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Risk of retinoblastoma is associated with a maternal polymorphism in dihydrofolatereductase (*DHFR*) and pre-natal folic acid intake:

Folic acid, DHFR and retinoblastoma risk

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Abstract

Background—Incidence of unilateral retinoblastoma varies globally suggesting possible environmental contributors to disease incidence. Maternal intake of naturally occurring folate from vegetables during pregnancy is inversely associated with risk of retinoblastoma in offspring.

Methods—Using a case-control study design, we examined the association between retinoblastoma risk and maternal variations in the folate-metabolizing genes, methylenetetrahydrofolate reductase (*MTHFR*677C>T, rs1801133) and dihydrofolate reductase (*DHFR* 19base pair deletion of intron 1a [*DHFR*19bpdel], rs70991108). In central Mexico, we enrolled 103 mothers of children with newly diagnosed unilateral retinoblastoma and 97 control mothers who had healthy children in an IRB approved study.

Mothers were interviewed regarding perinatal characteristics including use of prenatal vitamin supplements and gave peripheral blood samples used for PCR-based genotyping of rs1801133 and rs70991108.

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Results—The risk of having a child with unilateral retinoblastoma were associated with maternal homozygosity for *DHFR*19bpdel (OR=3.78, 95%CI:1.89,7.55; p=0.0002), even after controlling for child's *DHFR*19bpdel genotype (OR=2.81, 95%CI:1.32,5.99; p=0.0073). In a subgroup of 167 mothers with data on prenatal intake of supplements containing folic acid (a synthetic form of folate), *DHFR*19bpdel-associated risk was significantly elevated only among those who reported taking folic acid supplements. Maternal *MTHFR* genotype was unrelated to risk of having a child with retinoblastoma.

Conclusion—Maternal homozygosity for a polymorphism in the *DHFR* gene necessary for converting synthetic folic acid into biological folate is associated with increased risk for retinoblastoma. Prenatal ingestion of synthetic folic acid supplements may be associated with increased risk for early childhood carcinogenesis in a genetically susceptible subset of the population.

Keywords

DHFR; Folic Acid Intake; Retinoblastoma Risk; Childhood cancer

Introduction

Retinoblastoma, a malignant primitive neuroectodermal tumor, arises in immature retinal cells. Incidence of retinoblastoma varies geographically. The highest rates of incidence are found in some populations of Africa, South America, India and Scandinavia[1,2], while in the US, comparisons by ethnicity show that incidence is highest among Latinos.[3]

A US based case-control study in the 1980s suggested that factors associated with perinatal poverty such as lack of prenatal vitamins and lower maternal education were associated with having a child with retinoblastoma.[4] Global variation in incidence of retinoblastoma appears to be primarily among the sporadic unilateral form[2], which, in contrast to the bilateral form, involves only one eye and is not associated with a family history of the disease. Given that the median age at diagnosis for unilateral retinoblastoma is 23 months, the initiating events for tumor development are likely to occur during pregnancy and early infancy. In 85% of cases, the unilateral form is also associated with mutations in *RB1* occurring in somatic (but not germ) cells. Although the origin of these somatic *RB1* mutations is unknown, the majority involves methylated cytosines in CpG islands and results in stop codons and truncated *p*Rb.[5,6] A recent report has documented imprinting of a maternal *RB1* gene[7]. In a case-control study in Mexico (before folic acid fortification), we found that maternal consumption of vegetable derived folate during pregnancy was protective against having a child with unilateral retinoblastoma.[8]

Folate provides precursors for nucleotide biosynthesis and methyl groups, via methyl folate, for methylation reactions including DNA methylation. The potential role of folate in development of pediatric tumors derived from embryonal layers has not been well studied. Polymorphisms in genes encoding enzymes involved in metabolism of one-carbon donors have been studied in relation to development of birth defects such as neural tube defects (NTD) as well as cancer incidence in adults.[9–11] Because retinoblastoma is a primitive neuroectodermal tumor arising in the retina and therefore embryologically derived from the neural tube, we hypothesized that dysregulation of folate metabolism during early retinal development might influence tumorigenesis.

In the present study we examined the relationship between retinoblastoma risk and prevalence of maternal polymorphisms in two genes that encode two enzymes of folate metabolism, *MTHFR* 677C>T (*rs1801133*) and the *DHFR* 19basepair deletion of intron 1a, (rs70991108). Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-

methyltetrahydrofolate, which is used for homocysteine methylation. The *MTHFR* 677C>T (*rs1801133*) polymorphism has been studied extensively regarding increased susceptibility for development of NTD when folate availability is low[9] as well as for risk of cancer incidence.[11] In addition to its role in thymidylate synthesis, DHFR is responsible for the conversion of orally ingested folic acid (found in supplements and fortified foods) into folate (reduced), which can then be incorporated into the body's folate pool. The 19bp deletion in intron 1a of the human DHFR gene (*DHFR*19bpdel) was shown to be associated with an increased risk for NTD[12] as well as an increased risk for breast cancer among women taking folic acid supplements.[10] Homozygosity for *DHFR*19bpdel is associated with decreased efficiency in using folic acid[13,14] and is present in 17–19% of populations studied.[10,13,14]

We carried out a case-control study in central Mexico to examine whether the risk for having a child with retinoblastoma is associated with homozygosity for polymorphisms in *MTHFR* and *DHFR*. A second objective was to examine whether prenatal folate intake would be associated with risk of retinoblastoma.

Methods

Between January 2000 and December 2009, 108 mothers of children with newly diagnosed unilateral retinoblastoma at two adjacent referral hospitals in Mexico City were invited to participate in this case-control study, which was approved by IRBs of all participating institutions. Mothers of children with a known family history of retinoblastoma were not eligible to participate in the study. The study was designed to recruit 100 cases and 100 controls. Two mothers declined to participate. Case mothers (N=106) were enrolled during their child's initial visits to the treating hospital. Enrolled mothers were asked to refer a friend (not related by blood to the case mother) who had a child of the same age as their child (range ±1 year) to serve as a control mother. Eligibility criteria for control mothers also included not having a family member with retinoblastoma. Among the 97 control mothers, more than 81% were the first friend the case mother approached, while the remaining mothers were the second friend approached. Control mothers were enrolled during home visits in 18 states in central and southern Mexico. At the time of enrollment, all participating mothers gave signed consent. Blood samples were then obtained from all mothers and from most case and control children. Mothers were interviewed regarding supplement intake during the first trimester of pregnancy using a validated questionnaire.[15] If women reported taking any supplements, they were asked about the supplement's brand, dose and form of administration (tablet, powder etc.). Supplemental intake of folic acid was noted as present or absent after analyzing the folic acid content of the supplements reported using a nutrient content database for supplements available in Mexico.[16] Blood was drawn into Becton Dickinson CPT (Becton Dickinson, NJ) vials and kept at 4°C until centrifugation. Buffy coat were stored at −80°C until DNA extraction using standard non-organic methods (Qiagen, Valencia, CA). Genotyping for the *MTHFR* 677C>T and *DHFR* 19bpdel polymorphisms were performed by PCR amplification using RFLP and allelic specific methods, respectively.[9,10,12] Resulting PCR products were separated on 3% agarose gels and were visualized with ethidium bromide. About 20% of samples were randomly selected to be run in duplicate with 99% concordance. Laboratory personnel were blinded to sample origin. Batches contained equivalent proportions of case and control samples. Samples and questionnaires were linked through de-identified and bar-coded labels.

Statistical analysis

Quantitative variables

Maternal age and number of years of schooling completed were examined as continuous variables. Genotyping was examined by comparing the homozygous variant genotype with

the genotypes containing at least one wild type allele based on results from analyses done with the Framingham Offspring Cohort demonstrating impaired function with homozygous 19bp deletion for *DHFR*[13,14] and data demonstrating impaired *MTHFR* function with the homozygous TT genotype.[17] Vitamin supplement use was defined as whether or not mothers reported taking vitamins containing synthetic folic acid during their first trimester.

Statistical methods

We calculated summary statistics to describe sample characteristics and used Chi-square test and t-test to detect group differences in categorical variables and continuous variables, respectively. Variables with skewed distribution were transformed to meet assumptions for t-test. Based on distribution of mothers' genotype, we estimated allele frequency and tested for Hardy-Weinberg equilibrium in the control mothers. A logistic regression model was used to assess the association between maternal genotype and the odds of having a child with disease, with and without adjusting for control variables of child's genotype. The child's genotype variable had three categories with one category for "missing genotype" on 33 children. Odds ratios and 95% confidence intervals were derived from the model parameters and standard errors were used to aid interpretation.

Results

We recruited 106 mothers of children with unilateral retinoblastoma at time of initial diagnosis, of which, 103 were eligible for participation in the study (the child of one mother did not have retinoblastoma on histopathologic examination, while the children of two mothers were discovered to have family members with retinoblastoma and were thus excluded). All references to "case mothers" refer to mothers of children with unilateral retinoblastoma. All references to "control mothers" refer to mothers who had healthy children. All control mothers were healthy and met eligibility criteria to participate.

Analyses were performed on a dataset that contained measurements from 200 mothers (97 control mothers and 103 case mothers) who had data from their perinatal characteristics interviews as well as blood samples, and had genotype results for *DHFR*19bpdel (198 mothers) or *MTHFR*677C>T (197 mothers). Case mothers did not differ from control mothers in demographic characteristics such as age at delivery of the index child, child's birth weight, and maternal education or in the proportion reporting smoking during pregnancy, or folic-acid supplement use in their first trimester of pregnancy (Table 1). Supplement intake was missing for 31 mothers because they were lost to follow up before completing their dietary interview. DHFR and prenatal supplement data were thus available for 167 mothers (96 controls and 71 cases). Among mothers missing data on supplement use there was a significantly higher proportion who were mothers of case children, were homozygous for *DHFR*19bpdel, and had lesser education when compared with mothers that had data on supplement intake.

There was no significant departure from Hardy-Weinberg equilibrium detected in the 96 control mothers for either *DHFR* (p=0.99) or for *MTHFR* (p=0.06). Estimated allele frequency of *DHFR*19bpdel (rs70991108) was 0.61458. Genotypes for *DHFR* and *MTHFR* were not correlated in either control mothers (p=0.99) or in case mothers (p=0.42). The proportion of control mothers homozygous for *DHFR*19bpdel is similar to that reported in US cohorts.[10,14]

A higher proportion of case mothers were homozygous for *DHFR*19bpdel (*DHFR*19bpdel/del) (39.2%) compared with control mothers (14.5%); OR was 3.78, 95%CI: (1.89, 7.55), p=0.0002. After controlling for the child's genotype, the OR for the association between *DHFR* and having a child with retinoblastoma was 2.81, 95%CI (1.32, 5.99); p=0.0075).

The distribution of the homozygous genotype for *MTHFR*677C>T did not differ between case and control mothers (p=0.68).

Because we were interested in folate intake during initial retinal development, we included questions regarding folic acid supplement intake during their first trimester of pregnancy and during the three months prior to pregnancy. Case children were diagnosed at a median age of 24 months; thus, mothers were interviewed approximately three years after their pregnancy began. Other work done by Mejia et al. has shown that reliable information on vitamin intake can be assessed from Mexican mothers four to six years post-partum.[15] Only four mothers reported use of any supplements during the three months prior to their pregnancy. Therefore, we could not evaluate the impact of supplement use during this period. However, among the 169 mothers who were interviewed about use of vitamin supplements during their first trimester, 46.9% (n=81) took folic acid supplements in the first trimester. The proportion of folic acid supplement use in the first trimester among the mothers of unilateral cases was 50%, close to 46.4% among the mothers of controls, p=0.76 (Table 1), which is similar to the proportion (53%) reported for pregnant women surveyed as part of the 1999 Mexican national nutrition survey in Mexico.[16] Use of folic acid supplements in the first trimester was unrelated to *DHFR*19bpdel or maternal age at delivery, but those who used supplements had more schooling than those who did not, p=0.006 (Table 2).

Among those who reported taking folic acid containing supplements during the first trimester, women with the *DHFR*19bpdel/del genotype were more likely to have a child with unilateral retinoblastoma, OR= 3.58, 95%CI=(1.11, 11.55), p=0.03; while the association was insignificant among those not taking folic acid supplements in the first trimester, even after adjusting for the child's genotype (Table 3). However, there was no significant difference in the odds ratios for the DHFR-retinoblastoma risk association between mothers taking or not taking supplements (p=0.18). Odds of having a child with retinoblastoma were not associated with homozygosity of *MTHFR* 677 C>T, regardless of folic acid supplement use during pregnancy (Table 3).

Mothers of children born after 2001 were more likely to take folic acid supplements than those born before, which may be secondary to the greater attention given to promoting folic acid intake coinciding with the implementation of folic acid flour fortification. Adjusting for before and after full implementation did not affect the strength or direction of our results.

In summary, we have found that maternal homozygosity for *DHFR*19bpdel is associated with an increased risk of having a child with unilateral retinoblastoma. The *DHFR* associated risk is significantly elevated only among those who reported taking folic acid containing vitamin supplements during pregnancy.

Discussion

Results of this case control study suggest that risk for development of unilateral retinoblastoma is associated with maternal homozygosity for the 19bp deletion polymorphism in the intron 1a of the gene for *DHFR*.[14] When stratified by folic acid supplement use during the first trimester of pregnancy, *DHFR* associated risk for retinoblastoma is elevated only in those mothers taking supplements containing synthetic folic acid.

Folic acid, the synthetic form of naturally occurring folate, is found in fortified foods and vitamin supplements. Folic acid must be reduced to tetrahydrofolate (THF) by *DHFR* in order to enter the cellular metabolic pathway. It has been shown in humans that the capacity of *DHFR* to convert ingested folic acid into reduced folate is limited.[18] Ingestion of 200

micrograms (mcg) of folic acid in a single dose results in the appearance of circulating unmetabolized folic acid (cUMFA).[19]

The effect of the *DHFR*19bpdel polymorphism on the enzyme or its activity has not been characterized. Recent studies of the Framingham Offspring Cohort have shown that homozygotes for *DHFR*19bpdel have normal concentrations of plasma and red blood cell (RBC) folate, as well as plasma homocysteine, indicating that this polymorphism does not affect regeneration of THF from dihydrofolate (DHF).[14] But, when daily folic acid intake was below 250 mcg, homozygosity for the *DHFR*19bpdel was associated with significantly lower RBC folate compared to those without the polymorphism. In addition, among individuals whose daily folic acid intake exceeded 500 mcg, the prevalence of high concentrations of cUMFA was two-fold higher in *DHFR*19bpdel homozygotes than in those without the polymorphism.[14] Together, these Framingham data strongly imply that homozygosity for the *DHFR*19bpdel results in a diminished capacity of the body in converting ingested folic acid into reduced folate. This threshold level of intake is well below both the tolerated UL and the Dietary Reference Intake (DRI) for pregnant women (600 mcg daily).[13] Women who are homozygous for *DHFR*19bpdel have an increased risk for breast cancer, but this risk is increased only among women taking vitamin supplements, suggesting that the effect of the polymorphism on cancer incidence be apparent only when intake surpasses a threshold.[10]

The possibility that excessive intake of folic acid or presence of cUMFA is associated with adverse effects has been documented both in humans and in animal models. Presence of cUMFA is inversely related to natural killer cell cytotoxicity in older women.[20] In pregnant mice extreme folic acid intake (20 times the DRI) has been associated with embryonic delay and growth retardation[21] and with increased incidence of NTD.[22] Undoubtedly, the increased intake of folic acid through both food fortification and supplements has been beneficial particularly for preventing NTD, however, potential adverse effects even if involving rarer diseases need to be identified in order to inform future policies.

Because cUMFA results from the interaction between *DHFR*19bpdel and high folic acid intake, it is plausible that the increased retinoblastoma risk may be associated with cUMFA. Mothers who are homozygous for *DHFR*19bpdel and consume higher amounts of folic acid during pregnancy might have resulting high levels of cUMFA during a critical period of retinal development, potentially increasing tumorigenic risk. Increased levels of cUMFA have been associated with decreased Natural Killer (NK) cell cytotoxicity in older women.[20] Maternal NK cells are particularly active during the first trimester of pregnancy, when they contribute to building decidual tissue and formation of new vessels as well as to cytolytic immune surveillance.[23,24] Consequently, the developing fetus might be particularly susceptible to factors decreasing NK function.

Risk of retinoblastoma may be associated with less effective use of ingested folic acid during a key period in formation of these retinal tumors. There is contradictory evidence suggesting that it acts as both a protectant and a promoter of cancer. Both insufficient folate intake as well as increased folic acid intake has been associated with development of carcinomas in adults and with promotion of neoplastic lesions and genetic damage in rodent models.[25–28] Vitamin supplementation with folic acid has been associated with increased risk of breast cancer.[10,23] Prospective studies have shown that ingestion of folic acid at the tolerated upper limit (UL) of 1,000 mcg may contribute to tumor progression in colonic adenomas and to increased incidence of breast[29], prostate[30] and lung[31] cancer.

Flour fortification with folic acid is mandatory in approximately 42 countries, though levels of fortification vary and remain controversial.[32] Fortification has led to higher than

predicted ingestion of folic acid, in part, because consumption patterns for flour-containing foods have changed and are higher than envisioned when fortification was initially planned.[33,34] In Mexico, since 2001, wheat flour is fortified with 2,000 mcg folic acid per kilogram flour.[35,36] Supplements routinely contain high amounts of folic acid and even fortified foods often contain more than the required amount.[33] In the1999 pre-fortification Mexican national nutrition survey (ENSANUT), among non-pregnant women of child bearing age, 12% were consuming a daily dose above the UL.[13,16] Data from the 2006 ENSANUT demonstrated that poor Mexican women consume excessive levels of carbohydrates.[37] Some of these carbohydrates would likely originate from foods containing fortified flour. Together these data suggest that consumption at levels present in the diet of some Mexican women may plausibly be above levels associated with increased cancer risk.[29–31] Mothers of children with unilateral retinoblastoma may be consuming elevated quantities of folic acid, potentially through dietary consumption of foods made with folic acid fortified flour.

Our prior study in Mexico did not have access to parental bio-specimens.[8] Both that study and the one by Bunin in the US were performed before flour fortification with folic acid.[4] All the data suggesting geographical variations in incidence of retinoblastoma were compiled prior to widespread adoption of fortification.[1,2] Since fortification has begun, no new worldwide incidence data on retinoblastoma have been published though efforts are currently underway to collect such data and estimates are expected to be available in 2013.[38]

An inherent limitation of our study is that it is a retrospective study, a necessity because of the rarity of retinoblastoma. Because of the case-control design, there may be recall bias in the maternal recollection of prenatal vitamin intake; however, this recall bias would not be expected to vary with genotype. As shown in Table 1, our case and control mothers did not differ in the proportion consuming vitamins containing synthetic folic acid. One additional limitation is that we are unable to fully account for dietary intake because the nutrient database currently available in Mexico was updated in 2003, and reflects fortification of some industrialized foods, but does not reflect the synthetic folic acid content of non-industrialized products containing fortified wheat flour. Because these non-industrialized foods are consumed frequently by our population, accurate calculation of folic acid intake is not possible without accounting for the content of these foods. We are consequently unable to fully examine a threshold effect of synthetic folic acid intake with *DHFR*. We limited our examination of DHFR to the 19bpdel polymorphism in intron 1A because of its functional significance in the Framingham Offspring Cohort[14], its vitamin-specific carcinogenic effect in breast cancer[10] and its association with NTDs[12]. However, if there is linkage disequilibrium between DHFR19bpdel in intron1A and other DHFR polymorphisms, this might partially account for our findings. We were unable to fully examine the role of the child DHFR genotype or interactions between child and maternal DHFR genotypes because of our current sample size. Future plans to validate our findings include an expansion of our study population and a determination of dietary folic acid content in non-industrialized fortified foods, as well as collaboration with other groups to examine our hypotheses in other populations and expansion to include additional MTHFR and DHFR polymorphisms.

In summary, our study is the first to suggest that maternal metabolism of folic acid may affect the risk of a child developing retinoblastoma, and that maternal genotype for DHFR predicts risk for unilateral retinoblastoma. Our data is consistent with the hypotheses arising from the US and European trials showing increased incidence of cancer among adults taking folic acid supplements.

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References

1. Parkin, DM.; Kramarova, E.; Draper, GJ.; Masuyer, E.; Michaelis, J.; Neglia, J., et al. International incidence of childhood cancer. Lyon, France: IARC on Cancer Scientific Publications; 1998.
2. Stiller CA, Parkin DM. Geographic and ethnic variations in the incidence of childhood cancer. Br Med Bull. 1996; 52:682–703. [PubMed: 9039726]
3. Howe HL, Wu X, Ries LA, Cokkinides V, Ahmed F, Jemal A, et al. Annual report to the nation on the status of cancer, 1975–2003, featuring cancer among U.S. Hispanic/Latino populations. Cancer. 2006; 107:1711–42. [PubMed: 16958083]
4. Bunin GR, Meadows AT, Emanuel BS, Buckley JD, Woods WG, Hammond GD. Pre-and postconception factors associated with sporadic heritable and nonheritable retinoblastoma. Cancer Res. 1989; 49:5730–5. [PubMed: 2790788]
5. Rushlow D, Piovesan B, Zhang K, Prigoda-Lee NL, Marchong MN, Clark RD, et al. Detection of mosaic RB1 mutations in families with retinoblastoma. Hum Mutat. 2009; 30:842–51. [PubMed: 19280657]
6. Ohtani-Fujita N, Dryja TP, Rapaport JM, Fujita T, Matsumura S, Ozasa K, et al. Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. Cancer Genet Cytogenet. 1997; 98:43–9. [PubMed: 9309117]
7. Kanber D, Berulava T, Ammerpohl O, Mitter D, Richter J, Siebert R, et al. The human retinoblastoma gene is imprinted. PLoS Genet. 2009; 5:e1000790. [PubMed: 20041224]
8. Orjuela MA, Titievsky L, Liu X, Ramirez-Ortiz M, Ponce-Castaneda V, Lecona E, et al. Fruit and vegetable intake during pregnancy and risk for development of sporadic retinoblastoma. Cancer Epidemiol Biomarkers Prev. 2005; 14:1433–40. [PubMed: 15941952]
9. van-der-Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, van-den-Heuvel LP, et al. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet. 1995; 346:1070–1. [PubMed: 7564788]
10. Xu X, Gammon MD, Wetmur JG, Rao M, Gaudet MM, Teitelbaum SL, et al. A functional 19bp deletion polymorphism of dihydrofolate reductase and risk of breast cancer in multivitamin users. Am J Clin Nutr. 2007; 85:1098–102. [PubMed: 17413111]
11. Abuli A, Fernandez-Rozadilla C, Alonso-Espinaco V, Munoz J, Gonzalo V, Bessa X, et al. Case-control study for colorectal cancer genetic susceptibility in EPICOLON: previously identified variants and mucins. BMC Cancer. 2011; 11:339. [PubMed: 21819567]
12. Johnson WG, Stenroos ES, Spychala JR, Chatkupt S, Ming SX, Buyske S. New 19bp deletion polymorphism in intron-1 of DHFR: a risk factor for spina bifida acting in mothers during pregnancy? Am J Med Genet A. 2004; 124A:339–45. [PubMed: 14735580]
13. Food&Nutrition Board IOM. Dietary reference intakes for thiamin, riboflavin, niacin, vitaminB6, folate, vitaminB12, pantothenic acid, biotin, and choline. Washington, DC: National Academies Press; 1998.
14. Kalmbach RD, Choumenkovitch SF, Troen AP, Jacques PF, D'Agostino R, Selhub J. A 19-base pair deletion polymorphism in dihydrofolate reductase is associated with increased unmetabolized folic acid in plasma and decreased red blood cell folate. J Nutr. 2008; 138:2323–7. [PubMed: 19022952]
15. Mejía-Rodríguez F, Orjuela M, Amaya D, García-Guerra A, Neufeld LM. Validity of a semi-quantitative food frequency questionnaire to estimate nutrient intake during past pregnancies. Maternal and Child Health Journal. 2011

16. Mejia-Rodriguez F, Sotres-Alvarez D, Neufeld LM, Garcia-Guerra A, Hotz C. Use of nutritional supplements among Mexican women and the estimated impact on dietary intakes below the EAR and above the UL. J Am Coll Nutr. 2007; 26:16–23. [PubMed: 17353579]

17. deBree A, Verschuren WM, Bjorke-Monsen AL, van-der-Put NM, Heil SG, Trijbels FJ, et al. Effect of the methylenetetrahydrofolate reductase 677C-->T mutation on the relations among folate intake and plasma folate and homocysteine concentrations in a general population sample. Am J Clin Nutr. 2003; 77:687–93. [PubMed: 12600862]

18. 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. [PubMed: 19706381]

19. 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. [PubMed: 9174474]

20. 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. [PubMed: 16365081]

21. Pickell L, Brown K, Li D, Wang XL, Deng L, Wu Q, et al. High intake of folic acid disrupts embryonic development in mice. Birth Defects Res A Clin Mol Teratol. 2011; 91:8–19. [PubMed: 21254354]

22. Achon M, Reyes L, Alonso-Aperte E, Ubeda N, Varela-Moreiras G. High dietary folate supplementation affects gestational development and dietary protein utilization in rats. J Nutr. 1999; 129:1204–8. [PubMed: 10356088]

23. Vacca P, Moretta L, Moretta A, Mingari MC. Origin, phenotype and function of human natural killer cells in pregnancy. Trends Immunol. 2011; 32:517–23. [PubMed: 21889405]

24. LeBouteiller P, Siewiera J, Casart Y, Aguerre-Girr M, ElCosta H, Berrebi A, et al. The human decidual NK-cell response to virus infection: what can we learn from circulating NK lymphocytes? J Reprod Immunol. 2011; 88:170–5. [PubMed: 21277025]

25. 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. [PubMed: 17551129]

26. Kim YI. Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. Environ Mol Mutagen. 2004; 44:10–25. [PubMed: 15199543]

27. Ibiebele TI, Hughes MC, Pandeya N, Zhao Z, Montgomery G, Hayward N, et al. High intake of folate from food sources is associated with reduced risk of esophageal cancer in an Australian population. J Nutr. 2011; 141:274–83. [PubMed: 21178085]

28. Hirsch S, Sanchez H, Albala C, de-la-Maza MP, Barrera G, Leiva L, et al. Colon cancer in Chile before and after the start of the flour fortification program with folic acid. Eur J Gastroenterol Hepatol. 2009; 21:436–9. [PubMed: 19190501]

29. Stolzenberg-Solomon RZ, Chang SC, Leitzmann MF, Johnson KA, Johnson C, Buys SS, et al. Folate intake, alcohol use, and postmenopausal breast cancer risk in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Am J Clin Nutr. 2006; 83:895–904. [PubMed: 16600944]

30. Figueiredo JC, Grau MV, Haile RW, Sandler RS, Summers RW, Bresalier RS, et al. Folic acid and risk of prostate cancer: results from a randomized clinical trial. J Natl Cancer Inst. 2009; 101:432–5. [PubMed: 19276452]

31. Ebbing M, Bonaa KH, Nygard O, Arnesen E, Ueland PM, Nordrehaug JE, et al. Cancer incidence and mortality after treatment with folic acid and vitamin B12. Jama. 2009; 302:2119–26. [PubMed: 19920236]

32. Bentley TG, Weinstein MC, Willett WC, Kuntz KM. A cost-effectiveness analysis of folic acid fortification policy in the United States. Public Health Nutr. 2009; 12:455–67. [PubMed: 18590584]

33. Shakur YA, Rogenstein C, Hartman-Craven B, Tarasuk V, O'Connor DL. How much folate is in Canadian fortified products 10 years after mandated fortification? Can J Public Health. 2009; 100:281–4. [PubMed: 19722341]

34. Hoyo C, Murtha AP, Schildkraut JM, Forman MR, Calingaert B, Demark-Wahnefried W, et al. Folic acid supplementation before and during pregnancy in the Newborn Epigenetics STudy (NEST). BMC Public Health. 2011; 11:46. [PubMed: 21255390]

35. NOM-147. Disposiciones especificaciones sanitarias nutrimentales. Vol. 1996. Mexico: Comisión federal para la protección contra riesgos sanitarios; Aug 5. 1997 Norma Oficial Mexicana: NOM-147-SSA1-1996. Cereales y sus productos. Harinas de cereales.

36. Bourges, H.; Casanueva, E.; Rosado, JL. Recomendaciones Ingestion Nutrimentos. Mexico: Editorial Médica Panamericana; 2005.

37. Barquera S, Hernandez-Barrera L, Campos-Nonato I, Espinosa J, Flores M, Barriguete J, et al. Energy and nutrient consumption in adults: analysis of the Mexican National Health and Nutrition Survey 2006. Salud Publica Mex. 2009; 51 (Suppl 4):S562–73. [PubMed: 20464232]

38. IARC-WHO. International Incidence of Childhood Cancer. Vol. 3. Lyon, France: IARC-WHO; 2012. [cited 2012]; Available from: http://iicc.iarc.fr/about/index.php

Table 1

Comparison of frequency of genetic polymorphisms, demographic characteristics and vitamin supplement intake between mothers of controls and unilateral cases

	Control (n=97)	Unilateral case (n=103)
	%(n affected/n total informative)	%(n affected/n total informative)
DHFR19bpdel/del *	14.58 (14/96)	39.22 (40/102)
MHTFR677TT	26.04 (25/96)	29.70 (30/101)
Smoked during pregnancy	9.37 (9/96)	3.06 (3/98)
Folic acid supplement use during first trimester	46.39 (45/97)	50.00 (36/72)
	Median (range)	**Median (range)**
Age at delivery (years)	24.4 (14.4, 39.5)	25.2 (13.17, 38.66)
Years of schooling	9 (0,17)	9 (0,17)
Child's weight at delivery (kg)	3.3 (1.4, 4.8)	3.3 (2.1, 5.0)

* p = 0.0002, case control difference detected by Chi-square test.

Table 2

Comparison of genotypes and demographic characteristics of mothers by use of supplements containing synthetic folic acid in the 1st trimester

	Used folic acid containing supplements in the first trimester	Did not use folic acid containing supplements in the first trimester
	n = 81	n = 88
Characteristic	**Proportion affected % (n affected/n total informative)**	**Proportion affected % (n affected/n total informative)**
Homozygous for *DHFR*19bp del	20.25 (16/79)	20.45 (18/88)
Homozygous for *MTHFR*677C>T	31.65 (25/79)	21.84 (19/87)
Had child with unilateral retinoblastoma	44.44 (36/81)	41.91 (36/88)
	Median (range)	**Median (range)**
Age at delivery (years)	25.6 (14.4, 39.5)	24.5 (14.9, 39.5)
Years of schooling *	9 (4,17)	9 (0,17)

* p= 0.006, group mean difference detected by t-test.

Table 3

Odds ratios (OR) and 95%CI for gene-disease association by first trimester use of supplements containing synthetic folic acid

Genotype	All cases *vs* controls	Folic acid supplement intake in 1st trimester (n=79)	No Folic acid supplement intake in 1st trimester (n=88)	Difference between ORs
	OR (95%CI)	OR (95%CI)	OR (95%CI)	p-value
Maternal Homozygosity for *DHFR*19bpdel [+]	3.78 (1.89,7.55) [**]	3.58 (1.11,11.55) [*]	1.59(0.56,4.51)	0.31
Maternal Homozygosity for *DHFR*19bpdel [+†]	2.81(1.32,5.99) [**]	3.31 (0.95,11.50) [***]	0.98 (0.27,3.53)	0.18
Maternal Homozygosity for *MTHFR*677C>T [+]	1.20 (0.64, 2.24)	0.77 (0.30, 2.03)	1.45 (0.52,4.05)	0.38

[+] compared with heterozygotes, or homozygous non-variant allele;

[†] adjusted for Child's DHFR genotype;

[*] p=0.03;

[**] p<0.01;

[***] p<0.06.