DOI:
10.1158/1940-6207.CAPR-16-0278

Unmetabolized Folic Acid, Tetrahydrofolate and Colorectal Adenoma Risk

Judy R Rees, Carolyn B Morris, Janet L Peacock, Per M. Ueland, Elizabeth L Barry, Gail E

McKeown-Eyssen, Jane C. Figueiredo, Dale C. Snover, John A Baron

Department of Epidemiology, Geisel School of Medicine at Dartmouth: HB 7927, Dartmouth-Hitchcock Medical Center 1 Medical Center Drive, Lebanon, NH 03756 (J.R.R, E.L.B., J.A.B., J.L.P.).

Department of Medicine, University of North Carolina School of Medicine: CB #7555, 4160-A Bioinformatics Bldg., 130 Mason Farm Rd., Chapel Hill NC 27599 (J.A.B., C.B.M)

Division of Health and Social Care Research, King's College London, London, UK (J.L.P.)

Section for Pharmacology, University of Bergen, New Lab Building, 9th floor, Bergen, Hordaland, 5021Norway (P.M.U.)

Dalla Lana School of Public Health, University of Toronto, Toronto, Canada (G.M.E.)

Department of Preventive Medicine, Keck School of Medicine, University of Southern California: HNRT 1509J 1450 Biggy Street, Los Angeles CA 90089 (J.C.F.)

Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center: SOCCI AC1072 8700 Beverly Boulevard, Los Angeles CA 90048 (J.C.F.)

Department of Pathology, Fairview Southdale Hospital, Edina, MN (D.C.S.)

Running title

Folic acid, folate and colorectal adenoma

Key words

Unmetabolized folic acid, methylated folates, supplementation, colorectal adenoma, cancer

Funding

This work was supported in part by grants 5 R01 CA059005 and U54 CA100971 from the National Institutes of Health. Study tablets were provided by Wyeth Consumer Health

Care, Madison, NJ. J.L.P is supported by the NIHR Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London, UK.

Conflict of interest

The authors declare no potential conflicts of interest.

Corresponding author

Judy R Rees, Department of Epidemiology, Geisel School of Medicine at Dartmouth: HB 7927, Dartmouth-Hitchcock Medical Center 1 Medical Center Drive, Lebanon, NH 03756. Tel: 603 653 3683. Email: judy.r.rees@dartmouth.edu

Abstract word count: 251

Text word count: 3336

Abstract

In a randomized trial of folic acid supplementation for the prevention of colorectal adenomas, we previously found indications of increased risk during later treatment and follow-up. This could have been due to the unmetabolized folic acid or the natural reduced and methylated folates to which it is metabolized. In post hoc analyses, we measured methylated folates (mF, the sum of 5-methyl-tetrahydrofolate and 4-alfa-hydroxy-5-methyl-THF) and unmetabolized folic acid (UFA) concentrations in the serum of 924 participants. Using binomial regression models with a log link, we assessed the associations between plasma mF or UFA and adenoma occurrence. We found no association between plasma mF or UFA and overall adenoma risk. However, during later follow-up, the pre-specified, composite endpoint of high risk findings (advanced or multiple adenomas) was positively associated with plasma mF ($P_{linear\ trend}$=0.009), with a 58% increased risk for participants in the upper versus lowest quartile. An irregular association was seen with plasma UFA, with suggestions of an inverse trend ($P_{linear\ trend}$=0.049). A modest, significant inverse association was also seen between mF and risk of serrated lesions, with a 39% lower risk for upper vs lower quartile participants ($P_{linear\ trend}$=0.03). In conclusion, during the later follow-up period in which folic acid supplementation was previously seen to increase the risk of advanced and multiple adenomas, higher serum mF was associated with a higher risk of multiple and/or advanced adenomas, but no clear indication that UFA played a direct role. There were indications that higher mF was associated with reduced risk of serrated polyps.

Introduction

Extensive epidemiological and preclinical data have suggested that folates, a family of water-soluble vitamins that serve as cofactors for DNA synthesis and methylation reactions, exert substantial anti-neoplastic effects (1). Motivated by these findings, we conducted a randomized controlled trial to test the effect of 1g/day folic acid for the prevention of colorectal adenoma recurrence (2). Although we found no effect during the first three years of treatment, we saw unexpected indications of *increased risk* during subsequent follow-up and treatment: the relative risk for the occurrence of advanced adenomas was 1.67 (95% CI 1.00-2.80) and for 3 or more adenomas, 2.32 (95% CI 1.23-4.35) (2).

Although our finding of increased adenoma risk from folic acid supplementation may have been due to chance, there are other indications of increased cancer-related risks of folic acid, depending on dose, timing and folate status before supplementation. Whereas moderate doses may reduce risk, carcinogenesis may be enhanced by larger doses, or by supplementation after a neoplastic lesion has developed (3-6). The mechanisms for this adverse effect have not been fully elucidated but may include the provision of nucleotides for carcinogenesis (7).

Folic acid, is a stable, oxidized molecule rarely found in nature, though widely used for food fortification and in supplements. Folic acid is the molecular backbone of folates, but it is inactive until it is metabolized into the natural reduced forms, including 5-methyltetrahydrofolate (5m-THF), the prevailing circulating folate species (8). Since folic acid enters the folate metabolic cycles differently than 5m-THF and has a distinct

metabolism, it may have biological effects that differ from those of the naturally occurring folates. For example, some studies suggest that UFA reduces natural killer cell cytotoxicity in vitro (9, 10). Thus the increased risks associated with folic acid supplementation in our study could have been the result of folic acid itself, not the 5m-THF generated by it.

For both 5m-THF and folic acid, genetic variants in folate metabolism may also be pertinent. For example, the homozygous C677T variant of methylenetetrahydrofolate reductase (MTHFR), which metabolizes methylenetetrahydrofolate to mTHF, may modify the association of folate status with risk of colorectal carcinoma and adenoma (11-13). MTRR, methionine synthase reductase, maintains methionine synthase in its active form, and in this state maintains intracellular folate levels (14).

It is widely recognized that colorectal cancer is a heterogenous disease, with two distinct precursor pathways: the conventional pathway (accounting for about 60% of colorectal cancers), and the serrated pathway, which leads to CpG island methylated colorectal cancers (accounting for the remaining ~40%) (15, 16). Since these involve distinct genetic and epigenetic processes, it is possible that folates affect these pathways in different ways.

In a secondary, observational analysis, we previously found no consistent association between folate status and adenoma risk (17). However, during early follow-up, total folate intake and plasma total folate were inversely associated with adenoma risk among subjects randomized to folic acid placebo, while in later follow-up there were inconsistent suggestions of increased risks for dietary folate intake and red blood cell folate. In this analysis, we extend our analyses using separate measures of plasma UFA and

methylated folate (mF), and separate assessment of the conventional adenoma pathway and the serrated pathway. We hypothesized that long-term risk after folic acid supplementation is independently associated with plasma UFA levels.

Methods

Briefly, we conducted a double-blinded, placebo-controlled, randomized trial (NCT00272324) in a 3 x 2 factorial design to compare the effects of oral aspirin (81mg/day, 325 mg/day or placebo) with placebo or folic acid 1 g/day, in the prevention of colorectal adenomas in persons with a history of adenomas (2, 18). The trial's primary outcome was the occurrence of ≥1 colorectal adenomas. Secondary outcomes were the occurrence of advanced adenomas (tubulovillous or villous adenomas, or adenomas that were large (≥1cm) or had invasive cancer or high grade dysplasia); high risk findings (≥1 advanced adenomas or ≥3 adenomas); and serrated lesions (comprising hyperplastic polyps, sessile serrated adenomas/polyps, and traditional serrated adenomas). The recruitment period (1994-98) coincided with the beginning of folic acid food fortification in the U.S. and Canada that became mandatory in 1998 (19, 20). All participants provided written informed consent; the trial was approved by each participating center's institutional review board.

Participants aged 21-80 with a recent adenoma and no known remaining adenomas after a complete colonoscopy were identified at nine clinical centers, as described previously (18). Exclusion criteria included vitamin B_{12} deficiency, contraindication to aspirin treatment, or ongoing treatment with aspirin or related compounds (2, 18).

After a run-in phase of approximately 3 months, 1021 participants were randomized

to aspirin (81 mg, 325 mg or placebo) and folic acid (1 mg or placebo); 100 early participants were randomized only to aspirin/placebo. The initially planned study interventions continued until a follow-up colonoscopy anticipated approximately 3 years after the qualifying examination. This demarcated the end of the first follow-up interval and the beginning of the second interval, which lasted until 6 years later, one year longer than a 5-year colonoscopic surveillance cycle (Figure 1).

At the end of the first follow-up interval, participants were asked to continue placebo/folic acid treatment until their next surveillance colonoscopy, anticipated 3 to 5 years later; 729/1084 (67%) agreed to do so (Figure 1). Of the 355 who chose to discontinue randomized treatment, 278 (78%) agreed to observational follow-up. Participants still on study treatment were asked to stop taking study tablets by October 1, 2004 because folic acid supplementation had been associated with increased adenoma risk.

Plasma measures

Blood samples were collected at enrollment ("year 0", reflecting pre-randomization folate status), and shortly before the end of the first treatment interval ("year 3"), reflecting folate status on treatment. The blood draws were not timed to intake of food or study tablets. This study examined associations with circulating concentrations of folic acid and naturally-occurring folates shortly before the year 3 colonoscopy.

Plasma levels of 5m-THF, 4-α-hydroxy 5-methyl-THF (hm-THF), and unmetabolized folic acid (UFA) were analyzed at Bevital (21), using LC MS/MS (22). hm-THF is formed upon degradation of 5m-THF during storage so we pooled these measures for each sample and refer to the sum as methylated folate (mF) (22). The levels of detection were 0.27

nmol/L for UFA, 0.20 nmol/L for hm-THF and 0.07 nmol/L for 5m-THF (22). The intra-run CVs ranged from 5.0% to 9.9%, and the inter-run CVs from 3.3% to 9.5% (22). We identified the 677C>T, V222A (rs1801133) polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene and the 66A>G, I22M (rs1801394) polymorphism of methionine synthase reductase (*MTRR*) as previously described (23).

Follow-up

Participants taking study treatments were contacted every 4 months for information on adherence, use of personal nutritional supplements including folic acid, large bowel endoscopies and major medical events. During observational follow-up, they were contacted annually. For all tissue removed from the bowel, slides were obtained if possible and sent to a single, blinded pathologist for classification.

Statistical Analysis

We used conventional descriptive statistics to summarize characteristics of the study population. Correlations were assessed using Kendall's Tau b. We used the circulating folate measures at year 3 to characterize the folic acid and folate status of subjects during the first follow-up interval. In the analysis for the second follow-up interval, we included participants whose study folic acid treatment remained unchanged from that in the first interval: those randomized to folic acid who agreed to continue study treatment, those allocated folic acid placebo whether or not they agreed to continue study pills, and 100 participants randomized only to aspirin or placebo (*i.e.* no randomization to folic acid/placebo). Thus, apart from changes in diet, and non-compliance with the study

treatment regimen, the year 3 blood specimen represented the UFA and mF status during the second follow-up interval as well as the first.

We fit binomial regression models with a log link to describe the associations of circulating folate measures at year 3 with the occurrence of adenomas and serrated polyps during each of the surveillance intervals. The endpoints were: ≥1 adenomas; ≥1 advanced adenomas; high risk findings; ≥1 serrated lesions. All analyses were adjusted for age and sex, but not for treatment group (which would be reflected in the year 3 plasma measurements). Clinical center did not materially affect the analyses, and was excluded from all models. Personal folic acid supplement use during the second follow-up interval did not contribute usefully to the models for any of the measured outcomes, either as a covariate or as an effect modifier. It was therefore omitted from the analysis, and the same model was used for both intervals (i.e. age, sex, mF and UFA). Estimates for mF and UFA were mutually adjusted for each other so that the risk ratios reflected the independent associations of each analyte. mF was modeled in quartiles; UFA was modeled in four categories, undetectable (set to zero) plus tertiles of the remaining values. We assessed patterns of associations with a general test of heterogeneity (Wald test) and tests for trend using the linear and quadratic functions of the continuous measurements. This allowed testing of both monotonic trends and the possibility that modest increases in mF or UFA levels might be beneficial, while higher levels detrimental. Results are given as relative risks of outcome by mF or UFA category. The possibility of effect modification by randomized aspirin assignment (dichotomous) or by *MTHFR* or *MTRR* polymorphisms was assessed using likelihood ratio tests. All analyses were performed with SAS v9.4.

Results

Year 3 plasma measurements and colonoscopy data were available for 924 participants in the first interval, 680 of whom completed a further 3-6 years of colonoscopic follow-up (Figure 1, Table 1). At enrollment, the mean concentration of mF was 37.7 nmol/L (SD 22.4); at year 3 this had increased to 46.3 nmol/L (SD 21.7) in the placebo group and 84.8 nmol/L (SD 29.5) in the folic acid treatment group. At enrollment, UFA was detected in 19.3% of participants, with a mean concentration of 4.2 (6.5) nmol/L for the 170 with detectable levels. Just before the 3-year surveillance colonoscopy, detectable UFA was found in 23.2% of the placebo group and 73.0% of those in the folic acid group, with mean concentrations of 3.4 (8.5) and 21.7 (24.9) nmol/L for those with detectable levels, respectively. At year 3, mean plasma mF increased from 48.0 to 97.0 nmol/L within increasing categories of UFA (Supplementary Figure 1). The correlation between mF and UFA at year 3 was $r=0.46$ ($p<0.0001$).

There were no significant associations between circulating UFA or mF levels and overall adenoma risk during either follow-up interval (Table 2). However, during the second follow-up interval, there were indications of an association of mF with increasing risk of advanced adenomas and high risk findings (advanced or ≥3 adenomas) (Table 2). For high risk findings, there was a linear trend of increasing risk with increasing levels, with a 58% increased risk for participants in the upper quartile compared to the lowest (Table 2). There was a weaker, borderline-significant trend for advanced adenomas (25% increased risk for upper vs lowest quartile). During the second interval, UFA showed an erratic association with high risk findings, with a borderline significant inverse linear trend ($p = 0.049$). Analyses without mututal adjustment for mF and UFA showed broadly similar findings, though with non-significant trends (Supplementary Table 4).

There was no evidence for any differences between men and women in the association of mF or UFA with any of these adenoma endpoints (all $p_{interaction} > 0.15$, Supplementary Table 1). There was also little evidence for a quadratic relationship between mF or UFA and these outcomes during early or later follow-up (all p >0.15) (not shown). No significant interactions were seen for any outcome between randomized aspirin use (dichotomous) and either UFA or mF.

Associations for serrated pathway lesions differed markedly from those for adenomas. During the second surveillance interval, the risk of any serrated lesion was significantly inversely associated with plasma mF, with a 39% lower risk for upper vs lower quartile participants (Table 3). There was no association with plasma UFA (Table 3).

As reported previously for the entire study population (24), there were no significant associations between adenoma risk and polymorphisms of *MTHFR* or *MTRR* in either follow-up interval (Supplementary Table 2). There were also no indications of interactions between plasma levels of mF or UFA and genetic variants in either gene with regard to risk of all adenomas (Supplementary Table 2). However, in the corresponding analysis of high risk findings, there was an interaction of *MTHFR* with mF (p = 0.03) during the first follow-up interval, with higher mF-associated risk in CC participants than in those with the CT/TT genotype. (Supplementary Table 3). In the second interval, there was a broadly similar pattern, but the interaction did not reach statistical significance (p=0.08).

Discussion

In our clinical trial, we previously found that participants randomized to 1mg/day folic acid had an increased risk of advanced and multiple adenomas during longer follow-up (2). In

this post hoc analysis, high circulating levels of mF were positively associated with the composite high risk endpoint of advanced and/or multiple adenomas during this same period, while there was lower risk in the highest category of UFA, with a borderline significant trend. These findings suggest that the increased risks seen after folic acid supplementation (2) were due to the high mF levels, rather than due to a direct effect of UFA itself (Figure 2). In contrast, our findings for serrated lesions suggested a protective effect of higher levels of mF during later follow-up.

We found an interaction between *MTHFR* 677C->T and mF levels such that in the first follow-up interval there was a particularly elevated risk of high risk findings associated with high mF only among individuals homozygous for the CC allele. This is consistent with our finding from the second follow-up interval that mF is associated with increased risk, as the enzyme variant associated with the T allele generates 5 methyl-tetrahydrofolate from 5, 10-methylenetetrahydrofolate less efficiently than the variant associated with the C allele. However, during the second follow-up interval, the interaction was less marked.

Cho et al identified no overall association between circulating UFA and colorectal cancer, but with significant heterogeneity by sex, with higher risks among men and lower risks among women, with increasing UFA(25). In the second follow-up interval of our study, the directions of effects were similar to those seen by Cho et al, but the interactions were not statistically significant in our smaller study population. Cho et al also reported a significant interaction between UFA and *MTHFR* C677T, with a colorectal cancer OR of 2.20 (95% CI 1.22, 3.94) for the highest vs. lowest levels of UFA among those with CT or TT genotype. Increasing risks associated with the TT or CT genotype have been found

previously, either as main effects, or with increasing total folate levels (11, 26). We also found only slightly and non-significantly higher risk ratios in those with these genotypes at highest UFA levels.

Several laboratory studies indicate that folate or folic acid may enhance carcinogenesis in some settings. For example, in mouse models of colorectal cancer, low doses of dietary folic acid were protective if given before tumors developed, but promoted further tumor growth if given after tumors had emerged (27), and in a rat model of breast cancer, folate deficiency was protective (28, 29). Other *in vitro* studies have shown that folate repletion after deficiency has the potential to lead to carcinogenesis (5). Some argue that, because folate antagonists are effective anti-cancer drugs, the rationale for folate supplementation to prevent cancer may be flawed; indeed, the use of antifolates in leukemia was first prompted by the observation that large doses of folic acid made patients with leukemia substantially worse (30).

Although the inverse association between high risk findings and UFA may be a chance finding, there are reasons to speculate that synthetic folic acid might have different biological effects than naturally occurring folates (31, 32), some of them potentially harmful (33). UFA has been associated with neurological and cognitive effects in individuals with lower B_{12} status (34, 35) and with reduced natural killer cell cytotoxicity in some studies *in vivo* (9, 10), although not *in vitro* (36). There are differences in the carrier systems through which these natural folates and UFA enter the cell, the points at which they enter the folate cycle, and the bioavailability of folates from diet and folic acid supplements (31, 37). The assimilation of folic acid into tetrahydrofolate is catalyzed by dihydrofolate reductase (DHFR), whose activity varies considerably between individuals

and is inhibited by unmetabolized folic acid (38). In addition, UFA may inhibit MTHFR, the enzyme that produces methyltetrahydrofolate (39). Thus it is possible that UFA has tissue effects that decrease the bioavailability of intracellular mF levels following the supplementation. Further, after administration of radiolabeled folic acid to 10 volunteers, 40% of the increase in plasma total folate was due to mobilization of endogenous (unlabeled) folate rather than conversion of the ingested folic acid (40). The physiological differences between UFA and naturally occurring folate species are incompletely understood and require further study. The irregular risk ratios seen in the second interval of our study may also merit further investigation in future studies, to assess the possibility of a threshold effect.

After folic acid supplementation, 73.0% of our participants had detectable UFA, compared with 23.2% of controls; these figures are consistent with the wide range of detectable UFA seen in other studies (53-56). UFA has been found in the serum following boluses of dietary folic acid as low as 200 mcg in folate-replete women (34), suggesting that it should be expected in the serum of individuals taking 1 mg folate/day (57). A variety of factors influence the concentration of mF and UFA, including the time since ingestion of the supplement (58-60), variability in dietary intake including food fortification and DHFR polymorphisms. We did not control the timing of blood tests relative to pill-taking or meals in our study; variable timing would increase the variability of plasma measures and reduce our ability to identify associations if any exist. This would be particularly true for UFA, which has a short half life (~ one hour) (62) as compared to the more deep folate compartments that have previously been detected after chronic supplementation with

deuterium labelled folic acid (63). A future study should time blood draws consistently in relation to intake of folic acid from foods and supplements.

Our approach had the advantage of adjustment for serum mF while analyzing the effect of UFA and vice versa. By summing the measured concentrations of hm-THF and 5m-THF, we minimized the measurement error associated with 5m-THF breakdown to hm-THF in stored samples, (22). We also used a single blood sample during intervention (near the end of the first follow-up interval) to represent UFA or mF levels during two observation cycles; this is more reflective of in-trial levels than the pre-supplementation samples used in our previous report (17). However, a single measurement is an imperfect correlate of long-term exposure, and may cause regression dilution bias that would reduce our ability to identify associations(61). A further limitation is that our findings with respect to adenoma and especially serrated polyps apply only indirectly to the study of colorectal cancer. Finally, in exploring trends in these data, we performed many significance tests, with the possibility of type 1 errors, particularly for the quadratic trend tests that were conducted; one of 20 tests was statistically significant with P=0.04.

Conclusion

Our results indicate that the increased risk of advanced/multiple adenomas in later follow-up of our trial was likely related to higher levels of plasma mF, and not due to UFA. In contrast, findings regarding serrated lesions provide some suggestions of protection from mF in the serrated pathway. While our data do not indicate any significant harm associated with UFA itself, the associated high levels of mF seem to be detrimental with respect to adenoma risk. The widespread use of folic supplementation and food

fortification makes associations between the levels of folate species and disease an important avenue for further research.

Acknowledgements

The authors wish to acknowledge the participants and staff of the Polyp Prevention Study.

Table 1. Characteristics of trial participants included in serum analyses

Enrollment	Participants who completed Interval 1[a] N=924		Participants who completed Interval 2[a] N=680	
	Mean (SD)	N (%)	Mean (SD)	N (%)
Age	57.5 (9.3)		57.6 (9.0)	
Sex: Male		587 (63.5)		440 (64.7)
Female		337 (36.5)		240 (35.3)
Race/ethnicity:				
Non Hispanic white		784 (84.8)		591 (86.9)
Non Hispanic black		54 (5.8)		33 (4.9)
Hispanic		56 (6.1)		35 (5.2)
Asian/Pacific Islander		22 (2.4)		15 (2.2)
Native American		4 (0.4)		3 (0.4)
Other		4 (0.4)		3 (0.4)
Year 0	N=879		N=649	
mF[b] mean (SD) nmol/L	37.7 (22.4)		37.7 (22.4)	
Participants with detectable UFA[c] (%)		170 (19.3%)		119 (18.3%)
UFA mean (SD for those with detectable levels, nmol/L	4.2 (6.5)		4.5 (7.3)	
Year 3	N= 924		N= 680	
mF mean (SD), nmol/L	64.1 (32.0)		63.7 (31.7)	
Folic acid	84.8 (29.5)		87.0 (27.9)	
Placebo	46.3 (21.7)		46.3 (21.7)	
Number of participants with detectable UFA (%)		428 (46.3%)		314 (46.2%)
Folic acid		313 (73.0%)		230 (79.4%)
Placebo		115 (23.2%)		84 (21.5%)
UFA mean (SD) for those with detectable levels, nmol/L	16.8 (23.2)		16.7 (22.9)	
Folic acid	21.7 (24.9)		21.4 (24.5)	
Placebo	3.4 (8.5)		3.8 (9.2)	

[a] Interval 1 is year 0 through the year 3 colonoscopy. Interval 2 began after the year 3 colonoscopy and ended 6 years later.

[b] mF is methylated folate, the sum of measured 5-methyl-THF and 4-α-hydroxy 5-methyl-THF

[c] UFA is unmetabolized folic acid.

Table 2. Associations of plasma methylated folate (mF) and unmetabolized folic acid (UFA) levels with risk of adenomas and serrated lesions

	Interval 1[a]		**Interval 2[a]**	
All Adenomas[b]	N (events)	RR[c] (95% confidence interval)	N (events)	RR[c] (95% confidence interval)
mF nmol/L				
0-<37.35	218 (86)	1.00 (ref)	170 (62)	1.00 (ref)
37.35-<60.44	224 (91)	1.02 (0.79, 1.33)	157 (59)	1.00 (0.73, 1.39)
60.44-<85.50	221 (91)	1.04 (0.78, 1.38)	167 (68)	1.06 (0.75, 1.50)
≥85.50	224 (87)	1.02 (0.74, 1.42)	162 (60)	1.01 (0.68, 1.50)
		$P_{heterogeneity}$ = 0.99[d]		$P_{heterogeneity}$ = 0.98[d]
		$P_{linear\ trend}$ = 0.70		$P_{linear\ trend}$ = 0.69
UFA nmol/L				
0	475 (190)	1.00 (ref)	353 (130)	1.00 (ref)
>0-<3	163 (69)	1.04 (0.81,1.33)	111 (38)	0.88 (0.62, 1.25)
3-<20	120 (51)	1.00 (0.74,1.35)	91 (45)	1.31 (0.95, 1.82)
≥20	129 (45)	0.83 (0.58,1.18)	101 (36)	0.96 (0.64, 1.43)
		$P_{heterogeneity}$ = 0.64[d]		$P_{heterogeneity}$ = 0.14[d]
		$P_{linear\ trend}$ = 0.13		$P_{linear\ trend}$ = 0.81
High Risk Findings[e]				
mF nmol/L				
0-<37.35	214 (25)	1.00 (ref)	170 (15)	1.00 (ref)
37.35-<60.44	222 (35)	1.35 (0.87,2.11)	157 (19)	1.13 (0.71, 2.36)
60.44-<85.50	216 (26)	1.02 (0.61,1.71)	167 (25)	1.56 (0.84, 2.90)
≥85.50	223 (32)	1.32 (0.77,2.24)	162 (21)	1.58 (0.80, 3.09)
		$P_{heterogeneity}$ = 0.38[d]		$P_{heterogeneity}$ = 0.52[d]
		$P_{linear\ trend}$ = 0.96		$P_{linear\ trend}$ = 0.009
UFA nmol/L				
0	467 (63)	1	353 (38)	1
>0-<3	160 (21)	0.94 (0.60,1.46)	111 (16)	1.11 (0.65, 1.89)
3-<20	119 (20)	1.17 (0.73,1.88)	91 (18)	1.46 (0.86, 2.50)
≥20	129 (14)	0.73 (0.41,1.29)	101 (8)	0.59 (0.28, 1.24)
		$P_{heterogeneity}$ =0.45[d]		$P_{heterogeneity}$ = 0.10[d]
		$P_{linear\ trend}$ = 0.46		$P_{linear\ trend}$ = 0.049
Advanced Adenoma[f]				
mF nmol/L				

0-<37.35	214 (19)	1	166 (13)	1
37.35-<60.44	221 (24)	1.28 (0.78,2.10)	150 (14)	1.11 (0.59, 2.07)
60.44-<85.50	214 (18)	1.09 (0.62, 1.91)	162 (17)	1.18 (0.61, 2.28)
≥85.50	222 (23)	1.56 (0.88,2.77)	158 (15)	1.25 (0.61, 2.55)
		$P_{heterogeneity}$ = 0.39[d]		$P_{heterogeneity}$ =0.94[d]
		$P_{linear\ trend}$ = 0.73		$P_{linear\ trend}$ = 0.051
UFA nmol/L				
0	466 (48)	1	341 (28)	1
>0-<3	159 (16)	0.91 (0.56,1.47)	108 (13)	1.35 (0.76, 2.38)
3-<20	119 (11)	0.80 (0.44,1.41)	89 (13)	1.60 (0.88, 2.94)
≥20	127 (9)	0.60 (0.29, 1.09)	98 (5)	0.55 (0.23, 1.32)
		$P_{heterogeneity}$ = 0.40[d]		$P_{heterogeneity}$ = 0.07[d]
		$P_{linear\ trend}$ = 0.24		$P_{linear\ trend}$ = 0.07
Serrated Lesions[g]				
mF nmol/L				
0-<37.35	223 (74)	1	167 (52)	1
37.35-<60.44	222 (47)	0.63 (0.44 0.90)	155 (43)	0.88 (0.60, 1.28)
60.44-<85.50	219 (66)	0.87 (0.61, 1.25)	163 (44)	0.90 (0.60, 1.37)
≥85.50	226 (66)	0.85 (0.57, 1.26)	158 (28)	0.61 (0.36, 1.23)
		$P_{heterogeneity}$ = 0.09[d]		$P_{heterogeneity}$ = 0.30[d]
		$P_{linear\ trend}$ = 0.66		$P_{linear\ trend}$ = 0.03
UFA nmol/L				
0	478 (134)	1.00 (ref)	346 (98)	1.00 (ref)
>0-<3	162 (45)	1.03 (0.72, 1.45)	112 (34)	1.18 (0.81 1.73)
3-<20	121 (38)	1.12 (0.76, 1.66)	87 (17)	0.78 (0.46, 1.35)
≥20	129 (36)	0.98 (0.64, 1.50)	98 (18)	0.82 (0.47, 1.45)
		$P_{heterogeneity}$ =0.93[d]		$P_{heterogeneity}$ = 0.43[d]
		$P_{linear\ trend}$ = 0.66		$P_{linear\ trend}$ = 0.91

[a] Interval 1 is year 0 through the year 3 colonoscopy. Interval 2 began after the year 3 colonoscopy and ended 6 years later.

[b] Conventional adenomas: tubulovillous, villous or tubular adenomas (i.e. non serrated)

[c] Risk ratios are estimated for each quartile of mF [or UFA], with respect to the first quartile as referent, in the following model: colonoscopy findings = age + sex + quartiles of mF [or UFA] + continuous UFA [or mF]

[d] Wald test

[e] High risk findings: 3 or more adenomas or advanced adenoma

[f] Advanced adenoma: tubulovillous or villous adenomas, or adenomas that were large (≥1cm) or had invasive cancer or high grade dysplasia

[g] Serrated lesions: hyperplastic polyps, sessile serrated adenomas/polyps, and traditional serrated adenomas

Figure 1. Design of the trial and participants studied in this analysis (adapted from Cole et al, 2007 (2))

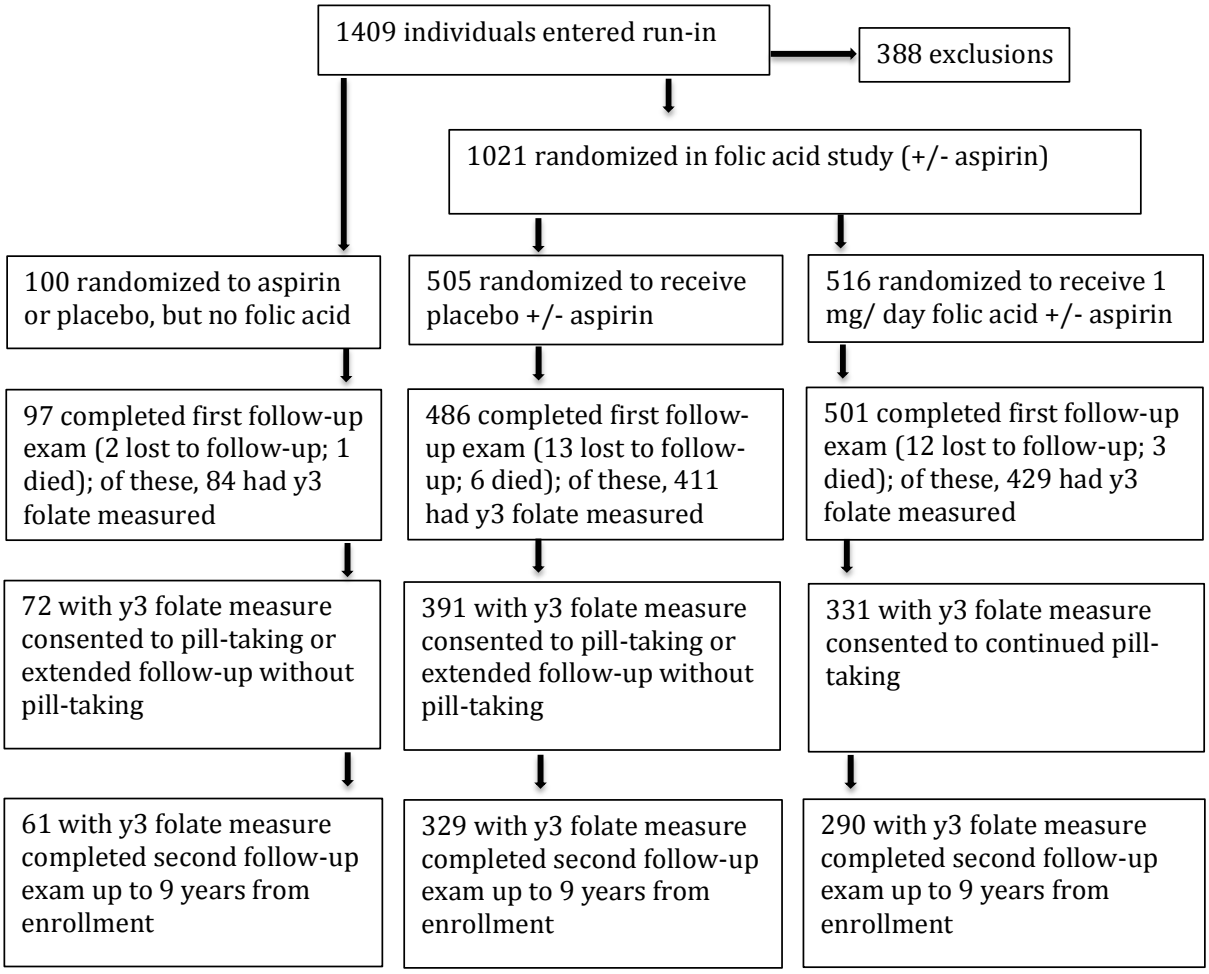
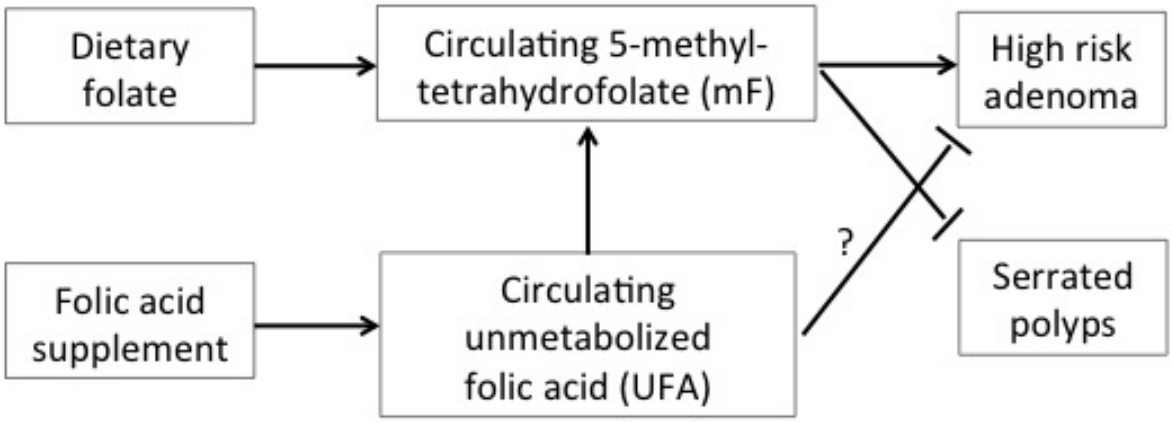


Figure 2. Possible causal pathways under investigation



References

1. Giovannucci E. Epidemiologic studies of folate and colorectal neoplasia: a review. J Nutr. 2002;132:2350S-5S.
2. Cole BF, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, et al. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. JAMA. 2007;297:2351-9.
3. Song J, Medline A, Mason JB, Gallinger S, Kim YI. Effects of dietary folate on intestinal tumorigenesis in the apcMin mouse. Cancer Res. 2000;60:5434-40.
4. Song J, Sohn KJ, Medline A, Ash C, Gallinger S, Kim YI. Chemopreventive effects of dietary folate on intestinal polyps in Apc+/-Msh2-/- mice. Cancer Res. 2000;60:3191-9.
5. Melnyk S, Pogribna M, Miller BJ, Basnakian AG, Pogribny IP, James SJ. Uracil misincorporation, DNA strand breaks, and gene amplification are associated with tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells. Cancer Lett. 1999;146:35-44.
6. Kim YI. Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. Environ Mol Mutagen. 2004;44:10-25.
7. Choi SW, Mason JB. Folate and carcinogenesis: an integrated scheme. J Nutr. 2000;130:129-32.
8. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF, 3rd, Mills JL, et al. Biomarkers of Nutrition for Development-Folate Review. J Nutr. 2015;145:1636S-80S.
9. Troen AM, Mitchell B, Sorensen B, Wener MH, Johnston A, Wood B, et al. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr. 2006;136:189-94.
10. Sawaengsri H, Wang J, Reginaldo C, Steluti J, Wu D, Meydani SN, et al. High folic acid intake reduces natural killer cell cytotoxicity in aged mice. J Nutr Biochem. 2016;30:102-7.
11. Chen J, Giovannucci E, Kelsey K, Rimm EB, Stampfer MJ, Colditz GA, et al. A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. Cancer Res. 1996;56:4862-4.
12. Ulrich CM, Kampman E, Bigler J, Schwartz SM, Chen C, Bostick R, et al. Colorectal adenomas and the C677T MTHFR polymorphism: evidence for gene-environment interaction? Cancer Epidemiol Biomarkers Prev. 1999;8:659-68.
13. Kim DH. The interactive effect of methyl-group diet and polymorphism of methylenetetrahydrofolate reductase on the risk of colorectal cancer. Mutation research. 2007;622:14-8.
14. Leclerc D, Wilson A, Dumas R, Gafuik C, Song D, Watkins D, et al. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci U S A. 1998;95:3059-64.
15. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;61:759-67.
16. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology. 2007;50:113-30.
17. Figueiredo JC, Levine AJ, Grau MV, Barry EL, Ueland PM, Ahnen DJ, et al. Colorectal adenomas in a randomized folate trial: the role of baseline dietary and circulating folate levels. Cancer Epidemiol Biomarkers Prev. 2008;17:2625-31.
18. Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, et al. A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med. 2003;348:891-9.

19. Food standards: amendment of the standards of identity for enriched grain product to require addition of folic acid. In: tration UDoHaHSFaDA-, editor. 61:8781. Fed Regist 1996.
20. Regulations Amending the Food and Drug Regulations (1066). Canada Gazette Part II 1998;132:SOR/98-550.
21. Mason JB, Choi SW. Folate and carcinogenesis: developing a unifying hypothesis. Adv Enzyme Regul. 2000;40:127-41.
22. Hannisdal R, Ueland PM, Eussen SJ, Svardal A, Hustad S. Analytical recovery of folate degradation products formed in human serum and plasma at room temperature. J Nutr. 2009;139:1415-8.
23. Figueiredo JC, Levine AJ, Grau MV, Midttun O, Ueland PM, Ahnen DJ, et al. Vitamins B2, B6, and B12 and risk of new colorectal adenomas in a randomized trial of aspirin use and folic acid supplementation. Cancer Epidemiol Biomarkers Prev. 2008;17:2136-45.
24. Levine AJ, Wallace K, Tsang S, Haile RW, Saibil F, Ahnen D, et al. MTHFR genotype and colorectal adenoma recurrence: data from a double-blind placebo-controlled clinical trial. Cancer Epidemiol Biomarkers Prev. 2008;17:2409-15.
25. Cho E, Zhang X, Townsend MK, Selhub J, Paul L, Rosner B, et al. Unmetabolized Folic Acid in Prediagnostic Plasma and the Risk of Colorectal Cancer. J Natl Cancer Inst. 2015;107.
26. Ma J, Stampfer MJ, Giovannucci E, Artigas C, Hunter DJ, Fuchs C, et al. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. Cancer Res. 1997;57:1098-102.
27. Kim YI. Folate and colorectal cancer: an evidence-based critical review. Mol Nutr Food Res. 2007;51:267-92.
28. Baggott JE, Vaughn WH, Juliana MM, Eto I, Krumdieck CL, Grubbs CJ. Effects of folate deficiency and supplementation on methylnitrosourea-induced rat mammary tumors. J Natl Cancer Inst. 1992;84:1740-4.
29. Kotsopoulos J, Sohn KJ, Martin R, Choi M, Renlund R, McKerlie C, et al. Dietary folate deficiency suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. Carcinogenesis. 2003;24:937-44.
30. Mason JB, Dickstein A, Jacques PF, Haggarty P, Selhub J, Dallal G, et al. A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. Cancer Epidemiol Biomarkers Prev. 2007;16:1325-9.
31. Ulrich CM, Potter JD. Folate supplementation: too much of a good thing? Cancer Epidemiol Biomarkers Prev. 2006;15:189-93.
32. Wright AJ, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr. 2007;98:667-75.
33. Obeid R, Herrmann W. The emerging role of unmetabolized folic acid in human diseases: myth or reality? Current drug metabolism. 2012;13:1184-95.
34. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr. 1997;65:1790-5.

35. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Circulating unmetabolized folic acid and 5-methyltetrahydrofolate in relation to anemia, macrocytosis, and cognitive test performance in American seniors. Am J Clin Nutr. 2010;91:1733-44.
36. Hirsch S, Miranda D, Munoz E, Montoya M, Ronco AM, de la Maza MP, et al. Natural killer cell cytotoxicity is not regulated by folic acid in vitro. Nutrition. 2013;29:772-6.
37. Ohrvik VE, Witthoft CM. Human folate bioavailability. Nutrients. 2011;3:475-90.
38. Bailey SW, Ayling JE. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. Proc Natl Acad Sci U S A. 2009;106:15424-9.
39. Christensen KE, Mikael LG, Leung KY, Levesque N, Deng L, Wu Q, et al. High folic acid consumption leads to pseudo-MTHFR deficiency, altered lipid metabolism, and liver injury in mice. Am J Clin Nutr. 2015;101:646-58.
40. Wright AJ, Finglas PM, Dainty JR, Hart DJ, Wolfe CA, Southon S, et al. Single oral doses of 13C forms of pteroylmonoglutamic acid and 5-formyltetrahydrofolic acid elicit differences in short-term kinetics of labelled and unlabelled folates in plasma: potential problems in interpretation of folate bioavailability studies. Br J Nutr. 2003;90:363-71.
41. Glynn SA, Albanes D, Pietinen P, Brown CC, Rautalahti M, Tangrea JA, et al. Colorectal cancer and folate status: a nested case-control study among male smokers. Cancer Epidemiol Biomarkers Prev. 1996;5:487-94.
42. Zschabitz S, Cheng TY, Neuhouser ML, Zheng Y, Ray RM, Miller JW, et al. B vitamin intakes and incidence of colorectal cancer: results from the Women's Health Initiative Observational Study cohort. Am J Clin Nutr. 2013;97:332-43.
43. Mason JB, Cole BF, Baron JA, Kim YI, Smith AD. Folic acid fortification and cancer risk. Lancet. 2008;371:1335; author reply -6.
44. Takata Y, Shrubsole MJ, Li H, Cai Q, Gao J, Wagner C, et al. Plasma folate concentrations and colorectal cancer risk: a case-control study nested within the Shanghai Men's Health Study. Int J Cancer. 2014;135:2191-8.
45. Song M, Garrett WS, Chan AT. Nutrients, foods, and colorectal cancer prevention. Gastroenterology. 2015;148:1244-60 e16.
46. Chuang SC, Rota M, Gunter MJ, Zeleniuch-Jacquotte A, Eussen SJ, Vollset SE, et al. Quantifying the dose-response relationship between circulating folate concentrations and colorectal cancer in cohort studies: a meta-analysis based on a flexible meta-regression model. Am J Epidemiol. 2013;178:1028-37.
47. Qin T, Du M, Du H, Shu Y, Wang M, Zhu L. Folic acid supplements and colorectal cancer risk: meta-analysis of randomized controlled trials. Scientific reports. 2015;5:12044.
48. Vollset SE, Clarke R, Lewington S, Ebbing M, Halsey J, Lonn E, et al. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50,000 individuals. Lancet. 2013;381:1029-36.
49. Ma J, Stampfer MJ, Christensen B, Giovannucci E, Hunter DJ, Chen J, et al. A polymorphism of the methionine synthase gene: association with plasma folate, vitamin B12, homocyst(e)ine, and colorectal cancer risk. Cancer Epidemiol Biomarkers Prev. 1999;8:825-9.
50. Jaszewski R, Misra S, Tobi M, Ullah N, Naumoff JA, Kucuk O, et al. Folic acid supplementation inhibits recurrence of colorectal adenomas: a randomized chemoprevention trial. World J Gastroenterol. 2008;14:4492-8.

51. Figueiredo JC, Mott LA, Giovannucci E, Wu K, Cole B, Grainge MJ, et al. Folic acid and prevention of colorectal adenomas: a combined analysis of randomized clinical trials. Int J Cancer. 2011;129:192-203.
52. Gao QY, Chen HM, Chen YX, Wang YC, Wang ZH, Tang JT, et al. Folic acid prevents the initial occurrence of sporadic colorectal adenoma in Chinese older than 50 years of age: a randomized clinical trial. Cancer Prev Res (Phila). 2013;6:744-52.
53. Obeid R, Kasoha M, Kirsch SH, Munz W, Herrmann W. Concentrations of unmetabolized folic acid and primary folate forms in pregnant women at delivery and in umbilical cord blood. Am J Clin Nutr. 2010;92:1416-22.
54. Obeid R, Kirsch SH, Kasoha M, Eckert R, Herrmann W. Concentrations of unmetabolized folic acid and primary folate forms in plasma after folic acid treatment in older adults. Metabolism. 2011;60:673-80.
55. Kalmbach RD, Choumenkovitch SF, Troen AM, D'Agostino R, Jacques PF, Selhub J. Circulating folic acid in plasma: relation to folic acid fortification. Am J Clin Nutr. 2008;88:763-8.
56. Bailey RL, Mills JL, Yetley EA, Gahche JJ, Pfeiffer CM, Dwyer JT, et al. Serum unmetabolized folic acid in a nationally representative sample of adults >/=60 years in the United States, 2001-2002. Food & nutrition research. 2012;56.
57. Centers for Disease Control. Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. . 1992.
58. Brouwer IA, van Dusseldorp M, West CE, Steegers-Theunissen RP. Bioavailability and bioefficacy of folate and folic acid in man. Nutrition research reviews. 2001;14:267-94.
59. Alemdaroglu NC, Dietz U, Wolffram S, Spahn-Langguth H, Langguth P. Influence of green and black tea on folic acid pharmacokinetics in healthy volunteers: potential risk of diminished folic acid bioavailability. Biopharmaceutics & drug disposition. 2008;29:335-48.
60. Willems FF, Boers GH, Blom HJ, Aengevaeren WR, Verheugt FW. Pharmacokinetic study on the utilisation of 5-methyltetrahydrofolate and folic acid in patients with coronary artery disease. British journal of pharmacology. 2004;141:825-30.
61. Hutcheon JA, Chiolero A, Hanley JA. Random measurement error and regression dilution bias. BMJ. 2010;340:c2289.
62. Loew D, Eberhardt A, Heseker H, Kubler W. [Plasma kinetics and elimination of folic acid]. Klin Wochenschr. 1987;65:520-4.
63. von der Porten AE, Gregory JF, 3rd, Toth JP, Cerda JJ, Curry SH, Bailey LB. In vivo folate kinetics during chronic supplementation of human subjects with deuterium-labeled folic acid. J Nutr. 1992;122:1293-99.