Substantial epidemiological evidence indicates that low folate intake or status is associated with increased risk of CRC[2–5] but this association is not consistent and some studies have failed to demonstrate a relationship[6,7].

The main metabolic role of folate is to carry one carbon unit[8] and, as such, folate is an essential cofactor in the purine and thymidylate synthesis and in the methylation of biological molecules, e.g. DNA. Due to evidence that loss of genomic integrity is fundamental to tumour development[9] a plausible case can be made that folate status may impact upon carcinogenesis. Aberrations in DNA synthesis, stability and repair due to a lack of folate have been associated with increased mutations, DNA strand breaks and impairments in DNA repair ability, all of which could increase the risk of neoplasia[10]. In addition, alterations in DNA methylation patterns have been reported in tumours at many sites including the colorectum[11]. Since DNA methylation is an important epigenetic determinant of gene expression, aberrations may lead to incorrect transcriptional control and thus increase tumour risk[12]. Aberrant DNA methylation also plays a role in the development of mutations, as well as affecting DNA integrity, DNA stability and chromosomal modifications[12].

Some human epidemiological studies support the hypothesis that supplemental folate, usually provided as folic acid, is protective against CRC development[2,13–15], but the evidence is equivocal and uncertainty remains about the effects of enhanced folic acid intake on CRC development[16]. Moreover, experiments using animal models have uncovered conflicting evidence about the relationship between folate supply and intestinal carcinogenesis. Cravo *et al.*[17] reported that moderate folate deficiency non-significantly enhanced the development of neoplasia in the colon of 1,2 dimethylhydrazine (DMH)-treated rats and Kim *et al.*[18] reported that supplementation with folate conferred protection against the development of microscopic to macroscopic foci in the same model. Furthermore, increased numbers of aberrant crypts have been observed in DMH-treated rats fed a folate-free diet compared with animals fed a normal folate diet (2 mg folic acid/kg diet)[19]. Feeding a folate-free diet to rats exposed to the mutagen tribromomethane in drinking water significantly increased aberrant crypt foci when compared with tribromomethane-exposed rats fed a normal folate diet (2 mg/kg)[20]. In contrast, in

Abbreviations: CRC, colorectal cancer; DMH, 1,2 dimethylhydrazine; RBC, erythrocyte; SI, small intestine.

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azoxymethane-treated rats, Le Leu *et al.*[21] reported that a folate-free diet reduced the development of colonic aberrant crypt foci over 12 weeks and reduced tumour number in the small and large intestine over 26 weeks compared with rats fed 8 mg folic acid/kg diet[22]. In adult *Apc* $^{+/Min}$ mice, which develop multiple intestinal neoplasms spontaneously, Song *et al.*[23] reported a protective effect of folate against small intestinal tumours, which was dose-dependent. In contrast, Sibani *et al.*[24] observed fewer tumours in *Apc* $^{+/Min}$ mice fed a diet deficient in folate and choline. Given the key role of folate in one carbon metabolism and cell division, it is conceivable that extra folate could enhance the growth of *in situ* tumours. Indeed, in the *Apc* $^{+/-}$*Msh2* $^{-/-}$ mouse, timing of folate supplementation was found to be critical to its effect upon tumorigenesis[25]. In mice given a folate-supplemented diet (8 mg folic acid/kg diet) from 3 weeks of age, intestinal and colonic adenomas were 2·7- and 2·8-fold lower than in folate-depleted mice (0 mg folic acid/kg diet). However, when folate was supplemented from 6 weeks of age, mice had 4·2-fold more small intestine (SI) adenomas than folate-depleted mice. Therefore, the protective (or otherwise) effects of folate in colorectal carcinogenesis remain to be characterised, with the likelihood that dose and timing may be critical to potential chemopreventative outcome.

Adequate folate supply is important during pregnancy. Moderately folate-deficient rats have a reduced litter size and birth weight compared with normal folate animals and severely deficient rats have an increased risk of fetal demise[26]. Folate deficiency during embryonic development caused increased rates of fetal resorption, malformations and post-partum death in mice[27]. Although the role of folate in spontaneous abortion in human females remains to be ascertained, low folate intake has been associated with low birth weight in human newborns[28] and the beneficial effect of supplemental folic acid in the prevention of neural tube defects is well documented[29]. Maternal nutrition post-partum is also important for the developing infant. Infant plasma folate concentration has been correlated directly with levels of folate in breast milk[30] and, although folate is taken up preferentially by the actively secreting mammary gland, a severe maternal deficiency could affect folate supply to the infant[31]. Maternal folate deficiency in rats has been associated with poor growth of pups during lactation[32].

There is increasing evidence to support the developmental origins of adult disease theory, in which it is hypothesised that exposures during early life may contribute to risk of disease in adulthood. The association between birth weight and adult diseases (such as type 2 diabetes, CHD and hypertension) has been attributed to poor nourishment *in utero*, under which condition the fetus adapts to enhance survival by altering metabolism and reducing body size[33]. The effects upon adult health may be due to the mechanisms that are used to convey these changes in the fetus. Given that increased incidence of CRC has been associated with low dietary folate status and that early life nutrition has a substantial influence on adult health, we hypothesised that folic acid depletion *in utero* and pre-weaning could affect tumorigenesis in adult *Apc* $^{+/Min}$ mice.

Materials and methods

Animal housing, husbandry and diets

Mice were housed in the Comparative Biology Centre (Newcastle University) at a temperature of 20–22°C and with 12 h light and 12 h dark cycles. Fresh water was available *ad libitum*. Experimental diets were based on the AIN-93G[34] and contained 0·26, 0·4 or 2 mg folic acid/kg diet. The 2 mg folic acid/kg diet was the control diet (containing the concentration of folic acid considered normal for rodents[34]) and the folic acid content was provided by the AIN93VX vitamin mix. The lower folic acid diets were prepared using a folic acid-free AIN93VX vitamin mix to which the appropriate amount of folic acid was added. The 0·4 mg folic acid/kg diet was fed to dams to induce folic acid depletion during pregnancy and lactation, whilst the 0·26 mg folic acid/kg diet was used to induce depletion of folic acid status in weaned mice as evidenced by significantly reduced erythrocyte (RBC) folate concentration.

Two C57BL/6J (Black 6) female mice were mated with each *Apc* $^{+/Min}$ male. Breeding mice were offered an experimental diet (6 g/d) containing either a normal folic acid (2 mg/kg) or depleted folic acid (0·4 mg/kg) concentration. Once females were observed to be pregnant (by presence of a vaginal plug and/or swollen abdomen) they were re-caged and the quantity of food offered was increased to 10 g/d. At 2 weeks post-partum, the diet was further increased to 20 g/d to ensure sufficient food supply for weanling pups and for the lactating female.

Sub-sets of pups and all dams were killed at weaning (mean 32 d post-partum). Blood was collected by cardiac puncture for RBC folate analysis and the intestines of the pups were weighed and measured. Following genotyping, the remaining offspring were re-caged (one to four animals per cage) and assigned at random to the normal (2 mg/kg) or depleted (0·26 mg/kg) folic acid diet (6 g/d). This resulted in four dietary intervention groups of adult offspring: normal folic acid pre- and post-weaning (NN); depleted folic acid pre- and post-weaning (DD); normal folic acid pre-weaning followed by depleted folic acid post-weaning (ND); depleted folic acid pre-weaning followed by normal folic acid post-weaning (DN). Body weights of animals were recorded weekly post-weaning. Folic acid depletion did not alter growth weights of animals compared with controls. After an average of 70 d on the post-weaning experimental diets, 148 mice (thirty-seven per treatment group) were killed for sample collection.

Genotyping

Pups were genotyped at a mean age of 29 d by a standard PCR procedure (using DNA extracted from a tail biopsy) followed by restriction digest[35].

Sample collection and analysis of gut tumours

Animals were anaesthetised using gaseous isoflurane. Blood was collected by cardiac puncture into an EDTA collection tube and protected from light. Total body, liver, SI, colon and caecum weights were recorded and the lengths of the SI and colon were measured. The SI was cut into two equal sections, the proximal and the terminal SI. The SI sections, colon

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and caecum were opened longitudinally and washed with PBS. Tumour size and location were recorded by a study-blinded technician.

Erythrocyte folate analysis

RBC folate concentrations were measured in haemolysate by the automated ion capture assay using the IMX folate system (Abbot IMx; Abbot Laboratories) as described by Basten *et al.*[36].

Statistical analysis

The effects of experimental diet were examined by ANOVA according to a 2 × 2 factorial design (maternal and weaning folate supply) with gender of mouse as a fixed effect factor.

Results

Effects of folic acid depletion on pregnancy outcome

Litter sizes of dams fed the depleted folic acid diet were reduced by 22 % ($P=0{\cdot}006$) compared with their normal folic acid counterparts, but there was no effect of feeding the folic acid-deplete diet on survival of pups during lactation. With both diets there was a slight excess (59 %) of males and no difference in the proportion of offspring carrying the Min genotype from folic acid-depleted compared with folic acid-normal dams (55 and 49 % respectively).

Effects of pre- and post-weaning folic acid depletion upon growth and organ dimensions

At weaning, pups from folic acid-depleted mothers were approximately 7·5 % lighter ($P=0{\cdot}093$) than controls but there were no significant ($P>0{\cdot}05$) effects of maternal folic acid supply on intestinal organ weights or organ lengths of pups at weaning (data not shown).

After 70 d of feeding the experimental diets to the offspring, there was little evidence of any gross differences in body size or in intestinal organ dimensions (Table 1). The exception was colon length, which was significantly ($P=0{\cdot}03$) greater in mice born to folic acid-depleted dams.

Effects of pre- and post-weaning folic acid depletion upon erythrocyte folate status of weaned and adult $Apc^{+/Min}$ offspring

At weaning, the offspring of the dams fed the low folic acid diet had significantly ($P=0{\cdot}011$) lower RBC folate concentration than the offspring of dams fed the normal folic acid diet (Fig. 1(A)). However, maternal folic acid depletion had no significant effect upon RBC folate concentrations in adult offspring (Fig. 1(B)). As expected, RBC folate concentration was reduced significantly ($P<0{\cdot}001$) in mice fed the depleted folic acid diet from weaning (Fig. 1(C)).

Effects of pre- and post-weaning folic acid depletion on tumour number and size in $Apc^{+/Min}$ mice

Tumour diameters ranged up to 10 mm with a mean of 1·8 mm and median 1·6 mm. On this basis, tumours 1 mm and under were classified as 'small' and those diameters over 1 mm being 'large'. The majority of tumours were found in the distal SI, regardless of dietary treatment (Table 2). In addition, small tumours made up a higher proportion of the total tumours in the distal SI when compared with the proximal SI and the colon (Table 2). There was no evidence that maternal folic acid depletion had any effect on total number, anatomical distribution or size of intestinal tumours (Table 2). In contrast, feeding the low folic acid diet to the weaned offspring resulted in fewer total gut tumours, with effects being concentrated in the distal half of the SI, but these differences were not statistically significant ($P>0{\cdot}05$) (Table 2).

Since smaller adenomatous polyps are less likely to develop into carcinomas in human subjects[37], the effect of dietary interventions on the size distribution of intestinal tumours was investigated by calculating the percentage of small (≤ 1 mm in diameter) tumours. This showed clearly that there was a greater proportion of small tumours in the gut of animals weaned on to the low folic acid diet ($P=0{\cdot}028$) with this reduction in tumour size being most apparent in the distal SI ($P=0{\cdot}023$) (Table 2).

Female mice had nearly twice as many intestinal tumours as male mice (Fig. 2) due to significantly more SI tumours ($P=0{\cdot}041$) with no difference in colonic tumours ($P=0{\cdot}322$). Accordingly, gender was included as a fixed factor in the statistical analysis of the tumour data (Table 2). Overall tumour burden, calculated as the total diameter of all gut tumours, was also higher in females, (29·6 mm compared with 17·6 mm for males) ($P=0{\cdot}056$).

Further inspection of the tumorigenesis data (Fig. 3) revealed significant ($P<0{\cdot}05$) differences in the responses of male and female mice to altered folic acid supply post-weaning. In male mice, reduced folic acid intake from weaning had no effect on total tumour multiplicity, SI tumour numbers, tumour burden or percentage of small tumours (Fig. 3(A)–(D)). In contrast, the low folic acid diet suppressed total and SI tumour number and tumour burden in female mice compared with females given a normal folic acid diet (Fig. 3(A)–(C)), so that females fed the folic acid-restricted diet had similar tumour numbers to males. However, the proportion of small tumours in females increased to almost twice that seen in males when the low folic acid diet was offered from weaning (Fig. 3(D)).

Discussion

As expected, in the present study, dietary folic acid depletion decreased RBC folate in both dams and weaned offspring. However, adult offspring exposed to maternal folic acid depletion did not have reduced RBC folate status, indicating that early folic acid depletion is not detrimental to RBC folate status in later life. Note that RBC folate concentrations in this study were higher than values described by Bird *et al.*[38] as being typical for mice and also higher than those reported by McDorman *et al.*[39] for mice fed adequate (5 mg folic acid/kg diet) or depleted (0 mg folic acid/kg) diets. In part,

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Table 1. Effect of maternal and post-weaning folic acid supply on body weight and organ dimensions of adult offspring†

Maternal diet…	Dietary folic acid regimen					Probability of effects		
	Normal		Depleted					
Post-weaning diet…	Normal (NN)	Depleted (ND)	Normal (DN)	Depleted (DD)	Pooled SEM	Maternal diet	Post-weaning diet	Maternal × Post-weaning diet
n	37	37	37	37				
Body mass (g)	22·3	21·6	23·4	22·2	0·09	0·364	0·316	0·793
SI weight (g)	1·31	1·20	1·31	1·27	0·006	0·404	0·102	0·480
SI length (cm)	28·5	27·6	27·1	27·6	0·07	0·052	0·778	0·803
Colon weight (g)	0·40	0·42	0·45	0·45	0·003	0·073	0·731	0·755
Colon length (cm)	5·00	5·06	5·36	5·27	0·020	**0·030***	0·932	0·537
Caecum weight (g)	0·45	0·45	0·48	0·47	0·002	0·324	0·791	0·762
Liver weight (g)	1·19	1·13	1·24	1·18	0·005	0·251	0·161	0·948

*$P < 0·05$.
† For details of diets and procedures, see Materials and methods.
SI, small intestine.

Fig. 1. Effects of pre- and post-weaning folate diets upon mean erythrocyte (RBC) folate concentrations. Error bars represent 95% CI. (A) Mean RBC folate of weanling pups of dams fed normal and depleted folate diets, (*n* 8 for both diet groups; *$P=0·011$); (B) mean RBC folate of adult offspring exposed to normal and depleted folate diets pre-weaning (*n* 28 and 24 respectively). Data for animals fed both normal and low folate diets from weaning have been pooled since there was no evidence for a maternal × weaning diet interaction; **(**C) mean RBC folate of adult offspring fed normal and depleted folate diets post-weaning. (*n* 25 and 27 respectively; *$P=<0·001$). Data for animals from dams fed both normal and low folate diets have been pooled since there was no evidence for a maternal × weaning diet interaction. For details of diets and procedures, see Materials and methods.

these differences are due to the significantly lower haematocrit values for *Apc* $^{+/Min}$ than wild-type mice (data not shown).

Folic acid depletion during pregnancy affected pregnancy outcome by significantly lowering the mean number of pups per litter. This effect of reduced maternal folate supply has been seen previously in rodents[26] and may be due to the

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Table 2. Effects of maternal and post-weaning folic acid supply on the number and size of gut tumours in adult *Apc*$^{Min/+}$ offspring§

Maternal diet…	Dietary folic acid regimen					Probability of effects		
	Normal		Depleted					
Post-weaning diet…	Normal (NN)	Depleted (ND)	Normal (DN)	Depleted (DD)	Pooled SEM	Maternal Diet	Post-weaning diet	Maternal × Post-weaning diet
n	37	37	37	37				
Total gut tumours	12·2	8·5	14	9·8	1·5	0·988	0·128	0·701
Proximal SI tumours	2·5	3·0	3·4	2·1	0·4	0·735	0·520	0·435
Distal SI tumours	9·4	4·8	10·3	7·3	1·2	0·869	0·077	0·443
Colonic tumours	0·3	0·7	0·3	0·4	0·1	0·385	0·105	0·295
Tumour burden‡ (mm)	26·8	17·2	28·9	18·6	3·2	0·711	0·057	0·690
% Small† gut tumours	21·9	28·2	21·3	38·7	3·1	0·370	**0·028***	0·403
% Small† tumours in proximal SI	0·0	10·0	3·3	11·7	2·5	0·426	0·056	0·859
% Small† tumours in distal SI	35·4	41·6	24·3	59·4	4·5	0·558	**0·023***	0·154
% Small† tumours in colon	0·0	11·1	0·0	0·0	3·7	0·409	0·524	0·648

*$P < 0·05$.
† Small tumours were defined as tumours ≤ 1 mm in diameter.
‡ Tumour burden was calculated as the sum of tumour diameters.
§ For details of diets and procedures, see Materials and methods.
SI, small intestine.

Fig. 2. Intestinal tumour multiplicity and tumour burden. ■, colon tumour number; □, small intestine tumour number. *$P=0·041$. For details of diets and procedures, see Materials and methods.

lack of folate required for DNA synthesis and therefore restricting cell division. Folate deficiency during pregnancy can cause fetal death and resorption, depending upon severity and length of the nutritional insult[40]. For example, mice given a folate-depleted diet and the antibiotic succinyl sulfathiazole, which reduces synthesis of folate by gut bacteria, from 4 weeks prior to mating had an increased incidence of resorptions at gestation days 11/12, 13–16 and 18 compared with mice given normal folate diets with and without succinyl sulfathiazole[27]. During the present study, fetal resorption was not investigated but is a possible mechanism for the reduced litter size with folic acid depletion. Although litter size was reduced by folic acid depletion during pregnancy, there was no preferential selection of either gender or genotype of the resultant litters from dams fed folic acid-depleted diets. In a recent study, maternal folate status was positively associated with infant birth weight in human subjects[41]. Although maternal folic acid depletion had no significant effect upon body weight or organ weights or lengths in either dams or offspring killed at weaning, it led to increased colon weight and length in adult mice. It has been hypothesised that nutrient deficiencies during development prepare the fetus for future harsh environments in which it may be subjected to further nutrient depletion[42]. Indeed, intrauterine growth retardation has been documented to restrict growth of the rat SI[43], ovine pancreas[44] and human liver and kidney[45], whilst nutrient restriction during pregnancy lowered kidney weight in rats at birth[46], inhibited growth of the ovine gastrointestinal tract during the first half of gestation[47] and increased liver weight in fetal sheep when restricted between 28–78 d gestation[48]. Reduced growth of some tissues may be due to increased growth of other organs, in a selective trade-off depending upon the relative importance of the organs for the survival of the offspring or due to the timing of the nutritional insult[42]. In the case of nutrient insults during development, it may be beneficial to increase gut length/capacity to increase lumenal surface area and to reduce food transit time, therefore increasing the capacity to absorb nutrients. If such a mechanism exists, the continued

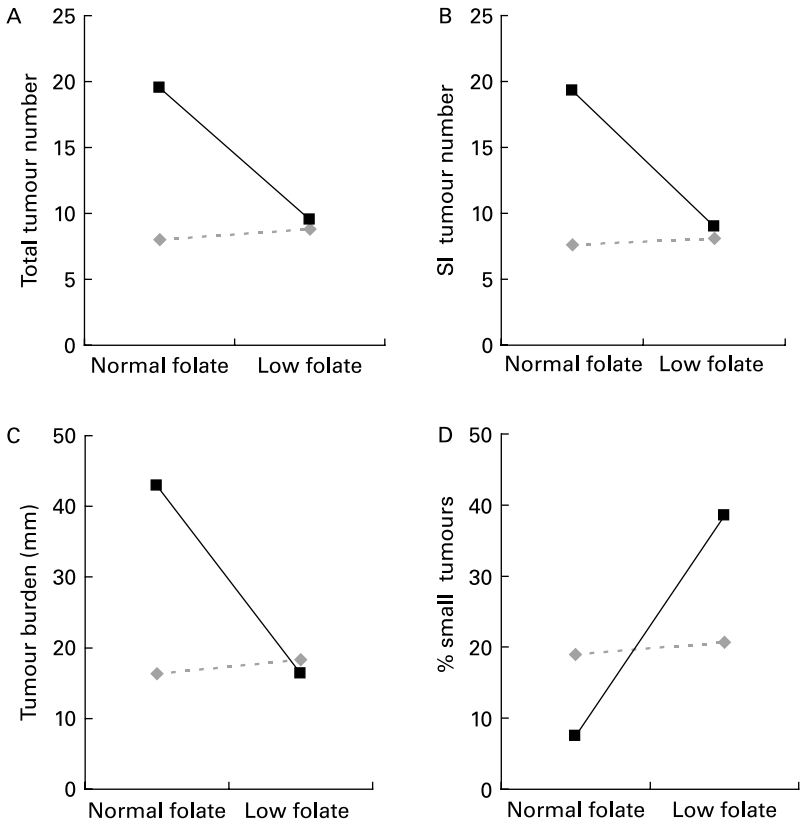

Fig. 3. Effects of gender and post-weaning folic acid supply on tumorigenesis in adult Apc$^{+/Min}$ offspring (- -◆- -, male; –■–, female). (A) Total tumours (P=0·077 for gender × diet interaction; (B) small intestine tumour number (P=0·075 for gender × diet interaction; (C) tumour burden (P=0·028 for gender × diet interaction); (D) % of total small tumours (P=0·047 for gender × diet interaction). For details of diets and procedures, see Materials and methods.

growth of gut organs may be pre-programmed due to the original developmental constraints to which the fetus was subjected[33]. Although organ growth was altered with folic acid depletion *in utero*, overall growth (measured by body mass) of mice was not affected by folic acid depletion *in utero* and/or post-weaning.

As observed previously in *Apc* $^{+/Min}$ mice[49,50], there were more tumours in the terminal SI when compared with the proximal SI and the colon. There were no significant effects of maternal folic acid supply on tumour number, incidence or size in adult *Apc* $^{+/Min}$ mice, indicating that folic acid depletion during *in utero* development had no effect on tumour initiation or tumour growth in these animals. This may be because the level of folic acid depletion may not have been sufficiently severe to impact upon tumorigenesis in the offspring and perhaps further dietary restriction of another methyl donor may have altered tumorigenesis in these mice. In the present study, birth weight was not measured and although offspring from folate-depleted dams tended to be lighter at weaning this difference was not statistically significant (P=0·093). It is not known whether the pups from folate-depleted dams experienced catch-up growth postpartum nor do we have any information on the effect, if any, of such catch-up growth on tumour development.

Female mice had a significantly (P=0·047) higher number of total tumours compared with males in the present study. Previous studies in *Apc* $^{+/Min}$ mice have observed differences in SI tumour number between gender. Steffensen *et al.*[49] reported that male mice had 18 % fewer tumours than females, whereas Paulsen *et al.*[51] reported that males had 50 % more tumours than females. However, we also observed a gender*post-weaning diet interaction for total tumour burden, in which normal folic acid females had a larger tumour burden than depleted females and both normal and folic acid-depleted males. These data indicate that a depletion of folic acid in these female mice was protective against tumorigenesis. Under the conditions of the current study, normal folic acid supply appears to be detrimental in respect of intestinal tumorigenesis in females, but not males. Several other studies have also indicated that gender can influence the efficacy of various interventions on intestinal tumorigenesis in *Apc* $^{+/Min}$ mice. For example, a rye bran-containing diet increased SI tumour number in female, but not in male *Apc* $^{+/Min}$ mice[52] and the addition of a vegetable–fruit mix to a low fat diet enhanced intestinal polyp multiplicity significantly in female *Apc* $^{+/Min}$ mice only[53]. Treadmill running reduced both intestinal polyp number and size in male but not in female mice[54]. This evidence indicates that gender is an important factor when investigating effects of environmental factors upon tumorigenesis in the *Apc* $^{+/Min}$ mouse model.

Two questions arise from the outcomes observed in this study: i) why were there more tumours in female mice compared with males? ii) why did females, but not males, respond to folic acid depletion? Gender-related hormones may have been one factor influencing the differences in tumour number between gender in this study. Indeed, ovariectomy in female *Apc* $^{+/Min}$ mice has been observed to increase

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intestinal tumour number[55,56] and subsequent treatment with 17β-oestradiol and coumestrol reduced tumour number similar to that found in non-ovariectomised control mice, indicating that female sex hormones can affect tumorigenesis in this model. However, this does not explain our findings. In contrast with Weyant *et al.*'s study[55], where oestrogen conferred protection against more aggressive tumorigenesis in females, we found enhanced tumorigenesis in intact females compared with males. This apparent gender effect and the discordant findings between studies require further investigation.

As summarised in a recent review by Kim [57], inconsistent findings on effects of altering dietary folate supply on intestinal tumorigenesis have been reported between different studies. With higher intakes of folic acid within the physiological range, i.e. 8 mg/kg diet, decreased incidence of microscopic and macroscopic neoplasms was observed in DMH rats[17,18]. However, azoxymethane-treated rats showed increased incidence of colonic aberrant crypt foci and tumours of the large and SI when fed 8 compared with 0 mg folic acid/kg diet[21,22]. Two studies used pharmacological concentrations of folic acid and found that 40 mg folic acid/kg diet increased tumorigenesis by 40 % compared with controls in DMH-treated rats[18], whereas increasing concentrations of folate up to 20 mg/kg in *Apc* $^{+/Min}$ mice led to a dose-dependent linear decrease in ileal adenoma and aberrant crypt foci[23]. It has been hypothesised that timing of folate depletion/supplementation may be critical in determining the effect on tumorigenesis. Song *et al.*[23] investigated the effects of folate depletion in *Apc* $^{+/-}$ *Msh2* $^{-/-}$ mice from both 3 and 6 weeks of age. Mice depleted from 3 weeks had more tumours compared with folate-supplemented animals, but folate depletion from 6 weeks of age reduced tumour number. Since tumour initiation has occurred by 6 (but not 3) weeks of age[23], this observation suggests that folic acid supplementation may be protective prior to tumour initiation but that folate depletion confers greater protection once tumour growth has started. Indeed in the present study, reducing folic acid supply from 4–5 weeks of age reduced tumorigenesis by lowering SI tumour numbers and increasing the mean percentage of small tumours in both the SI and colon compared with controls in female mice. The present study therefore supports the hypothesis that folic acid depletion post-tumour initiation (4–5 weeks of age) slows tumour progression, but did not indicate any beneficial or detrimental effect on tumorigenesis of folic acid depletion prior to tumour initiation (*in utero* and until 4–5 weeks of age).

The present study provides evidence that reduced folic acid supply may be protective against tumour progression in female *Apc* $^{+/Min}$ mice when imposed from 4–5 weeks of age. This is probably due to reduced availability of folic acid for DNA synthesis and cell division, thus slowing tumour growth. Indeed, in *Apc* $^{+/Min}$ mice given the chemotherapeutic drug, 5-fluorouracil, which acts upon thymidylate synthase causing a reduction in the conversion of dUMP to dTMP, folic acid depletion enhanced drug efficacy[58]. Further, folate depletion inhibited tumour recovery once the drug was withdrawn and, 6 weeks after withdrawal, folate-depleted mice had lower tumour numbers compared with age-matched controls[58]. This was possibly due to the continued depletion of nucleotides slowing tumour cell proliferation.

To our knowledge, this is the first study to test the hypothesis that folic acid depletion *in utero* and during lactation could influence tumorigenesis in later life. Although our dietary protocol was successful in reducing folate status whilst ensuring the production of viable offspring with no detriment in postnatal growth, there was no evidence that such maternal folic acid depletion affected tumorigenesis in the offspring. In contrast, folic acid depletion from weaning (4–5 weeks of age) may be protective against tumour development in female, but not in male, *Apc* $^{+/Min}$ mice. These observations support the hypothesis that a critical period of vulnerability to folic acid supply occurs after tumour initiation, especially in females. Further studies will be needed to confirm or refute this gender-specific effect of altered folic acid supply on intestinal tumour development and, if confirmed, to investigate the mechanism responsible for the greater sensitivity of females to this nutritional manipulation.

In summary, the present study shows that: i) there was no effect of maternal folic acid supply (within the range tested) on intestinal tumorigenesis; ii) reduced dietary folic acid supply from weaning inhibited the growth of neoplastic lesions in female *Apc* $^{+/Min}$ mice. The implications of these findings for the role of folic acid supply in human tumorigenesis remain to be established. Fortification (both voluntary and mandatory) of foods with folic acid is common in North America and in the UK. Whilst such fortification is likely to be beneficial for women of reproductive age in lowering the risk of neural tube defects, the implications for other population groups remain less certain. The important observation by Cole *et al.*[59] that those with a recent history of colorectal adenomas had a higher risk of having three or more adenomas and of non-colorectal cancer 3–5 years after being randomised to 1 mg folic acid/d suggests the need for further research to ascertain whether raised folic acid intake may increase the risk of colorectal neoplasia.

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