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INVITED MEDICAL REVIEW

Folic acid and orofacial clefts: a review of the evidence

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Orofacial clefts are common and burdensome birth defects with a complex genetic and environmental etiology. The contribution of nutritional factors and supplements to the etiology of orofacial clefts has long been theorized and studied. Multiple studies have evaluated the role of folic acid in the occurrence and recurrence of orofacial clefts, using observational and non-randomized interventional designs. While preventive effects of folic acid on orofacial clefts are commonly reported, the evidence remains generally inconsistent. This paper reviews the findings of the main studies of the effects of folic acid on orofacial clefts, summarizes study limitations, and discusses research needs with a focus on studying the effects of high dosage folic acid on the recurrence of oral clefts using a randomized clinical trial design. The role of folic acid in the prevention of neural tube defects is also briefly summarized and discussed as a reference model for orofacial clefts.
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Folic acid is a vitamin that has been shown to prevent the occurrence and recurrence of neural tube defects (NTDs) but findings for its effects on other common birth defects including orofacial clefts (OFCs) remain generally inconsistent. Multiple studies of various designs (primarily observational case-control studies) have evaluated the effects of folic acid and multivitamin use on OFCs with an overall suggestive evidence for a potential preventive role of folic acid, but the evidence remains largely inconclusive. In this article, we summarize the results of the previous main studies on the effects of folic acid on OFCs and also briefly summarize the results for NTDs. We also discuss the needs for future research in this area.

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Orofacial clefts

Orofacial clefts of the lip and palate are common birth defects of complex genetic and environmental etiology. Depending on geographic ancestry, OFCs affect about 1 in 500 (Asian or Amerindian ancestry) to 2500 births (African ancestry) (Mossey and Little, 2002). OFCs are one of the most prevalent birth defects in the United States, with about 20 400 children with oral clefts born between 1999 and 2001 (Centers for Disease Control and Prevention, 2006). Low socioeconomic status is also reported to increase the risk of OFCs (Murray *et al*, 1997; Clark *et al*, 2003; Durning *et al*, 2007).

Orofacial clefts are thought to result from a complex interplay of genetic and environmental factors. In humans, a finely choreographed cascade of gene expression, cell migration, cell transformation and apoptosis between 14 and 60 days postconception creates the soft and hard tissues of the face from the originating oropharyngeal membrane. By 48 days the upper lip is continuous and by 60 days palatal shelf fusion completes facial embryogenesis (Sperber, 2002). Disruption of any of the tightly regulated processes occurring in this time frame by environmental and/or genetic abnormalities may then predispose to cleft lip and/or palate. A few specific genetic contributors to cleft etiology have begun to be identified including variants in *IRF6* (Zucchero *et al*, 2004; Rahimov *et al*, 2008; *MSX1* (Lidral *et al*, 1997, 1998; Jezewski *et al*, 2003), *fibroblast growth factor* signaling pathway genes (Riley *et al*, 2007), *BMP4* (Suzuki *et al*, 2009) and a locus on 8q (Birnbaum *et al*, 2009) but the majority remain unexplained (see reviews in Lidral and Moreno, 2005 and Jugessur and Murray, 2005). Gene-environment interactions also contribute to OFCs with strong evidence for interaction between maternal smoking fetal variants of *GSTM1* and *GSTT1* (Lammer *et al*, 2005; Shi *et al*, 2007).

Orofacial clefts include cleft lip with or without the palate (CL/P) as well as cleft palate only (CP). CL/P and CP are sometimes differentiated in studies because of differences in embryologic origin and recurrence risks, but they are also combined in many studies due to potential common genetic and epidemiologic risks (van den Boogaard *et al*, 2000; Dode *et al*, 2003; Jezewski

et al, 2003; Zucchero *et al*, 2004; Jugessur and Murray, 2005). Recently the role for subphenotypes in clefts has also provided new insights into etiologies (Rogers *et al*, 2008; Suzuki *et al*, 2009). OFCs occur in both isolated and non-isolated forms. Isolated or non-syndromic forms involve no other major structural or developmental impairments and represent the majority of cases with CL/P (Jones, 1988; Marazita, 2002). The non-isolated or syndromic forms with CL/P occur because of more than 450 causes including chromosomal anomalies, single gene conditions, environmental exposures, and syndromes of unknown cause [OMIM (Online Mendelian Inheritance in Man), 2009]. OFCs impose significant health, psychosocial, and economic burdens, both at the individual and family levels (Berk and Marazita, 2002).

Folic acid and neural tube defects

There is strong evidence from clinical trials for a large preventive effect of folic acid on both recurrence and occurrence of NTDs. The strongest evidence for a preventive effect of high-dose folic acid supplementation on recurrence of NTDs comes from the Medical Research Council (MRC) double-blinded randomized study, which randomized women with a previous child with NTD into groups of 4 mg folic acid, vitamins other than folic acid, vitamins with 4 mg folic acid, and placebo, taken daily at preconception and throughout the first trimester of pregnancy (MRC Vitamin Study Research Group., 1991). The study reported a significant reduction of about 72% in the rate of NTDs in the groups supplemented with folic acid as compared with the other study groups. No significant decreases in NTD recurrence were observed in the group receiving vitamins without folic acid, indicating that preventive effects were attributable to the folic acid component (MRC Vitamin Study Research Group., 1991).

Multivitamin supplementation with a 0.8 mg folic acid at preconception and through at least 2 months postconception was also shown to lower the risk of first occurrence of NTDs by up to 100% in a randomized clinical trial in Hungary in a sample of women with no history of NTDs among their children (Czeizel and Dudas, 1992). This same study showed no decrease in the occurrence of OFCs though the overall rate of congenital anomalies was reported to have decreased with the multivitamin supplementation (Czeizel and Dudas, 1992). In a confirmatory study applying a two-cohort controlled design in Hungary with the interventional group receiving the same folic acid-containing multivitamin as Czeizel and Dudas (1992) study, Czeizel *et al* (2004) found a significant decrease in NTD occurrence by up to 89% and in cardiovascular defects (40%), but no decrease in OFCs.

Berry *et al* (1999) reported that the use of 0.4 mg folic acid before conception and in the first trimester of pregnancy reduced the occurrence of NTDs in China by up to 79% in a sample from the northern area with higher baseline rates of NTDs as compared with 16% in a sample from the Southern region sample with lower baseline rates. Several observational studies have also identified preventive effects of folic acid on NTDs [see a recent review by Wolff *et al*, (2009)].

The results of the studies described above strongly indicate that the preventive effects on recurrence and occurrence of NTDs are attributable to the folic acid component rather than the other vitamins, though interactive effects have not been thoroughly evaluated. The NTD research provides a model for developing clinical trials aimed at assessing the effects of folic acid on recurrence and occurrence of OFCs, which is of direct relevance for clinical practice. A connection between NTDs and OFCs can be inferred by the similarity of time of occurrence during embryogenesis, their status as defects involving the midline of the embryo, their near-identical population genetic characteristics (variable by geographic origin but with near-identical recurrence risks and very similar birth-prevalence rates overall), evidence of similar gene and environment contributions and the failure to identify major genetic factors for either.

The mechanisms by which folic acid might prevent NTDs or other birth defects remain unexplained. It might be secondary to the need to overcome pharmacogenetic deficiencies in women who require higher baseline intakes to reach therapeutic levels. One proposed mechanism relates to antibodies to the folic acid receptor (Rothenberg *et al*, 2004; Cabrera *et al*, 2008). The role of antibodies to the folate receptor is yet to be confirmed but could explain why some women respond to high doses of folic acid as this may be required to titer the effects of antibody bound to receptors. The pharmacologic rescue by high-dose folic acid has been reported in a rat model where folate receptor antibodies induced intracellular folate deficiency associated with birth defects (da Costa *et al*, 2003).

Folic acid and OFCs

The role of vitamins and especially folic has been of special interest in OFCs for over 20 years. We summarize below the main studies and designs that evaluated the role of folic acid in OFCs.

Observational studies of folic acid and OFCs

Some observational studies have reported a preventive effect of folic acid-containing supplements (mostly multivitamins) on OFCs (Botto *et al*, 2004). However, the evidence is mixed, likely caused by sample selection biases as well as differences in samples sizes/statistical power, populations, analytical models (including accounting for confounders), and folic acid measures. Several studies analyzed small samples that may have been underpowered to detect any significant potential effects of folic acid use on OFCs. However, these studies have provided important insights into the potential preventive effects of folic acid on OFCs. Below, we review the main observational studies in this area.

Using data from the Hungarian Congenital Anomaly Registry, Czeizel (2004) reported that use of high doses of folic acid (average of 6 mg) in the first month of pregnancy reduced CP risk by 50% but not CL/P risk.

Shaw *et al* (1995) reported a 50% decrease in CL/P with using folic acid-containing multivitamins in samples from California but found a smaller and insignificant effect for CP. van Rooij *et al* (2004) reported a similar reduction in CL/P risk with using folic acid supplements (mostly containing only folic acid) in a sample from the Netherlands. This study also reported a 74% reduction in CL/P risk with using the folic acid supplements in addition to a high folate diet (van Rooij *et al*, 2004). Wilcox *et al* (2007) reported a 39% decrease in CL/P risk with using folic acid supplements adjusting for the use of multivitamins and a 64% decrease when women used multivitamin/folic supplements with high folate diets. No preventive effects were observed for CP (Wilcox *et al*, 2007). Another recent case-control study of a smaller sample of affected births with OFCs and controls (as compared with the previous study) from Scotland and England found no effects of supplement and dietary folate on OFCs (including CL/P and CP; Little *et al*, 2008). Some studies found suggestive yet statistically insignificant effects of folic acid on OFCs (Bille *et al*, 2007), while others found no effects of folic acid (Shaw *et al*, 2006). Other studies of multivitamin use without specification of folic acid content have also reported a reduction in risks of CP (by 60%; Werler *et al*, 1999), CP and CL/P (by 40%; Loffredo *et al*, 2001), and CL/P (50%; Itikala *et al*, 2001). One observational study (Hayes *et al*, 1996) has reported an increased but statistically insignificant risk for CL/P with folic acid-containing supplements, yet their control group included children with birth defects (other than midline defects), which might reflect a potential severity reduction effect of folic acid for those anomalies.

With the relatively frequent number and mixed evidence of observational studies, meta-analyses of these studies may be helpful for estimating the average effects of folic acid across several studies and samples. In a metal-analysis including the most recent observational studies, Johnson and Little (2008) estimated a reduction of about 18% in the risk of CL/P with the use of folic acid-containing supplements, but no significant reduction in CP. This meta-analysis also found a reduction of about 23% in CL/P risk with using multivitamins (Johnson and Little, 2008). However, it is impossible to identify the effect of folic acid from the effect of multivitamin use in these studies given that most multivitamins may have contained folic acid. In an earlier meta-analysis, Badovinac *et al* (2007) estimated a reduction of approximately 28% and 20% in the risks of CL/P and CP respectively while using folic acid-containing supplements and/or multivitamins.

In summary, while there have been several studies that suggested a beneficial role of folic acid in decreasing OFC risk, results are often mixed in terms of the estimated effects of folic acid as well as whether CL/P or CP or both are affected. This is in part because of differences in the studied dose and definition of folic acid supplements (multivitamins, folic acid supplements, or both), measurement and sample selection biases, and statistical models including adjustment for confounders.

Folic acid fortification and oral clefts

A few countries have introduced folic acid fortification of grain and flour given the strong evidence for the preventive effect of folic acid on NTDs. Indeed, this evidence and its subsequent application to populations are considered to be one of the major public health successes in the field of birth defects. Unlike the case for NTDs, there is no converging evidence for significant changes in birth prevalence for oral clefts postfolic acid fortification. In the United States, where folic acid fortification of grain products was mandated on January 1, 1998, three studies reported non-significant reductions in CL/P prevalence by 3% in Texas (Hashmi *et al*, 2005), 5% in 23 states reporting to the National Birth Defects Prevention (Canfield *et al*, 2005), and 14% in Arkansas (Simmons *et al*, 2004), postfortification. Canfield *et al* (2005) reported a significant 12% reduction in CP prevalence. A recent study reported a significant 6% reduction in OFC prevalence based on birth certificate data from 45 states between the period 1990 and 2002 (Yazdy *et al*, 2007).

Ray *et al* (2003) reported a non-significant increase in the prevalence of OFCs after 2 years of fortification of cereal grain products (between 1998 and 2000) in Ontario, Canada. Also, in an evaluation of the effects of fortifying wheat flour with folic acid in Chile starting from 2000, Castilla *et al* (2003) reported no significant changes in prevalence of OFCs, while a significant reduction of 31% in NTDs was shown (López-Camelo *et al*, 2005). In a meta-analysis of fortification studies in the United States and Canada, Johnson and Little (2008) estimated a reduction of approximately 7% and 8% in the prevalence of CL/P and CP respectively.

Longer periods may be required for a more comprehensive evaluation of potential changes in prevalence of OFCs postfortification. However, given the evidence of NTD reduction of up to 50% in similar periods and across multiple populations (e.g. Liu *et al*, 2004; Canfield *et al*, 2005; López-Camelo *et al*, 2005; Williams *et al*, 2005; De Wals *et al*, 2007, 2008), these results suggest that low doses of folic acid may be inadequate to prevent occurrence of OFCs as also suggested by other studies (Czeizel *et al*, 1999; Czeizel, 2004). Further, the studies of changes in prevalence over time suffer from limitations including potential confounding by other simultaneously changing relevant factors and the lack of well-matched control groups.

Interventional studies of folic acid and oral cleft recurrence

Only few interventional studies have been conducted to study the effect of folic acid supplementation on recurrence of oral clefts in mothers with a child with OFCs or who are themselves affected. The decrease in OFCs recurrence among the folic acid groups reported in these studies, independent of statistical significance, ranges from about 24% to 100%. Conway (1958) reported no recurrent cleft cases among 59 births to mothers with history of OFCs in previous births who

received a multivitamin that included 0.5 mg of folic acid. The recurrence rate in a group of 78 births to mothers who did not receive the supplement was 5.1%. Peer *et al* (1964) reported a 53% reduction in the recurrence of OFCs in a group of 176 women who received a multivitamin in addition to 5 mg folic acid and 10 mg vitamin B6 during the first pregnancy trimester, as compared with a control group of 418 mothers ($P = 0.1$). In an extended study of Peer *et al* (1964) with more supplemented women, Briggs (1976) reported a 35% reduction in recurrence of OFCs ($P = 0.2$), but a 65% reduction in CL/P recurrence ($P = 0.06$). Tolarova (1982) reported an 84% reduction in recurrence of CL/P in a group of 80 women who received a multivitamin and 10 mg of folic acid during 3 months before and after pregnancy ($P = 0.02$), as compared with a control group of 202 women. Using data on a larger sample that included women with CL/P (40% of intervened sample) and mothers of a child with CL/P, and the same intervention as Tolarova (1982), Tolarova and Harris (1995) reported a 66% reduction in recurrence of CL/P ($P = 0.03$). Johnson and Little (2008) estimated a significant 67% reduction in CL/P recurrence based on these studies. These estimations are primarily descriptive given the array of interventions and populations used, but from an exploratory perspective, may be helpful for gauging expected treatment effects of folic acid to form hypotheses in clinical trials. The results of these studies are suggestive of potential preventive effects of high-dose folic acid on cleft recurrence.

Interventional studies of folic acid and oral cleft occurrence

The Hungarian randomized and cohort controlled trials of the multivitamin intervention (Czeizel and Dudas, 1992; Czeizel *et al*, 2004) support the notion of lack of preventive effects of low doses of folic acid on occurrence of oral clefts (Czeizel *et al*, 1999; Czeizel, 2004). These trials found no statistically significant effects on CL/P and CP (Czeizel, 2004).

Other studies

Other studies of micronutrient and folate exposures have also suggested associations with oral clefts in humans. Rouget *et al* (2005) reported a reduction in OFCs risk with an adequate folate diet (around 0.35 gm daily) in a French sample (Rouget *et al*, 2005). van Rooij *et al* (2003a) reported low maternal postpregnancy B12 levels and low infant serum folate among infants affected by OFCs.

Hernandez-Diaz *et al* (2000) reported that exposure to folic acid antagonists doubled the risks of OFCs. Animal studies also provide support for anti-teratogenic effects of folic acid supplementation and dietary folate on OFCs including studies in mice, rats and dogs (Peer *et al*, 1958; Fu *et al*, 1996; Elwood and Colquhoun, 1997; Paros and Beck, 1999; Bienengraber *et al*, 2001; Burgoon *et al*, 2002; Malek *et al*, 2003, 2004; Reynolds *et al*, 2003). These studies also provide suggestive results for a potential role of folic acid and possibly other micronutrients in OFCs etiology/prevention.

Folate gene interaction studies

Interactions between vitamin use and the folate metabolic pathway have also been intensively studied. Genes that code for folate metabolizing enzymes, such as Methylene tetrahydrofolate reductase (*MTHFR*), are optimal candidates for gene-folic acid interaction studies. Specific alleles in these genes, such as the T677C of *MTHFR*, may modify the effects of folic acid supplementation. Main candidate genes for interaction studies include *MTHFR*, *MTHFD*, *MTR*, *MTRR*, *RFC1*, *GCP2*, *CBS*, *BHMT*, *BHMT2* and *TS*.

There are numerous and often contradictory studies for the *MTHFR* T677C variant (Blanton *et al*, 2002; Jugessur *et al*, 2003; van Rooij *et al*, 2003b; Gaspar *et al*, 2004; Vieira *et al*, 2005; Verkleij-Hagoort *et al*, 2007; Chevrier *et al*, 2007; Boyles *et al*, 2008; Mills *et al*, 2008). Shelnutt *et al* (2003) reported that changes in folate and homocysteine levels with an increase in dietary folate varied by *MTHFR* 677 status. A potential interaction between vitamin use and *RFC1* has also been suggested (Shaw *et al*, 2003; Vieira *et al*, 2005), though no evidence has been observed in a recent study (Pei *et al*, 2006). In sum, there is as yet little consensus among the many studies of interaction between vitamin/folic acid use and genetic factors in the etiology of OFCs.

Limitations of previous studies

The studies described above are suggestive of protective effects of folic acid supplementation on OFCs risks, especially for CL/P, but they all suffered from data and design limitations. The interventional studies for human recurrence have serious limitations, particularly in lacking randomized assignment into treatment and control groups and in using interventions that combine folic acid with other supplements and prevent the identification of the effects of folic acid (Czeizel, 2002). The non-random assignment introduces the biases of self-selection into the treatment, which may confound the study results and introduce differences in outcomes between the treated and untreated groups that are not a result of the treatment. Most of the previous interventional studies also suffered from small sample size and power limitations.

Observational case-control studies also suffer from the problems of non-random self or provider selection of supplement use. The use of multivitamins and folic acid supplements during pregnancy is in part determined by perceived health risks that may also affect the risk for OFCs and other birth outcomes (Wehby *et al*, 2009). Specifically, women with unfavorable pregnancy histories or health problems may use more folic acid supplements but may also have a greater risk for adverse pregnancy outcomes including birth defects such as OFCs. Confounding bias in observational studies also results from the lack of data on health behaviors that may be correlated with both supplement use and OFCs. Other limitations include potential bias in self-reported use of supplements (both recall bias as well as biased report of use based on the pregnancy outcome such as OFC status in studies that measure use after pregnancy, which are the majority of studies in this

area) and the limited data on the folic acid content/dose and duration/intensity of use. Only double-blinded randomized clinical trials (RCTs) with sufficient sample sizes can identify the effects of folic acid.

Clinical trials for recurrence

The NTD model showing preventive effects of high- and low-dose folic acid on recurrence and occurrence respectively, and the suggestive results from interventional studies and observational studies for preventive effects of high doses on recurrence and occurrence of OFCs (summarized above) strongly indicate that large doses of folic acid are best suited for evaluation in RCTs of recurrence. The Oral Cleft Prevention Program (OCPP) was developed over the past 8 years as a double-blinded RCT to estimate the effect of periconceptual supplementation with high-dose folic acid (4 mg per day), which proved effective in preventing recurrences of NTDs (MRC Vitamin Study Research Group., 1991), *vs* low dose (0.4 mg per day) on prevention of CL/P recurrence among women who have CL/P or who have had a child with CL/P. The study was initiated under the sponsorship of the NIDCR, NICHD and the Gates Foundation, and is currently funded by the NIDCR. The OCPP has established an important infrastructure to implement a large-scale RCT to study the role of high-dose folic acid in prevention of CL/P recurrence including developing protocols for treatment provision and outcome measurement, data collection instruments, data management systems and quality-control procedures. The OCPP involves multiple craniofacial clinics in Brazil including the Hospital de Reabilitação de Anomalias Craniofaciais (Centrinho) in Bauru, Hospital de Clinicas de Porto Alegre (HCPA) in Porto Alegre, Hospital Santo Antônio-Centrinho-Obras Sociais Irmã Dulce (OSID) in Salvador, Instituto Materno Infantil de Pernambuco (IMIP) in Recife, Centro de Atendimento Integrado ao Fissurado Lábio Palatal (CAIF) in Curitiba and the Fundação para reabilitação das deformidades crânio-faciais (FUNDEF) in Lajeado. RTI International maintains the Data Center responsibilities for data management and storage.

There is a tremendous need for a double-blinded RCT, such as the OCPP, in order to identify the effects of folic acid on recurrence of OFCs. The double blinded randomized design will separate the effect of the intervention from the confounding effects that are inherent in the interventional and observational designs of previous studies. This design will also address a fundamental challenge in the clinical care of families with one or more individuals with a cleft; that is, how to manage recurrence prevention. Reducing the recurrence of OFCs is expected to have important reductions in the quality of life and economic costs of OFCs at the individual, family and societal levels.

Discussion and conclusions

There is some suggestive evidence for a possible role of folic acid in prevention of OFCs. However, several important questions remain unanswered including confirming whether folic acid prevents OFCs, whether it prevents occurrence or recurrence or both, whether it prevents CL/P, CP or both, and whether low or high doses are effective for prevention. Studies to date have provided mixed results particularly regarding whether low- or high-dose folic acid can prevent primary occurrence. Most case–control observational studies indicating a preventive effect are likely to have evaluated low to moderate doses of (<1 mg) folic acid though many did not measure or report the dose. The evidence is also mixed for the effects on OFC type. The treatment self-selection and confounding biases in addition to sample selection biases and measurement errors are likely to be the primary contributors to differences in results. Given that folic acid has been shown to prevent NTDs across different populations, it is unlikely that any potential real effect of folic acid on prevention of OFCs varies significantly across populations, though this remains to be established in future studies.

Given that low doses of folic acid are known to prevent NTDs, it would not be possible to conduct a randomized clinical trial to study the effects of low dose folic acid on occurrence of OFCs as a placebo control group would be unethical. Conducting an RCT to study the effects of high-dose folic acid on OFCs′ occurrence might not be the first research priority at this stage, given that low doses have not been ruled out to be ineffective for occurrence. Research should be focused on improving the quality of observational studies of OFCs occurrence including the use of larger and more representative samples and better measurement of folic acid use including timing, dose, and intensity of use as well as use of other dietary supplements that should be accounted for. The specification of analytical models can also be improved, including better measurement and accounting for confounders that are related to both folic acid use and OFC risks including nutrition, maternal health risks, family history of birth defects, health behaviors, and demographic and socioeconomic characteristics. Instrumental variable analyses with genetic instruments can also be employed to assess the effects of blood folate levels on OFC while accounting for unobserved factors that determine self-selection into folate supplement use and dietary patterns and that influence OFCs (Wehby *et al*, 2008). Further, meta-analyses of observational studies [such as Badovinac *et al* (2007) and Johnson and Little (2008)] should continue to be conducted to obtain improved estimates of the average effects of folic acid on OFCs′ occurrence.

The strong evidence of the effectiveness of high-dose folic acid in preventing the recurrence of NTDs (MRC Vitamin Study Research Group., 1991) and the preliminary evidence from the non-randomized interventional studies of OFCs recurrence suggest that identifying the effects of high-dose folic acid on OFCs′ recurrence is a high research priority. The effects of high-dose folic acid on OFCs′ recurrence can be identified through a double-blinded RCT design. The relatively low rate of OFCs′ recurrence (about 5%) implies that a large sample of births is needed to have adequate statistical power to identify the effect of folic acid. Specifically, about 1580 total births are required

for a one-sided hypothesis of 50% reduction in a baseline recurrence risk of 5%. Therefore, multi-site international collaborative efforts are needed to successfully conduct an RCT trial for OFCs′ recurrence. As a reference, the MRC Vitamin Study Research Group. (1991) trial for NTD recurrence was conducted over a course of about 8 years in 33 centers in seven countries.

The OCPP is a model RCT for OFCs recurrence that can be extended to multiple sites worldwide. The study has enrolled eligible at-risk women, regardless of whether they are planning a pregnancy or not over their years of participation in the study, in order to estimate the treatment effectiveness (overall population effect). However, this introduces the challenge of enrolling a much larger sample of study subjects than the minimum required number of live births, as only a small percentage of subjects may become pregnant during the study period. An alternative approach, similar to the MRC, involves enrolling at-risk women who are planning on becoming pregnant over the next year or two after enrollment. This design requires fewer resources but will estimate an effect that is specific to women who are planning their pregnancy. This tradeoff represents a real challenge for developing an RCT to study OFCs′ recurrence. However, given that the women who are planning their pregnancy will likely be the primary group who will utilize high-dose folic acid if found to be effective in reducing recurrence, estimating the treatment effects for this group seems appropriate given the large constraints of the alternative sampling approach. The OCPP can be developed in the future as a multi-country multi-site study with a more focused recruitment model that limits enrollment to women who are planning on becoming pregnant in order to obtain the required number of births with reasonable resources and timelines.

Finally, other micronutrients have also been implicated in OFCs though the evidence remains weaker than that for folic acid. B1 and B6 deficiencies were associated with an increased risk of OFCs (Krapels *et al*, 2004a; Munger *et al*, 2004; Tamura *et al*, 2007) as were myo-inositol and zinc (Krapels *et al*, 2004b; Tamura *et al*, 2005), though no effects of Zinc levels on OFCs have recently been reported in a sample from the United States (Munger *et al*, 2009). While these micronutrients could also be considered in other RCTs, the case for folic acid alone is far more compelling.

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References

Badovinac RL, Werler MM, Williams PL, Kelsey KT, Hayes C (2007). Folic acid-containing supplement consumption during pregnancy and risk for oral clefts: a meta-analysis. *Birth Defects Res A Clin Mol Teratol* **79:** 8–15.

Berk NW, Marazita ML (2002). Costs of cleft lip and palate: personal and societal implications. In: Wyszynski DF, ed. *Cleft lip and palate: from origin to treatment*. Oxford University Press: New York, pp. 458–467.

Berry RJ, Li Z, Erickson JD *et al* (1999). Prevention of neural-tube defects with folic acid in China. *N Engl J Med* **341:** 1485–1490.

Bienengraber V, Malek FA, Moritz KU, Fanghanel J, Gundlach KK, Weingartner J (2001). Is it possible to prevent cleft palate by prenatal administration of folic acid? An experimental study. *Cleft Palate Craniofac J* **38:** 393–398.

Bille C, Olsen J, Vach W *et al* (2007). Oral clefts and life style factors – a case-cohort study based on prospective Danish data. *Eur J Epidemiol* **22:** 173–181.

Birnbaum S, Ludwig KU, Reutter H *et al* (2009). Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. *Nat Genet* **41:** 473–477.

Blanton SH, Patel S, Hecht JT, Mulliken JB (2002). MTHFR is not a risk factor in the development of isolated nonsyndromic cleft lip and palate. *Am J Med Genet* **110:** 404–405.

van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK (2000). MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. *Nat Genet* **24:** 342–343.

Botto LD, Olney RS, Erickson JD (2004). Vitamin supplements and the risk for congenital anomalies other than neural tube defects. *Am J Med Genet C Semin Med Genet* **125:** 12–21.

Boyles AL, Wilcox AJ, Taylor JA *et al* (2008). Folate and one-carbon metabolism gene polymorphisms and their associations with oral facial clefts. *Am J Med Genet A* **146A:** 440–449.

Briggs RM (1976). Vitamin supplementation as a possible factor in the incidence of cleft lip/palate deformities in humans. *Clin Plast Surg* **3:** 647–652.

Burgoon JM, Selhub J, Nadeau M, Sadler TW (2002). Investigation of the effects of folate deficiency on embryonic development through the establishment of a folate deficient mouse model. *Teratology* **65:** 219–227.

Cabrera RM, Shaw GM, Ballard JL *et al* (2008). Autoantibodies to folate receptor during pregnancy and neural tube defect risk. *J Reprod Immunol* **79:** 85–92.

Canfield MA, Collins JS, Botto LD *et al* (2005). Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multi-state population-based study. *Birth Defects Res A Clin Mol Teratol* **73:** 679–689.

Castilla EE, Orioli IM, Lopez-Camelo JS, Dutra Mda G, Nazer-Herrera J, Latin American Collaborative Study of Congenital Malformations (ECLAMC) (2003). Preliminary data on changes in neural tube defect prevalence rates after folic acid fortification in South America. *Am J Med Genet A* **123:** 123–128.

Centers for Disease Control and Prevention (CDC) 2006. Improved national prevalence estimates for 18 selected major birth defects – United States, 1999–2001. *MMWR Morb Mortal Wkly Rep* **54:** 1301–1305.

Chevrier C, Perret C, Bahuau M *et al* (2007). Fetal and maternal MTHFR C677T genotype, maternal folate intake and the risk of nonsyndromic oral clefts. *Am J Med Genet A* **143:** 248–257.

Clark JD, Mossey PA, Sharp L, Little J (2003). Socioeconomic status and orofacial clefts in Scotland, 1989 to 1998. *Cleft Palate Craniofac J* **40:** 481–485.

Conway H (1958). Effect of supplemental vitamin therapy on the limitation of incidence of cleft lip and cleft palate in humans. *Plast Reconstr Surg* **22:** 450–453.

da Costa M, Sequeira JM, Rothenberg SP, Weedon J (2003). Antibodies to folate receptors impair embryogenesis and fetal development in the rat. *Birth Defects Res A Clin Mol Teratol* **67:** 837–847.

Czeizel AE. (2002). Prevention of oral clefts through the use of folic acid and the multivitamin supplements: Evidence and gaps. In: Wysynski DF, ed. *Cleft lip and palate: from origin to treatment*. Oxford University press: New York, NY, pp. 443–457.

Czeizel AE (2004). The primary prevention of birth defects: multivitamins or folic acid? *Int J Med Sci*. **1:** 50–61.

Czeizel AE, Dudas I (1992). Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* **327:** 1832–1835.

Czeizel AE, Tímár L, Sárközi A (1999). Dose-dependent effect of folic acid on the prevention of orofacial clefts. *Pediatrics* **104:** e66.

Czeizel AE, Dobo M, Vargha P (2004). Hungarian cohort-controlled trial of periconceptional multivitamin supplementation shows a reduction in certain congenital abnormalities. *Birth Defects Res A Clin Mol Teratol* **70:** 853–861.

De Wals P, Tairou F, Van Allen MI *et al* (2007). Reduction in neural-tube defects after folic acid fortification in Canada. *N Engl J Med* **357:** 135–142.

De Wals P, Tairou F, Van Allen MI *et al* (2008). Spina bifida before and after folic acid fortification in Canada. *Birth Defects Res A Clin Mol Teratol* **82:** 622–626.

Dode C, Levilliers J, Dupont JM *et al* (2003). Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nat Genet* **33:** 463–465.

Durning P, Chestnutt IG, Morgan MZ, Lester NJ (2007). The relationship between orofacial clefts and material deprivation in wales. *Cleft Palate Craniofac J* **44:** 203–207.

Elwood JM, Colquhoun TA (1997). Observations on the prevention of cleft palate in dogs by folic acid and potential relevance to humans. *N Z Vet J* **45:** 254–256.

Fu SS, Sakanashi TM, Rogers JM, Hong KH, Keen CL (1996). Influence of dietary folic acid on the developmental toxicity of methanol and the frequency of chromosomal breakage in the CD-1 mouse. *Reprod Toxicol* **10:** 455–463.

Gaspar DA, Matioli SR, de Cassia Pavanello R *et al* (2004). Maternal MTHFR interacts with the offspring's BCL3 genotypes, but not with TGFA, in increasing risk to nonsyndromic cleft lip with or without cleft palate. *Eur J Hum Genet* **12:** 521–526.

Hashmi SS, Waller DK, Langlois P, Canfield M, Hecht JT (2005). Prevalence of nonsyndromic oral clefts in Texas: 1995–1999. *Am J Med Genet A* **134:** 368–372.

Hayes C, Werler MM, Willett WC, Mitchell AA (1996). Case-control study of periconceptional folic acid supplementation and oral clefts. *Am J Epidemiol* **143:** 1229–1234.

Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA (2000). Folic acid antagonists during pregnancy and the risk of birth defects. *N Engl J Med* **343:** 1608–1614.

Itikala PR, Watkins ML, Mulinare J, Moore CA, Liu Y (2001). Maternal multivitamin use and orofacial clefts in offspring. *Teratology* **63:** 79–86.

Jezewski PA, Vieira AR, Nishimura C *et al* (2003). Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. *J Med Genet* **40:** 399–407.

Johnson CY, Little J (2008). Folate intake, markers of folate status and oral clefts: is the evidence converging? *Int J Epidemiol* **37:** 1041–1058.

Jones MC (1988). Etiology of facial clefts: prospective evaluation of 428 patients. *Cleft Palate J* **25:** 16–20.

Jugessur A, Murray JC (2005). Orofacial clefting: recent insights into a complex trait. *Curr Opin Genet Dev* **15:** 270–280.

Jugessur A, Wilcox AJ, Lie RT *et al* (2003). Exploring the effects of methylenetetrahydrofolate reductase gene variants C677T and A1298C on the risk of orofacial clefts in 261 Norwegian case-parent triads. *Am J Epidemiol* **157:** 1083–1091.

Krapels IP, van Rooij IA, Ocké MC, van Cleef BA, Kuijpers-Jagtman AM, Steegers-Theunissen RP (2004a). Maternal dietary B vitamin intake, other than folate, and the association with orofacial cleft in the offspring. *Eur J Nutr* **43:** 7–14.

Krapels IP, Rooij IA, Wevers RA *et al* (2004b). Myo-inositol, glucose and zinc status as risk factors for non-syndromic cleft lip with or without cleft palate in offspring: a case-control study. *BJOG* **111:** 661–668.

Lammer EJ, Shaw GM, Iovannisci DM, Finnell RH (2005). Maternal smoking, genetic variation of glutathione s-transferases, and risk for orofacial clefts. *Epidemiology* **5:** 698–701.

Lidral AC, Moreno LM (2005). Progress toward discerning the genetics of cleft lip. *Curr Opin Pediatr Dec* **17:** 731–739. Review.

Lidral AC, Murray JC, Buetow KH *et al* (1997). Studies of the candidate genes TGFB2, MSX1, TGFA, and TGFB3 in the etiology of cleft lip and palate in the Philippines. *Cleft Palate J* **34:** 1–6.

Lidral AC, Romitti PA, Basart AM *et al* (1998). Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. *Am J Hum Genet* **63:** 557–568.

Little J, Gilmour M, Mossey PA *et al* (2008). Folate and clefts of the lip and palate – a U.K.-based case-control study: Part I: Dietary and supplemental folate. *Cleft Palate Craniofac J* **45:** 420–427.

Liu S, West R, Randell E *et al* (2004). A comprehensive evaluation of food fortification with folic acid for the primary prevention of neural tube defects. *BMC Pregnancy Childbirth* **4:** 20.

Loffredo LC, Souza JM, Freitas JA, Mossey PA (2001). Oral clefts and vitamin supplementation. *Cleft Palate-Craniofac J* **38:** 76–83.

López-Camelo JS, Orioli IM, da Graça Dutra M *et al* (2005). Reduction of birth prevalence rates of neural tube defects after folic acid fortification in Chile. *Am J Med Genet A* **135:** 120–125.

Malek FA, Moritz KU, Fanghanel J, Bienengraber V (2003). Sex-related differences in procarbazine-induced cleft palate and microgenia and the anti-teratogenic effect of prenatal folic acid supplementation in rats. *Ann Anat* **185:** 465–470.

Malek FA, Moritz KU, Fanghanel J, Bienengraber V (2004). Reduction of procarbazine-induced cleft palates by prenatal folic acid supplementation in rats. *Pathol Res Pract* **200:** 33–40.

Marazita ML (2002). Segregation analysis. In: Wyszynski DF, ed. *Cleft lip and palate: from origin to treatment*. Oxford University Press: New York, NY, pp. 222–233.

Mills JL, Molloy AM, Parle-McDermott A *et al* (2008). Folate-related gene polymorphisms as risk factors for cleft lip and cleft palate. *Birth Defects Res A Clin Mol Teratol* **82:** 636–643.

Mossey PA, Little J (2002). Epidemiology of oral clefts: an international perspective. In: Wyszynski D, ed. *Cleft lip and palate: from origin to treatment*. Oxford University Press: New York, NY, pp. 127–144.

MRC Vitamin Study Research Group (1991). Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. *Lancet* **338:** 131–137.

Munger RG, Sauberlich HE, Corcoran C, Nepomuceno B, Daack-Hirsch S, Solon FS (2004). Maternal vitamin B-6 and folate status and risk of oral cleft birth defects in the Philippines. *Birth Defects Res (Part A) Clin Mol Teratol* **70:** 464–471.

Munger RG, Tamura T, Johnston KE, Feldkamp ML, Pfister R, Carey JC (2009). Plasma zinc concentrations of mothers and the risk of oral clefts in their children in Utah. *Birth Defects Res A Clin Mol Teratol* **85:** 151–155.

Murray JC, Daack-Hirsch S, Buetow KH *et al* (1997). Clinical and epidemiologic studies of cleft lip and palate in the Philippines. *Cleft Palate Craniofac J* **34:** 7–10.

OMIM (Online Mendelian Inheritance in Man) (2009). Available at: http://www.ncbi.nlm.nih.gov/omim [accessed on 22 May 2009].

Paros A, Beck SL (1999). Folinic acid reduces cleft lip [CL(P)] in A/WySn mice. *Teratology* **60:** 344–347.

Peer LA, Bryan WH, Strean LP, Walker JC Jr, Bernhard WG, Peck GC (1958). Induction of cleft palate in mice by cortisone and its reduction by vitamins. *J Int Coll Surg* **30:** 249–254.

Peer LA, Gordon HW, Bernhard WG (1964). Effect of vitamins on human teratology. *Plast Reconstr Surg* **34:** 358–363.

Pei L, Zhu H, Zhu J, Ren A, Finnell RH, Li Z (2006). Genetic variation of infant reduced folate carrier (A80G) and risk of orofacial defects and congenital heart defects in China. *Ann Epidemiol* **16:** 352–356.

Rahimov F, Marazita ML, Visel A *et al* (2008). Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. *Nat Genet* **40:** 1341–1347.

Ray JG, Meier C, Vermeulen MJ, Wyatt PR, Cole DE (2003). Association between folic acid food fortification and congenital orofacial clefts. *J Pediatr* **143:** 805–807.

Reynolds PR, Schaalje GB, Seegmiller RE (2003). Combination therapy with folic acid and methionine in the prevention of retinoic acid-induced cleft palate in mice. *Birth Defects Research (Part A) Clin Mol Teratol* **67:** 168–173.

Riley BM, Mansilla MA, Ma J *et al* (2007). Impaired FGF signaling contributes to cleft lip and palate. *Proc Natl Acad Sci USA* Mar 13 **104:** 4512–4517.

Rogers CR, Weinberg SM, Smith TD, Deleyiannis FW, Mooney MP, Marazita ML (2008). Anatomical basis for apparent subepithelial cleft lip: a histological and ultrasonographic survey of the orbicularis oris muscle. *Cleft Palate Craniofac J* **45:** 518–524.

van Rooij JA, Swinkels DW, Blom HJ, Merkus HM, Steegers-Theunissen RP (2003a). Vitamin and homocysteine status of mothers and infants and the risk of nonsyndromic orofacial clefts. *Am J Obstet Gynecol* **189:** 1150–1160.

van Rooij IA, Vermeij-Keers C, Kluijtmans LA *et al* (2003b). Does the interaction between maternal folate intake and the methylenetetrahydrofolate reductase polymorphisms affect the risk of cleft lip with or without cleft palate? *Am J Epidemiol* **157:** 583–591.

van Rooij IA, Ocke MC, Straatman H, Zielhuis GA, Merkus HM, Steegers-Theunissen RP (2004). Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. *Prev Med* **39:** 689–694.

Rothenberg SP, da Costa MP, Sequeira JM *et al* (2004). Autoantibodies against folate receptors in women with a pregnancy complicated by a neural-tube defect. *N Engl J Med* **350:** 134–142.

Rouget F, Monfort C, Bahuau M *et al* (2005). Periconceptional folates and the prevention of orofacial clefts: role of dietary intakes in France. *Rev Epidemiol Sante Publique* **53:** 351–360.

Shaw GM, Lammer EJ, Wasserman CR, O'Malley CD, Tolarova MM (1995). Risks of orofacial clefts in children born to women using multivitamins containing folic acid perconceptionally. *Lancet* **346:** 393–396.

Shaw GM, Zhu H, Lammer EJ, Yang W, Finnell RH (2003). Genetic variation of infant reduced folate carrier (A80G) and risk of orofacial and conotruncal heart defects. *Am J Epidemiol* **158:** 747–752.

Shaw GM, Carmichael SL, Laurent C, Rasmussen SA (2006). Maternal nutrient intakes and risk of orofacial clefts. *Epidemiology* **17:** 285–291.

Shelnutt KP, Kauwell GP, Chapman CM *et al* (2003). Folate status response to controlled folate intake is affected by the methylenetetrahydrofolate reductase 677C–>T polymorphism in young women. *J Nutr* **133:** 4107–4111.

Shi M, Christensen K, Weinberg CR *et al* (2007). Orofacial cleft risk is increased with maternal smoking and specific detoxification-gene variants. *Am J Hum Genet Jan* **80:** 76–90.

Simmons CJ, Mosley BS, Fulton-Bond CA, Hobbs CA (2004). Birth defects in Arkansas: is folic acid fortification making a difference? *Birth Defects Res A Clin Mol Teratol* **70:** 559–564.

Sperber GH (2002). Formation of the primary palate. In: Wyszynski DF, ed. *Cleft lip and palate: from origin to treatment*. Oxford University Press: New York, NY, pp. 5–13.

Suzuki S, Marazita ML, Cooper ME *et al* (2009). Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. *Am J Hum Genet* **84:** 406–411.

Tamura T, Munger RG, Corcoran C, Bacayao JY, Nepomuceno B, Solon F (2005). Plasma zinc concentrations of mothers and the risk of nonsyndromic oral clefts in their children: a case-control study in the Philippines. *Birth Defects Res A Clin Mol Teratol* **73:** 612–616.

Tamura T, Munger RG, Nepomuceno B, Corcoran C, Cembrano J, Solon F (2007). Maternal plasma pyridoxal-5′-phosphate concentrations and risk of isolated oral clefts in the Philippines. *Birth Defects Res A Clin Mol Teratol* **79:** 276–280.

Tolarova M (1982). Periconceptional supplementation with vitamins and folic acid to prevent recurrence of cleft lip. *Lancet* **2:** 217.

Tolarova M, Harris J (1995). Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and multivitamins. *Teratology* **51:** 71–78.

Verkleij-Hagoort A, Bliek J, Sayed-Tabatabaei F, Ursem N, Steegers E, Steegers-Theunissen R (2007). Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. *Am J Med Genet A* **143A:** 952–960.

Vieira AR, Murray JC, Trembath D *et al* (2005). Studies of reduced folate carrier 1 (RFC1) A80G and 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T polymorphisms with neural tube and orofacial cleft defects. *Am J Med Genet A* **135:** 220–223.

Wehby GL, Ohsfeldt RL, Murray JC (2008). 'Mendelian randomization' equals instrumental variable analysis with genetic instruments. *Stat Med* **27:** 2745–2749.

Wehby GL, Castilla EE, Lopez-Camelo JS, Murray JC (2009). Predictors of multivitamin use during pregnancy in Brazil. *Int J Public Health* **54:** 78–87.

Werler MM, Hayes C, Louik C, Shapiro S, Mitchell AA (1999). Multivitamin supplementation and risk of birth defects. *Am J Epidemiol* **150:** 675–682.

Wilcox AJ, Lie RT, Solvoll K *et al* (2007). Folic acid supplements and risk of facial clefts: national population based case-control study. *BMJ* **334:** 464. Epub 2007 Jan 26.

Williams LJ, Rasmussen SA, Flores A, Kirby RS, Edmonds LD (2005). Decline in the prevalence of spina bifida and anencephaly by race/ethnicity 1995–2002. *Pediatrics* **116:** 580–586.

Wolff T, Witkop CT, Miller T, Syed SB, U.S. Preventive Services Task Force (2009). Folic acid supplementation for the prevention of neural tube defects: an update of the evidence for the U.S. Preventive Services Task Force. *Ann Intern Med* **150:** 632–639.

Yazdy MM, Honein MA, Xing J (2007). Reduction in orofacial clefts following folic acid fortification of the U.S. grain supply. *Birth Defects Res A Clin Mol Teratol* **79:** 16–23.

Zucchero TM, Cooper ME, Maher BS *et al* (2004). Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* **351:** 769–780.