

# Trans-HHS Workshop: Diet, DNA Methylation Processes and Health

## Nutritional and Genetic Inefficiencies in One-Carbon Metabolism and Cervical Cancer Risk<sup>1</sup>

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**ABSTRACT** Folate deficiency has long been postulated to play a role in the etiology of cervical cancer, the third most frequent cancer among women worldwide. In a large, multiethnic community-based case-control study of invasive cervical cancer in five U.S. areas, we assessed accepted and postulated risk factors with an in-home interview and successfully obtained blood samples, at least 6 mo after completion of cancer treatment, from 51 and 68%, respectively, of interviewed cases and controls. Cases with advanced disease (6%) and/or receiving chemotherapy (4%) were excluded, leaving 183 cases and 540 controls. Serum and red blood cell folate were measured with both microbiologic and radiobinding assays. For all four folate measures, risk was moderately, but nonsignificantly, elevated for women in the lowest quartile, compared to the highest [fully adjusted relative risks (RR), including serologic human papillomavirus (HPV)-16 status = 1.2–1.6]. However, for women in the upper three homocysteine quartiles (>6.31  $\mu\text{mol/L}$ ), risk of invasive cervical cancer was substantially and significantly elevated (fully adjusted RR, including serologic HPV-16 status = 2.4–3.2;  $P$  for trend = 0.01). This strong relationship suggests that circulating homocysteine may be 1) an especially accurate indicator of inadequate folate, 2) an integratory measure of insufficient folate in tissues or 3) a biomarker of disruption of one-carbon metabolism. The contribution of common polymorphisms in one-carbon pathway genes, as well as inadequate vitamin B-6, vitamin B-12 and/or riboflavin, to elevated homocysteine, inefficient one-carbon metabolism and increased cervical cancer risk merits further exploration. *J. Nutr.* 132: 2345S–2349S, 2002.

**KEY WORDS:** • cervical cancer • epidemiology • folate • homocysteine • one-carbon metabolism

Worldwide, cancer of the uterine cervix is the third most frequent cancer among women, after breast and colorectal cancer, with ~371,000 new cases each year (1). Most cervical cancer cases (78%) occur in developing countries since screening programs have reduced its incidence in the developed world (1). Nonetheless, in the United States in 2001, an estimated 12,900 women will be diagnosed with cervical cancer and 4,400 women will die from this disease (2).

The evidence is increasing that human papillomavirus

(HPV)<sup>3</sup> is not just a primary cause of cervical cancer worldwide but a necessary cause (3). Several strains of HPV are oncogenic, with HPV-16 the most prevalent. However, only a small fraction of women infected with HPV will eventually develop cervical cancer. Additional steps involving viral DNA persistence, alteration of host genes that control viral gene expression, integration of viral DNA into the host genome and disruption of cell cycle regulation are required (4). Several cofactors that might facilitate this progression are under active investigation: hormonal factors such as oral contraceptive use and parity, immunologic factors, smoking, infection with other pathogens and poor nutrition (5).

For the last 30 y, there has been credible speculation that folate inadequacy might be a risk factor for cervical neoplasia. In 1973 Whitehead et al. (6) reported “megaloblastic” cervical abnormalities, similar to those seen in severe folate and vitamin B-12 deficiency, in 19% of women using oral contraceptives, although hematologic studies did not indicate systemic deficiency of either micronutrient. Similar cervical changes were not observed in women not using oral contraceptives. In

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<sup>3</sup> Abbreviations: CBS, cystathionine  $\beta$ -synthase; CI, confidence interval; HPV, human papillomavirus; MTHFR, methylenetetrahydrofolate reductase;  $r$ , correlation coefficient; RR, relative risk.

all eight women treated with pharmacologic doses of folate for 3 wk, the "megaloblastic" changes markedly improved or disappeared. It was hypothesized that oral contraceptives interfered with folate metabolism localized in cervical tissue. Nine years later, Butterworth et al. (7) postulated that localized problems in folate metabolism could encourage the development of cervical dysplasia. In a randomized, placebo-controlled trial among 47 women on oral contraceptives with cervical dysplasia, women given 10 mg of supplemental folate daily for 3 mo showed significantly improved cervical pathology (7). However, in a comparable but larger trial among 235 women, no significant differences were seen after 6 mo of supplementation (8). Like the clinical trials, the ~20 observational studies of folate and cervical dysplasia or cancer have also generated mixed results (9). While some studies have suggested a protective effect, most have not. However, dietary estimates of folate status are imprecise because the usual adult diet is hard to quantify and folate databases are limited by the multiple forms, instability and variable bioavailability of folate in foods (9). In addition, many of the observational studies have not been able to control for a history of HPV infection or for all of the postulated cervical cancer risk factors.

In the 1980s, the National Cancer Institute conducted a large, multiethnic, case-control study of invasive cervical cancer in five U.S. areas, centered around Birmingham, AL, Chicago, IL, Denver, CO, Miami, FL, and Philadelphia, PA (10,11). Eligible subjects were women, aged 20–74 y, with histologically confirmed, primary invasive cervical cancer diagnosed during a 21-mo interval. Up to two potential controls, matched by age, ethnicity and neighborhood, were identified by random digit dialing for each case. A total of 481 cases and 801 controls were interviewed (73 and 72% of those identified). Blood samples were obtained from 245 cases and 545 controls (51 and 68% of those interviewed). Of the cases that donated blood, 65% were Caucasian, 22% were African-American, and 9% were Hispanic. With five diverse communities serving as the source of cases and controls, this study population is reasonably representative of U.S. women.

Structured questionnaires were used to obtain detailed information on cervical cancer risk factors, including sexual behavior, reproductive and menstrual history, exogenous hormone use, personal and familial medical history, smoking and diet, including vitamin supplement use (12). Trained staff conducted the interviews in the subjects' homes. For the biochemical/molecular components of the study, nonfasting blood samples were drawn at least 6 mo after completion of treatment for cervical disease to minimize any effects on blood biomarkers. Treatment included surgery (44% of the cases), localized radiation (18%) or both (28%). All cases with advanced stage disease (6%) and/or receiving chemotherapy (4%) were excluded from biomarker analyses. Blood was al-

lowed to clot at room temperature for 40–60 min before being centrifuged. The aliquots for serum and red blood cell folate were stabilized with ascorbic acid. All aliquots were kept frozen at  $-70^{\circ}\text{C}$  until assayed.

Because it is not clear how best to assess folate status, serum and red blood cell folate were measured with both radiobinding and microbiologic assays (9). Red blood cell folate is considered a more reliable measure of folate status than serum folate because it integrates folate intake over several months whereas serum folate fluctuates with daily intake (13). Laboratory coefficients of variation, incorporating both within and between batch variability, were 5 and 12% for the serum radiobinding and microbiologic assays and 10 and 11% for the whole blood radiobinding and microbiologic assays. Serum homocysteine was measured by high performance liquid chromatography, with a laboratory coefficient of variation of 12% (14). HPV-16 seropositivity was tested using a well-characterized HPV-16 virus-like particle, enzyme-linked immunosorbent assay.

The risk of invasive cervical cancer was moderately, but nonsignificantly, elevated [fully adjusted, including HPV status, relative risk (RR) = 1.2–1.6] for women in the lowest folate quartile, compared with the highest folate quartile, for all four folate measures (Table 1). Comparison of various models suggested that there was little confounding by HPV-16 status and other cervical cancer risk factors. Women in the lowest folate quartile had serum folates of  $<12.5 \text{ nmol/L}$  by the microbiologic assay and  $<9.7 \text{ nmol/L}$  by the radiobinding assay and red blood cell folates of  $<319 \text{ nmol/L}$  by the radiobinding assay and  $<699 \text{ nmol/L}$  by the microbiologic assay, which tended to run high. To explore the gradient of risk over a wider range of folate status, RRs were recalculated by octile of each folate measure. For the radiobinding assays, but not the microbiologic assays, the RR between extremes was strengthened. Comparing the lowest to highest octile, RRs, adjusted for study design factors and HPV status, reached 2.58 [95% confidence interval (CI) = 1.3–5.3] for serum folate and 1.78 (95% CI = 0.9–3.7) for red blood cell folate.

Folate status based on the microbiologic and radiobinding assays was correlated for both the serum [correlation coefficient ( $r$ ) = 0.90] and red blood cell ( $r$  = 0.77) measures. This agreement was reassuring because it implies that, although absolute values might differ, both assays ranked individuals similarly and thus reliably. The correlation between serum folate and red blood cell folate was not as high ( $r$  = 0.72 with the microbiologic assay and  $r$  = 0.63 with the radiobinding assay). To integrate both serum measures and both red blood cell measures, risks were examined among subjects concurrently in the lowest quartile by both assay methodologies, relative to subjects concurrently in the highest quartile by both methodologies (Table 2). RRs were elevated, although

TABLE 1

Relative risks (95% CI) of invasive cervical cancer by folate status<sup>1</sup>

Folate status	Serum microbiologic assay	Serum radiobinding assay	RBC microbiologic assay	RBC radiobinding assay
Quartile 4	1.0	1.0	1.0	1.0
Quartile 3	0.85	0.68	1.04	0.87
Quartile 2	0.81	0.75	1.41	1.04
Quartile 1	1.27 (0.7–2.3)	1.63 (0.9–2.9)	1.18 (0.6–2.2)	1.49 (0.8–2.7)
P for trend	0.73	0.17	0.42	0.18

<sup>1</sup> Adjusted for age, ethnicity, study center, HPV-16 serologic status, number of sexual partners, age at first intercourse, years since last Pap smear, number of pregnancies, smoking status and intensity, oral contraceptive use, education and income. Included are 183 cases and 540 controls. CI, confidence interval; HPV, human papillomavirus; RBC, red blood cell.

TABLE 2

*Relative risks of invasive cervical cancer integrating microbiologic and radiobinding measures of folate status<sup>1</sup>*

	Cases/controls	Study design-adjusted, with HPV status, RR <sup>2</sup> (95% CI)	Fully adjusted, with HPV status, RR <sup>3</sup> (95% CI)
Serum folate			
Microbiologic/radiobinding			
High/high	30/94	1.0	1.0
Low/low	45/90	2.0 (1.1–3.7)	1.6 (0.7–3.6)
Red blood cell folate			
Microbiologic/radiobinding			
High/high	28/86	1.0	1.0
Low/low	35/80	1.7 (0.9–3.5)	1.5 (0.6–4.1)

<sup>1</sup> CI, confidence interval; HPV, human papillomavirus; RBC, red blood cell; RR, relative risk.

<sup>2</sup> Adjusted for age, ethnicity, study center and HPV-16 serologic status.

<sup>3</sup> Also adjusted for number of sexual partners, age at first intercourse, years since last Pap smear, number of pregnancies, smoking status and intensity, oral contraceptive use, education and income.

not significantly, in the low folate groups for both serum and red blood cell measures (fully adjusted, including HPV status, RR = 1.6 for serum folate and 1.5 for red blood cell folate).

Because of previous hypotheses linking oral contraceptive use to low folate status and thus increased risk of cervical abnormalities (6,7), these associations were closely investigated. Geometric mean serum and red blood cell folates, using either assay and adjusted for age, ethnicity and study site, were not significantly different between women who had used oral contraceptives and women who had not. In addition, although it had been hypothesized that oral contraceptive use would deplete cervical folate stores, no elevation in risk by folate status was observed among users of oral contraceptives.

Serum homocysteine is a sensitive indicator of folate status (15,16). Because of difficulties with the current assays for serum and red blood cell folate (17), serum homocysteine may be a preferable biomarker for folate inadequacy. In our study, women with homocysteine levels in each of the three highest quartiles, relative to women in the lowest quartile, had statistically significantly elevated risks of invasive cervical cancer (fully adjusted, including HPV status, RRs = 2.4–3.2, *P* for trend = 0.01, Table 3). The RRs were not substantially altered by addition of HPV-16 serologic status or other cervical cancer risk factors to the model, suggesting that there was little uncontrolled confounding. The homocysteine levels at which these two- to threefold increased risks were observed, >6.31  $\mu\text{mol/L}$ , are well within the range for U.S. women. In the third National Health and Nutrition Examination Survey, the 5th and 95th percentiles for adult women in various age groups were 4–5 and 10–12  $\mu\text{mol/L}$ , respectively.

To investigate when in the carcinogenic process elevated homocysteine might be critical, the RRs of invasive cervical cancer by homocysteine quartile were recalculated among women with a history of HPV infection (Table 4). All of the cases were assumed to have been infected at one time with an oncogenic strain of HPV. These cases were compared to controls seropositive for HPV-16 (15% of all controls). Risks remained substantially elevated for women in the upper three quartiles (fully adjusted RRs = 1.9–3.8), although smaller numbers of subjects led to less stable risk estimates and broader confidence limits. However, homocysteine levels were not predictive of detection of HPV-16 antibodies. Among the controls, adjusted RRs for the lowest to highest homocysteine quartiles were 1.0, 1.0, 1.0 and 1.3. Thus, elevated homocysteine, or the metabolic pattern it reflects, may facilitate the progression of cervical neoplasia following infection with oncogenic HPV.

The statistically significant 100–200% increase in risk of invasive cervical cancer observed among the women in our study with high serum homocysteine dwarfed the nonsignificant 20–60% increase in risk associated with low folate status. Three explanations are possible. First, as discussed earlier, serum homocysteine may simply be a more accurate measure of inadequate folate than serum or red blood cell folate because of limitations of the current folate assays (15,16,18). Radiobinding assays for folate do not detect all of the biologically active forms of folate, while microbiologic folate assays tend to be imprecise and hard to control. Second, serum homocysteine may be an integratory measure of tissue folate availability. Intracellular concentrations of homocysteine, a potentially

TABLE 3

*Relative risks of invasive cervical cancer by serum homocysteine<sup>1</sup>*

Serum homocysteine quartile	Range of homocysteine levels, $\mu\text{mol/L}$	Cases/controls <i>N</i> = 183/540	Study-design adjusted RR <sup>2</sup> (95% CI)	Fully adjusted, with HPV status, RR <sup>3</sup> (95% CI)
1	<6.3	20/134	1.0	1.0
2	6.3–8.0	44/135	2.20 (1.2–4.0)	2.40 (1.2–4.8)
3	8.1–10.5	60/135	3.04 (1.7–5.5)	3.22 (1.7–6.4)
4	>10.5	59/136	2.97 (1.7–5.4)	2.91 (1.5–5.9)
			<i>P</i> for trend = 0.001	<i>P</i> for trend = 0.01

<sup>1</sup> CI, confidence interval; HPV, human papillomavirus; RR, relative risk.

<sup>2</sup> Adjusted for age, ethnicity and study center.

<sup>3</sup> Also adjusted for HPV-16 serologic status, number of sexual partners, age at first intercourse, years since last Pap smear, number of pregnancies, smoking status and intensity, oral contraceptive use, education and income.

TABLE 4

Relative risks of invasive cervical cancer by serum homocysteine in women with a history of HPV infection<sup>1</sup>

Serum homocysteine quartile	Range of homocysteine levels, $\mu\text{mol/L}$	Cases <sup>2</sup> (N = 183)	HPV-16 seropositive controls (N = 79)	Fully adjusted RR <sup>3</sup> (95% CI)
1	<6.3	20	22	1.0
2	6.3–8.0	44	21	2.45 (0.9–7.1)
3	8.1–10.5	60	16	3.81 (1.3–11.2)
4	>10.5	59	20	1.93 (0.6–5.9)
				P for trend = 0.42

<sup>1</sup> CI, confidence interval; HPV, human papillomavirus; RR, relative risk.

<sup>2</sup> All cases assumed to have been exposed to oncogenic HPV.

<sup>3</sup> Adjusted for age, ethnicity, study center, number of sexual partners, age at first intercourse, years since last Pap smear, number of pregnancies, smoking status and intensity, oral contraceptive use, education and income.

cytotoxic sulfur amino acid, are kept low by catabolism of homocysteine to cystathionine and the excretion of excess homocysteine into the bloodstream (19). Thus, elevated circulating homocysteine may reflect continuing folate insufficiency and homocysteine accumulation in various tissues.

Third, elevated homocysteine could be a biomarker of disruption of one-carbon metabolism. Efficient one-carbon metabolism requires not only folate but also adequate levels of several other vitamins and optimal activity of multiple enzymes. In one-carbon metabolism, homocysteine accepts a methyl group from 5-methyltetrahydrofolate to form methionine in a vitamin B-12-dependent reaction; alternatively, homocysteine is degraded by transsulfuration to cystathionine in a vitamin B-6-dependent reaction (19). The formation of 5-methyltetrahydrofolate requires riboflavin (19). In U.S. populations, elevated levels of homocysteine have been associated with low levels of vitamin B-12, vitamin B-6 and riboflavin, as well as of folate (16,20). Methylene tetrahydrofolate reductase (MTHFR; EC 1.5.1.20) catalyzes the formation of 5-methyltetrahydrofolate, the source of the methyl group needed for homocysteine to be converted to methionine. A common polymorphism in the MTHFR gene, the C-to-T transition at position 677 (C677T), reduces enzyme activity, limits remethylation of homocysteine and thus raises circulating homocysteine levels (21). Cystathionine  $\beta$ -synthase (CBS; EC 4.2.1.22) catalyzes the catabolism of homocysteine to cystathionine. An increasing number of tandem repeats of a 31-base pair sequence that spans the exon-intron boundary of the CBS gene has recently been associated with reduced enzyme activity and increased circulating homocysteine (22). Thus, elevated homocysteine could be a sensitive biomarker of inefficient one-carbon metabolism caused by insufficient folate, vitamin B-12, vitamin B-6 and/or riboflavin, or by genetic variation in critical pathway enzymes.

We currently are exploring the contribution of several common polymorphisms in one-carbon metabolism genes to the risk of cervical cancer. Initially we are focusing on the MTHFR, CBS and methionine synthase (5-methyltetrahydrofolate:homocysteine S-methyltransferase; EC 2.1.1.13) genes, both independently and jointly. Methionine synthase, in a reaction requiring vitamin B-12, transfers a methyl group from 5-methyltetrahydrofolate to homocysteine to form methionine. Our initial hypothesis is that polymorphisms that reduce enzyme activity and make one-carbon metabolism less efficient will increase cancer risk. Ultimately, we hope to integrate the nutritional and genetic information to explore their interaction in cervical carcinogenesis.

We have questioned whether the increased cervical cancer risk associated with high serum homocysteine and low serum

and red blood cell folate in this study might be attributable to bias. Participation bias seems unlikely. While not all cases and controls agreed to give blood, those who did were similar in terms of demographic factors. In addition, the same patterns of cancer risk were seen in all subjects interviewed and in the subset that gave blood. Bias due to disease or treatment seems unlikely. Blood samples were collected from the cases at least 6 mo after completion of any treatment. The small number of women diagnosed with advanced disease (17 cases) or receiving chemotherapy (11 cases) were excluded from analysis. For the women included in the analyses, there was no evidence that stage (stage I or II) or treatment modality (surgery and/or localized radiation) significantly altered folate or homocysteine levels. Finally, bias due to deliberate dietary changes after diagnosis seems unlikely. At the time of the blood draw, only two cases reported increased vegetable, fruit or grain intake during the last 3 y in response to their illness, and only five cases reported a loss of appetite. Thus, we found no evidence that our findings were generated by differential participation or lingering effects of cancer or treatment. However, it is impossible to totally rule out these sources of bias in retrospective studies, and our results should be followed up in cohort studies using blood samples stored before diagnosis of disease.

Although the epidemiologic studies of folate and cervical dysplasia/cervical cancer—both the observational studies and the randomized trials—have not produced consistent or compelling results, all three studies of circulating homocysteine levels and cervical disease have demonstrated positive associations (13,23,24). In a small case-control study nested within the Washington County cohort (23), women in the highest tertile of serum homocysteine levels had more than twice the risk of cervical cancer of women in the lowest tertile (adjusted RR = 2.67, 95% CI = 0.8–8.8; P for trend = 0.05). Only 39 cases of cervical cancer (13 invasive and 26 in situ) were included in the analysis; therefore, the confidence limits of the result are wide. However, a strength of the study is that blood samples had been collected and stored prospectively (interval from blood collection to clinical diagnosis of disease: mean = 6 y, range = 1–20 y) and thus were unlikely to be affected by disease progression or treatment. Furthermore, in a case-control study including 294 cases of cervical dysplasia (24), plasma homocysteine was positively, but not significantly, associated with increased risk (adjusted RR for the highest, relative to lowest, quintile = 1.6, 95% CI = 0.7–3.6; P for trend = 0.06). In both these studies, as in ours, circulating homocysteine was directly related to increased risk of cervical dysplasia/neoplasia, although in these two studies the critical

concentration for homocysteine was somewhat higher than in ours (~9–10  $\mu\text{mol/L}$ , compared to ~6  $\mu\text{mol/L}$ ).

Only recently have epidemiologic studies begun to examine the importance of one-carbon metabolism in human carcinogenesis. The experimental research is far more extensive. Although the underlying mechanism(s) have not been conclusively proven, several plausible ones have been identified involving DNA/RNA synthesis, DNA repair and DNA/RNA methylation (25). Clearly, one-carbon metabolism is required for biosynthesis of the two purine nucleotides, adenylate and guanylate, and of the pyrimidine nucleotide thymidylate. The fidelity and efficiency of DNA synthesis and repair depend on the availability and balance of the deoxynucleotide triphosphates. For example, folate deficiency limits thymidylate synthesis from deoxyuridylate, and the nucleotide imbalance causes misincorporation of uracil bases into DNA (26). During the repair process, transient single-strand, and occasional double-strand, breaks develop, which lead to genetic instability and thus increased cancer risk (26). This faulty repair likely contributes to the chromosomal breaks at constitutive fragile sites that have been observed in thymidine-deficient human cell cultures (27). The common fragile site FRA3B, which is the site on human chromosomes most sensitive to folate stress, coincides with a site of HPV-16 integration in a primary cervical carcinoma (28). Thus, common fragile sites, in the presence of inefficient one-carbon metabolism, could provide targets for viral integration, an essential step in cervical carcinogenesis.

Inefficient one-carbon metabolism could also limit the production of *S*-adenosylmethionine, which donates its labile methyl group in numerous enzymatic reactions that methylate specific sites within DNA and RNA. Altered patterns of DNA methylation are believed to play an integral role in carcinogenesis (25). For example, hypomethylation of protooncogene DNA, whether due to global or gene-specific processes, could lead to increased gene expression, selective cell growth and transformation. Global DNA hypomethylation has been shown to be progressively increased in cervical dysplasia and cervical cancer, relative to normal cervical tissue (29,30). Furthermore, both serum folate and cervical tissue folate were inversely associated with degree of hypomethylation (30). Although hypomethylation may be only a biomarker of the disease process, further investigation of the role of DNA methylation in carcinogenesis is justified. Continued integration of epidemiologic, clinical and laboratory research promises to provide new insights into the role of one-carbon metabolism in carcinogenesis.

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