

## Dietary folate does not significantly affect the intestinal microbiome, inflammation or tumorigenesis in azoxymethane–dextran sodium sulphate-treated mice

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### Abstract

Inflammatory bowel disease (IBD) is a risk factor for the development of colon cancer. Environmental factors including diet and the microflora influence disease outcome. Folate and homocysteine have been associated with IBD-mediated colon cancer but their roles remain unclear. We used a model of chemically induced ulcerative colitis (dextran sodium sulphate (DSS)) with or without the colon carcinogen azoxymethane (AOM) to determine the impact of dietary folic acid (FA) on colonic microflora and the development of colon tumours. Male mice ( $n$  15 per group) were fed a FA-deficient (0 mg/kg), control (2 mg/kg) or FA-supplemented (8 mg/kg) diet for 12 weeks. Folate status was dependent on the diet ( $P < 0.001$ ) and colitis-induced treatment ( $P = 0.04$ ) such that mice with colitis had lower circulating folate. FA had a minimal effect on tumour initiation, growth and progression, although FA-containing diets tended to be associated with a higher tumour prevalence in DSS-treated mice (7–20 *v.* 0%,  $P = 0.08$ ) and the development of more tumours in the distal colon of AOM-treated mice (13–83% increase,  $P = 0.09$ ). Folate deficiency was associated with hyperhomocysteinaemia ( $P < 0.001$ ) but homocysteine negatively correlated with tumour number ( $r = -0.58$ ,  $P = 0.02$ ) and load ( $r = -0.57$ ,  $P = 0.02$ ). FA had no effect on the intestinal microflora. The present data indicate that FA intake has no or little effect on IBD or IBD-mediated colon cancer in this model and that hyperhomocysteinaemia is a biomarker of dietary status and malabsorption rather than a cause of IBD-mediated colon cancer.

**Key words:** Folate: Homocysteine: Inflammatory bowel disease: Colon cancer: Microflora

Ulcerative colitis (UC) and Crohn's disease are inflammatory bowel diseases (IBD) affecting 0.5% of the Canadian population, one of the highest prevalences in the world<sup>(1)</sup>. The development of IBD is complex involving the interaction of genetics and environmental factors, including the intestinal microflora and diet<sup>(2)</sup>. IBD has a significant and negative impact on the individual in terms of disability, co-morbidities and quality of life, and is a significant burden on the health care system<sup>(3–5)</sup>. Individuals with IBD are at an elevated risk of developing colorectal cancer (CRC) over their lifetime<sup>(6)</sup>. Chronic inflammation observed in IBD is considered a major promoter of cancer, as indicated by increasing cumulative risk with disease duration<sup>(7)</sup>, extent<sup>(8)</sup> and severity<sup>(9)</sup> of inflammation.

Folic acid (FA) is the synthetic form of folate, an essential water-soluble B vitamin that has been associated with sporadic and inflammation-mediated colon cancer<sup>(2)</sup>. The metabolism of folate is required for the *de novo* synthesis of purines and thymidylate and for the remethylation of homocysteine to form methionine<sup>(10)</sup>. The association of folate deficiency with sporadic CRC is attributed to its metabolic role in nucleotide synthesis and cellular methylation capacity, which has an impact on genetic mutations, genome stability and gene expression<sup>(2)</sup>. However, its role in inflammation-mediated colon cancer is not well understood. IBD patients often present with folate deficiency<sup>(11,12)</sup>. Dietary folate intake and folate status have been associated with protection from UC-associated CRC, suggesting that folate is chemopreventive in cases of chronic

**Abbreviations:** AOM, azoxymethane; CRC, colorectal cancer; DSS, dextran sodium sulphate; FA, folic acid; IBD, inflammatory bowel disease; OUT, operational taxonomic units; UC, ulcerative colitis.

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UC<sup>(13,14)</sup>. The use of folinic acid supplements, a reduced form of folate, by patients with chronic UC has been shown to decrease cell proliferation in the intestinal mucosa<sup>(15)</sup>. In addition, prevalence of SNP in methylenetetrahydrofolate reductase and methionine synthase genes, key regulatory enzymes involved in folate, vitamin B<sub>12</sub> and homocysteine metabolism, has been associated with IBD; all of which provide a link between altered folate metabolism and disease risk<sup>(11,16–19)</sup>.

IBD patients often demonstrate hyperhomocysteinaemia, a biomarker of folate and/or vitamin B<sub>12</sub> deficiency; B vitamin deficiency is frequently observed in IBD patients and has been attributed to malabsorption resulting from inflammation or use of sulfasalazine<sup>(11,19–23)</sup>. In addition to being a biomarker of folate and vitamin B<sub>12</sub> status, homocysteine can actively promote the inflammatory process<sup>(24)</sup>. For example, homocysteine has been suggested to promote oxidative stress, microvascular activation of the intestine, and the secretion of pro-inflammatory cytokines and chemokines<sup>(21,25,26)</sup>. Since homocysteine can promote the inflammatory process and IBD-related carcinogenesis is dependent on the extent, severity and duration of inflammation, homocysteine could play an active role in cellular transformation. Indeed, hyperhomocysteinaemia has been shown to be associated with IBD-related colon cancer<sup>(22,27)</sup>.

It has been difficult to tease apart the relationship among folate, homocysteine, inflammation and cancer risk in IBD, and the question remains whether reduced folate status is causative in inflammation-mediated cellular transformation, contributes indirectly to tumour initiation and growth by altering homocysteine metabolism, is a biomarker of active inflammation and nutrient malabsorption or alters the intestinal microflora to promote disease. The present study employed a mouse model of chemically induced UC to begin to distinguish the roles played by folate and homocysteine in inflammation-mediated colon cancer development and tumour growth, and their relationship with the intestinal microflora.

## Experimental methods

### Mice

All institutional and national guidelines for the care and use of animals were followed, namely the guidelines of the Canadian Council for Animal Care and all experimental procedures involving animals were approved by the Health Canada Ottawa-Animal Care Committee. Weanling male C57BL/6 mice aged 22–24 d were purchased from Charles River Laboratories. Mice were pair-housed in micro-isolators under specific pathogen-free conditions. Mice were allowed to acclimatise for 1 week before being randomly assigned to one of three experimental diets: control, FA-deficient or FA-supplemented. Each diet group consisted of fifty-three mice. The control diet was AIN-93G containing 2 mg FA/kg (Dyets, Inc.), the deficient diet was modified AIN-93G containing no FA (Dyets, Inc.) and the supplemented diet was modified AIN-93G containing 8 mg FA/kg (Dyets, Inc.). Mice were maintained on the diet for 2, 3 or 12 weeks, as outlined in the treatment regimen.

### Dextran sodium sulphate and azoxymethane–dextran sodium sulphate treatment

Colitis (dextran sodium sulphate (DSS)) and colitis-mediated colon cancer (azoxymethane (AOM)–DSS) were induced using a modified protocol<sup>(28)</sup>. After 2 weeks on their respective diets, fifteen mice from each experimental diet were assigned to the control, DSS or AOM–DSS treatment group. Mice assigned to the AOM–DSS treatment group were injected once with AOM (10 mg/kg in saline; Sigma-Aldrich Canada Limited). Mice assigned to the DSS or control group were injected once with saline. After 1 week, AOM–DSS and DSS mice were given DSS in their drinking water (1.5%, w/v; 36 000–50 000 molecular weight; MP Biomedicals) for 4 d. After 17 d, DSS and AOM–DSS mice were given DSS in their drinking water (1%, w/v) for 3 d. After 18 d, DSS and AOM–DSS mice were given DSS in their drinking water (1%, w/v) for 2 d. Control mice were given plain drinking water throughout the experiment. Mice were killed by cervical dislocation approximately 3 weeks after the last DSS cycle. In addition, four mice from each experimental diet were killed after 2 or 3 weeks on the diet to allow for an estimation of tissue folate status at the time of AOM injection and first DSS exposure, respectively.

### Tumour assessment

The entire colon from the caecal junction to the anus was removed. Colons were flushed with PBS, cut open longitudinally and laid flat with the lumen facing up. The number, distribution (proximal 1/3, middle 1/3 and distal 1/3) and diameter of lesions in the colon were noted using a Stemi 2000-C Stereo Microscope (Zeiss). The proximal and middle segments were flash-frozen in liquid N<sub>2</sub>. The distal third and identified tumours were fixed in 10% neutral buffered formalin. The tissues were trimmed, embedded in paraffin, cut in 5 µm-thick sections and stained with haematoxylin and eosin.

The classification of colonic tumours was performed following published guidelines<sup>(29,30)</sup>. Adenomas were characterised by well-demarcated and circumscribed areas of epithelial dysplasia which compressed adjoining mucosa. Adenomas were also graded on degree of dysplasia defined as low or high grade. Adenocarcinomas were characterised by epithelial cells invading the basement membrane into the lamina propria, into the submucosa, or deeper into the gut wall and beyond. A numeric grading system was developed to describe the leucocytic invasion for each tumour. Toluidine blue was used to highlight mast cell granules and mast cells in the colonic mucosa were counted. We did not attempt to classify individual crypts in the inflamed mucosa as dysplastic or regenerative, as it is well accepted to be difficult to differentiate dysplasia from regeneration based on histopathology at certain stages of crypt repair<sup>(31)</sup>.

### Tissue folate

Folate concentration of plasma and tissues was quantified using the *Lactobacillus casei* microbiological assay as described previously<sup>(32)</sup>.

### Plasma homocysteine

Plasma homocysteine was assessed using the homocysteine microtip reagent (Ortho Clinical Diagnostics) and analysed on the VITROS 5,1 FS Chemistry System (Ortho Clinical Diagnostics).

### 16S rRNA survey of faecal bacterial communities

Previously frozen ( $-80^{\circ}\text{C}$ ) pooled faecal pellets were weighed and homogenised in vials containing  $0.2\ \mu\text{m}$  ceramic beads (Bertin Technologies) and  $800\ \mu\text{l}$  ASL buffer (Qiagen) using the Precellys 24 homogeniser (Bertin Technologies) at 6800 rpm for 15 s for a total of three cycles. The vials were immersed in liquid  $\text{N}_2$  and allowed to thaw slightly in-between cycles to aid in the breakdown of Gram-positive cell walls. An additional  $400\ \mu\text{l}$  ASL buffer were added and bacterial genomic DNA was purified using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol for difficult-to-lyse bacteria. Genomic DNA concentrations were measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific). PCR products were generated using Ready-To-Go PCR Beads (GE Healthcare) and universal 16S rRNA primers, F44 ( $5'$ -RGTTYGATYMTGGCTCAG- $3'$ ) and R1543 ( $5'$ -GGTACCTTKTTACGACTT- $3'$ )<sup>(33)</sup>. Approximately 100 ng of genomic DNA were used as a template and  $1\ \mu\text{l}$  of each primer at  $25\ \mu\text{M}$  was used in the reaction. PCR conditions were  $94^{\circ}\text{C}$  for 2 min, twelve cycles at  $94^{\circ}\text{C}$  for 20 s,  $54^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 3 min, with a final elongation step at  $72^{\circ}\text{C}$  for 10 min. For each group, two PCR reactions were generated.

Shotgun cloning reactions were performed with the generated PCR products using the TOPO TA cloning kit (Life Technologies) according to the manufacturer's protocol for chemically competent cells. The PCR product ( $4\ \mu\text{l}$ ) was added to the cloning reaction. Thereafter, ninety-six white colonies from each cloning plate were picked and grown in Luria–Bertani media containing ampicillin ( $100\ \mu\text{g}/\text{ml}$ ). Plasmids were purified with the Wizard MagneSil Kit (Promega) and verified on ethidium bromide-stained 0.8% agarose gels. Sequencing reactions were performed using the BigDye version 3.1 cycle sequencing kit (Life Technologies) with M13 primers and an in-house designed internal primer, 16S-I ( $5'$ -TCACRRCACGAGCTGACGA- $3'$ )<sup>(34)</sup>. The reactions were purified using the Montage Seq96 Clean-up Kit (Millipore). The purified reactions were run on the ABI3130XL genetic analyser (Life Technologies). Sequence fragments were aligned and base-call corrected with Sequencher version 4.7 (Gene Codes). Data analysis was performed using Mothur version 1.20.1<sup>(35)</sup> and Mega<sup>(36)</sup>. Operational taxonomic units (OTU) were identified using the furthest-neighbour option of Mothur.

### Statistics

Data are presented as means with their standard errors. Differences among the diet groups were analysed by one-way ANOVA. All pairwise comparisons were assessed using Tukey's *post hoc* test for normally distributed data and Dunn's *post hoc* test for non-normally distributed data. When

possible, non-normally distributed data were normalised by square root transformation before analysis. Differences in proportions were assessed by the  $\chi^2$  test. Correlations between parameters were assessed by Spearman's rank order correlation test. Trends were assessed by multiple linear regression analysis.

Faecal bacterial communities from different diet and treatment groups were compared using several tests found within Mothur version 1.20.1<sup>(35)</sup>. The  $\beta$ -Libshuff statistic<sup>(37)</sup> uses the Cramer–von Mises statistic to test whether two communities differ across evolutionary distances. The parsimony statistic<sup>(38)</sup> tests whether the arrangement of phylotypes along the phylogenetic tree is evenly distributed among the communities of each experimental condition. The UniFrac statistic<sup>(39)</sup> measures the fraction of the phylogenetic tree branch length unique to each community to determine whether the communities evolved at similar rates<sup>(40)</sup>. Node analysis using UniFrac<sup>(41)</sup> was conducted to highlight potential differences between the libraries. The analysis of molecular variance is a non-parametric analogue of the traditional ANOVA, which tests the hypothesis that genetic diversity within two populations is not significantly different from the diversity of the pooled communities. The HOMOVA tests for HOMogeneity of MOlecular VARIance within the experimental communities using a non-parametric approach. The analysis of similarity tests whether the compositional dissimilarities between communities is greater than those within communities.

Non-metric multidimensional scaling analysis was performed as follows. Cluster analysis was performed using Sorensen distances<sup>(42)</sup> and matrices of fractional divergences between OTU pairs (generated by MOTHUR software). The resulting distance matrix was used to generate non-metric multidimensional scaling diagrams using Statistica Software.

Differences among groups or communities, or correlation between parameters were considered significant if  $P \leq 0.05$ .  $P$  values equal to or less than 0.10 are specified. All other  $P$  values are indicated as not significant. All ANOVA statistics were performed using SigmaPlot software, version 11.0 (Copyright © 2008 Systat Software, Inc.).

## Results

### *Effect of dietary folic acid and dextran sodium sulphate or azoxymethane–dextran sodium sulphate treatment on tissue folate and plasma homocysteine*

Plasma, liver and colon folate was analysed after 2 and 3 weeks on the diet to estimate tissue folate at the time of AOM injection and initiation of DSS, respectively. Plasma folate was significantly lower in deficient mice after 2 weeks in comparison with sufficient and supplemented mice and was further decreased after 12 weeks (Table 1,  $P < 0.001$ ). Plasma folate did not differ between the sufficient and supplemented mice. Plasma folate was significantly lower in AOM–DSS-treated mice and tended to be lower in DSS-treated mice compared with saline-treated mice (Table 1,  $P = 0.04$ ).

**Table 1.** Tissue folate and plasma homocysteine (Hcy)\*  
(Mean values with their standard errors)

Time on diet (weeks)	Treatment	Diet (mg/kg)	Folate (fmol/ $\mu$ g protein)							
			Plasma Hcy ( $\mu$ M)		Plasma		Liver		Colon	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
2	None	0	ND		16.2 <sup>a</sup>	5.3	50.5 <sup>a</sup>	2.4	9.8 <sup>a</sup>	0.5
		2	ND		22.8 <sup>b</sup>	0.3	60.0 <sup>a,b</sup>	2.6	19.8 <sup>b</sup>	1.9
		8	ND		26.5 <sup>b</sup>	0.5	70.5 <sup>b</sup>	6.4	17.5 <sup>b</sup>	1.4
3	None	0	ND		5.5 <sup>a</sup>	0.8	48.0 <sup>a</sup>	1.9	10.8 <sup>a</sup>	0.5
		2	ND		28.5 <sup>b</sup>	0.8	59.1 <sup>b</sup>	1.6	19.3 <sup>b</sup>	2.3
		8	ND		31.9 <sup>b</sup>	0.8	61.3 <sup>b</sup>	1.9	19.7 <sup>b</sup>	1.9
12	Saline	0	13.6 <sup>a</sup>	1.0	8.7 <sup>a</sup>	4.6	39.8 <sup>a</sup>	4.6	4.7 <sup>a</sup>	0.6
		2	6.4 <sup>b</sup>	0.4	60.0 <sup>b</sup>	6.7	57.4 <sup>a,b</sup>	3.4	12.5 <sup>b</sup>	0.6
		8	5.3 <sup>b</sup>	0.6	68.4 <sup>b</sup>	4.5	59.9 <sup>b</sup>	5.8	14.0 <sup>b</sup>	0.6
12	DSS	0	15.6 <sup>a</sup>	1.8	2.0 <sup>a</sup>	1.3	30.7 <sup>a</sup>	2.1	2.9 <sup>a</sup>	0.2
		2	5.2 <sup>b</sup>	0.7	31.6 <sup>b</sup>	3.2	65.4 <sup>b</sup>	4.8	13.5 <sup>b</sup>	0.7
		8	5.6 <sup>b</sup>	0.9	33.6 <sup>b</sup>	1.1	58.2 <sup>b</sup>	2.3	15.5 <sup>c</sup>	0.3
12	AOM–DSS	0	18.0 <sup>a</sup>	2.1	0.6 <sup>a</sup>	0.2	33.4 <sup>a</sup>	2.0	3.6 <sup>a</sup>	0.4
		2	6.6 <sup>b</sup>	0.3	27.1 <sup>b</sup>	1.8	55.2 <sup>b</sup>	1.7	13.2 <sup>b</sup>	0.3
		8	5.1 <sup>b</sup>	0.3	29.6 <sup>b</sup>	0.3	57.4 <sup>b</sup>	2.5	14.5 <sup>b</sup>	0.9
Main effect of time (saline or untreated mice), <i>P</i>			ND		<0.001 (2 and 3 v. 12)		0.054		<0.001 (2 and 3 v. 12)	
Main effect of diet (at 12 weeks), <i>P</i>			<0.001 (0 v. 2 and 8)		<0.001 (0 v. 2 and 8)		<0.001 (0 v. 2 and 8)		<0.001 (0 v. 2 and 8)	
Main effect of treatment (at 12 weeks), <i>P</i>			NS		0.043 (control v. AOM–DSS)		NS		NS	

ND, not determined; DSS, dextran sodium sulphate; AOM, azoxymethane.

<sup>a,b</sup> Mean values with unlike superscript letters within a column and within a treatment group were considered significantly different ( $P \leq 0.05$ ; one-way ANOVA and Tukey's *post hoc* analysis). No significant diet  $\times$  time or diet  $\times$  treatment interactions were observed.

\* Hcy  $n$  3–11 per group, plasma and tissue folate,  $n$  4 per group.

Liver folate was significantly lower in deficient mice compared with supplemented mice after 2 weeks (Table 1,  $P=0.03$ ). Liver folate did not differ between the sufficient and supplemented mice or among the treatment groups (Table 1).

Colon folate was significantly lower in deficient mice compared with sufficient and supplemented mice after 2 weeks (Table 1,  $P<0.001$ ) and further decreased after 12 weeks (Table 1,  $P=0.02$ ). Colon folate was not different between the sufficient and supplemented mice or among the treatment groups (Table 1).

Plasma homocysteine was significantly increased in deficient mice compared with sufficient and supplemented mice (Table 1,  $P<0.001$ ). Plasma homocysteine did not differ between the sufficient and supplemented mice or among the treatment groups (Table 1).

#### Folic acid and colitis-associated morbidity and colon cancer

We did not observe any morbidity in control mice injected with saline and given plain drinking water. We observed a low prevalence of morbidity among the DSS-treated mice ranging from 0 to 6.7%. Dietary FA was not associated with morbidity. We observed a non-significant lower prevalence of morbidity among the AOM–DSS-treated mice fed the FA-deficient and -supplemented diets (20 and 26%, respectively) compared with mice fed the FA-sufficient diet (40%).

Colon tumours were not observed in saline-treated mice. Low tumour prevalence was observed in DSS-treated mice (Table 2), of which all tumours were tubular adenomas with low-grade dysplasia. There tended to be an effect of diet on tumour prevalence in DSS-treated mice in that tumours were restricted to mice fed diets containing FA with three of the four tumour-bearing mice in the sufficient diet group ( $P=0.08$ ). No significant differences in colitis score, colonic mast cell number, or the prevalence or number of hyperplastic lymphoid aggregates were observed among the diet groups in DSS-treated mice (data not shown).

The AOM–DSS treatment was highly penetrant, resulting in the development of tumours in 94% of mice with no difference in prevalence among the diet groups (Table 2). The majority of the observed tumours were tubular adenomas with low-grade dysplasia. A low prevalence of adenocarcinomas was observed in approximately 20% of mice. Adenocarcinomas were restricted to mice fed FA-containing diets, but the difference was not significant (Table 2). Of the five observed adenocarcinomas, two were superficial and three demonstrated submucosal invasion; all were well differentiated and tubular. No significant differences in colitis score, tumour leucocyte infiltration score or the number of hyperplastic lymphoid aggregates (data not shown) were observed among the diet groups in AOM–DSS-treated mice.

The number of tumours per mouse tended to increase with increasing FA in AOM–DSS-treated mice (Table 2,  $P=0.10$ ). Differences in total tumour number were attributed to tumours in the distal colon (Table 2,  $P=0.09$ ). Tumour diameter and



**Table 2.** Death and tumour outcomes in dextran sodium sulphate (DSS) and azoxymethane (AOM)–DSS treated mice fed diets containing 0, 2 or 8 mg folic acid (FA)/kg diet (Mean values with their standard errors; number and percentages)

Treatment	Diet (mg FA/kg)	Tumour assessment (n)	Prevalence*						Tumour number per animal†			Tumour size and load																
			Tumour		Adenocarcinoma		Combined death and adenocarcinoma		Percentage of tumours > 5 mm <sup>2</sup> *			Total			Middle			Distal			Tumour diameter (mm)†			Tumour load (mm <sup>3</sup> )†				
			n	%	n	%	n	%	n	%	n	%	n	%	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE		
DSS	0	14	0	0	0	0	1	6.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	2	15	3	20	0	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	8	14	1	7	0	0	1	6.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
AOM–DSS	0	12	10	83	0	0	3	20	139	4.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	2	9	9	100	2	22	8	53.3	117	12.0	11.6	2.4	4.3	1.3	7.2	1.6	1.1	0.2	20.7	6.1	1.4	0.1	33.5	12.7	1.4	0.1	26.9	7.7
	8	11	11	100	2	18	6	40	159	7.5	17.7	4.2	4.5	1.0	13.2	4.2	1.3	0.1	26.9	7.7	1.3	0.1	26.9	7.7	1.3	0.1	26.9	7.7
		Effect of diet, P		NS		NS		0.02		0.004		NS		0.10		0.09		NS		NS		NS		NS		NS		

ND, not determined.

\*The effect of diet on prevalence was analysed by the  $\chi^2$  test. The expected results were based on that observed for the FA-sufficient diet.

†Diet effect was assessed by multiple linear regression analysis.

total tumour load did not differ among the diet groups (Table 2). The prevalence of large tumours with an area greater than 5 mm<sup>2</sup> was different among the groups, such that mice fed the sufficient diet were more likely to have large tumours than deficient or supplemented mice (Table 2,  $P=0.004$ ). Plasma, liver and colon folate did not correlate with tumour number or load as determined by Spearman's rank order correlation test.

### Homocysteine and inflammation-mediated colon cancer

A significant negative correlation was observed between plasma homocysteine and tumour number ( $r = -0.58$ ,  $P=0.02$ ) and tumour load ( $r = -0.57$ ,  $P=0.02$ ) as assessed by Spearman's rank order correlation test.

### Faecal bacterial community analysis

A total of 1599 bacterial 16S rRNA clones were distributed among six bacterial phyla with the majority (70%) falling within the Firmicutes. Rarefaction curves illustrated that adequate sampling had been conducted to assess relative changes in diversity as a function of the experimental treatments and diets (data not shown). The clones resolved into 178 OTU (phylotypes) assuming a 3% species cut-off<sup>(43)</sup> using MOTHUR with the furthest-neighbour setting. Clostridia were the dominant class among the Firmicutes, comprising 49% of all clones and phylotypes (Fig. S1(A) and Table S1, available online). Further analysis revealed that 64% of the total clones were attributable to seventeen of the OTU and that these clones dominated their respective families (data not shown), illustrating that bacterial diversity within the faecal communities was a function of both higher- and lower-abundance OTU. Diversity estimates as determined by three commonly used diversity measures (Chao1, ACE (abundance-based coverage estimation) and Shannon diversity index) ranged widely, having maximum values three to four times higher than minimum values with no clear pattern among the treatment or diet groups. However, averaging diversity across the treatments (control, DSS and AOM–DSS) showed a trend for greater diversity in the AOM–DSS group.

Non-metric multidimensional scaling analysis did not show a diet effect on library grouping, but showed that colitis-induced treatment did. In non-metric multidimensional scaling analysis, similar libraries group more closely together. The control and DSS groups were not different but AOM–DSS-treated mice grouped together (Fig. S1(B)). This analysis was supported by UniFrac analysis but other statistical tests tended to identify all groups as different (except for the 0 and 8 mg/kg control-treated groups). Additional analysis by principal components analysis separated the AOM–DSS group from the other treatment groups but did not identify a common pattern among the other treatments (data not shown).

The data were further analysed by examining the response of the seventeen most abundant genera (comprising 94% of all clones) to the treatment (data not shown). On the one hand, four genera increased with increasing inflammation/disruption of the colonic mucosa: *Bacteroides*, *Allobaculum*,

*Parasutterella* and *Alistipes*. On the other hand, eight genera decreased (seven associated with Ruminococcaceae) with increasing inflammation/disruption of the colonic mucosa. Changes in the remaining seven genera either indicated a maximum abundance in the AOM–DSS group or were approximately equal among all groups.

## Discussion

Altered folate metabolism is poised to play a number of roles in the pathogenesis of inflammation-mediated CRC. First, as proposed for sporadic CRC, limited folate-dependent *de novo* nucleotide and methionine synthesis may result in uracil-mediated genome instability and/or altered gene expression, respectively, laying the groundwork for cellular transformation. Second, hyperhomocysteinaemia resulting from folate deficiency may promote inflammation. Third, folate deficiency may be a consequence of intestinal inflammation and malabsorption resulting in increased tissue and circulating homocysteine, making a low folate status and hyperhomocysteinaemia biomarkers of active disease. Finally, folate could influence the microflora in such a way as to inhibit or promote cellular transformation. The results of the present study indicate that folate status is reduced during active inflammation and may decrease more with increasing intestinal damage (plasma folate: saline > DSS  $\geq$  AOM–DSS). In addition, dietary FA intake was not significantly associated with tumour prevalence or number nor was it associated with the degree of inflammation. However, folate deficiency and supplementation were associated with protection from tumour growth. Homocysteine was dependent on FA intake and was negatively associated with tumour number and size. Finally, folate intake did not have an impact on the intestinal microflora, rather the microflora responded to the induction of colitis.

Folate deficiency has often been associated with IBD and IBD-mediated cancer<sup>(11–14)</sup>; however, whether it is a risk factor for IBD-mediated cellular transformation or whether it results from intestinal inflammation-mediated malabsorption remains to be determined. The present data clearly demonstrate that inflammation in the colon, such as that induced by DSS and AOM–DSS, causes a reduction in circulating plasma folate. However, the colitis-inducing treatment and colitis score were not associated with reduced colon folate content; diet was the main determinant of colon folate. We observed a numeric but non-significant increase in tumour prevalence and number in mice fed FA-containing diets, but neither plasma nor colon folate correlated with tumour number. Contrary to the present findings, genetically induced folate deficiency in *Folbp*<sup>-/+</sup>/*RFC*<sup>-/+</sup> mice was associated with higher numbers of high multiplicity aberrant crypt foci after exposure to AOM and a single cycle of DSS treatment<sup>(44)</sup>. Aberrant crypt foci, and particularly high multiplicity aberrant crypt foci, are considered to be proxy indicators for the development of tumours<sup>(45)</sup>. However, *Folbp*<sup>-/+</sup>/*RFC*<sup>-/+</sup> mice did not demonstrate colon folate deficiency, as they were fed a control FA diet, and aberrant crypt foci, not tumours, were assessed, which could explain differences in the present

findings. Overall, we propose that folate status is mediated by inflammation-mediated malabsorption and is not associated with tumour initiation in this mouse model. Human studies should take into consideration that circulating folate does not necessarily represent colon folate status and may simply be an indicator of active or chronic inflammation and consequential folate malabsorption.

There is growing concern that FA supplementation may promote the growth of existing tumours and/or their transition to invasive adenocarcinoma. A randomised clinical trial aimed at preventing CRC recurrence observed an increased risk of recurrence for multiple and more aggressive adenomas in men taking 1 mg/d of FA supplement for approximately 7 years<sup>(46)</sup>. However, a second trial and a combined meta-analysis of three randomised clinical trials found that FA supplementation had no effect on CRC recurrence<sup>(47,48)</sup>. Here, we have distinguished tumour initiation (number) from tumour growth (size and load) and progression (lesion grade) to determine the effect of FA on IBD-associated CRC. We observed no effect of dietary FA on tumour load and an inverse U-shaped effect of FA on the percentage of large tumours, such that control diet-fed mice had the highest number of large tumours. These data suggest that FA deficiency and supplementation may inhibit tumour growth. Folate metabolism is required for *de novo* nucleotide synthesis; therefore, folate deficiency can limit tumour cell proliferation. On the other hand, supplementation of UC patients with folic acid, a reduced form of folate, has been shown to reduce cell proliferation abnormalities of colon epithelial cells<sup>(15)</sup>; supplemental FA may exert a similar anti-proliferative effect on colon cancer cells. The mechanism by which FA supplementation would provide such protection in transformed cells is unknown but may be related to its function in maintaining gene expression and genome stability.

In terms of tumour progression, we observed a numeric but insignificant increase in the incidence of adenocarcinoma among mice fed FA-containing diets, suggesting that diet had a limited or no effect. This is partly in line with observations made in a genetic model of colitis-mediated CRC, the IL-2- and  $\beta$ 2-microglobulin-deficient mouse, in which an inverse U-shaped effect of FA on high-grade lesions was observed such that control diet-fed mice had the highest incidence of high-grade lesions<sup>(49)</sup>. IL-2- and  $\beta$ 2-microglobulin-deficient mice also demonstrated an inverse U-shaped effect of FA on combined death and high-grade lesions, which was recapitulated in the present study. Dietary FA deficiency may prevent transition of tumours to more aggressive adenocarcinomas, perhaps by inhibiting cell proliferation, while supplementation may have a minimal protective or no effect on the development of invasive tumours.

Hyperhomocysteinaemia is repeatedly observed in patients with IBD. A meta-analysis has found that IBD patients have an OR of over 4 for hyperhomocysteinaemia<sup>(19)</sup>. Homocysteine has been shown to be pro-inflammatory and could play an active role in colitis. We demonstrate that FA-deficient mice have higher plasma homocysteine than mice fed FA-containing diets, as expected. However, plasma homocysteine was negatively associated with tumour number and load,

suggesting that homocysteine does not promote tumour development or growth in this model. There was no relationship between colitis score (a measure of inflammation) and homocysteine, indicating that homocysteine also does not promote inflammation in this model. Since patients with UC often demonstrate folate or other B vitamin deficiencies due to malabsorption<sup>(11,19,22,23)</sup>, we propose that hyperhomocysteinaemia results from reduced vitamin status and does not play a causal role in disease severity or progression.

The pathogenesis of colitis has been associated with changes in the intestinal microflora and certain bacterial species have been associated with disease risk. We did not observe a clear pattern associating dietary FA and changes in the microflora. The lack of an impact of FA, which may be considered a mild modifier of cancer initiation and progression, on the intestinal flora may be due to the highly penetrant effect of the AOM–DSS treatment. FA-dependent changes may simply have been masked by the AOM–DSS treatment. Indeed, the AOM–DSS treatment influenced the bacterial communities such that a greater percentage of clones from the *Bacteroides* and *Allobaculum* genera were observed in AOM–DSS-treated mice compared with control or DSS-treated mice. These obligate anaerobes have not been linked to bowel disease<sup>(50–52)</sup> or have been shown to be reduced<sup>(50,53)</sup>. However, Bacteroidetes were higher in irritable bowel subjects with mixed symptoms<sup>(54)</sup>. A prominent AOM–DSS-dependent pattern was a decrease in Ruminococcaceae, a potential response to DSS as it has been shown to reduce microbial richness<sup>(55)</sup>, but this response may be diet and animal model-dependent. Increased luminal cell turnover in response to AOM–DSS could also introduce more protein into the luminal space, giving preference to *Bacteroides* over Ruminococcaceae.

Overall, we observed a minimal effect of FA on tumour initiation, growth and progression, although the tendency for a higher tumour incidence and the development of more tumours in mice fed FA-containing diets remains a potential concern. Additional studies with greater power to detect FA-dependent differences are required to clarify these findings. We demonstrate that hyperhomocysteinaemia is probably a biomarker of dietary status and folate malabsorption rather than a causal factor in colitis-mediated colon cancer. Also, we did not observe an effect of FA on the intestinal microflora, but rather demonstrated that the microflora changes in response to inflammation and cellular transformation, as observed in AOM–DSS-treated mice.

### Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0007114512001857>

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