

1 **Does folic-acid supplementation prevent or promote colorectal cancer?**
2 **Results from model-based predictions**

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41 **Abstract**

42

43 Folate is essential for nucleotide synthesis, DNA-replication and methyl-group supply. Low-
44 folate status has been associated with increased risks of several cancer types, suggesting a
45 chemopreventive role of folate. However, recent findings on giving folic acid (FA) to patients
46 with a history of colorectal polyps raise concerns about the efficacy and safety of folate
47 supplementation and the long-term health effects of folate fortification. Results suggest that
48 undetected precursor lesions may progress under FA supplementation, consistent with folate's
49 role in nucleotide synthesis and cell proliferation.

50

51 To better understand the possible trade-offs between FA's protective effects due to decreased
52 mutation rates and possibly concomitant detrimental effects due to increased cell proliferation,
53 we used a biologically-based mathematical model of colorectal carcinogenesis. We predict
54 changes in cancer risk based on timing of treatment start and the potential impact of FA on cell
55 proliferation and mutation rates. Conclusion: changes in colorectal cancer risk in response to FA
56 supplementation are likely a complex function of treatment start, duration, and impact on cell
57 proliferation and mutations rates. Predicted colorectal cancer incidence rates under
58 supplementation are mostly higher than rates without FA supplementation unless
59 supplementation is initiated early in life (before age 20). To the extent to which this model
60 predicts reality, it indicates that the effect on cancer risk when starting FA supplementation late
61 in life is small, yet mostly detrimental. Experimental studies are needed to provide direct
62 evidence for this dual role of folate in colorectal cancer and to validate and improve the model
63 predictions.

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65

66 **Introduction**

67

68 The B-vitamin folate is essential for the synthesis of nucleotides as well as for the provision of
69 methyl groups for the maintenance of DNA methylation in dividing cells (1). Low intakes of
70 folate have been associated with increased risks of cancers of the colon, pancreas, esophagus,
71 and possibly breast (2-4). The biologic mechanisms ascribed to these associations include higher
72 mutation rates and reduced stability of DNA methylation patterns with a low folate status (1).
73 However, the protective role of folate in carcinogenesis has recently been questioned and may be
74 more complex and dependent on dose and timing of folate administration during the
75 carcinogenic process (2, 3, 5). Animal experiments show that folate supplementation prior to the
76 establishment of early neoplastic lesions reduces carcinogenesis, while administration after pre-
77 cancerous lesions are present appears to increase tumor growth (2). Similarly, epidemiological
78 evidence suggests that excessive levels of folate are not beneficial and may actually enhance
79 cancer risk (3, 6). The recent polyp-prevention trial by Cole et al. is a case in point (7). This trial
80 involved the administration of 1mg/day folic acid (the synthetic form of folate with greater
81 bioavailability) to patients with a history of colorectal polyps. It provides first human evidence
82 for a potentially detrimental effect of high dose folate in humans: not only was folic acid not
83 chemopreventive, but an increased risk of advanced and multiple adenoma was observed in the
84 intervention arm at 6-8 years of follow-up (advanced adenoma RR= 1.67; 95% CI: 1.00-2.80; \geq
85 3 adenomas RR=2.32; 95% CI: 1.23-4.35). These results suggest that undetected precursor
86 lesions were more likely to progress with folic acid administration (8).

87

88 The bimodal role of folate in carcinogenesis is thought to be attributable to its role in nucleotide
89 synthesis. Rapidly proliferating tissues, including tumors, have a greater requirement for folate.
90 Cancers often overexpress folate receptors and genes in nucleotide synthesis to meet their
91 increased need of sustained DNA synthesis and proliferation (9-11). Antifolate drugs have been
92 successfully used in cancer chemotherapy and in the 1940s folate has been described as inducing
93 an “acceleration phenomenon” if provided to children with leukemia, suggestive of increased
94 progression (12).

95

96 The question arises whether folate intakes in the population may approach levels that could cause

97 harm. Approximately 30-40% of adults in the United States use nutritional supplements that
98 contain folic acid, with a standard multivitamin containing 400 μg (13). Ironically, the group
99 with the highest supplement use is comprised of older individuals, who are also more likely to
100 have precancerous lesions. For example, colorectal polyps are thought to exist in about 30% of
101 adults 60 years and older, but in many fewer younger individuals (14, 15). In addition to
102 supplement use, a number of functional foods are fortified with folic acid, including nutrition
103 bars and drinks (often at 400 μg /serving), as well as fortified breakfast cereals. Finally, the
104 United States initiated folic acid fortification of grain products, allowable in 1996 and mandatory
105 as of Jan 1, 1998, to reduce the incidence of neural tube defects. This public health measure has
106 been highly effective in reducing neural-tube defects (16), yet has also increased the folic acid
107 intake universally in the US in the population (beyond the target group of women of childbearing
108 age) by about 100-200 μg /day (17). Biomarkers of folate intake suggest that a significant fraction
109 of the population has now folate levels that have been previously considered
110 “supraphysiological”, presumably due to a combination of fortification and supplement use (17).

111
112 The increase in folate status in the population and its potentially dual role in colorectal
113 carcinogenesis has raised the question whether folic acid fortification will prevent or promote
114 colorectal cancer (18). Considering the multiple, opposing effects on cancer risk, the answer is
115 not straightforward. We approached this complex question by using a mathematical model for
116 colorectal carcinogenesis. The model uses 4 stages to describe the progression to a colorectal
117 polyp that grows and transforms into a carcinoma. Results from this modeling strategy have
118 previously been shown to match Surveillance, Epidemiology and End Results (SEER) incidence
119 data of colorectal cancer (19). We utilize this model to investigate the “net impact” of effects of
120 folate supplementation on mutation rates and cellular proliferation on colorectal cancer rates in
121 the population.

122

123 **Model and methods**

124

125 To explore colon cancer risk in response to changes in folate status (e.g. folate supplementation)
126 we utilize a mathematical model that mirrors the multistage nature of colorectal cancer including
127 salient features of its pathogenesis. Specifically, we use the multistage clonal expansion model

128 developed by Luebeck and Moolgavkar (19), a model which has been shown to be consistent
129 with the observed incidence of colorectal cancer in the general population. The model stipulates
130 three distinct phases in the process of carcinogenesis. In the first phase, that of initiation, a
131 susceptible stem cell acquires one or more mutations resulting in an initiated cell, which has
132 partially escaped growth control. The second phase, that of promotion, is the clonal expansion of
133 initiated cells. Promotion is an extremely efficient way of bringing about carcinogenesis because
134 clonal expansion results in increased populations of cells that have already acquired some of the
135 genetic alterations on the pathway to malignancy. In the last phase, that of malignant conversion,
136 an initiated cell acquires another genetic change, one required to convert it into a malignant cell.
137 There is considerable evidence that most human malignancies go through these three phases and
138 that environmental agents, such as radiation and tobacco smoke, influence carcinogenesis via
139 their effects on one or more phases of this process (20, 21).

140

141 Fig.1 provides a schematic view of the colon carcinogenesis model. The model assumes that the
142 formation of an adenoma requires biallelic inactivation of a tumor suppressor (or 'gatekeeper')
143 gene, such as the APC gene. It also assumes sustained (asymmetric) stem cell divisions of a
144 mutant stem cell progenitor represented in the model by a high-frequency event. These divisions
145 represent the sustained generation of mutant progeny (via transient amplification) by a mutant
146 stem cell located at the bottom of a colonic crypt. Therefore, according to this model, a mutant
147 stem cell will only undergo clonal expansion (or promotion) once it leaves the protective
148 environment of the stem cell compartment and moves toward the top of the crypt – and the rate
149 of clonal expansion is determined by the net cell proliferation parameter $\alpha\beta$ (Fig.1) (22, 23).
150 Finally, a carcinoma develops from an adenoma in a single rate-limiting event (the 'adenoma to
151 carcinoma' transition), which in this context is also referred to as malignant transformation.

152

153 For the purpose of this study, we have extended the mathematical formulation of this model to
154 accommodate time-dependent model parameters reflecting the changes that might occur in an
155 individual's folate status. For simplicity, we assume that a change in folate intake from one level
156 to another immediately effects constant changes in the model parameters ignoring possible
157 delays in the cellular and enzymatic response to folate. Mathematical details of the model and a
158 derivation of the age-specific hazard function and tumor probabilities can be found in the

159 supporting information of Luebeck and Moolgavkar (19, 24). The extension of the hazard
160 function from constant parameters to piece-wise constant parameters is provided in the
161 Appendix. R-code for computing the hazard function and tumor probabilities can be obtained
162 from the authors upon request.

163
164 First, we use the mathematical model to explore the relative effects of folate-induced changes in
165 mutation rates and cell proliferation rates (of stem cells in adenomas) on colon cancer risk
166 (Figure 1). Here we are interested in quantifying the trade-offs between potentially opposing
167 effects of folate (or folate supplementation) on the carcinogenic process: reduction of mutation
168 rates (e.g., by reduced uracil misincorporation into DNA) versus stimulation of cell proliferation
169 of intermediate (adenomatous) cell populations at risk for malignant transformation. Specifically,
170 we assume that increases in the cell proliferation rate α (see Fig. 1) translate into proportionate
171 increases in the net proliferation (promotion) parameter $\alpha\beta$. This assumption is equivalent to
172 assuming that both cell proliferation and cell death (apoptosis) are equally affected by folate.
173 However, it is possible that folate reduces the rate of cell death yielding even a stronger effect on
174 tumor promotion. Thus, assuming that the percent increase in cell proliferation equals the percent
175 increase in net cell proliferation (or promotion), we make a conservative assumption concerning
176 a possible tumor-promoting effect of folate in carcinogenesis.

177
178 Second, we explore the predicted time course of colon cancer risk and its dependency on the age
179 at which folate supplementation begins. Finally, since the magnitude of both folate's effects on
180 mutation rates and cell kinetics are uncertain and likely depend on folate dose and genetic make-
181 up of the individual, we explore the sensitivity of the cancer risk to independent variations in
182 both mutation rates and net cell proliferation (promotion) rates. For simplicity, we assume that
183 all three mutation rates (μ_0 , μ_1 , and μ_2) in the model are equally affected by folate, an
184 assumption that seems plausible for the first two events representing mutations at the APC locus.
185 However, considering that the adenoma-carcinoma transition (rate μ_2) is a more complex
186 process that involves malignant transformation and the dynamics of tumor progression, there is
187 more uncertainty regarding the response of μ_2 to folate.

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189 **Results**

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Figure 2 (panel A) shows the impact of reduced mutation rates, alone or in combination with increased cell proliferation rates, on the relative risk of colon cancer as a function of age when supplementation commences early in life (age 2). For completeness we also show the risk when cell proliferation is increased without concomitant increases in mutation rates. Panel B shows the same scenarios as panel A, but for the predicted excess number of colon cancers per 100,000 individuals at risk. Assuming that folate has no effect on cell proliferation, a 20% reduction in mutation rates translates into an approximately 40% reduction in relative risk about 10-20 years after start of treatment. In contrast, a concomitant 20% increase in cell proliferation is seen to interfere with the protective effect from reduced mutation rates in a complex age-dependent manner. In this scenario, with the exception of an early (transient) reduction of relative risk before age 30, a significant/meaningful reduction in relative risk does not occur until much later in life (solid line). The potentially rapid drop in relative risk soon after start of folate supplementation (age 2 in Fig.2) can be attributed to the reduction in the adenoma-carcinoma transition rate, the rate-limiting event representing malignant transformation and (in the model here) immediate clinical detection of cancer. In spite of temporarily increased relative risks due to possible opposing effects of folate on mutation rates and rates of cell proliferation in colorectal adenoma (as shown in Panel A), the impact on the cumulative cancer risk (as shown in panel B) is much less pronounced but may still lead to several thousand excess cancer cases (per 100,000 individuals at risk) for individuals age 60 to 70. This example demonstrates that the effect of genomic protection that folate (or folic acid) is thought to exert on normal and cancerous cells could be canceled (at least partially) by an increase in cell proliferation of similar magnitude.

214 **Timing of folate supplementation.**

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Figure 3 shows the predicted number of excess cases of colon cancer (in the absence of competing causes) as a function of current age for 4 different ages at which supplementation begins (ages 2, 20, 40 and 60) assuming a constant folate dose. All scenarios assume that folate supplementation reduces the APC mutation rates (i.e. the rates of the first two rate-limiting events) and the malignant transformation rate by 20%, but increases the cell proliferation rate by

221 20% compared to (untreated) controls. It can be seen that the cancer risk is mostly higher than
222 the background risk (without supplementation) unless folate supplementation begins early in life.
223 On the other hand, it is intuitively clear that, when folate is given late in life, the effect on
224 cumulative risk will be small as the majority of cancer cases occur before treatment begins.
225 Qualitatively similar curves are obtained for a 10%/10% scenario (results not shown). The main
226 conclusion drawn here is that the risk of colon cancer would mostly be higher than the
227 background risk (without supplementation) unless folate supplementation is begun early in life
228 (well before age 20). When folate supplementation is started late in life, its effect on the
229 cumulative cancer risk appears to be small and mostly detrimental.

230

231 **Sensitivity of colorectal cancer risk to (hypothetical) variations of mutation and cell**
232 **proliferation rates.**

233

234 The magnitude of folate-associated anti-carcinogenic and carcinogenic effects is highly
235 uncertain. They likely depend on folate dose (e.g., fortification versus supplementation) and
236 genetic make-up of the individual (25, 26). To address this uncertainty on the level of the cell
237 (and within the framework of multistage carcinogenesis), we explore the sensitivity of the (life-
238 time) cancer risk to variations in both mutation rates and cell proliferation rates (Fig.4). To keep
239 the discussion simple, we assume (see Models and Methods) that the percent increase in cell
240 proliferation equals the percent increase in net cell proliferation (or tumor promotion) which is
241 the main determinant for tumor growth. The predicted cancer risk, again in terms of the expected
242 number of excess cases by age 70, responds almost linearly to moderate reductions (between 0
243 and 40%) in mutation rates and increases in proliferation in the same range, unless
244 supplementation is started early in life. When started early in life (Fig.4 top panel), the risk
245 surface responds exponentially to changes in mutation rates. The enhanced sensitivity of the
246 cancer risk with very early folate exposure reflects the more prolonged folate intake and higher
247 cumulative folate dose compared with later starting points. Comparison of the 3 scenarios shown
248 in Fig.4, especially in regard to the predicted (life-time) reductions in the number of colon cancer
249 cases in response to decreases in mutation rates, show that an efficient reduction in cancer risk –
250 in the presence of significant (putative folate-induced) increases in the growth rate of the
251 adenomatous polyps – likely occurs only when the supplementation starts very early in life.

252

253 Of particular interest is the nullcline (yellow curve in Fig.4), the curve for which reductions in
254 mutation rates and concomitant increases in tumor promotion cancel each other out yielding no
255 change in cancer risk. In addition, we also highlight two specific 'trade-off' scenarios (labeled 'A'
256 and 'B' on the surfaces shown in Fig.4) for which the percentage decrease in mutation rates
257 equals the percentage increase in net cell proliferation (or promotion). Specifically, the point 'A'
258 represents a 20/20 modulation, and the point 'B' a 30/30 modulation. Inspection of the respective
259 distances of points 'A' and 'B' from the nullclines (for the 3 scenarios shown in Fig.4) reinforces
260 the notion that, unless folate supplementation is begun very early in life, the risk of colon cancer
261 may well be increased if folate promotes premalignant lesions on the pathway to cancer.
262 Furthermore, the point 'B', which represents a higher sensitivity, is pushed toward higher risks
263 (away from the nullcline) compared to point 'A' when the onset of folate supplementation is
264 delayed for 2 or more decades.

265

266 Table 1 provides direct estimates for this sensitivity analysis, giving predicted rates of colorectal
267 cancer in the population depending on multiple scenarios. It illustrates how the expected number
268 of colorectal cancer cases changes depending on proliferation rate, mutation rate, and starting
269 age of folic acid supplementation use. For example, compared to the “baseline” of 3,319/100,000
270 cases per year, there are substantial reductions in the number of cases if the mutation rate is
271 reduced by 20%, independent of age group. If concomitantly proliferation increases (scenario:
272 +10% proliferation / -20% mutation rate), then only the youngest age group still benefits (-455
273 cases) whereas individuals who were 20 or 30 years old at the initiation of supplementation
274 would show increased cancer rates. If the proliferation increase is more substantial (scenario:
275 +20% proliferation / -20% mutation rate) then increases in cancer rates are expected in all age
276 groups (+419/100,000 for those who were 2 years old at supplementation begin, + 1053/100,000
277 for those who were 40-years old).

278

279 **Discussion**

280

281 Here we use a mathematical modeling strategy to investigate the “net effect” of folate
282 administration on colorectal cancer incidence in the general population, varying the age at which

283 supplementation is started as well as the putative effects of folate on mutation rates and net cell
284 proliferation. Naturally, this model can only provide predictions that need to be tested further in
285 experimental and epidemiologic settings. However, in consideration of potential harmful effects
286 of folate in subsets of the human population, many study designs may not be ethical or feasible.
287 The specific examples proffered here are clearly hypothetical, but construed to allow an
288 assessment of uncertainty and sensitivity of cancer risk in response to specific folate effects. Our
289 examples address two questions, (1) what are the relative biological effects of folate-induced
290 reductions in mutation rates versus increases in cell proliferation, either in isolation or
291 concomitantly, and (2), how does the cancer risk associated with folate supplementation vary
292 with age at the initiation of supplementation?

293
294 Results from our modeling suggest that the age of initiation of folate supplementation is critical.
295 Unless folate supplementation begins very early in life, there appears little benefit of folate
296 supplementation and possible harm, if one assumes equal effect sizes (e.g. 20% mutation
297 reduction and a 20% proliferation increase). How quickly any potential (population-level)
298 benefit may be lost, when folate supplementation does not start very early in life, can be gleaned
299 from Fig.3. When the start occurs between ages 20 and 40, the excess number of cancer cases
300 reaches about 2000/100000 at age 80, and is still at about 1000/100000 excess cases at age 80
301 when folate supplementation starts at age 60.

302
303 The sensitivity analyses that explore various effect sizes for folate on mutation reduction and on
304 cell proliferation illustrate the potency of a promotional effect of folate on proliferation. Even
305 when folate supplementation is started in young adulthood (around age 20) our model
306 calculations suggest that a 10-15% promotional effect of folate is sufficient to eliminate any
307 protective effect associated with a 40% reduction of all three critical mutation rates in the model
308 (i.e. the rates of both APC mutations and the rate-limiting event defining the adenoma-carcinoma
309 transition). These results should raise concerns, even if there is significant uncertainty in our
310 knowledge of the tumor- or growth-promoting effects of folate. Considering that a very modest
311 increase in cellular proliferation with folate may have significant adverse effects on colorectal
312 cancer rates in the population, we really need better quantitative data to establish the extent of
313 such effects. It is conceivable that such effects only occur at an overall excess dose, or that they

314 are more pronounced with folic acid rather than natural folates. It is unlikely that such data can
315 be generated from human studies, but may be generated from animal studies under controlled
316 environments and exposures (2).

317
318 Our model suggests that, in individuals who are older when supplementation is initiated, an
319 increase in colorectal cancer rates is expected to be seen after a period of about 10 years (see Fig.
320 3). These data appear consistent with recent data from the SEER cancer registries in the United
321 States and Canada presented by Mason et al. (27). In this ecological study, shortly after the
322 initiation of fortification, colorectal cancer rates increased in both countries resulting in an
323 annual excess of ~4 to 6 additional CRC cases per 100,000 individuals at risk. This abrupt
324 increase in cancer risk may simply reflect the accelerated development and growth of a nascent
325 malignancy into a clinically detectable or symptomatic cancer. Although our model does not
326 explicitly describe this process (which may be considered part of the adenoma-carcinoma
327 transition described by the last step in our model), it allows for accelerated growth of benign
328 adenomas on the pathway to cancer (promotion). Our model predicts an excess CRC risk that
329 increases with time about 10 years after supplementation is initiated (Fig. 3). Note, however,
330 Mason et al. only studied a period of 7 years (1996-2002) of folate fortification (27) and to
331 answer the question whether or not the observed increase in CRC risk is transient, constant, or
332 will eventually increase as predicted by our model will require more follow up data.

333
334 There are several uncertainties to our modeling. Although folate deficiency induces decreases in
335 cellular proliferation, there is no direct support for increased cell replication *in vivo* in the
336 presence of excess folate. However, *in vitro* studies show that cellular growth arrest induced by
337 folate deficiency can be reverted (after some delay) under acute folate repletion (28). Moreover,
338 possible changes in the rate of cell differentiation and apoptosis, which have been only poorly
339 studied in this context, may also play a decisive role in promoting tumor growth. Note that
340 accelerated tumor growth may result either from increases in the rate of cell replication, or from
341 decreases in the rate of apoptosis, or delays in cell differentiation. A recent study of folate
342 supplementation on mucosal cell proliferation in high risk patients for colon cancer is of interest
343 regarding the latter possibility (29). In this study it was found that folate supplementation mainly
344 decreased cell proliferation in the luminal part of the colorectal crypts, reflecting defective cell

345 differentiation control and delayed onset of normal cell differentiation and, ultimately, apoptosis.
346 Such delays may effectively increase tumor size.

347

348 The effects of folate on mutation rates are also not well quantified. Folate deficiency increases
349 the misincorporation of uracil into DNA damage, which can cause DNA strand breaks during
350 repair. However, because methylated CpG sites are mutational hotspots for C>T transitions,
351 moderate folate deficiency, which reduces, albeit slightly, genomic DNA methylation, may also
352 protect against this type of mutation, as has been suggested for the MTHFR 677 TT genotype
353 (30). Thus, the relationship between folate and mutation rates still needs to be better defined.

354

355 We are well aware of the hypothetical nature of this investigation. For one, the model used here,
356 although broadly consistent with important biological processes involved in colorectal cancer
357 and numerically consistent with the observed incidence of colorectal cancer in SEER (19), is
358 fraught with considerable uncertainty. For example, not all biological parameters of the model
359 can be estimated from incidence data alone making it necessary to fix one or the other model
360 parameter [see Appendix]. Furthermore, the number of clonogenic (transformable) stem cells in
361 colon remains elusive (with estimates from a few stem cells to hundreds of stem cells per colonic
362 crypt (31-33). More concerning, however, is the uncertainty due to multiple 'modes of action' of
363 folate on cancer initiation, cell proliferation and tumor progression. Much experimental and
364 clinical work remains to be done to define and to quantify the beneficial and detrimental effects
365 of folate on cancer risk, in particular how these effects are mediated on the cellular level. Our
366 calculations suggest that a focus be given to a careful study of possible proliferative effects of
367 folate on precursor lesions such as the adenomatous polyps in colon. We propose that this
368 question first be studied in a rodent model where the number and sizes of premalignant and
369 malignant lesions can be readily measured and cell proliferation kinetics ascertained with
370 immuno-histochemical techniques. Mouse endoscopy techniques provide now tools to evaluate
371 the impact of folate on existing polyps. As noted above, these cancer precursors are very
372 common (14), yet often go undetected due to a lack of appropriate colorectal cancer screening.
373 Only about 30% of US adults over age 50 have had a screening colonoscopy within the previous
374 5 years (34). As noted previously, the Aspirin/Folate Prevention Trial (7) only enrolled
375 individuals with prior resected adenoma and full colonoscopies. Thus, it does not provide

376 answers regarding the effects of folate on existing polyps, which may potentially be much
377 stronger than those on newly arising (or undetected) polyps, that were observed within the trial
378 during follow-up colonoscopy (8).

379

380 In the United States, some have suggested that a further increase in the amount of folic acid in
381 fortified foods is warranted. Similarly, several European countries are considering the
382 introduction of folic acid fortification for the prevention of neural tube defects. Our results add
383 another important piece to inform this public health policy debate. A key message is that – if
384 excessive folate has tumor-promoting effects, then those are likely to outweigh any beneficial
385 effects of folic acid supplementation on mutation rates with cancer rates predicted to rise in all
386 but those treated at a very young age. These results suggest caution when considering the
387 implementation of fortification until we have better data on the effects of folate on cell kinetics,
388 tumor promotion, and their quantitative effects by dose and type of folate.

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392 **References**

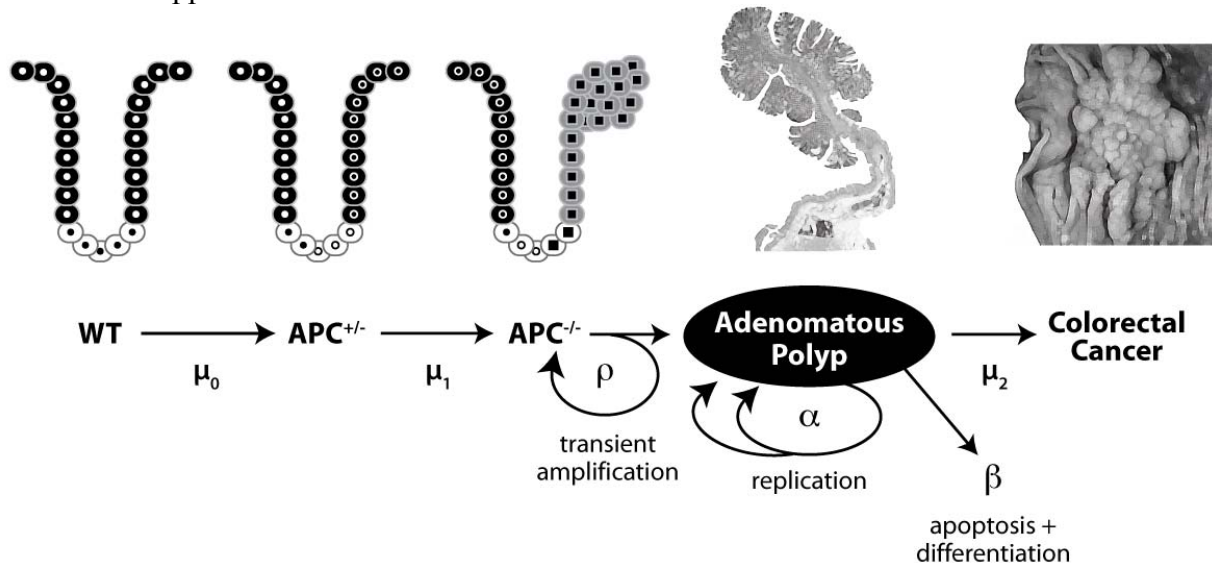
393

- 394 1. Choi, S. W., and Mason, J. B. Folate status: effects on pathways of colorectal
395 carcinogenesis. *J Nutr*, 132: 2413S-2418S, 2002.
- 396
- 397 2. Kim, Y. I. Folate and colorectal cancer: an evidence-based critical review. *Mol Nutr Food*
398 *Res*, 51: 267-92, 2007.
- 399
- 400 3. Ulrich, C. M. Folate and cancer prevention: a closer look at a complex picture. *Am J Clin*
401 *Nutr*, 86: 271-3, 2007.
- 402
- 403 4. Larsson, S. C., Giovannucci, E., and Wolk, A. Folate Intake, MTHFR Polymorphisms,
404 and Risk of Esophageal, Gastric, and Pancreatic Cancer: A Meta-analysis. *Gastroenterology*,
405 131: 1271-83, 2006.
- 406
- 407 5. Ulrich, C. M., and Potter, J. D. Folate supplementation: too much of a good thing? *Cancer*
408 *Epidemiol Biomarkers Prev*, 15: 189-93, 2006.
- 409
- 410 6. Stolzenberg-Solomon, R. Z., Chang, S. C., Leitzmann, M. F., Johnson, K. A., Johnson,
411 C., Buys, S. S., Hoover, R. N., and Ziegler, R. G. Folate intake, alcohol use, and postmenopausal
412 breast cancer risk in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. *Am J*
413 *Clin Nutr*, 83: 895-904, 2006.
- 414
- 415 7. Cole, B. F., Baron, J. A., Sandler, R. S., Haile, R. W., Ahnen, D. J., Bresalier, R. S.,
416 McKeown-Eyssen, G., Summers, R. W., Rothstein, R. I., Burke, C. A., Snover, D. C., Church, T.
417 R., Allen, J. I., Robertson, D. J., Beck, G. J., Bond, J. H., Byers, T., Mandel, J. S., Mott, L. A.,
418 Pearson, L. H., Barry, E. L., Rees, J. R., Marcon, N., Saibil, F., Ueland, P. M., and Greenberg, E.
419 R. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *Jama*, 297:
420 2351-9, 2007.
- 421
- 422 8. Ulrich, C. M., and Potter, J. D. Folate and cancer--timing is everything. *Jama*, 297: 2408-
423 9, 2007.
- 424
- 425 9. Weitman, S. D., Lark, R. H., Coney, L. R., Fort, D. W., Frasca, V., Zurawski, V. R., Jr.,
426 and Kamen, B. A. Distribution of the folate receptor GP38 in normal and malignant cell lines
427 and tissues. *Cancer Research*, 52: 3396-401, 1992.
- 428
- 429 10. Hough, C. D., Cho, K. R., Zonderman, A. B., Schwartz, D. R., and Morin, P. J.
430 Coordinately up-regulated genes in ovarian cancer. *Cancer Research*, 61: 3869-76, 2001.
- 431
- 432 11. Gates, S. B., Mendelsohn, L. G., Shackelford, K. A., Habeck, L. L., Kursar, J. D.,
433 Gossett, L. S., Worzalla, J. F., Shih, C., and Grindey, G. B. Characterization of folate receptor
434 from normal and neoplastic murine tissue: influence of dietary folate on folate receptor
435 expression. *Clin Cancer Res*, 2: 1135-41, 1996.
- 436
- 437 12. Farber, S. Some observations on the effect of folic acid antagonists on acute leukemia and

- 438 other forms of incurable cancer. *Blood*, 4: 160-167, 1949.
- 439
- 440 13. Radimer, K., Bindewald, B., Hughes, J., Ervin, B., Swanson, C., and Picciano, M. F.
- 441 Dietary supplement use by US adults: data from the National Health and Nutrition Examination
- 442 Survey, 1999-2000. *Am J Epidemiol*, 160: 339-49, 2004.
- 443
- 444 14. Cannon-Albright, L. A., Bishop, D. T., Samowitz, W., DiSario, J. A., Lee, R., and Burt,
- 445 R. W. Colonic polyps in an unselected population: prevalence, characteristics, and associations.
- 446 *American Journal of Gastroenterology*, 89: 827-31, 1994.
- 447
- 448 15. Rutter, C. M., Yu, O., and Miglioretti, D. L. A hierarchical non-homogenous Poisson
- 449 model for meta-analysis of adenoma counts. *Stat Med*, 26: 98-109, 2007.
- 450
- 451 16. Honein, M. A., Paulozzi, L. J., Mathews, T. J., Erickson, J. D., and Wong, L. Y. Impact
- 452 of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA*,
- 453 285: 2981-6, 2001.
- 454
- 455 17. Pfeiffer, C. M., Caudill, S. P., Gunter, E. W., Osterloh, J., and Sampson, E. J.
- 456 Biochemical indicators of B vitamin status in the US population after folic acid fortification:
- 457 results from the National Health and Nutrition Examination Survey 1999-2000. *Am J Clin Nutr*,
- 458 82: 442-50, 2005.
- 459
- 460 18. Kim, Y. I. Will mandatory folic acid fortification prevent or promote cancer? *Am J Clin*
- 461 *Nutr*, 80: 1123-8, 2004.
- 462
- 463 19. Luebeck, E. G., and Moolgavkar, S. H. Multistage carcinogenesis and the incidence of
- 464 colorectal cancer. *Proceedings of the National Academy of Sciences of the United States of*
- 465 *America.*, 99: 15095-100, 2002.
- 466
- 467 20. Hazelton, W. D., Luebeck, E. G., Heidenreich, W. F., and Moolgavkar, S. H. Analysis of
- 468 a historical cohort of Chinese tin miners with arsenic, radon, cigarette smoke, and pipe smoke
- 469 exposures using the biologically based two-stage clonal expansion model. *Radiat Res*, 156: 78-
- 470 94, 2001.
- 471
- 472 21. Little, M. P., Haylock, R. G., and Muirhead, C. R. Modelling lung tumour risk in radon-
- 473 exposed uranium miners using generalizations of the two-mutation model of Moolgavkar,
- 474 Venzon and Knudson. *Int J Radiat Biol*, 78: 49-68, 2002.
- 475
- 476 22. van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van
- 477 der Horn, K., Battle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Fong, M., Moerer, P., van den
- 478 Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. The beta-catenin/TCF-4
- 479 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell*, 111: 241-50,
- 480 2002.
- 481
- 482 23. Battle, E., Henderson, J. T., Beghtel, H., van den Born, M. M., Sancho, E., Huls, G.,
- 483 Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T., and Clevers, H. Beta-catenin and

- 484 TCF mediate cell positioning in the intestinal epithelium by controlling the expression of
485 EphB/ephrinB. *Cell*, *111*: 251-63, 2002.
- 486
- 487 24. Meza, R., Luebeck, E. G., and Moolgavkar, S. H. Gestational mutations and
488 carcinogenesis. *Math Biosci*, *197*: 188-210, 2005.
- 489
- 490 25. Ulrich, C. Genetic variability in folate-mediated one-carbon metabolism and cancer risk.
491 *In*: S. W. Choi and S. Friso (eds.), *Nutrient-Gene Interactions in Cancer*, pp. 75-91. Boca Raton,
492 FL: Taylor & Francis Group, 2006.
- 493
- 494 26. Ulrich, C. M. Nutrigenetics in cancer research--folate metabolism and colorectal cancer. *J*
495 *Nutr*, *135*: 2698-702, 2005.
- 496
- 497 27. Mason, J. B., Dickstein, A., Jacques, P. F., Haggarty, P., Selhub, J., Dallal, G., and
498 Rosenberg, I. H. A temporal association between folic acid fortification and an increase in
499 colorectal cancer rates may be illuminating important biological principles: A hypothesis. *Cancer*
500 *Epidemiol Biomarkers Prev*, *16*: 1325-9, 2007.
- 501
- 502 28. Melnyk, S., Pogribna, M., Miller, B. J., Basnakian, A. G., Pogribny, I. P., and James, S. J.
503 Uracil misincorporation, DNA strand breaks, and gene amplification are associated with
504 tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells. *Cancer*
505 *Letters*, *146*: 35-44, 1999.
- 506
- 507 29. Khosraviani, K., Weir, H. P., Hamilton, P., Moorehead, J., and Williamson, K. Effect of
508 folate supplementation on mucosal cell proliferation in high risk patients for colon cancer. *Gut*,
509 *51*: 195-9, 2002.
- 510
- 511 30. Ulrich, C. M., Curtin, K., Samowitz, W., Bigler, J., Potter, J. D., Caan, B., and Slattery,
512 M. L. MTHFR variants reduce the risk of G:C->A:T transition mutations within the p53 tumor
513 suppressor gene in colon tumors. *J Nutr*, *135*: 2462-7, 2005.
- 514
- 515 31. Bach, S. P., Renehan, A. G., and Potten, C. S. Stem cells: the intestinal stem cell as a
516 paradigm. *Carcinogenesis*, *21*: 469-76, 2000.
- 517
- 518 32. Potten, C. S., Booth, C., Tudor, G. L., Booth, D., Brady, G., Hurley, P., Ashton, G.,
519 Clarke, R., Sakakibara, S., and Okano, H. Identification of a putative intestinal stem cell and
520 early lineage marker; musashi-1. *Differentiation*, *71*: 28-41, 2003.
- 521
- 522 33. Yatabe, Y., Tavaré, S., and Shibata, D. Investigating stem cells in human colon by using
523 methylation patterns. *Proc Natl Acad Sci U S A*, *98*: 10839-44, 2001.
- 524
- 525 34. American Cancer Society. *Colorectal Cancer Facts and Figures Special Edition 2005*.
526 Atlanta: American Cancer Society, 2005.
- 527
- 528 35. Parzen, E. *Stochastic Processes*: Society for Industrial and Applied Mathematics, 1999.
- 529

530 **Figure 1.** CRC model: biallelic inactivation of the APC gene is assumed to occur in colonic stem cells in two rate-limiting steps with rates μ_0 (for the first allele) and μ_1 (for the second allele).
 531 After the first step the crypt may contain a mixture of APC-wild-type and APC^{+/-} cells (nuclei
 532 represented by filled and open circles, respectively). Transient amplification with production rate
 533 ρ populates the proliferative zone above the stem cell compartment with APC^{-/-} progeny (grey
 534 cells with square-shaped nuclei). Unresponsive to changes in Wnt-signaling, this progeny
 535 remains in a proliferative state as it enters the differentiation zone near the top of the crypt
 536 leading to rapid accumulation of APC^{-/-} cells. Subsequent clonal expansion of APC^{-/-} cells,
 537 which divide with rate α and die or differentiate with rate β , describes the growth of an adenoma.
 538 The final event in the model represents the adenoma-carcinoma transition, which occurs with
 539 rate μ_2 . A reduction in the mutation rates with folate supplementation is modeled by a %
 540 decrease of μ_0 , μ_1 and μ_2 . Effects on replication rates by folate supplementation are modeled
 541 with a % increase in the replication rate α . Folate supplementation is assumed to decrease the
 542 mutation rates μ_0 , μ_1 and μ_2 , but increases the cell division rate α and the transient amplification
 543 rate ρ . The ratio β/α is assumed constant, thus only the net cell proliferation rate $\alpha - \beta$ increases
 544 with folate supplementation.
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546
 547

Figure 2. A) Relative risk of colon cancer as a function of current age for when supplementation begins at age 2 with different combinations of reduced mutation rates and increased cell proliferation rates. **B)** Predicted excess number of colon cancers per 100,000 individuals at risk as a function of current age for when supplementation begins at age 2 with different combinations of reduced mutation rates and increased cell proliferation rates.

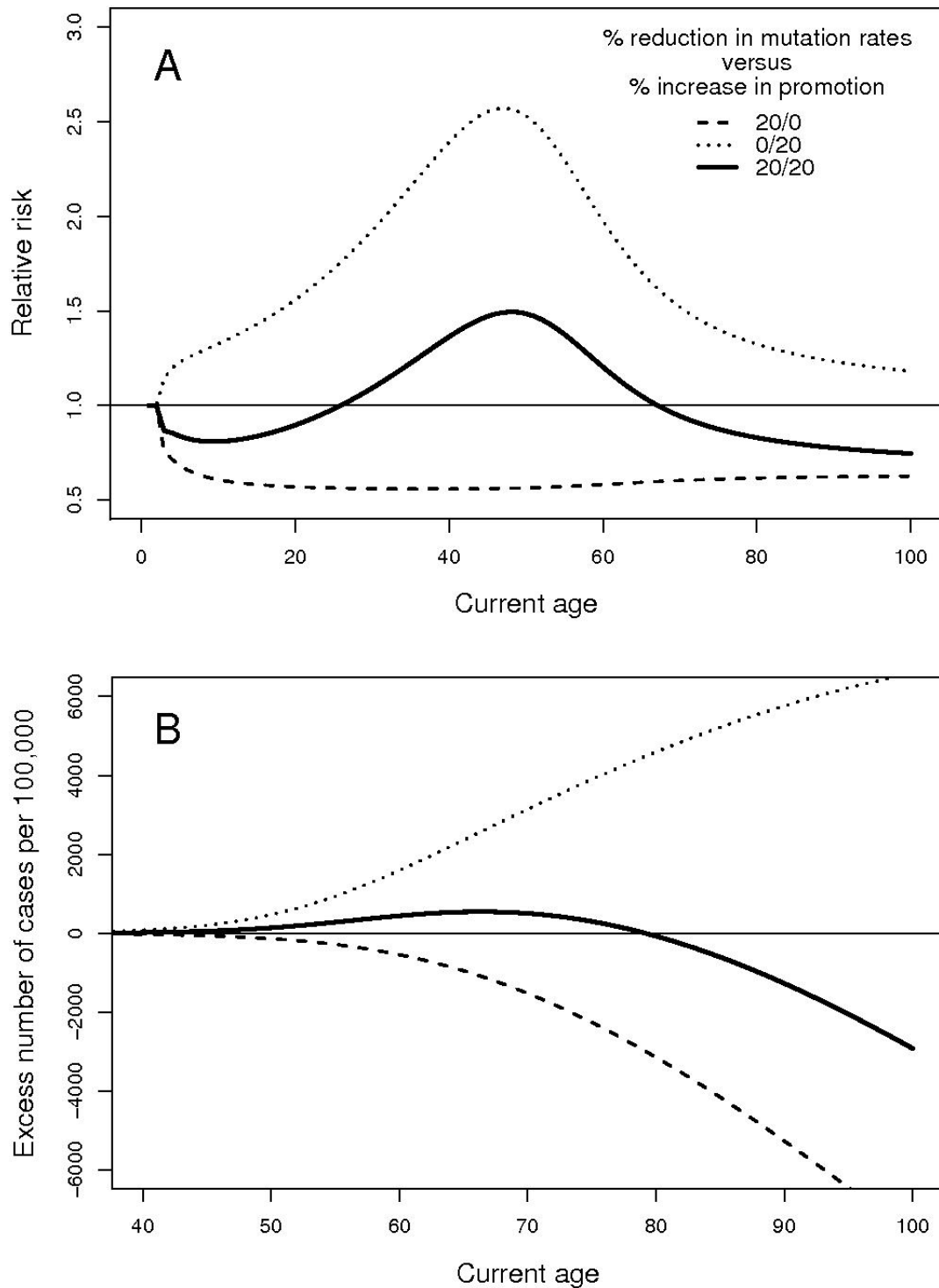


Figure 3. Predicted number of excess cases of colon cancer as a function of current age for different ages at which supplementation begins.

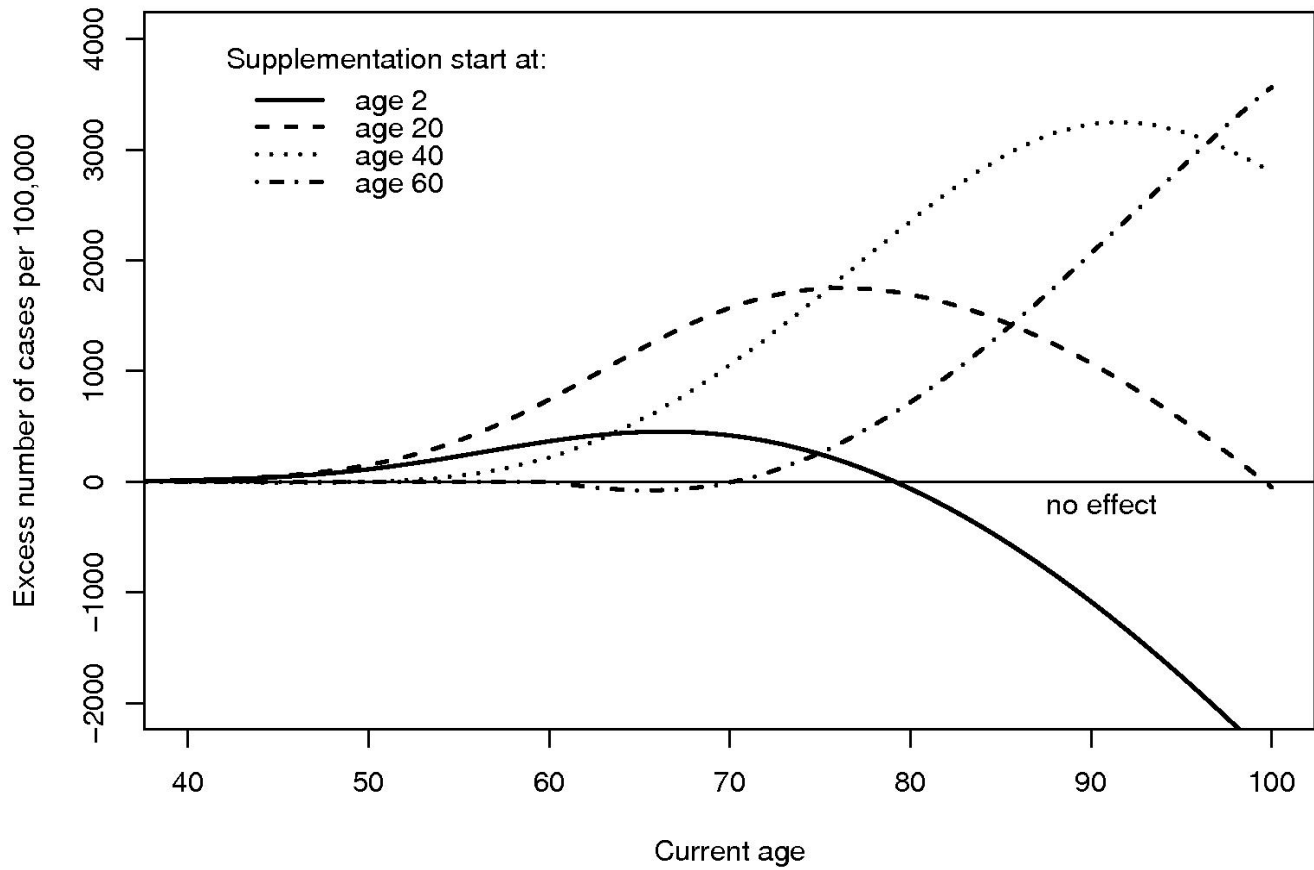


Figure 4. Predicted number of excess cases of colon cancer as a function of both mutation rates and cell proliferation rates for different ages at which supplementation begins.

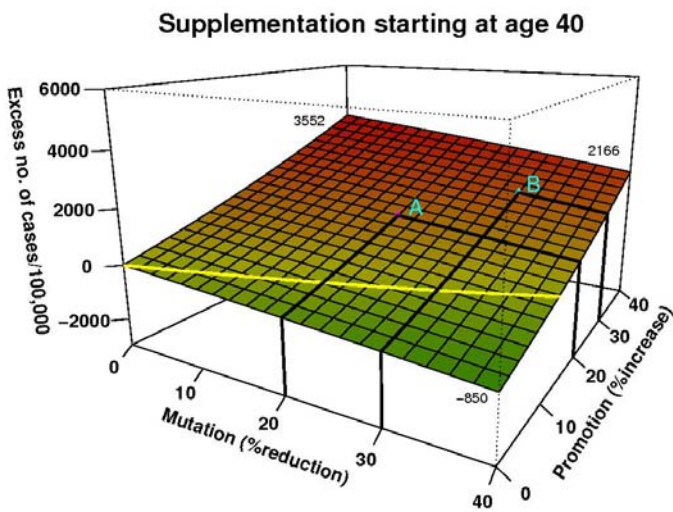
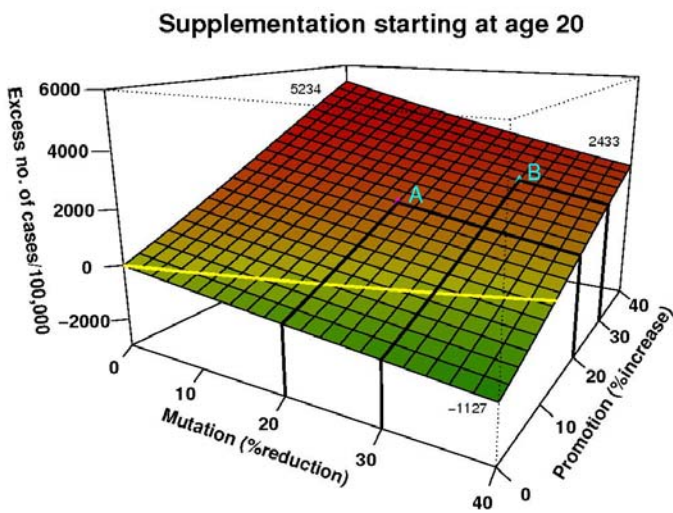
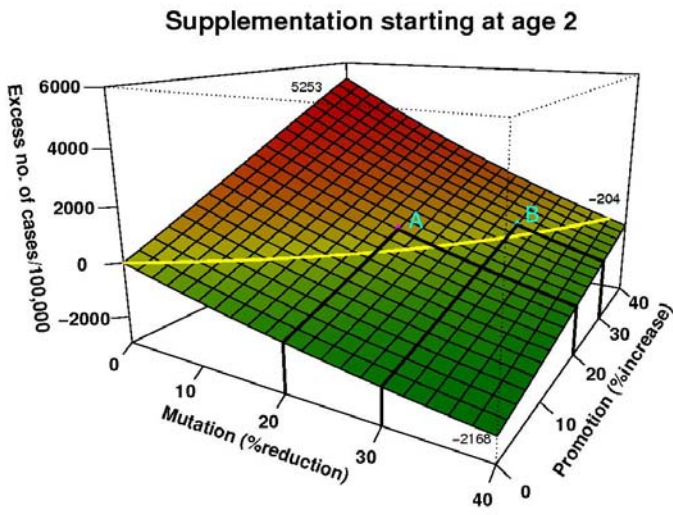


Table 1. Expected number of colorectal cancer cases depending on proliferation rate, mutation rate, and starting age of folic acid supplementation use.

Proliferation Increase (%)	Mutation Reduction (%)	Folic acid supplementation starting at age		
		2	20	40
<i>Expected number of colorectal cancer cases per 100,000 individuals at risk</i>				
0	0	3319	3319	3319
<i>Change in colorectal cancer cases</i>				
0	10	-687	-298	-193
0	20	-1274	-583	-397
10	0	+1286	+1223	+732
10	10	+353	+826	+511
10	20	-455	+449	+275
20	0	+2631	+2565	+1571
20	10	+1451	+2051	+1320
20	20	+419	+1570	+1053

Appendix

For the class of models where initiation of a premalignant lesion (such as an adenomatous polyp in the colon) requires a number of rate-limiting events to occur in specific order, as for the model presented in Fig.1, the tumor survival function is readily derived from the probability generating function of a filtered Poisson process (35). Specifically, let $S_k(s,t)$ represent the survival function of a filtered (non-homogeneous) Poisson process that starts off at time s for a model with k steps. Assume that cells of type 0 (normal cells) emit cells of type 1 with rate μ_0 , cells of type 1 emit cells of type 2 with rate μ_1, \dots , until cells of type $k-2$ emit cells of type $k-1$, which may undergo clonal expansion before giving rise to malignant progeny (type k). For this model class, it is easy to show that the survival function can be computed recursively:

$$S_j(s_{k-j}, t) = \begin{cases} \exp\left[-\int_{s_{k-j}}^t ds_{k-j+1} \mu_{k-j} (1 + X\delta_{j,k})(1 - S_{j-1}(s_{k-j+1}, t))\right] & \text{if } 2 < j \leq k \\ \left[(q-p)/(qe^{-p(t-s_{k-j})} - pe^{-q(t-s_{k-j})})\right]^{\mu_{k-j}/\alpha} & \text{if } j = 2 \end{cases}$$

Here, δ_{ik} is the Kronecker symbol, X is the number of normal stem cells in the tissue, and the parameters p and q are related to the biological parameters α (the cell division rate), β (the cell death rate), and the malignant transformation rate μ_{k-1} via

$$p = \frac{1}{2} \left((-\alpha + \beta + \mu_{k-1}) - \sqrt{(\alpha + \beta + \mu_{k-1})^2 - 4\alpha\beta} \right)$$

$$q = \frac{1}{2} \left((-\alpha + \beta + \mu_{k-1}) + \sqrt{(\alpha + \beta + \mu_{k-1})^2 - 4\alpha\beta} \right).$$

Note, $g \equiv -(p + q) = (\alpha - \beta - \mu_{k-1})$, is approximately equal to the net cell proliferation rate for the clonal stage (stage $k-1$) of the multistage process. The parameter q is approximately equal to $\mu_{k-1} / (1 - \beta/\alpha)$, which may be viewed as an upper bound for the malignant transformation rate. See Heidenreich et al. (1997) for more details.

The age-specific hazard function $h_k(t)$, required for the analysis of cancer incidence in populations, can now be derived from the survival function by computing

$$h_k(s_0, t) = \frac{\partial}{\partial t} \ln S_k(s_0, t),$$

which readily yields

$$h_k(s_0, t) = \int_{s_0}^t ds_1 \mu_0 X S_{k-1}(s_1, t) h_{k-1}(s_1, t).$$

Again, the first time argument of (s, t) makes explicit the time origin of the stochastic process in question. Thus, for a 4-stage model, we have (now dropping the argument $s_0 = 0$)

$$h_4(t) = \int_0^t ds_1 \mu_0 X S_3(s_1, t) \int_{s_1}^t ds_2 \mu_1 S_2(s_2, t) h_2(s_2, t),$$

where h_2 and S_2 are the hazard function and survival function of the two-stage clonal expansion (TSCE) model, respectively. See Heidenreich, et al. (1997) for explicit formulas for h_2 and S_2 for the case of constant or piecewise constant parameters. For constant parameters, setting $\mu_0 = \mu_1 = \nu$ (i.e. equality of the two APC mutation rates), the hazard function for our colon cancer model (see Fig.1) simplifies to

$$h_4(t) = \nu X \left(1 - \exp \left\{ \int_0^t du \nu (S_2(u, t) - 1) \right\} \right).$$

When the parameters are time-dependent, as may be the case with drug treatment or exposures to chemo-preventions such as NSAIDs or folate, then we use the more general formula for $h_4(t)$ and evaluate the time integrals numerically by integrating from time t to 0 for efficiency.

Note, X , the number of normal stem cells, always appears in combination with $\mu_0 (= \nu)$, and therefore cannot be determined without further assumptions or constraints on the parameters. For more details on parameter identifiability see e.g. Hanin and Yakovlev (1996) and Heidenreich et al. (1997).

We have fitted the 4-stage model to the incidence of colorectal cancers reported in the SEER registry (1973-2000) following the approach first described in Luebeck and Moolgavkar (2002). Our parameter estimates differ only slightly from those of the earlier analysis. We now include 4 additional calendar years (1997-2000). Specifically, for the calculations presented here, we use the estimates $\nu = 1.4910^{-6}$, $\rho (= \mu_2)/\alpha = 1.84$, $p \approx (\alpha - \beta) = 0.155$, and $q = 1.205 \cdot 10^{-5}$. As in the earlier analysis, we assume a constant number of stem cells in the colon, $X = 10^8$, which is also similar to the value provided in Potten et al. (2002).