Folic Acid Deficiency During Late Gestation Decreases Progenitor Cell Proliferation and Increases Apoptosis in Fetal Mouse Brain[1]

Corneliu N. Craciunescu, Elliott C. Brown, Mei-Heng Mar, Craig D. Albright, Marie R. Nadeau* and Steven H. Zeisel[2]

Department of Nutrition, School of Public Health and School of Medicine, University of North Carolina-Chapel Hill, 27599 and U.S. Department of Agriculture Human Nutrition Center on Aging, Tufts University, Boston, MA 02111

ABSTRACT In mice and rats, maternal dietary choline intake during late pregnancy modulates mitosis and apoptosis in progenitor cells of the fetal hippocampus and septum. Because choline and folate are interrelated metabolically, we investigated the effects of maternal dietary folate availability on progenitor cells in fetal mouse telencephalon. Timed-pregnant mice were fed a folate-supplemented (FS), control (FCT) or folate-deficient (FD) AIN-76 diet from d 11–17 of pregnancy. FD decreased the number of progenitor cells undergoing cell replication in the ventricular zones of the developing mouse brain septum (46.6% of FCT), caudate putamen (43.5%), and neocortex (54.4%) as assessed using phosphorylated histone H3 (a specific marker of mitotic phase) and confirmed by bromodeoxyuridine (BrdU) labeling of the S phase. In addition, 106.2% more apoptotic cells were found in FD than in FCT fetal septum. We observed 46.8% more calretinin-positive cells in the medial septal-diagonal band region of FD compared with pups from control dams. FS mice did not differ significantly from FCT mice in any of these measures. These results suggest that progenitor cells in fetal forebrain are sensitive to maternal dietary folate during late gestation. J. Nutr. 134: 162–166, 2004.

KEY WORDS: • *folic acid* • *brain development* • *nutrition* • *calretinin* • *septum*

During the perinatal period, progenitors of neurons and glia divide, unnecessary cells die by apoptosis, whereas others migrate to reach their final destinations within various brain regions, creating the structures of the brain and setting the stage for brain function or dysfunction later in life. During this period, the brain is sensitive to the supply of essential nutrients. Maternal dietary supplementation with folic acid in the periconceptional period significantly reduces the risk of neural tube defects (1–3). Folate plays a central role in DNA synthesis through de novo purine and thymidine biosynthesis necessary for mitotic cell division, and folate is important in the transfer of methyl groups (4). Although the importance for normal brain development of folate intake early in pregnancy is well accepted, the requirements for folate intake late in fetal gestation are not well understood.

Folate is interrelated metabolically to choline metabolism; both methyltetrahydrofolate and betaine (derived from choline) can methylate homocysteine to produce methionine (5–9). We observed that maternal dietary choline intake during late pregnancy modulated mitosis and apoptosis in progenitor cells of the fetal rat hippocampus and septum (10,11) and altered the differentiation of neurons in the fetal hippocampus (10,12–14). Mothers fed choline-deficient diets during late pregnancy had offspring with diminished progenitor cell proliferation and increased apoptosis in the fetal hippocampus (10,11), insensitivity to long-term potentiation when they were adult animals (15), and decremented visuospatial and auditory memory (16). Because choline and folate metabolism are interrelated, we tested the hypothesis that maternal dietary folate intake during late gestation might similarly influence neurogenesis in developing mouse brain.

MATERIALS AND METHODS

Timed-bred animals. Timed-pregnant C57Bl/6J mice were ordered from Jackson Laboratory (Bar Harbor, ME) and were housed individually in cages in a temperature-controlled room at 24°C and exposed to a 12-h light:dark cycle. Mice were fed an AIN-76A pelleted diet (Dyets, Bethlehem, PA) (17) with the standard 2 mg folic acid/kg diet and 1% succinyl sulfathiazole (kills intestinal bacteria able to synthesize folate) (18). The pregnant mice consumed the diet and water ad libitum until d 11 of gestation when they were randomly assigned to one of 3 treatment groups: folate-deficient (FD),[3] control (FCT) or folate-supplemented (FS). The FD group was fed an AIN-76A diet with 0.0 mg folic acid/kg diet and 1% succinyl sulfathiazole (this diet is standard in folate deficiency studies), the

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[2] To whom correspondence should be addressed.
E-mail: steven_zeisel@unc.edu.

[3] Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; CDKI, cyclin-dependent kinase inhibitors, p15Ink4B and p27Kip1; DAPI, 4′,6-diamidino-2-phenylindole, nuclear DNA staining; E, embryonic (gestation) day; FCT, FD, FS, control, folate-deficient, and folate-supplemented diet; GABA, γ-aminobutyric acid.

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TABLE 1

Folate concentrations in plasma of timed-pregnant mice fed a folate-supplemented (FS), control (FCT) or folate-deficient (FD) AIN-76 diet from d 11–17 (E11–E17) of pregnancy and in fetal brain and liver[1]

	FD	FCT	FS
Maternal plasma,[2] *ng/mL*	8.33 ± 0.99[a]	63.45 ± 2.05[b]	286.33 ± 14.62[c]
Fetal (E17) brain, *ng/g tissue*	14.7 ± 2.5[a]	23.8 ± 1.9[b]	24.5 ± 0.7[b]
Fetal (E17) liver, *ng/g tissue*	119 ± 23	161 ± 24	176 ± 31

[1] Values are means ± SEM, $n = 3$. Means in a row without a common letter differ, $P < 0.05$ by ANOVA and Tukey-Kramer test.
[2] To convert ng/mL folate to nmol/L, multiply by 0.002265.

FCT group was fed an AIN-76A diet with 2 mg folic acid/kg diet (this amount is the recommended composition for rodent diets (17) and 1% succinyl sulfathiazole, and the FS-group was fed an AIN-76A diet with 20 mg folic acid/kg diet and 1% succinyl sulfathiazole. These diets were ingested until the dams were killed on embryonic (gestation) day 17 (E17). All dams were injected intraperitoneally on E15 with bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdU; Sigma, St. Louis, MO), dissolved in 1 mL of 0.007 mol/L sodium hydroxide in 9 g/L sterile saline, 50 mg/kg body weight. This BrdU dose is not cytotoxic, and labeled cells retain the ability to differentiate and migrate (19).

Tissue collection. Fetal brains were collected using the modified technique of Park et al. (20). Pregnant dams from the three treatment groups were anesthetized with a subcutaneous injection of 0.03 mL of ketamine (100 g/L) and 0.02 mL of xylazine (20 g/L) (Henry Schein, Melville, NY). The anesthetized dam was placed on a heating pad, and a small incision was made into the abdominal wall to expose the uterine horn. The embryos were removed one at a time. The chest cavity of the embryo was opened to expose the heart for in situ fixation. A 30-gauge needle was inserted into the right atrium, and then 2 mL of fixative containing 4% formaldehyde and 0.2% glutaraldehyde (Polysciences, Warrington, PA) was injected into the left ventricle. The fixed embryo was placed on ice and the brain was removed and placed into the perfusion buffer overnight. This procedure was repeated for all of the embryos. The residual fetal bodies were kept on ice for later use in sex determination. The next morning, the brains were removed from the postfixative and placed into 70% ethanol. Tissue was automatically processed overnight and embedded in paraffin. Coronal sections (5 μm) were made using a Leica microtome and applied to glass slides. Because there is a posterior to anterior gradient of neurogenesis in fetal mouse brain, the paraffin sections were reviewed at the time of sectioning to ensure that they included anatomically reproducible areas of the septum (21–23). Plasma was collected from dams and used to determine folate concentrations.

Determination of total plasma and tissue folate. Total folate concentration in dams' plasma was determined using a radioassay kit (Solid Phase No Boil Dualcount radioassay; DPC Diagnostic Products, Los Angeles, CA) as per manufacturer's directions.

Fetal liver and brain samples were analyzed after they were boiled for 5 min in 1 mL of 2% ascorbic acid/2% β-mercaptoethanol and then storing them at −80°C. Tissue folate concentration was measured using a modified *Lactobacillus casei* assay of Wilson et al. (24).

Assessment of mitosis. Coronal brain sections were probed with an antibody that recognizes phosphorylated histone H3, the core protein of the nucleosome; it becomes phosphorylated at the end of prophase, an event that is essential for the maintenance of mitosis-associated chromosome condensation (25). Slides were prepared and histone H3 phosphorylation assessed using anti-phospho-histone H3 antibody (Upstate, Lake Placid, NY) as previously described (26). The incidence of phospho-histone H3–labeled cells was measured at the ventricular surface of the ventricular zone beginning at the junction of the septum and caudate putamen striatum (septal fork), and extending toward and then to both the cingulated cortex and frontal neocortical ventricular zone. Cells were counted hemilaterally in four consecutive serial sections and the values were averaged to obtain a single value per section (region) per mouse. Calibrated 50X magnification images of the same regions were captured to measure the length of the septal ventricular zones with an internal macro of NIH Image program version 1.61.

Assessment of BrdU labeling. Slides were deparaffinized, treated for antigen retrieval and then counterstained and mounted using the same procedures as for mitosis assessment Additional antigen retrieval was performed in BD Retrievagen A (BD Biosciences Pharmingen, San Diego, CA) followed by heating in a microwave oven for 10 min at 90°C. Nonspecific sites were blocked for 1 h at room temperature with M.O.M. Mouse Ig Blocking reagent and Avidin D solution (Vector Laboratories, Burlingame, CA). Sites of BrdU incorporation were detected using Fluorescein Vector M.O.M. immunodetection kit (Vector Laboratories). As primary antibody, we used mouse monoclonal anti-bromodeoxyuridine containing a nuclease (Amersham Biosciences, Piscataway, NJ) to produce single-stranded DNA that was easily accessible without the need for chemical denaturation. The number of positive BrdU labeled cells was counted for the whole septal section beginning at the junction of the septum and caudate putamen striatum (septal fork, nucleus accumbens) and extending toward the corpus callosum. Cells were counted hemilaterally in four consecutive serial sections and the values were averaged to obtain a single value per 100 μm length of the lateral ventricular section per mouse. Calibrated 50X magnification images of the same regions were used to measure the length of the septal ventricular zones with an internal macro of NIH Image program version 1.61.

Assessment of apoptosis. Apoptosis was assessed hemilaterally in hematoxylin and eosin–stained coronal sections of the septum using morphological criteria as described previously at a final magnification of 200X (10,26).

Immunohistochemical localization of calretinin. Calretinin protein was localized using an immunoperoxidase technique as described previously (14). Calretinin positive cells were counted hemilaterally in the septum at a final magnification of 200X.

Image analysis. For the assessment of mitosis, the image analysis of fetal brain slices was performed on a Zeiss Confocal Laser Scanning Microscope LSM 210 (Carl Zeiss, Thornwood, NY) equipped with an Optronics DEI 750 low light level integrating CCD camera (Optronics Engineering, Goleta, CA) connected to an Apple Macintosh G3 computer utilizing a Scion CG7 image capture card for digital image capture of standard and epi-fluorescence, images) and the public domain NIH Image program version 1.61. Images obtained from the same field with fluorescent filters optimized for observing DAPI (blue), and Cy3 conjugates (red) signals were subsequently overlapped or merged. For BrdU studies, image acquisition was performed on a Nikon FXA microscope (Nikon, Garden City, NY) using the public domain NIH Image program version 1.61.

Statistical analysis. Statistical differences from group means were calculated using analysis of variance (ANOVA) and Scheffé's *F*-test, or Tukey-Kramer test as indicated in the figures (JMP Version 2, SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$.

RESULTS

Folate in maternal plasma and in fetal brain and liver. Maternal plasma folate concentrations were lowest in the FD group, intermediate in FCT and highest in the FS group (**Table 1**). In fetal brain, folate concentrations were signifi-

cantly lower in the FD than in the FCT or FS groups. There were no difference between groups in fetal liver folate concentrations.

Mitosis and proliferation in fetal mouse forebrain. We measured the incidence of phospho-H3-labeled cells at the ventricular surface of the ventricular zone, adjacent to the septum, caudate putamen-striatum and both cingulated cortex plus frontal neocortex within the fetal mouse telencephalic portion of the forebrain (**Fig. 1**). On E17, the number of progenitor cells in mitotic phase (as assessed using phosphorylated histone H3) in the ventricular zones of the developing mouse brain septum (46.6% of FCT), caudate putamen (43.5%), and neocortex (54.4%) was lower in the FD group; the FS did not differ from the FCT group (**Fig. 2**). To confirm the effects of maternal dietary folate levels on the replication of progenitor cells, we measured the number of cells that were in S phase at the moment of BrdU pulse-labeling on E15 and

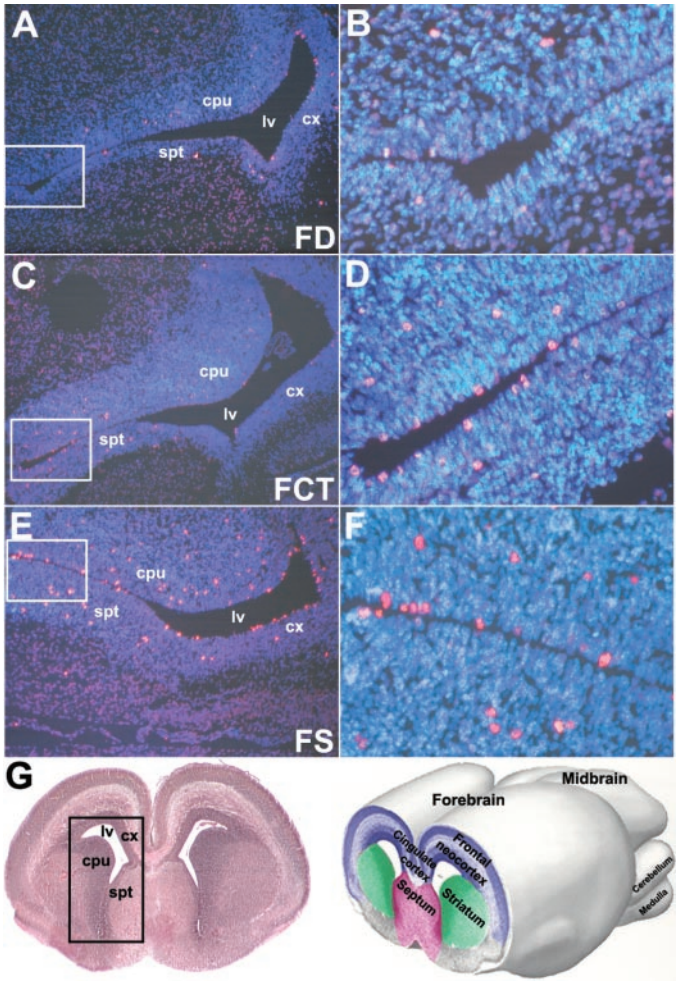

FIGURE 1 Dietary folate availability in timed-pregnant mice fed a folate-supplemented (FS), control (FCT) or folate-deficient (FD) AIN-76 diet from d 11–17 (E11-E17) of pregnancy as assessed by histone H3 phosphorylation in the periventricular zones corresponding to septum (spt), striatal caudate putamen (cpu), and neocortex (cx). In sections of fetal mouse brain, the DAPI nuclear DNA counterstaining is blue, whereas the Cy3 conjugated secondary antibody bound to the anti-phosphorylated histone H3 (Ser10) primary antibody stains red. *Panels* A, *C*, *E*: 50X magnification of representative sections from FD, FCT or FS fetuses, respectively; *Panels B*, *D*, *F*: 200X magnification of the boxed areas in the *left panels*; *Panel G*: A schematic diagram showing the location of the regions of the brain studied.

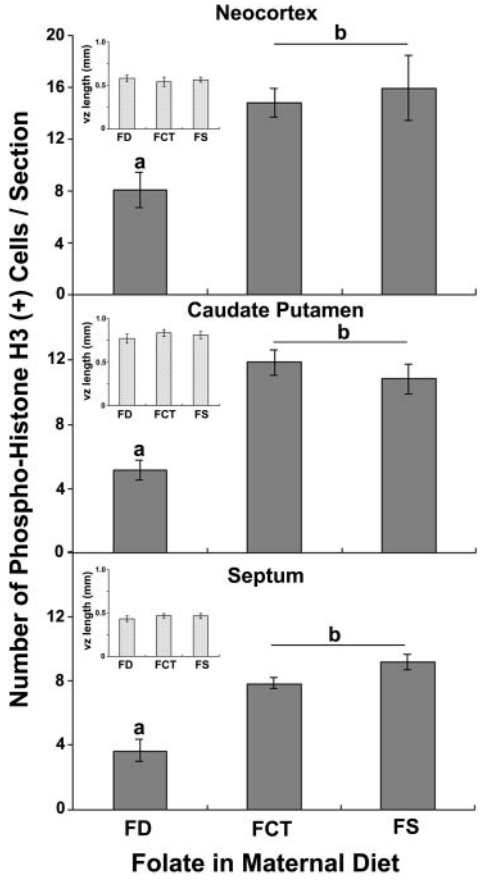

FIGURE 2 Maternal folate deficiency in timed-pregnant mice fed a folate-supplemented (FS), control (FCT) or folate-deficient (FD) AIN-76 diet from d 11–17 (E11-E17) of pregnancy decreased the number of phospho-histone H3 positive cells in fetal mouse forebrain. Incidence of phospho-histone H3 positive cells was quantified in regions described in Figure 1 legend. Values are means ± SEM , n =5–6 of the total number of phospho-histone H3 positive cells/region from 4 to 6 consecutive sections/fetal brain . Means in a row without a common letter differ, $P < 0.05$ by ANOVA and Tukey-Kramer test. The number of phospho-histone H3 positive cells was significantly lower in FD mice in all regions studied. *Insets*: The length (mm; mean ± SEM) of the ventricular zone in corresponding anatomical regions of developing mouse forebrain. The length of ventricular zone was nearly identical across diet groups for a given region of brain.

subsequently continued proliferation or accumulated as measured on E17 in mouse septum. The number of BrdU positive cells in the FD group (38.5 ± 3.6 BrdU positive cells/100 μm length of lateral ventricular zone) was lower ($P < 0.05$ by ANOVA and Tukey-Kramer test) than those in the FCT (59.2 ± 2.67) and FS (60.3 ± 4.8) groups. The BrdU-positive cells were located almost exclusively in the ventricular and subventricular zones adjacent to the lateral septum.

Apoptosis. In the developing septum on E17, apoptotic cells were located mainly in the dorsal regions of developing septum; the number of apoptotic cells was 106.2% greater in the FD group than in the FCT group and 109.4% greater in FS mice ($P < 0.01$, **Fig. 3**).

Calretinin positive cells. In E17 mouse basal forebrain septum, calretinin protein was restricted to cells in the region of the medial septal-diagonal band. The number of calretinin positive cells in the FD group was 46.8% greater than in the FCT group, and 113.3% greater than in the FS group ($P < 0.01$, **Fig. 4**).

DISCUSSION

Previous studies showed that altering maternal dietary choline availability from E12 through E18 affected progenitor cell mitosis and apoptosis in the developing rat hippocampus and septum (10,11) as well as in the mouse hippocampus (26). We now report that dietary folic acid availability also influences progenitor cell mitosis and apoptosis in the fetal mouse telencephalic portion of the forebrain.

Feeding the FD diet, like a choline deficient diet, delayed cell cycle transit as evidenced by decreased numbers of neuronal progenitor cells expressing the mitotic marker phosphorylated histone H3 (Fig. 2), and corroborated by decreased numbers of progenitor cells having incorporated BrdU (DNA synthetic phase). Mitotic cell division in brain progenitor cells is regulated by cyclin-dependent kinase inhibitors (CDKI) (27). In previous studies in fetal rat brain, we showed that feeding a choline-deficient diet increased, whereas a choline-supplemented diet decreased the expression of CDKI, correlating with reciprocal changes in mitotic cell division by progenitor cells in the ventricular zone (12). Here we show that feeding a FS diet did not affect cell cycle transit, as shown by BrdU and phosphorylated histone H3 labeling. This may be because the FCT diet contained sufficient folic acid to permit maximum cell proliferation.

Apoptosis is a regulated form of cell suicide (28) that is important in normal brain development (29–32); in neurons, it is modulated by survival factors such as neurotrophins, sex hormones and neuronal activity (33,34), and is induced by choline deficiency (35–38). Here we show that FD increased apoptosis in E17 dorsal lateral mouse septum (Fig. 3). We extensively characterized the choline-regulated intermediate signals that mediate apoptosis (35,38–41) and suggest that FD and choline deficiency may share a common apoptosis execution pathway.

We reported previously that pups from choline-deficient dams had more calretinin protein (threefold increase) and pups from choline supplemented–dams had less calretinin protein (70% decrease) than did pups from control dams (14). Importantly, decreased calretinin protein was still detectable in hippocampus in aged, 24-mo-old mice, born of choline-supplemented dams and fed a control diet since birth (14). Calretinin is a calcium-binding protein present in γ-aminobutyric acid (GABA)ergic neurons of the ventral forebrain's medial septal-diagonal band regions (42), and these neurons

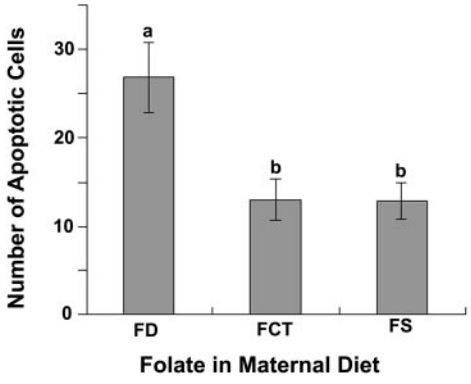

FIGURE 3 Maternal folate deficiency in timed-pregnant mice fed a folate-supplemented (FS), control (FCT) or folate-deficient (FD) AIN-76 diet from d 11–17 (E11-E17) of pregnancy increased apoptosis in E17 mouse septum. Values are means ± SEM , n =5–7; bars without a common letter differ, $P < 0.01$ by ANOVA and Scheffé's test.

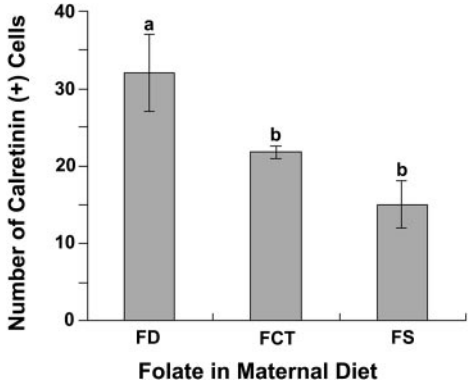

FIGURE 4 Maternal folate deficiency in timed-pregnant mice fed a folate-supplemented (FS), control (FCT) or folate-deficient (FD) AIN-76 diet from d 11–17 (E11-E17) of pregnancy increased the number of calretinin positive cells in E17 mouse septum. Values are means ± SEM , n =5–7; bars without a common letter differ, $P < 0.01$ by ANOVA and Scheffé's test.

are important modulators of sleep, attention and memory (43,44). In addition to defining a subgroup of GABAergic neurons, calcium-binding proteins buffer intracellular free calcium (45) and are thought to attenuate cell damage associated with nonphysiologic increases in intracellular calcium, thus promoting selective cell survival. Because the changes induced by manipulation of choline were so profound, we chose to examine this biomarker in folate-manipulated mice. Here we show that FD increased the number of calretinin-positive cells in the medial septal-diagonal band region of the mouse ventral forebrain (Fig. 4). Thus, both choline deficiency and FD have similar effects on calretinin expression.

Choline deficiency and folate deficiency may share some common underlying mechanism that explains our observations on brain development. As noted earlier, the metabolism of choline, homocysteine and methyl-folate are closely interrelated. Perturbation of the metabolism of one of the methyl-donors results in compensatory changes in the other methyl-donors due to the intermingling of these metabolic pathways (6–8). Diminished phosphatidylcholine concentrations were observed in brains from folate-deficient adult rats (46). Choline nutriture is marginal during pregnancy (47); folate supply is also limiting. Folate concentrations in serum and RBC decline during pregnancy to the point that pregnant women can become clinically folate deficient (48,49). Folate deficiency may be more common in women with genetic polymorphisms of folate metabolism that increase dietary requirements for folate (50–52).

To date, the majority of scientific investigations about dietary folate requirements during pregnancy have focused on folate's role in preventing neural tube defects. This has led to recommendations that pregnant women take supplemental folic acid before and during the first weeks of pregnancy (53). The data presented here suggest that folate availability affects brain development long after neural tube closure, and indicates that it may be very important that women ingest adequate intakes of folic acid throughout pregnancy. This may be especially important in those women with genetic polymorphisms in genes of folate metabolism.

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